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Laboratory Studies of *Pseudomonas Sp*. in Bioremediation of Lubricant Oil Pollution

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Abstract: ***Wide varieties of microorganisms present in soil are indigenous to remediate the polluted environment. Used engine oil pollution causes a devastating effect on both terrestrial and aquatic environment. The microbes are isolated based on their ability to utilize the used engine oil as sole carbon source and energy. Pseudomonas species isolated from oil contaminated site showed major growth proliferation on used engine oil as sole carbon source. Isolated Pseudomonas Sp. degraded more than 95% of hydrocarbons present in the used engine oil within the period of 28 days. The growth and pH parameters of the organism were studied between different mediums containing used engine oil as sole carbon source. The percentage degradation of residual oil was evaluated using Gas chromatography (GC) equipped with Flame Ionization Detector. Presence of heavy metals was also detected before and after remediation by Energy dispersion Spectroscopy (EDS) and characterized using Scanning Electron Microscope (SEM). This in-vitro study on remediating used engine oil pollution under stimulated condition can be used as efficient approach on reclaimination and restoration of oil polluted environment by microorganisms.***

Keywords: Engine oil pollution, Hydrocarbon utilizers, Bioremediation, hydrocarbons and heavy metals.

1. Introduction

Pollution due to used engine oil which is accidentally or deliberately released into the environment causes serious effects on both biotic and abiotic ecosystems such as mutagenicity and carcinogenicity [1]. Moreover, effects of oil pollution breaks open only after prolong exposure to the contaminant, which can even affect the food chain of an ecosystem. It is also dependent on increasing concentrations of the toxicants which can cause immediate damage. Major contamination occurs from unorganized releases of petroleum products above the ground, storage tanks, and leakage during transportation. These accidental or unorganized industrial processes affect soil and ground water resulting in a serious threat to both animals and human health in long term [2].

The used engine oil consists of common and complex pollutant, it mainly consists hydrocarbon ranging from C_9 to C_{23} and lots aromatic compounds also. The accidental impact of oil pollutant not only affects humans but also plant growth through direct or indirect toxic effects. Thus, it sequentially affects the productivity of many ecosystems [3]. However, when it comes to natural environment there are wide ranges of microorganisms which are able to degrade hydrocarbon contamination, though it is a slow process bioremediation is the best approach to restore and detoxify the hydrocarbon contamination [4].

2. Literature Survey

Bioremediation technique can be divided as in situ or ex-situ remediation. In situ bioremediation involves on-site treatment of contaminated areas, while ex-situ involves the treatment in laboratory conditions [5]. The limiting factor of this work is that bioremediation takes some couple months to totally remediate. The process of bio-stimulation or bio augmentation of the soil or water pollution by the addition of nutrients or multiple microorganism with potential degrading ability to enhance the process of bioremediation.

Modern waste disposal methods using a consortium of microorganism in bio remediation, oil and heavy metal pollution in the environment were conducted [6] and statistical statements revealed that microbe's are potential agents for degradation of heavy metals like Zn, Cu and Pb from various sources of contaminated oil and soil samples.

Cleaning up of hydrocarbon content of the environment by microbial degradation process was extensively studied [7] with various physical and chemical parameters. Study on Pseudomonas Species showed degradation potential compared to other isolates on degrading the hydrocarbon present. The toxicity and fertility of the soil were also studied before and after microbial treatment and the lipase activity showed the enzyme pathway in bio remediation hydrocarbon pollution in soil.

Upon this background survey, the current study deals with the bacteria involved in bio remediating oil pollution under in vitro conditions. The organisms are been isolated based on their ability to grow on used oil as sole carbon source. The study also focused on bio remediation, heavy metals present in the contaminated sample. Residual oil mass was measured at the regular time interval based on their specific growth rate in different mediums. Isolates from oil contaminated sites are grown in Minimal Salt Media with used oil for evaluation of degradation ability.

3. Methodology

3.1 Source of engine oil sample

Contaminated Oil and soil samples were collected from polluted areas near the mechanical workshop. Used oil sample was collected from an old used oil storage tank, while soil samples was collected randomly from oil sludgy top soil up to 10 cm depth, stored in sterile glass bottles for oil sample, polythene bags for soil sample and preserved in a cool dry place. Then the samples were analyzed for microbiological and some physical- chemical parameters such as temperature, pH and moisture content (Mc) according to APHA standards [8].

$$Mc = \frac{wet\ weight - dry\ weight}{wet\ weight} \times 100$$

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3.2 Isolation of petroleum Hydrocarbon Utilizing bacteria

Hydrocarbon utilizing bacteria were isolated from the collected contaminated sample by enriching it in Nutrient broth for 7 days. After 7 days of incubation, 1 ml of sample was serially diluted to 10^{-8} and plated in nutrient agar plates at 37°C for 24 hours. The colonies were randomly picked and isolated based on their ability to grow on used oil as sole carbon source. Pure strains are isolated by the quadrant streak plate method and stored in glycerol stock for further studies. The isolated organisms were identified by staining and biochemical analysis based on Bergey's manual [9], [10].

3.3 Growth medium and culture condition

Modified minimal salt medium (MSM) [11] and oil dispensed distilled water medium was used as a growth medium for the organisms containing 5% used engine oil has the sole carbon source. The pH of the medium was adjusted to 7.2 for bacterial growth. The medium with oil was autoclaved for 121°C at 15 psi before inoculation. An experiment was conducted in both bacterial optimized incubator temperature (37°C) and Room temperature (25 ± 2°C). Bacterial growth was monitored by withdrawing a sample from culture medium every 7 days interval. The sample was analyzed in UV visible spectrophotometer at 600 nm analyzing their growth on used engine oil.

3.4 Efficiency of bacteria in consuming used engine oil

The selected microorganisms are isolated in pure form for further studies. So the selected strains are sub cultured in 10ml nutrient broth for 24 hours. Then 1ml of pre-cultured sample was inoculated in both MSM and oil dispensed distilled water medium containing 5 ml of used engine oil and incubated at room temperature (25 ±2°C). The experiment was conducted in duplicates at two different temperatures also for the period of 28 days with 7 day intervals. The efficiency can be calculated compared with the weight of used oil at the end of each 7 days interval. Control was also maintained without inoculation. The formula used for calculating percentage oil reduction is [12]

$$\%\ Reduction = \frac{Initial\ weight - final\ weight}{Initial\ weight} \times 100$$

3.5 Hexane Extraction method

Residual oil was extracted by liquid- liquid extraction by adding 2 ml of hexane to the broth culture in a flask and shake for 30 minutes [13]. After removing the aqueous phase with separating funnel, the residual oil concentration was determined by Gas Chromatography. Similarly, other sample and control flasks were also extracted. GC analysis was done by HP GC equipped with flame ionization detector (FID) with capillary DB column. Helium gas was used as carrier gas. 1 µl of sample was injected into the injection port at 260°C; column temperature maintained at 50°C for 5 min, then increased to 260°C for 15 min with pressure at 90.7 mmHg. The samples were analyzed in split less mode for 3 cycles. The consecutive peaks and area would show the presences of hydrocarbons in the sample compared with the negative control.

4. Results

4.1 Isolation, screening and characterization of petroleum hydrocarbon utilizing bacteria

Microorganisms were isolated based on the ability to grow on used oil as single carbon source. 7 strains of hydrocarbon utilizing bacteria have been isolated from the enriched oil and soil samples. Microscopic observation showed rods and coccus forms. And Gram straining results showed Gram negative for all isolates. Biochemical analysis based on Bergey's manual of determinant microbiology as showed similarity to *Bacillus Sp., Enterobacter Sp and Pseudomonas Sp*.

Table1: Information of isolates from different polluted sites

S. No	Source of oil sample	Isolates identification
1.	Car workshop	C1, C2, C3, C4
2.	Bike workshop	T1, T2, T3

4.2 Analysis of growth and pH parameters

The growth curve of selected organism both in the MSM and Oil Dispensed distilled water medium at Incubator temperature

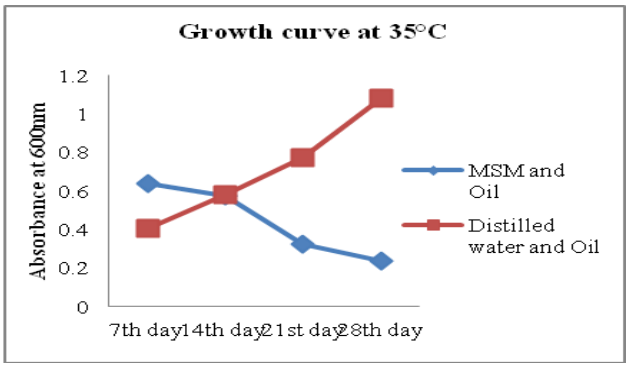

Figure 1: growth of T2 at 35°C for 7 days interval for period of 28 days

Growth curve of selected organism in MSM and Oil dispensed distilled water medium at room temperature 25°C

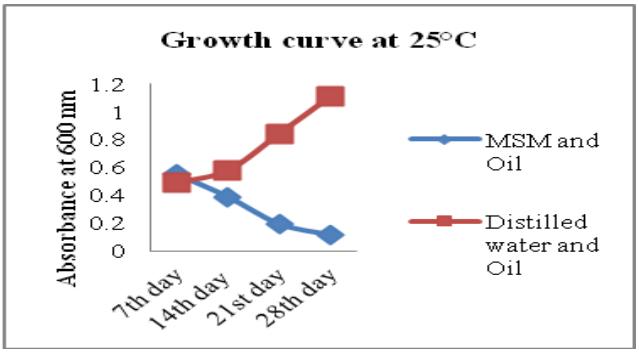

Figure 2: growth of T2 at 25°C for 7 days interval for period of 28 days

Comparative analysis of pH on selected organism in MSM and Oil dispensed distilled water medium after the every intervals

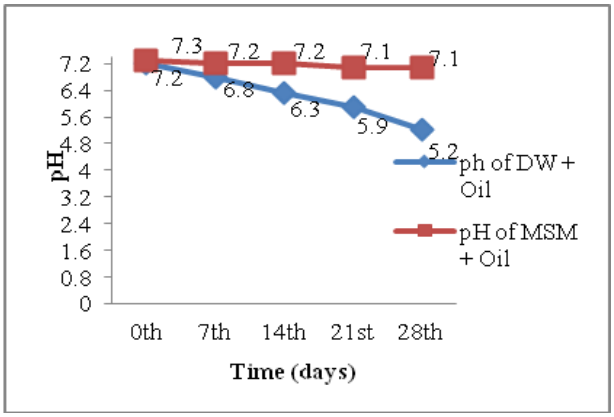

Figure 3: T2 showing reduction in pH in oil dispensed medium

4.3 Biochemical analysis

Table 2: Biochemical and Morphological characterization of the isolates

Biochemical test	C2	T2
Gram's Reaction	-	-
Indol	-	+
Citrate	+	+
Methyl red	-	+
Vp	+	+
Fermentation	+	-
H_2S	-	-
Catalyze	-	-
Morphology	Rods	Short Rods
Tentative identification of Species	Enterobacter Sp.	Pseudomonas Sp.

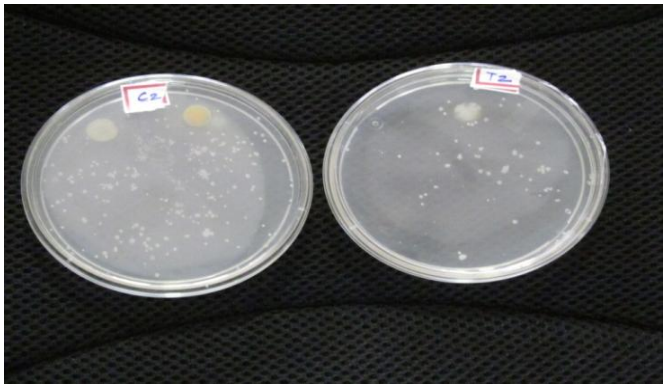

Figure 4: Growth of T2 (a) in Cetrimide Agar plate (Pseudomonas specific media) at room temperature

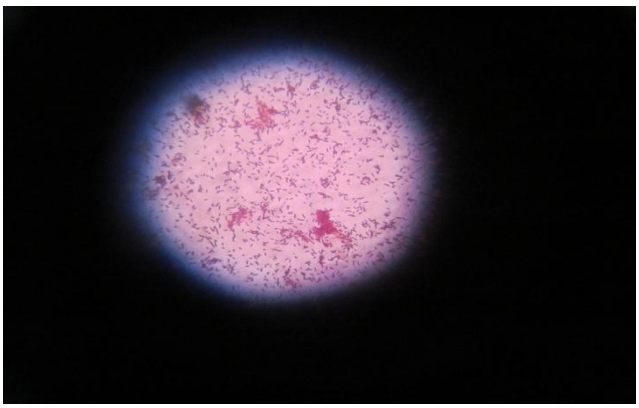

Figure 5: microscopic view of T2 showing gram negative short rods

4.4 Ability of selected microorganism in utilizing used oil

Table 3: Weight reduction

Sample Name	Initial weight	Final weight				% reduction
		7th day	14th day	21st day	28th day	
C2	4.5	4.3	4.2	4.1	4.0	11.1%
T2	4.5	4.0	3.5	3.1	2.7	40%

4.5 GC analysis of used oil before and after bioremediation

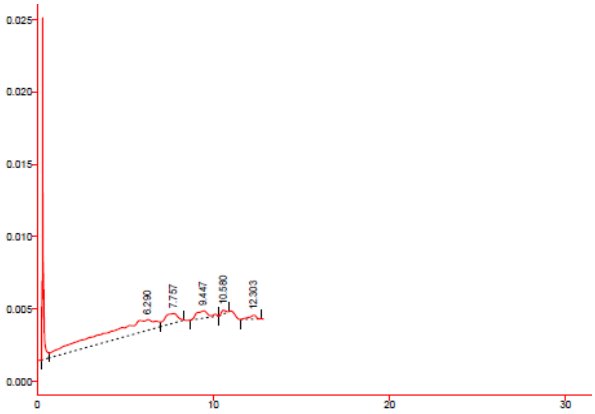

Figure 6: GC spectra of control used oil (before degradation)

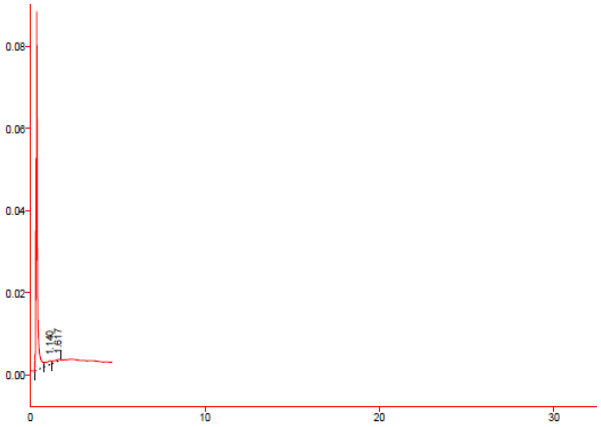

Figure 7: GC spectra for T2 in Minima Salt media after 28 days fermentation at room temperature.

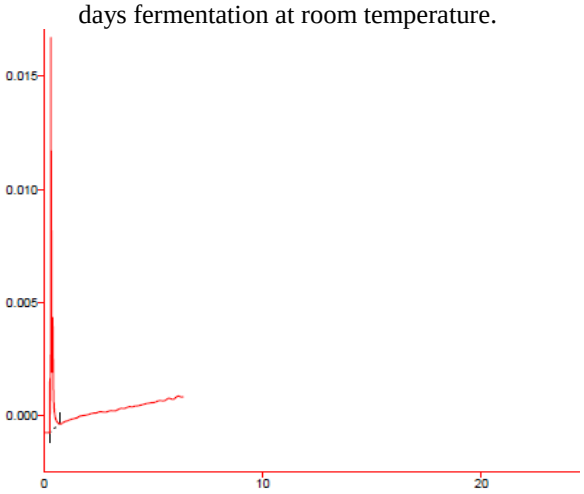

Figure 8: GC spectra for T2 in Oil dispensed distilled water media after 28 days fermentation at room temperature.

4.6 Heavy Metal Detection

Electron Dispersion spectroscopy (EDS) analysis showed the presence of heavy metals in the control as well as in the sample indicating low percent of degradation, which was also characterized by the SEM images [14]. Where the metallic clumps have been dispersed all over the oil sample.

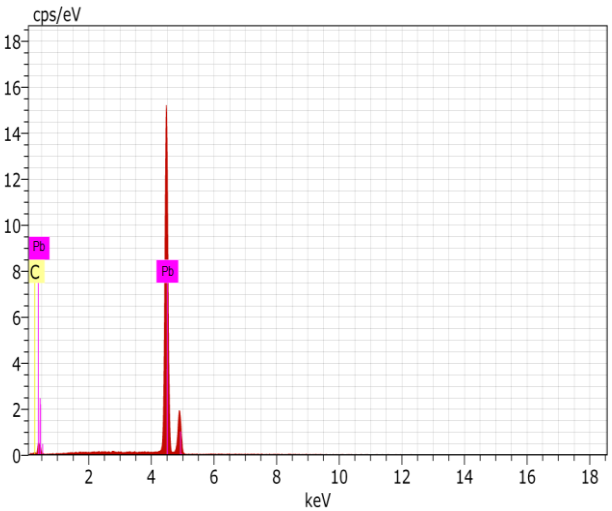

Figure 9: EDS chromatogram of Used engine oil (before degradation) showing peaks of Lead

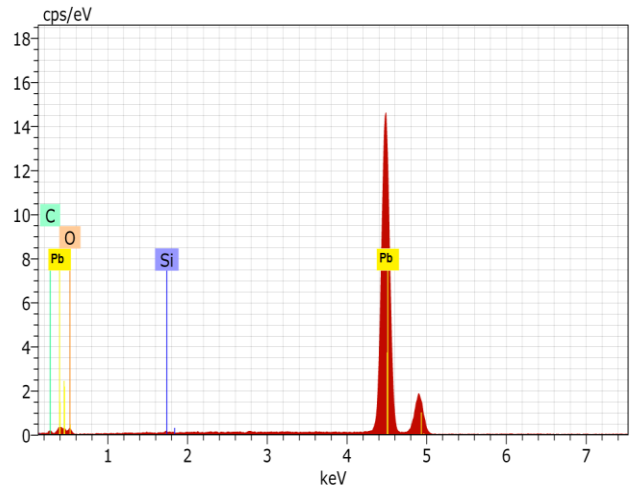

Figure 10: EDS chromatogram of Used engine oil after 28 days degradation by T2

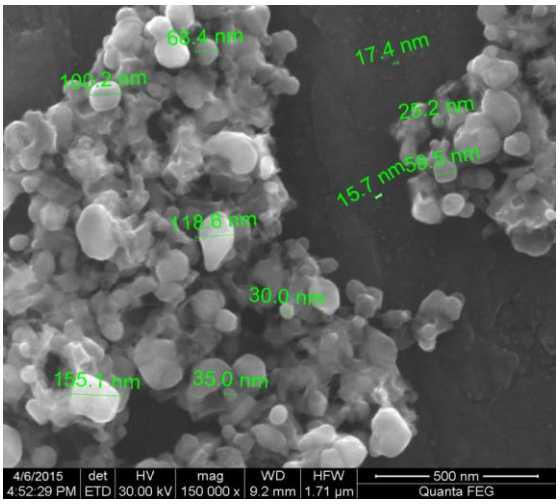

Figure 11: SEM images of used oil containing clumps of Heavy metals

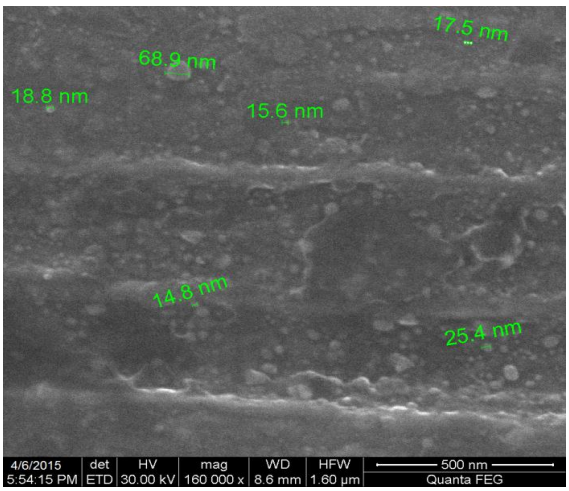

Figure 12: SEM images of used oil were Heavy metals are dispersed after 28 days fermentation of by T2

5. Discussion

Petrochemical pollution is a major problem in developing countries like India and the modern world. These wastes cause a major pollutant in rivers, ponds, lakes, etc and is

more resistant to normal water treatments, hence polluting both water and soil ecosystems. Over the past few decades, extensive work has been carried out on employing microbes in cleaning up polluted environment called bioremediation.

This study is preliminarily based on screening and identification of efficient microorganism involved in bio remediating hydrocarbon waste or pollution and also determining the best condition for degradation process. Organisms obtained in this study were isolated directly from the contaminated sites near mechanical workshops. 2 pure strains of bacteria, including *Enterobacter sp*.(C2), *Pseudomonas Sp.(T2),* has been isolated and selected for biodegradation under in-vitro conditions. Optimization of Physio-chemical parameters for the degradation of used oil under stimulated conditions for each organism has been studied. From results it showed that oil dispensed distilled water media was more suitable for the microorganisms to attach on the oil surface and there by degrading it without short intervals of time. pH reduction must be further studied because in the was constant in MS media, whereas in oil dispensed media the pH has reduced consistently.
Comparative evaluation of the time course and extent of biodegradation of used oil in 2 different mediums was conducted with the above mentioned organisms. In which *Pseudomonas Sp.* C2 showed maximum reduction in oil weight about 40% within the period of 28 days under stimulated conditions compared with [12]. And the pattern of degradation was observed by GC analysis for it shows 90.8% degradation of hydrocarbon content in used oil after fermentation for 28 days in MS media and about 96% was degradated in Oil dispensed media. The results were comparable with [7] where the *Pseudomonas Sp*. showed 92% degradation in minimal media, whereas in the present work Oil dispensed distilled water medium containing used oil showed maximum results of above 96% degradation of hydrocarbons.

Heavy metals in the oil sample were analyzed using Energy dispersion Spectroscopy (EDS) over silica plate showed peaks indicating Lead in the sample though it was not degraded metallic components are dispersed in the oil in smaller particle size. And this dispersion of heavy metals characterized by Scanning Electron Microscope (SEM) at 500nm

6. Conclusion

The present study indicates that organisms isolated from the contaminated site have degradation potentials on used engine oil under stimulating conditions. The growth on oil media showed major degradation potential than in minimal media. GC analysis of used oil compared with control and after remediation indicated maximum percentage of degradation in oil dispensed media. The percentage area in hydrocarbon reduction showed adsorption of carbon molecules present in the oil. These potentials of the isolated microorganism confirm a promising efficiency in bio remediating petroleum hydrocarbon polluted sites, particularly in lakes, ponds etc.

7. Future Scope

The isolated strains can be fermented in different crude substrate mediums such as organic waste, forestry waste etc to enhance the efficiency of microbial degradation of very petroleum hydrocarbons and heavy metals.

8. Acknowledgement

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