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# Bioremediation of Soil Contaminated with Petroleum Products Using Associated Microbes

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## ABSTRACT

The present study was undertaken to determine the bacterial species present in the petroleum contaminated soils of various workshops and to analyze the rate of utilization of petroleum products by the isolates. The soil samples were collected from 5 different mechanic shops located in Tiruchirappalli Corporation, Tamil nadu, India. Soil samples were plated on to nutrient agar, desoxycholate citrate agar, brilliant green agar, blood agar etc., to isolate the bacterial species from the soils contaminated with petroleum products. The bacterial isolates isolated from the soil contaminated with petroleum products were *Pseudomonas sp., Micrococcus sp., Bacillus sp., Salmonella sp., E.coli sp*., *Klebsiella sp*., *Streptococcus sp*. Among the varied bacterial isolates *Bacillus sp., Pseudomonas sp., Streptococcus sp., Micrococcus sp.,* utilized all the test substrates as petrol, diesel and kerosene. The study demonstrates that *Bacillus sp., Pseudomonas sp., Streptococcus sp., Micrococcus sp.,* could utilize petroleum products in the soil environment.

**KEYWORDS**: bioremediation; contaminated soils; bacterial species; petroleum products; Tiruchirappalli
**Running Title:** Bioremediation of petroleum contaminated soil

## INTRODUCTION

Bioremediation, a process that utilizes the capability of microorganisms to degrade toxic waste, is emerging as a promising technology for the treatment of soil contamination. The soils near mechanic workshops are found to be contaminated with petroleum products such as engine oil, petrol, diesel and kerosene which are used daily in varied forms. In industrialized nations substantial volumes of soil have been contaminated with petroleum products. When the oil in motor cars, bikes, generators etc., is changed and disposed into the gutters, water drains, open vacant plots and farmlands, by motor and generator mechanics large amounts of petroleum products are liberated into the environment[1]. Petroleum products consist primarily of complex mixtures of molecules called hydrocarbons. These hydrocarbon molecules are highly toxic to many organisms including human beings [2]. The soil contamination with hydrocarbons cause extensive damage to local systems, as bioaccumulation of hydrocarbons in animal and plant tissues might cause death and mutations [3]. The dominance of petroleum products in the world economy has aided in release of large amounts of these toxins in areas having high human populations as well as other ecosystems around the world. Hydrocarbons from engine oil contribute a potential threat to human, animals and vegetation etc., by prolonged accumulation as fat soluble components and cause the development of liver and kidney diseases and possible damage to the bone marrow and increase the risk of cancer [4,5]. One of the modes of decontamination of the environment loaded with petroleum derivatives is employing methods based on metabolic activities of the microorganisms [6]. Hence, bioremediation has become an alternate way to remedy the petroleum product polluted sites, by addition of specific microorganism or enhancement of microorganism already present to improve the biodegradation efficiency [7]. Hydrocarbon utilizing microorganisms play an important role in combating the problem of petrol product polluted environment [8]. It is found that microorganism`s have an ability to utilize mineral oils as their energy source [2].The microorganisms with multi cytochrome $P_{450}$ enzyme systems degrade n-alkane and aliphatic hydrocarbons as a sole source of carbon and energy [9,10]. The specificity of degradation process is related to the genetic potential of particular microorganism to introduce molecular oxygen into hydrocarbon and to generate the intermediate that subsequently will enter the general energy yielding metabolic pathway of the cell [11] .Microorganism produce enzymes in the presence of carbon source which are responsible for attacking hydrocarbon molecules. Many different enzymes and metabolic pathways are involved to degrade hydrocarbons contained in

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petroleum. But lack of an appropriate enzyme will either prevent attack or will act as a barrier to complete hydrocarbon degradation [12].

Therefore, the present study is aimed at determining the bacterial species present in the petroleum product contaminated soils collected from various mechanic workshops, and to analyse the rate of utilization of petroleum products by the isolates.

## MATERIALS AND METHODS

***Study sites***

The study sites were 5 different mechanic workshops situated at different locations in the Tiruchirappalli Corporation. The locations includes: a. mechanic workshop near a filing station at Sempattu, Tiruchirappalli ($S_1$); b. a mechanic workshop besides CSI Hospital, Woraiyur, Tiruchirappalli ($S_2$); c. a mechanic workshop along market road in Tiruchirappalli ($S_3$); d. a mechanic workshop near Mahatma Gandhi Higher Secondary School ($S_4$) and e. a mechanic workshop at Tiruchirappalli Junction ($S_5$).These sites were chosen as they were indiscriminately seen dumping waste engine oil into the environment.

***Sample Collection***

The soil samples were collected from the mechanic shop at the above mentioned sites in Tiruchirappalli Corporation. Soil samples weighing about 250 g was collected by digging up the soil with a hoe and transferred into clean, sterile containers and taken to microbiology laboratory in the School of Engineering and Technology (now named as Anna University-Bharathidasan Institute of Technology) campus for analyses.

***Determination of physical parameters of the soil***

Physical properties of the soil such as texture (by simple field test/ Finger test), temperature and pH were determined [12].

***Isolation and Identification of the soil bacteria***

The soil bacteria were isolated by serial dilution technique using pour plate method. The medium used was nutrient agar and incubated at 30 ºC for one week. Samples that were turbid were sub cultured into nutrient agar for determination of total bacterial count, desoxycholate citrate agar for isolation of *Escherichia coli,* Brilliant green agar for isolation of *Pseudomonas* and *Salmonella*, blood agar for isolation of *Bacillus, Streptococcus, Mycobacterium, Klebsiella and Micrococcus* [13]. The plates were prepared and inoculated in duplicates. They were incubated at 35º C for 24 hrs. The plates were observed under research microscope and the microorganisms were identified by their morphological characteristic as described by in the revision of Bergey`s manual of determination bacteriology [14].

***Utilization of Petroleum products by isolates***

A loopful of isolates on agar plates were picked and inoculated on Agar-Agar. A quantity of 0.3ml of each test substrate (kerosene, petrol, diesel, and engine oil) was measured and spread on the surface of the agar-agar. The plates were incubated at 35ºC for 24 - 48 hrs and the number of colony forming units (cfu) was determined [15].

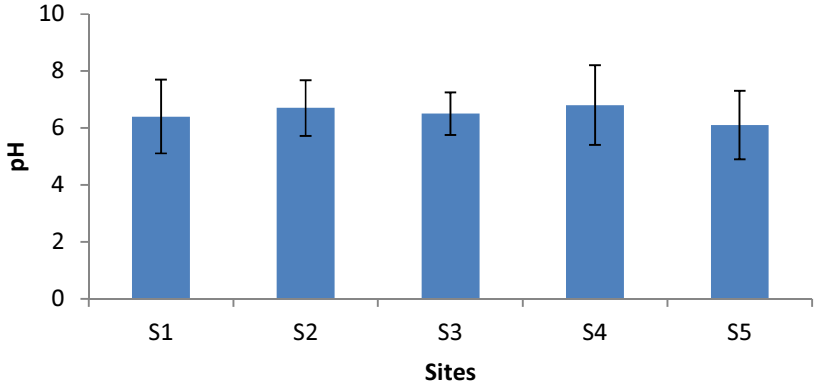
***Statistical Analysis***

The five replicates of soil collected from the mechanic shops at the above mentioned sites were analysed for various parameters and were expressed as Mean ± SD.

## RESULTS AND DISCUSSIONS

The physicochemical analysis of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation showed that the highest pH 6.8 was observed in mechanic workshop near Mahatma Gandhi Higher Secondary School and low pH 6.1 in mechanic workshop at Tiruchirappalli Junction (Fig.1).

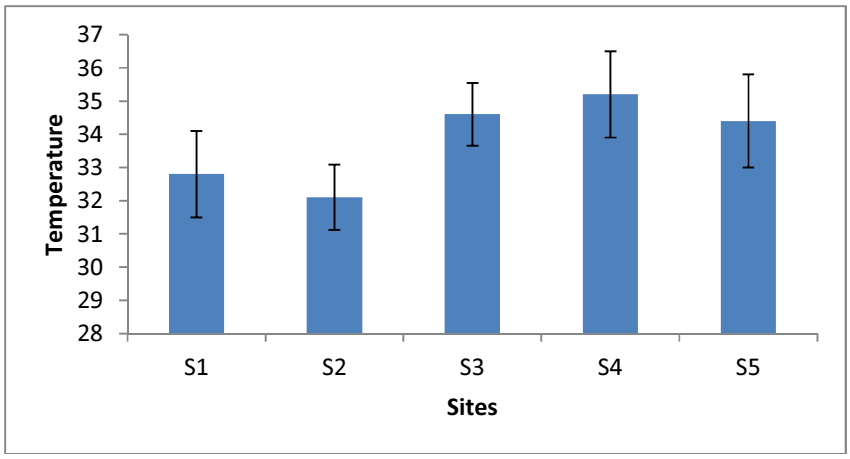
**Fig.1.** Physicochemical characteristics (pH) of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation.




$S_1$- mechanic workshop near a filing station at Sempattu, Tiruchirappalli $S_2$- mechanic workshop besides CSI Hospital ,Woraiyur, Tiruchirappalli, $S_3$- mechanic workshop along market road in Tiruchirappalli, $S_4$- mechanic workshop near Mahatma Gandhi Higher Secondary School, Woraiyur, Tiruchirappalli $S_5$- mechanic workshop at Tiruchirappalli junction.

The texture of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation were similar *i.e.,* sandy loamy. The temperature varied from 32.1ºC at mechanic workshop besides CSI Hospital Woraiyur to highest of 35.2ºC at mechanic workshop near Mahatma Gandhi Higher Secondary School (Fig.2).

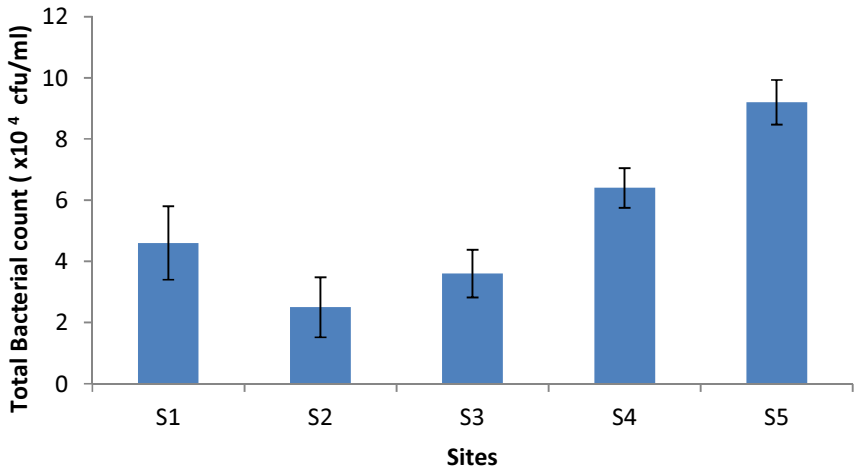
**Fig.2.** Physicochemical characteristics (Temperature) of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation.




$S_1$- mechanic workshop near a filing station at Sempattu, Tiruchirappalli $S_2$- mechanic workshop besides CSI Hospital Woraiyur, Tiruchirappalli, $S_3$- mechanic workshop along market road in Tiruchirappalli, $S_4$- mechanic workshop near Mahatma Gandhi Higher Secondary School, Woraiyur, Tiruchirappalli $S_5$- mechanic workshop at Tiruchirappalli junction.

The results of the bacterial counts of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation showed that the mechanic workshop at Tiruchirappali Junction showed the highest count of 9.2 x $10^4$ / cfu/ml, while the mechanic workshop besides CSI Hospital, Woraiyur showed the lowest count of 2.5 x $10^4$ / cfu/ml (Fig.3).

**Fig.3.** Bacterial counts of petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation.




S1- mechanic workshop near a filing station at Sempattu, Tiruchirappalli S2- mechanic workshop besides CSI hospital Woraiyur, Tiruchirappalli, S3- mechanic workshop along market road in Tiruchirappalli, S4- mechanic workshop near Mahatma Gandhi Higher Secondary School, Woraiyur, Tiruchirappalli S5- mechanic workshop at Tiruchirappalli junction.

The bacterial isolates from the soil contaminated with petroleum products from 5 different locations in Tiruchirappalli Corporation were *Pseudomonas sp., Micrococcus sp., Bacillus sp., Salmonella sp., E.coli* sp., *Klebsiella* sp., *Streptococcus* sp. The highest percentage of occurrence was shown by *Bacillus sp.*,(80%) followed by *Pseudomonas* (70%), *Streptococcus* (60%), *Micrococcus* (50%), *Klebsiella* (24%), and *E.coli* and *Salmonella* (10%) (Table.1).

**Table.1.** Bacterial isolates from petroleum product contaminated soil samples from 5 different locations in Tiruchirappalli Corporation

| S.No. | Isolates | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | % of Occurrence |
|---|---|---|---|---|---|---|---|
| 1 | *Pseudomonas sp.* | + | + | + | + | + | 70 |
| 2 | *Micrococcus sp.* | + | + | + | - | + | 50 |
| 3 | *Bacillus sp.* | + | + | + | + | + | 80 |
| 4 | *Salmonella sp.* | - | + | - | - | - | 10 |
| 5 | *Escherichia coli* | - | - | + | - | - | 10 |
| 6 | *Klebsiella sp.* | - | + | - | - | + | 24 |
| 7 | *Streptococcus sp.* | + | - | + | + | + | 60 |

$S_1$- mechanic workshop near a filing station at Sempattu, Tiruchirappalli $S_2$- mechanic workshop besides CSI Hospital Woraiyur, Tiruchirappalli, $S_3$- mechanic workshop along market road in Tiruchirappalli, $S_4$- mechanic workshop near Mahatma Gandhi Higher Secondary School, Woraiyur, Tiruchirappalli $S_5$- mechanic workshop at Tiruchirappalli junction. + = Present; - = Absent.

The results of utilization of petroleum products by the isolates showed that most of the bacterial isolates have the ability to utilize the test substrates. *Bacillus sp., Pseudomonas, Streptococcus, Micrococcus sp.,* utilized all the test substrates unlike *E.coli* and *Salmonella sp.*, which did not utilize any of the test substrates (Table. 2).

**Table.2.** Results of utilization of petroleum products by various isolates

| S.No. | Isolates | Test substrates | | |
|---|---|---|---|---|
| | | Petrol | Diesel | Kerosene |
| 1 | *Pseudomonas sp.* | + | + | + |
| 2 | *Micrococcus sp.* | + | + | + |
| 3 | *Bacillus sp.* | + | + | + |
| 4 | *Salmonella sp.* | - | - | - |
| 5 | *Escherichia coli* | - | - | - |
| 6 | *Klebsiella sp.* | + | - | - |
| 7 | *Streptococcus sp.* | + | + | + |

- = No growth; + = Growth

Our data shows an obvious influence of petroleum products on the physicochemical and microbiological properties of soil. The low bacterial counts observed in certain samples can be attributed to the unfavourable environment effected out by the petroleum products [16] . A high concentration of the petroleum contaminants could be toxic to microbes and inhibit their degradation suggesting the possibility of bacterial degradation at a contaminant concentration below threshold level [17] as the contaminant are the only source of carbon for bacteria and other microbial populations . The ability to isolate high numbers bacteria can be taken as evidence that these microbes might be active degraders of petro-products. An attempt to establish different microflora in petroproduct contaminated soils is itself an remedy to overcome soil pollution [18,19]. It is reported that oil contaminated soils are dominated by gram negative bacteria [20]. The present study contradicts to this statement by showing the existence of both gram negative (*Pseudomomnas*) and also gram positive (*Bacillus and Micrococcus*) bacteria. Among the isolates present in the petroproduct contaminated soil, 4 isolates namely *Pseudomonas sp., Micrococcus sp. Bacillus sp.* and *Streptococcus sp.* are active degraders of petroleum products, a result which corroborates with the similar other reports [2,8,13,21] . The mechanisms of toxicity to microbial membranes caused by hydrocarbons have been well studied and comprehensively reviewed[22]. The accumulation of toxic hydrocarbons in the membrane increases membrane fluidity (allowing the leakage of macromolecules such as RNA, phospholipids, proteins), increases membrane swelling, and reduces the normal functioning of membrane-associated proteins [20,22]. The accumulation of hydrocarbons results in the disruption of bilayer stability and membrane structure, causing a loss of membrane function and ultimately cell death. Despite this extreme toxicity, hydrocarbon-tolerant Gram-negative and Gram-positive bacteria that are capable to grow in a two-phase water-hydrocarbon system have been isolated [22]. Many of these tolerant bacterial species, including the first strain isolated, were Gram-negative bacteria such as *Pseudomonas putida* or closely related *Pseudomonas* sp. Gram-positive bacteria such as *Bacillus*, *Rhodococcus*, *Staphylococcus*, *Exiguobacterium* have also been found to be hydrocarbon-tolerant, although limited investigation has occurred towards understanding the mechanisms of their hydrocarbon tolerance. Because of the highly impermeable outer membrane of Gram-negative bacteria, it was generally accepted that this type of bacteria are more tolerant to hydrocarbons than Gram-positive bacteria [20, 22].

A single microorganism can degrade only certain types of petroleum compounds, but a mixed population – microbial community enables a higher level of degradation. A single bacterium usually has only a relatively small degradation range, and thus not all fractions of the mineral oil hydrocarbon can be degraded by a single species[5]. It is reported that petroleum biodegradation to be mostly enhanced in the presence of a consortium of bacterial species compared to monospecies activities[23]. Moreover, some substances can be decomposed only by co- metabolism. In natural conditions, the presence of microorganisms that use the products of primary degradation is of particular importance in bioremediation studies. Further, decomposition of crude oil is stimulated by the removal of its degradation products. The speed and efficiency of bioremediation of a soil contaminated with petroleum and petroleum products depends on the number of hydrocarbon-degrading microorganisms in the soil. The most important factors for population growth are temperature, oxygen, pH, content of nitrogen and phosphorus, hydrocarbon class and their effective concentration. Temperature is found to play an important role in biodegradation of hydrocarbons, by its direct effect on chemistry of the pollutants and by its effect on physiology and diversity of microorganisms. Microbes have maximal and minimal range for survival and optimal temperature for substrate utilization [8,23]. It is reported that at low temperatures the viscosity of the oil increases while the volatility of toxic low molecular weight hydrocarbons is reduced, thereby delaying the onset of biodegradation [24] . The rate of biodegradation decreases with decreased temperature. Highest degradation rate generally occurs between the range of 30-40°C in soil environments. , 20-30°C in some freshwater environment and 15-20°C in some marine environments [25,26]. Biological activity in the soil can be affected by the pH. Some rnicroorganisrns can survive in a wide range of pH, but others are sensitive to small variations. The bacteria grow better in pH values between 6.5 and 8.5 [27]. Bioremediation is therefore favoured by near neutral pH values (6-8). Soi1 pH can be adjusted if necessary

to enhance microbial activity. In the present study it is observed that all the study sites provided a favourable environment in terms of temperature and pH for the biodegradative action of microbes on hydrocarbons. The degree and rate of biodegradation are influenced by the type of soil in which the process occurs[28]. Hence, an attempt using microbial consortium could be more effective in bioremediation of petroleum contaminated soils.

**Conclusions**

The present investigation provides information on the physicochemical requirements for optimum degradation of petroleum products by these bacteria and also gives a lead for the selection of bacterial species that could be employed in the bioremediation of environments polluted with petroleum products. This can be exploited for large oil-spill cleanup campaigns. A more directed study of community dynamics related to petroleum degrading microbes have potentials to enhance our understanding about the role played by microbes in natural genesis of long term effect on petroleum product pollution and to determine new remediation systems.

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