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Biodegradation of Hydrocarbon Pollutant Soil by Indigenous Microbes

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ABSTRACT

Petroleum commonly referred to as oil, is a naturally occurring liquid found in rock formations. It consists of a complex mixture of hydrocarbons of various molecular weights, plus other organic compounds. Spilling of oil is the release of a liquid petroleum hydrocarbon into the environment, it is toxic to almost all forms of life. The main cause for the water and soil pollution is due to petroleum based products. Degradation is a treatment method used to remediate a variety of contaminants, including soil contaminated with petroleum hydrocarbons. Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment. The hydrocarbon contaminated soil samples were collected from different regions (Coimbatore and Pollachi) and collected soil samples were determined for the hydrocarbon content by cold extraction method. The microorganisms were isolated from the sample through serial dilution method and then different colonies were picked and inoculated in petrol amended medium (1%) and incubated for 24 hours. After the incubation period the isolates that showed better growth was selected. The selected isolates were nearly identified according to the biochemical test were found to be as *Psuedomonas sp, Corynebacterium* sp.and *Eshrechia* sp. These isolates were amended with 1% petrol in the MSM medium and incubated for 24 hours and under shaking condition, after the incubation period the liquid phase was collected and analysed spectrometrically at 420nm for degradation study. An edible mushroom *Pleurotus ostreatus* was cultivated, in the collected petroleum contaminated soil and the compost was prepared the isolated strain was added to the spent mushroom compost (SMC) to stimulate the activity of indigenous microflora. After 15 days the spent soil used were determined for TPH analysis and *Psuedomonas sp* showed a high level of decreased TPH rate when compared with other two organisms .

Keywords: Petroleum Hydrocarbons, contaminated soil, Microbial degradation, mushroom cultivation.

INTRODUCTION

Petroleum is a crude yellow to black liquid found beneath the earth. It is natural material with complex hydrocarbons and other organic compounds which been refined and used as fuels. The petroleum industry includes the global processes of exploration, extraction, refining, transporting (often by oil tankers and pipelines) and marketing petroleum products(Liodakis *et al*., 2011).The environmental impact of petroleum is often negative because it is toxic to almost all forms of life. Petroleum, commonly referred to as oil, is closely linked to virtually all aspects of present society, especially for transportation and heating for both homes and for commercial and industrial activities. Spilling of oil is the release of a liquid petroleum hydrocarbon into the environment, may cause water and soil pollution, this may cause damage to the organisms, plants and humans by inhaled (or) consumption (Lingering, 2015).Petroleum hydrocarbons can be grouped into 3 classes: alkanes and aromatic hydrocarbons (Dragun, 1998) differ in their susceptibility to microbial attack and have generally been ranked in the following order of decreasing susceptibility: n-alkanes >branched alkanes > low molecular weight aromatics > cyclic alkanes (Leahy and Colwell, 1990). Removing (or) treating petroleum contaminated soil is especially urgent because the hydrocarbons can leach into the underlying groundwater and move into human residential areas.

Degradation is a treatment method used to remediate a variety of contaminants, including soil contaminated with petroleum hydrocarbons. Bioremediation has emerged in recent decades as a response to this threat.Bioremediation is the process of using microbes to degrade organic substances into simpler substances or demineralization process (Udiharto, 1992). Bioremediation is an engineered process where the natural biodegradation of petroleum hydrocarbons by indigenous soil bacteria, fungi and protozoa is accelerated (Bunkim chokshi, 2003). One of the important factor that limit biodegradation of oil pollutants in the environment is their limited availability of microorganisms. Microbes are primary agents for the degradation of organic contaminants in soil. Increasing microbial density and activity can accelerate degradation of contaminants (Namkoong *et al*., 2002).

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment. Several bacteria are even known to feed exclusively on hydrocarbons (Atlas, 1981). There are various methods used for degradation of hydrocarbons such as, Bioremediation, Biostimulation, and Bioaugumentation. Bioremediation is the promising technology for the treatment of contaminated sites since it is cost effective and will lead to complete mineralization. The remediation of oil contaminated soil environment is difficult because petroleum is a complex mixture of chemical compounds, and their degradation whether chemical or biological is not easy as different class of compounds needs different treatments (Nilanjana Das, 2011). Biostimulation is the process of adding nutrient, electron acceptor and oxygen to stimulate existing bacteria involve in bioremediation. This is the process of optimizing the environment condition of the remediation site. Additives are usually added the subsurface through injection wells. Subsurface characteristics such as ground water velocity, hydraulic conductivity of the subsurface, and lithology of the subsurface are important in developing a biostimulation system. The indigenous microorganisms present in the soil are responsible for degradation of the pollutant, but biostimulation can be improved by bioaugumentation (Vidali, 2001). Bioaugumentation is the addition of a group of indigenous microbial strains (or) genetically engineered microbes to treat the contaminated soil. It is effective where native microorganisms are not identified in the soil or do not have the metabolic capability to perform the remediation process (Bijay *et al*, 2012).

The present study has been attempted to use the indigenous microorganism for the degradation of hydrocarbons in contaminated soil by cultivating the mushroom.

Materials and Methods

Collection of samples

The samples were collected from different petrol bunks from different sites in Coimbatore and Pollachi. The samples were collected in autoclaved bags and processed immediately for further studies in the laboratory.

TPH analysis of the soil:

Collected soil samples were determined for the hydrocarbon content by cold extraction method (Adesodum and Mbagwu, 2012). Soil (5g) samples were weighed into 25mL flask and 10mL of toluene was added. After 30 minutes, shaking the liquid phase was taken and measured calorimetrically at 420nm.

Isolation and selection of organism:

Collected samples were serially diluted, $10^{-2}$to $10^{-7}$and 1ml was inoculated in the agar plates and kept in the incubation for 24 hours. After the incubation period colonies were counted and recorded. Different colonies were picked and selected colonies were inoculated in petrol amended medium (1%) and incubated for 24 hours. After the incubation period the isolates that showed better growth was selected (Nilanjan Das and Preethy Chandran, 2010).

Identification of the isolates:
The isolates were identified based on the colony morphology and phenotypic characterization (Gram staining) and biochemical methods (IMVIC Test,Urease test, Catalase test,Oxidase test, Gelatin test). .

Degradation studies:
Selected isolates were amended with 1% petrol in the MSM medium and incubated for 24 hours. Under shaking condition, after the incubation period the liquid phase was collected and analyzed spectrometrically at 420nm (O.P.Abioye *et al*, 2012).

Mushroom cultivation:
An edible mushroom *Pleurotus ostreatus* was cultivated, in the collected petroleum contaminated soil and the compost was prepared and used for further degradation studies. A bacterial strain was added to the spent mushroom compost (SMC) to stimulate the activity of indigenous microflora(Ibiene*et al*, 2011).

TPH analysis from the spent mushroom soil:
5g of petrol contaminated soil was weighed and mixed with rice husk and spawn was inoculated to the compost and humidity was maintained for 15 days. After 15 days, the spent soil used were determined for TPH analysis.
10g of soil sample was weighed into 50ml flask and 20ml of toluene added and kept in shaking condition at 37ºC for 24 hours. After the incubation period, culture was taken and centrifuged at 5,000 rpm for 5 minutes. The 1ml of aqueous phase was taken and diluted with 1:1 ratio with toluene (equal amount).

RESULT:
Total five sites were selected from Coimbatore to Pollachi. The soil samples from the petrol contaminated area in different regions were collected in both the surface and 15 cm depth. The petrol contaminated soil was found to be black and brown in colour (Table 1).
Five samples chosen from the study region were designated as CP1, CP2, CP3, CP4 and CP5. The samples plated in the nutrient broth showed numerous colonies (Fig. 1). To study the tolerance of the indigenous organism different percentage of petrol were amended in the medium. The colonies grown were selected for further studies (Fig .2).
The selected three isolates (CP1, CP2 and CP3) were identified morphologically by Gram's staining. The result showed that all the selected isolates were Gram negative bacteria (Table 2).

Biochemical test:
The selected isolates (CP1, CP2 and CP3) were nearly identified according to the biochemical test were found to be as CP1- *Psuedomonas sp,* CP2 *Corynebacterium* sp.and CP 3-., and *Eshrechia* sp. The results were tabulated in table 3.

Total Petroleum Hydrocarbon (TPH) analysis:
Soil sample that showed maximum colonies were selected for TPH analysis. The Total Petroleum Hydrocarbon rate of the soil sample CP1, CP2 and CP5 were analyzed and tabulated. The result was expressed in optical density at 420 nm, calorimetrically (CP1- 1.8 nm, CP2- 0.8 nm and CP5- 0.5 nm) (Table 4). The selected three areas and indigenous bacterium isolated were targeted for the degradation of the hydrocarbon compounds.

Degradation studies:
The three isolates selected were inoculated in petrol amended medium and the result showed that after 24 hrs. The rate of degradation was found to be 62 % for CP1, 22 % for CP2 and CP3 for 42 %. The results were illustrated in Fig 6&7.

Mushroom cultivation
The growth of the mushroom in the petrol polluted soil was observed after 21 days (Fig 3&4).

TPH analysis of mushroom spent soil
TPH rate was observed in the mushroom spent

petrol contaminated soil. The spent soil was analysed for the TPH rate and CP1 showed a high level of decreased TPH rate when compared with other two CP2 and CP3 organisms

DISCUSSION

Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environment, particularly from petroleum-contaminated sites. Bacterial strains that can degrade aromatic hydrocarbons have been repeatedly isolated mainly from soil. In the present study hydrocarbons are used as a substrate for the growth by the microorganism. The consortium of bacteria, *Pseudomonas sp.*, *Micrococcus sp.*, and *Baillus sp.*, were used in degradation. The isolated bacteria were identified according to the criteria on the basis of colony morphology, Gram staining and biochemical characteristic. These are usually gram negative bacteria; most of them belong to the genus *Pseudomonas, Micrococcus* and *Corynebacterium*. The biodegradative pathways have in bacteria from the genera Mycobacterium, *Corynebacterium*, *Aeromonas*, *Rhodococcus* and Bacillus. These organisms have the capacity to degrade the hydrocarbons.

The similar study has done by Boonchan et al. (2000) and Strauss and Plessis, (2000) they concluded that the major microorganism responsible for biodegradtion of petroleum hydrocarbons have been found to be bacteria and fungi. The genera to which hydrocarbon degrading bacteria belong are *Alcaligenes, Micrococcus, Nocardia, Corynobacterium, Rhodococcus, Enterobacter, Eschrechia, Arthrobacter, Baillus, Streptomyces, Clostredium,* and *Proteus*.

In the present study, the average initial petroleum hydrocarbon is 0.66 and after inducing the indigenous isolates in the spent mushroom compost and the total petroleum hydrocarbon was determined by taking the values in spectrophotometer at 420 nm and then the average value is 0.53 and it shows that the microorganisms degrade the petroleum hydrocarbons. Therefore, the isolated microorganism has the capacity to degrade hydrocarbons effectively.

CONCLUSION

The present study states that degradation ability of CP1 was found to be maximum hydrocarbon degrading bacteria. Spent mushroom cultivation decreased the TPH rate of the soil which indicates cultivated mushroom utilizes hydrocarbon as its “c” source.

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Table 1: Colony morphology and screened isolates from petrol contaminated soil

Sample	Soil type	Tested dilution	Colonies observed	Color	Selected dilution	Selected colony
1.	Black	10^{-3}	20	Milky white, light orange	10^{-5}	Milky white
2.	Brown	10^{-3}	12	Pale white	10^{-5}	Pale white
3.	Black	10^{-3}	10	Yellow, pale white	10^{-5}	Pale white
4.	Black	10^{-3}	7	Milky white, pale white	10^{-5}	Milky white
5.	Brown	10^{-3}	10	Yellow, milky white	10^{-5}	Yellow

TABLE 2: Phenotypic characterization of screened isolates

Isolates	Morphology	Gram staining
CP1	Rod	Gram negative
CP2	Rod	Gram negative
CP3	Rod	Gram negative

TABLE 3: Biochemical characterization of screened isolates

Biochemical test	CP 1	CP 2	CP 3
Indole test	+	-	-
Methyl red test	+	-	-
VP test	-	-	-
Simmon citrate test	-	+	-
Urease test	-	+	-
Catalse test	+	+	+
Oxidase test	-	+	-
Gelatin test	-	-	-

TABLE 4: TPH rate analysis after inducing bacteria to the soil sample in day 1 (24 hours):

Sample	OD (420 nm)
CP 1	0.46
CP 2	0.75
CP 3	0.43

In day 2 (48 hours):

Sample	OD (420 nm)
CP 1	0.41
CP 2	0.69
CP 3	0.40

In day 3 (72 hours):

Sample	OD (420 nm)
CP 1	0.38
CP 2	0.60
CP 3	0.36

TABLE 5: Initial and final TPH determination by taking O.D values

Sample	Concentration (ml)	Initial (OD)	Final (OD)
CP 1	0.1	1.92	1.84
	0.2	1.79	1.65
	0.3	1.70	1.62
CP 2	0.1	0.86	0.64
	0.2	0.86	0.56
	0.3	0.79	0.48
CP 3	0.1	0.16	0.10
	0.2	0.21	0.18
	0.3	027	0.23
CP 4	0.1	0.44	0.39
	0.2	0.48	0.45
	0.3	0.51	0.49
CP 5	0.1	0.06	0.01
	0.2	0.19	0.11
	0.3	0.21	0.17

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Fig.1 Plate showing the colonies isolated from petrol contaminated sites

Fig: 2 Plate showing the colonies in the petrol amended medium

Fig: 3 Spawn inoculated at 0^{th} day in petrol polluted soil

Fig 4Spawn inoculated at 21st day in petrol polluted soil

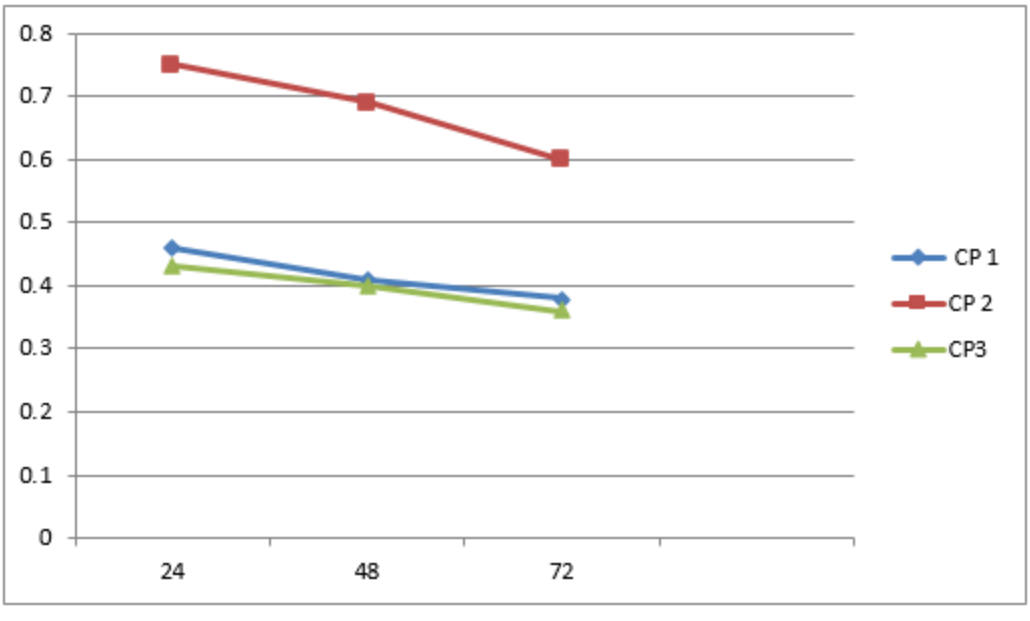

Fig.5. OD values are taken at 420nm after inducing the isolates in the contaminated soil

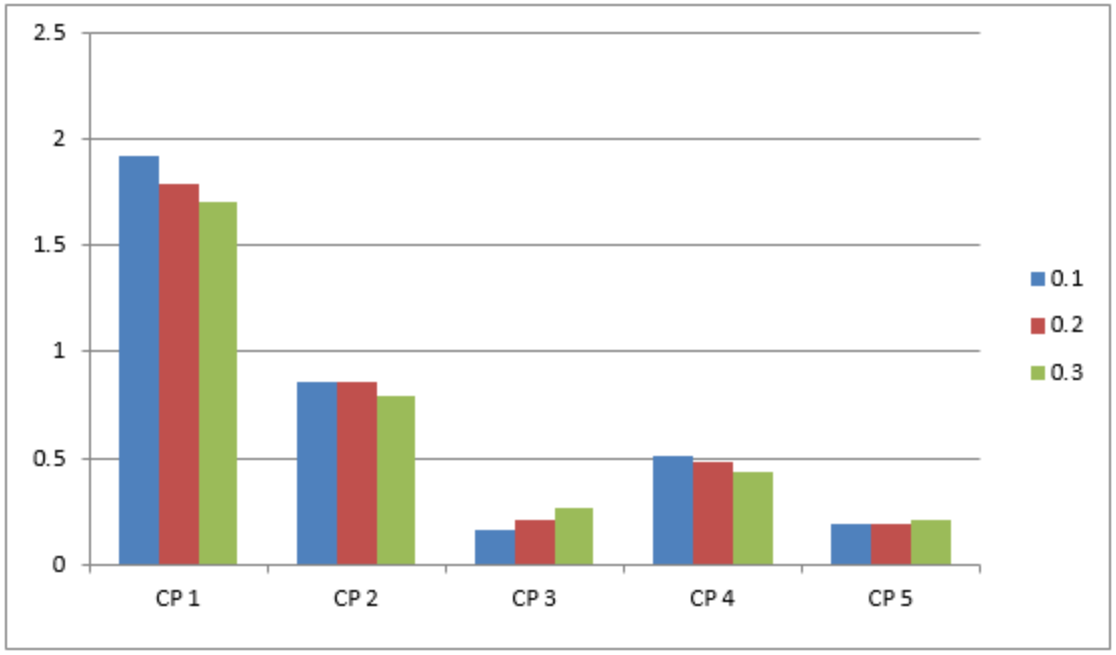

Fig 6. Initial TPH determination

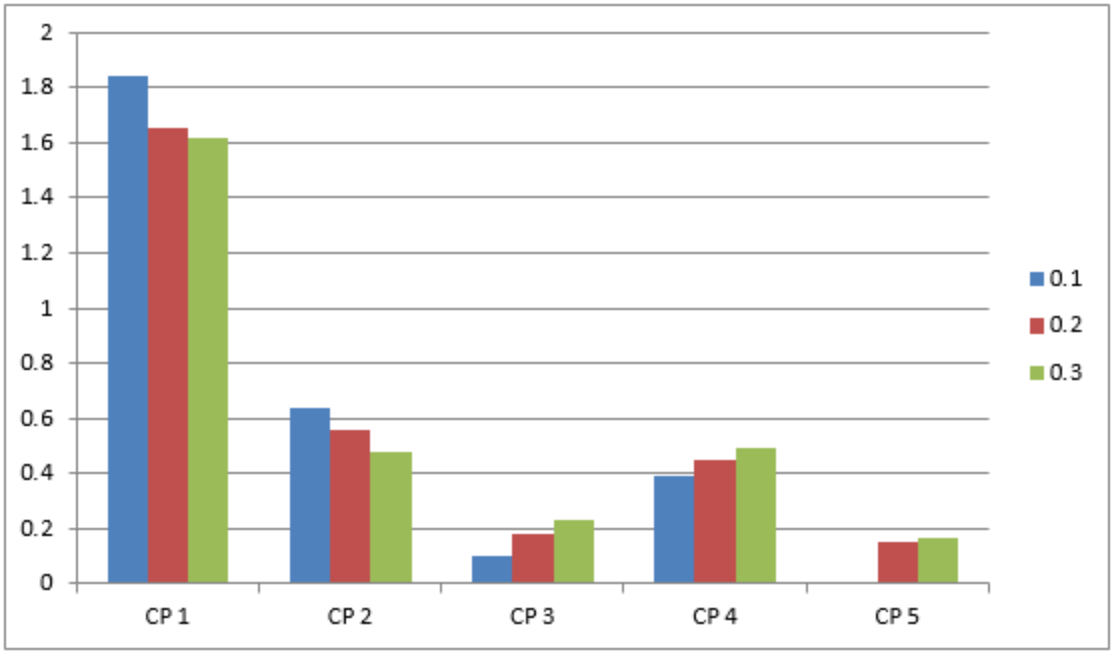

Fig.7.Final TPH determination