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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF MICRORGANISM FROM DIESEL AND PETROL CONTAMINATED SOIL

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Manuscript Info *Abstract*

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A biological treatment was used to determine the potential microbes to degrade the pollutants. The present study was designed to identify microorganism present in the agricultural contaminated petrol and diesel treated soil sample and study their degradation. The identified Colonies subjected to biochemical test revealed that they were *Acinetobacter baumamii, Azotobacter tropicalis, Bacillus subtilis, Corynebacterium variabilis, Flavobacterium lutescens, Micrococcus luteus, Plesiomonas shigelloides, Pseudomonas fluorescens* and *Xanthomonas oryzae.* The pH of the petrol and diesel was increased as the degradation percentage increased. Petrol contaminated soil has degradation percentage of 34.89%. The diesel contaminated has degradation percentage of 23.46%.

Introduction

Natural environment makes our living ecosystem to have healthy life. Because of highly increasing population there is a need in use of different types of commodities to overcome the needs of the living. The things such as increase in usage of the vehicles and production of products by machine used in day today life leads to the increase in import and export of the petroleum products. This also increases the oil spillage in oceans, seas which affects the aquatic environment. Unfortunately petroleum products contaminate the agricultural lands affects the soil fertility and the growth of the plant materials Varjani*et al*., (2013). The deposited polyaromatic hydrocarbons in the soil become more toxic when it reacts with the sunlight. Bioremediation is a method by which the toxic substances are converted into the non toxic/ less toxic substances by microorganisms. Some of the bacteria having the capacity of hydrocarbon degradation isolated from the hydrocarbon contaminated sites are *Pseudomonas stutzeri, Pseudomonas alcaligenes, Pseudomonas aeruginosa, Stenotrophomona smaltophilia, Serattiaodorifera, Achromobacte rxylosoxidans, etc.,* Ebrahimi *et al.,* (2012). The organic nutrients such as chicken droppings, cow dung for the bioremediation also reported (Iiah and Antai, 2003; Obire*et al.*, 2008). Our present study investigated to identify the different types of bacteria present in the contaminated soil and its change in pH.

Material and Methods

Agricultural soil samples were collected from in around Namakkal district. Soil samples were contaminated with diesel and petrol by 4% v/w in 100 gm soil samples and maintained for 16 days. Soil samples were collected after 1st, 6th, 11th and 16th days for enumeration of bacterial population.

Enumeration of bacterial population

Nutrient agar (NA) medium was prepared according to manufacturer's instruction, sterilized and poured into Petri dishes. One gram of soil sample was diluted serially until fifth dilutions and 0.1ml aliquot from the fifth dilution was inoculated on the freshly prepared media, incubated at 37°C for 24 hours and observed for growth. Colonies were counted and recorded as colony forming units per gram of soil (cfu/g). Isolates were subcultured repeatedly to obtain pure isolates and characterized according to the methods described by **Holt *et al.*, (1994)**.

Bacterial characterization

The bacterial species that were isolated from the soil samples were characterized further using morphological, physiological and biochemical properties that included Gram reaction, shape, indole production test, Methyl red test, VogesProsakaeur test, Citrate test, Nitrate reduction, Catalase test, Oxidase test, Glucose utilization test, Triple sugar ion test and urease test were determined according to standard methods of Bergey's Manual of Determinative Bacteriology.

Degradation rates of utilization of petroleum products by 0.1 ml soil bacteria

Minimal salts medium (MSM) containing; 0.27g K_2HPO_4, 0.6g NH_4Cl, 0.03g $MgSO_4.7H_2O$, 0.015g NaCl, 0.0015g $NaSO_4$.7H2O and 150ml distilled water with 1% refined petroleum product (diesel, petrol) as the only source of carbon. The MSM was inoculated with 0.1ml of five time's serially diluted agricultural soil samples. The setup was incubated at 30°C for 16 days. Turbidity produced as a result of microbial growth was monitored visually at 1st, 6th, 11th and 16th day's incubation period and their absorbance reading at 540nm on spectrophotometer were determined.

Results

Isolation of Microorganisms

From the treated soil sample was serially diluted and plate on a nutrient agar plate using spread plate technique and the microbial growth was observed

Biochemical Test

Four isolates (P1, P5, P7 and P9) showed negative gram staining and five isolates showed positive (P2, P3, P4, P6 and P8) in which two cocci (P1 and P6 and seven rod shaped bacteria (P2, P3, P4, P5, P7, P8 and P9) were observed. Indole test showed positive cherry red colour in the isolates P2 and P7, other organisms are negative. Methyl red test showed a positive result a red or pink in colour in the isolates P2, P3, P6, and P8, negative in P1 and P4. Voges-proskauer test showed a positive result brown or red colour in the isolates, negative in P1, P3, P4, P6 and P8 and positive in P2. Citrate utilization test showed a positive result deep Prussian blue in the isolates positive in P1, P2, P6 and P8, negative in P3 and P4.

Urease test showed a positive result pink colour in the isolates P8, P6 and P2 negative in P1, P3 and P4. Triple sugar iron agar test showed a positive result red colour in the isolates P4 and P8, negative in P1 and P3. Nitrate reduction test showed a positive result red colour in the isolates P2, P4 and P8, negative in P1, P3, P5, P6 and P9. Catalase test showed a positive result in air bubbles to adding of hydrogen per oxide in the isolates P1, P2, P3, P4, P5, P6 and p8. Oxidase test showed positive result blue colour in the isolates P2, P3, P6 and P8, negative in P1, P5, P7 and P9 (Table 1).

Diesel

The result bacterial count showed that diesel treated soil had the highest count in *Bacillus subtilis* after treatment of 16 days. In the other organisms such as *Acinetobacter baumamii, Azotobacter tropicalis, Corynebacterium variabilis, Flavobacterium lutescens, Micrococcus luteus, Plesiomonas shigelloides, Pseudomonas fluroescens* . *Xanthomonas oryzae* showed increase and decreasing population. Figure 1

Petrol

The result bacterial count showed that petrol treated soil had the highest count in *Pseudomonas fluroescens* after treatment of 16 days. In the other organisms such as *Acinetobacter baumamii, Azotobacter tropicalis, Bacillus subtilis, Corynebacterium variabilis, Flavobacterium lutescens, Micrococcus luteus, Plesiomonas shigelloides, Xanthomonas oryzae* showed increase and decreasing population. Figure 2

pH of the Diesel and Petrol contaminated soil

The pH of the petrol contaminated soil ranges from 4.89 to 7.2. The pH was 4.89 in the 1st day and the pH was 7.2 in the 16th day. The pH of the diesel contaminated soil ranges from 5.12 to 7.37. The pH was 5.12 in the 1st day and the pH was 7.37 in the 16th day. The pH was tremendously decreased during the 1st day and increased higher in the 16th day in diesel and petrol (Figure 3 and 4).

Degradation of Petrol and Diesel in Agricultural soil

The petrol contaminated soil was analyzed for the degradation activity. The degradation percentage was low in the 1st day with 1.28% and the percentage of degradation was higher in the after 16 days with 34.89%. The diesel contaminated soil was analyzed for the degradation activity with degradation percentage was low in the 1st day with 0.98% and the percentage of degradation was higher in the after 16th days with 23.46% (Figure 5 and 6).

TABLES AND FIGURES

Table 1. Biochemical analysis of isolated strains

S.No	Bacterial species	Gram stain	Shape	Indole	MR	VP	Citrate	Catalase	Oxidase	Nitrate	Glucose	Tsi	Urease
P1	*Acinetobacter baumannii*	-	Cocci	-	-	-	+	+	-	-	+	-	-
P2	*Azotobacter tropicalis*	+	Rod	+	+	+	+	+	+	+	+	+	+
P3	*Bacillus subtilis*	+	Rod	-	+	-	-	+	+	-	-	-	-
P4	*Corynebacterium variabilis*	+	Rod	-	-	-	-	+	-	+	-	+	_
P5	*Flavobacterium lutescens*	-	Rod	-				+		-	-		
P6	*Micrococcus luteus*	+	Cocci	-	+	-	+	+	+	-	-		+
P7	*Plesiomonas shigelloides*	-	Rod	+					-		+		
P8	*Pseudomonas fluorescens*	+	Rod	-	+	-	+	+	+	+	-	+	+
P9	*Xanthomonas oryzae*	-	Rod					+	-	-			

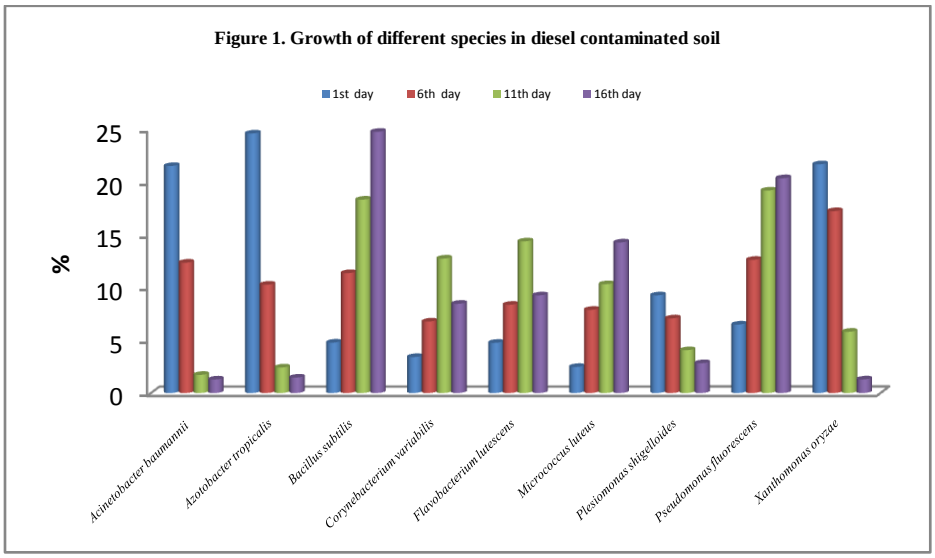
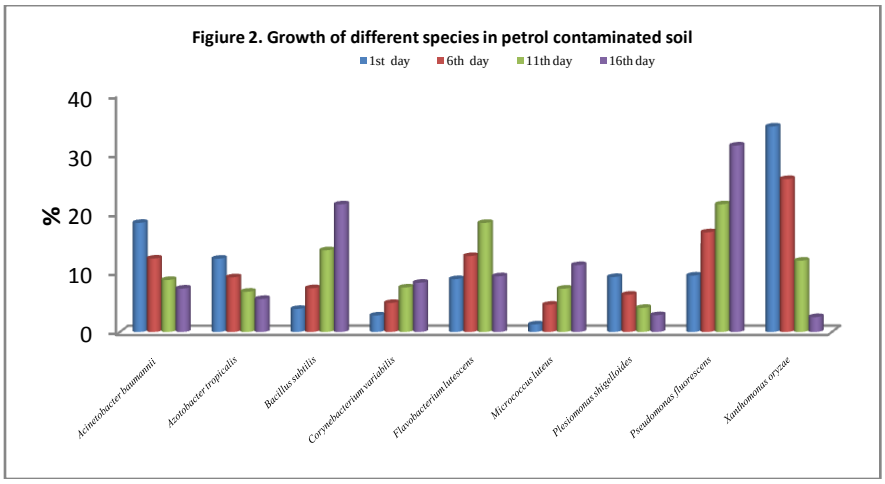


Figure 1. Growth of different species in diesel contaminated soil


Figiure 2. Growth of different species in petrol contaminated soil
1st day
6th day
11th day
16th day
%
40
30
20
10
0
Acinetobacter baumannii
Azotobacter tropicalis
Bacillus subtilis
Corynebacterium variabilis
Flavobacterium lutescens
Micrococcus luteus
Plesiomonas shigelloides
Pseudomonas fluorescens
Xanthomonas oryzae

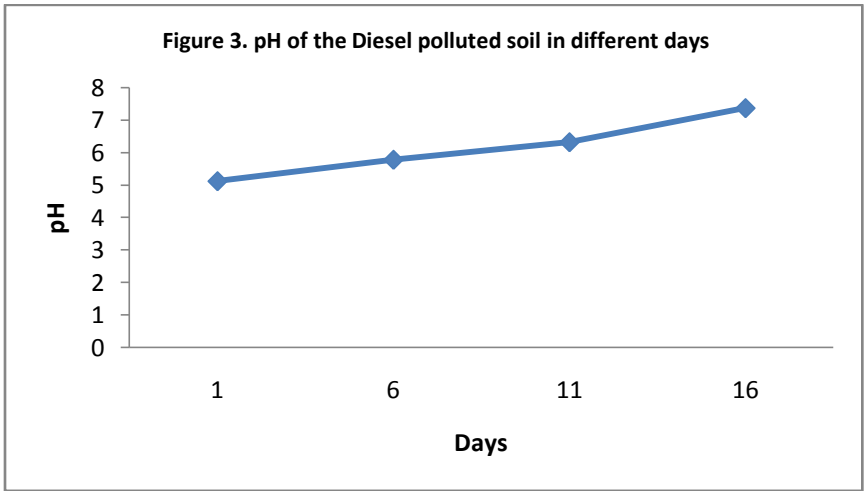


Figure 3. pH of the Diesel polluted soil in different days
pH
8
7
6
5
4
3
2
1
0
1
6
11
16
Days

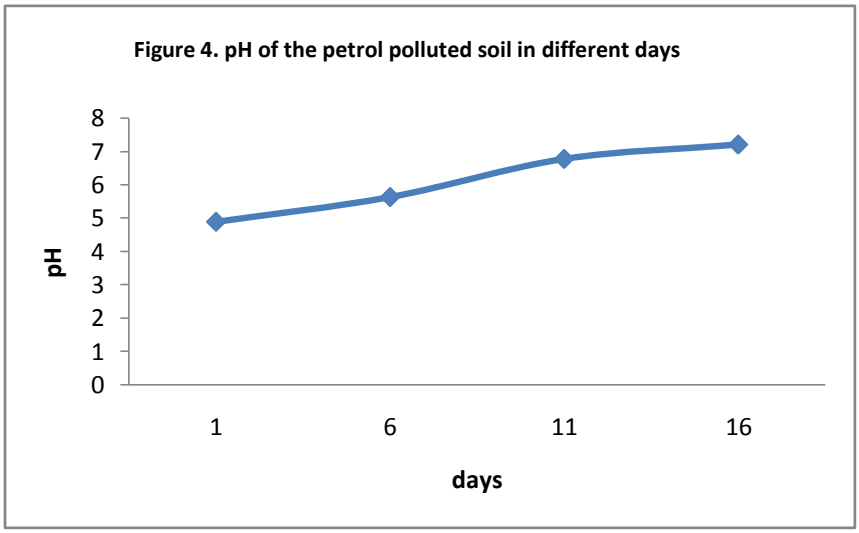


Figure 4. pH of the petrol polluted soil in different days
pH
8
7
6
5
4
3
2
1
0
1
6
11
16
days

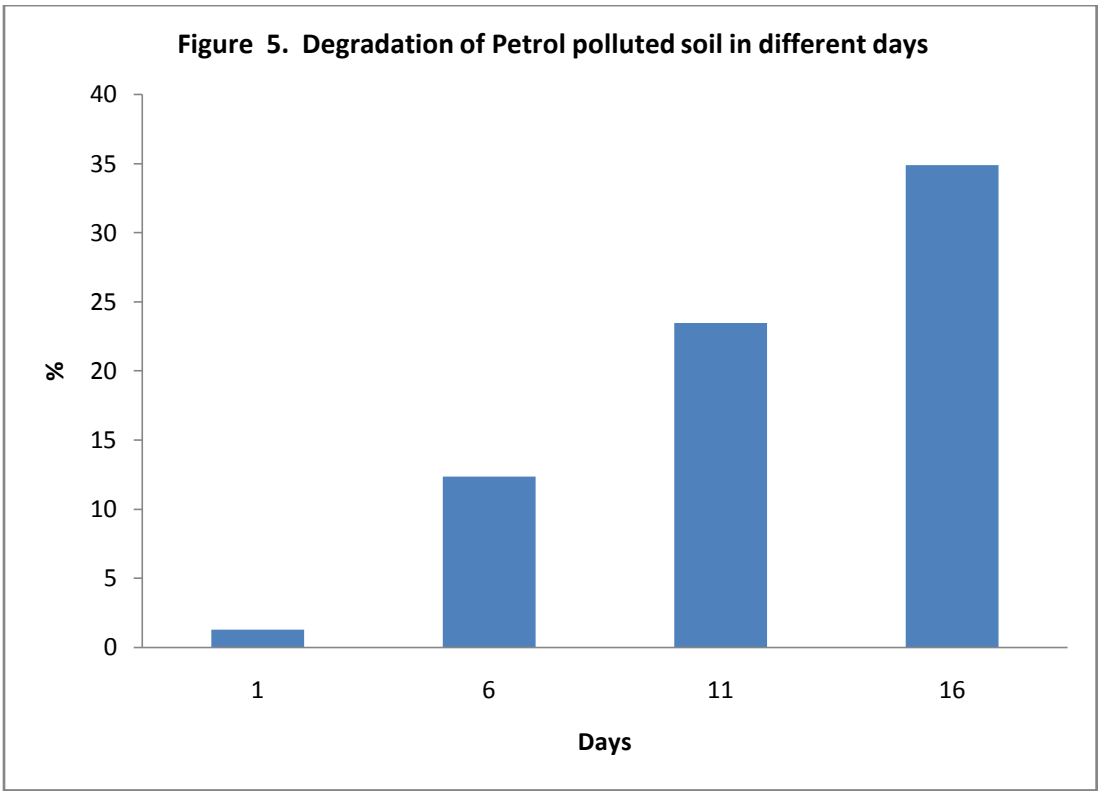


Figure 5. Degradation of Petrol polluted soil in different days
40
35
30
25
20
15
10
5
0
%
1
6
11
16
Days

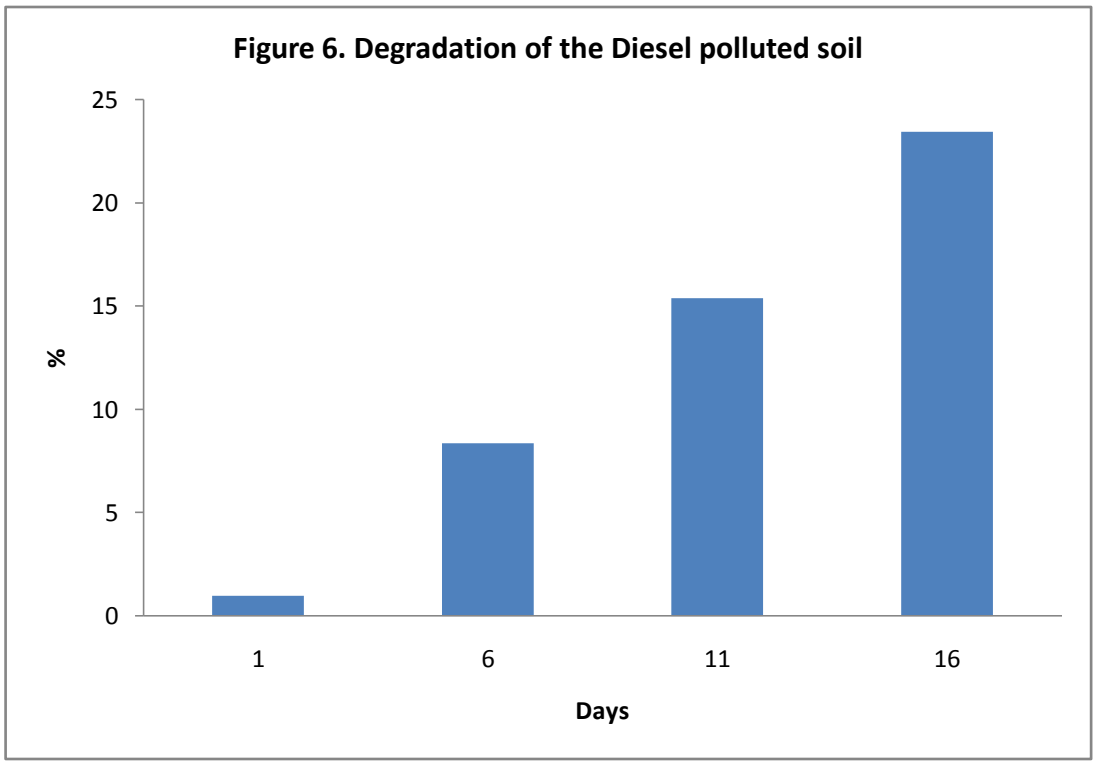


Figure 6. Degradation of the Diesel polluted soil
25
20
15
10
5
0
%
1
6
11
16
Days

Discussion

From the study the petrol and diesel contaminated agricultural soil different types of petrol and diesel 9 different isolates were identified as *Acinetobacter baumannii, Azotobacter tropicalis, Bacillus subtilis, Cornybacterium variabilis, Flavobacterium lutescens, Micrococcus luteus, Plesiomonas shigelloides, Pseudomonas fluorescens, Xanthomonas oryzae.* In which *Xanthomonas oryzae* was higher in the 1st day and *Psudomonas fluorescens* was higher in the 16th day. Olalemi and Arotupin 2012 identified *Bacillus thurigiengis* present in the agricultural petroleum product contaminated soil which able to degrade 20 to 62% of hydrocarbons. It is one of the potential organisms for naturally degrading hydrocarbon contamination (Bujang*et al* 2013). The 7(*Micrococcus, Pseudomonas, Flavobacterium, Serratia, Moraxella, Bacillus and Klibesella*) different species of potential hydrocarbon degrading organism which utilizes hydrocarbon has sole carbon source for their growth was identified from hydrocarbon contaminated soil collected in Mexico (Santhini*et al.,* 2009). Some of the researchers have reported that biodegradation of soil bacteria ranges from 0.13(Jones e*t al* 1970) to 50% (Pinholt *et al*., 1979), and marine bacteria ranges from (0.003% (Hollaway, *et al.*, 1980) to 100% (Mulkins and Phillips 1974). Bacillus sp was effective hydrocarbon degradation (Amund and Adebiyi, 1991; Atlas and Bartha 1992; Nwachuku and Ugoji, 1995; Nwachuku, 2001; Benkacaker and Ekundato, 1997; Diaz et al 2000). Bacillus sp identified from hydrocarbon contaminated soils has a potential to degrade benzene, crude, decanol, ethyl-benzene, n-tetradecanol and xylene.(Ghazali *et al*., 2004). Kebria *et al.,* 2009 identified Diesel degrading microorganism *Bacillus Cereus* and *Bacillus thurigenesis.* Yulani *et al* 2012 isolated 4 different *Bacillus sp* have capability to degrade pyrene and phenanthrene and they posses dioxyrenese *nidA* and *nahAc* gene which are responsible for initial attack of PAHs degradation. DeRito et al., 2005 and Shukor*et al*., 2009) identified *Staphylococcus sp* which has hydrocarbon-degrading capacity.

Pseudomonas sp., Micrococcus sp. and mixed consortium of this has been used has bioremediation of diesel oil (Nikhil 2013). *Pseudomonas aeruginosa* had shown 49.93% of diesel oil degradation in 20days against 0.5% of diesel oil. (Panda *et al*., 2013).

Conclusion

Hydrocarbon degradation in the agricultural soil is the important factors to protect our soil from the pollution. Pollutants by hydrocarbon changes the pH, nutrient content, and microbial activity of the soil and makes the agricultural land to infertile and affect the growth of the plants. To protect our environment by microbial degradation is one of the methods to degrade the pollutant to less toxic and which can able protecting our soil from infertility. From the present study we have identified the following species *Bacillus subtilis* showed more number in the diesel polluted soil sample. *Pseudomonas fluorescens* showed more number in the petrol polluted soil. Petrol contaminated soil has degradation percentage of 34.89%. The diesel contaminated has degradation percentage of 23.46%.

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