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Screening of Bacteria from Hydrocarbon Contaminated Soil in and around Mayiladuthurai with Reference to Biosurfactant Production and Bioremediation

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Petroleum is a complex mixture of hydrocarbons and other organic compounds. Oil pollution is hazardous to terrestrial and marine ecosystems. The contaminated soil samples were collected and analyzed for various physico - chemical parameters. The hydrocarbon degrading bacteria were isolated from contaminated soil. The selected organisms were tested for biosurfactant and BATH assay. The degradation potential of *Pseudomonas, Flavobacterium, Klebsiella, Micrococcus* and *Bacillus sp* were analyzed using BHA medium added with Petrol and Diesel as a substrate and was monitored by emulsification activity.

Key words: Bacteria, Hydrocarbon, Bioremediation, Biosurfactant, Emulsification.

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most notably complexion vanadium and nickel. The natural accumulations of hydrocarbon in oil contaminated sites are often characterized by the absence of molecular oxygen. Petroleum fuel spills have resulted in accumulation of petroleum products at refineries fuel storage areas, airports and gasoline service stations and hazardous to terrestrial and marine ecosystems.

The physico - chemical remedial strategies to clean up contaminated sites are not considered as an effective method. Therefore research is increasingly being suggested on these pollutants by biological method of degradation to eliminate them. The ability of microbes to degrade hydrocarbons was first pointed out by Zobell (1950). The current applied research on petroleum microbiology encompasses oil spill remediation, fermentor and wet land based hydrocarbon treatment, biofiltration of volatile hydrocarbons, microbial enhanced oil recovery, oil and fuel

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upgrading through desulphurization and denitrogenation, coal processing fine chemical production and microbial community based site assessment, biosurfactant etc, (Mac *et al*., 1999).

Bacteria with selected petroleum – metabolizing enzymes amenable to being linked to electronic interfaces are being engineered and developed as biosensors. These systems have applications in monitoring environmental contaminant concentrations and toxicities during implementation of remedial processes and also have potential applications in control of environmental processes. Bio degradation of petroleum oil by *Pseudomonas, Flavobacterium, Klebsiella, Micrococcus* and *Bacillus sp.*

MATERIAL AND METHODS

For the present study soil samples were collected from automobile workshops in Mayiladuthurai.

Collection of samples

Samples were collected at a depth with in 5 cm from the surface of the soil. They were collected in sterile polythene bags and carefully transferred to the laboratory for analysis.

Physio- chemical properties of contaminated soil

The soil physico-chemical parameter was analyzed. Particle size analysis was carried out using the hydrometer method (Bouyoucos, 1951). Soil texture was sandy loam. Soil pH was determined using a pH meter (Elico instruments, India). The temperatures of the soil samples were determined using a Mercury Thermometer. The electrical conductivity of the soil suspension was measured using the Electronic Digital conductivity meter (Elico instruments, India).

Total nitrogen was determined by Kjeldahl digestion and steam distillation method (Sankaran,1966). Available phosphorous was determined by the method of Olsen *et al*., 1954. Available potassium was determined by using the Flame photometer (Sankaran, 1966). Available micronutrients were determined by the method of Lindsay and Norwell 1978 .

Isolation of bacteria and characterization

Isolation of bacteria was performed by soil dilution plate technique using Bushnell – Hass agar media. The isolates were characterized as described by Holt *et al.,* (1994)

Biosurfactant production

The ability of the bacterial isolates to produce biosurfactants activity was tested in liquid culture by supplementing with different carbon sources to Bushnell – Hass agar medium.

Biosurfactant analysis

The biosurfactant activity in the microbial culture was analyzed by the method of Banat 1993.

Bath assay

The organisms were tested for BATH assay as described by Rosenberg *et al.,* 1980. The optical density of cells in nutrient broth medium was determined initially at 660nm.

Growth potential of hydrocarbon utilising bacteria

Minimal salts medium (100 ml, pH-10) supplements with trace elements solution were put into 500ml Erlenmeyer flasks. The hydrocarbon substrate (10% v/v diesel and petrol) were used as sole carbon sources. The media were inoculated with cells previously grown for 24 hours in nutrient broth and washed 4 times in phosphate buffer (pH 7) to remove trace of nutrient. Incubation was carried out at room temperature (30ºC ± 2ºC). Growths of the organisms were assayed after 48 hours by optical density (O.D) measurement at 600 nm. Inoculated minimal salts medium without hydrocarbon served as control.

Characterisation of degradation potential

A single colony of the isolate was inoculated by nutrient broth at 30º C over night. The over night culture was centrifuged for 15 minutes at 35000 rpm. The cell pellet was washed twice and was resuspended with Bushnell – Hass medium until OD600 was equivalent to one. 1 ml of bacterial inoculums (1 OD600 equivalent) was transferred into 5ml Bushnell – Hass medium with 0.5ml of Petrol for one set and 0.5 ml of diesel for another set. They were incubated at 30º C at 160 rpm for 21 days. A control devoid of the bacterial isolate was prepared for each set of experiments. The level of hydrocarbon degradation was monitored using emulsification activity.

RESULTS AND DISCUSSION

For the present study, the bacterial flora was isolated from the soil sample of the three different automobile workshops. The physico-chemical properties of the soil of contaminated field were taken for analysis and the results were recorded. Bacteria were isolated from the soil samples by serial dilution techniques and were identified through number of various biochemical tests and antibiotic sensitivity test. The biosurfactant production and analysis were carried out with *Pseudomonas , Flavobacterium* , *Klebsiella, Micrococcus* and *Bacillus sp*. The organisms were tested for BATH assay process. Biodegradation potential of different hydrocarbon substrates like petrol and diesel with all organisms showed goods results. The biodegradation process was identified by the emulsification activity.

Table 1. History of sites and characteristics of soils used for isolation of hydrocarbon utilizing bacteria

Sample	Soil characteristics and history
S1	Murugan lorry service, Kaveri nagar, Contaminated for over 25 years.
S2	Guruchandra Car Mechanics, Koranadu, Contaminated for over 20 years.
S3	Balavalli Automobile Workshop, Koranadu.Contaminated for over 20 years

Table 2. Physico-chemical properties of contaminated soil samples

S. No.	Properties	S1	S2	S3
1	Texture	Sandy loam	Sandy loam	Sandy loam
2	Temperature	50	40	50
3	pH	8.5	8.1	7.4
4	Total Nitrogen(Kg/hect)	40.6	23.8	37.8
5	Total phosphorous(Kg/hect)	3.3	3.3	3.3
6	Total potassium(Kg/hect)	80	140	153
7	Zinc (ppm)	4.20	5.05	4.91
8	Copper (ppm)	2.31	14.49	21.06
9	Iron (ppm)	2.46	8.08	19.44
10	Manganese(ppm)	2.88	5.71	15.02
11	Electrical conductivityEc (dSM^{-1})	0.20	1.25	1.28

Table 3. Bacterial isolates from hydrocarbon contaminated soil samples

Isolates	S1	S2	S3
Micrococcus .	+	+	+
Pseudomonas	+	+	+
Flavobacterium	+	-	+
Klebsiella	-	-	+
Bacillus	+	+	+

[+ Present; - Absent]

Huston (1994) reported that microbial diversity constitutes the most extra ordinary reservoir of life in the bioshpere that we have only just begun to explore and understand. Diversity is composed of two elements: richness and eveness, so that the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (eveness). In the present study 6 species of bacteria were screened in all the tested soil samples. They were *Pseudomonas, Flavobacterium , Klebsiella, Micrococcus* and *Bacillus sp.*

The biosurfactant producing genera were obtained from the screened soils, of these the isolation of *Flavobacterium* MTN 11 ab a surfactant producers is novel and the biosurfactant is currently being purified for structural elucidation (Burd and Ward,1994)

The bacterial species were few of the well known degraders of aliphatic fractions of petroleum hydrocarbon (Bhattacharya *et al.,* 2003). This study correlates the present study of degradation ability was found to be in the order.

Table 4a. Physiochemical charcteristics of bacterial species

Identification chacracters Gram staining	*Micrococcus* Gram positive	*Pseudomonas* Gram negative	*Flavobacterium* Gram negative	*Klebsiella* Gram negative	*Bacillus* Gram positive
Shape	Cocci	Rods	Rods	Rods	Rods
Motility	Non-Motile	Motile	Gliding motility	Non-Motile	Motile
Indole production	-	-	-	+	-
Methyl red	-	-	-	+	-
Voges Proskauer	-	-	-	+	-
Citrate utilization	+	+	-	+	-
H2S production	-	-	-	-	-
Urea hydrolysis	-	+	-	+	-
Oxidase test	+	+	-	-	Variable
Catalase test	+	+	+	+	+
Carbohydrate fermentation testGlucose	+	+	+	+	+
Maltose	+	-	-	+	-
Sucrose	+	-	-	+	-

[+ = Positive, - = Negative]

Table 4b. Antibiotic sensitivity test for various bacteria

Antibiotics	Zone of inhibition in mm				
	Micrococcus	*Pseudomonas*	*Flavobacterium*	*Klebsiella*	*Bacillus*
Neomycin	19	18	19	21	25
Chloramphenical	24	10	20	10	25
Gentamicin	15	25	11	21	24
Rifampicin	18	resistant	20	21	20
Amikacin	20	13	24	25	22

Table 5a. Biosurfactant analysis from various bacteria in Petrol medium

Organisms	Optical Density at 660 nm
Micrococcus	1.12
Pseudomonas	1.05
Flavobacterium	0.82
Klebsiella	0.91
Bacillus	0.79

Table 5b. Biosurfactant analysis from various bacteria in Diesel medium

Organisms	Optical Density at 660 nm
Micrococcus .	1.62
Pseudomonas	1.94
Flavobacterium	0.83
Klebsiella	0.75
Bacillus	0.71

Table 6. BATH assay for various bacteria

Organisms	Petrol		Diesel	
	Control	Final	Control	Final
Micrococcus .	0.68	1.68	0.68	1.79
Pseudomonas	0.41	1.87	0.41	1.93
Flavobacterium	0.36	1.65	0.36	1.69
Klebsiella	0.40	1.67	0.40	1.75
Bacillus	0.29	1.59	0.29	1.77

Table 7. Growth potential of hydrocarbon utilizing bacteria on petrol and diesel

Organisms	Control	Petrol	Diesel
Micrococcus.	+	++	++
Pseudomonas	+	+++	+++
Flavobacterium	+	++	++
Klebsiella	+	++	++
Bacillus	+	++	++

+ - Low OD; ++ - Moderate OD
+++ - High OD

Pseudomonas > *Flavobacterium* > *Bacillus* > *Klebsiella*> *Micrococcus* .

Thus, the biodegradation process can be augmented and approached by the use of microorganisms that are present in the soil with the increase of specific microbial community and nutrient addition. This approach reduces clean up time substantially. Thus, the biosurfactant producing ability of the bacteria has great concern to ensure rapid degradation of the pollutant thereby controlling environmental pollution.

Table 8a. Degradation of Petrol by various bacteria

Days	Degradation effect of various bacteria				
	Micrococcus	*Pseudomonas*	*Flavobacterium*	*Klebsiella*	*Bacillus*
5th day	+	+	+	+	+
15th day	+	++	+	+	+
21st day	++	+++	++	++	++

- = Nil, +=Low, ++=Normal, +++=High

Table 8b. Degradation of Diesel by various bacteria

Days	Degradation effect of various bacteria				
	Micrococcus	*Pseudomonas*	*Flavobacterium*	*Klebsiella*	*Bacillus*
5th day	-	-	-	-	-
15th day	+	++	+	+	+
21st day	++	+++	++	+++	++

- = Nil, +=Low, ++=Normal, +++=High

CONCLUSIONS

The contaminated soil samples were collected and analyzed for various physico – chemical parameters. The hydrocarbon degrading bacteria were isolated from contaminated soil. The selected organisms were tested for biosurfactant production and BATH assay. The degradation potential of *Pseudomonas , Flavobacterium , Klebsiella, Micrococcus* and *Bacillus* were analyzed using BHA medium, added with petrol and Diesel as a substrate. The hydrocarbon degradation was monitored by emulsification activity.

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