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Studies on diversity of actinomycetes in oil contaminated soil

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ABSTRACT

Petroleum hydrocarbons are typically a complex mixture of aliphatic and aromatic organic compounds. Soil contaminated with petroleum derivatives are potentially carcinogenic and mutagenic. The soil contaminated with PAHs are remediated using a diverse set of physical and chemical methods. Inaddition to physical and chemical methods , biodegradation has become more accepted over the last two decades. The contaminated soil samples were collected and analyzed for various physico - chemical parameters. The hydrocarbon degrading Actinomycetes were isolated and identified from the isolated from contaminated soil. The selected organisms were tested for biosurfactant production and biosurfactant analysis.

Key words: Hydrocarbons, Biodegradation, Biosurfactant.

INTRODUCTION

The ecological functions of soil in turn depend on the maintenance of a healthy and dynamic community of soil biota. Soil and its biotic component have been described as " our most precious non-renewable resource" (Marshall *et al.* 1982) and linking the diversity of the soil biota to ecosystem functioning remains a key ecological question.

A number of soil microbiological parameters, notably microbial biomass carbon and basal respiration have been suggested as possible indicators of soil quality and employed in national and international monitoring programs. More recently, microbial diversity (Community structure) has also been recommended as a biological indicator of soil quality.

Because of the markedly increased exploration for oil and related energy sources. Public attention has been directed to environmental effects of such exploration, particularly with respect to potential contamination of environment with oil. Hence, enumeration of petroleum degrading microorganisms is important both to determines their the potential for removal of oil via microbes and to asses the amount of oil pollution that has occur. If the population of petroleum degrading microorganisms prove to be related to the concentration of polluting oil present.

Petroleum hydrocarbons are typically a complex mixture of aliphatic and aromatic organic compounds. They can be fractionated by distillation into saturates, aromatics, asphaltenes, and resins (Solomon,1996). Contribution of petroleum hydrocarbon to the environment results from

burning of fossil fuels and subsequent atmospheric deposition, primarily from industrialized countries (Sims and Overcash, 1983). In addition,many industrial activities associated with processing, production, and disposal of petroleum hydrocarbons contribute to the overall environmental load. Soils contaminated with petroleum are classified hazardous and are of great importance because certain petroleum derivatives are potentially carcinogenic and mutagenic (Song and Bartha, 1990; Wilson and Jones, 1993). Remediation of soils contaminated with petroleum hydrocarbons is a global problem that consumes economic resources from both industrial and government offers. Soils contaminated with PAHs are remediated using a diverse set of physical and chemical methods that strip contaminants from the soil. In addition to physical and chemical treatment methods, biodegradation has become more accepted over the last two decades. Biodegradation, in general, is the decomposition of compounds by living organisms. Degradation of PAHs may be accomplished by complete mineralization, cometabolic degradation, and /or radial oxidation (Mahro *et al.,* 1994). Complete mineralization is the total breakdown of organic compounds to water and carbondioxide. Factors that affect biodegradation include pollutant concentration and preexposure time. Microbial communities present in previously contaminated soil can metabolize PAHs at greater rates than soil microbial communities found in uncontaminated soils. Comprehensive knowledge of the diversity of indigenous microbial communities and their activities is considered important when assessing the strategy and outcome of bioremediation; yet, little is known about the component of functional diversity responsible for degradation of PAHs in field situations. Actinomycetes are gram positive bacteria frequently filamentous and sporulation with DNA rich in G+C from 57-75%. Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive, bodacious and charming filamentous gram positive bacteria. They make up in many cases, especiallyunder dry alkaline conditions, a large part of the microbial population of the soil (Athalye *et al.,* 1981; Goodfellow and Williams, 1983; Lacey, 1973; Lacey, 1997; Nakayama,1981; waksman,1961). Mycelial fungi and actinomycets have filamentous (threadlike and branching) growth habits that may make them better suited as early exploiters of relatively large fragment of lignified plant residue, because they can transport various nutrients among zones of enrichment. Over 350 bacterial strains have been documented with their genomic and functional diversity. Site-specific bioremediation strategies have been developed and successfully tested at different TPH (total petroleum hydrocarbon) contaminated sites of India.

- By keeping all these in mind the present study is aimed at,
- To analyze various the physico-chemical characteristics of Hydrocarbon
- To study the biodiversity of actinomycetes in Hydrocarbon polluted soil
- To know the biosurfactant activity of actinomycetes.

MATERIAL AND METHODS

For this study the soil samples were collected from six different places of oil contaminated sites at pattukkottai (S1,S2 and S3) and Orathanadu Taluk (S4,S5and S6) Thanjavur District, Tamilnadu, India. Soils samples were used to analyze various physico-chemical characteristics such as pH, ECsc, N,P, K,Zn,Mn,Fe and organic carbon and to isolate actinomycetes.

Collection of soil samples

Soil sample were collected from the study site at random during the study period. The samples were made at a depth within 5 cm from the surface of the soil. The collected soil samples were brought to the laboratory in sterilized polythene bags, hand picked , air dried and stored in deep freezer for future use.

The soil physico-chemical parameters were analyzed. Particle size analysis was carried out using the hydrometer method (Bouyoacos, 1951). Soil pH was determined using a pH meter (Elico instruments, India) temperature of the soil samples were determined using a mercury thermometer. The electrical conductivity was determined using Electronic Digital conductivity meter (Elico instruments, India). Total nitrogen was determined by Kjeldare digestion and steam distillation method (Sankaram, 1966). Available phosphorous was determined by the method of

Olsen *et al.,* 1954. Available potassium was determined using Flame photometer (Sankaram, 1966). Available micronutrients were determined by the method of Lindsay and Norwell , 1978.

Isolation of actinomycetes

Isolation and enumeration of actinomycetes were performed by soil dilution plate technique using Difco Glycerol – Yeast Extract Agar. Olsen (1960) formulated Actinomycetes isolation Agar for isolating and cultivating actinomycetes from soil and water.

Isolation procedure

One gram of dried soil was taken in 9 ml of distilled water, agitated vigorously and preheated at 50°C for 30 mins. Different aqueous dilutions,10-3,10-5 and 10-7 of the suspension were applied onto plates and 20 ml of melted medium at around 50°C was added to it. After gently rotating, the plates were incubated at 27°C for 7 to 14 days. Selected colonies (rough , Chalky) of actinomycetes were transferred from mixed culture of the plates onto respective agar plates and incubated at 27°C for 7 days. Plates containg pure culture were stored at 4°C until further examinations.

Taxonomic grouping of active actinomycetes isolates

Actinomycetes colonies were characterized morphologically and physiologically following the direction given by the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Locci, 1989).

Cultural characteristics of pure isolates in various media were recorded after incubation for 7 to 14 days at 27°C. morphological observation were made with a light microscope (Model SE; Nicon) by using the method of Shirling and Gottlieb (1966). Active purified isolates of actinomycetes were identified up to the species level by comparing their morphology of spore chain with the actinomycetes morphologies, as described in Bergey's, manual (Cross, 1989; Lechevalier, 1989; Locci, 1989; Wendisch and Kutzner, 1991; Williams *et al.,* 1989). This was done by using cover-slip method (Cross, 1989) in which individual cultures were transferred to the base of cover slips buried in medium for photomicrographs. Carbon utilization was determined on plates containing ISP basal medium 9 to which tantalization –sterilized carbon sources were added to a final concentration of 1.0%. The plates were incubated at 27C and growth was read 7,14,21 days using glucose as positive control (Shirling and Gottlieb,1966). The ability to utilize nitrogen sources was determined in a basal medium containing glucose 10, $MgSO_4.7H_2O$ 0.5 g, Feso4.7H2O 0.01g, K2HPO4 1.0 g, NaCl 0.5 g , agar 3.0 g and distilled water 200ml; results were determined after 15 days. Other physiological and biochemical characteristics were determined by the method described by shirling and Gottlieb (1966). All tests were performed at 27°C.

Biosurfactant production

The ability of isolate to produce biosurfactant activity was tested in liquid culture. Culture medium (100 ml in 250 ml conical flask) was prepared by supplementing with different carbon sources (1%) to Bushell – Hass medium. The medium was inoculated with 1 ml of 12 hrs old culture of growth nutrient broth, washed and resuspended in sterile distilled water. The flask were incubated in an orbital shaker at 120rpm and 28C. the cultures were set up in triplicate. Samples measuring 4 ml were collected at 24 hrs intervals for 144 hrs.

Biosurfactant analysis

The biosurfactant activity in the selected microbial cultures was analyzed by the methods of (Bannat,1993). To 5 ml Tris-HCl (2mM, pH 8.0) was added 35 ml of culture suspension or buffer (control) and 35 ml xylene. The mixture was vigorously mixed for 45 second and allowed to stand at room temperature (29°C) for 20 mins. The optical density of the aqueous phase was measured at 660 nm. The activity has been reported as arbitrary units (AU). The results of protein and biosurfactant activity represent the mean of three replicates.

RESULTS

The present study was undertaken to assess the distribution and occurrence of actinomycetes from hydrocarbon polluted soils, which were collected from Pattukkottai and Orathanad Taluk, Thanjavur district, Tamil Nadu,India.

Table 1: Physico-chemical characteristics of soil

S. No	Parameters	Pattukkottai soil samples			Orathand soil samples		
		S1	S2	S3	S4	S5	S6
1	pH	9.2	8.7	8.9	7.4	8.2	8.5
2	Ecdsm-1	0.13	0.19	0.18	0.19	0.18	0.21
3	Organic carbon %	0.38	0.34	0.45	0.33	0.36	0.38
4	Nitrogen (Kg/ac)	88.1	91.8	94.5	81.5	78.8	75.0
5	Potassium (Kg/ac)	77.9	80.5	78.9	69.5	57.6	61.0
6	Phosphorus (Kg/ac)	8.9	7.5	9.1	6.3	6.1	6.5
7	Zinc (ppm)	0.89	0.87	0.91	0.80	0.76	0.74
8	Copper (ppm)	0.50	0.48	0.42	0.37	0.33	0.36
9	Iron (ppm)	4.15	5.05	4.90	3.15	4.05	3.78
10	Manganese (ppm)	9.10	9.26	9.05	8.13	8.19	8.91

Table 2: Morphological, physiological and biochemical characteristics of isolates

characteristics	Isolates								
	1	2	3	4	5	6	7	8	9
Aerial mycelium	+	+	+	+	+	+	+	+	+
Melanin production									
Peptone yeast	+	-	+	-	-	-	-	-	-
Iron agar	+	-	+	-	-	-	-	-	-
Tyrosine agar	+	-	+	-	-	-	-	-	-
Mycelium fragmentation		-	-	-	-	-	-	-	-
Growth with (%W/v)									
Nacl (7%)	+	+	-	+	-	-	+	-	+
Nacl (10%)	-	+	-	-	-	-	-	-	-
Utilization of									
L-cysteine	+	+	+	+	+	+	+	+	+
L-valine	+	+	+	+	+	+	+	+	+
L-histidine	+	-	+	+	+	+	-	+	+
L-lysin	+	+	+	+	+	+	+	+	+
L-thyrosine	+	+	+	+	+	+	+	+	+
L-asparagines	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-	-	-	+
Mesoinosital	+	+	+	+	-	-	+	+	+
Mannital	+	+	-	+	+	+	+	-	+
Adonital	+	+	-	+	+	+	-	+	+
Sorbital	+	-	-	-	+	-	-	-	-
Glucose	+	-	-	+	+	-	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Xelose	+	+	+	+	+	-	-	-	-
Galactose	+	+	+	+	+	-	-	-	-
Lactose	+	+	+	+	+	-	-	-	-

+ - Positive; - Negative; ND-Not Determined

Physiochemical characteristics of soil

The physiochemical characteristics of soils samples of all the six study sites are given in (Table 1). The pH of the study localities is given in the Table 1. the highest pH (9.2) value was reported in the soil sample of Pattukkottai (SI) and the lowest pH (7.4) value was reported in Orathanadu (S4). The electrical conductivity was very low in all the study sites (0.13 to 0.22). The percentage of organic carbon is given in the Table.1. the high percentage of organic carbon was recorded in the soil sample (S3) of Pattukkottai (0.45) and it was other soil samples. The amounts of macronutrients present in the study sites are given in the Table 1. The physico-chemical properties of the soils varied considerably among samples of study sites particularly in macro nutrient level. The total amount of N,P and K were maximum in the soil samples of Pattukkottai. The least amount of N,P and K was observed in Orathanad soil samples.

The micronutrients such as zinc, copper, Iron and Manganese were present in moderate level in all the six soil samples. Among the six soil samples, all the micronutrients were maximum in the Pattukkottai when compared to Orathanad soil samples (Table 1).

Isolation of actinomycetes

The physiological and biochemical characteristics of the strains are summarized in Table 2. A total of 9 isolates of actinomycetes were isolated from six different soil samples (Table 3). All the nine species of actinomycetes were not recorded in all the soil samples. The Nocardia farcinia was not recordedin Pattukkottai soil samples. Similarly Microbiospora sp was not recorded in Orathanad soil samples. In general, among the actinomycetes the Streptomyces present in the maximum no of species (3). Similarly Nocardia and Actinomadura were recorded with two species each (Table 3).Most of the isolates were presumed to be of the genera Streptomyces as they showed good sporulation with compact, chalk – like dry colonies of different colours. A few pigmented strains, unique to individual sites were observed. All the isolates were grouped into 3 colour groups (white series,grey series and brown series) based on the colour of aerial mycelium on oatmeal agar, after 14 days incubation at 28°C. Majority of the strains was of the grey series, followed by white series and brown the least.(Table 3). The grey series include pale grey, light grey,medium grey and dark grey; white colour group includes yellowish white, milky white and orange white while brown colour group includes grayish orange, brownish orange and grayish brown.

Table 3: Various Actinomycetes species isolated from Hydrocarbon polluted soil samples

S. No.	Name of the Organism	Pattukkottai soil samples			Orathanad soil samples		
		S1	S2	S3	S4	S5	S6
1	*Actinomadura cremea*	+	+	-	+	+	-
2	*A.echnispora*	+	-	+	-	+	-
3	*Corynebacterium* sp.	+	+	+	+	+	+
4	*Nocordia asteroids*	+	+	+	-	-	-
5	*N.farcinia*	-	-	-	+	+	+
6	*Streptomyces* sp.	+	+	+	-	-	+
7	*S.griseus*	-	-	-	+	+	-
8	*S.fradiae*	-	+	+	-	-	+
9	*Microbispora* sp	-	-	+	-	-	-

DISCUSSION

Scientists have intensively pursued screening of microorganisms for the treatment of waste for many years. Actinomycetes have the capability to synthesize many different biologically

active secondary metabolites used in waste treatment. Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive, bodacious and charming filamentous gram-positive bacteria. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil (Athalye *et al.*, 1981; Goodfellow and Williams, 1983; Lacey, 1973; Lacey, 1997; Nakayama, 1981; Waksman, 1961). Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. Diversity is composed of two elements: richness and evenness, so that the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994). In the present study totally nine species of actinomycetes were recorded in all the soil samples. The *Nocardia farcinia* was not recorded in Pattukkottai soil samples. Similarly *Microbiospora sp* was not recorded in Orathanad soil samples. In general, among the actinomycetes the *Streptomyces* present in the maximum no of species (3). Similarly *Nocardia* and *Actinomadura* were recorded with two spcies each. Comparing these results with those of other authors, it could be said that members of *Streptomyces* family are the most common among the isolates from polluted regions and from plants, inhabitating polluted waters (Ellaiah *et.al.*,1996; Kim *et.al.,* 1999; Amoroso *et. al.,*2001;). This is, probably, due to their remarkable resistance to bad environmental conditions and to different pollutants. The sites contaminated with hydrocarbons are ecologically important locations as one may encounter microbial flora of diverse nature, which may be potential candidates for important industrial processes. There is a plethora of cultivable microbes with the ability to utilize hydrocarbons as sole source of carbon or to transform them to a less toxic form (Leahy and Colwell, 1990; Kanaly and Harayama, 2000). Earlier reports based on cultivable bacteria suggested that hydrocarbon contaminated soil is predominated by Gram-negative bacteria. In this present study biosurfactant activity was measured in some selected actinomycetes species and recorded. Secretion of surface-active agents by selective actinomycetes when grown on different times was evaluated. The biosurfactant activity in the cultures. Shows When actinomycetes was grown on sugar there was very little surfactant activity, but when grown in hydrocarbons there was a significant amount of surfactant activity seen. Among the species tested the *Nocardia* having higher activity than other organisms tested.. Although many of the PAH-degrading bacteria described are actinomycetes, a variety of non-actinomycete bacteria have also been reported. The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of bioremediation interest. The search will be more promising if diverse actinomycetes are sampled and screened. For this reason, soils were specifically collected under stress environment. This is based on the hypothesis that actinomycetes diversity may be influenced by the diversity of oil polluted area. Furthermore, different type of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinomycetes. However, adaptation has in turn leaded the actinomycetes to produce their own secondary metab.

CONCLUSSION

For this present study the soil samples were collected from six different places of oil contaminated sites at Pattukkottai (S1, S2 and S3) and Orathanad Taluk (S4, S5 and S6) Thanjavur District, Tamilnadu, India. Soils samples were used to analyze various physico-chemical characteristics such as pH, EC_{sc}, N, P, K, Zn, Cu, Mn, Fe and organic carbon and isolate actinomycetes.

The significant findings of the present study are given below

- The pH of soils of all the twelve study sites was varied from 7.4 to 9.2.
- The electrical conductivity (EC_{se}) was low to moderate (EC_{se} 0.13-0.22).
- The organic carbon was maximum in Pattukkottai sites and minimum in Orathanad sites.
- The available P-content of the soils ranged from 5.5 to 9.5kg/ac.
- The available N and K content were invariably high.
- The other soil nutrients such as Zn (0.74ppm to 0.99ppm), Cu (0.33 ppm to 0.50ppm) Mn

(8.13ppm to 10.01ppm) was relatively low in Orathanad.

- Totally nine species of actinomycetes were isolated from six different soil samples.
- Among the actinomycetes *Streptomyces* was recorded maximum with three species.
- Biosurfactant activity was tested in four different actinomycetes species. Among the species tested the *Nocardia* having higher activity than other organisms tested.

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