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Isolation and Screening of Crude Oil and n-Hexadecane Degrading Microflora from Selected Sea Ports in India.

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ABSTRACT

In the present study the occurrence of crude oil and n-Hexadecane degrading microflora isolated from sea ports were checked. Selected ports were Mumbai port (P16), Mormugao Port (P17), Karwar port (P18), Mangalore port (P13), Cochin port (P19), Tuticorin port (P20), Chennai port (P9), Nagapattinam port (P21) and Pondicherry port (P22). Total 64 samples (water and sediment) were collected from 9 locations. Total plate count in Soya bean casein digest agar by serial dilution technique showed more heterotrophic microflora in Cochin port and Mangalore port. Enumeration of crude oil and n-Hexadecane degraders by most probable number procedure showed more total and alkane degraders in Cochin port, Mangalore port and Karwar port. Morphological and biochemical analysis revealed the organisms to be *Pseudomonas* spp. Isolates from Cochin port, Mangalore port and Karwar port were found to be more efficient crude oil and n- Hexadecane degrader than the isolates from other selected sea port regions. Isolates P13 and P19 were able to degrade 40% of crude oil in 21 days. Isolate P19 was able to degrade 56% of n-Hexadecane in 24 days.

Keywords: Crude oil. n- Hexadecane. Cochin port. Karwar port. Pseudomonas.

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INTRODUCTION

The earth has faced many problems that have been caused by human species throughout the history. Today one of the most important disasters jeopardizing marine environments would be marine oil spills. Oil reaches sea as a result of oil tanker disaster, oil rig disaster, leaks during oil extraction etc. Oil spills make up about 12% of the oil that enters the ocean. Everyone has seen pictures of dying birds with feathers soaked in oil that cannot fly anymore or bays of dead fish because they cannot breathe or feed anymore [1].

Hydrocarbons are the simplest organic compounds consisting of hydrogen and oxygen. 95% of crude oil contains hydrocarbons. They can be straight chain, branched chain, or cyclic molecules. Carbon tends to form four bonds in a tetrahedral geometry. Hydrocarbon derivatives are formed when there is a substitution of a functional group at one or more of these positions. Polycyclic aromatic hydrocarbons (PAHs) are formed as a result of fossil fuel combustion and by product waste from industrial activities are the prevalent contaminants in the environment [2].

Pseudomonas is a genus of gram negative, aerobic, gamma proteobacteria which belongs to Pseudomonadaceae family. It is a free living bacteria commonly found in the soil and water. Almost all strains are motile by a polar flagellum.The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches [3]. *Pseudomonas* is tolerant to wide variety of physical and chemical conditions. It is resistant to high concentrations of salts, dyes, weak antiseptics and common antibiotics [4].

Ability of *Pseudomonas* to biodegrade hydrocarbons and other materials are mainly due to its genome. A major problem faced by scientists in bioremediation of oil polluted site is the inability of microorganism to stick to the materials they need to degrade. Since *Pseudomonas* has the biofilm layer along with the fimbriae it is able to attach to the material itself [5].

The present study was conducted on various sea port regions in India. The main aim of this study was to check the presence of crude oil and n-Hexadecane degrading microflora on these selected locations.

MATERIALS AND METHODS

The present study was conducted to know the distribution of total and alkane degraders on the major sea ports of South India. Selected ports were Mumbai port (P16), Mormugao Port (P17), Karwar port (P18), Mangalore port (P13), Cochin port (P19), Tuticorin port (P20), Chennai port (P9), Nagapattinam port (P21) and Pondicherry port (P22).

Sample collection

Oil contaminated water and sediment samples (64) were collected in pre-sterilized bottles from selected regions using aseptic techniques. The water samples were collected from the surface without air bubbles. The sediment samples were collected using simple sampling techniques [6]. Care was taken to avoid contamination of the samples and carried to the laboratory for bacterial isolation as early as possible. In all the selected regions the sea was subjected to oil pollution by discharges from ships.

Isolation of total heterotrophic flora

Isolation of total heterotrophic flora was done by serial dilution technique [7] in Soya bean casein digest agar using sediment sample collected.

Microbial enumeration using most probable number method

Improved most probable number method was done for direct count of oil degrading microorganisms [8]. Serial dilution of samples were inoculated into Bushnell Hass (BH) medium) supplemented with 3% NaCl and adjusted to pH 7.8. Crude oil degraders and Hexadecane degraders were enumerated adding crude oil and n-hexadecane separately as sole source of carbon and energy.

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Identification of Bacteria

The total and alkane degraders were identified by observing morphological characters and performing basic biochemical tests. Different biochemical tests were done such as Gram staining, indole test, methyl red test, Voges Proskauer test, citrate test, triple sugar iron test, starch hydrolysis, O-F test, gelatin liquefaction, oxidase test, catalase test and sugar fermentation test. From the positive tubes 0.1ml is transferred to nutrient agar plates and spread plating was done. From these colonies were taken and streaked on to nutrient agar plate, soya bean casein digest agar and cetrimide agar plates. Morphological features such as cell morphology, colony morphology and structural appearance were noted [9].

Isolation and cultivation of crude oil and n-Hexadecane degrading bacteria

From the dilution, 0.1 mL of each one is spread onto BH agar and supplied with crude oil as sole carbon and energy source by placing it in a vapour tube (cut of micro pipette tip, sealed at one end with heat in the lid of the plate). Control plates without substrates were also inoculated. The plates were sealed with paraffin, incubated at 25°C for one month and colony characteristics were studied. From this to obtain pure culture sub culturing was done on nutrient agar, soya bean casein digest agar and cetrimide agar [10, 11].

Biodegradation assay by spectrophotometric analysis

The bacterial isolates of three selected regions Mangalore port (P13), Cochin port (P19) and Karwar port (P18) were used for further studies. Isolates from overnight culture were transferred to 250 mL conical flasks, each containing 100 mL of mineral salt medium with (2% v/v) crude oil and n-hexadecane separately. The experiment was carried out in duplicates. All flasks were incubated at 22°C, 200 rpm and pH 7 for 30 days. Crude oil degradation and microbial growth were determined spectrophotometrically at 510 nm in selected intervals of 3, 6, 9, 12, 15, 18, 21 and 24 days [12, 13].

RESULTS AND DISCUSSION

Water and sediment samples (64) from various locations were scr*e*ened for crude oil and n-Hexadecane degradation by MPN procedure. Out of the 9 isolates obtained, 3 isolates showed better degradation ability. All the isolates were identified as *Pseudomonas spp.* based on basic biochemical tests, Gram staining and colony characteristics in cetrimide agar.

Quantitative analysis – Total plate count

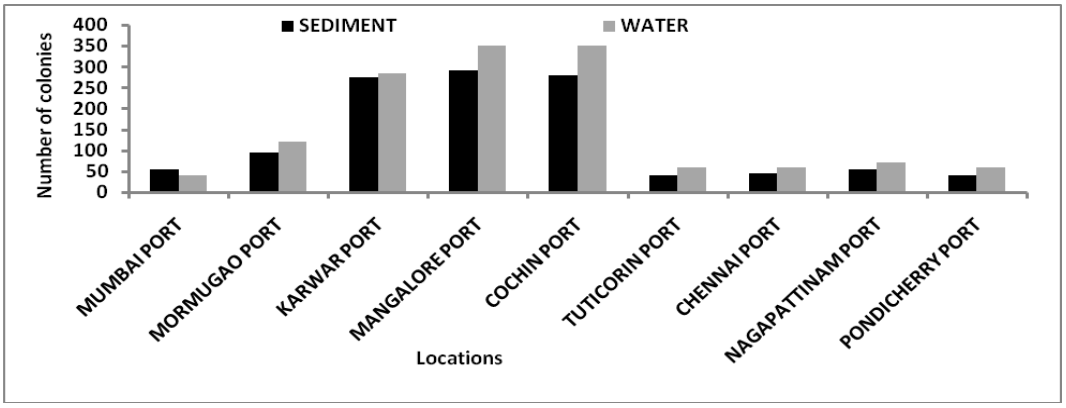

Figure 1: Total plate count - 10^{-3} dilution

For pure culture isolation, total heterotrophic plate count in soya bean casein digest agar by serial dilution technique has been done and total density was estimated. Out of the 9 selected locations more heterotrophic flora were present in Cochin port and Mangalore port. Mangalore port and Cochin port showed heterotrophic count of greater than 300 CFU/mL in 10^{-4} dilution from sediment sample (Fig. 1 and Fig. 2). Bacteria that cannot grow on selective substrates do not produce false positive responses even when the

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inoculum density is very high. Presence of enzymatic digests of casein and soya bean meal which provided amino acids and other nitrogenous substances making it a nutritious medium for a variety of organisms.

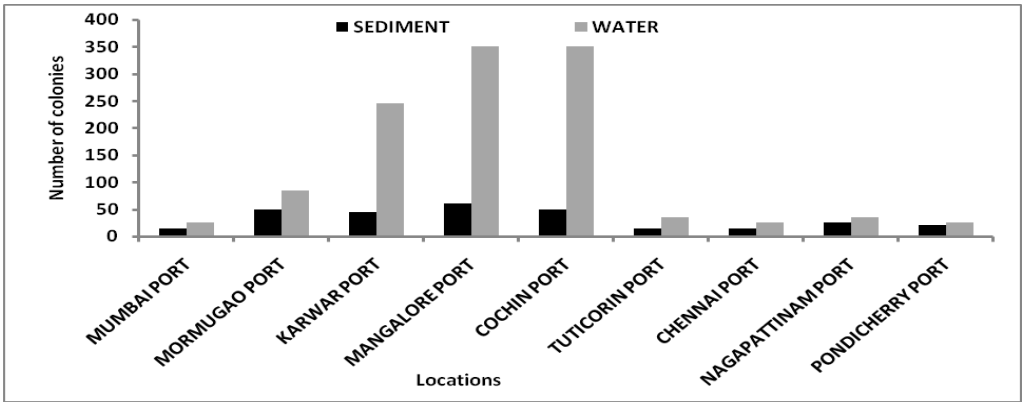

Figure 2: Total plate count - 10^{-4} dilution

Microbial enumeration using most probable number method

For the enumeration of hydrocarbon degrading organisms crude oil and n-Hexadecane were given respectively as the sole source of carbon. Out of the nine isolates, three isolates from Karwar port (P18), Mangalore port (P13) and Cochin port (P19) showed maximum MPN index. P18, P13 and P19 isolates showed an MPN index of $11x10^3$, $13x10^3$,$13x10^3$ for crude oil and $11x10^3$, $13x10^3$, $13x10^3$ for n-Hexadecane respectively (Table 1, Table 2 & Fig. 3.)

Table 1: Microbial enumeration – MPN method for crude oil

MICROBIAL ENUMERATION-MPN METHOD								
FOR CRUDE OIL DEGRADATION								
S. No.	STATIONS	SAMPLE	DILUTION					MPN INDEX
			10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
1	Mumbai Port	Sediment	5	5	5	4	2	0	$2.2x10^3$
		Water	5	5	5	4	2	0	$2.2x10^3$
2	Mormugao Port	Sediment	5	5	5	5	2	1	$7x10^3$
		Water	5	5	5	5	2	1	$7x10^3$
3	Karwar Port	Sediment	5	5	5	5	3	1	$11x10^3$
		Water	5	5	5	5	3	1	$11x10^3$
4	Mangalore Port	Sediment	5	5	5	5	4	0	$13x10^3$
		Water	5	5	5	5	4	0	$13x10^3$
5	Cochin Port	Sediment	5	5	5	5	4	0	$13x10^3$
		Water	5	5	5	5	4	0	$13x10^3$
6	Tuticorin Port	Sediment	5	5	5	3	1	0	$1.1x10^3$
		Water	5	5	5	3	1	0	$1.1x10^3$
7	Chennai Port	Sediment	5	5	5	4	2	0	$2.2x10^3$
		Water	5	5	5	4	2	0	$2.2x10^3$
8	Nagapattinam Port	Sediment	5	5	5	5	3	0	$7.9x10^3$
		Water	5	5	5	5	3	0	$7.9x10^3$
9	Pondicherry Port	Sediment	5	5	5	2	1	0	$0.68x10^3$
		Water	5	5	5	2	1	0	$0.68x10^3$

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Table 2: Microbial enumeration – MPN method for n-Hexadecane

MICROBIAL ENUMERATION-MPN METHOD FOR n-HEXADECANE DEGRADATION									
S.No.	STATIONS	SAMPLE	DILUTION						MPN INDEX
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
1	Mumbai Port	Sediment	5	5	5	4	3	0	$2.7x10^{3}$
		Water	5	5	5	4	3	0	$2.7x10^{3}$
2	Mormugao Port	Sediment	5	5	5	5	2	1	$7x10^{3}$
		Water	5	5	5	5	2	1	$7x10^{3}$
3	Karwar Port	Sediment	5	5	5	5	3	1	$11x10^{3}$
		Water	5	5	5	5	3	1	$11x10^{3}$
4	Mangalore Port	Sediment	5	5	5	5	4	0	$13x10^{3}$
		Water	5	5	5	5	4	0	$13x10^{3}$
5	Cochin Port	Sediment	5	5	5	5	4	0	$13x10^{3}$
		Water	5	5	5	5	4	0	$13x10^{3}$
6	Tuticorin Port	Sediment	5	5	5	3	1	0	$1.1x10^{3}$
		Water	5	5	5	3	1	0	$1.1x10^{3}$
7	Chennai Port	Sediment	5	5	5	4	3	0	$2.7x10^{3}$
		Water	5	5	5	4	3	0	$2.7x10^{3}$
8	Nagapattinam Port	Sediment	5	5	5	5	3	0	$7.9x10^{3}$
		Water	5	5	5	5	3	0	$7.9x10^{3}$
9	Pondicherry Port	Sediment	5	5	5	3	1	0	$1.1x10^{3}$
		Water	5	5	5	3	1	0	$1.1x10^{3}$

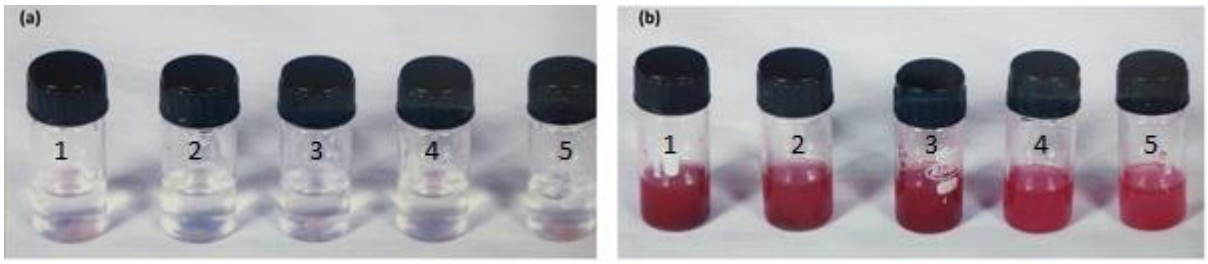

Figure 3: MPN method for n-Hexadecane degradation (a) control tubes (b) 10^{-3} dilution

The tubes which showed turbidity and disruption to the film of oil on the surface of the medium were scored as positive. When 1% TTC was added, a deep red colour developed on the positive tubes due to Formazan formation. The presence of viable hydrocarbon degrading cells was revealed by the reduction of TTC to a coloured formazan due to microbial dehydrogenases activities. These respiratory activities are present in aerobic, facultative and anaerobic microorganisms [14].

Isolation of crude oil and n-Hexadecane degrading bacteria

After conducting MPN, the positive tubes were taken and were plated onto BH agar and hydrocarbon degrading bacteria were detected. Large, flat, spreading, irregular, yellowish green colonies appeared on BH agar where as large, flat, spreading, irregular, bluish green colonies appeared on nutrient agar (Data not shown).

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On Soya bean casein digest agar large, irregular, yellowish brown colonies with pigment diffuses into the medium was observed, and on cetrimide agar large, flat, spreading and irregular greenish and yellowish fluorescent colonies with pigment diffuse into the medium was observed (Fig. 4). *Pseudomonas* spp. produces two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. But the fluorescent pigment production depends on the nature of medium for its manifestations [15].

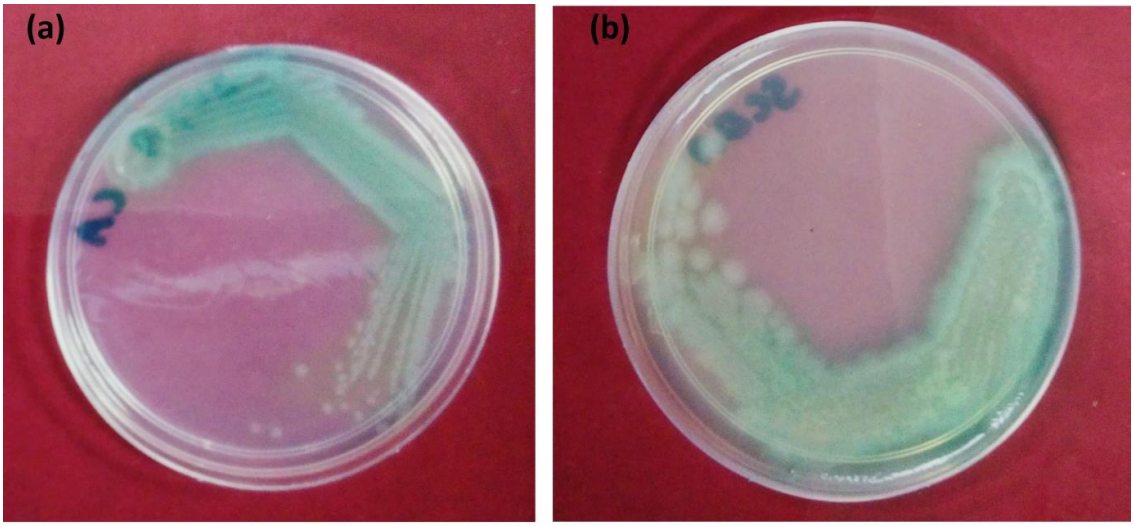

Figure 4: Colony morphology of *Pseudomonas* (a) Cetrimide agar (b) Soya bean casein digest agar.

Identification of bacteria

Based on basic biochemical tests and colony characteristics the isolates were identified as *Pseudomonas* spp. The isolates were found to be gram negative non sporing rods. Starch hydrolysis was found to be negative, with no clear zone outside the area of growth. All the isolates were found to be positive for gelatine liquefaction, indicated the proteolytic potential of the isolates and they were able to reduce nitrate (Table 3). *Pseudomonas* spp. was the only organisms among the 60 different microbial species described by Zobell and Upahm [16] which reduced nitrate to free nitrogen.

Pseudomonas spp. can catabolize a wide range of organic molecules, including organic compounds such as benzoate. This makes *Pseudomonas* a very ubiquitous microorganism [17].

Table 3: **Biochemical analysis**

BIOCHEMICAL POTENTIAL OF *Pseudomonas* FROM WATER AND SEDIMENTS																			
S.No.	BIOCHEMICAL ACTIVITY	STATIONS																	
		P16		P17		P18		P13		P19		P20		P9		P21		P22	
		W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S
1	Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Indole Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	Methyl red Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	VogesProskeur Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Citrate Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Triple Sugar Iron Test	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS	AKS
7	Starch Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	O-F Test	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
9	Gelatine Liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	Oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	Sugar Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

AKS is Alkaline Slant

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Biodegradation assay by spectrophotometric analysis

Biodegradation efficiency of the selected isolates was done spectrophotometrically at 510nm. Isolates P13 and P19 were able to degrade 40% of crude oil whereas isolate P18 was able to degrade 36% of crude in 21 days. Isolate P19 was able to degrade 56% of n-Hexadecane in 24 days whereas isolates P13 and P18 were able to degrade 51% in 24 days (Fig. 5 and Fig. 6).

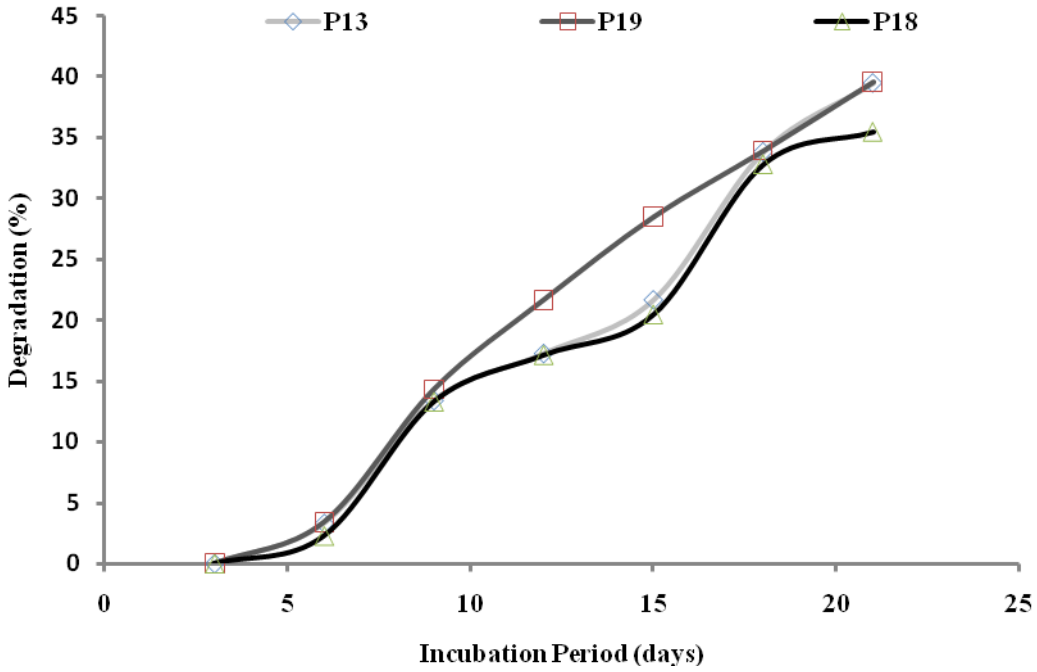

Figure 5: Biodegradation assay for crude oil

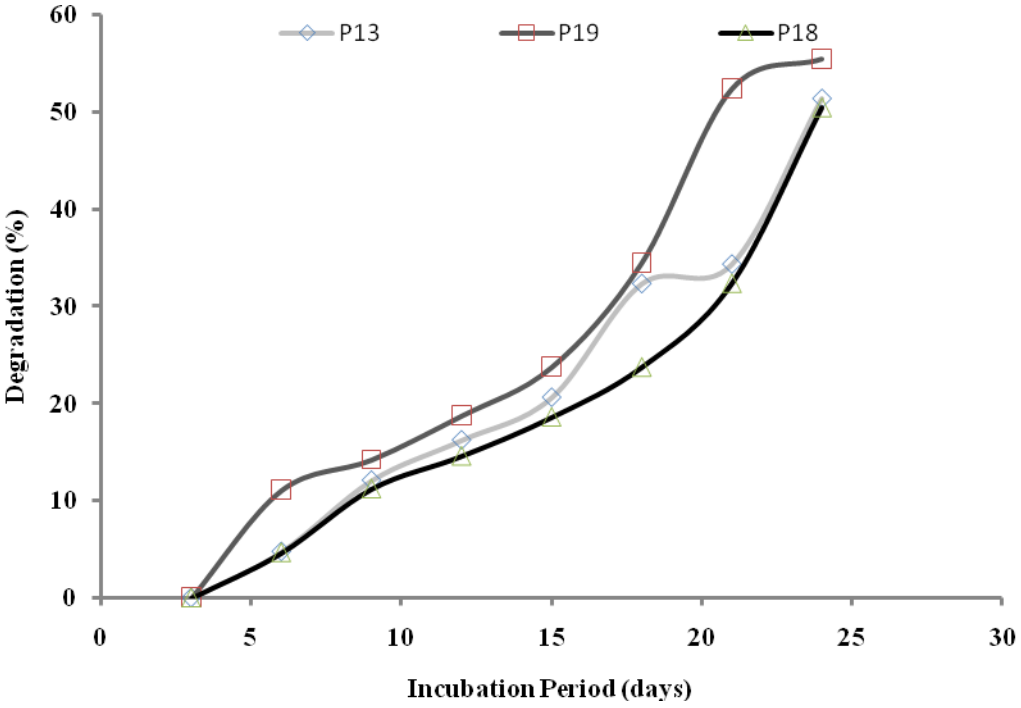

Figure 6: Biodegradation assay for n-Hexadecane

Pseudomonas aeruginosa, as well as many other *Pseudomonas* spp., can degrade aromatic hydrocarbons such as methylbenzenes, which are the by-products of petroleum industries and are commonly used as solvents for enamels and paints as well as in the production of drugs and chemicals [18]. *Pseudomonas* spp. can break down toluene, the simplest form of methylbenzene through the oxidation of the methyl group to aldehyde, alcohol, and an acid, which is then converted to catechol. Hence, *Pseudomonas* spp. can be used in pollution control [19].

Raghavan and Vivekanandan [20] studied bioremediation of oil spilled sites through seeding of naturally adapted *Pseudomonas putida.* Norman [21] informed that *Pseudomonas aeruginosa*, alkane degrader is frequently isolated from petroleum contaminated sites and produce factors that enhance its competitiveness and survival in many environments.

CONCLUSION

Increasing petroleum exploration refining and other allied industrial activities have led to the large scale contamination of most of the swamps, creeks, rivers, streams and oceans. The development of petroleum industry into new frontiers leads to more spillage, which usually occurs during routine operations and records of acute accidents during transportation suggests for more studies into oil pollution problems.

Biodegradation of oil spills is a major problem because it usually occurs in marine water surface and seeding with bacteria becomes difficult. Besides, there is no single bacterium that can degrade all the components of oil which are petroleum products. Isolates from sea ports were able to degrade both crude oil and n- Hexadecane.

It is concluded from the results that crude oil and n-Hexadecane degrading *Pseudomonas* spp. are present on theselected sea ports. Presence of these organisms on the sea ports will reduce the pollution in water by accidental oil spills to some extent. In accidental oil spill cases by the addition of nutrients (biostimulation) growth of indigenous organisms can be stimulated to prevent oil pollution.

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