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ISOLATION OF OIL DEGRADING BACTERIA FROM ENGINE OIL CONTAMINATED SOIL

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author MJJ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author FBR managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Contamination of soil surface by used lubricating oil is a common occurrence in most developing countries. These hydrocarbon pollutants have harmful effects on the environment and human beings. Using hydrocarbon degrading microbes can be an alternative green technology for remediation of contaminated soil. The purpose of the present work is to evaluate the effectiveness of microorganisms indigenous to the soil in remediating the soil pollutant. Used engine oil contaminated soil samples were collected from service stations and motor garages located in Kirathoor, Mankadu and Nithiravilai of Kanniyakumari District. Hydrocarbon utilizing bacteria was isolated by its lipase producing activity. Oil utilization efficiency is estimated by counting total cell number of microbes at 24 h and oil displacement assay. A total of thirteen hydrocarbanoclastic bacteria were isolated from Kirathoor, Mankadu and Nithiravilai workshops engine oil contaminated soil. Among the thirteen bacteria only seven are identified as lipase producing bacteria and are identified as *Acenetobacter*, *Pseudomonas* and *Proteus*. Maximum of oil utilization efficiency was observed in *Acenetobacter* and *Pseudomonas* species at 2% and 4% of oil supplementation respectively. *Proteus* and *Acinetobacter* species showed positive result in oil displacement assay indicating the secretion of biosurfactant to degrade spilled oil. From the present work it is concluded that biosurfactant producing bacteria can be used for remediating oil spill area.

Keywords: Bioremediation; hydrocarbanoclastic; oil displacement assay; biosurfactant.

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1. INTRODUCTION

Engine oil is a thick mineral liquid applied to a machine to reduce friction Shahida [1]. Used engine oil represents the oil that has undergone destructive changes in its property, when subjected to combustion and high temperature, its viscosity also changes as well as additive depletion and oxidation. The engine oil is composed of long-chain saturated hydrocarbons (base oil) and additives used in motor engines. Their abundance in several polluted environmental areas had been reported Mohammed [2]. Spent engine oil is a common and toxic environmental contaminant not naturally found in the environment Dominguez-Rosado and Pichtel [3]. Large amount of them is liberated into the environment and disposed into the soil Achuba and Perehemo-Clarke [4], during engine use and due to engine leaks Osubor and Anoliefo [5]. The oil released from motor engines causes environmental concern and attracts public attention had been Roling [6]. The causes of engine oil pollution can be numerous, but the consequences are the same. Used motor oil contains aromatic hydrocarbons (PAHs) that could contribute to chronic health hazards. The accumulation of hydrocarbons, especially polycyclic aromatic hydrocarbons (PAH), in the environment is a major health concern. These compounds have been shown to exhibit toxic, mutagenic, and carcinogenic effects in man Lin and Mandri [7]. Traditional hydrocarbon waste management methods are often difficult and costly processes. In the past decade bioremediation has proven to be a relatively inexpensive and effective alternative to traditional waste management techniques. Bacteria are ubiquitous and incredible microorganisms on earth by having the ability to degrade the natural or synthetic materials. This activity is utilized to clean up the hydrocarbon polluted environment.

Microbial degradation is the major mechanism for the elimination of used petroleum products from the environment Bartha [8]. Soils contain very large number of hydrocarbons utilizing bacteria and fungi suggested Namkoong [9] capable of breaking down many complex molecules by their degradative enzyme system Boonchan [10]. The process of microbial degradation of hydrocarbons has four steps, i) emulsification of petroleum hydrocarbons by surfactants secreted by the microorganism, ii) adsorption of emulsified hydrocarbons to the cell surface membrane of the microorganism, iii) penetration of the adsorbed hydrocarbons through the cell membran iv) enzymatic degration of hydrocarbon to provide energy to the microorganism**.** Bioremediation is, therefore, the application of naturally occurring process by which microorganisms transform environmental contaminants into harmless end products Abdulsalam [11]. Therefore, the objective of this study is to determine the biodegradation ability of bacteria isolated from used engine oil contaminated soil.

2. MATERIALS AND METHODS

2.1 Sample Collection

Engine oil contaminated soil samples were collected over a period of one month from service stations and motor garages located at Kirathoor, Mankadu and Nithiravilai of Kanniyakumari District. The samples were collected from the sub surface soil layers at a depth from 5 to 30 cm. The soil is stored at 4ºC in pre-sterilized bags after the removal of surface litter by sieving through 2 mm sized sieve.

One gram of soil from each workshop was suspended in 1.0 ml of distilled water. After the soil particles setteled down the supernatant was inoculated on mineral salt agar medium enhanced with 1% engine oil. Another mineral salt agar media without 1% engine oil used as negative control. Heterotrophic bacteria present in the soil samples were enumerated by serial dilution (10^{-5}) and spread plating on Mineral salt agar medium. The mineral salt agar plates incubated for 24-48 h at 37^0 C for the growth of microorganisms. Microbial colonies which appeared on minimal agar plates were identified by the morphology. The morphological characteristics are studied based on size, shape, colors, and margin of colony. Morphologically different colonies were isolated on mineral salt medium (broth) (MSM) and mineral salt medium (agar) (MSM) with 1% oil and without oil as control.

2.1.1 Qualitative screening for lipolytic activity

The microbial colonies on minimal agar plates were subjected to qualitative screening for lipolytic (lipase/esterase - producing) activity using tributyrin agar (TBA), rhodamine olive oil agar (ROA) media at neutral pH 7.0. The experiment was repeated two time.

2.1.2 Tributyrin agar (TBA) plate assay

Tributyrin agar media plates were prepared according to the direction given by the manufacturer. Lipase/esterase producing microorganisms produced a zone of clearance (hydrolysis) when their appropriate dilutions were inoculated on the TBA media plates and incubated at 37°C.

2.1.3 Rhodamine olive oil agar plate assay

Rhodamine olive oil agar media plates were prepared according to the direction given by the manufacturer. The bacterial colonies were inoculated and incubated for 48 h at 37°C. Lipase-producing strains were identified on these spread plates by the formation of orange, fluorescent halos around the bacteria.

Both Rhodamine and Tributyrin agar plate assay length and width was measured using graph sheet and the area of halo region was calculated using the formula,

Area= L x W

L= Length;

W= Width;

2.2 Colony Forming Unit (CFU) and Oil Utilization Ability

Cell growth is measured by counting total cell number of the microbes present in the broth at 24 h duration by using a Petroff - Hausser counting chamber. All cells are counted in large square and total number per ml of sample is measured. The cell culture is 1:1 dilution using culture and buffer solution. Oil utilizing efficiency was calculated by calculating the percent increase or reduction in tota lcount numberof bacteria in the cultured medium using the formula,

Increase / decrease in CFU/ml (%) = ((Total CFU in control -Total CFU in experiment) / Total CFU Control) X 100

Negative value indicates the increased efficiency of utilization and vice versa.

2.3 Oil Spread or Oil Displacement Assay

The Oil spreading experiment was performed using the technique developed by Morikawa [12]. Modification to the method as follows, 0.1ml of oil is added to 0.1ml of distilled water taken in an embryo cup. 20 µl of cell free culture broth was added to the oil surface. The oil will be displaced with oil free clearing zone if biosurfactant is produced by the bacteria into the broth. The diameter of the clear zone formed is measured and is converted to percentage area displaced in the embryo cup using the formula.

$$\text{Area displaced (\%)} = \frac{\text{Area of clear zone in experiment}}{\text{Total area of embryo cup}} \text{ X } 100$$

The diameter of clear zone on the oil surface correlates to surfactant activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed, and Tween 20 was used as the positive control. The percent increase or decease in oil displacement activity of biosurfactant is calculated by using the formula.

$$\text{\% increase / decrease of displacement activity} = \frac{\text{Oil displacement in experiment}}{\text{Oil displacementincontrol}} \times 100 - 100$$

2.3.1 Statistical analysis

The results were compared using One–way Analysis of Variance to find the differences between the measurement means at 5% (0.05) significance level using Excel Software.

3. RESULTS AND DISCUSSION

Total of thirteen hydrocarbanoclastic bacteria were isolated from the engine oil contaminated workshops soil of Kirathoor, Mankadu and Nithiravilai. Of these thirteen species, five from Mankadu (M1 to M5) and Nithiravilai (N1 to N5) area and only three species are isolated from Kirathoor (K1 to K3) (Table 1). Through biochemical analysis, these bacterial sps were identified as *Acinetobacter* (K1, N4 & N5), *Pseudomonas* (K3 and M4) and *Proteus* (K2, M1, M2, M3, M5, N1, N2 and N3) (Table 1). Khouloud [13] explained that the number and type of bacterial species varying in contaminated sites. In the present study also different bacterial sps were observed which may be due to varying composition of engine oil or organic matter of the soil. Different oil degrading bacteria such as *Bacillus*, *Pseudomonas, Staphylococcus*, *Micrococcus* and *Proteus* species from hydrocarbon-polluted environments as discussed by Stephen [14]. Earlier reports supported the findings of present study.

Lipase assay was conducted to identify potent biosurfactant producer as in reported by Deepa [15] and Anuradha & Aruna [16]. Among the thirteen bacteria only seven are identified as lipase producing bacteria in Tributyrin agar (TBA) and Rhodamine olive oil Agar (ROA) medium (Fig.1). Seven bacteria (N1, N2, N3, M1, M2, M3 and M5) were considered as negative for lipoytic activity. Maximum clear zone was exhibited by *Pseudomonas sps.* (K3) and *Acinetobacter* sps (N4) (Plate 1).

Table 1. Morphology of bacteria obtained from oil contaminated sites of Kirathoor (K1, K2 & K3) Mankadu (M1 – M5) and Nithiravilai (N1 – N5)

PHYSICAL ANALYSIS								BIOCHEMICAL ANALYSIS								
COLONY	**SIZE**	**FORM**	**COLOUR**	**MARGIN**	**ELEVATION**	**SURFACE**	**SHININGNESS**	**MANNITOL**	**GLUCOSE**	**LACTOSE**	**SUCROSE**	**H_2S PRODUCTION**	**CITRATE**	**UREASE**	**OXIDASE**	
K1	Small clusters	Circular	Pale colonies	Entire	Flat	Smooth	Dull	-	-	+	-	-	+	-	-	*Acinetobacter*
K2	Small	Circular	Cream	Entire	Convex	Smooth	Shiny	-	-	+	+	+	+	+	-	*Proteus*
K3	Large	Oval	Light White	Irregular	Convex	Smooth	Dull	-	-	-	-	-	+	-	+	*Pseudomonas*
M1	Small	Circular	Cream	Entire	Convex	Smooth	Shiny	-	-	-	-	-	+	-	+	*Proteus*
M2	Small	Irregular	Half white	Undulate	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
M3	Small	Circular	Orange	Smooth	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
M4	Large	Oval	White	Irregular	Convex	Smooth	Dull			+	+	+	+	+		*Pseudomonas*
M5	Small	Rhizoid	Half white	Filamentous	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
N1	Small	Circular	White	Smooth	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
N2	Large	Irregular	Light White	Lobated	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
N3	Small	Circular	Orange	Smooth	Flat	Smooth	Dull	-	-	-	-	-	+	-	+	*Proteus*
N4	Small	Circular	Pale Colonies	Entire	Flat	Smooth	Dull	-	-	+	-	-	+	-	-	*Acinetobacter*,
N5	Small	Circular	White	Entire	Flat	Smooth	Dull	-	-	+	-	-	+	-	-	*Acinetobacte*,

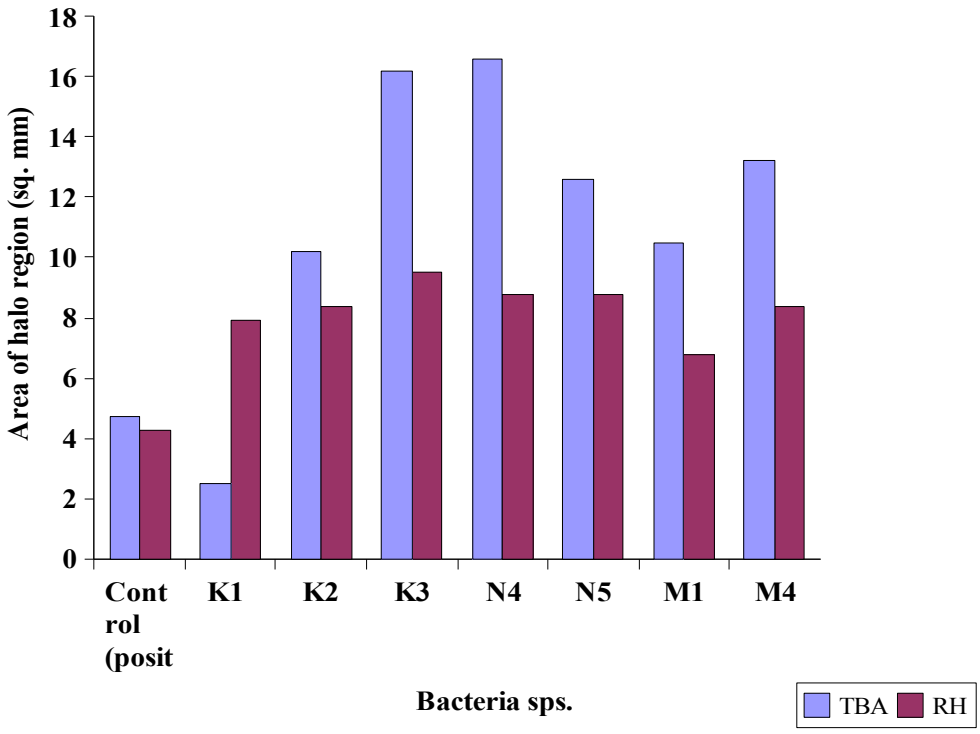

Fig. 1. Lipolytic activity of selected bacteria of kirathoor, mankadu and nithiravilai workshop soil
RH- Rhodamine oil agar assay; TBA- Tributyrin agar assay

Venosa [17] reported that microorganisms with the appropriate metabolic capabilities when exposed to hydrocarbons become adapted and multiply within hours to exhibit higher rates of hydrocarbon biodegradation. *Acinetobacter sps.* and *Pseudomonas sps.* of Kirathoor, Mankadu and Nithiravilai area exhibits maximum number of Colony forming units (CFU) at 2% and 4% oil supplementation respectively (Fig. 2A; 2B and 2 C). Compared to control, increased number of CFU/ml was observed in all the experimental categories. Of all the three species the *Acinetobacter sps* of Kirathoor produces highly significant number of CFU /ml (df = (1, 8); F (5.32) = 225.04; $P < (.05)$ followed by *Pseudomonas* (df = (1,8); F (5.32) =12.91; $P < (.05)$. The number of CFU/ ml was significantly high in 3% (df = (1, 24); F (4, 26) = 26.4; $P < (.05)$ and 4% (df = (1, 24); F (4.26) = 15.6; $P < (.05)$ supplementation of oil at 96 hrs of exposure. Higher number of bacteria in oil enriched medium might be the result of utilization of hydrocarbon pollutants as energy source which is the necessary character for biodegradation activities.

Maximum of oil utilization efficiency was observed in *Acinetobacte*r and *Pseudomonas* species at 2 % and 4% oil supplementation respectively (Fig. 3). The increased number of CFU/ml correlates the oil utilization efficiency. Compared to control the *Pseudomonas* (df = (1,8); F (12.91) = 5.32; $P<(.05)$ of Kirathoor and *Acinetobactor* (df = (1,8); F (10.83) =5.32; $P=$ (.05) of Nithiravilai exhibit highly significant oil utilization efficiency. The oil utilization ability was increased from 24 to 96 hours of exposure (Fig. 3). The reason for the low percentage of oil degradation during initial days might be attributed to the toxicity of oil to microbial flora of the soil; high concentration of oil might likely to have negative effects on the biodegradative activities of the microbial population in the contaminated soil. This initial trend of low biodegradation in high oil concentration has also been reported in Ijah and Antai [18] article. Adenipekun and Isikhuemhen [19] and Shukor [20] reported that removal of hydrocarbons increases as the days of incubation increases. Adenipekun [21] reported hydrocarbon decreases after two months from fresh contaminated soil samples.

All seven isolates were screened for oil displacement test for biosurfactant production, maximum of 2.25 mm^2 clear zone area was observed in *Acinetobacter* sps. followed by *Pseudomonas* sps and *Proteus* sps. The result of oil displacement assay against positive control was given in Table 2. This technique was used in the studies of Bola [22] to show the ability of bacteria for utilizing crude oil.

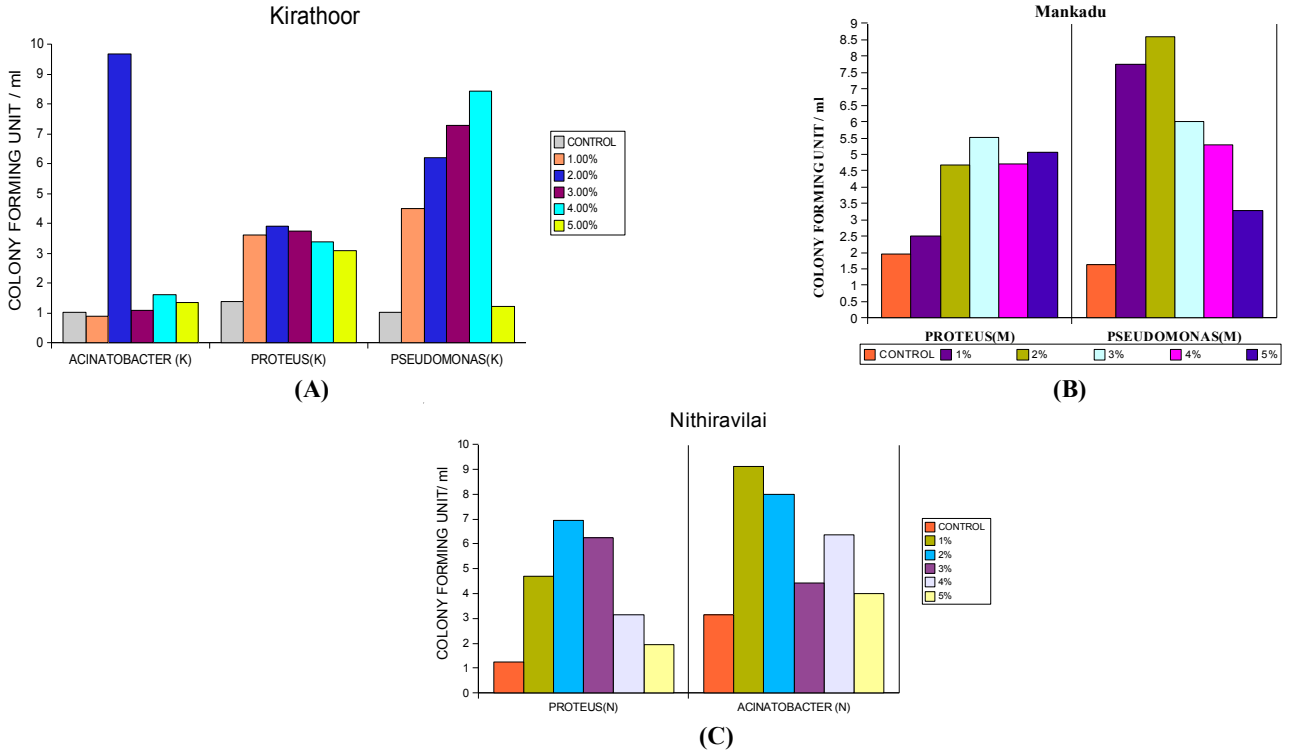

Fig. 2. Number of CFU/ml of bacteria obtained from Kirathoor (K1, K2 & K3) Mankadu (M1, M2, M3, M4 and M5) and Nithiravilai (N1, N2, N3, N4 &N5) workshop soil contaminated with oil

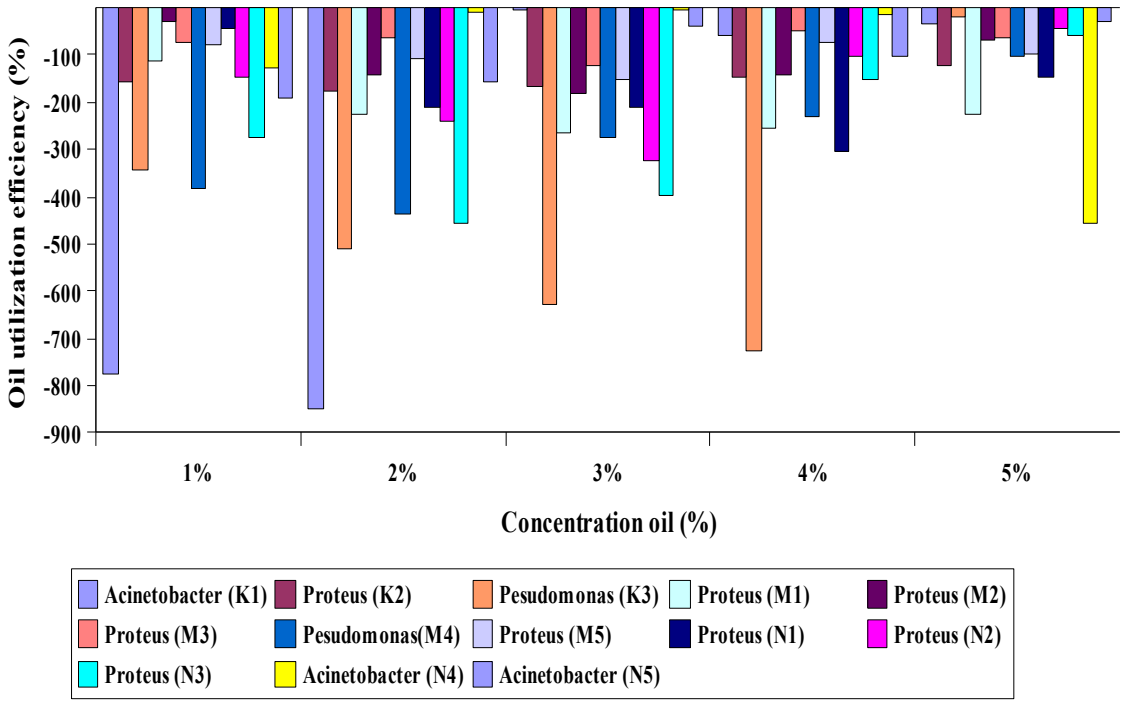

Fig. 3. Oil utilizing ability of hydrocarbanoclastic bacteria in a medium supplemented with different concentration of engine oil

Table 2. Clear zone area (mm^2) of control $^+$, control and experimental categories

Treatment/Microbe	7th day	14th day	21st day	30th day	60th day
Control	11	11	11	11	11
Control$^+$	1.21	1.23	1.21	1.23	1.23
Acinetobacter (K1)	1.4	1.98	2.02	2.22	2.25
Proteus(K2)	1.37	1.21	1.23	1.26	2.02
Pseudomonas (K3)	1.24	1.25	1.33	2.17	2.21
Proteus (M1)	1.17	2.08	2.12	2.17	2.21
Pseudomonas (M4)	1.31	1.35	1.46	1.48	2.10
Acinetobacter (N4)	1.3	2.15	2.17	2.19	2.23
Acinetobacter (N5)	1.08	2.06	2.08	2.10	2.17

11 mm^2 – the size of cavity of the embryo cup

Various researchers like Vijaya Kumar [23] and Sunkar [24] reported that the oil spreading test is used as a standard screening test for biosurfactant. As Youssef [25] mentioned in his work the oil displacement area in the oil spreading assay was directly proportional to the biosurfactant concentration. The clear zone size was increased as the time of incubation was increased from 24 to 48hrs. Niraj Prasad [26] reported that oil displacement technique was more suitable for detecting low levels of biosurfactant production. The isolated bacteria degrade crude oil because they utilize engine oil as their energy source discussed by Akhavanet [27]. In this study by using the same oil spreading test as a standard screening test for biosurfactant allowed viable results to be gathered from the various regions. Percent increase / decrease of oil displacement was shown in Fig. 4. Negative values show high oil displacement. Very high negative deviation from control was observed in *Proteus* sps. of Mankad, *Pseudomonas* sps. of Kirathoor and *Acienetobacter* sps. of Nithiravilai shows positive deviation (Fig. 4). Significantly very high displacement was exhibited by *Proteus sps.* of Mankad [M1- df = (1,4); F (7.7)= 26.9; $P < (.05)$ & M5 (df = (1,4); F (7.7)= 58.4; $P < (.05)$] and *Acinetobacter* sps. of Nithiravilai (df = (1, 4); F (7.7) = 26.1; $P< (.05)$. Oil displacement activity was significantly high upto 21 days (df = 1, 7); F (3.73) = 3.78; $P = (.05)$ after that it was reduced (Plate1).

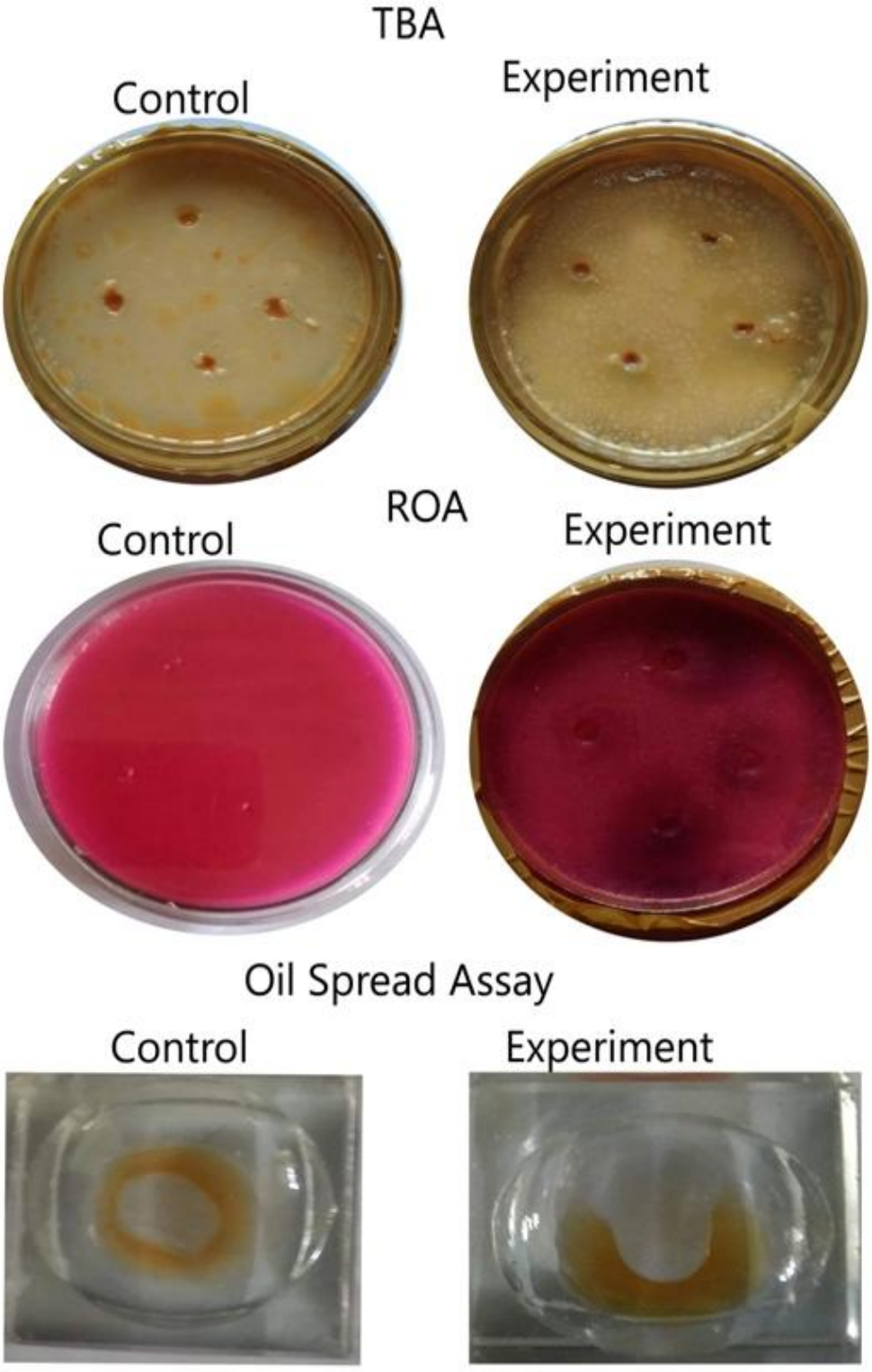

Plate 1. Lipase activity assay in tributyrin assay (TBA) and rhodamine oil assay (ROA) with clear zone in experimental categories and oil spread assay

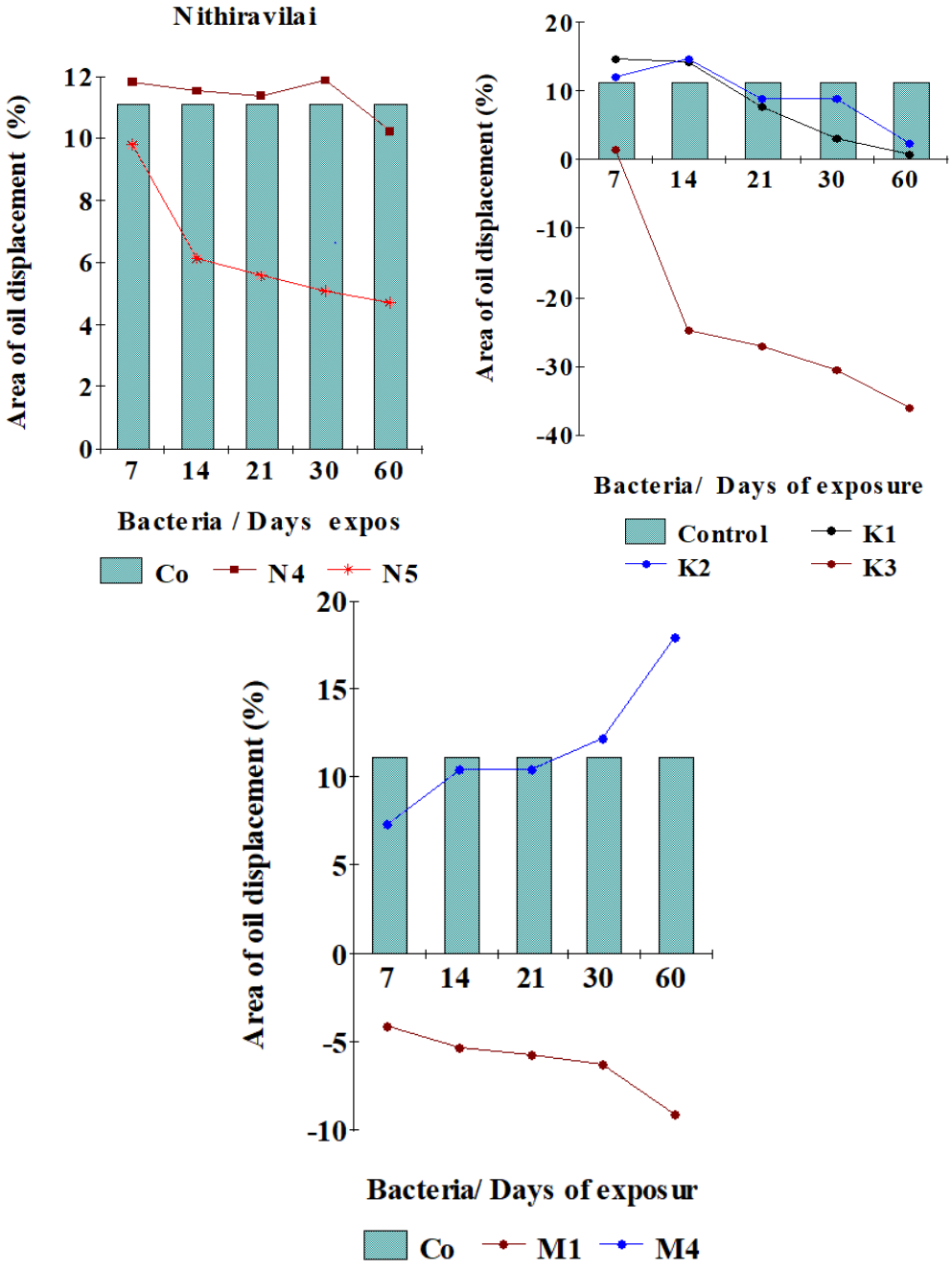

Fig. 4. Percent oil displacements by selected bacterial strains of workshop soil
N4, N5&K1 – *Acnetobacter;* K2 &M1 –*Proteus;* M4 &K3 – *Pseudomonas*

4. CONCLUSION

The above mentioned studies emphasize the availability of hydrocarbanoclastic bacteria in the workshop soil. Variation in number and bacterial sps were observed. The bacteria isolated were proved to have the ability of hydrocarbon degradation by oil utilization and oil displacement assay. The bacteria isolated were multiply within hours in an engine oil enriched medium indicates its utilization of hydrocarbon as its energy source necessary for hydrocarbon degradation. Oil spreading test is used as a standard screening test for biosurfactant, in this study oil spreading test confirms that and *Acinetobacter sps., Pseudomonas sps* and *Proteus sps.* secreted biosurfactant. The oil degradation efficiency

is very low at initial period and is increasing with the increasing days of exposure. *Proteus* sps., *Pseudomonas sps.,* and *Acinetobacter sps.* are exhibiting high degradation efficiency from 2 to 4% of oil contamination.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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