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Biodegradation of Kerosene by Bacterial Isolates

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Abstract: **In the present study, an attempt was made to examine the ability of bacterial isolates to degrade kerosene. Oil-contaminated soil samples from various locations at Kottayam were used for isolating oil degrading microorganisms. Production of biosurfactants and increased emulsification index by the isolates enhanced their oil degrading ability. The biosurfactant producing ability of the microorganisms was determined by hemolytic assay, drop collapse test and methylene blue agar plate method. The isolated organisms which showed increased biosurfactant production and emulsification index were identified as *Pseudomonas* sp., *Serratia* sp. and *Bacillus* sp. based on their biochemical characteristics. *Pseudomonas* sp. reduced kerosene from 25ml to11.8ml, *Bacillus* sp. From 25ml to 12ml and *Serratia* sp. from 25ml to 13.2ml within a period of 15days. The emulsifying bacteria were able to transform kerosene into an emulsion. These bacterial isolates can be used to remove oil from oil-polluted areas.**

Keywords: **Kerosene, biodegradation, bio surfactant, emulsification, hydrocarbon, contamination.**

1. INTRODUCTION

Petroleum-based products are primarily obtained from natural crude oil with a complex mixture of organic molecules, hydrocarbons with varied chemical and physical properties (Kvenvolden & Cooper, 2003). The major constituents of kerosene are paraffin (alkanes) and cycloparaffins (cycloalkenes) (68.6%), benzene (13.7%) and naphthalenes (17.7%) (Irwin *et al*., 1997). Hydrocarbon or oil biodegradation process by microbial flora employs the enzymatic capabilities of microorganisms (Atlas, 1981). Two main approaches to oil bioremediation or biodegradation are Bioaugmentation and Biostimulation.

One of the most important characteristics of hydrocarbon-degrading-bacteria is the ability to emulsify hydrocarbons by surface active agents such as biosurfactants (Hommel, 1990; Neu, 1996; Desai and Banat, 1997; Bredholt *et al*., 1998). Biosurfactants are directly involved in the process of hydrocarbon removal from the environment through increased biodegradation by direct cell contact (Hommel, 1990; Leahy and Colwell, 1990; Deleu *et al*., 1999; Banat *et al*., 2000). Addition of biosurfactants increase the availability of long chain hydrocarbons to microbes and renders them more accessible to microbial enzyme system for utilization (Banat *et al*., 2000; Hommel, 1990).

Kerosene has many toxic effects on plant, animal and humans. The present study was undertaken with the objectives of isolation and identification of oil degrading bacteria from oil contaminated soil, degradation potential of the isolates, and characterization of the biosurfactant production during degradation and to study the rate and extent of active microbial degradation of the hydrocarbon waste.

2. MATERIALS AND METHODS

Isolation of microorganisms:

Oil contaminated soil samples near workshops and petrol pumps were collected from four different locations at Kottayam and were enriched with sterile mineral salt medium (MSM) containing kerosene and incubated at 37°C for 48 hours. After

incubation period, the samples were serially diluted and plated using nutrient agar. After incubation, bacterial isolates were selected based on their colony morphology and were subjected to further studies.

Screening for biosurfactant production

Hemolytic activity:

Pure bacterial isolates were streaked on freshly prepared blood agar and incubated at 37°C for 48-72 hours. Results were recorded based on the type of clear zone observed such as, α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear zone and γ-hemolysis when there was no change in the medium surrounding the colony (Carrillo *et al*., 1996).

Drop collapsing test:

Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Bodour and Maier (2004). 2µl of kerosene was applied to the wells of micro plates and left to equilibrate for 24 h. 5µl of the 48 hours culture, before and after centrifugation at 12,000 rpm for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production (Youssef *et al*., 2004)

Blue agar plate (Bap) method:

Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant (Satpute *et al*., 2008). 30µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer. The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the isolate was considered positive for anionic biosurfactant production.

Emulsification test (E24):

2 ml of pure cultures in mineral salt medium was mixed with 2 ml of hydrocarbon, vortexed at high speed for 1 min and allowed to stand for 24 h (Bodour *et al*., 2004). The emulsion index was calculated using the formula,

$$\text{Emulsification index (E24)} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Determination of biosurfactant:

The biosurfactant activity was estimated using orcinol assay method (Tuelva *et al*., 2002). The orcinol assay was used for the direct assessment of the amount of glycolipids in the sample. To 100 µl of each sample, 900 µl of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80ºC, the samples were cooled to room temperature and the OD at 421 nm was measured. Control was prepared with distilled water. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mgmL-1).

Purification of biosurfactant:

The biosurfactant was extracted from culture medium after cell removal by centrifugation at 12,500 rpm for 30 min. The pH of the supernatant was adjusted to 2.0 mL with 6 M HCl, and an equal volume of ethyl acetate was added in a separation funnel. The mixture was vigorously shaken for several minutes and allowed to set until phase separation. The organic phase was collected by repeating the above procedure 2 to 3 times using anhydrous sodium sulphate. The water was removed and concentrated using a rotary evaporation. The resulting product was considered as the crude biosurfactant.

Thin layer chromatography:

Preliminary characterization of the biosurfactant was done by TLC method. A portion of crude biosurfactant was separated on silica gel plate using $CHCl_3$:CH_3OH:H_2O (70:10:0.5, V/V/V) as developing solvent system with different

colour developing reagent. Ninhydrin reagent was used to detect lipopeptide biosurfactant as red spot and anthrone reagent was used to detect glycolipid biosurfactant as yellow spot.

Identification of the isolates:

The selected isolates were identified based on their morphological, microscopic and biochemical characteristics.

Degradation of kerosene:

25ml of kerosene was added to 75ml of nutrient broth and pH was maintained at 7. After sterilization, it was inoculated with 1ml of 24 hours old culture. The inoculated flasks were incubated at 37^0C for 15 days. The degradation was quantified by measuring the amount of oil added. Degradation rate was also determined by the OD value at 600nm.

TLC after degradation of kerosene:

The components of degraded kerosene were detected by TLC method. Kerosene after degradation was compared with nondegraded kerosene by separating the components on silica gel plate in benzene:ethyl acetate:methanol (85:13.1.5,V/V/V) as developing solvent system.

3. RESULTS AND DISCUSSION

Eight morphologically different isolates were isolated from different samples and the sample near workshop at Nagampadam showed more bacterial population (Fig.1). Initially the isolates were named as S1, S2, S3, S4, S5, S6, S7 and S8 and except S7, all others are able to produce haemolysis on blood agar (Fig.2 and table1). The isolates that are able to produce β-hemolysis were scored as potential producers of biosurfactant as described by Bicca *et al*. (1999).

The flat drop in the microtitre plate considered as positive biosufactant production. The isolates *Pseudomonas* sp *1*, *Bacillus* sp *1*, *Serratia* sp., *Bacillus* sp *2*., and *Pseudomonas* sp *2* showed flat drops in microtiter well which was considered as positive biosurfactant production (Fig.3 and table 2). The flat drop appearance suggested by Jain *et al*. (1991) proving the use of drop collapse method as a sensitive and` easy method to test for biosurfactant production.

The blue agar plate was performed for detecting anionic biosurfactant. The dark blue halo zone around the well was considered as positive result for anionic biosurfacant. The isolates produced dark blue halo zone around the well which produce anionic biosurfactant (Fig.4 and table 2). Blue agar plate method was developed for the detection of anionic biosurfactant like extracellular rhamnolipids and glycolipids (Siegmund and Wagner, 1991). Extracellular anionic biosurfactants developed an insoluble ion pair with the cationic tenside cetyltrimethyl ammonium bromide (CTAB) and the basic dye methylene blue and productive bacterial colonies were surrounded by dark blue halos on the light blue agar plates.

All of the eight isolates have the ability to emulsify oils (Fig. 5 and 6). The emulsification produced by *Pseudomonas sp*, *Bacillus sp* and *Serratia sp* enhances the biodegradation of hydrocarbons by increasing their bioavailability to the microorganisms involved in the process. The efficiency of the biosurfactants to make the oil available to the bacterial cells was studied by measuring the emulsification activity (E24 index) of the biosurfactants as described by Bodour *et al.* (2004).

Biosurfactant from the isolates were separated and concentrated using hot air oven. The resulting product considered as biosurfactant. The isolates *Pseudomonas sp*, *Bacillus sp* , *Serratia sp* produce biosurfactant (Fig. 7 and 8).

Orcinol assay method was used for detection of amount of glycolipid and rhamnolipid in the sample. The isolates of *Pseudomonas* sp and *Bacillus* sp showed high rhamnolipid and glycolipid concentration. Lipopeptide was produced by *Bacillus* sp showed by red spots in TLC plates (Fig.9 and 10).

Pseudomonas sp 1 and *Bacillus* sp 2 showed highest degradation capacity when compared with *Serratia* sp (Fig. 11, Table 2 and 3). These organisms utilize oil components as a source of energy for their metabolism and growth. Screening of purified culture for degradation of oil was described by Jahir Alam Khan and Hasan Abbas Rivzi (2011). After the degradation study, the growth rate of the isolates was also determined. All of the 5 isolates showed high growth rate.

In degradation study TLC was used for find out the components separated from kerosene due to degradation. Kerosene inoculated with *Pseudomonas* sp 2 showed two bands (Blue and Red colours) (Fig.12), whereas others showed only blue

colour band. This was assumed that the blue colour was the dye added to the kerosene and the other band may be the separated degraded compound.

4. CONCLUSION

Bacteria are considered to represent the predominant agency of hydrocarbon degradation in the environment and hydrocarbon degrading bacteria are ubiquitous. Though quantum of literature on oil degradation is high, the study on kerosene degradation is very limited.

In conclusion, *Pseudomonas sp*, *Bacillus sp* and *Serratia sp* isolated from oil contaminated soil showed biosurfactant producing ability. The isolates used kerosene as carbon source for their growth and produce biosurfactant. In this study, *Pseudomonas sp* and *Bacillus sp* species showed high degrading capacity than *Serratia sp*.

By adding these selected bacterial isolates into oil contaminated areas we can disperse the oil as small oil droplets that can be further degraded by indigenous bacteria in the environment. In particular, such bacteria may be used to remove crude oil from polluted areas.

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APPENDIX - A

FIGURES TABLES AND PHOTOS:

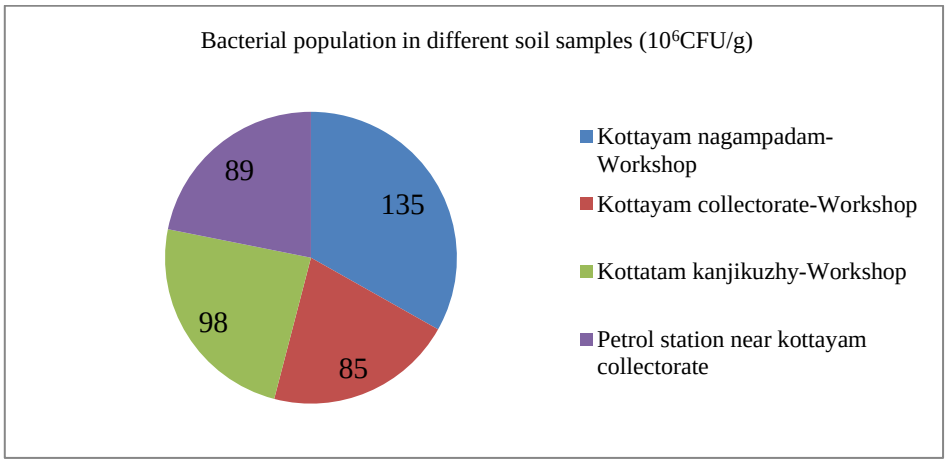

Fig 1: Bacterial population

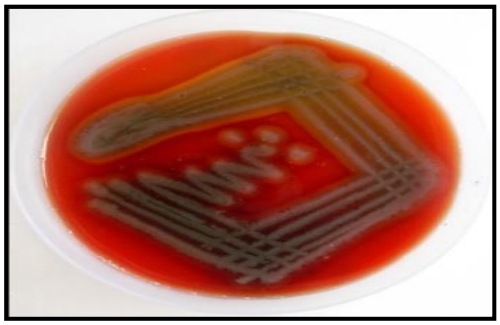

Fig 2: Haemolysis test

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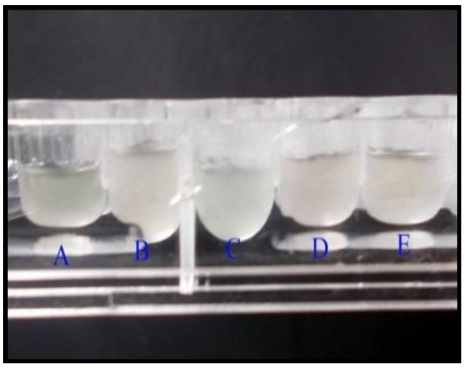


A- *Pseudomonas sp.*
B- *Bacillus sp.*
C- *Serratia sp.*
D- *Bacillus sp.*
E- *Pseudomonas sp.*

Fig 3: Drop collapsing test

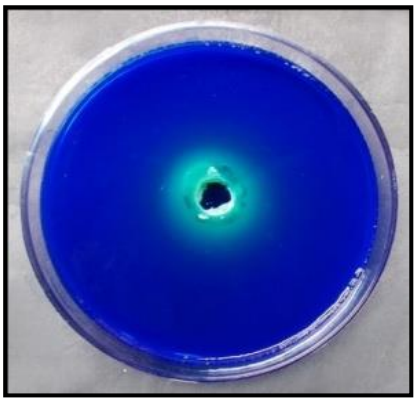
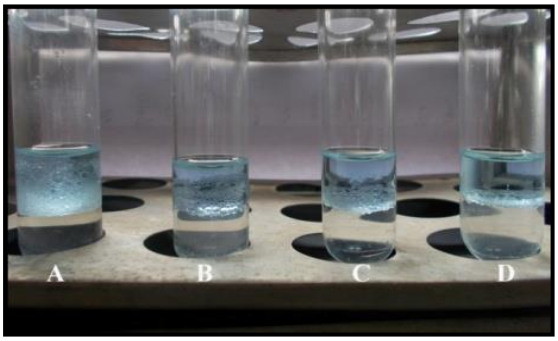


Fig. 4: Blue agar plate method



A- *Pseudomonas sp.*
B- *Bacillus sp.*
C- *Serratia sp.*
D- *Pseudomonas sp.*

Fig. 5: Emulsification test

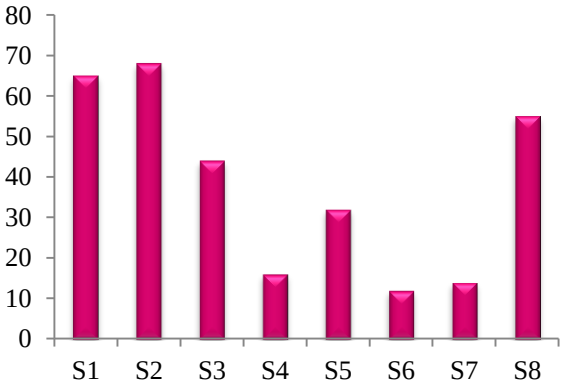


Fig. 6: Emulsification index

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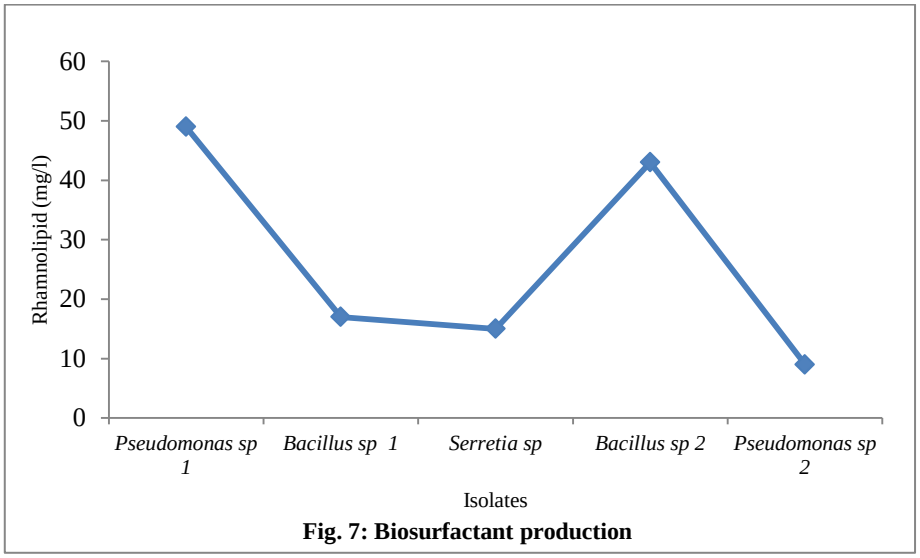

Fig. 7: Biosurfactant production

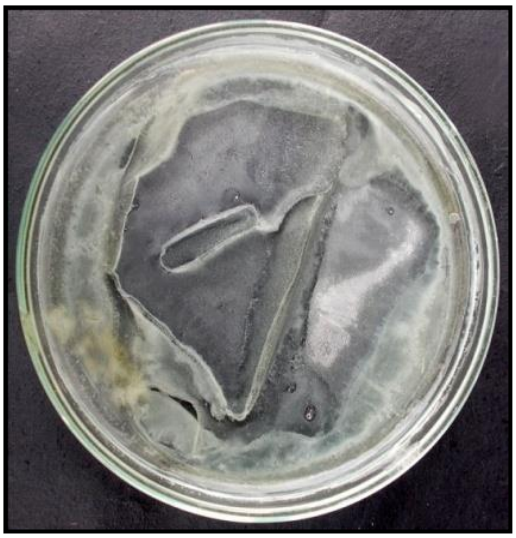

Fig. 8: Biosurfactant

Detection of Biosurfactant by TLC method:

Fig.9: Glycolipid produced by Bacillus sp

Fig. 10: Lipopeptide produced by Pseudomonas sp

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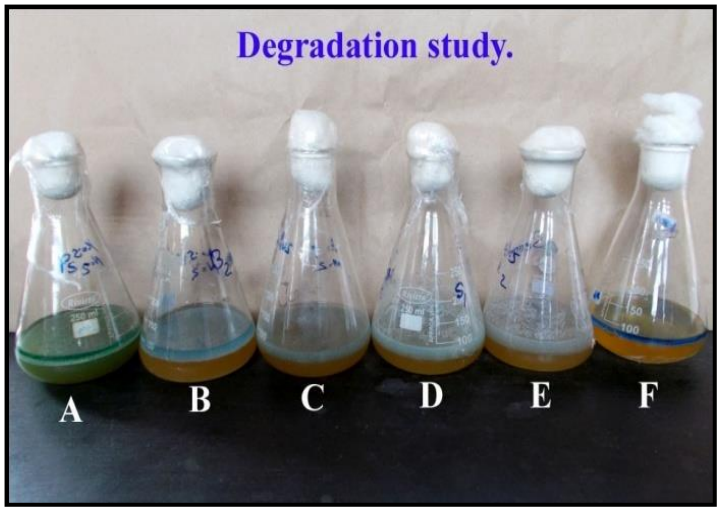

A- *Pseudomonas sp.*
B- *Bacillus sp.*
C- *Bacillus sp.*
D- *Pseudomonas sp.*
E- *Serratia sp.*
F- *Control.*

Fig. 11: Kerosene degradation by different isolates

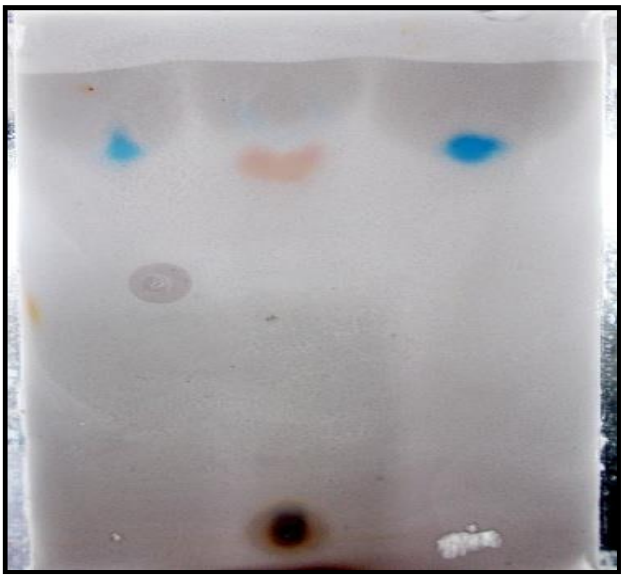

Fig. 12: Detection of components of degraded Kerosene by TLC.

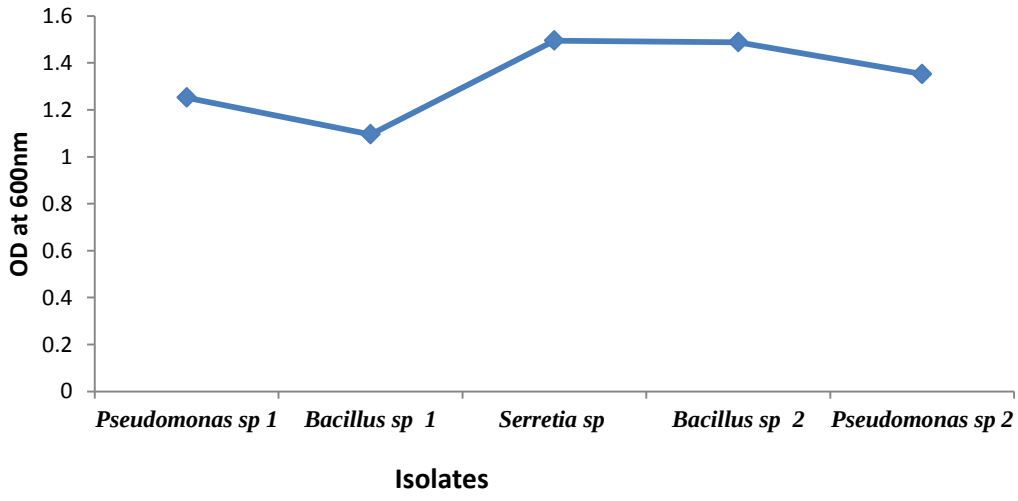

Fig. 13: Growth rate of the isolates after degradation

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Table 1: Screening for biosurfactant production

Test isolates	Hemolytic assay	Drop collapsing test	Blue agar plate method
S1	+	+	+
S2	+	+	+
S3	+	+	+
S4	+	-	-
S5	+	+	+
S6	+	-	-
S7	-	-	-
S8	+	+	+

Table no.2: Oil degradation studies (width)

No	Isolates	Width of oil on zero day	Width of emulsification on 1-3 weeks		
			1 week	2week	**3week**
1	*Pseudomonas* sp 1	0.5	0.5	0.7	**1**
2	*Bacillus* sp 1	0.5	0.5	0.8	**1**
3	*Serratia* sp	0.5	0.5	0.6	**1**
4	*Bacillus* sp 2	0.5	0.5	0.7	**0.9**
5	*Pseudomonas* sp 2	**0.5**	**0.5**	**0.5**	0.7

Table no.3: Oil degradation study (oil recovery)

No	Isolates	Volume of kerosene on zero day(ml)	Volume of kerosene on 3rd week(ml)
1	*Pseudomonas sp* 1	25	**11.8**
2	*Bacillus sp* 1	25	**12.9**
3	*Serratia sp*	25	**13.2**
4	*Bacillus sp* 2	25	**12**
5	*Pseudomonas sp* 2	25	**14.9**