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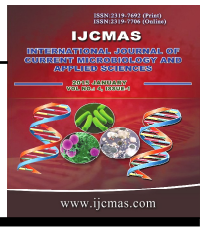


Original Research Article

Biodegradation of petroleum and crude oil by *Pseudomonas putida* and *Bacillus cereus*

C.Vinothini[1]*, S.Sudhakar[2] and R.Ravikumar[3]

[1]Department of Microbiology, Shrimathi Indira Gandhi College, Tiruchirappalli, India
[2]Department of Botany, A.A. Government Arts College, Attur, Salem, India
[3]P.G and Research Department of Botany, Jamal Mohamed College, Tiruchirappalli, India
**Corresponding author*

Keywords

Crude oil, Degrading, Germination

A B S T R A C T

Microbial degradation of petroleum hydrocarbons is one of the major practices in natural decontamination process. In the present study crude oil degradation were analyzed using isolated bacterial strains. Bacterial strains were isolated from the crude oil contaminated soil sample. The selected two bacterial strains were named as DB1 and DB2. These organisms were identified based on the cultural, morphology and biochemical characteristics and results of DB1 (*Pseudomonas putida*) and DB2 (*Bacillus cereus)*. The crude oil degradation of isolated bacterial strains was analyzed based on the growth of crude oil containing medium. The growth ability was measured at 1st and 7th days after inoculation. The crude oil degrading ability of isolated strains was analyzed. The effect of crude oil on the growth of *Vignamungo* by pot culture experiments were analyzed the germinating and growth ability in different experimental groups showed that *Pseudomonas putida* have more efficient than *Bacillus cereus.*

Introduction

Environmental impacts are mainly attributed to the petroleum industry (Pala and Freire, 2002). Petroleum contaminated soil causes organic pollution of local ground water (Wang and Fingas, 2008). Individual microorganisms are capable of degrading only a limited number of crude oil depends on the presence of metabolically diverse microbial communities (Atlas, 1981). Determination of fate of hydrocarbon degradation disappearances of individual hydrocarbons and or total hydrocarbon (Okora, 2008). The success of bioremediation technologies applied to hydrocarbon-polluted environments highly depends on the biodegrading capabilities of native microbial populations or exogenous microorganisms used as inoculants (Venosa and Zhu, 2003). Oil biodegradation of subsurface does not require oxygen, it does require certain essential nutrients (e.g., nitrogen, phosphorus, potassium), which can be provided by dissolution of minerals in the water lake. Hydrocarbon biodegradation can occur over a wide range of temperature,

the rate of bio degradation generally decrease with decreasing temperature. Highest degradation rates generally occur in the range of 30 to 40^{o}C in soil environments, 20 to 30^{o}C in some freshwater environments, and 15 to 20^{o}C in marine environments. The effect of temperature is also complicated by other factor such as the composition of the microbial population (Zhu *et al.,* 2001).

Bioremediation have been investigated as alternative tools for residues oil clean up (Sergy *et al.,* 2003). The rate of biodegradation depends on both physicochemical and biological variables (Churchill *et al.,* 1995).

Material and Methods

Sample collection

Soil samples were collected randomly from crude oil and petroleum contaminated polluted soil from an automobile workshop in Thajavur, Tamil Nadu,

Isolation and identification of microbes from soil samples

The microbial strains were isolated from the collected sample by serial dilution technique. Selected colonies identified by using morphological, cultural and biochemical characteristics (Aneja, 2002) Gram's staining (Bailey and Scott, 1966) motility test Biochemical confirmation test Bergey's manual of systemic bacteriology classification.

Screening of crude oil degradation

Crude oil degradation ability was screened based on the growth efficiency on 2 % crude oil**.** The bacterial strains such as (DP1) and (DP1) were used for crude oil degradation.

Growth efficiency in crude oil as carbon source in liquid medium

Inoculum flask containing 50 ml for two selected bacterial strains were prepared were inoculated sterile nutrient broth with 2% crude oil concentration. Control containing 50 ml of medium with 2ml crude oil. Cell turbidity was measured by 540 nm using colorimeter (Kumar *et al.,* 2006; Lin *et al.,* 2005; Ziad *et al.,* 2005).

Assay of protein in bacterial biomass

Protein content of the cell biomass in the medium determined by Folin's -Lowry's method (Lowry *et al.,* 1951)

Crude oil extraction from control flask

Flask containing 50ml medium with 2% crude oil was acidified with HCL. 10 ml hexane was added and flask was placed on shaker at 120 rpm for 20 min. This solution was then transferred in separating funnel, mixed well and then the aqueous the hexane phases were allowed to separate. The lower layer of H_2O was drained and the extraction was repeated with 10 ml of solvent. Temperature at 72°C

Crude oil extraction from test flask

Cells and crude oil was centrifuged at a speed of 5000 rpm for 35 min such that the biomass settled at the bottom and the supernatant aqueous phase containing bulk of the oil separated biomass pellet was extracted by adding 2 ml of hexane, The procedure were repeated twice and reduction amount of crude oil.

Standardizing time required for evaporation of Hexane

Take two beakers. In the first beaker, 20ml

of pure hexane second beaker 20ml 0f hexane along with 1ml crude oil was taken and kept in an oven at 72˚C.The weight of both beakers was measured overtime until there be no further in weight. At 72˚C, 20 ml of hexane is evaporated in 126 min, Crude oil degradation percentage calculate by formula

$$\%\text{ of crude oil degradation} = \frac{\text{Initial conc. of crude oil - Final conc. of crude oil}}{\text{Initial conc. of crude oil}} \times 100$$

Assay Well Diffusion method (Saddoun, 2002)

Growth response of the selected bacterial strains was wells punched in the medium. The control wells were filled with hexane and incubated at 37°C. The growth response was monitored by measuring the diameter of zone of exhibition around the wholes (Saddoun, 2002; determined by solid media with 2ml test culture (and assayed by adding 50 µl of sterile crude oil (1-6% prepared in hexane) in 6 mm diameter Ziad *et al.*, 2005).

Extraction of Biosurfactants

The biosurfactant production of *Bacillus species* and *Pseudomonas putida*were estimated by Itoh and Suzuki (1972) method. Surfactants were extracted following Itoh and Suzuki (1972).The cells were first separated from supernatant by centrifugation at 6,800 x g.

The supernatant was precipitate by centrifugation (12,100x g) and the extracted three times with a chloroform ethanol (2:1) mixture, which was then, evaporated away leaving behind relatively pure rhamnolipids having an oil-like appearance (Zhang and Miller, 1992).

Analysis of Morphometric parameters

Pot Culture Experiment

Effects of crude oil on the growth of plant were analyzed by pot culture experiment. The following treatment was made for this study. 1. Control – (sterile soil + seeds). 2. Effect of crude oil– (sterile soil + crude oil + seeds). 3. Degradation ability of microbes - (sterile soil+ crude oil + microbes + Seeds). 4. Effect of microbes – (sterile soil + microbes + seeds).

Percentage of Germination

The percentage of seed germination was calculated from the each treated pot after one week from the sowing.

Root and Shoot Length

Plants were collected from each treated pot after 10 day from the seed sowing. The length of the root and shoot was measured individually for plant and expressed in cm. The formula for calculating mean is $\bar{X} = \sum x/N$ where $\sum x$- Sum of variable N – Total number of frequency in mean and standard deviation was calculated by using formula, Mean - $SEM = \frac{\sigma}{\sqrt{N}}$,

Standard deviation- $\sigma = \sqrt{\sum(X - \bar{X})^2/n - 1}$

Results and Discussion

Isolation of bacteria

Bacterial strains were isolated from the crude oil contaminated soil sample using Nutrient agar medium respectively. Among this study 55 bacterial colonies were noted in 10^{-6} and 10^{5} dilution (Table 1).

Identification of bacterial strains

The selected two bacterial strains were named as DB1 and DB2. These organisms were identified based on the cultural, morphology and biochemical characteristics (Table 2). And results of DB1 and DB2 were compared with Bergey's manual of systemic bacteriology classification.

Screening of crude oil degradation

The crude oil degradation of isolated bacterial strains was analyzed based on the growth of crude oil containing medium among the study, all the test organisms' growth in the respectively medium. At the same time highest growth observed *Pseudomonas putida*. The low growth observed *Bacillus cereus* (Table 3).

Determination of microbial resistance to crude oil

The selected two bacterial strains crude oil resistance were analyzed based on the growth ability on the crude oil contain medium. The growth ability was measure at 1^{st} and 7^{th} days of after inoculation. Among the two bacterial strains *Pseudomonas putida* (0.55/7^{th} day) have highest growth compared than *Bacillus cereus* (0.20/7^{th} day). The results were showed in Table 4 and Figure 1.

Assay of protein in bacteria

The total proteins were estimated in bacterial strains biomass. This study highest total protein was recorded in *Pseudomonas putida* (5.50 mg/g) compared than *Bacillus cereus* (2.3 mg/g). The results were showed in Table 5 and Figure 2.

Assay of crude oil degradation in liquid medium

The crude oil degradation capability of both bacterial strains was analyzed by liquid medium. Among this study highest crude oil degradation were noted in *Pseudomonas putida* (55 %) compared than *Bacillus cereus* (40 %). The results were showed in Table 6, Figure 3.

Assay of crude oil degradation by plate method

The crude oil degradation ability of bacterial strains was analyzed by plate assay method. Among this study highest zone of exhibition was noted in *Pseudomonas putida* (8mm in diameter) compared than *Bacillus cereus* (6mm in diameter) (Shrivastava and Gupta, 2008). The results were presented in Table 7, Figure 4.

Assay of biosurfactants

The bio surfactant production of *Pseudomonas putida* and *Bacillus cereus* were estimated by Itoh and Suzuki (1972) method. The investigated results were presented in (Table 8) and Figure 5. The maximum level of biosurfactants was noted in *Pseudomonas putida* than *Bacillus cereus.*

Analysis of germination and growth ability

The crude oil degrading ability of isolate and the effect of crude oil on the growth of *Vigna mungo* also analyzed by pot culture experiments. The germinating and growth ability were analysed in different experimental groups using *Pseudomonas putida* and *Bacillus cereus.* The results were showed in Table 9. *Pseudomonas putida* (75%) groups have maximum germinating and growth ability noted compared to *Bacillus species* (62%) inoculated groups.

In this study the degradation of crude oil were analyzed using two bacterial isolates.

The crude oil degradation also analyzed by pot culture experiments. The investigated results were discussed with previous theoretical and biostatistical values.

From this studies crude oil contaminated soil were collected and isolate two different bacterial colonies were isolated which were identified using cultural characteristics, cell morphology and biochemical characteristics. The isolates were confirmed as *Bacillus species* and *Pseudomonas species*. Bhadauria (1997) also collected agricultural soil irrigated with petroleum refinery effluent, then 15 species of bacteria have been identified where the bacterial count range between 66 and 860×10^6. In this crude oil degradation screening study two bacterial isolates were selected based on the ability of utilization of crude oil as an energy source. *Pseudomonas species* was highly degrading the crude oil compared than *Bacillus species*.

Leahy and Cowell (1991) demonstrated that microorganisms are the main degrades of petroleum hydrocarbons in contaminated ecosystem. Enrichments to isolates using crude oil as sole carbon and energy source provides various microorganisms from contaminate soil (Jirasripongpun, 2002). The use of pure cultures in this study, in addition provides practical advantages by eliminating the ambiguity associated with constantly fluctuating microbial populations (Ghazali *et al.,* 2004).

Oil products not only modify physico chemical and biological properties of the soil, but also contribute to limitation of the productive ability of crop (Wyszkowskwa *et al.,* 2002). Microorganisms possess mechanisms by which they degrade the crude oil compounds by utilizing them as carbon and nitrogen sources. The pattern of degradation varies for different degrading microorganisms because different microorganisms possess different catabolizing enzymes (Penet and Marchal, 2006). Microorganisms capable of surviving on these highly reduced organic compounds have been identified (Penet and Marchal, 2006).

In growth efficiency study, *Pseudomonas species* have high growth level and also high protein content compared than other test organisms. Several studies have reported on the roles of *Bacillus cereus* more tolerant to high level of hydrocarbon in soil due to their resistance endospore (Annweiller *et al.,* 2000). Chosson *et al.* (1991) showed that of 73 aerobic bacteria ability to degraded petroleum hydrocarbon.

Amund and Akaqngbou (1993) showed that crude oil fraction with lower amount of saturated hydrocarbons were more resistance to microbial degradation than the fraction containing higher amount of saturated hydrocarbons. The Escravos crude oil blend used in this experiment has been show to contain 69. 74% saturated hydrocarbon, 22.05% aromatics 2.56%, asphaltenes and 5.65% residues. This could possibly have accounted for the slow rate of degradation of the oil.

Rahman *et al.* (2002) reported that *Pseudomonas* species degradation of hydrocarbon was achieved at 5% oil concentration. Nutritional and environmental factors affecting petroleum degradation have been evaluated by applying Placketl-Burman design, where K_2HPO_4, inoculum size and pH were the most significant variables. The degradation of hydrocarbon was achieved at 5% oil concentration and proved a maximum petroleum oil degradation of 98.8%.In this study growth resistance to crude oil were analysed *Pseudomonas species* was noted

high growth efficiency. This observation agrees with that of (Atlas, 1984; Bartha and Atlas, 1977) reported that refined petroleum plot. Supply only carbon and energy to resident microorganisms while crude oil supplies, in addition to carbon and energy, mineral nutrients such as nitrogen, sulphur and heavy metals. Atlas and Bartha (1972) reported that the application of crude oil to arctic tandra soil caused overall increase in microbial numbers compared to un-oiled reference (Control) soil. In this pot culture study, stunted plant growth, minimum level of chlorophyll and protein content were recorded in crude oil only inoculated treatment. The increasing level of growth, chlorophyll and protein content were observed in crude oil and bacteria inoculated treatment.

In this study analyzed by the variation in protein content in this study (Ziad *et al.,* 2005) reported a noticeable decline in seed germination percentage of in the presence of the crude oil in soil and the similar results were obtained in this study, which reveals that the crude oil have inhibition of *Vigna mungo* seed germination potential in it.

Westlake (1983) supported this work, which reported on 10 pots of oil contaminated soil was prepared with tomato and alfalfa plants of *Pseudomonas species*, to test on microbial community at various time intervals. The hypothesis that the *Pseudomonas species* promotes the rhizosphere bioremediation of oil contaminated soil by increasing the composition of the microbial community. The symbiotic relationship between the soil microbes and in *Pseudomonas spp.*, may be responsible for the degradation of oil contaminates. The Alfalfa sample with *Pseudomonas species* worked best at removing hydrocarbon from the soil.

In present study the crude oil degradation were analysed by plate assay method. The highest zone of exhibitions was noted in *Pseudomonas species*. The similar results was also reported as Shrivastava and Gupta (2008) *Pseudomonas sps.*, showed best growth as well as metabolic activity in broth culture, to prove the versatility of its growth was studied in solid medium in response to a range of crude oil concentration. Growth response of the isolated at increasing crude oil concentration from 1% to 6% and as clearly evident from the zone exhibition.

The effect crude oil on the crop plant were analysed in pot culture experiment. In the study maximum germinating and growth ability recorded in crude oil done experimental groups. At the same time *Pseudomonas* species (75%) inoculated groups have maximum germinating and growth ability noted compared the *Bacillus* species (62%) inoculated groups. The similar results also reported as Ziad *et al.* (2005) reported a noticeable decline in seed germination percentage of *Alfalfa* in the presence of the crude oil in soil and the similar results were obtained in this study, which reveals that the crude oil have inhibition of *Vigna mungo* seed germination potential in it.

Table.1 Total No. of bacterial count in crude oil contaminated soil sample

S.No.	Dilution	Colony forming unit (Cfu)
1	10^{-6}	55
2	10^{-7}	35

Table.2 Morphological characteristics

S.No	Morphological characteristics	Isolated bacterial colonies	
		DB1	DB2
1.	Colour of the colony	White	White
2.	Shape of the cell	Bacillus	Rod
3.	Gram's Staining	Positive	Negative
4.	Motility	Motile	Motile

Table.3 Biochemical characteristics

S.No	**Biochemical characteristics**	**Isolated bacterial colonies**	
		DB1	**DB2**
1.	Indole	Negative	Negative
2.	Methyl red	Negative	Negative
3.	Voges-proskauer test	Negative	Negative
4.	Citrate utilization test	Positive	Negative
5.	Triple sugar iron agar test	A/A (Gas)	K/K
6.	Urease hydrolysis	Variable	Negative
7.	Oxidase	Variable	Positive
8.	Catalse	Positive	Negative
9.	Carbohydrate fermentation test		
	Glucose	A/G	A
	Dextrose	A/G	-
	Maltose	A/G	A

DB1 - Degrading Bacteria 1, DB2 - Degrading Bacteria 2, A/A – Acid butt and Acid slant, K/K – Alkaline butt and Alkaline slant, A/G – Acid /Gas, A – Acid

Table.4 Determination of microbial resistance to crude oil

S.No	Incubation days	Growth ability measured at 540 nm	
		Pseudomonas putida	*Bacillus cereus*
1.	1st day	0.22	0.19
2.	7th day	0.55	0.20

Table.5 Assay of total protein in bacterial

S.No	Test organisms	Total protein mg/g
1.	*Pseudomonas putida*	5.5 ±0.53
2.	*Bacillus cereus*	2.3 ±0.21

Table.6 Degradation of crude oil

S.No	Test organisms	1st day in crude oil conc. ml (Initial)	7th day in crude oil conc. ml(Final)	% of crude oil degradation
1.	Control	2	2	-
2.	*Pseudomonas putida*	2	1.2	65
3.	*Bacillus cereus*	2	0.7	40

Table.7 Plate assay method

S.No	Test organisms	Zone of exhibition (mm in dm)
1.	*Pseudomonas putida*	8 ± 0.92
2.	*Bacillus cereus*	6 ± 0.19

Table.8 Assay of Bio surfactants

S.No	Test organisms	**Biosurfactants (mg)**
1.	*Pseudomonas putida*	0.340 ± 0.005
2.	*Bacillus cereus*	0.220 ± 0.007

Table.9 Germination and growth of *Vigna mungo* after 7 Days

S.No	Growth ability (%)	Control	Crude oil +seed	Crude oil + (seed) *Pseudomonas putida*	Crude oil + (seed) *Bacillus cereus*
1	Germination	92%	50%	75%	62%
2	Shoot length	5.4±0.67	2.5±0.49	21.8±0.60	5.7±1.40
3	Leaf length	4.4±0.35	2.1±0.15	3.6±0.10	2.7±0.5

Values are Mean ± Standard deviation

Fig.1 Determination of microbial resistance to crude oil

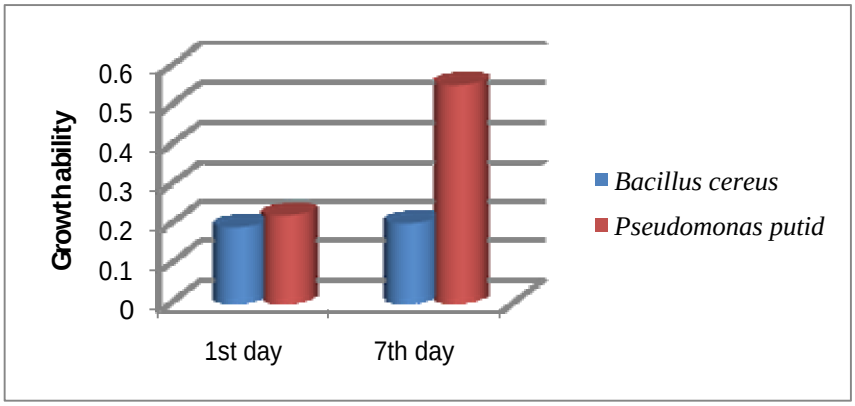


Fig.2 Assay of total protein in bacterial

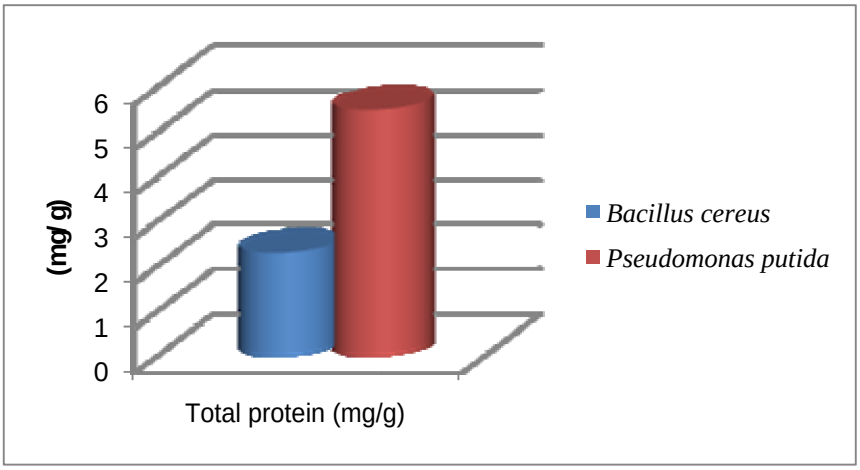


Fig.3 Degradation of crude oil

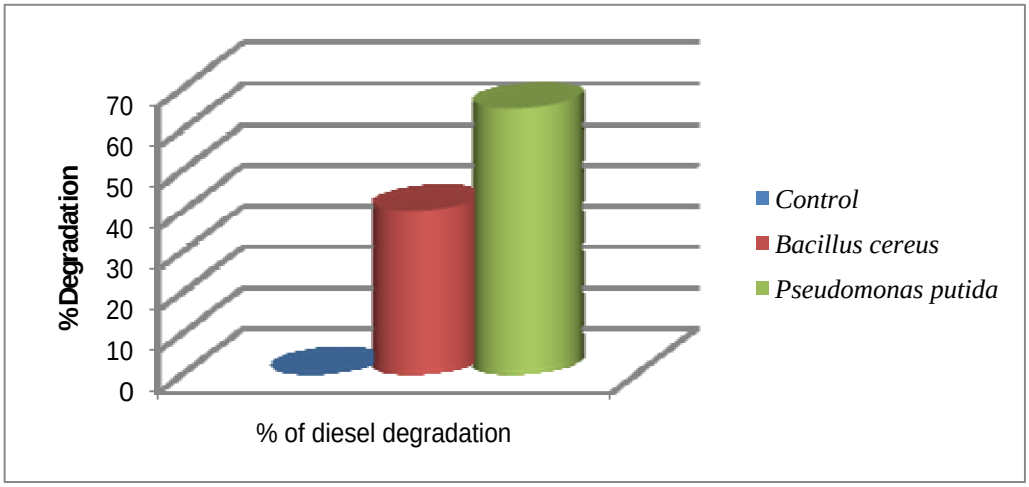


Fig.4 Plate assay method

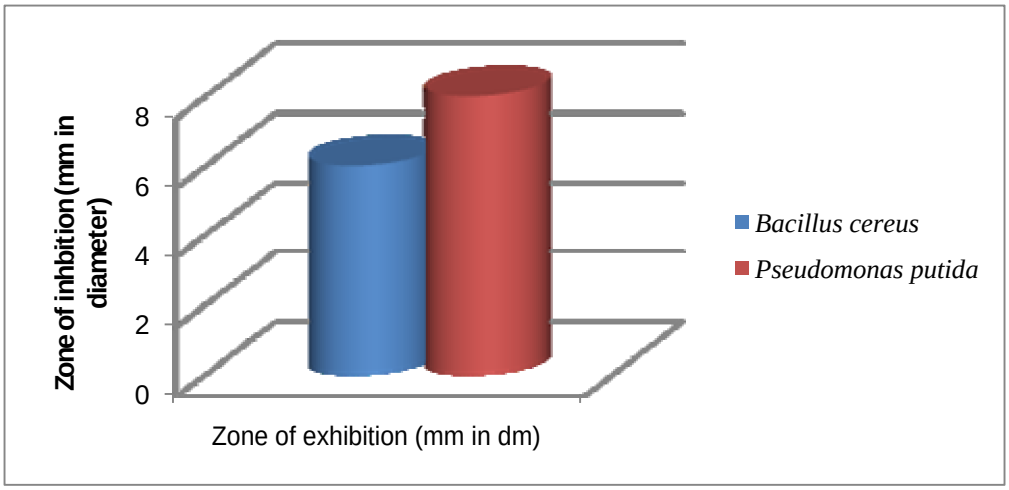
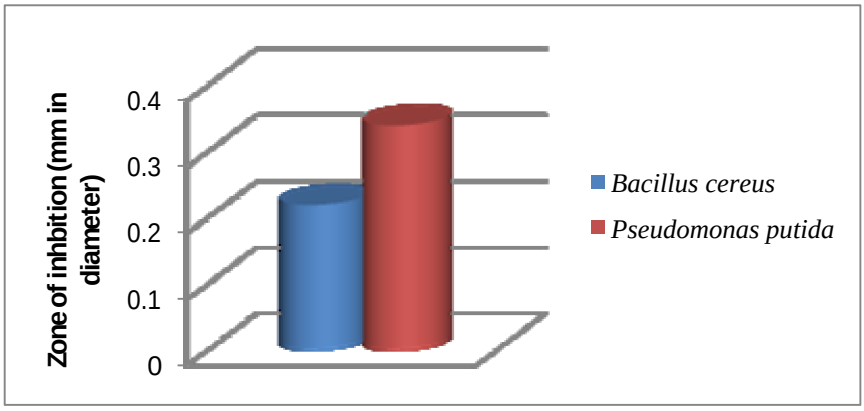


Fig. 5 Assay of Biosurfactants



The symbiotic relationship between the soil microbes and in *Pseudomonas sps.*, may be responsible for the degradation of oil contaminates. The *Alfalfa* sample with *Pseudomonas species* worked best at removing hydrocarbon from the soil.

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