Research Article

IN – VITRO SCREENING OF PETROLEUM HYDROCARBON DEGRADING ABILITY OF BACTERIA ISOLATED FROM CONTAMINATED SOILS

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A R T I C L E   I N F O

A B S T R A C T

Microbial degradation is the major and ultimate natural mechanism that can clean up the petroleum hydrocarbon pollutants from the environment, whereas the contamination of soil is due to release of hydrocarbons in environment and causes extensive damage to local ecosystem. During present investigation isolated and identified bacteria were characterized the petroleum hydrocarbon degrading indigenous bacteria from soil contaminated with petrol / petroleum products that facilitate the candidacy as bioremediation agents. Out of 63 samples, five bacterial strains were isolated, identified and initially screened as petroleum hydrocarbon degraders at different concentration of petrol i.e. 0.5%, 1%, 1.5% and 2% concentration, which exhibited utilization of petroleum hydrocarbon rich crude oil in mineral. Out of five isolated bacteria Bacillus sp. and Pseudomonas sp. were screened with significant ability to degradation of petroleum and its product. Ultimately findings revealed the extents to which the isolate could degrade crude oil hydrocarbon.

INTRODUCTION

Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution and the contamination of soils and groundwater with petroleum compounds is among the most prevalent problems in environments worldwide (Alquati *et al.,* 2005). Many microorganisms have the power to use hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are ubiquitous and widely spread in the nature (Jyothi *et al.,* 2012). In situ biodegradation is one of the primary mechanisms by which petroleum and other hydrocarbons are eliminated from the environment. Hydrocarbon-degrading bacteria are widely distributed and their use in bioremediation of hydrocarbon-contaminated soils, which exploits their ability to degrade and/or detoxify organic contaminants, has been established as an efficient, economical, versatile and environmentally sound treatment (Margesin and Schinner, 1997).

Petroleum compounds consist of four fractions: saturated hydrocarbons, aromatic hydrocarbons, nitrogen-sulphur-oxygen containing compounds and asphaltenes. Normally, of the saturated hydrocarbons, the straight-chain *n*-alkanes

are most susceptible to biodegradation, whereas branched alkanes are less vulnerable to microbial attack. Polycyclic aromatic hydrocarbons occur extensively as pollutants in soil & water and are important environmental contaminants because of their recalcitrance. These compounds also constitute a potential risk to human health, as many of them are carcinogens (Deziel, *et al.,* 1996). Liquid petroleum has become one of the most prevalent pollutants in industrialized and developing countries (Joshi and Pandey, 2011). Its transportation and global usage has increased the tendency to pollute the environment (Tyagi *et al.,* 2011). The source of pollution is usually accidental spills, uncontrolled landfills, leaking underground storage tanks or improper storage crude oil (Plohic *et al.,* 2002). Due to oil mobility, it may cause considerable damage not only in soil, but also in water intakes or ground water reservoirs (Jina *et al.,* 2014). The rate of oil spillage reported in the country has been rising with a corresponding increase in petroleum production (Onifade *et al.,* 2007). Oil spills pose serious environmental challenges due to the possibility of air, water, and soil pollution (Trindade *et al.,* 2005). These oil spills are dangerous for health, drinking water, natural resources and disturb the economy (Gesinde *et al.,* 2008). When petroleum products are burned as fuel, they give off carbon dioxide, a greenhouse gas that is linked with the global warming.

Bioremediation has become an alternative way of remediation of oil contaminated sites, where the addition of specific microorganisms (Bacteria, Cyanobacteria, Algae,

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Fungi, Protozoa) or enhancement of microorganisms already present can improve biodegradation efficiency in both *in-situ* and or *ex-situ* (In reactors) procedures. Physical, chemical and biological factors have complex effects on hydrocarbon biodegradation in soil (Bossert and Campeau 1995).

Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environments, particularly from petroleum-contaminated sites (Whyte, *et al.*1997). Evaluations of indigenous microorganisms are needed so that microbial community composition can be correlated with ability to degrade target pollutants (Alquati *et al.*, 2005).In the present course of investigation we have isolated Bacteria from soil (contaminated with petroleum & petroleum product) samples and assessed the petroleum product degrading ability of microbial isolates.

MATERIALS AND METHODS

Sampling

Contaminated Samples were collected from different sampling sites, i.e., Soil of ground of automobile garages and petrol pumps, located at different sites of city where spilled of diesel, kerosene, petrol, grease and motor oil had occurred over a period of 10 years. The samples were stored in refrigerator maintaining below 10^oC temperature for isolation & identification of Bacteria and further investigation.

Isolation and identification of soil Bacteria

For the selection of the experimental material all species of bacteria isolated applying proper media (NAM, Mac-Conkey, EMB and Mannitol Broth) and isolated bacteria were identified. Spread plate method, Pour plate method and Streak plate method were employed for isolation of bacterial strains. Identification of bacterial samples was made on the basis of different biochemical characterisation based on different biochemical tests and morphological features of colonies and Gram staining.

Selection of test bacteria

All isolated bacterial cultures were taken in culture plates. Petrol / Mobil oil has been placed on the surface of Nutrient Agar plates (NAM) with glass rod. Screening was done by well diffusion method. The wells were saturated with 50 μl bacterial strains and incubated for 48 hours at 37^oC. At interval of 24 & 48 hours the control (without supplemented with Petrol / Mobil oil) and radically growing colonies (Supplemented with Petrol / Mobile oil) were examined for growth.

Assessment of bacteria growth in petroleum product

Qualitative determination of bacteria, growing on hydrocarbon incorporated NB (Nutrient Broth) respectively, was determined by culturing of bacteria 0.1ml, 0.5ml,1ml,1.5ml, 2ml petrol and mobile oil was poured in 100ml individual conical flask containing NB and inoculated bacteria with one control each which were subjected to a period of 3 hrs. at 37^oC respectively, the control and radically growing colonies were examined for their growth and degrading ability.

RESULTS AND DISCUSSION

To determine the bacteria inhabiting to the soil, the entire samples were examined using their concerned media for *in-vitro* culture and identifying methodology. The bacterial examination of soil samples reveal the occurrence of total 5 species of bacteria i.e. *Bacillus sp. , Pseudomonas sp., Klebsiella sp. , Staphylococcus sp. and Enterococcus sp.,* have been isolated and identification of bacterial isolates was made on the basis of biochemical tests (Table 1). *Bacillus sp.* and *Pseudomonas sp.* was found more frequent than *Klebsiella sp., Staphylococcus sp. and Enterococcus species* (Plate 1, Fig.-i to v)

For taking test organism the bacterial isolates have been screened regarding their ability to degrade petrol/petroleum product as mention in Table - 2 and shown in Plate -2, Fig. – a. To assess the petrol/petroleum product degrading ability of bacteria, their growth pattern was determined by optical density within stipulated period.

The increase in biomass, and increase in OD, between 1 to 3 hrs. in all the concentrations, strongly suggests that *Bacillus* and *Pseudomonas sp.* has got a potential to degrade both petrol and Mobil oil. Mobil oil was found as a most suitable carbon and energy source for the test bacteria. The bacterial was able to multiply within an hours of study, indicating that it was able to degrade and utilize the oil for its growth and development, hence the concomitant increase in the concentration of the broth (turbidity). The gradual increase in the absorption of the broth indicates bacterial growth, hence degradation of hydrocarbons, decline in the decrease in OD suggests a reduction in the bacterial population and that the hydrocarbon has been degraded, mostly between 1 and 3 hours.

Accidental releases of petroleum products are of particular apprehension in the environment. Currently conventional disposal methods of incineration or burial insecure landfills can become prohibitively costly when amounts of contaminants are large. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to\other simpler organic compounds by biological agents like microorganisms. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants.

The rate of crude oil biodegradation in the soil seems to be more rapid, that may be due to the microorganisms in the soil have ability to utilize the residual crude oil as a source of carbon and energy. Crude oil contains hydrocarbon and does not resist attack by microorganisms. This study proposes degradation of petroleum hydrocarbons using indigenous microorganisms isolated from the hydrocarbon contaminated sites .The isolate have shown very good growth on crude oil hydrocarbons. This investigation had achieved its primary objective of designing microbial formulation that could be employed in the bioremediation of soil polluted by crude oil. So many investigators have found various bacterial sps. such as *Bacillus sp.,*

Table 1 Characterization & Identification of Bacterial isolates

Appearance and Biochemical Tests	Enterococcus sp.	Pseudomonas sp.	Bacillus Sp.	Klebsiella sp.	Staphylococcus sp.
Shape	Spherical	Rod shaped	Rod shaped	Rod shaped	Spherical
Gram test	+	-	+	-	+
Indole test	-	-	-	+	-
Methyl red test	-	+	-	+	-
VP	+	-	-	-	-
Citrate	-	+	+	-	-
Catalase	+	+	+	+	+

Fig.-i. *Staphylococcus sp.*   Fig.-ii. *Pseudomonas sp.*   Fig.-iii. *Enterococcus sp.*

Fig.- v. *Klebsiella sp.*   Fig.-iv. *Bacillus sp.*
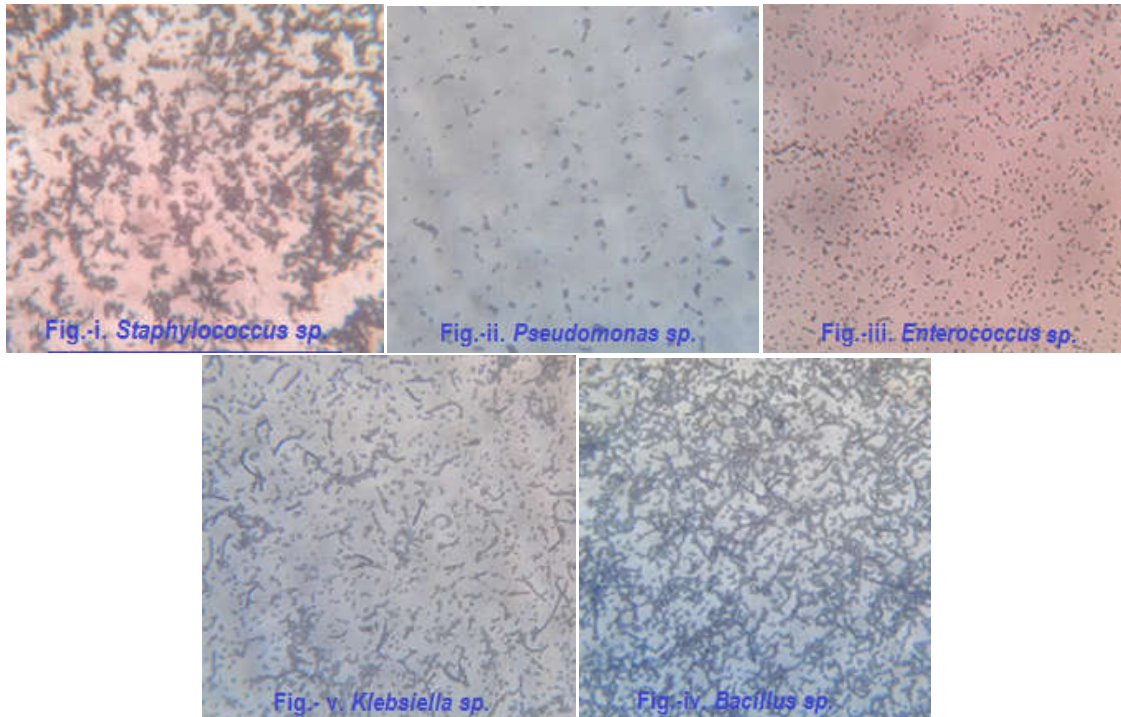
Plate 1 **Fig. i – v**

Table 2 Bacterial growth (mm.) after 24 & 48 hour incubation in NAM supplemented with / without petrol & mobile oil.

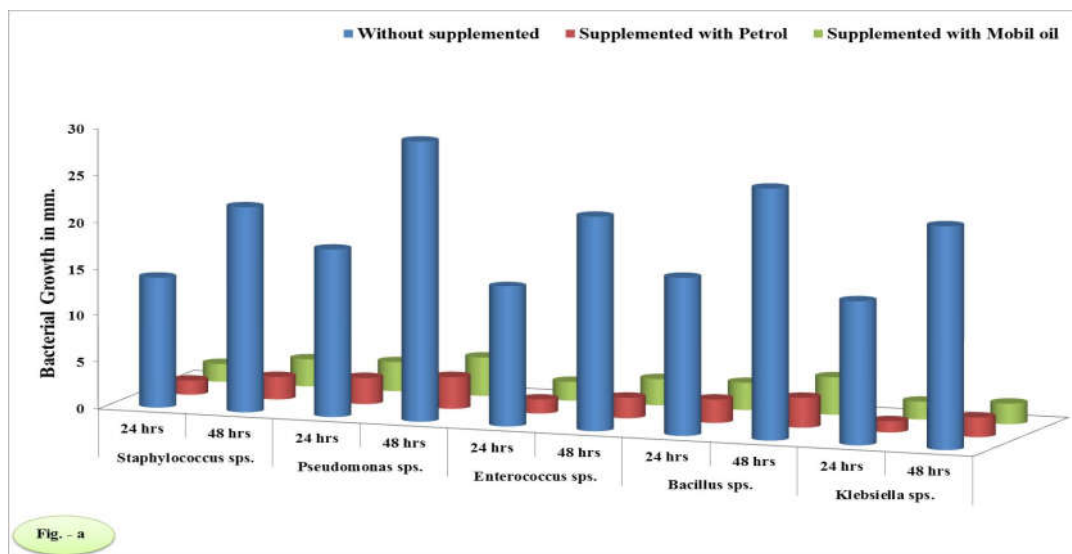| Bacterial isolates	Growth of isolates: Size of colony in mm (value ±SD)					
	Without supplemented		Supplemented with Petrol		Supplemented with Mobil oil	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
Staphylococcus sps.	14±0.62	22 ±0.55	1.5 ±0.15	2.4 ±0.17	1.9 ±0.11	2.9 ±0.16
Pseudomonas sps.	18±0.73	30 ±0.65	2.8 ±0.19	3.4 ±0.20	3.1 ±0.17	4.1 ±0.22
Enterococcus sps.	15±0.60	23 ±0.54	1.5 ±0.14	2.2 ±0.19	2.0 ±0.20	2.8 ±0.22
Bacillus sps.	17±0.68	27 ±0.21	2.5 ±0.20	3.2 ±0.22	2.9 ±0.19	4.0 ±0.18
Klebsiella sps.	15±0.54	24 ±0.44	1.2 ±0.21	2.1 ±0.25	1.9 ±0.19	2.2 ±0.22

Plate 2 **Fig. –a**

Table 3 Effect of different concentration of Petrol and Mobile oil on growth of Bacterial isolates–*Bacillus sp.*

Petrol & Mobile oil	Concentration	Growth of *Bacillus sp.* (Optical Density at 580 nm)						
		Initial OD	30 min	60 min	90 min	120 min	150 min	180 min
Petrol	0.5%	0.07 ±0.12	0.09 ±0.16	0.16 ±0.19	0.28 ±0.13	0.39 ±0.12	0.41 ±0.21	0.38 ±0.18
	1%	0.07 ±0.15	0.07 ±0.11	0.14 ±0.10	0.24 ±0.18	0.35 ±0.23	0.37 ±0.26	0.33 ±0.14
	1.5%	0.07 ±0.11	0.09 ±0.08	0.13 ±0.12	0.21 ±0.13	0.29 ±0.17	0.31 ±0.21	0.28 ±0.15
	2%	0.07 ±0.16	0.09 ±0.23	0.11 ±0.27	0.14 ±0.12	0.19 ±0.20	0.21 ±0.28	0.13 ±0.23
Mobile Oil	0.5%	0.07 ±0.13	0.08 ±0.09	0.10 ±0.12	0.13 ±0.16	0.17 ±0.14	0.18 ±0.11	0.12 ±0.18
	1%	0.07 ±0.11	0.07 ±0.13	0.09 ±0.17	0.12 ±0.18	0.14 ±0.25	0.15 ±0.12	0.11 ±0.19
	1.5%	0.07 ±0.14	0.07 ±0.12	0.08 ±0.17	0.10 ±0.07	0.12 ±0.15	0.13 ±0.19	0.09 ±0.11
	2%	0.07 ±0.16	0.06 ±0.08	0.07 ±0.10	0.09 ±0.13	0.11 ±0.21	0.10 ±0.24	0.06 ±0.16
Without Supplemented (Control)		0.07 ±0.12	0.11 ±0.21	0.20 ±0.15	0.35 ±0.26	0.54 ±0.28	0.62 ±0.21	0.63 ±0.31

Table 4 Effect of different concentration of Petrol and Mobile oil on growth of Bacterial isolates– *Pseudomonas sp.*

| Petrol & Mobile oil	Concentration	Growth of *Pseudomonas sp.* (Optical Density at 580 nm)						
		Initial OD	30 min	60 min	90 min	120 min	150 min	180 min
Petrol	0.5%	0.07 ±0.13	0.09 ±0.15	0.13 ±0.17	0.21±0.22	0.31 ±0.15	0.39 ±0.13	0.37 ±0.15
	1%	0.07 ±0.09	0.08 ±0.21	0.11 ±0.15	0.19 ±0.14	0.27 ±0.22	0.31±0.13	0.29 ±0.22
	1.5%	0.07 ±0.06	0.08 ±0.14	0.10 ±0.11	0.15 ±0.16	0.23 ±0.16	0.27 ±0.22	0.24 ±0.21
	2%	0.07 ±0.16	0.07 ±0.22	0.09 ±0.22	0.14 ±0.21	0.20±0.14	0.22 ±0.11	0.13 ±0.16
Mobile Oil	0.5%	0.07± 0.13	0.09 ±0.12	0.15 ±0.25	0.24 ±0.22	0.38 ±0.12	0.49 ±0.21	0.48 ±0.11
	1%	0.07± 0.11	0.08 ±0.21	0.12 ±0.09	0.20 ±0.15	0.32 ±0.14	0.41 ±0.16	0.40 ±0.22
	1.5%	0.07± 0.14	0.08 ±0.24	0.10 ±0.13	0.17 ±0.21	0.27 ±0.22	0.37 ±0.18	0.36 ±0.14
	2%	0.07± 0.05	0.06 ±0.13	0.08 ±0.14	0.14 ±0.17	0.22 ±0.16	0.30 ±0.21	0.29 ±0.23
Without Supplemented (Control)		0.07±0.12	0.07 ±0.12	0.10 ±0.06	0.16 ±0.15	0.28 ±0.10	0.45 ±0.11	0.53 ±0.21
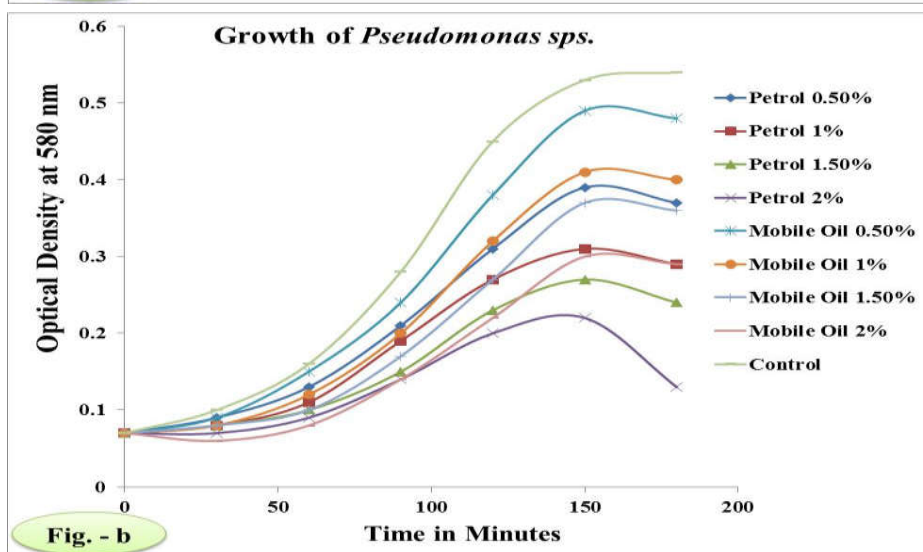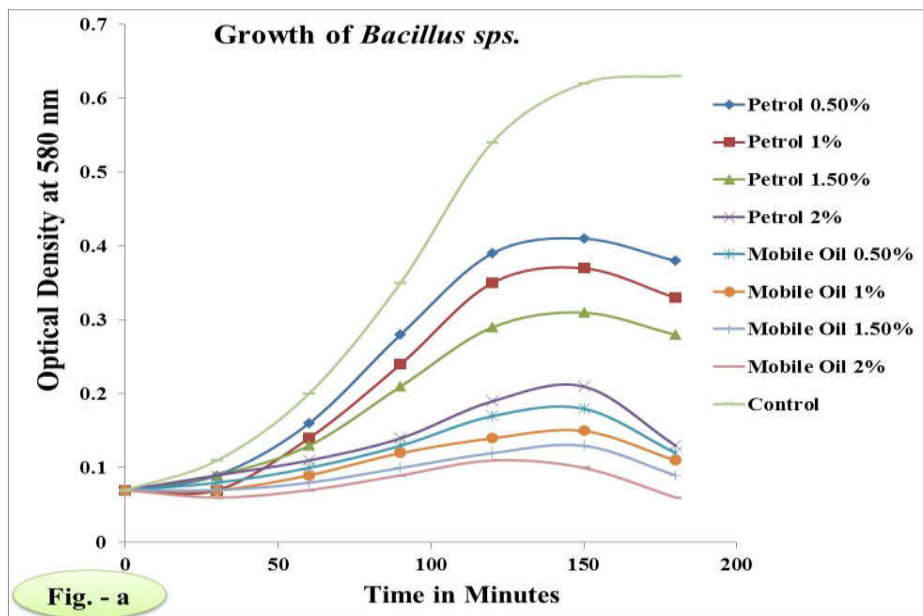
Plate 3 Fig. - a & b
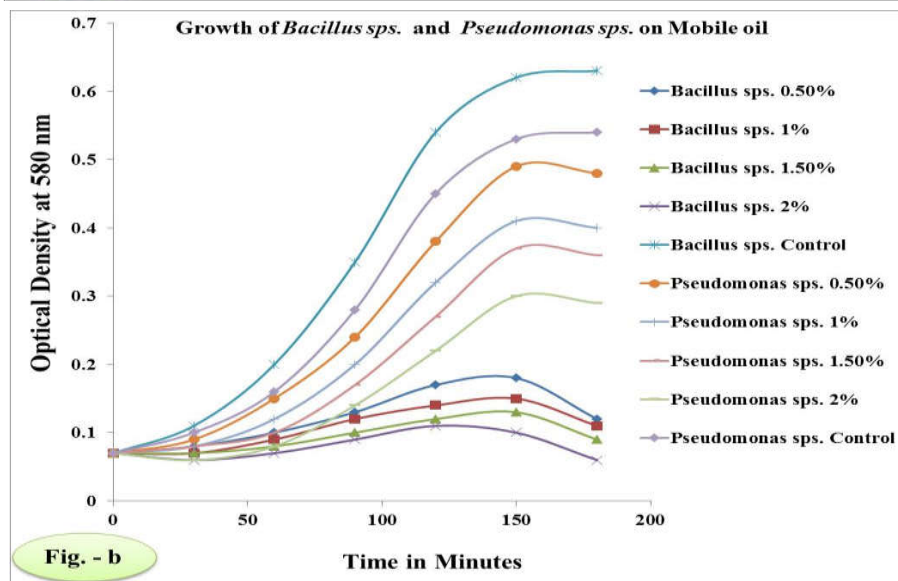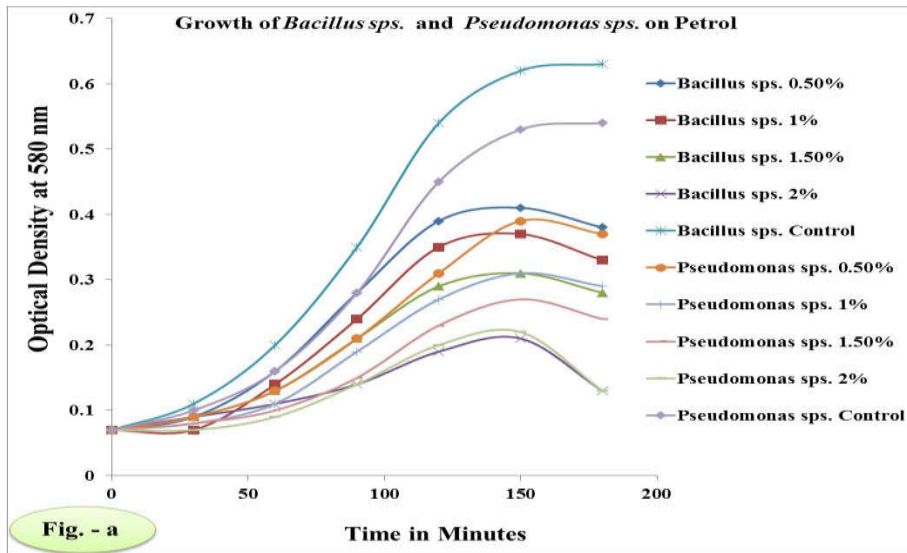
Plate 4 Fig. - a & b

Pseudomonas sp., Klebsiella sp., Staphylococcus sp. and *Enterococcus sp.* were found to be more predominant in the polluted soil, as Panda *et al.,* (2013) was also found the biodegradation ability of *Pseudomonas aeruginosa and Bacillus sp.*

The increase in optical density indicates the degradation of petrol and petroleum product (mobil oil) by the bacteria indirectly. The increase in optical density with the increase in treatment period but a decline with the increase in petrol concentration has strongly suggest that both bacteria i.e. *Pseudomonas sp. & Bacillus sp.* have got a potential to degrade petrol and petroleum products (diesel) (Table – 3 & 4, Plate – 3, Fig: a & b and Plate – 3 Fig: a & b). Panda *et al.* (2013) found the biodegradation ability of both *Pseudomonas aeruginosa and Bacillus* sp. in 2% of petrol and petroleum product (Mobil oil).

CONCLUSION

Soil is the natural material which consists of both organic and inorganic compounds which support the characteristics of the soil to maintain equality in all conditions.

But due to the man-made pollution, such as oil spills, hydrocarbon contamination and all the xenobiotic there is a change in the alterations of the nutrients present in the soil and use of microbial colonies has been affected. Alliteration makes the soil to release of more CO_2 from them it leads to the cause of environmental pollution such as climate change and global warming etc. The biological method of degradation is useful to degrade the toxic pollutant to nontoxic chemical. Therefore the present work has been performed, to identify petrol and petroleum product degrading microorganism from the petroleum polluted soil. All the strains isolated from the soil were capable of consuming Petrol and Petroleum product as a sole carbon source. Environmental pollution caused by released of a wide range of compound as a consequence of industrial progress has assumed serious proportions. To prevent development of hazardous waste the process of bioremediation has been followed. Our present study follows the isolation of hydrocarbon degrading bacteria from the contaminated soil with petrol and diesel oil. Samples were collected from contaminated soil sites and identification of isolated bacterial strains were done on the basis of gram staining, morphology and biochemical tests.

The isolates were screened for their oil degrading capacity. Bacillus species, Klebsiella species, Pseudomonas species, Enterococcus species and Staphylococcus species were identified from the Petroleum contaminated soil. From the identified organisms, Pseudomonas species and Bacillus species were further experimented and found to be capable of degrading Petrol and Petroleum product. Finding of the present investigation revealed that the biological method of degradation is useful to degrade the toxic pollutant to non-toxic chemical, whereas such bacterial strains would be more significant. Hence these bacterial strains can be used in cleaning oil polluted sites and controlling the soil pollution caused by petroleum product through biodegradation of pollutants.

Acknowledgment

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