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BIODEGRADATION POTENTIAL OF *BACILLUS SPECIES* ISOLATED FROM OIL CONTAMINATED SITE

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ABSTRACT
The discharge of oil contaminated soil to environment caused serious damages to human, animal and environment and bioremediation are an attractive alternative to chemical method for removal of hydrocarbon from industrial effluents. This study has demonstrated a very good biodegradation capability of oil contaminated soil by *Bacillus species.* In spite of the complex composition of oil contaminated soil from three different automotive workshops, biodegradation could be accomplished by *Bacillus species*. Soil samples were collected from automotive workshops and subjected to serial dilution and plating. From the developed bacterial colonies, one was selected and identified as *Bacillus species* based on the biochemical tests. The isolated strain was able to grow in minimal broth along with 2, 7, 10 and 15% concentrations of oil which indicated the capability of the organism in degrading oil and utilizing it as a source of growth. The isolated strains efficiency was determined by analyzing the parameters pH, optical density and CO_2 released during petrol degradation. HPLC analysis also confirmed the degradation of oil by *Bacillus species.* The isolate *Bacillus species* has the ability to tolerate the oil concentrations and grow on them. Hence, this strain can be used in cleaning oil polluted sites.

KEYWORDS: Biodegradation, *Bacillus species*, Oil contaminated soil, Hydrocarbon, HPLC analysis.

INTRODUCTION
Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Apparently inevitable spillages, which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field (Okoh, 2003). Oil spill have become a global problem in industrialized and developing countries. Attention has been focused on the marine environment, because of the largest and most dramatic spills (Cooney, 1984).

These microorganisms utilize these hydrocarbons as their nutrient sources. Such prominent isolated microbes include *Mycobacterium*, *Streptococcus*, *Corynebacterium* etc., Bioremediation employs biodegradation process to clean up the environment (Ogunbayo *et al*., 2012).

Petroleum hydrocarbons can be introduced into the environment via oil spills, leaking or unplugged oil wells, the disposal ponds of waste petroleum products, abandoned oil refinery sites, pipe line ruptures, incomplete combustion of fossil fuels and accidental discharge during transport in tanks and ships failures. Petroleum production, drilling operations and improperly sealed abandoned wells have caused major contamination of surface, ground waters and soils (US Environmental Protection Agency (USEPA), 1987; Richter and Kreitler, 1993; Kharaka *et al*., 1995; Kharaka and Hanor, 2003).

Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Evidently inevitable spillages, which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field (Okoh, 2003). Crude oil is a complex mixture of hydrocarbons and other organic compounds mainly composed of alkanes, cycloalkanes and aromatic alkanes, which constitute about 50% to 80% of the oil content. The ability of microorganisms to utilize hydrocarbons in oil contaminated environments has been documented (Obuekwe *et al.*, 2009). Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amount of oil by various physical and chemical methods (Ljah and Okang, 1993).

Crude oil contamination of water and soil poses a severe ecological and environmental threat. This problem is of

great concern to the oil industry. Oil refineries generate huge volume of oily sludge and effluent during the refining of crude oil (Hadibarata and Tachiban, 2009). Extensive petroleum hydrocarbon exploration activities often result in the pollution of environment which will lead to disastrous consequences for the biotic and abiotic components of the ecosystem (Ockoh, 2006) and (Mueller *et al.,* 1992). Improper disposal of this oil contaminated water causes serious hazard to the ecosystem. The traditional treatment of oily wastewater, such as containment and collection using floating booms, adsorption by natural or synthetic materials, etc., cannot degrade the crude oil thoroughly (Ollis, 1992).

Among the major hydrocarbon products, benzene is of major concern as it is a stable, water-miscible, highly mobile, poisonous and cancer-causing aromatic compound. Successful degradation of benzene by microorganisms in an aerobic environment has been reported; however, under anaerobic conditions its rate of biodegradation is observed to be very slow and poor (Vogt *et al*., 2011; Singh *et al.,* 2009). The common bacterial genera exploited for benzene bioremediation are *Pseudomonas, Bacillus* (Mukherjee and Bordolai, 2012), *Acinetobacter* (Kim and Jeon, 2009), *Gammaproteobacteria* (Sei and Fathepure, 2009), and *Marinobacter* (Berlendis *et al*., 2012). The other bacterial species identi ed for diesel biodegradation were *Pseudomonas aeruginosa* (Mariano *et al*., 2010) and *Staphylococcus aureus* (Shukor *et al.,* 2009). So the aim of this study was to isolate local bacterial species which have the ability to biodegrade crude oil and compare its biodegradation abilities in case of single use with mixed consortium. In the present work oil degrading microorganisms were isolated from oil.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from oil contaminated site of three different automotive workshop in Thiruthuraipoondi, Thiruvarur district, Tamil Nadu. Samples were labelled and transported to the laboratory and stored in the refrigerator at temperature 4^0C prior to analysis.

Physico-chemical properties of the soil

Soil moisture, pH and temperature were determined as described by Mishra (1968). The total organic carbon and the total organic matter were estimated by rapid titration methods of Walkley and Black (1934). The total organic matter was calculated by multiplying the organic carbon with constant factor 1.7241 as it is presumed that the organic matter of soil contains 58% carbon (Robinson and Garret, 1969) total organic nitrogen was estimated by the Micro-kjeldahl distillation method (Jackson, 1958).

ISOLATION OF OIL DEGRADING BACTERIA (Ronald Atlas, 1998)

The collected oily waste soil were serially diluted up to 10^{-7} and 0.1ml from the dilution 10^{-5} and 10^{-6} were spread plate on bushnell hass mineral salt medium (magnesium sulfate 0.2 g, calcium chloride 0.02 g, mono potassium phosphate 0.2 g, dipotassium phosphate 1.0 g, ammonium nitrate 1.0 g, ferric chloride 0.05 g), containing 2.5% of petrol. The plates were incubated at 37^OC for 24 hours and from the developed colonies, One was selected for further experiments.

IDENTIFICATION OF OIL DEGRADING BACTERIA (Bailey and Scott, 1966)

The organism was identified by gram staining, motility test and biochemical test.

BIODEGRADATION OF OIL (Darsa *et al.*, 2014)

One hundred ml of Minimal medium (dextrose 1g, ammonium sulphate 1g, dipottassium phosphate 7g, mono pottassium phosphate 2g, sodium citrate 0.5g, and magnesium sulphate 0.1g) with petrol and 1ml inoculum from the overnight culture maintained in nutrient broth during the logarithmic phase were added to 250ml Erlenmeyer flasks which were subjected to a period of sixteen days. Flasks were incubated in shaker at 30^0C at 100 rpm. After specified time, flasks were taken out. The ability of the isolated strain to degrade petrol was studied by determining the parameters p^H, OD and CO_2 in the culture medium every 4 days.

pH estimation

Sample from the culture medium was checked for pH after (4, 8, 12 and 16) days of treatment with the pH meter.

Optical density (growth rate) determination

The optical density of the sample from culture medium was determined at 610 nm after (4, 8, 12 and 16) days of treatment using a spectrophotometer.

Estimation of carbon dioxide

Each sample concentration, 1 ml of sample was taken after 4, 8, 12 and 16 days of treatment and titrated against 0.05N NaOH solution. Phenolphthalein was used as the indicator and appearance of stable pink color was considered as the end point. The amount of CO_2 was calculated.

$$\text{Free } CO_2 \text{ (mg } l^{-1}) = \frac{\text{Titrate value} \times \text{Normality of NaoH} \times 1000 \times 44}{\text{Volume of the sample}}$$

Optimization of biodegradation by physical and chemical parameters

Effect of temperature

The percentage of degradation was influenced by temperature. The rate of biodegradation of crude oil was estimated at different temperature ranges from (25, 30, 35 and 40^OC). The bacterial isolates were incubated at

each specified temperature in Mineral Salt Medium with 1% crude oil for 30 days.

Effect of pH
The presence of degradation was influenced by pH. The effect of pH was evaluated by incubating the culture at optimum temperature with various pH (4, 6, 7 and 9) for 30 days. The pH of Mineral Salt Medium with crude oil (1%) was maintained using 1N HCL and 1M NaOH. To maintain the pH citrate - phosphate buffer (pH 4-6), phosphate buffer (pH 7-8) and carbonate bicarbonate buffer (pH 9) were used. Every 5 day internal, pH for maximum oil degradation was checked. The optimum pH for maximum oil degradation was determined.

Incubation period
To find the optimum condition for biodegradation activity, the production media were prepared after sterilization, 1% of inoculum were added into different flasks containing medium and the flasks were incubated at various incubation periods for 48, 60 and 72 hrs.

Concentration of crude oil on biodegradation
The influence of concentration of crude oil was studied by varying the concentration of crude oil (1,5, 10, 15%) in Mineral Salt Medium at optimum temperature and pH for same incubation period.

STATISTICAL ANALYSIS (Zar, 1984)
Standard deviation
All analysis were performed in triplicates and results were presented here by the mean of triplicate ± standard deviation.

High performance of liquid chromatography (HPLC)
The separation and identification of compounds were made through High performance of liquid chromatography (HPLC). The extracts used for HPLC analysis were passed through a 0.45 µm filter (Millipore, MSI, Westboro, MA) before injection into a HPLC column of 150 mm length (Agilent technologies 1200 series). The mobile phase was acidified water containing 0.1% formic acid (A) and acidified acetonitrile containing 0.1% formic acid (B), eluted in gradient. The flow rate was 0.8 mL/min and the wavelengths of detection were set at UV 300 nm, temperature at 30°C, injection volume = 20 µl and analysis time was 60 min. Reference substances is a mixture of gallic acid, vanilic acid, ascorbic acid, quercetin, caffeic acid, catechin and coumaric acid(solutions in methanol, each of them 0.5 mg/ml).

Column Specification
Reverse phase HPLC (Cyberlab, USA) analysis was carried out in a C 18 column (250mm ×4.6mm) version (lake forest, CA USA) equipped with a c 18 curved column. The components were eluted with an isocractic elution of acetonitrile vs water at the flow rate of1 ml /min and absorption recorded at 680nm.

Sample preparation
One ml of the samples was centrifuged (at3000rpm for 15 minutes) and dissolved in specific solvent of HPLCgrade and filtered through 0.22 micro filter. The filtrate was collected and degassed using sonicator for 50 times at 4°c.

Solvent preparation
Solvent was prepared using aceto nitrile and water in the radio 65:35 and degassed using sonicator for 15 times at 4°c.

Column equilibration
Column equilibration was done using 65% aceto nitrile in water until zero base line.

Sample injection
Twenty micro litre of the sample was injected in to the injection head using injection needle. Required time and wavelength were set and the purification profiles were seen on the screen that shows the degraded components with its retention time.

RESULTS
The present study was carried out with the oil contaminated soil collected from three different location of automotive workshop in Thiruthuraipoondi. From the sample, bacterial species were isolated and identified. The bacterial isolates were selected to assess the efficiency of biodegradation oil of contaminated soil.

ANALYSIS OF PHYSICO-CHEMICAL PARAMETERS
The physical factors such as pH 5.8, organic carbon 13.0. The chemical factors of soil such as nitrogen 0.1, phosphorus 3.0, zinc 0.2, copper 0.8, iron 4.0 and magnesium 4.5 were analysed.

ISOLATION AND IDENTIFICATION OF OIL DEGRADING BACTERIA
The bacterial species were isolated from oil contaminated soil sample by serial dilution techniques. Different bacterial colonies were observed in Mineral Salt Medium. The population density was observed from three different location of automotive workshop from Madapuram, Edaiyur and Velur in Thiruthuraipoondi taluk. The population density of Madapuram site was observed by the 350×10^7 colonies, Edaiyur site was observed by the 340×10^7 colonies and Velur site was observed by 330×10^7 colonies. The bacterial colonies were identified by Gram staining and biochemical tests. Based on the morphological and biochemical characteristics, the isolates were confirmed as *Pseudomonas spp, E.coli, Staphylococcus spp, Bacillus subtilis* and *Bacillus cereus.* The results were presented in (Table 1).

Table – 1: Identification of Bacterial Species from Different Location of Thiruthuraipoondi Taluk Identification of Bacteria.

Organisms	Shape	Indole	MR	VP	Citrate	Urease	Catalase	Oxidase
E.coli	Rod	-	-	-	+	-	+	+
Pseudomonas sp	Rod	+	+	-	-	-	+	+
B.subtilis	Rod	-	+	-	-	-	+	+
B.cereus	Rod	-	+	-	-	-	+	+
Staphylococcus sp	Cocci	-	+	-	-	-	+	-

(+) - indicates positive
(-) - indicates negative

BIODEGRADATION OF OIL

The maximum biodegradation activity was recorded in pH 9, when compared with other pH range. The minimum biodegradation activity was recorded in pH 5, when compared with other pH range. Maximum optical density was observed for 7.5% oil concentration after eight days of treatment. Minimum optical density was observed for 5%. During degradation of 10% oil by *Bacillus subtilis* and *Bacillus cereus*, more amount of carbon dioxide was released on 12^{th} day of incubation, CO_2 released during petrol degradation showed an increase during initial period and later remained in the asymptote level except for 5% oil concentration. Highest level was found for 10% petrol concentration after twelve days of treatment.

Table – 2: Biodegradation of pH, Optical Density and Carbon Dioxide Estimation.

Biodegradation study	Maximum	Minimum
pH	9	5
Optical density	7.5	5
Carbon dioxide estimation	10%	5%

Optimization of biodegradation by physico- chemical parameters

The maximum biodegradation activity was recorded in (40^{o}C), when compared with other temperature and minimum biodegradation activity was recorded in (30^{0}C). The optimum pH for maximum oil degradation was determined, the maximum biodegradation activity was recorded in (pH 9), when compared with other pH range and minimum biodegradation activity was recorded in (pH 7). The maximum biodegradation activity was recorded in 72 hours, when compared with other incubation periods and minimum biodegradation activity was recorded in 60 hours. The maximum biodegradation activity was observed in (15%) of crude oil followed by the minimum biodegradation activity was observed in (10%) of crude oil.

Table – 3: Optimization Studies of Temperature, pH, Incubation Period and Concentration of Crude Oil.

S.No	Optimization study	Days			
		4^{th}	8^{th}	12^{th}	16^{th}
1	Temperature (^{O}C)	25^{O}C	30^{o}C	35^{O}C	40^{o}C
2	pH	4	6	7	9
3	Incubation period	48	60	68	74
4	Concentration of crude oil (%)	2%	0.7%	10%	15%

HPLC ANALYSIS

The variations due to oil concentration and treatment period were statistically significant for the factors optical density and carbon dioxide released, while they were not significant for PH. Figure 5 shows the HPLC analysis report for control having 10% of oil without inoculum. It shows only the three peaks with the retention time of 4.209, 6.113 and 12.056 min. In Fig. 6, HPLC analysis report for 10% oil treated with *Bacillus subtilis* after sixteen days is shown. Both the peaks observed in the control were missing and the appearance of several new peaks with different retention time, indicate the degradation of petrol.

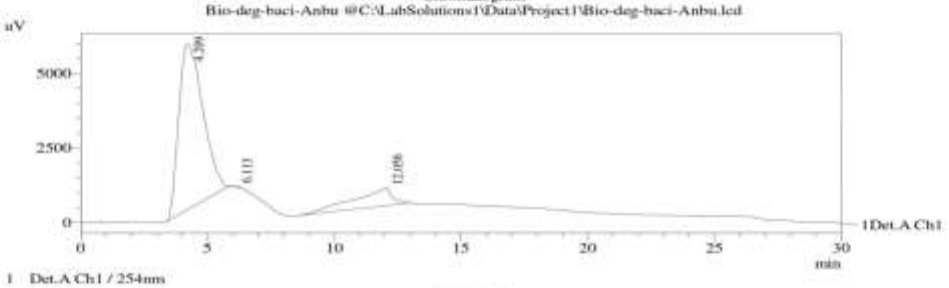
HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: Bio-deg-baci-Anbu
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: Bio-deg-baci-Anbu.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 31-03-2017 PM 04:53:07
Data Processed	: 31-03-2017 PM 05:40:37

Chromatogram

Bio-deg-baci-Anbu @C:\LabSolutions\Data\Project1\Bio-deg-baci-Anbu.lcd



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.209	365361	5550	84.548	89.365
2	6.113	870	47	0.201	0.757
3	12.056	65906	613	15.251	9.878
Total		432137	6210	100.000	100.000

ANALYSED BY

APPROVED BY

HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: bio-deg-oil-anbu
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: bio-deg-oil-anbu.lcd
Method Filename	: SJC-Law-Method.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 28-03-2017 PM 03:27:04
Data Processed	: 28-03-2017 PM 03:52:05

Chromatogram

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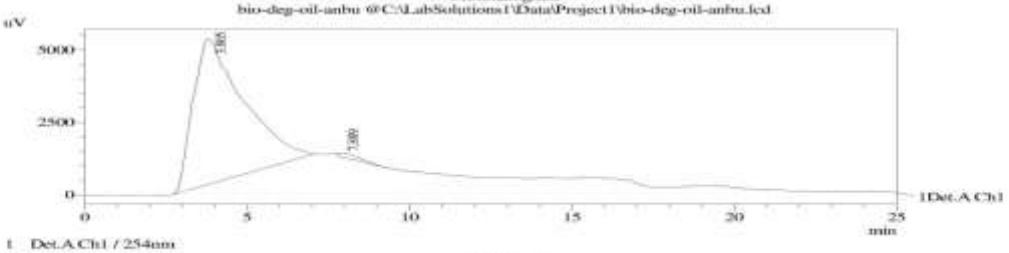

1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.805	538583	5029	98.654	97.505
2	7.889	7346	129	1.346	2.495
Total		545929	5158	100.000	100.000

ANALYSED BY

APPROVED BY

DISCUSSION

The present study was undertaken with the oil degradation by *Bacillus sp.* The results were discussed with the previous research. *Bacillus subtilis* and *Bacillus cereus* were isolated from the oil contaminated soil. The isolated colonies were identified by cultural, morphological and biochemical test and compared with Bergey's manual of bacteriological classification. Based on the results, the isolated colonies were confirmed as *Bacillus subtilis* and *Bacillus cereus* respectively.

Microbial populations increase rapidly in the water after an oil spill. Oil spilled on the ground can rapidly disappear completely under optimal conditions often within a year or two as a result of microbial oxidation of the hydrocarbons. Consequently, it may be useful to employ the natural microflora in cleaning up of oil spills (Robertson *et al*., 1973). The population density of Madapuram site was observed by the 350×10^7 colonies, Edaiyur site was observed by the 340×10^7 colonies and Velur site was observed by 330×10^7 colonies. The bacterial colonies were identified by Gram staining and biochemical tests.

Few researchers have reported on *Bacillus sp* for oil degradation potential. *Bacillus subtilis* is most tolerant to high levels of oil due to the resistant endospores. There is a growing evidence that *Bacillus subtilis* could be effective in clearing oil spills (Khan *et al.,* 2011). The maximum biodegradation activity was observed in (15%) of crude oil followed by the minimum biodegradation activity was observed in (10%) of crude oil.

HPLC analysis also offers further confirmation exhibiting new peaks which represent the metabolites of petrol degradation. *B. subtilis* showed an increase in degradation up to eight days. When relating this trend to the increase in microbial cell count, it was observed that there was a rapid increase in the cell biomass of *B. subtilis* in the first eight days of incubation. The initial high rate of biodegradation observed is attributed to the increase in microbial biomass and nutrient availability (Dibble and Bartha, 1991).

From this it can be understood that the efficiency of the organism and its stage of growth should be taken into consideration in clean up or removal of oil from the environment. This study has also revealed that the removal of oil from an environment is effective within the first two weeks of microbial growth. Survival of microorganisms in oil medium after their inoculation is a key deciding factor in the rate of biodegradation of oil either in soil or in liquid phase (Ramos et al., 1991).

The necessity for oil degrading bacteria might have arisen from the fact that introduction of efficient oil degraders would be essential in order to effectively degrade the oil mixture (Atlas, 1977). Figure 5 shows the HPLC analysis report for control having 10% of oil without inoculum. It shows only the three peaks with the retention time of 4.209, 6.113 and 12.056 min. In Fig. 6, HPLC analysis report for 10% oil treated with *Bacillus subtilis* after sixteen days is shown.

CONCLUSION

Oil industries contributes to major industrial pollution. Various preventive measures are taken care by the industries to minimize the environmental pollution. Bioremediation has been found to be most environment friendly method for treatment of oil contamination generated due to various petroleum industries. Bioremediated soil by found to be non toxic and has no adverse effect on seed germination. Bioremediation technology has helped the Indian oil refineries in disposal of their waste oily sludge in an environment friendly manner.

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REFERENCE

1. Atlas, R.M., 1977. Stimulated petroleum Authority, pp: 1-24. biodegradation. CRC Crit. Rev. Microbiol., 5: 371-386.
2. Atlas. Microbial ecology: Fundamentals and application. Pearson education India, 1998; 4: 704.
3. Bailey, W.R. and E.G. Scott. Diagnostic Microbiology, Second Edition. Toppan Company Ltd., Japan, 1966; 342 pp.
4. Berlendis, S., Cayol, J.L., Verh´e. F., Laveau, S., Tholozan, J.L., Ollivier, B., Auria, R. First evidence of aerobic biodegradation of BTEX compounds by pure cultures of Marinobacter. Appl Biochem Biotechnol. 2012; 160: 1992–1999.
5. Cooney, J.J. The fate of petroleum pollutants in fresh water ecosystem, in Petroleum Microbiology, edited by Atlas, R.M. (Macmillan Pub Co, New York), 1984; 399.
6. Darsa, K.V., Joseph, A., Thatheyus and Ramya. D. Biodegradation of Petroleum Compound Using the Bacterium *Bacillus subtilis*, 2014; 20-25.
7. Dibble, J.T. and R. Bartha, 1991. Rehabilitation of Pollutants oil-inundated agricultural land: A case history. Soil USA., Sci., 123: 56-60.
8. Hadibarata, T. and Tachibana, S. Microbial Degradation of n-Eicosane by Filamentous Fungi. Interdisciplinary Studies on Environmental Chemistry, Environmental Research in Asia. 2009.
9. Jackson, M.L. "Soil Chemical Analysis" Prentice-Hall Inc. Englewood Cliffs, New Jersey. 1958.
10. Khan, J.A., Rizvi, A. and Hasan, S. Isolation and characterization of microorganism from oil contaminated sites. Applied Sci., 2011; 2: 455-460.
11. Kharaka, Y.K., Thordsen, J.J., Ambats, G. Degradation associated with exploration for and production of energy sources in U.S.A., 1995; 8: 25-30.

12. Kharaka, Y.K. and Hanor, J.S. Deep fluids in the continents: I. Sedimentary basins, in Drever JI ed, Treatise on Geochem, 2003; 5: 499-540.
13. Kim, J.M. Jeon, C.O. Isolation and characterization of a new benzene, toluene and ethylbenzene degrading bacterium, Acinetobacter sp. B113. Curr Microbiol, 2009; 58: 70–75.
14. Ljah, U.J.J. and Okang, C.N. Petroleum Degrading capabilities of bacteria isolated from soil. Appl. Chem., 1993; 38: 9-15.
15. Mishra, R. Ecology Work Book, Oxford and IBH Publishing Co., New Del., 1968.
16. Mueller, J.G., Resnick, S.M., Shelton, M.E., Pritchard, P.H. 1992. Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil. J. Indst. Microb., 1968; 10: 95-102.
17. Mukherjee, A.K. and Bordoloi, N.K. Biodegradation of benzene, toluene and xylene (BTX) in liquid culture and in soil by Bacillus subtilis and Pseudomonas aeruginosa strains and a formulated bacterial consortium. Environ Sci Poll Res Int., 19: 3380–3388.
18. Mariano, A.P., Tomasella, R.C., Martino, C.D., Morais, E.B., Filho, R.M, Seleghim, M.H.R., Contiero, J., Tornisielo, S.M.T. and Angelis, D.F. 2010. Aerobic biodegradation of butanol and diesel oil blends. Afr J Biotechnol. 2012; 9: 7094–7101.
19. Ollis, D. Slick solutions for oil spills. Nature., 1992; 358: 453–454.
20. Okoh, A.I. Biodegradation of Bony light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow station saver pits in Nigeria, African Biotech, 2003; 104.
21. Obuekwe, C.O., Al-Jadi, Z.K. and E.S. Al-Saleh, Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. Int. Biodeterior. Biodegrad., 2009; 63: 237- 297.
22. Okoh, A.I. Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnology and Molecular Biology Review., 2006; 1: 38–50.
23. Ogunbayo, A.O., Bello, R. A. Nwagbara, U. Bioremediation of engine oil contaminated site. Journal of Emerging Trends in Engineering and Applied Sciences, 2012; 3: 483-489.
24. Richter, B.C. and Kreitler, W.C. Geochemical techniques for identifying sources of ground water salinization, Boca Raton, Florida, C.K. Inc, CRC press, New York, 1993. ISBN 1-56670-000-0.
25. Ramos, J.L., E. Duque and M.I. Ramos-Gonzalez, 19. Survival in soils of an herbicde-resistant debris. Land farming of oil and oily Debris: Marine Pseudomonas putida strain bearing a recombinant tol plasmid. Applied Environ. Microbiol., 57: 260-266.
26. Robinson, P.M. and Garret, M.K. Identification of volatile sporostatic factors from cultures of Fusarium oxysporum, Trans. Brif. Mycol. Soc., 1969; 52: 293-299.
27. Robertson, B., Arhelger, S., Kinney, P.J. and Button, D.K. Hydrocarbon degradation in Alaskan Waters. Louisiana state University, Baton Rouge, LA., USA., 1973; 171-184.
28. Singh, A., Sar, B., Bennet, G.N. Isolation and characterization of benzene degrading bacteria from gasoline contaminated water. Clean Technol, 2009; 15: 286–289.
29. Sei, A. Fathepure, B.Z. Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake. J Appl Microbiol. 2009; 107: 2001–2008.
30. Shukor, M.Y., Dahalan. F.A., Jusoh, A.Z., Muse, R., Shamaan, N.A. and Syed, M.A. Characterization of a diesel-degrading strain isolated from a hydrocarbon-contaminated site. J Environ Biol., 2009; 30: 45–50.
31. U.S. EPA (U.S. Environmental Protection Agency), Underground storage tank corrective action technologies. Office of Research and Development, Washington, D.C. 1987; 625: 9-87.
32. Vogt, C., Kleinsteuber, S., Richnow, H.H. Anaerobic benzene degradation by bacteria. Microb Biotechnol. 2011; 4: 710–724.
33. Walkley, A. and Black, I.A. An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic Tasmania. Austral. J. Soil Res. 1934; 34: 545–553.
34. Zar, J.H. One-sample hypotheses. Biostatistical Analysis, ed 2. Englewood Cliffs, NJ Prentice-Hall, Inc., 1984; p 97-121.