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Bioremediation of total petroleum hydrocarbons using cotton plant (*Gossypium hirsutum*)and applying augmentation teqnique by inoculation with *Pseudomonas aeruginosa* and *Penicillium expansum*.

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Abstract : Cotton plant (*Gossypium hirsutum*) was used to remediate crude oil polluted soil applying augmentation teqnique. Results of physical and chemical analysis of soil revealed that soil was sandy loam, slightly alkaline pH, poor of total phosphorus. *Gossypium hirsutum* and its roots associated microorganisms applied to treat polluted soil withcrude oil (rhizoremedation) applying augmentation teqnique by inoculating polluted soil with *Pseudomona aeruginosas* bacteria and *Penicilliumexpansum* fungi. Total CFU count of bacteria was increased with time while total CFU fungal count was decreased. The best rhizoremediation value after two months was 97% of the treatment with combination of bacterial and fungal inoculum while the lowest value 89.5% was of polluted non-treated soil.
Key words: Total hydrocarbons, Rhizoremediation, Augmentation, *Gossypium hirsutum, Pseudomonas aeruginosa, Penicillium expansum.*

Introduction:

Bioremediation is the process of using organisms to remove different pollutants from the environment such as soil and ground water [1].In the last decades, physical, chemical, and thermal treatment methods had failed to decrease the problems of pollution because these techniques may just change pollution to another phase such as air pollution [2]. Bioremediation technology which leads to breakdown of contaminants, could be a profitable and environmentally beneficial alternative that could be a source of economic profit [3].

Crude oil will be transported for long distances from producing refineries by water ports in tankers or on land pipeline and both ways could be prone to accidents and petroleum spills. The amounts of natural petroleum seepage is estimated about 600,000 metric tons per year, sometimes more than that [4].

Petroleum hydrocarbons accumulation in the environment is consider as a serious direct threat for living organisms and bio-ecosystems [5], especially when they enter the food chain, and human health including: prenatal toxicity, lung cancer, skin diseases, leukemia, and adverse effects on the reproduction, because of most of them are more persistent compounds, carcinogenic as in aliphatic hydrocarbons and some of polycyclic aromatic hydrocarbons (PAHs), causing long term environmental effects [6,7].

There are two main approaches for the bio-remediation of petroleum contaminated soil: microbial remediation and plant remediation (phytoremediation) [8].Several isolated fungal and bacterial species have been reported as an effective petroleum hydrocarbons degraders even polycyclic aromatic hydrocarbons [9].The microbial diversity makes it possible to break down a large number of different organic chemicals [10], is correlated with the genetic potential of the certain microorganism to introduce oxygen molecule into the

hydrocarbon and then to generate intermediate compounds that enter the general metabolic pathway to yield energy for the cell subsequently [11].

Also researchers reported that phytoremediation of petroleum contaminated soils has shown that some plants (including *Gossipum hirsutum*) could contain, translocate and/or volatilize petroleumhydrocarbons as they grow on petroleum contaminated soils, although not without growth problems like leaf burn, wilting and stunted growth [12,13].

The rhizosphere region is the region of vegetative roots, the use of rhizoremediation to detoxify hydrocarbons has increasing acceptance as a viable cleanup technology [14,15]. The synergism between plants and their associated microbes resulted in higher rate of degradation of petroleum contaminants than microbial remediation and phytoremediation [16]. Plant roots secrete organic and inorganic materials to their ambient environment during metabolism process. These exudates will act as substrates for soil microbes, therefore enhancing the breakdown of toxic organic chemicals [17].

The current study aimed to evaluate the ability of cotton plant and some species of microorganisms to remediate total petroleum hydrocarbons from crude oil polluted soil.

Materials and methods:

1. Collection of uncontaminated soil:

Uncontaminated soil were collected from Al-Tajiyah region, Hilla city, Babylon Province, Iraq, taken from the upper layer (25-30 cm in depth) of the soil, dried by air and sieved.

2. Collection of crude oil:

It was collected from Al-Najaf Petroleum Refinery, Iraq.

3. Addition of crude oil to uncontaminated soil:

Medium crude oil was obtained from Al-Najaf Oil Refinery, 75 gm of crude oil has been added to each kg of uncontaminated soil, mixed very well and let for two weeks to dry by air to allow volatilization of a volatile compounds. After that, pots were underlined with aluminum foil. 5 kg of oil contaminated soil were put in each pot, all pots were firstly watered to full extent with water and then laid for 3 days in order to make fully blended of the petroleum, soil and water to reach stable state [18].

4.Analysis of physical and Chemical properties of soil:

Physical and chemical properties of soil were measured three times: at the beginning of the experiment, after one month and after two months at the end of the experiment.

a. Temperature:

Temperature of soil was measured by soil celosias thermometer. The thermometer was inserted into the soil up to depth of 5 cm and allowed to stay for 10 minutes, after which the temperature reading was obtained.

b. Soil texture: It was measured according to [19] by using bouyoucos method.

c.Moisture: It was calculated depending on the difference between wet and dry weight according to [19].

d. pH and salinity: It was measured according to [20], by using pH meter (Hanna / 214)in the soil extract 1:1.

e. Total phosphorus: It was measured according to [20] by digesting with $HCLO_4$ 60%.

5. Inoculum build-up:

Pseudomonas aeruginosa and *Penicillium expansum* were isolated from Al-Najaf Oil Refinery polluted soil, as the most dominant species of bacteria and fungi in crude oil polluted soil of the refinery which can live and utilize hydrocarbons.

A. Bacterial inoculum:

*Pseudomonasaeruginosa*was inoculated into nutrient broth and incubated at 37° C. The bacterial count was carried out by measuring absorbance using spectrophotometer at an absorbance of 560 nm wavelength, until a cell concentration of $1.5 * 10^8$ colony forming units (CFU)/ml (1 McFarland Standard) was achieved [21].

B. Fungal inoculum:

It was prepared according to [22]by removing spores of *Penicillium expansum*from the surface of cultivatedpotato dextrose agar with a sterilized needle to be suspended, then filtrate or centrifuged and using direct method to calculate the spores concentration using haemocytometer and applying the equation:

*No. of spores/ml = average of spores number in four sq. * 10^4*

6. Experimental design:

Experiments were involved cultivating polluted soil with *Gossypium hirsutum* plant and addition of bacterial and fungal inoculation. Sampling was each two weeksfor two months from April to May, 2016. The treatments with three replicates as follows:

1. Unpolluted soil (control) cultivated with*G. hirsutum.*
2. Crude oil polluted soilcultivated with*G. hirsutum.*
3. Crude oil polluted soilcultivated with*G. hirsutum*inoculated with *Pseudomonas aeruginosa.*
4. Crude oil polluted soilcultivated with*G. hirsutum*inoculated with *Penicillium expansum.*
5. Crude oil polluted soilcultivated with*G. hirsutum*inoculated with combination of *Pseudomonas aeruginosa*and *Penicillium expansum.*

7. Dilution of samples and Microbial population count:

1 g soil from rhizosphere region were taken each two weeks with three replicates for each treatment. The samples were processed using soil dilution plate method, dilution was up to $10^{10}$to avoid crowded growth in the plate, and then 0.1 ml of dilution was taken and added to 20 ml of nutrient agar medium for bacteria and potato dextrose agar for fungi, in 90 mm diameter sterile Petri dishes.

Soil samples after serial dilution plates were incubated with 37S℃ for 48 hours to grow the bacterial colonies properly and with 25℃ for7 days for fungi, then enumerated. Colony Forming Units (CFU) were counted by using a colony counter[21], then applying the following equation:

$$CFU / ml = number\ of\ colonies \times \frac{1}{dilution\ \ factor} \times plating\ \ \ factor$$

8. Extraction of total hydrocarbons from Soil:

The crude oil polluted soil was extracted using 10 ml of 1:1 Ethanol/Chloroform mixture to extract the crude oil from 1 g of each soil sample which is collected from rhizosphere region. Then using silica gel column to remove other compounds. The collected were left at room temperature for 30 minutes and the optical density (absorbance) was read at the wavelength of 520 nm using spectrophotometer [23].

Results and Discussion:

1. Physical and chemical properties of soil:

Physical and chemical properties of soil were measured three times, at the beginning of the experiment at 25℃, after one month at 29℃ and after two months at 43℃. The measured properties were pH, salinity, moisture, and total phosphorus. Results revealed that soil is sandy loam composed of clay 28%, silt 32% and sand 40%, negligible concentrations of total phosphorus and it is slight alkaline.

The results showed there is a graduated decrease from the beginning of the experiment until the end of the experiment for all parameters measured for polluted soil cultivated with *Gossypium hirsutum* except of temperature and moisture content were increased with time. There is a significant decrease of moisture content and a significant increase of pH and nitrate concentration in comparison with unpolluted soil at the beginning of the experiment (table 1).

After one month results revealed also a significant increase of pH and salinity, and a significant decrease of moisture in comparison with unpolluted soil (table 2).

At the end of the experiment results indicated that there is a significant difference between unpolluted soil and polluted soil for salinity and moisture as in table (3).

Table 1: physical and chemical properties of soil at the beginning of the experiment at 25°C

No.	Treatments	pH	Salinity ‰	Moisture %	Total Phosphorus%
1	polluted soil + *G. hirsutum* (control)	7.73 ± 0.58	1000.3 ± 0.58	19.47± 0.15	Nill
2	polluted soil + *G. hirsutum*	7.93± 0.58	1000.4± 0.40	10.70 ± 0.21	Nill
3	polluted soil +*G. hirsutum* + *P. aeruginosa*	7.96± 0.58	1000.4 ± 0.53	10.14± 0.04	Nill
4	polluted soil+*G. hirsutum*+ *P. expansum*	8.03± 0.58	1000.3 ± 0.58	10.66± 0.06	Nill
5	polluted soil +*G. hirsutum* + *P. aeruginosa*+ *P. expansum*	8.0± 0.10	1000.4 ± 0.53	10.38 ± 0.15	Nill
6	LSD (0.05)	0.1	N.S	0.14	N.S

* Each value represents mean ± standard deviation.
* Nill = negligible value.
* N.S = non-significant difference.

Table 2: physical and chemical properties of soil after one month at 29°C.

No.	Treatments	pH	Salinity ‰	Moisture %	Total Phosphorus%
1	polluted soil + *G. hirsutum* (control)	7.70± 0.00	718.33± 0.06	19.80± 0.40	Nill
2	polluted soil + *G. hirsutum*	7.93± 0.06	822± 5.57	11.08± 0.17	Nill
3	polluted soil +*G. hirsutum* + *P. aeruginosa*	7.83± 0.06	778.67±5. 13	15.48± 0.25	Nill
4	polluted soil+*G. hirsutum* + *P. expansum*	7.93± 0.12	829± 4.00	12.04± 0.61	Nill
5	polluted soil +*G. hirsutum* + *P. aeruginosa*+ *P. expansum*	7.93± 0.06	737.33±27 .32	15.36± 0.45	Nill
6	LSD (0.05)	0.09	19.5	0.5	N.S

* Each value represents mean ± standard deviation.
* Nill = negligible value.
* N.S = non-significant difference.

Table 3: physical and chemical properties of soil after two months at 43°C

No.	Treatments	pH	Salinity ‰	Moisture %	Total Phosphorus %
1	polluted soil + *G. hirsutum* (control)	7.66± 0.06	506.33±17 .21	18.16± 0.38	Nill
2	polluted soil + *G. hirsutum*	7.83± 0.06	629.33±17 .01	13.26± 0.41	Nill
3	polluted soil +*G. hirsutum* + *P. aeruginosa*	7.73± 0.15	536.67±25 .5	16.36± 0.58	Nill
4	polluted soil+*G. hirsutum* + *P. expansum*	7.83± 0.06	599.33±19 .14	13.46± 0.42	Nill
5	polluted soil +*G. hirsutum* + *P. aeruginosa*+ *P. expansum*	7.76± 0.06	539.67±28 .02	16.30± 0.36	Nill
6	LSD (0.05)	N.S	32.33	0.6	N.S

* Each value represents mean ± standard deviation.
* Nill = negligible value.
* N.S = non-significant difference.

Petroleum pollution, has a negative effects on agricultural soils by changing the physical and chemical properties of the soil and hence significant negative effects on plant life[24].

Soil texture is an important to study soil because amount and type of clay which is present in the soil can affect the soil matrix, and therefore, microorganisms presence and activity. [25] examined the effect of petroleum spills on soil properties, the soil chemical parameters indicated that phosphorus decreased from 15ppm in control to between 7.34 and 5.42 in the soil contaminated with elevated concentrations of petroleum.

pH could affects growth of plant byeffecting on the availability of nutrients . Low or high pH causes deficiencies of nutrients which are essential for plants growth. Soil pH increase after pollution attributed to microorganisms degradation of petroleumwithin anaerobic conditions of soil, indicating the effect of petroleum spills to raise soil pH. The released CO2 contributed to the alkalinity in the treatment medium [26]. While the oil may have had some direct impact in lowering the pH, it is also possible that microbial actions through metabolic process contributed to changes in pH by producing organic acids [27].

From results, salinity was decreased with time, it is effecting bioremediation rate as explained by [28]that biodegradation rate will drop off if soil salinity increases beyond an optimum level.

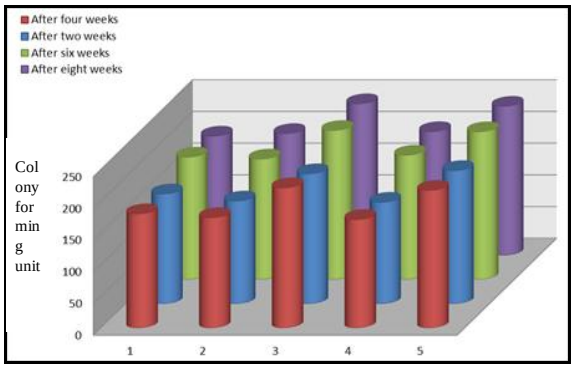
Soil moisture decreases with oil pollution and can influence the biodegradation rate due to its effects on hydrocarbon bioavailability, diffusion processes, transfer of produced gases, oxygen availability in the soil, and soil toxicity level [29].

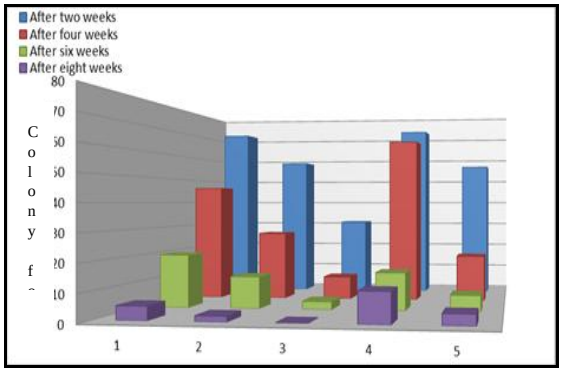
Results indicated that soil was poor of phosphorus, as a reason of alkaline pH, alkaline soils demonstrate deficiencies in phosphorus [30].

2. Total bacterial and fungal count:

The results revealed that total bacterial count of crude oil polluted soil cultivated with *Gossypium hirsutum* was decreased significantly at the untreated polluted soil and polluted soil treated with fungi in comparison with total bacterial count of unpolluted cultivated soil (control), while the total count increased significantly at the treatments polluted soil treated with bacteria and polluted soil treated with bacteria and fungi (figure 1).

Total fungal count of crude oil polluted soil was decreased significantly at untreated crude oil polluted soil, treated with bacteria and the treatment with bacteria and fungi in comparison with total fungal count of cultivated unpolluted soil (control), the significant lowest value was for treatment with bacteria. While the total count increased significantly at polluted soil treated with fungi (figure 2).





LSD (0.05) treatment = 2.73 , LSD (0.05) time= 2.44LSD (0.05) treatment = 1.98, LSD (0.05) time = 1.77

Figure1: total bacterial count x 10^9 cfu/ml of the treatments (1: unpolluted soil, 2: untreated polluted soil, 3: polluted soil treated with *P. aeruginosa*, 4: polluted soil treated with *P. expansum*, 5: polluted soil treated with *P. aeruginosa* and *P. expansum*) during two months.

Figure 2 (reversed): total fungal count x 10^9 cfu/ml of the treatments (1: unpolluted soil, 2: untreated polluted soil, 3: polluted soil treated with *P. aeruginosa*, 4: polluted soil treated with *P. expansum*, 5: polluted soil treated with *P. aeruginosa* and *P. expansum*) during two months.

The results of rhizosphere total CFU bacterial count of crude oil polluted soil indicating a significant decrease of total count for polluted soil which is not treated with bacteria in comparison with control of polluted soil. These results are in line with [18] who treated soil with 75gm/kg crude oil and cultivating soil with *Zea mays* for two months, she found that the total CFU bacterial count of polluted soil was less than that of unpolluted soil of rhizosphere. According to [31], if crude oil contacted with the soil the result is the damage of the plants and agricultural lands with their microorganisms.

Some microorganisms will be effected by reduced permeability of their cell membrane, entirely blocking or lowering the ability to take nutrients which finally leads to the starvation and death. Another effects is by direct exposure to the toxic and growth inhibiting chemicals [32].

The total CFU count of bacteria is effected by soil chemical and physical parameters and by hydrocarbon's type. Also soil composition represents an important factor effecting the function, activity and diversity of soil microorganisms. It is reported that clay prevents the negative effects of crude oil from effecting soil microorganisms, hydrocarbons may lead to decrease the total count from unpolluted soil. High clay content, make hydrocarbons tend to sorption resulted in decreasing its bioavailability which lead tohighCFU total count in comparison with sandy soil, or textured soil [33],while the results of this study revealed that soil texture was sandy loam(28% for clay),which express the effect of hydrocarbons on total CFU count.

Soil pH represents an important factor whicheffectingthe diversity of soil microorganism. Low pH values are related with high heterotrophic total bacterial CFU count [34], in comparison with the high values of soil pH of current study, it was slightly alkaline pH $\geq$ 7.7.

Total CFU bacterial count were increased with the passage of time, this is as a result of increased moisture content and temperature. Temperature effecting rates of bio-chemical reactions, and the rates of several reactions are double for each 10 °C elevation of temperature, also vialable water is essential for microorganisms [35].

Treatments with bacteria (bioaugmentation) revealed a higher total count than unpolluted soil as a result of addition of *Pseudomonas* inoculum and success of this bacteria to live in crude oil polluted soil. Native or indigenous microorganisms are found in small quantities which cannot prevent the contamination from being spread because they don't have the ability to breakdown a particular contaminant or they can be in an inactive metabolic form in their environments, this explains why bioaugmentation is favorable over biostimulation [36]. *Pseudomonas aeruginosa* can live and survive in high concentrations of hydrocarbons (up to 50% v/v) and utilizinghydrocarbons,the capacity of *Pseudomonas aeruginosa* to degrade crude oil compounds make it the most active hydrocarbons utilizer [37].

Total CFU fungal counts of polluted soil were decreased significantly with the passage of time even at treatments with *Penicillium expansum* the lower significant value was for the treatment with *Pseudomonas*.

These results are similar to the results of the study of[38] who investigated the ability of two fungal species *Aspergillus* and *Penicillium* species predominated at acidic and alkaline soil pH. He found that the lowest growth of*Aspergillus* was at alkaline pH (8 - 8.5), while *Aspergillus* was predominant and *Penicillium* was not observed. Conversely, fungal populations were greatest at acidic soil pH =5.5. While studied soil pH was alkaline which is not suitable for many fungal species includes *Penicillium.*

Total CFU count were decreased with the increased temperature. Moisture content and temperature arevery important factors that effecting microorganism's life and activity in soil, fungi are adapted to tolerate low soil moisture than bacteria [39]. [40] found that bacterial and fungal growth need to live in temperature ranges between 25 and 30 C, while at high temperature total count will be decreased. Fungi would be effected more than bacteria, which resulted in high enumeration of bacteria and less for fungi. Fungi tends to live in low temperatures.

Another important cause for decreasing total CFU fungal count especially of soil treated with bacteria, is the antagonism relationship between *Pseudomonas* bacteria and *Penicillium expansum*. This was documented by [41] when used *Pseudomonas* as a biocontrol of *Penicillium expansum*, the results showed that bacteria inhibited fungal growth to 78.5%.

3. Total hydrocarbons:

Results showed a significant decrease of total hydrocarbons concentrations for all treatments each two weeks. A significant decrease was statistically resulted of polluted cultivated soil treated with bacteria, fungi and the combination from untreated polluted cultivated soil. The best remediation percentage was for the treatment with combination (bacteria + fungi) 97% followed by bacteria 96.82% and fungi 93.7% in comparison with untreated polluted cultivated soil 89.5 %.

Table 4: Total hydrocarbons concentrations mg/gm in crude oil polluted soil cultivated with *Gossypium hirsutum* during two months

No.	Treatments	After two weeks	After four weeks	After six weeks	After eight weeks	Remediation %
1	polluted soil + *G. hirsutum* (control)	N.D	N.D	N.D	N.D	0
2	polluted soil + *G. hirsutum*	0.076± 0.004	0.057± 0.002	0.048± 0.005	0.04± 0.030	89.5
3	polluted soil + *G. hirsutum* + *P. aeruginosa*	0.054± 0.001	0.031± 0.001	0.019± 0.001	0.015± 0.001	96.82
4	polluted soil+ *G. hirsutum* + *P. expansum*	0.058± 0.005	0.042± 0.003	0.034± 0.003	0.024± 0.001	93.7
5	polluted soil + *G. hirsutum* + *P. aeruginosa* + *P. expansum*	0.05± 0.030	0.035± 0.004	0.026± 0.001	0.011± 0.001	97
6	LSD (0.05) Treatment= 0.006 Time = 0.006 Inter =0.0134					2.285

*Each value represents mean ± standard deviation

* N.D = not detected

Results of rhizoremediation showed that there are numerous pollutants of crude oil solvents and products are remediated faster in plant cultivated soils because of processes of water transpiration, transport of oxygen, bio-stimulation in the rhizosphere region and the uptake of chemicals by plants are considered to be an influencing processes[42].

The soil pollution with hydrocarbons results in a rapid changes in the structure of microbial community, an increasing number of hydrocarbons degraders and a rapid rate of petroleum breakdown, which referred to the presence of a preadapted, petroleum degrader microorganisms and sufficient levels of nutrients [43], which explains the ability of microorganisms of untreated cultivated soil (without augmentation) to breakdown hydrocarbons.

The remediation rates by microbes and phytoremediation greatly differ. Microbial remediation can be achieved by several microorganisms' species that are either indigenous in the soil or added as good degraders(bioaugmentation)[8]. [16]referred to that the synergism between plants and their microorganisms(rhizoremediation) revealed a higher bio-degradation rate of hydrocarbons than microbial remediation and phytoremediation.

Several plant species are effective in degrading total petroleum hydrocarbons (TPH). The TPH degradation rates among various plant species depends on the microbial population in the rhizosphere of these plants [44]. Rooting type and intensity is the key factor leading to high TPH loss rates, and root development is important in evaluating the phytoremediation potential [45].

Conclusion:

Bioremediation is a good method to treat crude oil polluted soil also augmentation increases the efficiency of bioremediation.

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References:

1. Chehregani A, Noori M, Yazdi LH.Phytoremediation of heavy metal polluted soils: screening for new accumulator plants and evaluation of removal ability. J Ecotoxicol Environ Safety,2009, 72:1349–1353.
2. Abioye PO. Biological remediation of hydrocarbon and heavy metals contaminated soil. InTech, 2011, 8: 142.
3. Iranzo M, Sainz-Padro I, Boluda R, Sanchez J, Mormeneo S. The Use of Microorganisms in Environmental Engineering, Annals of Microbiology, 2011, 51: 135-143.
4. Kvenvolden, K.A., Cooper, C.K. (2003). Natural seepage of crude oil into the marine environment. Geo-Marine Letters 23(3-4): 140 – 146.
5. Bonnet S, Guieu C, Chiaverini J. Impact of atmospheric inputs on the autotrophic communities in a low nutrient low chlorophyll system. Limnol. Oceanogr, 2005, 50(6): 1810–1819.
6. Evans M, Singh. DNA economic evaluation of three varieties of pearl millet compared to grain sorghum in diets for laying hens and broilers. Proceedings of the 17th Australian Poultry Science Symposium, Sydney, New South Wales, Australia, 2005, 301-302.
7. Chakroun H, Mouillot F, Nasr Z, Nouri M, Ennajah A, Ourcival JM. 35 Perfomance of LAI MODIS and the influence on drought simulation in a Mediterranean 36 forest. Ecohydrology. 2005. DOI 10.1002/eco.1426 .
8. Tang JC, Wang RG, Niu XW, Wang M, Chu HR, Zhou QX. Haracterisation of the rhizoremediation of petroleum-contaminated soil: effect of different influencing factors Biogeosciences, 2010, 7: 3961–3969.
9. Marquez-Rocha FJJ, Olmos-Soto. Rosano-Hernandez CM. Muriel-Garcia M. Determination of the hydrocarbon-degrading metabolic capabilities of tropical bacterial isolates. Int. biodeterior. Biodegrad., 2005, 55: 17-23.
10. Malik P. India's Universal Service for telecom: Policy and Regulatory Gaps. Forthcoming in Connectivity: Beyond Technology, Samarjiva R, Zainudeen (eds.), Sage Publication, India, 2006.
11. Millioli VS, Servulo EC, Sobral LGS,Carvalho DD. Bioremediation of crude oil bearing soil: evaluation the effect of Rhamnolipid addition to soil toxicity and to crude oil biodegradation efficiency. Glubal NEST Journal, 2009, 11(2): 181-188.

12. Wiltse CC, Rooney WL, Chen Z, Schwab AP, Banks MK. Greenhouse evaluation of agronomic and crude oil-phytoremediation potential among alfalfa genotypes. J. Environ. Qual.,1998, 27: 169–173.
13. Ayotamuno MJ, Kogbara RB, Agunwamba JC. "Bioremediation of petroleum-hydrocarbon polluted agricultural soil at various levels of soil tillage in Port Harcourt, Nigeria. " Nigerian Journal of Technology. 2006, 25 (1): 44-51.
14. Alkorta I, Garbisu C. Phytoextraction: A cost effective plant-based technology for the removal of metals from the environment. Biores Technol. 2001, 77(3):229–236.
15. Arthur L, Beecher B, Death E, Dockett S,Farmer S. Programming and Planning in Early Childhood Settings, (3 rd ed.). Southbank, Victoria: Thomson. 2005.
16. Gurska J, Wang WX, Gerhardt KE, Khalid AM, Isherwood DM, Huang XD, Glick BR, Greenberg, BM. Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste, Environ. Sci. Technol., 2009, 43(12): 4472– 4479.
17. Anderson TA, Guthrie EA, Walton BT. Bioremediation in the rhizosphere. Env Sci Technol. 1993, 27:2630–2636.
18. Aburas MM. Enhancing Bioremediation of Saudi Desert Soils Polluted with Hydrocarbons. Doctoral thesis, University of Surrey. 2010.
19. Bashour II, Sayegh AH. Methods of Analysis for Soils of Arid and Semi-Arid Regions. 1st Edn., FAO, USA. ISBN: 2007, 978-92-5-105661-5 p. 119.
20. Ryan J,Estefan J. Rashid A. Soil and Plant Analysis Laboratory Manual. Jointly published by the International Centre Agricultural Research in Dry Areas (ICARDA), Aleppo, Syria and National Agric. Res. Centre (NARC), Islamabad. 2001.
21. Nandhini B, Mary Josephine R. A study on bacterial and fungal diversity in potted soil. *Int.J.Curr.Microbiol.App.Sci*2013, 2(2): 1-5
22. Sarhan, ART. Practical Mycology. First Edition. 2012.
23. Ezeonu CS. Phytoremediation and crude oil bioaccumulation potential of *Zea mays L*. Jour.of Environ.Sci. Toxicol.and Food Techno.2013,**7**: 24-26.
24. Agbogidi OM, Eruotor PG, Akparabi SO. Effects of Time of Application of Crude Oil to Soil on the Growth of Maize (Zea mays L.) Res. Environ. Toxicol. 2007,1(3): 116- 123.
25. Wokocha GA, Emeodu D, Ihenko S. Impact of Crude Oil Spillage on Soil and Food Production in Rivers State, Nigeria.Journal of Money, Investment and Banking. 2001, ISSN 1450-288X Issue 19.
26. 26.Essien, OE, John IA. Impact of crude-oil spillage pollution and chemical remediation on agricultural soil properties and crop growth. J. Appl. Sci. Environ. Management. 2010, 14 (4): 147 – 154.
27. Manahan SE. Environmental Chemistry.CRC Press, Inc. Florida. 1994, pp. 811.
28. Walworth J, Pond A, Snape I, Rayner J, Ferguson S, Harvey P. Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. 2007.
29. Yang SZ, Jin HJ, Wei Z, He RX, Ji YJ, Li XM,Yu SP. Bioremediation of oil spills in cold environments: A review. Pedosphere, 2009, 19 (3):371-381.
30. Hopkins B, Ellsworth. Phosphorus availability with alkaline/calcareous soil. Western Nutrient Management Conference. Salt Lake City, UT. 2005.vol. 6.
31. Onuoha CI, Arinze AE, Ataga AE. Evaluation of growth of some fungi in crude oil polluted environment. Glob. J. Agric. Sci. ISSN 2003, (2): 1596-2903.
32. Pezeshki, SR, Hester MW, Lin Q, Nyman JA. The effects of oil spill and clean-up on dominant US Gulf coast marsh macrophytes: a review. Environmental Pollution, 2000, 108: 129-139.
33. Labud V, Garcia C, Hernandez T. Effect of hydrocarbon pollution on the microbial properties of a sandy and a clay soil. Chemosphere, 2007, 66: 1863-1871.
34. 34.Killham. K. Soil Ecology. (Fourth ed.)Cambridge University Press, Cambridge, UK. 2006.
35. Thapa SB. Evaluation of twenty bermudagrass cultivars for their drought resistance. Master thesis. Oklahoma State University. Nepal. 2011.
36. Annibale A, Sermanni GG, Federici F, Petruccioli M. Olive-mill wastewaters: a promising substrate for microbial lipase production. Bioresour. Technol. 2006, 97: 1828 – 1833.
37. Prakash B, Irfan M.*Pseudomonas aeruginosa* is Present in Crude Oil Contaminated Sites of Barmer Region (India). J Bioremed Biodegrad 2011, 2:129. doi:10.4172/2155-6199.1000129.
38. Pawar RM. The Effect of Soil pH on Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHS). J Bioremed Biodeg, 2015, 6:291. doi:10.4172/2155 6199.1000291.
39. Paul EA, Clark FE. Soil Microbiology, Ecology, and Biochemistry. USA: Academic Press; 1996.

40. Pietikainen, M, Valiheikki A, Oravisjarvi K, Kolli T, Huuhtanen M, Niemi S, Virtanen S, Karhu T, Keiski RL. Particle and NOx emissions of a non-road diesel engine with an SCR unit: The effect of fuel, Renewable Energy. 2015, 77: 377-385.
41. Etebarian HR, Sholberg PL, Eastwell KC, Sayler RJ. Biological control of apple blue mold with Pseudomonas fluorescens Can. J. Microbiol. 2005, 51: 591–598.
42. Trapp SA, Ucisik S, Romano D,Larsen M. "The Role of Plants and Bacteria in Phytoremediation-Kinetic Aspects Bioremediation of Soils Contaminated with Aromatic Compounds in NATO Science Series IV," Earth Environmental Science, 2007, 76: 41-49.
43. Hamamura N, Olson SH, Ward DM, Inskeep WP. Microbial population dynamics associated with crude-oil biodegradation in diverse soils. Appl Environ Microbiol 2006, 72: 6316–6324.
44. Shirdam R, Zand AD, Bidhendi GN, Mehrdadi N. Phytoremediation of hydrocarbon-contaminated soils with emphasis on the effect of petroleum hydrocarbons on the growth of plant species, Phytoprotection, 2008, 89, 21–29,.
45. Hou, FS, Milke MW, Leung DW, MacPherson DJ. Variations in phytoremediation performance with dieselcontaminated soil, Environ. Technol., 2001, 22(2): 215–22.

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