International Journal of Environmental Bioremediation & Biodegradation, 2016, Vol. 4, No. 1, 8-12
Available online at http://pubs.sciepub.com/ijebb/4/1/2
© Science and Education Publishing
DOI:10.12691/ijebb-4-1-2

Enhanced Removal of Crude Oil in Soil by Mixed Culture of *Bacillus Megaterium* UL05 and *Pseudomonas Aeruginosa* UL07

Maryam L. Riskuwa-Shehu[1,*], Udeme J.J. Ijah[2]

[1]Department of Microbiology, Usmanu Danfodiyo University Sokoto, Sokoto state, Nigeria
[2]Department of Microbiology, Federal University of Technology, Minna, Niger state, Nigeria
*Corresponding author: mlriskuwa@hotmail.com

Abstract Oil degrading bacteria were isolated from polluted soil from Eremu, Delta State, Nigeria and identified as species of *Bacillus, Pseudomonas, Alcaligenes* and *Micrococcus.* The bacteria were able to utilize Ubefan (Nigerian) light crude oil as a source of carbon and energy. Two isolates, *Bacillus megaterium* UL05 and *Pseudomonas aeruginosa* UL07, which had very high ability in utilizing the crude oil, were used as mixed culture for bioremediation studies. Results showed that addition of the mixed bacterial culture to oil polluted soil caused a decreased in pH of the soil and a reduction in the peaks of the soil components as revealed by the GLC analysis. The hydrocarbon components ($C_5 - C_{12}$) were extensively degraded in all treatments. In oil polluted soil inoculated with bacteria, C14 – C38 were highly degraded as compared to the control non-bioaugmented soil. These results suggest that *Bacillus megaterium* UL05 and *Pseudomonas aeruginosa* UL07 can be useful in treating oil spills in tropical soil.

Keywords: *bioremediation, enhanced removal, bacterial, crude oil, mixed culture, soil*

Cite This Article: Maryam L. Riskuwa-Shehu, and Udeme J.J. Ijah, "Enhanced Removal of Crude Oil in Soil by Mixed Culture of *Bacillus Megaterium* UL05 and *Pseudomonas Aeruginosa* UL07." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 4, no. 1 (2016): 8-12. doi: 10.12691/ijebb-4-1-2.

1. Introduction

Biodegradation is the natural process whereby microorganisms breakdown organic molecules into other substances such as fatty acids, carbondioxide and water. This process helps to rid the environment of pollutants. However, the natural process of biodegradation is slow due to inadequate nutrients (nitrogen and phosphorus), temperature, unfavorable pH and insufficient competent microorganisms to degrade the pollutant. In terms of use of bacteria for bioaugmentation, bacterial inocula from the temperate region may not degrade oil spills efficiently in the tropics. Therefore there is the need to develop microbial inocula indigenous to an area where spills occur. Bioremediation of oil spills is important in regions where there are frequent oil spills and difficult terrain for cleanup operation by mechanical means.

Although many microbial isolates from the environment have been reported to degrade crude oil [1,2,3,4] the need to screen the microbial inocula still arises. This will allow for the selection of more competent microbial inocula for oil spill remediation in polluted soil. Studies in our laboratory indicate that when oil polluted soil was inoculated with *Flavobacterium* sp., 58.5% of the oil was degraded as compared to 30% oil degradation in soil not seeded with bacteria [5]. Other investigators [6,7,8,9,10] have recorded a lot of success with the use of single or mixed microbial culture for oil spill remediation. In most instances, the microbial inocula are used in conjunction with nutrients, particularly nitrogen and phosphorus before realizable results can be achieved. The aim of the present study was to evaluate the potential of a mixed culture of *Bacillus megaterium* UL05 and *Pseudomonas aeruginosa* UL07, without the addition of nutrients to speed up oil biodegradation in soil. The patterns of soil biodegradation were also discerned. This is a simple and less expensive way of tackling oil spills in the creeks and other areas of unreaching difficult terrain in Nigeria and other parts of the world. Besides, this means of rehabilitation does not pose ecological problems.

2. Materials and Methods

2.1. Collection of Sample

Oil polluted soil samples were collected from different points at the oil spilled site of Eremu, Delta State, Nigeria and bulked. Equally, non-oil polluted soil samples were collected. The soil samples were transported to the laboratory for the isolation of bacteria. Crude oil used was Ubefan (Nigerian) light crude oil. The oil was collected from the Shell Petroleum Development Company of Nigeria Limited, Warri, Delta State, Nigeria.

2.2 Isolation and Identification of Bacteria

Serially diluted soil samples were plated on Nutrient agar (NA) and incubated at 35^0C for 48 hours. Colonies which developed on the plates were characterized based on Gram's reaction and biochemical tests including carbohydrate utilization profiles. The microorganisms were identified using the schemes of Bergey's Manual of Determinative Bacteriology [11].

2.3. Screening of Bacteria for Potential to Utilize Crude Oil

Twenty bacterial isolates were screened for potential to utilize crude oil. The isolates were grown in Nutrient broth (NB) and incubated at 35°C for 24 hours. Then 1ml (10^8 cells) of the NB grown culture was introduced into test tubes containing 5ml of oil broth (1.7g K_2HPO_4, 1.32g KH_2PO_4, 1.26g NH_4CL, 0.01g $MgCL_2.6H_2O$, 0.02g $CaCl_2$, 100ml diluted water, 1ml of filtered sterilized crude oil, pH 7.4). The inoculated tubes were incubated undisturbed at 35°C for 7 days. After the incubation the extent of oil utilization by the isolates were assessed by visual observation of the turbidity of the medium [12]. The extent of bacterial growth was represented as maximum growth (+++), moderate growth (++), minimal growth (+) and no growth (-).

2.4. Enhanced Biodegradation Studies

Two hundred grams of soil was placed in plastic containers (CP) and treated with 30% crude oil and mixed bacterial culture (*Bacillus megaterium* UL05 and *Pseudomonas aeruginosa* UL07). The control soil had no added bacteria. The experiments set up in duplicates were incubated at 35°C for 10days. At 2 days interval, the residential crude oil was extracted from the soil using an organic solvent (diethyl ether) and the level of microbial degradation of the oil was determined using the weight loss method [13]. The percentage weight loss was calculated using the formula below:

$$\%\text{Weight loss of crude oil} = \frac{\left(\begin{array}{l}\text{weight of crude oil (control)} \\ -\text{weight of crude oil (degraded)}\end{array}\right)}{\text{weight of crude oil (control)}} \text{X100.}$$

After 10 days, the residual oil was also subjected to gas chromatographic analysis. One microlitre (1µL) of the extractable crude oil was diluted with 1µL of n-pentane and analyzed on a 25-m Cpsip CB capillary column (Chrompack, The Netherlands) installed in a capillary gas chromatograph (Packard Instruments, Delft, The Netherlands) equipped with a flame ionization detector (FID). Split injector was used with helium as carrier gas. The oven temperature was initially set at 45°C for 2min. and increased at rate of 10°C/min to 280°C. The hydrocarbons were identified by comparing with standard. The pH of the oil polluted soil was also monitored using pH meter (Jenway 3015, UK).

3. Results

3.1. Identification of Isolates and Their Crude Oil Utilizing Potential

Analysis of soil samples from oil polluted soil of Eremu, Delta State, Nigeria revealed twenty bacterial isolates belonging to four genera: *Bacillus (*13 isolates), *Pseudomonas* (4 isolates), *Micrococcus* (2 isolates), *Alcaligenes* (1 isolate) (Table 1). Testing the isolates for potential to utilize Ubefan light crude oil as a source of carbon and energy revealed that thirteen isolates (65%) were able to utilize the oil to varying extent (Table 1). Two isolates (10%), *Bacillus megaterium* UL05 and *Pseudomonas aeruginosa* UL07) utilized the crude oil at a maximum rate and were able to grow in the oil medium after 24 hours of incubation while 5 isolates (25%) utilized the oil moderately. Six isolates (30%) utilized the oil minimally whereas 7 isolates (35%) were unable to utilize the oil as a source of carbon and energy. The non-oil utilizing species belong to the genera, *Bacillus (*5 isolates), *Alcaligenes* (1 isolate) and *Micrococus* (1 isolate).

Table 1. Extent of Growth of Bacteria in Crude Oil Medium

Coded bacterial isolates	Growth in the crude oil medium
Bacillus brevis UL01	-
Bacillus brevis UL02	-
Pseudomonas deminuta UL03	+
Bacillus circulans UL04	-
Bacillus megaterium UL05	+++
Pseudomonas aerogenosa UL06	++
Pseudomonas aerogenosa UL07	+++
Bacillus thuringiensis UL08	++
Bacillus coagulans UL09	++
Bacillus alvei UL10	++
Alcaligenes faecalis UL11	-
Pseudomonas putida UL12	+
Bacillus macerans UL13	+
Pseudomonas mallei Ul14	+
Bacillus mycoides UL15	+
Bacillus brevis UL16	-
Micrococcus leteus UL17	-
Bacillus mycoides UL18	+
Bacillus panthothermicus UL19	-
Micrococcus varians UL20	++

+++: Maximum growth, ++: Moderate growth, +: Minimal growth, -: No growth.

3.2. Biodegradation of Crude Oil in Soil

Table 2. Weight Loss Of Crude Oil In Soil Inoculated With Mixed Bacterial Culture

Time (days)	Weight loss of crude oil (%)	
	Oil polluted soil	Oil polluted soil + mixed bacterial culture
2	8.5	15.6
4	15.8	26.8
6	20.4	40.5
8	35.6	65.8
10	48.2	75.5

The results (Table 2) revealed that, in both inoculated and uninoculated oil polluted soil, the rates of oil biodegradation increased from the 2nd to the 10th day of inoculation. 48.2% of the oil was degraded in uninoculated soil (not sterile control) after 10 days whereas 75.5% oil degradation was recorded in soil inoculated with bacteria over the same period. Statistical analysis of the data using Analysis of Variance (ANOVA) revealed significant differences ($p < 0.05$) in the extent of

oil degradation between the uninoculated and bacterial inoculated oil polluted soils.

pH of the soil was also monitored and the results revealed that the pH of the crude oil free soil ranged from 6.83 to 7.67 while that of the oil polluted soil inoculated with mixed bacterial culture ranged from 6.68 to 7.67 (Table 3).

Table 3. pH Of Crude Oil Polluted Soil Inoculated With Mixed Bacterial Culture

Time (days)	Crude oil free soil	Crude oil polluted soil	Crude oil polluted soil + mixed bacterial culture
0	6.83	7.82	7.15
2	7.41	6.93	6.68
4	7.50	7.34	7.67
6	7.49	7.84	6.84
8	7.67	7.20	6.80
10	7.42	6.90	6.70

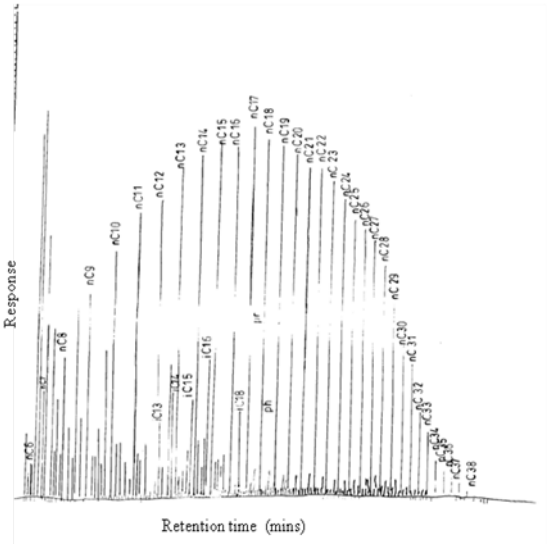

Figure 1. Chromatographic profile of Ubefan light crude oil (Undegraded)

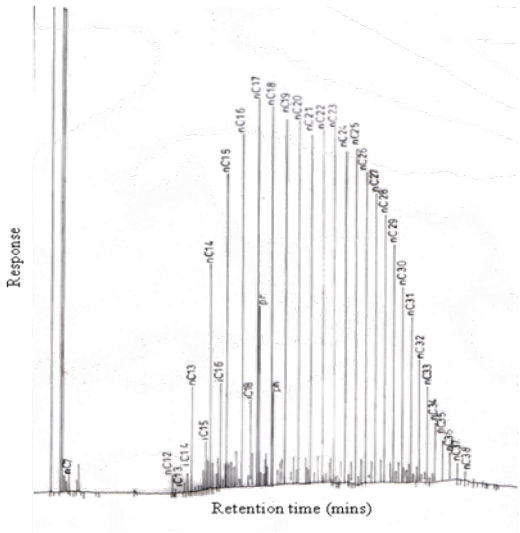

Figure 2. Chromatographic profile of Ubefan light crude oil after contact with unsterilized oil polluted soil for 10 days

The GC analysis of Ubefan light crude oil is presented in Figure 1 and showed a battery of chromatograms (C_8 – C_{38}). After exposure of the oil to microorganisms in the soil for 10 days, some components of the oil, particularly C_8 – C_{11} were completely lost while C_{12} –C_{15} were reduced in peak height, suggesting that degradation of the components has occurred (Figure 2). Other carbon components of the oil were intact. Figure 3 shows the GC analysis of the oil extract recovered from unsterilized oil polluted soil. While Figure 4 shows the chromatograms of oil extracted from sterilized oil polluted soil inoculated with bacteria. In both treatments the hydrocarbons were degraded but more hydrocarbon degradation occurred in unsterilized soil inoculated with bacteria (Figure 4) than the sterilized soil (Figure 3). It was also found that the isoprenoids (pristine and phytane) were degraded.

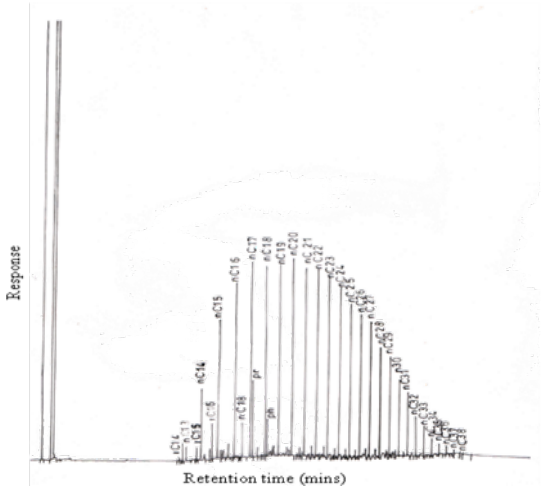

Figure 3. Chromatographic profiles of Ubefan light crude oil after contact with unsterilized soil plus bacteria for 10 days

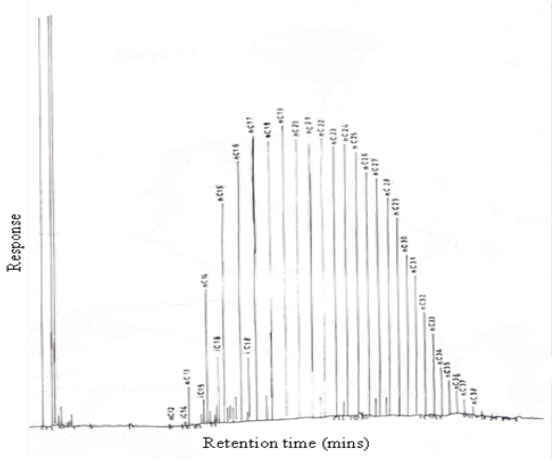

Figure 4. Chromatographic profiles of Ubefan light crude oil after contact with sterilized oil polluted soil plus bacteria for 10 days

4. Discussion

A lot of oil exploration and exploitation activities take place in oil producing areas. Consequently, the environment is continuously being polluted with oil leading to selective enrichment of the crude oil degraders. The microorganisms identified in this study are naturally occurring populations that participate in soil processes and having inherent ability to degrade petroleum hydrocarbons [14]. Similar organisms were also isolated by other investigators [7,10,15]. The varying capabilities of the isolates in metabolizing the oil may be due to their

hydrocarbon degradation enzyme systems. While some isolates may have strong affinity for the hydrocarbon substances, some may have to undergo a long period of lag due to inhibitory components of the oil before onset of biodegradation. These results are similar to those obtained in our earlier study [2,4] and recently by [15].

The considerably high ability of *Bacillus* sp. Strain UL05 and *Pseudomonas* sp strain UL07 in utilizing the oil prompted the choice of these two isolates for the bioremediation study. Species of *Bacillus* and *Pseudomonas* have been consistently isolated from oil polluted soil and implicated in crude oil biodegradation [7,16,17]. Most of the studies indicated that the effective ability of *Bacillus* and *Pseudomonas* species in crude oil biodegradation is caused by the active enzyme system for hydrocarbon metabolism. Besides, *Bacillus* species produce spores which help the bacteria to withstand harsh conditions and germinate when conditions become favorable.

These results indicate that the added mixed bacterial culture has enhanced the rate and total extent of crude oil biodegradation in the soil. Other investigators [17,18,19] have reported enhanced crude oil biodegradation in soil caused by inoculation of microbial slurry. Reference [10] emphasized that there is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil. Moreover, in nature biodegradation of crude oil typically involves a succession of species within the consortia of microbes present. Therefore, study of biodegradation by bacterial consortia is encouraged because such mixed cultures display metabolic versatility and superiority to pure cultures [20,21].

The reduction in pH to slight acidic range in oil polluted soil inoculated with MBC could be attributed to acidic metabolites resulting from oil biodegradation. However, the pH range observed in the present study still fall within the pH range suitable for microbial growth indicating that these isolates exhibited optimal growth at pH range of 6.0 to 8.0. Reference [22] reported that the growth of most microorganisms is usually greatest within a pH range of 6 to 8.

Isoprenoids have been reported to resist microbial attack [2,21]. These results indicate that the *Bacillus* sp. Strain UL05 and *Pseudomonas* sp. UL07 are competent oil degraders and could be useful in clearing oil spills in tropical environment. Reference [22] reported the removal of upto 75 to 100% of pristane and phytane by non-defined mixed culture obtained by enrichment in artificially weathered crude oil.

Most bioremediation studies involve the use of a combination of microorganisms and nutrients (nitrogen and phosphorus) but in the present study, the microbial inoculum functioned effectively without added nutrients, probably because there has been sufficient nutrient in the soil and therefore do not need additional nutrients to be added from external sources. The need for nutrients depends on the amount of nutrients in the soil [23]. This, in practical forms, will reduce cost and labour associated with bioremediation of oil spilled soil. The results also suggest that the added bacterial slurry was not inhibited by biodegradation process. In our laboratory, we have found in a separate study that *Bacillus* and *Pseudomonas* species can degrade crude oil and produce biosurfactants, which make them more effective in oil biodegradation. Mixed bacterial cultures capable of degrading Nigerian light crude oil have considerable potential in the bioremediation of crude oil, polluted soil.

References

[1] Ijah, U.J.J., and Ukpe, L.I., "Biodegradation of crude oil by *Bacillus* strains 28A and 61B isolated from oil spilled soil," *Waste Manag.* 12(1). 55-60, 1992.

[2] Ijah, U.J.J., Antai, .S.P., "Removal of Nigerian light crude oil in soil over a 12 month period, *Int. Biodeterior. Biodegrad.* 51, 93-99, 2003.

[3] Adebusoye, S.A., Ilori, M.O., Amund, O.O., Teniola, O.D., Olatope, S.O., "Microbial degradation of petroleum hydrocarbons in a polluted tropical stream," *W. J. Microbiol. Biotechnol.* 23, 1149-1159, 2007.

[4] Ibrahim, M.L., Manga, S.B., Rabah, A.B., Ijah, U.J.J., "Biodegradation of Escravos light crude oil by bacteria isolated from the rhizosphere of *Eucalyptus camaldulensis*, *Lablab purpureus* and *Moringa oleifera*," In *Proceedings of Biotechnology for Improved Production of Oil and Gas in the Gulf of Guinea* 3, 4-14.

[5] Ijah, U.J.J. "Accelerated crude oil biodegradation in soil by inoculation with bacterial slurry," *J. Environ. Sci.* 5, 38-47, 2002.

[6] Salleh, A.B., Mohamad Ghazali F., Abd Rahman, R.N.Z. and Basri, M., "Bioremediation of petroleum hydrocarbon pollution," *Ind. J. Biotechnol*. 2, 411-425, 2003.

[7] Satishkumar, M., Banupriya, A.R., Baik, S. and Yun, S., "Biodegradation of crude oil by individual bacterial strains and a mixed consortium isolated from hydrocarbon contaminated areas," *Clean* 36, 92-96, 2008.

[8] Singh, C. and Lin, J. "Bioaugmentation efficiency of diesel degradation by *Bacillus pumilus* JL_B and *Acintetobacter calcoaceticus* LT_1 in contaminated soils," *Afr. J. Biotechnol.* 9, 6881-6888, 2010.

[9] Chang, L.K., Ibrahim, D., Ibrahim, C.O., "A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C," *Afr. J. Biotechnol*. 5, 2609-2615, 2011.

[10] Malik, Z.A. and Ahmed, S., "Degradation of petroleum hydrocarbons by oil field isolated bacteria consortium," *Afr. J. Biotechnol.* 1, 650-658, 2012.

[11] Holt, J.D., "Bergey`s Manual of Determinative Bacteriology," Williams CO. Baltimore, USA, p 983. 1994.

[12] Okpokwasili, G.C. and Okorie, B.B., "Biodegradation potentials of microorganisms isolated from engine lubricating oil, *Tribol. Int.* 21, 215-217, 1988.

[13] Bossert I., and Bartha, R., "The Fate of Petroleum in Soil Ecosystem: In Atlas, R.M. (Ed.) *Petroleum Microbiology.* Macmillan Publishing Company, New York, 1984, pp. 435-473.

[14] Arvanitis, N., Katsifas, E.A., Chalkou, K.I., Meintanis, C. and A. D. Karagouni, A.D. "A refinery sludge deposition site: presence of nahH and alkJ genes and crude oil biodegradation ability of bacterial isolates," *Biotechnol. Lett.*, 30, 2105-2110, 2008.

[15] Jasmine J. and Mukherji, 2014 "Evaluation of bioaugmentation and biostimulation effects on the treatment of refinery oily sludge using 2n full factorial design," *Environ. Sci.: Processes Impacts*, 16, 1889-1896, 2014.

[16] Agarry, S.E., Ogunleye, O.O., "Factorial designs application to study enhanced bioremediation of soil artificially contaminated with weathered Bonny light crude oil through biostimulation and bioaugmentation strategy," J. Environ. Protect. 3, 748-759, 2012.

[17] Vinithini, O., Sudhakar, S. and Ravikumar, R., "Biodegradation of petroleum and crude oil by Pseudomonas putida and Bacillus cereus," Int.J. Curr. Microbiol. App. Sci. 4(1). 318-329, 2015.

[18] Ismailov, N.M. "Biodegradation of oil hydrocarbons in soil inoculated with yeasts," M*icrobiologiya* 54, 670-675, 1985.

[19] Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., "Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation," *Bioresour. Technol.* 96, 1049-1055, 2005.

[20] Hamme, J.D., Odumeru, J.A., Ward, O.P., "Community Dynamics of a Mixed-Bacterial Culture Growing on Petroleum Hydrocarbons in Batch Culture," *Can. J. Microbiol.* 46, 441-450, 2000.

[21] Al-Wasify, R.S., Hamed, S.R., "Bacterial biodegradation of crude oil using local isolates," *Intern. J. Bacteriol* 2014.

[22] Eweis, J.B., Ergas, S.J., Chang, D.P.Y., Schroeder, E.D., "*Bioremediation principles*," McGraw-Hill Companies, Inc., pp 296, 1998.

[23] Treshner, M. and Wehner, H. "Chromatographic investigations on biodegraded crude oil" *Chromatographic* 20, 407-416, 1985.

[24] Ibrahim, M.L. and Ijah, U.J.J., "*Biodegradation by rhizosphere microorganisms* In: Daniels A.J. Eds *Advances in Environmental Research* vol 35, Nova Science publishers, Inc. New York, USA pages 153-172, 2014.

[25] Miller, H. "Biostimulation as a form of bioremediation of soil pollutants," *Basic Biotechnol.* 6, 7-12, 2010.