Biodegradation of Crude Oil, Refinery Effluent and Some Petroleum Components by *Penicillium Sp.* and *Mortierella Sp*. Isolated From Oil Contaminated Soil in Auto Mechanic Workshops

A.E. Okougbo and N. De*
Department of Biological Sciences
Covenant University
Ota, Ogun State, Nigeria
okougboakhere@gmail.com; manadina93@gmail.com

Y.M. Bello
Departmentof Microbiology
Federal University of Technology, Yola

***Abstract*—Ten isolates of five different types of fungi identified as Penicillium sp. (B101F, B202F and B302F), Aspergillus sp. (B102F, B104F and B304F), Fusarium sp. (B501F), Trichoderma sp. (K602F and K561F) and Mortierella sp. (B1002F) were isolated from contaminated soil samples obtained from auto mechanic workshops in Minna, Nigeria. Mycelial extension rate measurement method was used for the isolation of the best crude oil, refinery effluent and some other petroleum hydrocarbons degraders. Two fungal strains B101F and B1002F were selected based on their ability to degrade (Lagoma light) crude oil and some other petroleum components as both carbon and nitrogen sources in mineral salt medium (MSM). The biodegradation study showed Penicillium strain B101F and Mortierella strain B1002F performed degradation at an optimum pH and temperature of 5.0 and 280C respectively. The optimum concentration of (Lagoma light) crude oil, refinery effluent and other hydrocarbons in mineral salt medium (MSM) for fungal growth was 0.5% within an incubation period of 21 days. When complex medium such as maize bran was utilized as nitrogen source with crude oil in mineral salt medium (MSM) omitting NaNO3, the optimum growth was attained on the 14th day of fermentation for both the isolates. With respect to the selected strains ability to degrade crude oil, these organisms have shown significance in reducing pollution that arise from oil spills in our environments.**

Keywords—Biodegradation, Mycelial extension rate, Lagoma light, Mineral Salt Medium (MSM).

I. INTRODUCTION

Bioremediation, microbial decomposition of petroleum and petroleum products, is of considerable economic and environmental importance. Because petroleum is a rich source of organic matter and the hydrocarbons within it are readily attacked aerobically by a variety of microorganisms, bioremediation employs microorganisms capable of degrading toxic contaminants [1]. By augmenting the contaminated site with an appropriate inoculum of microorganisms is a promising technique to enhance the biodegradation of hydrocarbons. It is known that petroleum hydrocarbons can be removed by microorganisms such as fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium, Amorphotheca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium*, yeasts which includes *Candida*, *Yarrowia* and *Pichia* and microalgae [2]. Filamentous fungi play an important role in degrading diesel and kerosene by producing capable enzymes, because of their aggressive growth, greater biomass production and extensive hyphal growth in soil, fungi offer potential for biodegradation technology [3].

Reference [4] have reported that single cultures of fungi have been found to be better than mixed cultures of fungi and bacteria and more recently, fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria [5]. Forty five isolates belonging to 12 genera were purified and five isolates namely *Alternariaalternata*, *Aspergillusterreus*, *Cladosporiumsphaerospermum*, *Eupenicilliumhirayamae* and *Paecilomycesvariotii*as well as their consortium were found to be able to grow in association with petroleum oil as sole carbon source under *in vitro* conditions [6]. *Aspergillusniger* and *Rhizopusstolinifer* were capable of consuming kerosene as a sole carbon source [7]. The white rot fungus Polyporus sp. S133 collected from petroleum contaminated soil was tested for its ability to grow and degrade crude oil obtained from petroleum industry. Degradation at 93% was observed at crude oil concentration of 1000 rpm for 60 days [8].

In this study, an investigation was made on the biodegrading capability of crude oil and other petroleum components by fungal species isolated from oil-contaminated soil of mechanic workshop dumps and the effects of some

environmental parameters on the biodegrading capability of these fungal isolates.

II. MATERIALS AND METHODS

A. Collection of Samples

Soil samples were collected from three auto mechanic workshops located in three areas namely Bosso, KeteranGwari and Northern bye-pass, Minna, Nigeria where the mechanics generally dump lubricating oil, gasoline and diesel. Three samples were collected at each site for microbiological and biochemical purposes.

The crude oil (Lagoma light) was collected from Chemical Refining Laboratory, NNPC, Kaduna.Refinery effluent was collected at a discharge point where the effluent runs off pavement freely in Kaduna Refinery, Nigeria. Gasoline and diesel were collected from a filling station whereas engine oil was collected from a lubricating shop, all in Nigeria.

B. Isolation and Identification of Microorganisms

One (1) g of soil from each sample was transferred into 9 ml of sterile distilled water, and aseptically, serial dilution as described by [9] was performed to obtain soil suspension up to 10^{-9}. 1ml of each dilution (10^{-1} -10^{-9}) was inoculated on potato dextrose agar medium using a duplicate method: spread plate method and pour plate method. The plates were incubated at 28^0C for 48 hours. Distinct colonies were selected to re inoculate into PDA slants for identification purpose. Pure culture of fungal isolateswere examined under the microscope. The microscope study of each colony was carried out by wet-mount method [9]. The somatic and reproductive structures were observed. The relationship of the hyphae, size and shape of the reproductive structures were also noted.

C. Selection of Crude Oil, Refinery Effluent and Other Petroleum Hydrocarbon (Diesel) Degraders

Sterile PDA plates were inoculated with 2mm of the mycelium of each of the isolated fungus and cultures were incubated for 4 days at 28^0C. Mycelial plugs measuring 5 mm in diameter were cut with a sterile cork borer from the margin of the fungal colonies on PDA and placed at the center of Modified Czapek (MSA) plates containing the crude oil at different concentrations [10]. About 18 - 20ml of the solid medium was poured unto sterile plates and dried at room temperature for 3 - 4 hours before the plates were coated with crude oil. Crude oil at concentrations of 0.1, 0.5, 1.0, 2.5 and 5.0% were used. Plates with MSA medium without incorporation of crude oil were served as controls. The plates were incubated at 28^0C and mycelium extension were recorded for seven days.

The same experiment was repeated for refinery effluent and diesel samples.

D. Determination of Optimum Cultural Conditions for the Biodegradation of Crude Oil, Refinery Effluent and Other Hydrocarbons by Strains B101F and B1002F.

From previous experiments, it has been shown that B101F and B1002F were best degraders compared to others with the optimum concentration of the hydrocarbons being 0.5%. So these were selected for further work. For the development of inoculum, each of the isolates was grown for 7 days in 50ml Potato Dextrose medium. After 7 days of fermentation, the cells were harvested and washed twice thoroughly with distilled water and then 30ml of sterile water was added to the cells to make a cell suspension. The optimum pH (initial) of fermentation medium was determined by carrying out the fermentation at different pH values (3.0, 5.0, 7.0 and 9.0) of the medium. For this purpose, 5ml of inoculum was added to 50ml of Modified Czapek Liquid Medium (MSLM) at different pH with 0.5% crude oil in each of the 250ml Erlenmeyer flasks. The flasks were incubated at 28^0C for 28 days and the dry cell weight (wt.) was determined at definite time intervals. For determination of dry wt., the cells were harvested and washed twice thoroughly with distilled water and then transferred to a constant weight aluminum cup, dried at 60 - 70^0C for 24 hours. For determination of optimum temperature, fermentation was carried out at 28^oC and 37^oC. Dry cell wt. was determined on different days of fermentation. Then the optimum incubation period was determined by carrying out the fermentation for 28 days using 0.5% crude oil incorporated in 50ml MSLM in 250ml Erlenmeyer flask and the dry wt. was determined at definite interval.

E. Effect of Complex Nutrients on Biodegradation of Crude Oil

The materials used were beans husk extract (BN) and rice (RN) and maize bran (MN) extracts. Twenty grams of each of the materials was suspended in 200ml hot water in a 500ml flask. The suspensions were kept at 90^0C for 24 hours. The hot extracts were filtered through Whatman no. 1 filter paper. The solid content (%) of rice, maize, corn and beans were determined to be 3.43, 4.12, 3.91 and 0.98 respectively [11, 12].

To first set, each of the complex nutrients at 0.1% level was added in the crude oil incorporated MSLM (omitting $NaNO_3$) as nitrogen source. To second set, only crude oil at 0.5% level was incorporated into $NaNO_3$ containing MSLM medium. In each case, 5ml inoculum was added for 50ml fermentation medium in 250ml Erlenmeyer flask. Dry cell wt. was calculated as described before.

Same thing was repeated with refinery effluent and diesel samples.

F. Utilization of Gasoline, Kerosene, Diesel, Engine Oil and Refinery Effluent

For refinery effluent, 0.5% of the hydrocarbon was incorporated into Minimal Salt Liquid Medium. 50ml of the medium was dispensed into 100 ml conical flask and inoculated with B101F. The experiment was also done using B1002F. For each isolate, the experiment was done in triplicate.

For other hydrocarbons, the same experiment was done while in case of refinery effluent, other hydrocarbons were used. Crude oil was used as control. All the flasks were incubated at 28 1^0C for 4 weeks. At the end of the incubation period, the cell mass of the fungal degraders were calculated and expressed as g/l.

III. RESULTS

A. Characteristics of Soil Samples

The values of pH of collected soil samples in three sites were in the ranges of 4.8 - 6.4 and the moisture content (%) of the samples were in the ranges of 8 - 14.

B. Isolation and Identification of Microorganisms

Ten isolates belonging to five different types of organisms namely *Penicillium sp.*, *Aspergillus sp.*, *Fusarium sp.*, *Trichoderma sp.* and *Mortierella sp.*were identified.

C. Selection of Crude oil Degraders

All the isolated fungi were capable of utilizing crude oil, refinery effluent and other hydrocarbons at 0.5% concentration but B101F and B1002F (Fig. 1, Fig. 2 and Fig. 3) were capable of utilizing the hydrocarbons more efficiently than the other isolates. So these two isolates were selected for further studies.

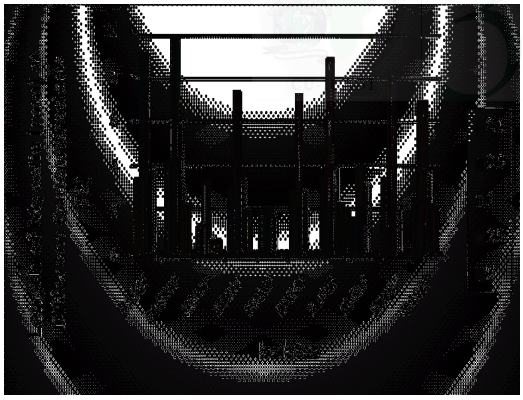

Fig. 1: Biodegradation of Crude Oil by the Isolates

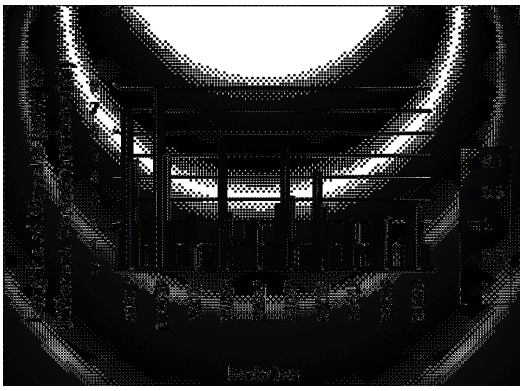

Fig. 2: Biodegradation of Refinery Effluent by the Isolates

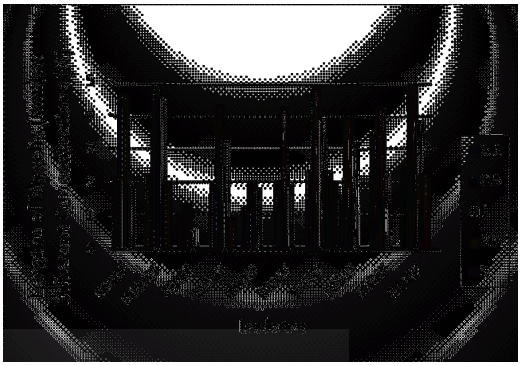

Fig. 3: Biodegradation of Diesel Oil by the Isolates

D. Determination of Optimum Cultural Conditions

The optimum pH and the optimum temperature for the degradation of crude oil by B101F and B1002F were 5.0 and 2.8^0C (Fig. 4 and Fig. 5). The optimum incubation period was the 21st day of fermentation. The results are shown in Table 1.

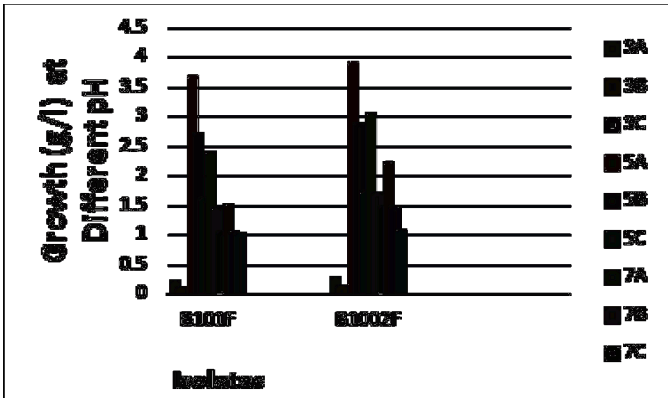

Fig. 4: Effect of pH and Temperature on Utilization of Crude Oil by B101F and B1002F (28°C)

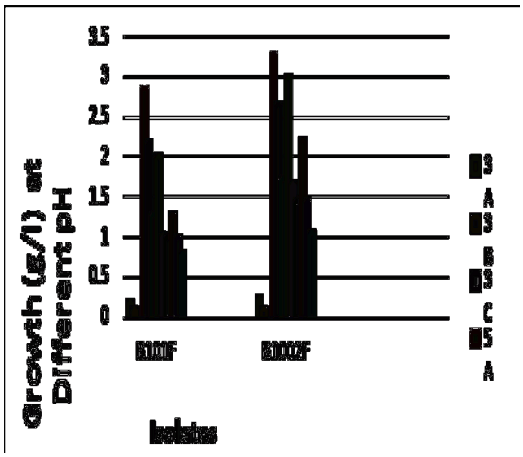

Fig. 5: Effect of pH and Temperature on Utilization of Crude Oil by B101F and B1002F (37^{0}C)

TABLE 1: DETERMINATION OF OPTIMUM INCUBATION PERIOD

Dry Cell Wt. (g/l) at Different Days of Incubation	B1002F	B101F	CONTROL
7th			
A (Crude oil)	2.03	0.71	0.11
B(Refinery effluent)	1.19	0.89	0.14
C(Diesel)	0.80	0.60	0.14
14th			
A	3.87	1.49	0.20
B	1.49	1.32	0.16
C	1.20	1.02	0.15
21st			
A	4.17	2.28	0.19
B	1.49	1.49	0.22
C	1.02	1.02	0.20
28th			
A	4.18	2.33	0.19
B	1.75	1.48	0.18
C	1.24	1.30	0.19

E. *Effect of Complex Nutrients on biodegradation of Crude oil*

The results in Fig. 6 indicate that maize bran extract in crude oil incorporated MSLM gave the maximum growth on the 14th day of fermentation whereas in case of crude oil incorporated MSLM medium, the optimum growth was reached on the 21st day of fermentation. RN-rice bran extract incorporated inthe crude oil incorporated MSLM omitting $NaNO_3$; BN-beans husk in the crude oil incorporated MSLM omitting $NaNO_3$ as nitrogen source; MN-maize bran extract in the crude oil incorporated MSLM omitting $NaNO_3$ as nitrogen source.

F. *Crude Oil as both Carbon and Nitrogen Source*

Both the isolates can utilize crude oil at 0.5% concentration both as carbon and energy sources in MSLM medium without incorporation of $NaNO_3$, the conventional nitrogen source. The results are shown in Table 2.

G. *Growth of Penicillium sp. (B101F) and Aspergillus niger (B1001F) on some petroleum hydrocarbons*

The results are expressed in Figure 7.

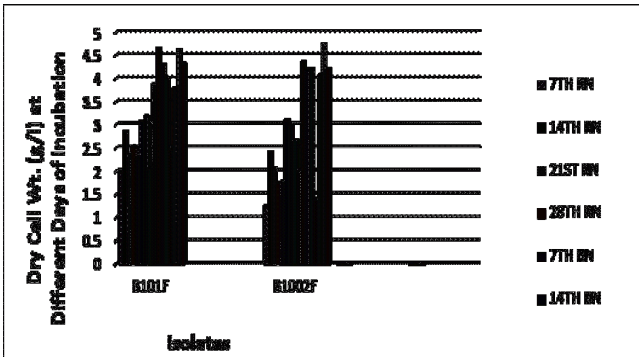

Fig. 6: Effect of Complex Nutrients on Biodegrading Capability of B101F and B1002F

TABLE 2: UTILIZATION OF CRUDE OIL AS BOTH CARBON AND NITROGEN SOURCE

Isolates	Crude oil	Dry Cell Wt. (g/l) at Different Days of Incubation			
		7th	14th	21st	28th
B101F	P-	2.20	3.81	4.64	4.40
	P+	2.44	3.61	4.60	4.33
B1002F	P-	1.44	3.99	4.25	4.68
	P+	1.36	4.00	4.45	4.32

P+ with $NaNO_3$, P- without $NaNO_3$

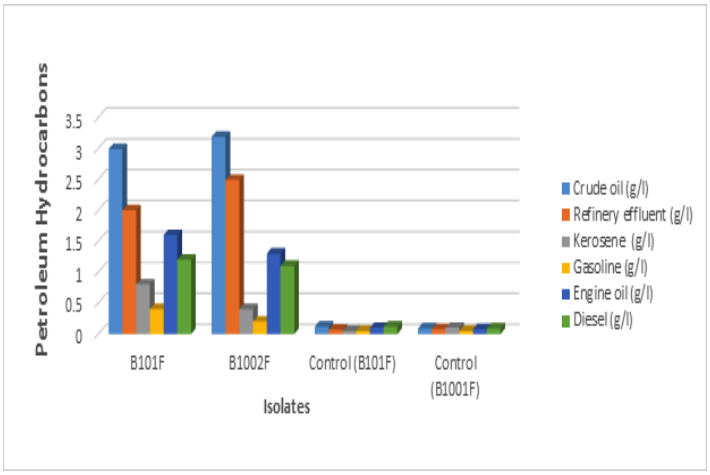

Fig. 7: Growth of Fungal Isolates on Some Petroleum Hydrocarbons

IV. DISCUSSION

The soil samples were collected at a depth between 15 - 20cm because of the bactericidal effect of sunlight, and inadequate moisture due to evaporation on the surface. Here the fungi were isolated from petroleum contaminated soil to ensure that the organisms have a higher tolerance to the toxicity of hydrocarbons and are resistant to variations in the environment (Dibble and Bartha, 1979). Reference [8] examined the biodegrading capability of the crude oil by white rot fungus Polyporus sp. S133 collected from petroleum contaminated soil. The ability of Polyporus sp. S133 pre-grown on wood meal to degrade crude oil was measured. Maximal degradation (93%) was obtained when Polyporus sp. S133 was incubated in 1000 ppm of crude oil for 60 days, as compared to 19% degradation rate in 15000 ppm. Increased concentration of crude oil decreased the degradation rate. In this study, the optimum concentration of Lagoma light crude oil in MSLM for fungal degradation was 0.5%. This is in contrast to a report by [13] that stimulation of microbial activity is enhanced up to 5% level of hydrocarbon. The increase in concentration of crude PHC likely interfered with medium aeration, hence degradation could not occur because it is an oxidative process.

Also at higher concentration, the PHC could be toxic to the microorganisms. It agrees with the findings of [8] that increased concentration of crude oil decreased the degradation rate. Despite the differences in cell mass, the fungal isolates had same optimum period of incubation of 21 days. The fungal isolates utilized crude PHC at all pHs tested with maximum at pH 5.0. This supports earlier observations of [13] and Debbie and Bartha (1979) that fungi degrade oil most efficiently in acidic medium. At low temperatures, fungi degradative capability was arrested. Optimum growth was obtained at low temperature (28^0C) and higher above that oil degrading capabilities reduced. This is in line with acclaimed work Debbie and Bartha (1979) and [14] that best condition of temperature for maximal fungal activity is the mesophilic range. When complex nutrients were added as nitrogen source with crude oil in MSLM omitting $NaNO_3$, the growth of isolates were almost same as of crude petroleum used as carbon source. However, the optimum growth period was different. The effect of complex nutrients on the biodegradation of crude oil may be due to the presence of inorganic materials and organic nitrogenous substances in the complex nutrients. Crude petroleum could serve as both carbon and nitrogen source for these isolates. Lack of mineral elements as nitrogen, phosphorus and sulphur is said to be a limitation in PHC biodegradation [15, 16] but crude PHC contains small amounts of nitrogen, oxygen and sulphur containing components [17].

When a comparison was made between the degradation of petroleum and other petroleum hydrocarbons, it was observed that the PHCs were utilized as Crude oil > Refinery effluent >Engine oil > Diesel> Kerosene> Gasoline by both the isolates involved. This study investigated the ability of two fungi to utilize kerosene. Reference [7] studied the biodegradation of kerosene by *Aspergillusniger*and*Rhizopusstolinifer*. The highest percentage loss of kerosene concentration by the cultures of fungi was 93% by *A.niger* after 28 days of biodegradation, but the loss of kerosene concentration in the culture of *R.stolinifer* reached 88% after 28 days. Both strains *A.niger* and *R.stolinifer* were capable of consuming kerosene as a sole carbon source. Reference [6] studied on biodegradation of diesel fuel hydrocarbons by mangrove fungi from Red Sea Coast of Saudi Arabia.Five fungal isolates namely *Alternariaalternata*, *Aspergillusterreus*, *Cladosporiumsphaerospermum*, *Eupenicilliumhirayamae* and *Paecilomycesvariotii* displayed rapid diesel degradation ability, and when used together as a consortium, there was a synergistic effect that enhanced the degradation process.

V. REFERENCES

[1] M. Eriksson, G. Dalhammar and A. Borg-Karson. "Aerobic degradation of hydrocarbon mixture in natural contaminated potting soil in indigenous microorganisms at 20ºC and 6ºC." Appl. Microbiol. Biotechnol.vol.51,pp. 532-535, 1995.

[2] F. Chaillan, A. Fleche and Bury, E. "Identification and biodegradation potential of typical aerobic hydrocarbon degrading microorganisms." Res. Microbiol. vol.155: pp. 587-595, 2004.

[3] E. H. Kenneth."Mechanisms of polycyclic aromatic hydrocarbon degradation by ligninolytic fungi."Environ. Health Perspec.vol.103,pp. 41-43, 1995.

[4] P. O. Okerentugba and Ezeronye, O. O. "Petroleum degrading potentials of single and mixed microbial cultures in Nigeria isolated from rivers and refinery effluents." African Journal of Biotechnology vol.2 (9), pp. 288-292, 2003.

[5] C. D. Batelle. Mushrooms: higher macro fungi to clean up the environment. Batelle Environmental Issues, Fall, 2000.

[6] F. Ameen, M. Moslem, S. Hadi and A. Al-Sabri. "Biodegradation of diesel fuel hydrocarbons by mangrove fungi from Red Sea Coast of Saudi Arabia". Saudi Journal of Biological Sciences vol. 23(2), pp. 211-218, 2006.

[7] I. F. Hasan. "Biodegradation of kerosene by *Aspergillus niger* and *Rhizopus stolinifier"*. Journal of Applied and Environmental Microbiologyvol.2(1):pp. 31-36, 2014.

[8] T. Hadibarata and S. Tachibana, S. "Degradation of crude oil by fungi pre-grown on wood meal. Interdisciplinary Studies on Environmental Chemistry - Environmental Research in Asia. Eds. Obayashi, Y., Subramanian, A., Suzuki, S. and Tanabe, S., Terrapub. 2009, pp. 317-322.

[9] H. Benson. Microbiological Applications 9th edition. McGraw Hill Education New York. 2005.

[10] J. T. Dibbie and R. Bartha. "Effect of environmental parameters on the biodegradation of oil-sludge." Applied Environmental Microbiology vol.37(4), pp. 729-739, 1979.

[11] I. Saadoun. "Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel."J. Basic Microbiol.vol.42 (6), pp. 420-428, 2002.

[12] D. E. Silas, C. S. Dunah and N. De."Protein enrichment of crushed spent sorghum using *Candida* species and *Saccharomyces cerevisiae."* Nigerian Journal of Biotechnology vol.15 (1): pp. 22-29, 2004.

[13] I. Bossert and R. Bartha. The fate of petroleum in soil ecosystem. Petroleum Microbiology, Ed. Atlas, R. M., Macmillan London Press. 1984, pp. 435-473.

[14] G. J. Mikkins-Philips and J.E. Stewart. "Effect of environmental parameters on bacterial degradation of bunker C oil, crude oil and hydrocarbons." Applied Microbiology vol.28(6), pp. 915-922, 1974.

[15] R. M. Atlas. "Microbial degradation of petroleum hydrocarbon: an environmental perspective". Microbiological Reviews vol.**45**: pp. 180-209, 1981.

[16] I. Saadoun. ‘‘Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel’’.J. Basic Microbiol. *v*ol42 (6), pp: 420 – 428, 2002.

[17] Bartha, R. “Biotechnology of petroleum pollutants biodegradation”. Microbiology Ecology vol.12, pp. 155-172, 1986.

