Nig J. Biotech. Vol. 31 (2016) 1 – 8
ISSN: 0189 1731
Available online at http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: http://dx.doi.org/10.4314/njb.v31i1.1

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Molecular Identification of Bacteria Involved in Degradation of Crude Oil

Nwakanma, C.[1], Obih, E.C.[2] and Onyia, O.[3]
[1]Department of Environmental Management and Toxicology, Michael Okpara University of Agriculture, Umudike, Abia State. Nigeria, [2]Department of Biological Sciences (Biotechnology unit), Godfrey Okoye University Enugu, Enugu State, Nigeria, [3]Microbiology Department, University of Nigeria, Nsukka, Enugu State.

(Received : 14 :12 :2015 ; Accepted : 29 :06 :2016)

Abstract
In this present study, bacteria were isolated from soil obtained from oil contaminated mechanic site in Enugu. Out of the 206 forming colonies, 16 colonies were selected based on observable morphological differences and screened for crude oil degraders. Out of the 16 isolates, 11 isolates were assumed to be degraders because they formed maximum clear zones on mineral salt media. At the end of 14 days incubation, 14 out of the 16 isolates degraded the crude oil ranging from 5% to 60%. Isolate 1, 5 and 14 had the highest percentage degradation of 60%, followed by isolate 13 with 50% degradation. Isolate 3 was the least degrader with 5% degradation while isolate 2 and 16 were not able to degrade the crude oil. An attempt to identify the isolates was done by sequencing the 16s rRNA gene of the isolates using DNA sequencing technique. Isolate 2, 3, 4, 5, 6, and 7 were identified as Escherichia coli, Streptococcu anginosus strain SK52, Lactobacillus spp. G12, Bacillus cereus strain ABc17, Paracoccus spp. KF89 and Lactobacillus spp. G22 respectively. However, isolate 1, 8, 9, 10, 11, 12, 13, 14, 15 and 16 did not produce any result. This study shows that microorganisms isolated from a non native crude oil contaminated site can be utilized for bioremediation.

Key words: Bioremediation, 16s rRNA gene, DNA sequencing, Biotechnology
Correspondence: dr.nwakanmac@gmail.com

Introduction
Crude oil is a naturally occurring complex mixture of hydrocarbon and non-hydrocarbon compounds which at appropriate concentration, possesses a measurable toxicity towards living systems. The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination (Obire and Anyanwu, 2009). Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products, the release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution. One of the greatest challenges to humanity today is the endangering of biota as a result of environmental pollution from crude oil. Oil spills have become a global problem particularly in industrialized countries and developing countries like Nigeria. The shift in economic base of coal to crude oil and petroleum products, more especially after the World War II, greatly increased the volume of these commodities being transported across the high seas (Onwurah et al., 2007).

Interest in the microbial biodegradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments (Diaz, 2008 and Koukkou, 2011). According to Best and Seiyefa (2014), an average of 240,000 barrels of crude oil are spilled in the Niger delta every year, mainly due to unknown causes (31.85%), third party activity (20.74%), and mechanical failure (17.04%). The spills contaminated the surface water, ground water, ambient air, and crops with hydrocarbons, including known

carcinogens like polycyclic aromatic hydrocarbon and benxo (a) pyrene, naturally occurring radioactive materials, and trace metals that were further bioaccumulated in some food crops. The oil spills could lead to a 60% reduction in household food security and were capable of reducing the ascorbic acid content of vegetables by as much as 36% and the crude protein content of cassava by 40%. These could result in a 24% increase in the prevalence of childhood malnutrition. Animal studies indicate that contact with Nigerian crude oil could be hemotoxic and hepatotoxic, and could cause infertility and cancer (Nwakanma and Hart, 2012). Over the decades, the after effect of oil spillage has left our land desolate, bleak and barren. This has put virtually every plant species and animals located at these contaminated sites in grave danger thereby interfering with the ecological system and the environment at large. This has left man with the only option of searching for possible methods to remediate the situation and one of these methods is the use of microorganisms isolated from oil contaminated sites to degrade the oil spills. Although recent research findings by scientists has shown that microorganisms isolated from crude oil contaminated sites possess the ability to biodegrade crude oil and remediate oil spillage, it's still important to investigate the ability of microorganisms isolated from other environment to biodegrade crude oil and also the possibility of utilizing these organisms as possible agents of bioremediation. Physical and chemical methods have been employed in remediating the effects of oil spillage but these methods have not proven to be cost effective and do not remediate the environment back to its original state. There is therefore a need to investigate new frontiers in this pressing area of research which will be more cost effective, safer and reliable.

Raed and Shimaa (2014) assessed the efficiency of Pseudomonas aeruginosa, Bacillus subtilis, and Acinetobacter lwoffi isolated from petroleum contaminated water and soil samples to degrade crude oil, separately and in a mixed bacterial consortium. Capillary gas chromatography was used for testing the effect of those bacterial species on the biodegradation of crude oil. Individual bacterial cultures showed less growth and degradation than did the mixed bacterial consortium. At temperature 22^0C, the mixed bacterial consortium degraded a maximum of 88.5% of Egyptian crude oil after 28 days of incubation. This was followed by 77.8% by Pseudomonas aeruginosa, 76.7% by Bacillus subtilis, and 74.3% by Acinetobacter lwoffi. The results demonstrated that the selected bacterial isolates could be effective in biodegradation of oil spills individually and showed better biodegradation abilities when they are used together in mixed consortium. Also, Okerentugba and Ezeronye (2003) tested the ability of fungi (Penicillin spp., Aspergillus spp. and Rhizopus spp.) and bacteria Bacillus spp., Micrococcus spp. and Proteus spp isolated from rivers and refinery effluent in Nigeria to degrade crude oil. The results showed changes in pH, optical density and total viable count for the bacterial isolates after a 17-day period. There was an increase in biomass for the fungal isolates after a 35-day period. It was observed that these organisms were able to utilize and degrade the crude oil constituents, with bacterial isolates showing increase in cell number and optical density as pH decreases. Single cultures were observed to be better crude oil degraders than the mixed cultures (bacteria or fungi). It was also observed that oil degraders could be isolated from a non-oil polluted environment, although those from oil-polluted environments have higher degradation potentials. Latha and Kalaivani (2012) investigated about the isolation of bacteria from crude oil contaminated site using gravimetric analysis of degradation in which, two bacterial isolates formed maximum clearing zone on mineral salt medium. Among these isolate numbered as S2 was identified as Bacillus subtilis which showed maximum growth (0.85mg/ml) and degradation on seventh day of incubation, followed by S10 (Pseudomonas aeruginosa) that showed maximum growth (0.92mg/ml) and degradation. The total viable count of Bacillus subtilis and Pseudomonas aeruginosa were $257x10^6$ Cfu and $248x10^3$ Cfu respectively. An increase in oil degradation was correlated to an increase in cell number indicating that the bacterial isolates were responsible for the oil degradation. Their results demonstrate the potential for biodegradation of these isolates in situ and/or ex situ. The main aim of this study was to isolate bacteria from oil contaminated site of an automobile mechanic workshop, to identify the isolated bacteria using 16s DNA gene sequencing technique and to test for the

ability of the isolated bacteria to degrade crude oil.

Material and Methods

The principal location involved in this study is a mechanic workshop located at Emene junction close to the temporary site of Godfrey Okoye University, Thinkers Corner area of Enugu State, Nigeria. The convenient sampling technique that was used is Random selection. Randomization was adopted because each soil bacterium species/family was given a chance to be represented in the bacteria population within the geographical location of the study area. The population of this study currently identifies approximately 206 different colonies on the different serial dilution plating out. To determine the sample size of the study, the researcher selected 16 distinctive colonies based on morphological observation from different locations on the dilution plate to form a 7.7% ratio of the population of the study. The researcher went to the location of target to observe, take notes and collect the soil samples. Soil samples were collected using sterilized spatula at a tillage depth of 2 cm randomly from 10 core points. The soil samples were mixed and a sub-soil collected into sterile re-sealable bags and transferred to the laboratory at 4^0C within 30 minutes. The laboratory bench was cleaned with cotton wool soaked in ethanol, this is done to avoid contamination and enhance aseptic conditions. 28g of nutrient agar powder was weighed into a 2000ml conical flask and then mixed with 1000ml of distilled. The media was homogenized by agitating and then sterilized by autoclaving at 121 ^{0}C for 15 minutes, after which it was aseptically poured into sterile Petri dishes containing the diluted solution of the soil samples and allowed to gel on the bench. Slants were also prepared by pouring 8ml of the media aseptically into sterile Bijou bottles and then slanted and allowed to gel. For testing of the ability of isolates to degrade crude oil, mineral salt media was prepared. The mineral salt media (MSM) contain (10g of NaCl, 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 2.0g of $(NH_4)SO_4$, 0.53g of KH_2PO_4 and 0.10g of $MgSO_47H_2O$). The media was prepared by mixing the MSM with 1000ml of distilled water and then homogenized using a homogenizer. The $_P$H was adjusted to 7.2 and the media was autoclaved at 121 ^{0}C for 15 minutes. In a test to confirm which of the isolates that were degraders, mineral salt agar media was prepared. The mineral salt media (MSM) contain (10g of NaCl, 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 2.0g of $(NH_4)SO_4$, 0.53g of KH_2PO_4, 0.10g of $MgSO_47H_2O$). 20g of agar-agar powder was also added to the MSM in order to solidify it. The media was prepared by mixing the MSM with 1000 ml of distilled water and then homogenized using a homogenizer. The $_P$H was adjusted to 7.2 and the media was autoclaved at 121 ^{0}C for 15 minutes. Aseptically, 9ml of distilled water was pipette into five (5) different test tubes and labelled accordingly from (10^1 to 10^5). 1 g of the soil sample A was weighed and transferred into the test tube labelled 10^1, and then from 10^1, 1ml was pipette into 10^2 and the process was repeated at each dilution factor using a different pipette to avoid cross contamination. The test tubes were shook for proper homogenization. The steps above were then repeated for the remaining nine (9) soil samples.The method used was pour plate inoculation method. 0.1ml of the diluted sample was aseptically pipette into the labelled Petri dish plates. The dilution factor (10^1, 10^3 and 10^5) was used. An already prepared nutrient agar media at 45 ^{0}C was then poured into all the plates and carefully swirled to mix. The plates were then incubated in an incubator at 37 ^{0}C for 24 hours. The growth of the microorganism was observed on the plates. The distinct colonies were picked (with wire loop flamed to red hot and allowed to cool) from the nutrient agar. The distinct colonies was streaked onto fresh prepared nutrient agar plates and labelled properly to be able to identify each pure colony after growth and the plate where it was pre-inoculated. The plates were then incubated in the incubator for 24 hours at 37 ^{0}C. Slant culture was prepared using 8ml nutrient agar in a Bijou bottle; the slant was inoculated to preserve the isolated colonies for future analysis. Then the slants were refrigerated at 4 ^{0}C to reduce the rate of growth and nutrient consumption.

5ml of nutrient broth was prepared in test tubes and inoculated with the isolates. It was then inoculated incubated at 37°C for 2 days. After incubation, 2ml of broth culture was transferred into 20ml mineral salt medium (using different pipette tip in order to avoid cross contamination) and incubated at room temperature. Growth was confirmed by plating out after three (3) days and also by the change in the appearance of mineral salt

medium from transparent to opaque. 5ml of nutrient broth was prepared in test tubes and inoculated with the isolates. It was then inoculated incubated at 37°C for 2 days. After incubation, 2ml of broth culture was transferred into 18ml mineral salt medium (using different pipette tip in order to avoid cross contamination) and incubated at room temperature. After incubation, 10µl of the mineral salt medium culture was plated in mineral salt medium using spread plate technique. 500µl of crude oil was uniformly sprayed over the surface of the agar plate and spread rod used to spread the crude oil uniformly on the agar surface. The plates were incubated at room temperature for 3 days. The organisms that formed clear zones around the colonies were considered as crude oil degraders. For examining the degradation of oil, Bushnell Haas medium (BHM) supplemented with 10% v/v of crude oil was used. About 20ml medium was dispensed in 100ml conical flasks. The media was inoculated with 0.1ml of crude oil degrading bacteria isolate culture (the bacteria isolate culture from the screening of crude oil degrading bacteria) and incubated at room temperature for 14 days. The conical flasks were shook regularly in the absence of a rotary shaker. For estimating the growth of the isolates during the biodegradation test two different methods were employed: the number of colony forming units of each isolate and measuring optical density using UV spectrometer. To determine the number of colony forming units, 10µl of each isolate sample was obtained and a $10^{-}8$ fold serial dilution was carried out. 100µl was plated out on nutrient agar plate and incubated at 37^{0}C for 24 hours. For estimation of growth by measuring optical density, 500µl of each isolate sample was collected using sterile pipette tips and released into 1.5ml labeled eppendorf tubes. The tubes were centrifuged at 16,000rpm for 2 minutes, after which the supernatants were decanted. The cells were washed using 1ml TBE buffer three times and re-suspended in 1ml of the buffer. The cell absorbance of each of the isolate was measured at 620nm wavelength. For estimation of oil degradation rates by gravimetric analysis, 2ml of n-hexane was added to above flasks and shook. The contents were transferred to a separating funnel and extracted. Extraction was carried out twice to ensure complete recovery of oil. The extract was treated with 0.4g of anhydrous sodium sulphate to remove the moisture and decanted into a beaker leaving behind sodium sulphate. This was evaporated to dryness in an oven under low temperature. The amount of residual oil was measured after extraction of oil from the medium and evaporating it to dryness in an oven. The volume of extracted oil was deducted from the previously weighed beaker. The colony characteristics and cellular morphology of the isolated, staining reactions, physiological characteristics were examined by standard methods and the isolates were identified by 16s RNA gene sequencing technique. Firm (smear) of each of the isolate were prepared by picking a small portion of microbial growth from the plates with the aid of a sterilized wire loop into a drop of normal saline on glass slide and after making the smear, it was allowed to air dry before the slides were heat fixed by carefully passing them over a Bunsen burner flame. The heat fixed smears were stained with crystal violet for 1 minute, washed off with distilled water and drained, then flooded with Lugol's iodine for 1 minute, washed off gently with distilled water and drained. The slides were rinsed with acetone for 3 seconds, after which they were rinsed with distilled water and drained. The slides were then counter stained with safranin for 1 minute and the stain was washed off with distilled water. The slides were air dried. Immersion oil was dropped on the smears and examined microscopically using x100 oil immersion objective of the microscope. After confirming the extraction of DNA from the isolates and the amplification of the 16s rRNA gene using specific primer for the polymerase chain reaction and running the samples in 2% agarose gel, the PCR products were properly packed and supported with enough ice packs to help maintain the cold chain and prevent the DNA samples from denaturing. The samples were then sent to Genewiz 115 Corporate Blvd. SOUTH Plainfield, NJ 0708 in the United States for sequencing.

Results

Bacterial strains isolated in this study were identified among hydrocarbon degrading microorganisms for crude oil in relation to works by Friello et al., (2001). In this study, the results obtained clearly showed that the microorganisms had biodegradable abilities and values of degraded crude oil varied after

incubation at day 7 and day 14. The total viable counts of the microbes present in the soil sample in Table 1 varied from soil sample A to soil sample J. Viable counts of microorganisms in sample C, E, G, H and I had flooded growth at 10^1 (number of colonies) while viable count of soil sample B, E,F, G and I had flooded growth at 10^3 (number of colonies). At 10^5 (number of colonies), soil sample F and I had flooded growth. It was observed that only sample I had flooded growth all through the period of the viable count of microbes present in the soil sample.

Table 1: Viable counts of the microbes present in the soil samples

SOIL SAMPLE	10^1 (NUMBER OF COLONIES)	10^3 (NUMBER OF COLONIES)	10^5 (NUMBER OF COLONIES)
A	19	5	3
B	16	F.G	7
C	F.G	124	14
D	72	8	3
E	F.G	F.G	20
F	12	F.G	F.G
G	F.G	F.G	11
H	F.G	3	2
I	F.G	F.G	F.G
J	23	8	5

Key: F.G = Flooded growth

The result of colony forming units of the isolates for day 7 and day14 are presented in Table 2 below. At day 7, isolate number 6 had the highest colony forming units (240 x 10^6cfu/ml) and isolate number 3 had the lowest colony forming units (72 x 10^6cfu/ml). Isolate 11, 12, 13 and 14 had flooded growth while isolate 16 was contaminated. At day 14, isolate number 1 had the highest colony forming unit (198 x 10^8cfu/ml), while isolate 8 and 9 had the same colony forming unit (162 x 10^8cfu/ml) respectively. Isolate 10 and 14 had flooded growth and there was occurrence of contamination at day 14.

Table 2: Colony forming units of the isolates after plating out

ISOLATES	DAY 7 cfu/ml	DAY 14 cfu/ml
1	156 X 10^6	198 X 10^8
2	108 X 10^6	96 X 10^8
3	72 X 10^6	90 X 10^8
4	126 X 10^6	60 X 10^8
5	36 X 10^6	144 X 10^8
6	240 X 10^6	96 X 10^8
7	168 X 10^6	94 X 10^8
8	42 X 10^6	162 X 10^8
9	114 X 10^6	162 X 10^8
10	180 X 10^6	F.G
11	F.G	132 X 10^8
12	F.G	136 X 10^8
13	F.G	98 X 10^8
14	F.G	F.G
15	90 X 10^6	60 X 10^8
16	C	84 X 10^8

$$\text{CFU} = \frac{No\ of\ colony\ x\ Dilution\ factor}{Volume\ of\ inoculated\ sample}$$

KEY:
C = Contaminated, F.G = Flooded growth

The results of Plate 1 to 5 indicated below showed the sequenced blast isolate. The result clearly showed that the bacterial culture could carry out a maximum degradation percentage for crude oil after 14 days of incubation from Escherichia coli (100%), Streptococcus anginosus (92%), Lactobacillus sp (97%), Bacillus cereus (100%), and Paracoccus sp (100%).

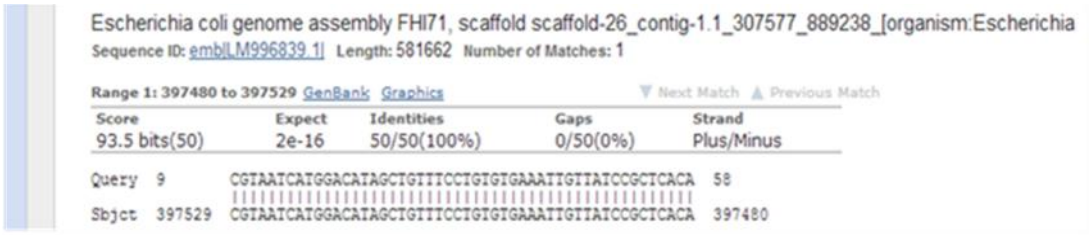

Plate 1: Sequence blast result for isolate 2

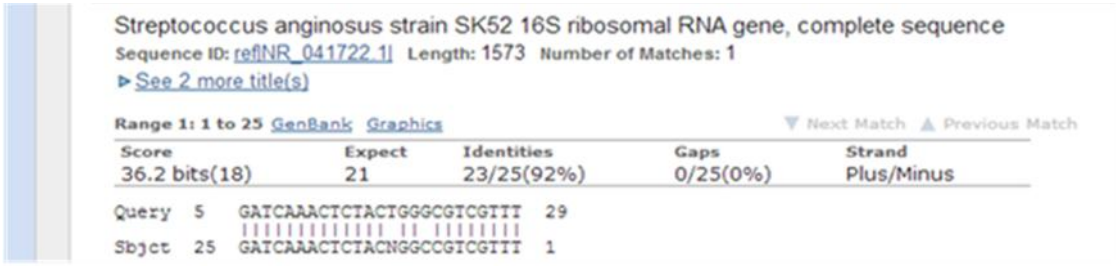

Plate 2: Sequence blast result for isolate 3

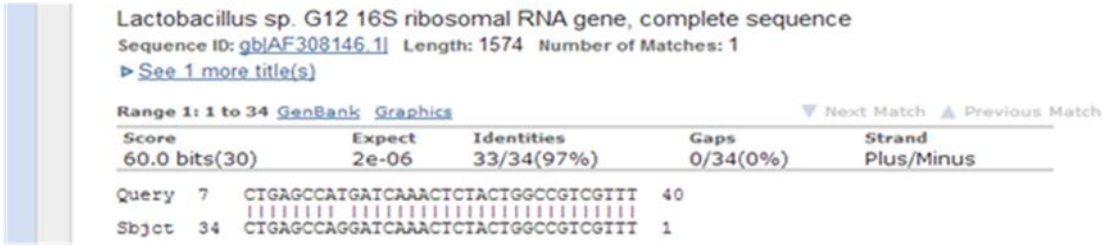

Plate 3: Sequenced blast result for isolate 4

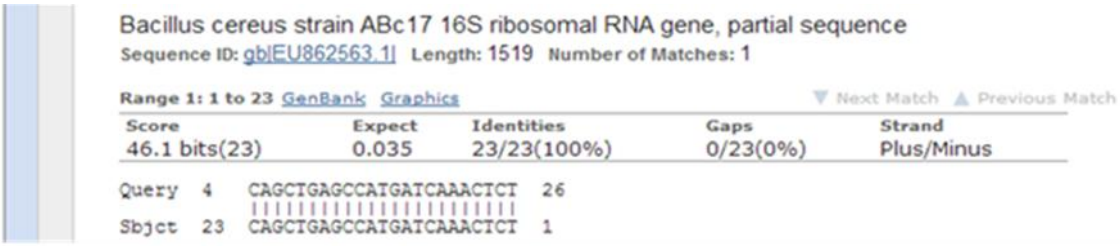

Plate 4: Sequenced blast for isolate 5

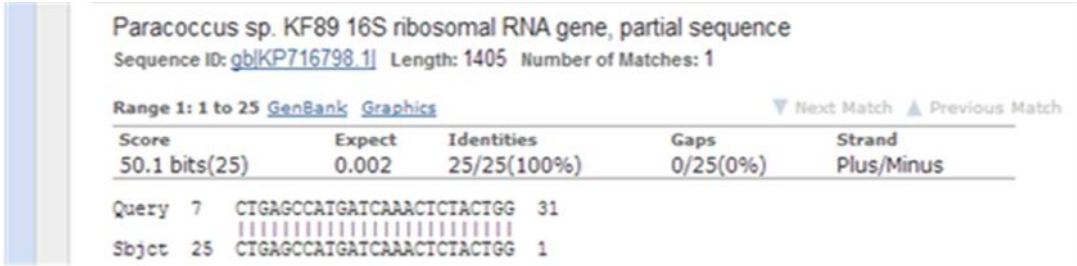

Plate 5: Sequenced blast for isolate 6

Discussion

In this study, the efficiency of crude oil degraded by the individual bacterial cultures was determined. The findings of the degradation percentage of crude oil in Plate 1 to 5 are in line with several workers (Dasgupta et al., 2013; Rahman et al., 2002; Vasudevan and Rajaram, 2001). They reported the ability of mixed bacterial consortia to degrade 28 - 51% of saturates and 0 – 18% of aromatics present in crude oil or up to 60 – 78% crude oil after 20 days of incubation. The presence of petroleum hydrocarbons has been reported to influence the biodiversity, distribution and pollution of microorganisms in an environment. Crude oil, because of its characteristics is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Mishra et al., 2001; Lloyd and Cacket, 2001). The environment of microorganisms in the degradation of petroleum and its products has been established as an efficient, economic, versatile and environmentally sound treatment. In this study, soil samples were collected from 10 different core locations within a mechanic workshop located in Thinkers' Corner, Emene Enugu state. The ability of the isolates to grow utilizing crude oil as their main carbon source was examined by inoculating the isolates into a 5ml nutrient broth prepared in a test tube. All the isolates were able to growth utilizing the crude oil as their carbon source. This corresponds to the findings of Okerentugba and Ezeronye (2003) and Latha and Kalaivani (2012).

The ability of the isolates (including the isolates that didn't produce clear zones during screening for crude degraders) to degrade crude oil was then tested. Isolate 1, 5, 6, 7, 8, 9, 12, 13, 14 and 15 all produced clear zones ranging from 3 or 5 clear zone areas to multiple clear zones. Isolate 8 and 12 produced the most clear zone areas. The crude oil degraders screening test agrees with the finding of Latha and Kalaivani (2012). Isolate 2, 3, 4, 5, 6, and 7 were identified as Escherichia coli, Streptococcu anginosus strain SK52, Lactobacillus spp. G12, Bacillus cereus strain ABc17, Paracoccus spp. KF89, Lactobacillus spp. G22 respectively. While isolate 1, 8, 9, 10, 11, 12, 13, 14, 15 and 16 did not produce any result. The reason for the negative result for some of the isolates could be attributed to low quality (concentration) of the isolated DNA samples or due to the break in the cold chain during the storage of primers before usage due to power failure or the denaturation of some of the DNA samples due to break in cold chain also. This might have occurred while the samples were being transported from Nigeria to the United States for sequencing. It is therefore recommended to the federal government of Nigeria that they should invest more into the area of biological sciences research and establishment of research centers across the nation which will provide advanced molecular biology services such as DNA sequencing at an affordable rate. More research should be carried out to investigate the possibility of utilizing microorganisms from a non native crude oil contaminated sites in the biodegradation of crude oil. Also, molecular techniques should be employed in the identification of these microorganisms down to strain level and in investigation of the excreted enzymes which degrade petroleum hydrocarbons. Cleaning up of petroleum hydrocarbons in the subsurface environment is a real world problem due to impact of petroleum discharge pollutants which cause decline of environment health species health. Currently the nature became more familiar with biological control solutions to remove hazardous from the environment. Although disposal methods can become prohibitively expensive when the amounts of pollutants are huge, using microbial remediation process is successful and safe way to enhance environment health in particular with low cost, technique and high public acceptance to cleaning up aquatic ecosystems from oil spills. From the results above, it is observed that the most of the isolated microorganisms are crude oil degraders and that; they can be utilized as agents for bioremediation.

References

Best, O. and Seiyefa, B. (2013). The human health implications of crude oil spills in the Niger delta, Nigeria: An interpretation of published studies. Nigeria Medical Journal 54(1): 10-16.

Dasgupta, D., Ghosh, R., and Sengupta, T. K. (2013). Biofilm-mediated enhanced crude oil degradation by newly isolated Pseudomonas

species. ISRN Biotechnology, vol. 2013, Article ID 250749, P. 13.

Diaz, E. (2008). Microbial Biodegradation: Genomics and Molecular Biology (1st ed.). Caister Academic Press, New York, USA.

Friello, D. A., Mylroie, J. R., and Chakrabarty, A. M. (2001). Use of genetically engineered multi-plasmid microorganisms for rapid degradation of fuel hydrocarbons. International Bio-deterioration and Biodegradation, vol. 48, no. 1–4, pp. 233–242.

Gardner, G. R., Yevich, P. P., Harshbarger, J.C. and Malcolm, A. R. (1991). Carcinogenicity of Black Rock Harbor sediment to the eastern Oyster and trophic transfer of Black Rock Harbor carcinogens from the blue mussel to the winter flounder. Environmental Health Perspectives 90: 53-66.

Latha, R. and Kalaivani, R. (2012). Bacterial Degradation of Crude Oil by Gravimetric Analysis. Advances in Applied Science Research 3(5): 2789-2795.

Lloyd, C. A. and Cackette, T. A. (2001). Diesel Engines: Environmental Impact and Control Air and waste management Association 51: 805.

Margesin, R. and Schinner, F. (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl Microbiol Biotechnol 56: 650-663.

Mehdi, H. and Giti, E. (2008). Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. International Biodeterioration and Biodegradation 62(2): 170-178.

Mishra, S., Jyot J., Kuhad R. C. and Lal, B. (2001). In situ bioremediation potential of an oily sludge-degrading bacterial consortium. Appl. Environ. Microbiol. 67: 1675.

Nwakanma, C., and Hart, A. I. (2012). The effects of oil based on the survival of Periopthalmus barbarus after 96hrs. Current Research Journal of Biological Sciences, 4(6), 682-683.

Obire, O. and Anyanwu, E. C. (2009). Impact of various concentrations of crude oil on fungal populations of soil. International Journal of Environmental Science Technology 6: 211-218.

Okerentugba, P.O. and Ezeronye, O.U. (2003). Petroleum Degrading Potentials of Single and Mixed Microbial Cultures Isolated from Rivers and Refinery Effluent in Nigeria. African Journal of Biotechnology 2(9): 288-292.

Onyefulu, K. O. and Awobajo, O.A. (1979). Environmental aspects of the petroleum industry in the Niger Delta, problems and solutions. In: The Petroleum Industry and Niger Delta. Proceedings of the NNPC Seminar on Environment. Port-Harcourt, Nigeria, 2p.

Orisakwe, O. E., Akumka, D. D., Njan, A. A. and Afonne, O. J. (2004). Testicular toxicity of Nigerian bonny light crude oil in male albino rats. Reproductive Toxicology 18(3): 439-442.

Rahman, K. S. M., Thahira-Rahman, J., Lakshmanaperumalsamy, P. and Banat, I. M. (2002). Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresource Technology, vol. 85, no. 3, pp. 257–261.

Vasudevan, N., and Rajaram, P. (2001). Bioremediation of oil sludge-contaminated soil. Environment International, vol. 26, no. 5-6, pp. 409–411.