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Biodegradation Assessment of *Bacillus subtilis Isolated from Locust Beans* on Crude Oil

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Abstract

The potential of *Bacillus subtilis* isolated from locust beans to degrade crude oil was assessed. The microorganism exhibited a very high degradation capability towards crude oil. Gas Chromatograph attached to a Flame Ion Detector (GC-FID) was used to measure the concentration of aliphatic and aromatic hydrocarbon contents in the crude oil. The *Bacillus subtilis* was found to be able to degrade about 95 %, almost all of the total hydrocarbon in the crude oil in 20 days. The result further showed that the *Bacillus subtilis* irrespective of its source of isolation can be used for degradation.

Keywords: *Bacillus subtilis; Biodegradation; Hydrocarbons; Crude oil; Locust beans.*

1. Introduction

The environmental impact of petroleum in Nigeria and other oil producing countries like Saudi Arabia, Iran, Iraq, Kuwait and others has been on the increase. The environmental concern is the destruction caused by oil spill both on cultivated and virgin lands. Oil spills are destructive to both vegetations and animals in the soil not only as a result of their toxicity but also because hydrocarbons in the soil reduce the oxygen in the soil and hence enhances anaerobiosis which is harmful to plant roots [1-3]. It is also the most serious form of water pollution and has been used in connection with losses of crude oil or petroleum products to the marine environment; about 10 % of the spilling into the sea comes from tanker accidents [2-3,4]. The frequency of large scale spillages of petroleum and its products in the last two decades has been alarming and the pollution it has caused has resulted in adverse ecological effects [5]. Crude oils from different parts of the world differ considerably in their physical and chemical properties. These differences become important in relation to the behaviour of the spilled oil to the environment and subsequent clean-up technique [2-3,6]. The growing awareness of the high level of toxicity of crude petroleum products has prompted many investigations to assess their impacts on the environment with the aim of finding possible solutions. The need to reclaim environmentally petroleum-products polluted sites has led to the development of a number of techniques which are physical, chemical or biological in approach [7].

The biodegradation of pollutants in the environment is a complex process whose quantitative and qualitative aspects depend on the nature and amount of the pollutants present, the ambient and seasonal environmental conditions. The constitution of the indigenous microbial community is also an important factor [8]. Organic compounds can be degraded aerobically with oxygen or anaerobically without oxygen. Aerobic pathology is the most effective strategy for bioremediation. The initial steps in the catabolism of aliphatic, cyclic and aromatic hydrocarbon by bacteria and fungi involve the oxidation of the substrates by oxygenases for which molecular oxygen is required. Aerobic conditions are therefore necessary for the microbial

oxidation of hydrocarbon in the environment [8]. The availability of oxygen in soils is dependent on rates of its consumption microbially, type of soil, the extent of water logging of the soil and the presence of utilizable substrates leading to oxygen depletion. Anaerobic degradation rate of hydrocarbon is slower than the aerobic. During biodegradation, crude oil is used as a source of organic carbon in a microbial process resulting in the breakdown of crude oil components to components of lower molecular weight. The microorganisms convert the hydrocarbons into carbon IV oxide and water with the release of energy and cell mass which are essential to the microbial growth, development and activities [9].

Microorganisms produce certain enzymes that have the capability to attack hydrocarbon molecules thereby causing degradation. The degradation of oil relies on having sufficient microorganisms to degrade oil through the microbial metabolic pathways, which are series of steps by which degradation occurs. Incidentally, nature has evolved many microorganisms to do this job [10]. There are over seventy known genera of microorganisms that are known to degrade hydrocarbons. These microorganisms usually account for less than 1 % of natural population of microorganisms, but can account for more than 10 % of the population in polluted eco-system. When microorganisms are not present in a system, they can be added to promote biodegradation. The added microorganisms can be cultures grown from other contaminated area or they can be microorganisms genetically engineered to degrade oil. However, even when microorganisms are present, degradation of hydrocarbons can take place only if all other basic requirements of the microorganisms are met. Microorganisms have the naturally occurring microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons. Most microorganisms use the soil as their habitat [11].

The inherent biodegradability of different components of crude oil is a reflection of their chemical structures, but also strongly influenced by the physical state and toxicity of the compounds. For instance, the C_5 to C_{10} homologous have been shown to be inhibiting the activity of majority of hydrocarbon degraders. The homologues as solvents disrupt lipid membrane structures of microorganisms, similarly, alkanes in the C_{20} to C_{40} range are hydrophobic solids at physiological temperatures, and hence it is the physical state that strongly influences their biodegradation. As in the case of alkanes, the monocyclic compounds, cyclopentane, cyclohexane and cycloheptane have strong solvent effect on lipid membranes and are toxic to the majority of the hydrocarbon degrading microorganisms. Highly condensed cycloalkane compounds resist biodegradation due to their structure and physical state [12].

Ekpo and Udofia [13] carried out a research on the rate of biodegradation of crude oil by microorganisms isolated from oil sludge environment. According to their report, mineral salt medium supplemented with crude oil was used and the most abundant species isolated from crude oil soil sludge. The microorganisms selected for the degradation tests include *Micrococcus varians* and *Pseudomonas aeroginosa* and were found to degrade 97.2 % and 85.7 % respectively. The degradation of diesel by *Bacillus subtilis* was also reported [14]. The study was designed to isolate, characterize and identified diesel degrading microorganisms. The isolation of *Bacillus cereus, Trichoderma harzanium* and *Trichotercium roseum* was also reported in the study. The study revealed the degrading ability of these microorganisms with *Bacillus subtilis* having the greatest potential of them all hence the possibility of crude oil fractions been degraded. Bello and Isinguzo [15] reported the rehabilitation of polluted soil in Nigeria using genetically modified microbial inoculums of a bacterium, *Bacillus sp* and a fungus, *Aspergillus niger*. The experiment was carried out in-situ with Nigerian crude oil, Bonny light in polluted teak soil using anoxic cultures of the bacterium and fungus.

There are two distint biological mechanisms known for the degradation of water-insoluble hydrocarbons and aromatic compounds. The first case involves the use of microorganisms as emulsifiers to overcome poor solubility of hydrocarbons and aromatic compounds, whilst the other is to increase cell surface hydrophobicity which enhanced their adherent capacity to the hydrocarbon [16]. Most of the case, microorganisms use both mechanisms with one mechanism acting dominantly with the dominant mechanism been easily figured out by centrifugation of the cell broth after cultivation using hydrocarbons or oils. If the increase of cell surface hydrophobicity were the dominant mechanism, most of the cells would exist in the interface of

aqueous and oil phase after centrifugation. Most of the cells would be in the bottom of the aqueous phase in the opposite case [17]. Factors that affect biodegradation of hydrocarbons in the soil include; soil temperature, type and amount of hydrocarbons present, nutrients, soil pH, aeration, type and population of hydrocarbon degrading microorganisms present or introduced into contaminated soil. The manipulation and optimization of these factors is needed to get the best result [18].

In this work, the potential of *Bacillus subtilis* to degrade crude oil was investigated.

2. Materials and methods

2.1. Sampling

The samples used in this study were bought at the popular Tejuosho Yaba Market in Yaba, Lagos, Nigeria. It is the popular Ekiti Locust beans called 'Iru Ekiti' The Locust beans samples were collected in a sterile polythene bags and taken to the laboratory for isolation of microorganisms by serial dilution technique and agar plating method [19-20] in the Microbiology laboratory of the Yaba College of Technology, Yaba, Lagos, Nigeria. The crude oil was gotten from Chevron Nigeria Limited, Warri, Nigeria.

2.2. Isolation and identification of bacteria from soil samples

The isolation of bacteria species from the contaminated soil samples was done by serial dilution technique and agar plating method. 1g of soil sample was taken and then dissolved in a bottle containing 99 ml sterile dilute saline solution to make a dilution 10^{-2}. This was placed in the laboratory shaker for about 3hrs at 30°C to allow for homogenization of the solution. Exactly 0.1 mL water was transferred into 9 ml sterile dilute saline solution to make a dilution of 10^{-3}. This was done subsequently to produce a dilution 10^{-4}, 10^{-5}, and 10^{-6} [19-20]. Then 1 mL of each saline dilution was plated on Bushnell-Hass agar having composition

$$(1\,g\ KH_2PO_4, 1\,g\ K_2HPO_4, 1\,gNH_4NO_3, 0.05\,g\ FeCl_3, 10.0\,ml\ Crude\ Oil, 15\,g\ agar, 0.2\,g\ MgSO_4.7H_2O, 0.02\,g\ CaCl_2.H_2O, 0.3g\ Cholesterol, 1000ml\ Distilled\ water, pH7.2)$$

The inoculation was done by spreading method. The chemicals were purchased from Sigma-Aldrich. The plate was later incubated for 36 hrs at 37°C.

2.3. Identification of bacteria

The *Bacillus subtillis* colonies were identified and characterized based on the results of their gram reaction test, morphological features and biochemical characteristics compared with Bergey's Manual of Determinative Bacteriology [19-21].

2.4. Preparation of innoculum

The inoculums' was made by transferring the 40 mL of culture from nutrients agar slants in Bushmell Hass medium into 100 mL flask. Then 2 mL of crude oil was introduced into the flask and allowed to incubate at 35°C in a rotary shaker at 150 rpm for 30 days. The samples was removed after 30 days and centrifuged for about 20 mins at 5000 rpm so as to allow the biomass and the supernatant that contains the crude oil to be separated. About 2 Ml of hexane was added to extract oil [19-21]. Control experiment was also prepared.

2.5. Gas chromatography analysis

The concentrations of various hydrocarbon compounds were determined by gas chromatography with flame ionization detector (GC-FID, Agilent 4890). The GC was equipped with a glass column (3.2mm x 1m) that had been packed with activated silica gel and anhydrous sodium sulphate. The flow rate of the nitrogen carrier gas was 30 mL/min and the column temperature was programmed from 80°C through 250°C at 5°C/min. The injector and detector temperature was maintained at 250°C and 280°C respectively.

3. Results and discussion

The *Bacillus subtilis,* also known as the hay *Bacillus* or grass *Bacillus,* a member of the *Bacillus* genus family *Bacillus subtilis* is a rod shaped, gram-positive, Catalase-positive and has the ability to form a tough, protective endospore that allows the organism to tolerate extremes of environmental conditions of heat, acid and salinity. The endospore is formed at times of nutritional stress, thus allowing the organism to persist in the environment until conditions become favourable. Prior to the production of the spore, the bacterium may become motile through the production of flagella and also take up DNA from the environment. The bacterium commonly found in soils [22]. *Bacillus subtilis* is used as soil inoculants in horticulture and agriculture. It can also convert explosives into harmless compounds of nitrogen, carbon IV oxide and water [23]. In this paper, its biodegradability potential is being investigated.

The chromatogram of the control experiment is shown in Figure 1. The crude oil used was shown to contain 1.836×10^5 ppm. of aliphatic hydrocarbon and 1.387×10^5 ppm of aromatic hydrocarbon as revealed by the gas-chromatography analysis. The retention times for the aliphatic hydrocarbons were found to vary between 8.036 to 8.230 minutes and 18.552 to 18.629 minutes for the aromatic hydrocarbon contents.

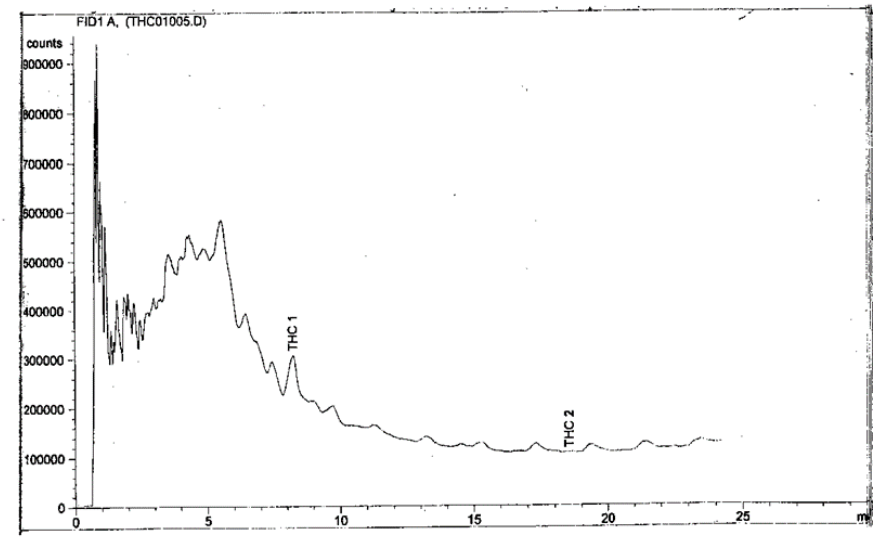

Fig. 1. GC-FID chromatogram showing the total aliphatic and aromatic hydrocarbon in crude oil before biodegradation

Figure 2 shows the chromatogram of the analysis at 20 days. On comparative look of the two chromatograms i.e the control and the inoculated sample, it was observed that the peaks of the crude oil contents for the inoculated sample whose retention time are above 8 minutes have drastically reduced. This shows that their contents have reduced as a result of degradation while the peaks before a retention time of 8 minutes have increased. Although the amount of aliphatic hydrocarbons decreased compared to the control sample, the increase in the peak may be due to the higher aliphatic compounds and aromatic compounds that have broken down to smaller compounds.

The trend of biodegradation of the aliphatic hydrocarbon content of the crude oil by the *Bacillus subtilis* is shown in Figure 3. It can be seen that the *Bacillus subtilis* was able to degrade the hydrocarbon very well. Initially, the rate as was exemplified at the onset of the process was slow up to the sixth day or thereabout. This may be attributed to the need of the organism to adapt to the new non aqueous environment and the time required by the hydrocarbon to diffuse to the water-oil interface where the biodegradation can take place.

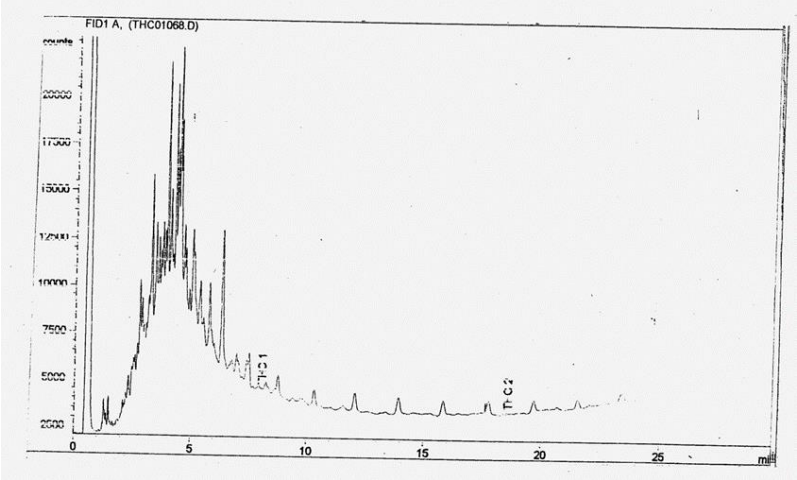

Fig. 2. The GC-FID chromatogram showing the total aliphatic and aromatic hydrocarbon in crude oil after biodegradation

There was an appreciable increase in the rate of disappearance of the aliphatic hydrocarbon after the eight day till when the hydrocarbon was almost exhausted, this time the plausible explanation is that the organism has acclimatized with the new environment and is using the hydrocarbon as well as other nutrients which are not of interest to this study but are available for growth as well as for rapid multiplication and other metabolic activities [24]. Further, the operating temperature at which the biodegradation experiment was set up is also the optimal incubation temperature of the *Bacillus* thus the biodegradation was expected to increase tremendously [8]. At about 16 days from commencement of the reaction, almost all the hydrocarbon has been exhausted.

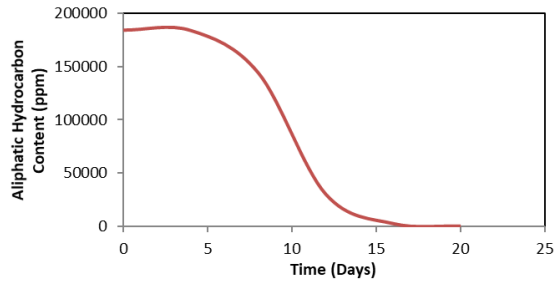

Fig. 3. Aliphatic hydrocarbon content biodegradation against time

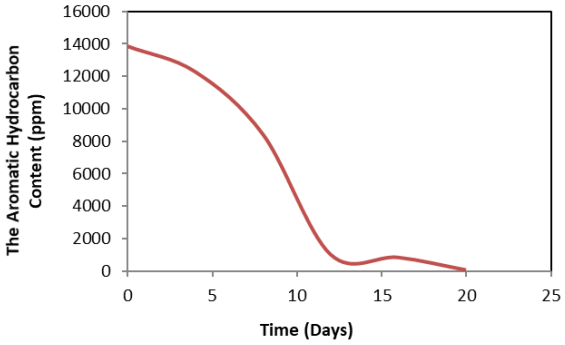

Fig. 4. Aromatic hydrocarbon content biodegradation against time

The consumption pattern of the aromatic hydrocarbon content of the crude oil is illustrated in Figure 4. The consumption pattern of the aromatic hydrocarbon content of the crude oil is not as rapid as that of the aliphatic. The amount of aromatic hydrocarbon content in ppm degraded per day compared to that of aliphatic is small. It is a known fact that the aliphatics are easily degraded relative to the aromatics. Generally, the *Bacillus* degraded the two classes of hydrocarbon considered in this work and thus a good candidate in the biodegradation of crude oil.

4. Conclusions

This paper describes the biodegradation of crude oil by *Bacillus subtilis*. The microorganism was found to have the capability of degrading about 95 % of each of the aliphatic and aromatic hydrocarbon components of the crude oil. The result further showed that the *Bacillus subtilis* irrespective of its source of isolation can be used for degradation

References

[1] Atlas RM. Microbial Degradation of Petroleum Hydrocarbons. An Environment perspective. Microbiol. Rev, 1981;45 **:**180 – 120.

[2] Das N , Chandran P. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. Biotechnology Research International, 2011: 1 – 13.

[3] Brewer R, Nagishima J, Kelley M, Heskett M, Rigby M. Risk-Based Evaluation of Total Petroleum Hydrocarbon in Vapour Intrusion Studies. International Journal of Environmental Research and Public Health, 2013; 10 (6): 2241 – 2467.

[4] Atlas RM and Bartha R. Hydrocarbon Biodegradation and Oil Spill Bioremediation, Adv. Microb. Ecol., 1992; 12 : 278 – 338.

[5] Amadi A, Dickson A, Mater G. Water, Air and Soil pollution, J. Nig. Soc. Chem. Eng., 1993; 10: 76 – 79.

[6] Ferrari MD, Neirotti E, Albornol C, Mostazo MR, Cozzo M. Biotreatment of Hydrocarbons from Petroleum Tank Bottom Sludges in Soil Slurries, Biotechnology*,* 1996; 18: 1241 – 1246.

[7] Jordan RE, Payne JR. Fate and Weathering Petroleum Spills in the Marine Environment, Ann. Arbour Sci*.,* 1990: 405 – 409.

[8] Leahy JG, Colwell RP. Microbial Degradation of Hydrocarbons in the Environment, *M*icrobiol. Rev., 1990; 54 **:**305 – 540.

[9] Rozas LP, Minello TJ, Henry CB. An Assessment of Potential Oil Spill Damage to Salt Marsh Habitats and Fishery Resources in Galveston Bay, Texas. Marine Pollu. Bull*.,* 2000; 40(12): 1148 -1160.

[10] Nakano MM, Zuber P. Anaerobic Growth of a Strict Aerobe (*Bacillus subtilis*), Annu. Rev. Microbial., 1998; 52(1): 65 – 90.

[11] Dusseault MB. Comparing Venezuela and Canadian Heavy Oil and Tar Sands, Canadian International Petroleum Conference, 2001.

[12] Alboudwarej A. Highlighting Heavy Oil. Oilfield Review*,* 2006; 5 (4) : 93 -102.

[13] Ekpo MA, Udofia US. Rate of Biodegradation of Crude Oil by Microorganisms Isolated from Oil Sludge Environment, African Journal of Biotechnology, 2008; 7 (24) : 4495 – 4499.

[14] Nwaogu LA, Onyeze GOC, Nwabueze RN. Degradation of Diesel Oil in a Polluted Soil Using Bacillus subtilis. Afr. J. Biotechnol. 2008; 7 :1939 – 1943.

[15] Isinguzo NJ, Bello OS. Polluted Soil Rehabilitation Using Genetically Modified Mix Microbial Inoculums. Journal of Food Agriculture and Environment. 2005; 3(2) :299 – 301.

[16] Moran AC, Olivera N, Commedatore M, Esteves JL, Sineriz F. Enhancement of Hydrocarbon Waste Biodegradation by the Addition of a Biosurfactant from Bacillus subtilis 09. Biodegradation, 2000; 11**:** 65 – 71.

[17] Goswami P, Singh HD. Different Modes of Hydrocarbon Uptake by Two Pseudomonas species, Biotechnol. Bioeng. 1991; 37:1—11.

[18] Admon S, Green M, Avnimelech Y. Biodegradation of Hydrocarbon in Soil during Land Treatment of Oil Sludge, Bioremediation Journal ,2001; 5(3): 193 – 209.

[19] Prakash B, Irfan M. Pseudomonas aeruginosa is Present in Crude Oil Contaminated Sites of Barmer Region (India). Journal of Bioremediation and Biodegradation. 2011; 2(5) : 1 – 2.

[20] Gopinathan R, Prakash M, Bharathirajan R. An Experimental Study of Crude Oil Biodegradation in Contaminated Soil, International Journal of Current Microbiology and Applied Sciences. 2012; 1(1) : 12 – 16.

[21] Vinothini C, Sudhaker S, Ravikumar R. Biodegradation of Petroleum and Crude Oil *by Pseudomonas pitida* and *Bacillus cereas*. International of Current Microbiology and Applied Sciences. 2015; 4(1): 318 – 329.

[22] Madigan MT, Jung DO, Karr EA, Sattley WM, Achenbach LA, van der Meer TJ. Diversity of anoxygenic phototrophs in contrasting extreme environments, in Geothermal Biology and Geochemsistry in Yellowstone National Park, eds, Inskeep W. P., McDermott T. R., editors. (Bozeman: Montana State University Publications;), 2005: 203–21.

[23] Euzeby JP. Classification of domains and Phyla – Hierarchical Classification of Prokaryotes (Bacteria) in List of Prokaryotic Names with standing in Nomenclature (LSPN,), 2008.

[24] Namkoong W, Hwang, E, Park J, Choi, J. Bioremediation of Diesel Contaminated Soil with Composting, Environmental Pollution. 2003; 119: .23 – 31.

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