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A Microcosm-Based Study on the Biodegradation of Nigerian Crude Oil by *Bacillus subtilis* Isolated from a Hydrocarbon-Impacted Soil

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A B S T R A C T

Crude oil spill serves as a source of contamination to soil and ground water leading to hazards that can negatively affect humans, health and the environment. In this study, soil samples were collected from an auto mechanic workshop, and hydrocarbon-degrading bacteria were isolated following an enrichment culture procedure. Three strains of *Bacillus subtilis* were characterized among the bacteria isolated and subjected to growth on Bushnell Haas medium to determine their hydrocarbon degrading potential. The isolate, *Bacillus subtilis* BS2 showed the best potential and was further subjected to biodegradation studies for a period of sixteen (16) days. It's crude oil degrading ability was monitored by determining the optical density and hydrocarbon utilizing bacterial (HUB) count at regular intervals. The OD was observed to progressively increase with the highest value taken on day sixteen (16), this increase in optical density (OD) was also confirmed by the values of hydrocarbon utilizing bacterial (HUB) count. *Bacillus subtilis* BS2 was able to degrade crude oil with HUB counts in the range hence, this strain can be used in cleaning of oil polluted sites.

Keywords: Crude oil; *Bacillus subtilis*; biodegradation; hydrocarbon; Nigeria.

1. Introduction

The world is moving towards an era that is facilitated by the use of renewable sources of energy such as bioenergy, solar, hydropower, wind geothermal and nuclear energy, etc. While the last decade has witnessed the transition from the use of non-renewable energy and fossil fuels[1], there is a need to mitigate the menace such as carbon risk, pollution that affects large area surfaces and subsurface water, abnormal chemical concentration on lands, oil spillages, etc. that has stemmed (and still stemming) from the current use of a non-renewable source of energy, petroleum, and its fractions[2].

A sustainable solution is required to address petroleum spillage that many oil-producing countries face. According to Bang and Lahn[3], petroleum resources is central to the development of many nations' economies around the globe, including Nigeria. However, from this natural resource comes negative impacts that not only affect the political, economic, and geopolitical sphere of living but also the environment, social life and quality of people's living. Numerous bacteria can break down some of the simpler chemicals, but fewer types of bacteria have the capacity to break down hydrocarbons. Instead, each type of bacterium specialized in a small number of hydrocarbons as preferred food sources, making it impossible for one type of bacterium to produce all the diverse enzymes[4].

Petroleum, during the process of drilling, refining, storage, exploration and transportation can spill and seep into the environment thus causing pollution and contamination of water bodies and land[5]. Studies have shown that petroleum contains hepatotoxic and haemotoxic substances, as well as carcinogens such as radioactive materials, polycyclic aromatic hydrocarbons and heavy metals including lead and cadmium. These compounds are usually accumulated in agricultural products from areas that are polluted and when consumed are detrimental[6,7].

The process of bioremediation is eco-friendly, and more cost-effective than other conventional mechanical or chemical procedures[8]. Optimisation of bioremediation involves a

complicated system with numerous variables, the availability of contaminants to the microbial population, the presence of oxygen or other electron acceptors in the environment (soil type, temperature, pH, and nutrients), as well as the existence of a microbial population capable of decomposing the pollutants[9]. Biosurfactants (BS) are substances produced by some hydrocarbon-degrading bacteria that aids the breakdown of petroleum hydrocarbons. Specific types of microorganisms use substrates including simple sugars, oils, and hydrocarbons to produce biosurfactants, which are chemically active surface compounds. These microbes thrive in contaminated settings. They lessen the surface tension interface between liquids and solids, which raises the bioavailability of organic contaminants, such as those found in crude oil, and thus promoting biodegradation[10].

According to[11], it was established that bioremediation may hold the potentials to solve the perils of petroleum contamination in the environment as this process involves the exploitation of microorganisms that can degrade petroleum[12]. The aim of the study is to evaluate the potential of *Bacillus subtilis* isolated from an automobile workshop in the biodegradation of crude oil.

2. Materials and Methods

2.1 Sample Collection

Twenty grams (20g) of soil sample with a long history of petroleum contamination was collected from a motor repair garage located in Samaru, Zaria, Kaduna State, Nigeria. The soil sample was transported to the laboratory in the Department of Microbiology, Ahmadu Bello University, Zaria for further analysis. One hundred milliliters (100mLs) of crude oil used for this study was collected from the National Research Institute for Chemical Technology (NARICT) Zaria, Kaduna State, which was donated to them for research purposes by Kaduna Refinery and Petrochemical Company (Kaduna, Nigeria). Crude oil was collected in a sterile bottle of 750 mL capacity and transported to the laboratory in the Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

2.2 Isolation of *Bacillus subtilis*

Isolation was done according to the method of Singh and Singh (2020) with slight modification. Ten grams (10g) of soil sample was added to 90mL of sterile distilled water and heat shocked for 15 minutes at 70°C using a water bath (model number: DK-420), after which 0.1ml was spread on the surface of sterile nutrient agar plates, incubated at 35°C for 24-48 hours. After incubation, suspected colonies having rough with waxy growth (1-4mm diameter), white opaque flat colonies with irregular spreading edge was examined by Gram staining procedure as outlined below[13]

Gram staining procedure was performed according to Tripathi and Sapra[14], the endospore staining was carried out following the protocols of Schaeffer and Fulton[15].

2.3 Biochemical Characterization of the Isolates

Biochemical tests that include catalase test, citrate test, Voges-Proskaeur test, methyl red test, indole test and oxidase test was carried out for identification and characterization of *Bacillus subtilis*[16].

2.4 Screening of Isolates for Hydrocarbon Utilisation

Based on the outcome of the biochemical characterization, three strains tentatively identified as *Bacillus subtilis* were selected for a 48-hour screening biodegradation experiment. This was conducted by streaking colonies of pure isolates of the selected strains on the surface of freshly prepared Bushnell Haas medium (In 1L of distilled water: Magnesium sulphate 0.2g, Calcium chloride 0.02g, Ferric chloride 0.005g, Dipotassium phosphate 1g, Ammonium nitrate 1g and Monopotassium phosphate 1g), overlaid with a thin layer of crude oil, incubated at 37°C for 48 hours. After the period of incubation, the strain showing the most proficient growth was selected for further biodegradation experiment[4].

2.5 Biodegradation Studies

Using a slightly modified method described by Atta[17], one milliliter of crude oil was added to 10mL of mineral salt medium (In 1L of distilled water: Dextrose 1g, Ammonium sulphate 1g, Dipotassium phosphate 7g, Monopotassium phosphate 2g, Sodium citrate 0.5g and Magnesium sulphate 0.1g), the selected strain of *Bacillus subtilis* was suspended in 4mL sterile normal saline, and the turbidity was adjusted to match 0.5 MacFarland turbidity standard which is equivalent to $1.5x10^8$ bacterial density. An aliquot of one millilitre (1mL) of the inoculant was added into mineral salts medium and an abiotic control (uninoculated) was also included in the set-up. The experimental set-up was incubated at ambient temperature on an orbital shaker at 150rpm for 16 days. At intervals of 4 days, the crude oil was dissolved with chloroform and extracted along with the mineral salt medium of each treatment and assessed for hydrocarbon removal by taking the absorbance at 600nm and comparing it with the control setup[19]. Hydrocarbon utilizing bacterial counts were also conducted during same intervals.

2.6 Hydrocarbon utilizing bacterial (HUB) count

Hydrocarbon utilizing bacterial count was determined by carrying out a ten-fold serial dilution up to 10^{-5} using a 1mL aliquot from the tubes in the setup. Aliquot of 0.1mL was aseptically transferred to the surface of freshly prepared nutrient agar using spread plate technique. The inoculated media were incubated at 37°C for 24 hours, colonies observed were counted using a colony counter[20].

3. Results

The bacteria isolated are as presented in Table 1. The hydrocarbon-degrading potential of the isolates, *Bacillus subtilis* BS1, *Bacillus subtilis* BS2 and *Bacillus subtilis* BS3 are as shown in Table 2, *Bacillus subtilis* BS2 showed the greatest potential.

Table 1: Biochemical and Characterization of strains of *Bacillus subtilis*.

Test	*Bacillus subtilis* BS1	*Bacillus subtilis* BS2	*Bacillus subtilis* BS3
Gram staining	Gram positive rods	Gram positive rods	Gram positive rods
Endospore	+	+	+
Catalase	+	+	+
Citrate	-	-	-
Voges Proskaeur	-	-	-
Methyl red	+	+	+
Indole	-	-	-
Oxidase	-	-	-

+: positive, - : negative.

As observed in Figure 1, there was an increase in optical density (OD) of *Bacillus subtilis* BS2 when compared with the

control, this indicates the utilization of crude oil for metabolic activities, and this is further confirmed by the increase in hydrocarbon utilizing bacterial (HUB) count as seen in Figure 2.

Table 2: Hydrocarbon utilizing bacterial count of bacteria during preliminary screening.

Isolate	Counts (CFU/g)
Bacillus subtilis BS1	9.40 x 10^4
Bacillus subtilis BS2	2.89 x 10^5
Bacillus subtilis BS3	3.20 x 10^4

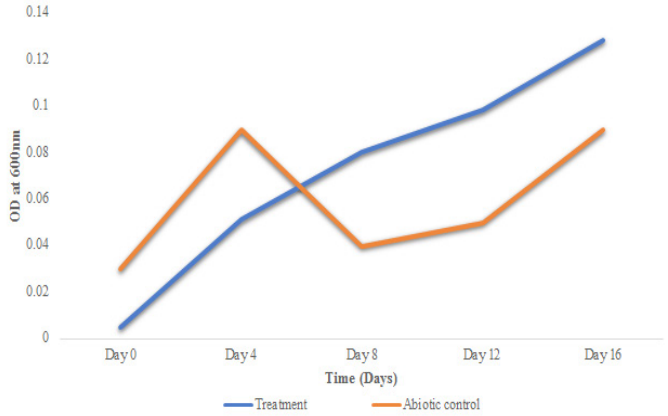

Figure 1: Optical density of growth medium during crude oil degradation by *Bacillus subtilis.*

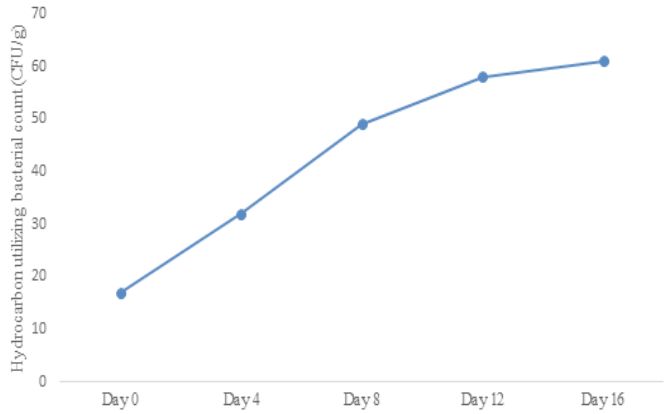

Figure 2: Hydrocarbon Utilizing Bacterial Count of *Bacillus subtilis* during degradation of crude oil.

4. Discussion

The soil sample collected for the isolation of *Bacillus subtilis* is very rich in hydrocarbons due to its history of exposure to different crude oil factions thus, the microorganisms associated with the soil are adapted to utilizing these compounds and they can serve as a source of carbon, nutrient or energy which they utilize for their metabolic activities[21].

Using the natural microflora in contaminated soils to clean up oil spills may be beneficial[22] since the microbes dwelling in those environments may have already adapted to using the hydrocarbons for metabolism. Bacteria may use the carbon molecules in oil as a vital source of energy and have a significant capacity to adapt to their surroundings by using a unique enzymatic system. Hydrocarbons come in a wide variety, and over millions of years, bacteria have developed catalytic enzymes that are unique to various breakdown processes[21]. Since all of the bacteria in the current study were isolated from a petroleum-impacted soil, they survived and adapted to the oil-contaminated environment very easily as reported by other authors[23]. Microorganism survival in petroleum hydrocarbons medium after their inoculation is a key determining factor in the rate of biodegradation of hydrocarbons either in soil or in liquid phase[24].

Based on the canonical biochemical tests carried out as seen in Table 1, three (3) strains of *Bacillus subtilis* were isolated and screened for their potential to degrade crude oil, each strain exhibited different capabilities as seen in Table 2, the difference exhibited might be due to difference in genetics of each individual strains which resulted in different growth rate and utilization capacities of crude oil by the bacteria. The strain, *Bacillus subtilis* BS2 showed the highest number of colonies after incubation, this could be because it possesses a more robust metabolic capability compared to the other two strains.

The biodegradation of crude oil by *Bacillus subtilis* BS2 showed a constant increase in the optical density (OD) as seen in Figure 1, the reason for this increase in the optical density (OD) is due to the increase in the number of cells of the inoculants as a result of their capacity to be able to utilize the hydrocarbons present in the crude oil for their metabolic activities. The abiotic control was shown to have less values than the treatment thus, implying that loss of hydrocarbons by the bacterial inoculant is more pronounced than that due to other possible physical factors such as photooxidation and evaporation. However, on day 4 where the abiotic control was observed to have a higher optical density than the treatment, it could be as a result of the bacterial inoculant adjusting to the growth medium hence it's slow growth before eventually surpassing the control in subsequently. Some researchers have reported that *Bacillus subtilis* degradation may be due to its highly resistant endospore[4]. Due to the high concentration of crude oil during the initial period of treatment period of treatment, *Bacillus subtilis* BS2 exhibited an increase in biomass that confirms the strain being able to breakdown and utilize crude oil leading to an increase in hydrocarbon utilizing bacterial count as observed in Figure 2. Increase in bacterial growth is linked to nutrient availability and as such increase in hydrocarbon utilizing bacterial count (HUB) due to utilization of the crude oil as a source of carbon and energy. The effectiveness of *Bacillus subtilis* in breaking down crude oil has been examined in several laboratory investigations. For instance, *Bacillus subtilis* was found to be capable of utilising up to 75% of the total petroleum hydrocarbons present in crude oil after incubation, according to a study by Safdari[25]. Similar findings were made in a 2015 study by Sakthipriya[26], who discovered that *Bacillus subtilis* could break down 80% of the total petroleum hydrocarbons in crude oil after only 10 days of incubation. These investigations show that *Bacillus subtilis* can degrade crude oil in a laboratory setting. Bacterial consortium containing *Bacillus subtilis* and *Pseudomonas aeruginosa* indicated a very significant reduction in total petroleum hydrocarbon level in contaminated soil (76% degradation) when compared to the control soil (3.6% degradation) after 180-day post-inoculation at remediating locations that have been contaminated with crude oil, according to field research[27].

5. Conclusion

Bacillus subtilis possess the potential to degrade hydrocarbon compounds because of its ability to grow in high concentration of crude oil. This is confirmed by the increase in optical density (OD) and hydrocarbon utilizing bacterial (HUB) count, respectively. The benefit of bioremediation is the mineralization of the pollutants, which leads to an ultimate formation of H_2O,

CO_2 and biomass; these byproducts make bioremediation environmentally friendly and cost effective.

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