Bionatura Journal
Ibero-American Journal of Biotechnology and Life Sciences

Article

Biodegradation efficiencies of Low Pour Fuel Oil by *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates.

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ABSTRACT

This study explored the efficiencies of *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates in the degradation of a derivative of crude oil, Low Pour Fuel Oil (LPFO), commonly known as Black oil. The comparison was carried out on the effects of nutrient stimulation on the degradation of LPFO by the selected organisms. After a 14-day treatment, correlational analysis of the biodegradation test showed a significant solid correlation between organisms and different treatments at $p<0.01$. There was an increase in the counts of *B. licheniformis* and *P. aeruginosa* during the degradation process. The susceptibility of the hydrocarbon compounds to microbial degradation varied with the type and size of the hydrocarbon molecules. Alkanes of intermediate chain length (C_{10}–C_{24}) were degraded rapidly compared to long-chain alkanes (C_{20}–C_{34}). There was a significant increase in degradation when the LPFO was inoculated with *B. licheniformis* and *P. aeruginosa*, while there was no significant effect of nutrient amendment on the hydrocarbon degradation compared to treatments with individual microorganisms alone. The average Degradation Efficiency was 99.9%. *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates were influential in the degradation of LPFO and can be employed in the remediation of contaminated soil.

Keywords: biodegradation, bio-stimulation, biotechnology, hydrocarbon, low-pour fuel oil

INTRODUCTION

Increasing industrialization associated with economic growth and demand for more new derivatives of crude oil and consequent spillages have presented more challenges to applications of biotechnology in the remediation of hydrocarbon-contaminated environment[1]. Varying chemical compositions and structures of these different new derivatives of hydrocarbons may affect bioavailability[2], biodegradability[3], and remediation efficiencies[4] of microbes. It may further pose challenges to selecting consortia of microbes for remediation, considering environmental factors, and resulting in microbial growth inhibition. [5,1]

The Low Pour Fuel Oils (LPFOs), called Black oil, are products derived from crude oil with major components residuals of petroleum refining operations[6, 7]. LPFOs, also known as Light oil, Marine oil, or Furnace oil, are a fraction obtained from petroleum distillation, either as a distillate or residue. LPFOs are fundamental input in steam generation in many labor-intensive industries like textiles (coloring), construction (cement), food (sugar), and beverages (sterilizing). Residual or distillate oil is one of the lowest-value petroleum products from a refinery. It is a by-product of light products like Premium Motor Spirit or Regular Motor Spirit (Petrol), Dual Purpose Kero or Household Kero (Kerosene), and Naphthalene [6,7]. LPFO is blended with other petroleum products to meet customer specifications[6].

According to Ohijeagbon et al., increased density of emissions from boiler operations of LPFOs would lead to increasing greenhouse effects and global warming by releasing such greenhouse gases as carbon dioxide, nitrous oxide, and water vapour[8]. Studies have shown that the shear strength of different soil types is reduced with increasing soil contamination with LPFO[9,7]. According to Otunyo and Anele, more damaging effects on the soil properties caused by LPFO spillage included an increase in consolidation settlement for used engine oil-contaminated laterite, which, in the case of clay soil, generally decreases with all the contaminants [9]. Effects of spillages of crude oil derivatives have silently constituted a major environmental concern, especially outside the areas of oil exploration[10]. The spillage may result from leakage of pipes, disruption of storage tanks, and failures in processing plants, refineries, and transport systems [10]. These derivatives may be discharged in large pools or areas easily dispersed by other environmental events. These derivatives may, through penetration, reach more profound levels of the earth's crust, constituting remediation challenges, especially in agricultural lands.[11] The increasing usage of most derivatives of crude oil like LPFOs and its by-products may be responsible for the uncontrolled dispersal of unused portions, spent oils, and their mixture in the environment.

Spillages occur during the production process, and environmental damages are evidently observed on vegetation and soil[12]. There might also be surface and groundwater contamination through rainwater runoffs on the surfaces of contaminated soils.[13, 14]

Restoration of the polluted soil can be attributed to employing bioremediation methods.[15,16,17] This can be achieved through the informed introduction of microbial agents and nutrient enhancement to optimize bioremediation of the contaminated soil[18]. According to Xu et al., the reduction of chemical compounds by biological catalysis is known as biodegradation. [19] Furthermore, Xue et al. defined microbial degradation of organic compounds into minute units as biodegradation. [20] Invariably, biodegradation is a process of mineralization of complex compounds. The rate of biodegradation has been established to depend on essential factors like biotic, concentration of contaminant or substrate[19], the amount of "catalyst" or microbial population[21], environmental and abiotic factors[22,] and genetic or species of microbes[19]. Biodegradation has been employed extensively to remove environmental contaminants[23,] especially those classified as intermediate refinery products that pose significant challenges.

These may be everyday experiences at spill sites of LPPFOs at Okari Jetty, a unit at Port Harcourt Refinery, Rivers State, and other oil-producing areas of Niger Delta, South-southern Nigeria. This study, therefore, explored the efficiencies of *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates in the degradation of LPFO in a laboratory test experiment. Biostimulation experiments were also carried out to compare the effectiveness of nutrient addition to laboratory treatments in the degradation of LPFO by *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates. These will further enhance the cleanup and remediation efforts of LPFOs' contaminated environment and prevent environmental consequences of LPFOs' pollution.

MATERIAL AND METHODS

Sample collection

Triplicate samples of Low Pour Fuel Oil were collected with the aid of sterilized 1 Litre amber glass bottles, with Teflon-lined lids below the point source of the discharge at the spill sites at Okari Jetty, a unit at Port Harcourt Refinery, Rivers State, Nigeria. The sample bottles were securely closed and packed carefully to prevent leaking or breakage, labeled, and immediately transported to the laboratory for analysis.

A composite mixture of fifty grams (50g) of materials from the gastrointestinal contents of five slaughtered cows was collected with sterile transport containers at an abattoir in Owerri, Imo State, Nigeria. Drops of sterile saline were added to keep small pieces of materials moist. The samples were then transported to the microbiology laboratory. At the laboratory, one gram (1g) of the gastrointestinal materials was transferred into a 9ml test tube of sterilized distilled water and subjected to a 10-fold serial dilution and bacterial isolation and characterization.

Media Preparation

Preparation of Pseudomonas P agar

Pseudomonas P agar (Sigma-Aldrich, Darmstadt, Germany) was prepared per the manufacturer's directives. A 46.4 g medium was suspended in 1000 ml of demineralized water containing 10 ml of glycerol. To completely dissolve the suspension, it was heated to boiling with agitation. The solution was sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. The sterilized solution was dispensed into Petri dishes to cool at room temperature.

Preparation of Bushnell-Haas broth

Bushnell-Haas broth was prepared according to the method outlined by Bushnell and Haas[24] (1941). A 3.27g Bushnell Haas broth (HiMEDIA, USA) was suspended in 1000 ml of distilled water. The suspension was boiled for 1 minute to dissolve the medium completely. The mixture was sterilized by autoclaving at 15 lbs. pressure at 121°C for 15 minutes.

Isolation and identification of Bacillus licheniformis

Isolation and identification of *Bacillus licheniformis* from samples of gastrointestinal materials of cows was performed according to the method by Wang and Shih[25]. Wang and Shih[25] composed Basic Growth Medium with a Minimum Growth Medium (MGM) made of (in gl^{-1}): NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4 and $MgSO_4.7H_2O$, 0.1 at pH 7. Further identification of *Bacillus licheniformis* was carried out through biochemical characterization as described by Al-Dhabaan[26].

Plate count and enumeration

Triplicate plates of each dilution were used to achieve a plate count of between 30 – 300 colonies forming units/ml (cfu/ml) as described by Prescott[27].

Colonial and Microscopic Characteristics of Test Cultures

Gram staining

The Gram Staining method of Cruickshank[28] was adopted to determine the Gram staining reactions of all bacterial isolates. With the help of a sterile wire loop, individual isolate was smeared onto clean microscope

slides, air-dried, and fixed. Crystal violet solution was poured on the smear and, after 30 seconds, washed in gentle running water for 5 seconds, covered with iodine solution, and washed again. The stain-smear was then decolorized using 95% ethanol, washed with water, and finally counter-stained with 3% Safranin solution for 15 seconds. The smear was washed in clean tap water, air–dried, and observed first under X60 magnification and finally under an oil immersion object lens (X100).

P. aeruginosa was isolated and identified with the aid of the Cetrimide Agar medium, which is selective for *P. aeruginosa* and other species of *Pseudomonas.* Cetrimide Agar medium is made up of Cetrimide, which is known for inhibiting the growth of numerous bacteria, including gram-positive bacteria, while *Pseudomonas* species, especially Pseudomonas aeruginosa, proliferate.

Biochemical characterization of bacterial isolates

Further identification was carried out through biochemical characterization as follows.

Nitrate reduction test

Test tubes with nitrate broth and inverted Durham's tube were autoclaved. These were allowed to cool at room temperature. The bacteria isolates from fresh (24 hours old) cultures were then inoculated into the tubes using a sterile inoculating loop. The tubes were thereafter incubated at 37ºC for 24 hrs. The presence of gas in the Durham's tube showed a positive result.

Methyl red (MR) and Voges Proskaur (VP) test

After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35 °C for up to 4 days, the change of color to red indicates a positive MR result [29].

Oxidase test

This test was carried out by smearing a filter paper earlier saturated with freshly prepared oxidase reagent with bacterial colony. A positive oxidase test was recorded as the development of a blue-purple color within 10 s [30].

Test for fermentation of sugars

Bacterial isolates were tested for their ability to ferment sugars like maltose, lactose, glucose, and sucrose. The medium fermentor was thoroughly mixed, and 9ml was dispensed into each clean, dry test tube containing Durham tubes and autoclaved at 121^0C for 15 minutes. A 10% stock solution of each of the sugars to be tested was prepared and sterilized by autoclaving at 115^0C for 10 minutes. One milliliter of sterile 10% sugar solution was put in each fermentation medium. A 0.1ml isolated organism emulsified in sterile peptone water was used to inoculate such a tube and incubated at 37^0C for 24 hours. Positive sugar fermentation was indicated by a change from purple to the yellow color of the medium due to acid production accompanying sugar fermentation. In tubes where fermentation was accompanied by gas production, such gas will accumulate in the inverted Durham tubes.

Catalase test

A catalase test was carried out by adding a purified bacterial culture to 5 ml hydrogen peroxide solution. Detection of gas bubbles within 10 seconds after adding bacterial culture indicated a positive catalase test[30].

Coagulase test

A tube containing ½ ml rabbit plasma was inoculated with the bacterial inoculum. This was incubated at 37º C and checked at ½ hour or the next lab period by tipping the slide at an angle. The absence of coagulation is considered a negative test for the lack of coagulase enzyme.

Urease test

Bacterial colonies were added to slanted bijou bottles filled with two millilitres of urea medium and incubated at room temperature. Red-pink color in the medium was considered as a positive test for urease induction[30].

Indole test

Bacterial colony added to SIM media was applied with 0.5 ml of Kovac's reagent and incubated at 35 °C for 24 h. The appearance of bright red and yellow colors indicated positive and negative results, respectively[30].

Simmons Citrate test

Simmons Citrate test was performed via inculcating Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures, then incubated at 37 °C up to 48 h. changing the media color from green to bright blue indicates a positive reaction.

Methyl red (MR) test

After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubating at 35 °C for up to 4 days, changing color to red indicates MR test positive- the appearance of tested bacteria[29].

Gelatin hydrolysis

The nutrient gelatin stab method was applied according to Edison[31]. Heavy inoculums of a test bacterium inoculated into tubes containing nutrient gelatin, gelatin liquefaction is the positive result for bacterial gelatin hydrolysis.

Serial Dilution of Low-Pour Fuel Oil

Nine milliliters (9 ml) of sterile distilled water (without nutrients) and 9 ml of sterile Bushnell Haas medium (with nutrients) were prepared in duplicates, and the test tubes were covered with sterile cotton wool to prevent contamination.

Analysis of hydrocarbon concentrations

Reagents: HPLC grade hexane (Spectrum Chemical, USA), HPLC grade Dichloromethane (Sigma-Aldrich, China)**,** Analytical grade Acetone (Alliance Chemical, Texas USA)**,** Anhydrous Sodium tetra-oxosulphate (vi) (Na_2SO_4) (Sigma-Aldrich, Germany)**,** Chromic Acid (Sigma-Aldrich, Germany)**,** Glass wool (Sigma-Aldrich, Germany) and Silica Gel (Sigma-Aldrich, Germany).

Apparatus/Equipment

The equipment used was HP 5890 Gas Chromatography equipped with Chem Station software workstation, capillary column, Flame Ionization Detector (FID) and chemstation software workstation, rotary evaporator**,** vials for storing extracts (2ml), and separatory funnel**.**

Procedure:

A 1:1 solvent mix of acetone/ Dichloromethane (DCM) was prepared, and 10 ml of low-paste fuel oil sample was added to an acid-washed acetone beaker devoid of water. Fifty milliliters (50ml) of Acetone/DCM solvent mix was added into the beaker. The sample was then placed in a sonicator and sonicated for 15 minutes at 65°C and was centrifuged for 10 minutes at 55°C. Samples were then carefully extracted by adding 5g of anhydrous sodium sulfate, and the samples were concentrated into 2 ml using a rotary evaporator. Components of samples were then fractionated into Aliphatic and Aromatic hydrocarbons using column chromatography packed with glass wool and silica gel; the packed column was then preconditioned with hexane through the procedure of Gas Chromatography-Mass Spectrometry (GC-MS) [32].

Test for pH

Each treatment sample was immersed with a clean, sterile electrode at a sufficient depth to allow immersion. The treatment samples were mixed by shaking and stirring for a few minutes and allowed to stand for 15 minutes. After a steady reading of the meter, the pH of the treatment was recorded.

Test for Electrical Conductivity (µS/cm)

The Electrical Conductivity (EC) meter electrode was immersed into the slurry, and the needle drift was waited to cease. The EC value was recorded for each sample.

Biodegradation treatments

Duplicate test tubes of 9 ml of sterile distilled water (without nutrients) and 9ml of sterile Bushnell Haas medium (with nutrients) broth were prepared in duplicates, as recommended by the Society for Industrial Microbiology (SIM) Committee on Microbiological Deterioration of Fuels[33]. A 0.1ml *Bacillus licheniformis* and *Pseudomonas aeruginosa* inocula were inoculated directly into the Bushnell Haas broth in duplicate test tubes. The Bushnell Haas broth was then overlaid with 1ml of sterile LPFO. These were also repeated with sterilized distilled water (without nutrients), devoid of Bushnell-Haas broth as the nutrient, to compare the effects of nutrient stimulation in the degradation of LPFO. These inoculated broths were incubated aerobically at 25-30°C for two weeks (14 days). The tubes were examined daily for growth by growth count and recorded every two days.

Initial values of total hydrocarbon concentration before inoculation with *Bacillus licheniformis* and *Pseudomonas aeruginosa* inocula and after incubation for 14 days were determined and recorded according to the different experiments.

Computation of degradation efficiency

Degradation efficiency of bacterial isolates was calculated using the following equation:

$DE = (I_c - F_c)/I_c \times 100\%$ ……Equa. (1)

Where, DE = Degradation efficiency (%)

F_c = Final concentration of hydrocarbon after treatment

I_c = Initial concentration of hydrocarbon before treatment

Statistical analysis

The results were subjected to descriptive statistics, Analysis of Variance, Duncan Multiple analysis, and correlation.

RESULTS

Bioremediation Treatments

In Table 1, the pH values of the biodegradation experiments after degradation were slightly acidic; degradation with *P. aeruginosa* (without nutrients, P) at pH 6.65±0.01; with *P. aeruginosa* (with nutrients, Po) at pH 6.41±0.01; with *B. licheniformis* (without nutrients) at pH 5.87±0.01; and with *B. licheniformis* (with nutrients, Po) at pH 5.95±0.01. The pH varied significantly from all the treatments at $p < 0.05$.

The Electrical Conductivity (EC) of the biodegradation experiments was thus: with degradation with *P. aeruginosa* (without nutrients, P) at 112.3±0.1 µ*S*/cm; *P. aeruginosa* (with nutrients, Po) at 108.6±0.10 µ*S*/cm; degradation with *B. licheniformis* (without nutrients) at 134.4±0.10 µ*S*/cm;

and *B. licheniformis* (with nutrients, Po) at 129.8±0.10 µ*S*/cm. The EC varied significantly from all the treatments at $p < 0.05$.

The Total Petroleum Hydrocarbon (TPH) concentrations in the initial concentration of LPFO (35560mg/L) were after 14 days thus, with *B. licheniformis* (without nutrients) to

2.3682±.0001 mg/L; with *P. aeruginosa* (without nutrients, P) to 9.9021±.0001 mg/L; with *B. licheniformis* (with nutrients, Po) to 2.878±.0001 mg/L and with *P. aeruginosa* (with nutrients, Po) to 9.9879±.0001 mg/L. The degradation efficiencies were thus: degradation with *P. aeruginosa* (without nutrients, P) at 99.97%; *P. aeruginosa* (with nutrients, Po) at 99.97%; degradation with *B. licheniformis* (without nutrients) at 99.99%; and *B. licheniformis* (with nutrients, Po) at 99.99%, with an overall average treatment of 99.9% efficiency.

Parameters	14P	14Po	14B	14Bo	Black Oil
pH	6.65±0.01	6.41±0.01	5.87±0.01	5.95±0.01	-
Conductivity, µS/cm	112.3±0.10	108.6±0.10	134.4±0.10	129.8±0.10	-
TPH(mg/L) / DE	**9.9021±.0001 (99.97%)**	**9.9879±.0001 (99.97%)**	**2.3682±.0001 (99.99%)**	**2.878±.0001 (99.99%)**	**35560**

Legends: P = *P. aeruginosa* only, Po = *P. aeruginosa* with nutrient, B = *B. licheniformis* only, Bo = *B. licheniformis* with nutrient, TPH = Total petroleum hydrocarbon, DE = Degradation Efficiency

Table 1: Final levels of the qualities of treatment and Degradation Efficiencies

The chromatographs in Fig. 1 were used as the control, which showed the initial concentrations of the petroleum hydrocarbon compounds, with the peaks of individual components of carbon chains, before biodegradation of LPFO with any of the bacterial isolates. Fig. 2 shows the levels of the final concentration of the petroleum hydrocarbon compounds, with the peaks of individual components of carbon chains, *Bacillus licheniformis* (without nutrients), after 14 days of biodegradation of LPFO. Fig. 3 shows the levels of the final concentration of the petroleum hydrocarbon compounds, with the peaks of individual components of carbon chains, *Pseudomonas aeruginosa* (without nutrients), after 14 days of biodegradation of LPFO. Fig. 4 shows the levels of the final concentration of the petroleum hydrocarbon compounds, with the peaks of individual components of carbon chains, *Bacillus licheniformis* (nutrients), after 14 days of biodegradation of LPFO.

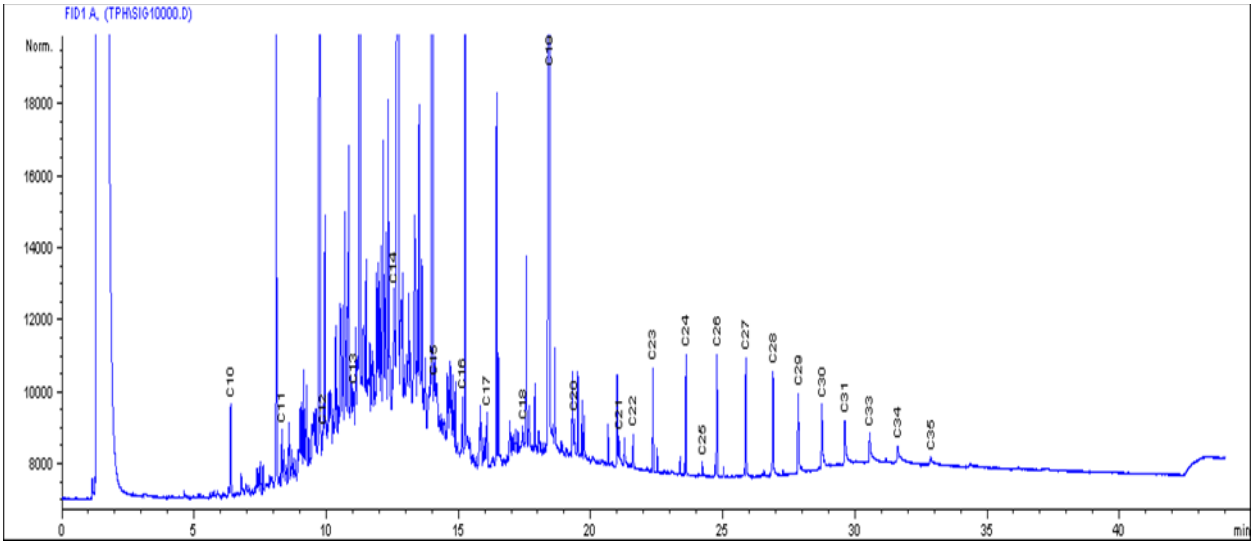

Figure 1: Initial values of LPFO in the sample before degradation

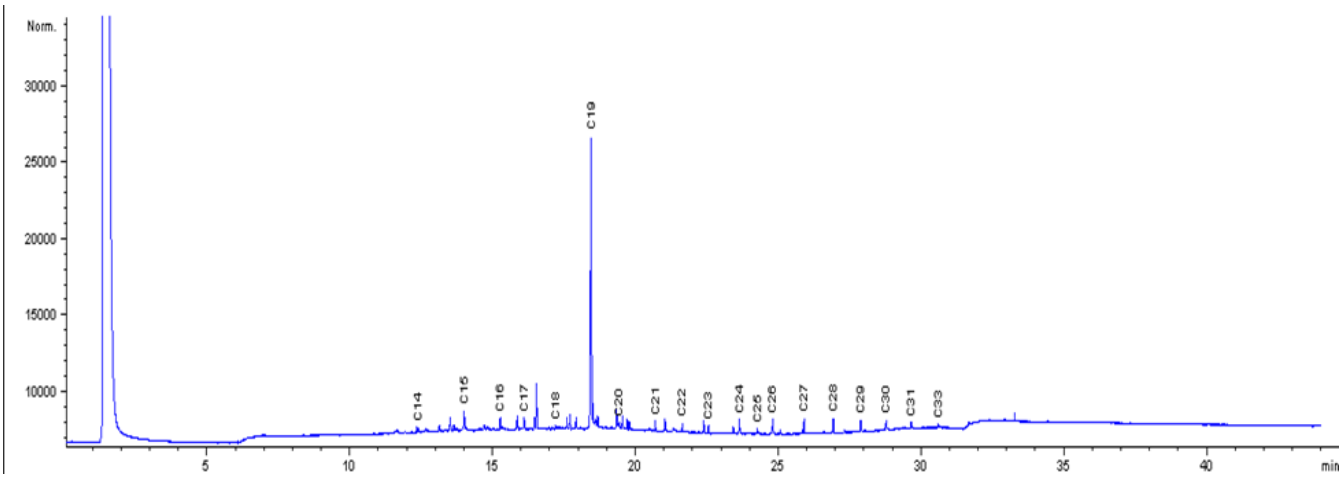

Figure 2: Degradation of LPFO by *Bacillus licheniformis* without addition of nutrient

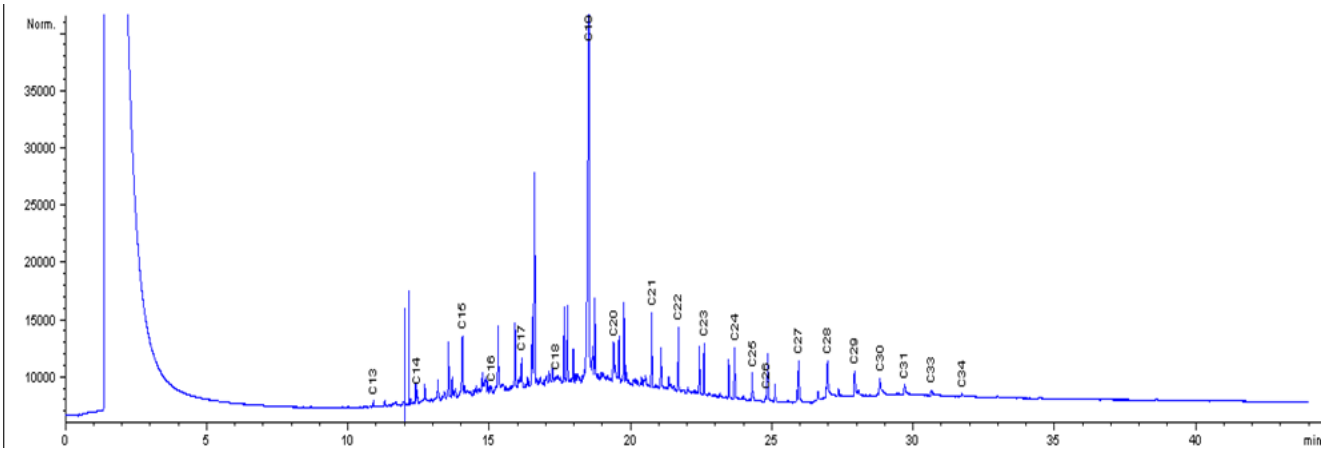

Figure 3: Degradation of LPFO by *Pseudomonas aeruginosa* without the addition of nutrient

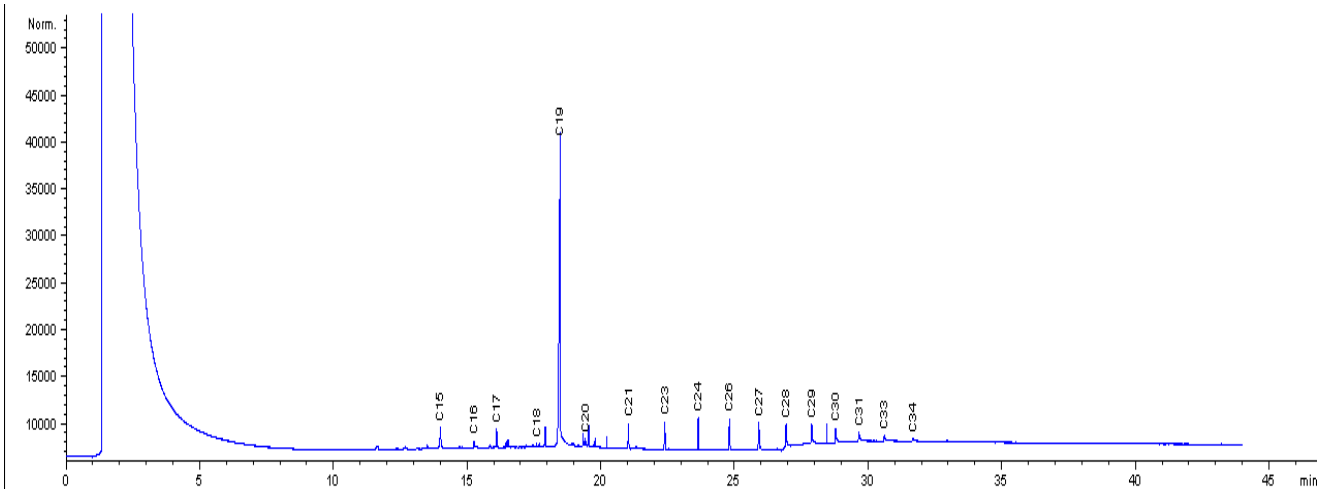

Figure 4: Degradation of LPFO by *Pseudomonas aeruginosa* with nutrient

C-group	LPFO (mg/L) with *B. licheniformis*	LPFO(mg/L) with *P. aeruginosa*	LPFO (mg/L) with *B. licheniformis* + Amendment	LPFO (mg/L) with *P. aeruginosa* + Amendment	LPFO (mg/L) before Treatment
C_{10}	-	-	-	-	12.1486
C_{11}	-	-	-	-	10.2658
C_{12}	-	-	-	-	42.5682
C_{13}	-	0.0024	-	-	12.4587
C_{14}	0.2215	0.0214	-	0.0041	51.2487
C_{15}	0.1458	0.6548	0.0254	0.5985	21.5641
C_{16}	0.1125	0.0357	0.0352	0.0258	21.2543
C_{17}	0.0985	0.7569	0.0451	0.8962	18.2652
C_{18}	-	0.0067	-	0.0325	14.2145
C_{19}	1.0115	4.7256	1.2548	2.9854	30.5648
C_{20}	0.0995	0.3124	0.1958	1.0102	11.2654
C_{21}	0.0274	0.6252	0.1152	0.0087	8.2415
C_{22}	0.0852	0.4145	-	0.0096	1.2104
C_{23}	0.0452	0.3254	0.1620	0.1242	10.2248
C_{24}	0.0958	0.2514	0.1626	0.8852	15.4412
C_{25}	-	0.0420	-	0.0045	-
C_{26}	0.1255	0.1125	0.3567	0.9652	17.5642
C_{27}	0.1128	0.2251	0.2054	0.7485	13.5487
C_{28}	0.1222	0.1254	0.1956	0.6521	10.2451
C_{29}	0.0987	0.1122	0.0639	0.5226	8.9654
C_{30}	0.0478	0.0985	0.0514	0.2948	2.6635
C_{31}	0.0311	0.0421	0.0089	0.1128	1.5621
C32	-	-	-	-	-
C_{33}	-	0.0112	-	0.0985	0.4105
C34	-	0.0066	-	0.0085	0.1025

Table 2: Hydrocarbon Concentrations after 14 days Different Treatments

Treatments	*Pseudomonas*	*Pseudomonas* + Nutrient	*Bacillus*	*Bacillus* + Nutrient
Pseudomonas	1	0.958**	0.969**	0.980**
Pseudomonas + Nutrient	0.958**	1	0.917**	0.944**
Bacillus	0.969**	0.917**	1	0.965**
Bacillus + Nutrient	0.980**	0.944**	0.965**	1

** = Significant at $p < 0.01$

Table 3: Correlation of different efficiencies of treatments in the biodegradation of LPFO

In Table 2, the initial concentration of LPFO (35560mg/L), after 14 days of treatment, individual carbon compounds were reduced thus: with *Bacillus licheniformis* (without nutrients), C_{14}(51.2487 to 0.2215mg/L), C_{15}(21.5641 to 0.1458mg/L), C_{16}(21.2543 to 0.1125mg/L), C_{17}(18.2652 to 0.0985mg/L), C_{18}(14.2145 to 0mg/L), C_{19}(30.5648 to 1.0115mg/L), C_{20}(11.2654 to 0.0995mg/L), C_{21}(8.2415 to 0.0274mg/L), C_{22}(1.2104 to 0.0852mg/L), C_{23}(10.2248 to 0.0452mg/L), C_{24}(15.4412 to 0.0958mg/L), C_{26}(17.5642 to 0.1255mg/L), C_{27}(13.5487 to 0.1128mg/L), C_{28}(10.2451 to 0.1222mg/L), C_{29}(8.9654 to 0.0987mg/L),C_{30}(2.6635 to 0.0478mgL), C_{31}(1.5621 to 0.0311mg/L), C_{33}(0.4105 to 0mg/L) and C_{34}(0.1025 to 0mg/L); with *Pseudomonas aeruginosa* (without nutrients) reduced to C_{13}(12.4587 to 0.0024mg/L), C_{14}(51.2487 to 0.0214mg/L), C_{15}(21.5641 to 0.6548mg/L), C_{16}(21.2543 to 0.0357mg/L), C_{17}(18.2652 to 0.7569mg/L), C_{18}(14.2145 to 0.0067mg/L), C_{19}(30.5648 to 4.7256mg/L), C_{20}(11.2654 to 0.3124mg/L), C_{21}(8.2415 to 0.6252mg/L), C_{22}(1.2104 to 0.4145mg/L), C_{23}(10.2248 to 0.3254mg/L), C_{24}(15.4412 to 0.2514mg/L), C_{25}(0 to 0.0420mg/L), C_{26}(17.5642 to 0.1125mg/L), C_{27}(13.5487 to 0.2251mg/L), C_{28}(10.2451 to 0.1254mg/L), C_{29}(8.9654 to 0.1122mg/L), C_{30}(2.6635 to 0.0985mgL), C_{31}(1.5621 to 0.0421mg/L), C_{33}(0.4105 to 0.0112mg/L) and C_{34}(0.1025 to 0.0066mg/L); with *Bacillus licheniformis* (nutrients) reduced to C_{14}(51.2487 to 0mg/L), C_{15}(21.5641 to 0.0254mg/L), C_{16}(21.2543 to 0.0352mg/L), C_{17}(18.2652 to 0.0451mg/L), C_{18}(14.2145 to 0mg/L), C_{19}(30.5648 to 1.2548mg/L), C_{20}(11.2654 to 0.1958mg/L), C_{21}(8.2415 to 0.1152mg/L), C_{22}(1.2104 to 0mg/L), C_{23}(10.2248 to 0.1620mg/L), C_{24}(15.4412 to 0.1626mg/L), C_{26}(17.5642 to 0.3567mg/L), C_{27}(13.5487 to 0.2054mg/L), C_{28}(10.2451 to 0.1956mg/L), C_{29}(8.9654 to 0.0639mg/L),C_{30}(2.6635 to 0.0514mgL), C_{31}(1.5621 to 0.0089mg/L), C_{33}(0.4105 to 0mg/L) and C_{34}(0.1025 to 0mg/L); and with *Pseudomonas aeruginosa* (nutrients) reduced to C_{14}(51.2487 to 0.0041mg/L), C_{15}(21.5641 to 0.5985mg/L), C_{16}(21.2543 to 0.0258mg/L), C_{17}(18.2652 to 0.8962mg/L), C_{18}(14.2145 to 0.0325mg/L), C_{19}(30.5648 to 2.9854mg/L), C_{20}(11.2654 to 1.0102mg/L), C_{21}(8.2415 to 0.0087mg/L), C_{22}(1.2104 to 0.0096mg/L), C_{23}(10.2248 to 0.1242mg/L), C_{24}(15.4412 to 0.8852mg/L), C_{26}(17.5642 to 0.9652mg/L), C_{27}(13.5487 to 0.7485mg/L), C_{28}(10.2451 to 0.6521mg/L), C_{29}(8.9654 to 0.5226mg/L),C_{30}(2.6635 to 0.2948mg/L), C_{31}(1.5621 to 0.1128mg/L), C_{33}(0.4105 to 0.0985mg/L) and C_{34}(0.1025 to 0.0085mg/L).

Variations in microbial counts of different samples subjected to treatment

Microbial counts of the samples subjected to treatments with *Pseudomonas* ($1.21 \times 10^9 \pm 2.07 \times 10^9$cfu/ml) and *Pseudomonas* with nutrient ($1.3 \times 10^9 \pm 1.66 \times 10^9$cfu/ml) did not vary significantly at $p > 0.108$; *Pseudomonas* and *Bacillus* ($4.5 \times 10^8 \pm 5.86 \times 10^8$cfu/ml) varied significantly at $p < 0.001$; *Pseudomonas* and *Bacillus* with nutrient ($3.51 \times 10^9 \pm 5.585 \times 10^9$cfu/ml) did not vary considerably at $p > 0.184$; *Pseudomonas*

with nutrient and *Bacillus* varied significantly at $p < 0.044$; *Pseudomonas* with nutrient and *Bacillus* with nutrient varied significantly at $p < 0.00$; while *Bacillus* and *Bacillus* with nutrient did not differ considerably at $p > 0.069$.

Table 3 below compares the initial concentrations of LPFO and their final concentrations after 14 days of treatment. It was observed after 14 days that the concentration of the petroleum hydrocarbon (PH) compounds reduced significantly when treated with microbial cultures and the addition of nutrients. There was a significant reduction in the concentration of the compound C_{19} when treated with *B. licheniformis* and *B. licheniformis* + nutrient compared with the concentration of LPFO before the samples were subjected to treatment. This same trend was observed in most of the component carbon compounds of the LPFO.

Colonial Characteristics	**Spore Formation**	**Motility**	**Gram Reaction**	**Identity of Isolates**
Small circular, colorless, moist, and shiny colonies on Cetrimide Agar and bluish-green pigment produces in Nutrient Agar.	-	+	-R	*Pseudomonas aeruginosa*
Color ranges from opaque to white, irregular, rough, and wrinkled with hair-like growth with undulate and fimbriate margins.	+	+	+R	*Bacillus licheniformis*

Legends: R= rod-shaped; + = positive test; − = negative test

Table 4: Colonial and Microscopic Characteristics of Test Cultures

NO_3 reduction	**Cit**	**Ure**	**Oxi**	**Cat**	**Coag**	**In**	**MR**	**VP**	**S**	**L**	**G**	**M**	**Identity of isolates**
+	-	-	+	+	-	-	+	-	+	-	+	+	*Pseudomonas aeruginosa*
+	+	v	-	+	-	-	+	-	+	+	+	+	*Bacillus licheniformis*

Legends: NO_3 = nitrate reduction test; Cit = citrate utilization test; Ure = Urease; Oxi = oxidase test; Cat = Catalase test; Coag = coagulase test; In = indole test; MR = Methyl Red test; VP = Voges Proskaeur test; S = sucrose; L = lactose; G = glucose; M = maltose; v = variable

Table 5: Microscopic and Biochemical Characteristics of Test Cultures

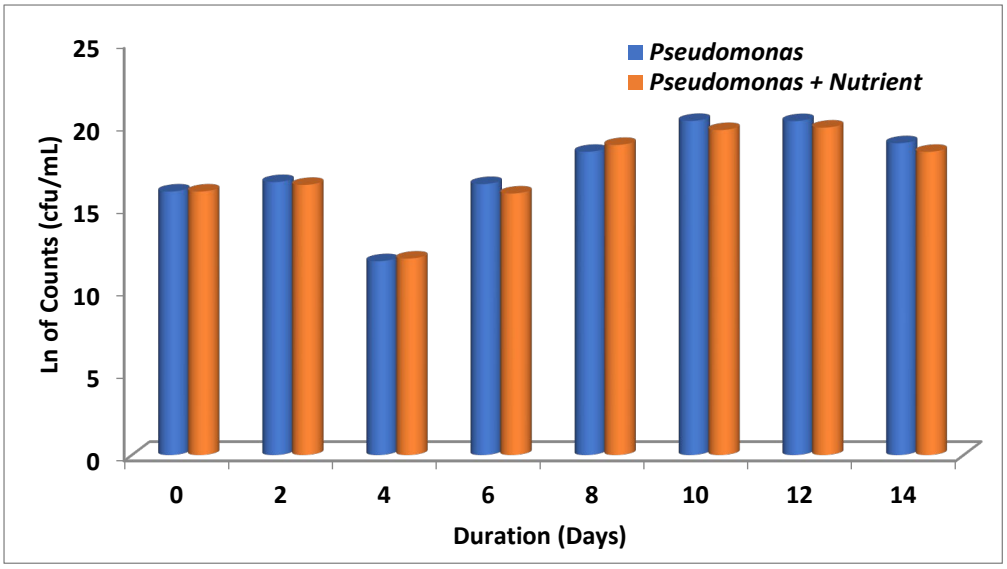

Figure. 5: Average bacterial counts in different treatments over 14 days

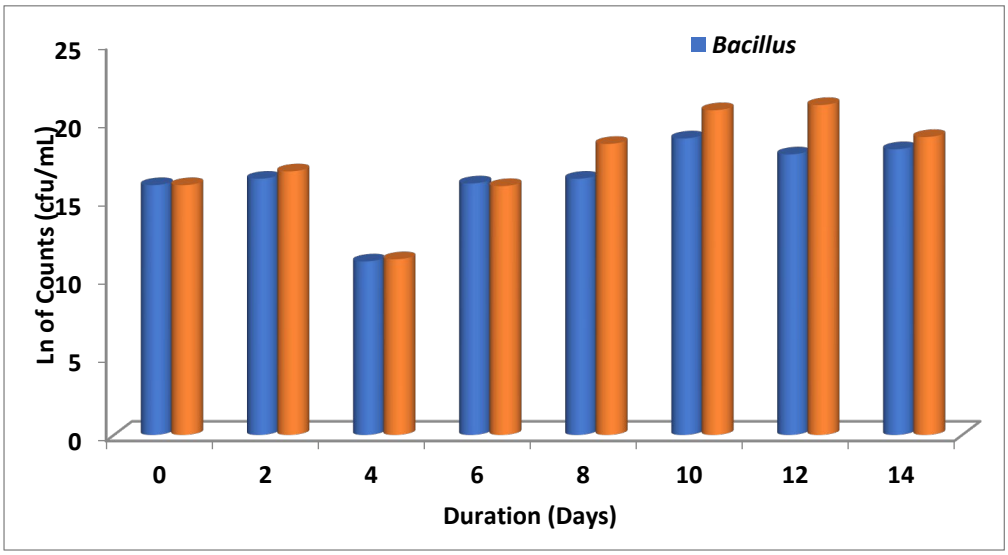

Figure. 6: Average bacterial counts in different treatments over 14 days

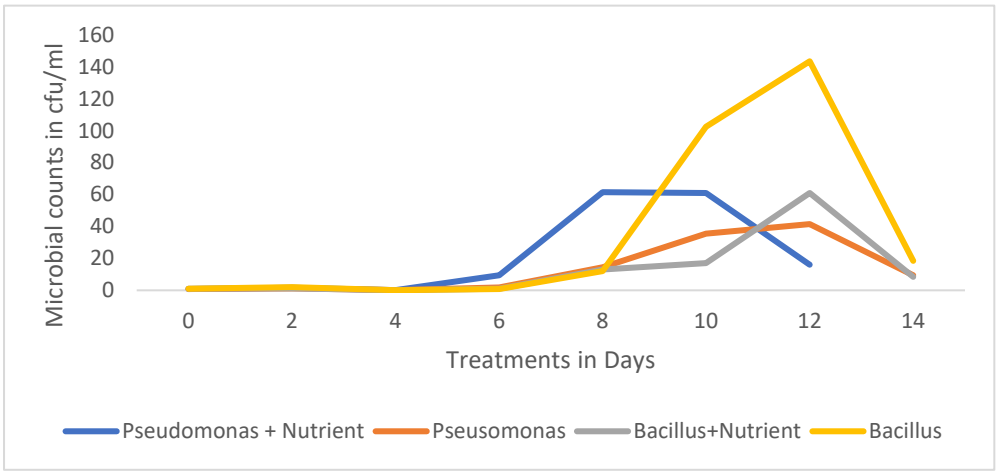

Figure 7: Bacterial growth curve for the different treatments

DISCUSSION

The influence of environmental factors rather than the genetic capability of a microorganism has been reported to limit the degradation of pollutants.[34] pH is an essential factor influencing microbiological metabolic activity and microorganism growth. Different microorganisms can grow over a wide pH range, and organisms have their tolerance levels. Studies have shown that fat, oil, and grease-degrading organisms have optimum growth between pH 5.5 and 8.0, with maximization at 7.5. [35] From the observed results in this study (Table 1), the isolates actively degraded the hydrocarbons at approximately pH 6. According to Bala et al. and Xu et al., the influence of environmental factors rather than the genetic capability of a microorganism has been reported to limit the degradation of pollutants [34,19]. According to Chen et al., the co-existence of hydrocarbon-degrading microbial consortia may increase the degradative efficiency [36], with greater tolerance to acidic pH and subsequent decontamination of polluted soils.

The results from Table 3 show the comparison of the initial concentrations of LPFO and their final concentrations after 14 days of treatment. It can be observed that the susceptibility of the hydrocarbon compounds to microbial degradation varies with the type and size of the hydrocarbon molecule. In line with the findings of Lina et al. and Liu et al., alkanes of intermediate chain length (C_{10}–C_{24}) were degraded rapidly compared to very long chain alkanes (C_{20}–C_{34}) [37, 38]. According to Nzila[35] and Constanza[39], aliphatic compounds are degraded as linear aliphatic compounds > branched aliphatic compounds >cyclic aliphatic compounds, respectively. Nzila et al. observed that the biodegradation of short and middle-chain aliphatic compounds is more extensive than the long-chain hydrocarbons [40].

There was a significant increase in degradation when the LPFO was inoculated with *B. licheniformis* and *Pseudomonas aeruginosa*. Ferreira et al. and Nayak et al. reported the ability of *B. licheniformis* and other *Bacillus sp* to degrade engine oil. [41,42]

Adding inorganic or organic nitrogen-rich nutrients (biostimulation) is a practical approach to enhance the bioremediation process.[42] Positive effects of nutrient amendment on microbial activity and/or petroleum hydrocarbon degradation have been widely demonstrated.[43,44] However, in this study, there was no significant effect of nutrient amendment on hydrocarbon degradation compared to when the individual microorganisms acted alone.

The degradation of n-alkanes higher than C_9 increases with the chain length.[45] Various microorganisms readily degrade the longer chain aliphatic hydrocarbons under aerobic conditions. However, in this study, the n-alkanes C_{19} showed resistance to degradation by both the organisms and when amended with nutrients. According to Pandolfo and Ghosal et al., the ability to degrade polycyclic aromatic hydrocarbons or their fractions is not limited to particular species but occurs over a broad group of bacterial strains [46, 47] and increasing the microbial consortia involved in this study may increase the degradation [36] of the n-alkanes C_{19}. Xu et al. noted that individual organisms often prefer to metabolize a limited range of hydrocarbon substrates. That may be the reason for the observed non-degradation of C_{19}. [48]

There was an increase in the bacterial count of B. licheniformis, followed by Pseudomonas aeruginosa during the degradation process, which demonstrated the ability to utilize LPFO as this organism's energy source. Wang et al. reported an increase in the cell number of *B. stearothemophilus* during the degradation process of engine oil.[49] Microorganisms have different rates at which they utilize and degrade hydrocarbons in the soil

or water.[50] This rate is reflected in the multiplication of isolated organisms and colony-forming units (cfu). According to Bala et al., the number of microbes that use the contaminants as carbon and energy sources increases during active contaminant biodegradation. [34]

The average bacterial counts of samples with different treatment options are shown in Figures 5 and 6, respectively. The lowest bacterial counts were observed on the 4th day in all the treatments (*Bacillus*, *Bacillus* + nutrient and *Pseudomonas*, *Pseudomonas* + nutrient, respectively). This was also observed as we studied the microbial growth curve of different treatments (Figure 7). Highest bacterial count was observed when treated with Bacillus + nutrient on the 10th and 12th day of treatment. As observed in this study, the decline in the population of degrading microbes might be due to inhibitory metabolites 51, which are produced during the degradation of LPFO. Heras-Martínez et al. reported that degradation increased with the incubation period and peaked after 30 days, where nearly 91% of the hydrocarbon was degraded. [51]

CONCLUSIONS

Based on the observations made in this study, it was concluded that *Bacillus licheniformis* could degrade LPFO than *Pseudomonas aeruginosa*. It was also observed that degradation increased with the incubation period, and on average, 99.9% of the hydrocarbon was degraded. It can be observed that the susceptibility of the hydrocarbon compounds to microbial degradation varies with the type and size of the hydrocarbon molecule. Interestingly, adding nutrient supplements showed no remarkable increase in LPFO degradation. Further work may include a field study using a diverse microbial consortium to ascertain the synergistic effect of different microorganisms. Such studies may also include bioassays on the potential toxicity of degradation products.

Author Contributions: Conceptualization, Chinedu Emeka Ihejirika, Joseph Ifeanyichukwu Garricks and Ejeagba Okorie Imo; **methodology**, Chinedu Emeka Ihejirika, and Ejeagba Okorie Imo; **software**, Joseph Ikechukwu Nwachukwu; **validation**, Chinedu Emeka Ihejirika, and Ejeagba Okorie Imo; **formal analysis**, Ihuoma Ezichi Mbuka-Nwosu; **investigation**, Chinedu Emeka Ihejirika; **resources**, Etienne Chukwuma Chinakwe; **data curation**, Obenade Moses.; **writing—original draft preparation**, Chinedu Emeka Ihejirika and Ejeagba Okorie Imo.; **writing—review and editing**, Chinedu Emeka Ihejirika and Ejeagba Okorie Imo; **visualization**, Christopher Chibuzor Ejiogu; **supervision**, Ursula Ngozi Nwaogwugwu; **project administration**, Chinedu Emeka Ihejirika, Joseph Ifeanyichukwu Garricks and Ejeagba Okorie Imo.; **funding acquisition**, Joseph Ifeanyichukwu Garricks. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Acknowledgments: We express our profound gratitude to the Laboratory Technologists of the Department of Environmental Management, Federal University of Technology, Owerri, Imo State, Nigeria, for providing technical support during the study.

Conflicts of Interest: The authors declare no conflict of interest.

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Received: June 5, 2024 / **Accepte**d: September 3, 2024 / **Published:** December 15, 2024

Citation: Ihejirika C E, Ifeanyichukwu Garricks J, Okorie Imo E, Ikechukwu Nwachukwu J, Ezichi Mbuka-Nwosu I, Chukwuma Chinakwe E, Ngozi Nwaogwugwu U, Chibuzor Ejiogu C, Moses O. Biodegradation efficiencies of Low Pour Fuel Oil by *Pseudomonas aeruginosa* and *Bacillus licheniformis* isolates. Bionatura journal. 2024;1(4):15. doi: 10.70099/BJ/2024.01.04.15

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Peer review information. Bionatura thanks anonymous reviewer(s) for their contribution to the peer review of this work using https://reviewerlocator.webofscience.com/

ISSN.3020-7886