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Screening for Biodegradative Activity of Diesel Oil by Microorganisms Isolated from Petroleum Polluted Soil of a Mechanic Workshop at AKAD Community, Akure, Nigeria

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ABSTRACT: This study was carried out to determine the bacterial and fungal flora of soils contaminated with used automobile engine oil at a mechanic workshop at AKAD community, Akure, Ondo State, Nigeria, with the aim of testing their ability to degrade diesel oil. Isolation and identification of microbes were done using standard microbiological techniques. Oil degrading activity of the isolates were assayed for using Bushnell Haas broth (BHB) supplemented with 1% diesel oil. The growths and activities of the bacterial isolates in the Bushnell-Haas broth (BHB) were monitored for seven days by taking the optical density, pH changes, and free CO_2 produced in the broth by the microbes. The bacterial species isolated and identified were *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. All of the isolates showed degradative activity with the increase in optical density and free carbon dioxide (CO_2) produced in the broth. However, the most effective diesel degrader with respect to free CO_2 produced was *Proteus mirabilis*, while *Pseudomonas aeruginosa* was most effective with respect to optical density of the broth. The fungal species isolated and identified were *Aspergillus terreus*, *Candida* species, and *Aspergillus niger*. The most effective diesel degrader with respect to free CO_2 produced is *Aspergillus terreus*, while *Candida* species was most effective with respect to optical density of the broth. Also, the growth of all bacterial and fungal isolates increased with decreasing pH. Overall, the bacterial isolates had higher degrading activities over fungal isolates throughout the incubation period. The results of this investigation revealed that hydrocarbon polluted soils harbour beneficial microorganisms, which can have obvious environmental implications because they carry out activities such as the biodegradation of diesel oil. Thus, further investigations can be carried out to validate their ability to remove hydrocarbon from the polluted environment.

Keywords: Oil Polluted Soil; Biodegradative Activity; Diesel oil Degrader; Optical density

Introduction

Several countries in the developed world depend heavily on fossil fuel to satisfy their fundamental energy needs. Indeed, anthropogenic activity is reliant on oil to meet its energy demands. Accidental release of hydrocarbons into the environment through anthropogenic activities (Jayanthi and Hemashenpagam, 2015) and its attendant detriments is not restricted to oil producing regions alone, but other areas due to tanker accidents and leakage from ruptured pipelines (Akpoveta *et al*., 2011). However, majority of oil spills are due to poor maintenance and monitoring by the oil companies (Aljazeera, 2012). In addition, it was also pointed out that some of the drilling facilities used by the oil companies are outdated and the procedures employed for preventing oil spills are ineffective (Egwu, 2012). Oil spill can also occur through mechanical failure, corrosion of pipelines, operational error, natural hazard, third party activity and sabotage (Aroh *et al.*, 2010). Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel engine oil by some vehicles and generators, greater quantities are being transported over long distances. Therefore, diesel engine oil can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanical workshops. Diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the

reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants (Baker, 1982), and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil (Zahir *et al*., 2001). Also, in pursuance of the ideals of the millennium development goals initiative increased access to good and high-quality potable water is considered paramount in attainment of the human development index. The continued deterioration in quality of fresh aquatic system by oil through sabotaging act is one major factor identified as working against full realization of this goal (Aroh *et al.*, 2010).

The process of removing these pollutants from oceans and soil is very cumbersome and expensive. Current conventional disposal methods of petroleum products include physiochemical techniques such as photo-oxidation, burying, dispersion, washing, incineration, thermal conversion, and other pyrolysis techniques (Lam and Chase, 2012). Many of these methods are, however, expensive and can result in incomplete decomposition of oil products. In addition, physicochemical methods such as volatilization, photo-oxidation and chemical oxidation are rarely successful in the rapid removal of hydrocarbon contaminants, especially the aromatics (Hu *et al*., 2013). Among several clean-up techniques available to remove petroleum hydrocarbons from the soil and groundwater, microbial biodegradation processes are gaining ground due to their simplicity, higher efficiency and cost-effectiveness when compared to other technologies (Mariano *et al*., 2007). Microbial remediation of a hydrocarbon contaminated site is achieved by the help of a diverse group of microorganisms applying enzymes in their metabolism, especially the indigenous soil bacteria (Olukunle *et al*., 2015). These microbes completely degrade or mineralize petroleum compounds into non-toxic end products that include carbon dioxide, water, or organic acids and methane, through oxidative/reductive processes (Geetha *et al*., 2013). Also, bioremediation can be performed either through addition of the oil degrading microbes into the soil in a process referred to as bioaugmentation or through provision of appropriate conditions and/or amendments (e.g. supplying oxygen, moisture, and nutrients) for the growth of the microorganisms, a process known as bio-stimulation (Das and Chandran, 2010). Bioremediation is an effective technique, environmentally friendly and cost effective for treatment of oil pollution (Geetha *et al*., 2013).

This study was designed to isolate, characterize and identify diesel oil-degrading microorganisms with respect to turbidity and free CO_2 in the broth.

Materials and Methods

Sample collection: Three soil samples were obtained from portions of chronically polluted soil at a Mechanic workshop at AKAD community, Akure, Nigeria. Five (5) soil were randomly collected in sterile plastic bags. Diesel oil was obtained from AKAD Petroleum Company, in Akure South Local Government Area of Ondo State. All samples were transported within 24 hours to the Microbiology Laboratory of the Federal University of Technology, Akure, Nigeria. The soil samples were kept under aseptic condition inside the refrigerator until use.

Isolation of bacterial and fungal cultures from soil samples: The soil samples were microbiologically analyzed using the pour plate method (Fawole and Osho, 2001). One gram (1g) of the moist soil was used to make ten-fold serial dilutions. One milliliter (1 ml) each from dilution 10^{-5} and 10^{-6} were seeded into Nutrient agar for bacteria, and 1 ml each from dilution 10^4 and 10^5 inoculated into potato dextrose agar plates for fungi. Nutrient agar plates were incubated at 37°C for 24 hours and potato dextrose agar plates at 25°C for 72 hours. Pure isolates for bacteria and fungi were obtained by sub culturing while streak technique was used for bacteria. All the microbial isolates obtained were stored at 4°C in agar slants.

Identification of bacterial isolates: Identification of the bacterial isolates was based on morphological characteristics and biochemical tests. Cultural characteristics observed include size, shape, edge, surface elevation, colour of colonies and optical characteristics of the isolates. Biochemical tests were carried out using standard microbiological procedures and isolates were identified using Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

Identification of fungal isolates: A drop of cotton blue-in-lactophenol was placed on a clean grease-free slide, a tiny mycelium/yeast colony was picked from the pure culture with an inoculating needle and placed on the clean grease-free slide, teased out to make a smear. The slide was covered with a cover slip and examined under the microscope with x10 and x40 objective lens for morphological structures. The fungal isolates were also characterized based on the colour of the colony and vegetative parts (Larone, 1995; Samson *et al.*, 2002).

Preparation of Diesel oil sample: The diesel oil sample was carefully passed through a membrane filter with a pore size of 0.22µm to sieve out and remove bacteria, debris, and other particles, thereby, making it free of contaminants and components not inherent to the diesel oil itself.

Screening for degradative activity of the bacterial isolates: The degrading activities of each isolates were obtained by using Bushnell-Haas broth (BHB) in which 1% of diesel oil was added and incubated in a rotary shaker (with a speed of 110 oscillations per minute) for seven days. Incubation was carried out at a temperature

of 37ºC. The optical density using a wavelength of 620nm was read using the spectrophotometer daily, while at the same time taking the pH value, and carrying out titrations to obtain titratable acidity (TA) value which was used to calculate the free CO_2 concentration. The activities of the bacterial isolates in the Bushnell-Haas broth (BHB) were monitored by taking the optical density, pH changes, and free CO_2 produced in the broth by the bacterial.

The Bushnell-Haas medium (BHM) was used for bacteria. The composition of the BHM is as follows: K_2HPO_4 (1 g/L); KH_2PO_4 (1 g/L); NH_4NO_3 (1g/L); $MgSO_4$ (0.2 g/L); $CaCl_2$ (0.02 g/L); $FeCl_3$ (0.05 g/L); Agar (15 g/L); carbon source (1%); and distilled water (1L) with pH 7.0 ± 0.2. The medium was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) filter sterilized diesel oil to serve as the only source of carbon and energy. The medium was incubated at 37ºC for 7 days (Bushnell and Haas, 1941).

Screening for degradative activity of the fungal isolates: The degrading activities of each isolates were obtained by using the Czapek-dox broth in which 1% of diesel oil was added and incubated in a rotary shaker (with a speed of 110 oscillations per minute) for seven days. Incubation was carried out at a temperature of 25ºC. The optical density using a wavelength of 540nm was read using the spectrophotometer daily, while at the same time taking the pH value, and also carrying out titrations to obtain titratable acidity (TA) value which was used to calculate the free CO_2 concentration in the broth. The activities of the fungal isolates in the CDM broth were monitored by taking the optical density, pH changes, and free CO_2 produced in the broth by the fungi.

The Czapek-dox medium (CDM) was used for fungi. The composition of the CDM is as follows: K_2HPO_4 (1 g/L); Sucrose (30g/L); $NaNO_2$ (2g/L); KCl (0.5g/L); $MgSO_4$ (0.5 g/L); Fe_2SO_4 (0.01 g/L); Agar (15 g/L); Carbon source (1%); and distilled water (1L) with pH 7.0 ± 0.2. The medium was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) filter sterilized diesel oil to serve as the only source of carbon and energy. The medium was incubated at 25ºc for 7 days (Czapek, 2017).

Results

Identification of bacterial isolates: Five (5) bacterial isolates that were identified in this study include: *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Proteus mirabilis,* and *Pseudomonas aeruginosa*. All the bacteria isolated were gram positive except *Proteus mirabilis* and *Pseudomonas aeruginosa.* Only *Bacillus subtilis* was able to form endospore. The morphological and biochemical characteristics of the bacterial isolates are presented in Table 1.

Table 1: Morphological and Biochemical characteristics of the bacterial isolates

Isolates	**A**	**B**	**C**	**D**	**E**
Elevation	Convex	Convex	umbonate	convex	umbonate
Margin	Entire	Entire	undulate	entire	Wavy
Pigmentation	White	Yellow	cream	cream	green
Gram stain	+	+	+	-	-
Shape	Cocci	Cocci	rods	rods	rods
Spore stain	-	-	+	-	-
Coagulase test	+	-	-	-	-
Urease test	-	-	-	-	-
Motility test	-	-	+	+	+
Catalase test	+	-	-	-	+
Citrate test	+	+	-	+	+
Indole	-	+	+	-	+
Glucose	AG	AG	AG	AG	AG
Mannitol	AG	AG	AG	AG	A
Maltose	-	A	A	A	AG
Sorbitol	-	-	-	-	-
Lactose	-	A	A	-	AG
Galactose	-	-	A	-	-
Probable organism	*Staphylococcus aureus*	*Micrococcus luteus*	*Bacillus subtilis*	*Proteus mirabilis*	*Pseudomonas aeruginosa*

Key: + = Positive, - = Negative, A = Acid production, G = Gas production, AG = Acid and Gas production

***Identification of fungal isolates*:** The three (3) fungal colonies isolated were: *Aspergillus terreus, Candida* species, and *Aspergillus niger*. Table 2 below shows the morphological and staining characteristics of the fungal isolates.

Table 2: Morphological and staining characteristics of the fungal isolates

Isolates	**A**	**B**	**C**	**D**
Morphological characteristics	Brown-black mould colonies	Whitish-pink creamy colonies	Carbon black mould colonies	Carbon black mould colonies
Microscopic Examination	Compact, biseriate, yellowish conidia, ascospores produced	Single cluster of blastoconidia which is round and elongate	Wide, septate hyphae brownish conidia, ascospores produced	Wide, septate hyphae brownish conidia, ascospores produced
Probable organism	*Aspergillus terreus*	*Candida* sp.	*Aspergillus niger*	*Aspergillus niger*

Degradative activity of bacterial isolates: The activities of the bacterial isolates in Bushnell-Haas broth were monitored by taking the optical density, free CO_2, and pH changes produced in the broth by the bacteria.
The values obtained for the optical density continued to increase as the incubation period increased. The optical density of the broth was at the peak for all the isolates on day 7 except for *Bacillus subtilis* which had its peak on day 6. *Pseudomonas aeruginosa* had the highest optical density value with *Proteus mirabilis* following closely, while *Bacillus subtilis* had the least optical density value. The optical density of the bacterial isolates is presented in Figure 1.

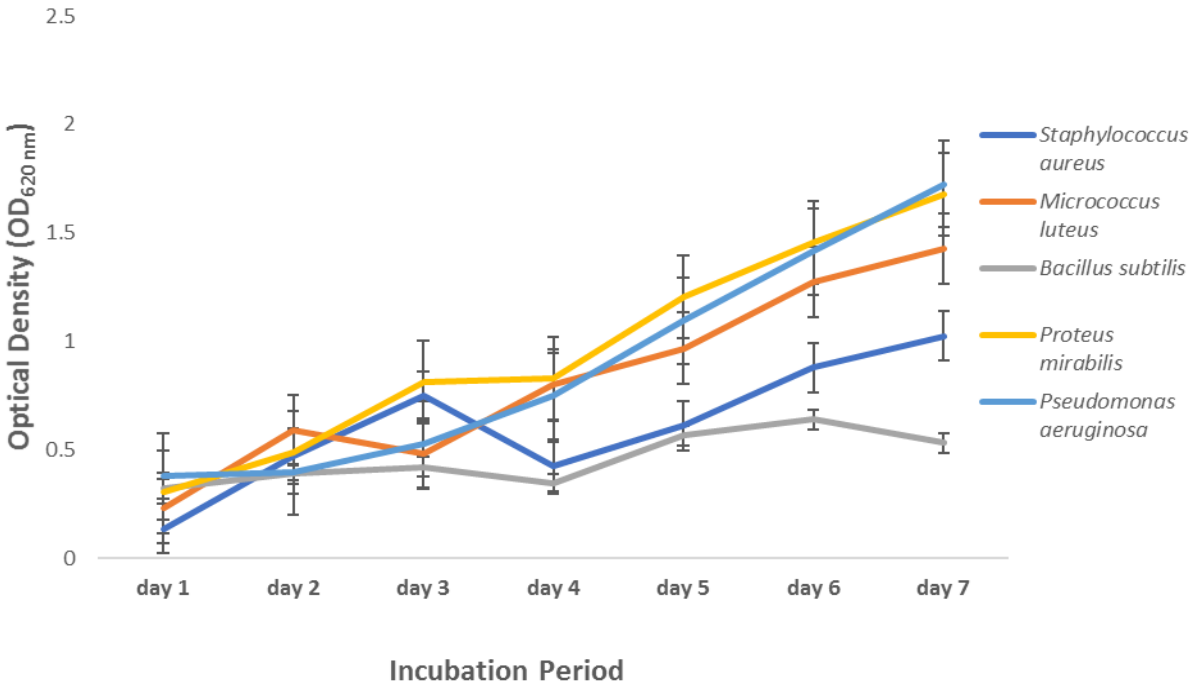

Figure 1: Optical density of bacterial isolates

The amount of free CO_2 produced fluctuated for all the isolates as the incubation period increased with *Pseudomonas aeruginosa* and *Proteus mirabilis* having its peak on day 4 while *Micrococcus luteus* had its peak on day 7. *Staphylococcus aureus* and *Bacillus subtilis* had their peak on day 3. The highest amount of free CO_2 produced was by *Proteus mirabilis* and *Pseudomonas aeruginosa* which had their peak on day 7 while *Staphylococcus aureus* had the least amount of free CO_2 produced. The amount of free CO_2 produced by the bacterial isolates are shown in Figure 2.

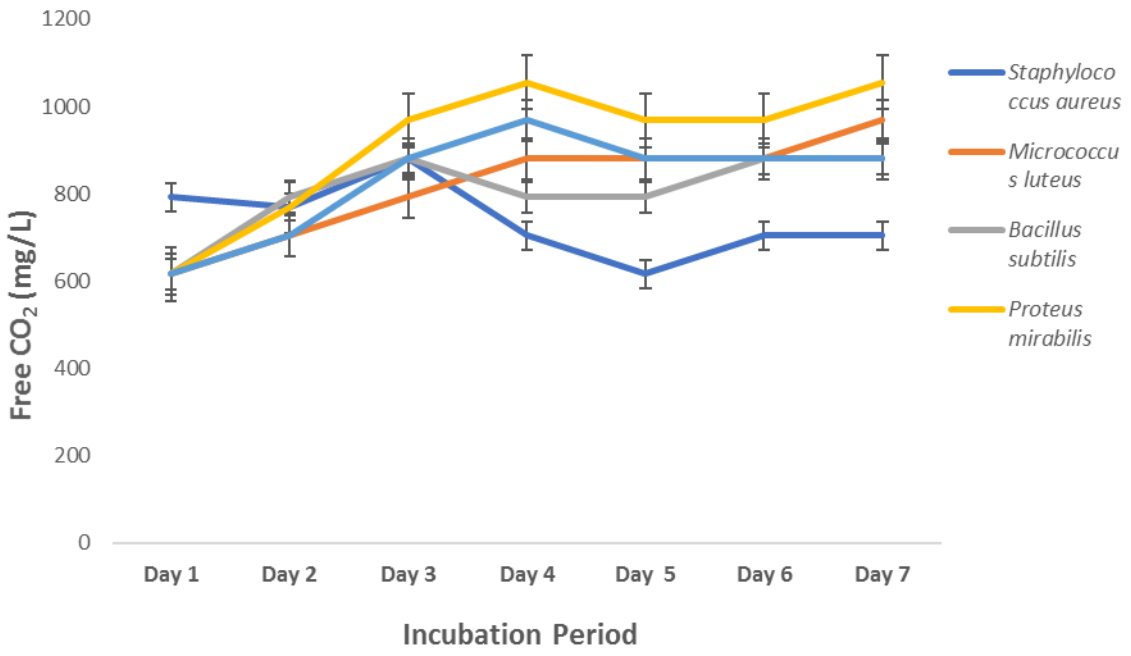

Figure 2: Free CO_2 produced by the bacterial isolates.

The pH of the broth decreased with increase in incubation time. The pH decreased throughout day 1 to day 7 for all the isolates with negligible fluctuations. These are represented in Figure 3.

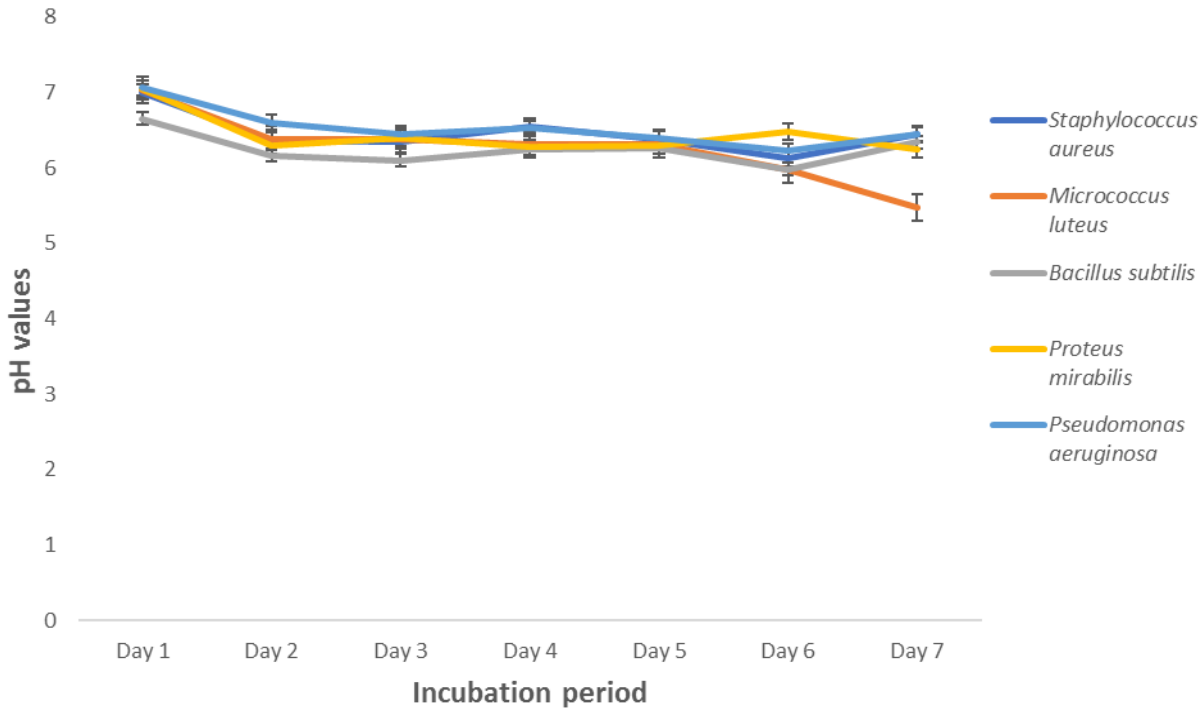

Figure 3: pH of the bacterial isolates.

Degradative activity of fungal isolates: The activities of the fungal isolates in Czapek-dox broth were monitored by taking the optical density, free CO_2, and pH changes produced in the broth by the fungi. The values of the optical density continued to increase as the incubation period increased. The optical density of the broth was at its peak for all the isolates on day 7 except for *Aspergillus terreus* which has its peak on day 6, with *Candida* sp. having the highest optical density.

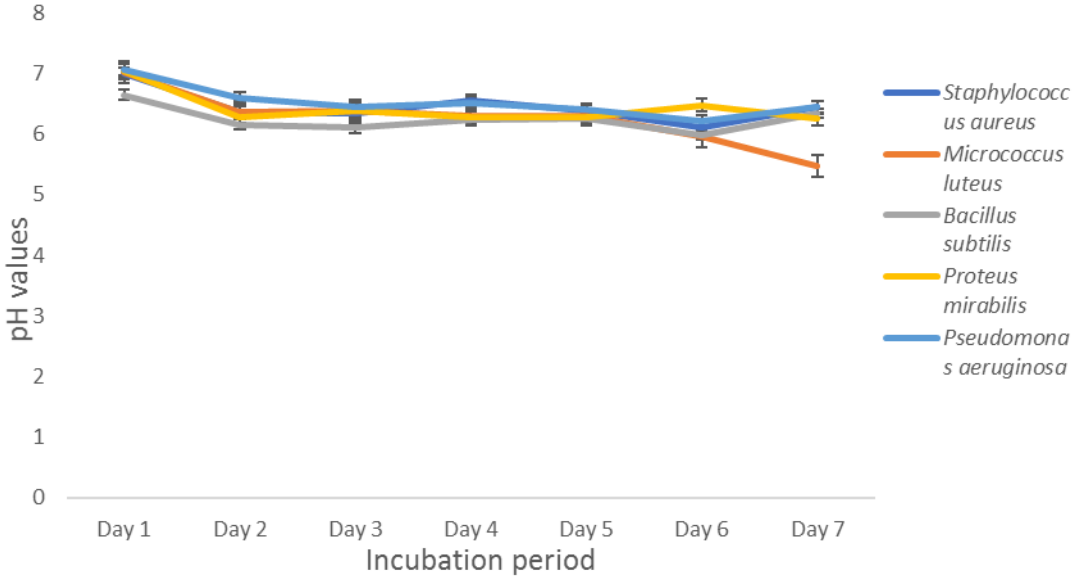

Figure 4: Optical density of fungal isolates.

The amount of free CO_2 produced fluctuated for all the isolates as the incubation period increased. The highest amount of free CO_2 produced was by *Aspergillus terreus* which had its peak on day 6.

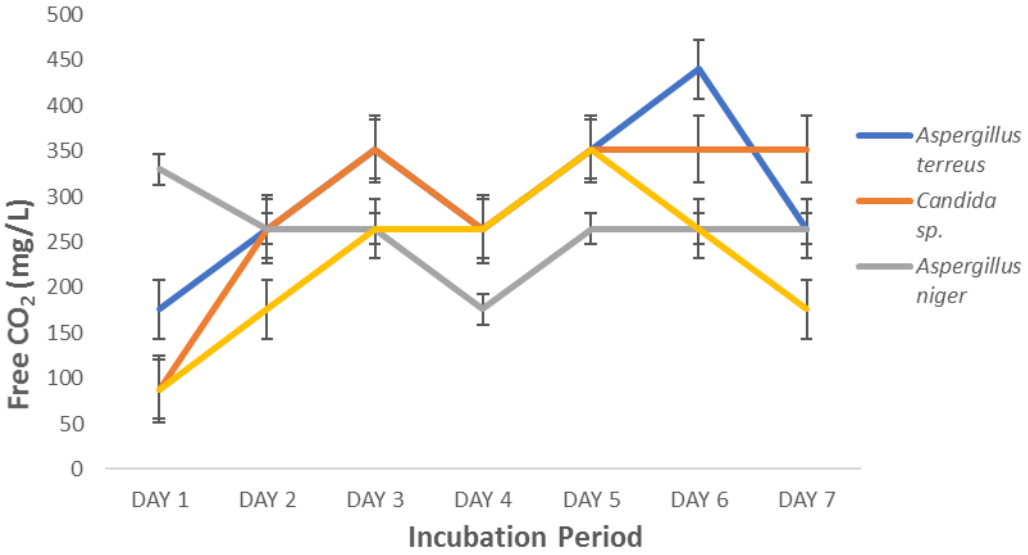

Figure 5: Free CO_2 produced by the fungal isolates

The pH of the broth decreased with increase in incubation time. The pH decreased sharply from day 1 to day 2 followed by a minimal decrease throughout day 3 to day 7. This is represented in Figures 6.

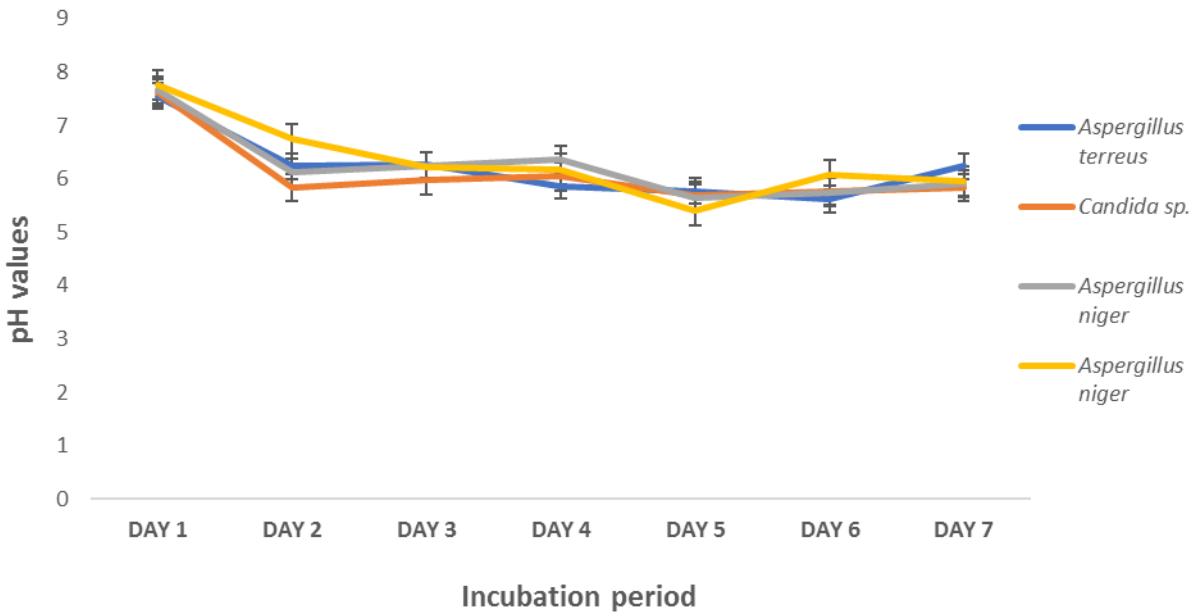

Figure 6: pH of the fungal isolates.

Discussion

Several studies have revealed that hydrocarbon polluted soils harbor beneficial microorganisms, which have obvious environmental implications because they carry out important activities, especially the bioremediation of diesel oil spills (Jahangeer and Kumar, 2013). It is obvious from this study that when the environment (broth) was contaminated with diesel, the proportion of hydrocarbon-degrading microorganisms increased rapidly. High numbers of certain hydrocarbon-degrading microorganisms from an environment implies that those organisms are the active degraders of that environment (Lima, *et al.*, 2019). The result obtained in this work agrees with that of Jayanthi and Hemashenpagam, (2015) and Lima *et al.* (2019) who recorded that *Pseudomonas, Staphylococcus, Bacillus, Micrococcus* species were isolated from oil contaminated sites in the Coimbatore region of India, and Southern Amazon of Brazil respectively. This result is also in conformity with Ebakota *et al* (2017), who isolated *Pseudomonas* sp. and *Staphylococcus* sp. from road automobile workshops and reported them to be implicated in hydrocarbon degradation in automobile workshops located in Benin Metropolis. Also, Ekanem and Ogunjobi (2017), isolated *Bacillus* sp. and *Pseudomonas* sp. from spent lubricating oil contaminated soils. Boboye *et al*. (2010), Xue *et al*. (2015) and Uddin *et al*. (2016) also reported similar results with fungi such as *Aspergillus*. Most of these bacteria and fungi belong to a group of microorganisms known as hydrocarbonoclastic microbes or hydrocarbon degraders due to their ability to metabolize hydrocarbons as their sole carbon and energy source. They are known to be abundant in the environment both in the soil and air where they carry out their metabolic activities (Schulz *et al.*, 2013).

The bacterial cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the oil for their growth and development, hence the concurrent increase in the concentration of the Bushnell-Haas broth (optical density and free CO_2 produced). All of the isolates showed degradative activity with the increase in optical density and free carbon dioxide produced in the broth. However, the most effective diesel degrader with respect to free CO_2 produced was *Proteus mirabilis*, while *Pseudomonas aeruginosa* was most effective with respect to optical density of the broth. This is in agreement with the findings of Ekanem and Ogunjobi (2017), who also found *Pseudomonas* sp. to show the highest potential for hydrocarbon utilization.

The fungal species isolated and identified were *Aspergillus terreus, Candida* sp, and *Aspergillus niger*. The most effective diesel degrader with respect to free CO_2 produced is *Aspergillus terreus*, while *Candida* sp. was most effective with respect to optical density of the broth.

Most of the isolates obtained from this study were mostly innate microorganisms that were constantly exposed to different hydrocarbon contaminants in the soil. The bacteria and fungi isolated from the diesel contaminated

soil confirms their ability to degrade the polluted soil because the microorganisms were able to use the diesel as carbon and energy sources. The presence of diesel-degrading organisms in the polluted soil is a confirmation that the innate microbes were metabolizing the diesel. This action carried out by these innate microorganisms could be responsible for the bioremediation of the environment (Boboye *et al*., 2010).

Conclusion

One of the major problems today in the environment is hydrocarbon contamination resulting from activities related to the petrochemical industries. On the basis of the experimental evidence obtained, this study showed that hydrocarbon degrading organisms can be isolated from hydrocarbon polluted sites and that the degrading ability demonstrated by the microorganisms is a confirmation that they can be applied in the bioremediation of the diesel contaminants.

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