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A LABORATORY BASED EVALUATION OF THE HYDROCARBON DEGRADATION CAPABILITIES OF SINGLE AND MIXED CULTURE OF *PSEUDOMONAS PUTIDA* AND *BACILLUS MEGATERIUM*

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Abstract Effluents released from petroleum refining typically contain microbes that are naturally adapted to using these hydrocarbons for their metabolic needs. In this study, the ability of *Pseudomonas putida* and *Bacillus megaterium* isolated from a petroleum refinery effluent to degrade diesel and spent engine oil was assessed; they were identified among the bacteria isolated using conventional biochemical and phenotypic tests. The strains (*Pseudomonas putida* C15a and *Bacillus megaterium* N9a) showing the highest potential were selected for the biodegradation studies; they were tested individually and in consortium for their ability to utilize the hydrocarbons by monitoring the hydrocarbon utilizing bacterial counts (HUB) and percentage hydrocarbon degradation. The consortium performed least efficiently than the individual strains; *Pseudomonas putida* C15a degraded 98.3% of diesel hydrocarbons with a HUB count of 1.85 x 10^7CFU/mL. The strain of *Pseudomonas putida* (C15a) isolated in this study is a very good candidate for bioremediation studies of petroleum hydrocarbons.

Key words: Biodegradation, *Pseudomonas putida*, *Bacillus megaterium*, diesel, spent engine oil

Introduction

The continued usage of petroleum as lubricating agents and to power many machinery and equipment leads to the inevitable presence of these organic compounds as pollutants in the environment. Although oil pollution is difficult to treat, petroleum hydrocarbon-degrading bacteria have evolved as a result of existing in close proximity to naturally occurring petroleum hydrocarbons in the environment. Such organisms are candidates for the treatment of oil pollutants (Margesin *et al*., 2003; Ron and Rosenberg, 2014; Lea Smith *et al*., 2015). Therefore, bacteria have been screened and utilized to degrade waste products produced by the food, agricultural, chemical and pharmaceutical industries. In recent years, the use of bacteria to deal with environmental pollutants has become a promising technology because of its low cost and eco-friendly nature (Guerra *et al*., 2018).

Petroleum refining just like other industrial processes causes the eventual release of effluents containing potentially toxic compounds such as phenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls and heavy metals. Most petroleum hydrocarbons encountered in the environment are ultimately degraded or metabolized by indigenous bacteria because of their energetic and carbon needs for growth and reproduction, as well as the requirement to relieve physiological stress caused by the presence of petroleum hydrocarbons in the microbial bulk environment (Hazen, 2010; Kleindienst *et al*., 2015).

Indeed, many studies have revealed that there is a large number of hydrocarbon-degrading bacteria in oil-rich environments, such as oil spill areas and oil reservoirs (Hazen, 2010; Yang *et al*., 2015) and that their abundance and quantity are closely related to the types of petroleum hydrocarbons and the surrounding environmental factors (Fuentes *et al*., 2015; Varjani and Gnansounou, 2017).

Many normal and extreme bacterial species have been isolated and utilized as hydrocarbon-degrading communities for tackling petroleum contamination. The degradation pathways of a variety of petroleum hydrocarbons (e.g., aliphatics and polyaromatics) have been shown to employ oxidizing reactions; however, these pathways differ greatly because of the specific oxygenases or dioxygenases found in different bacterial species. For instance, some bacteria can metabolize specific alkanes, while others breakdown aromatic or resin fractions of hydrocarbons. This phenomenon is related to the chemical structure of petroleum hydrocarbon components (Xu *et al*., 2018).

Bioremediation is the use of living organisms to degrade or detoxify hazardous wastes into harmless substances such as carbon dioxide, water and cell biomass. It is one of the most promising technological approaches to the problem of hazardous waste, which relies on microorganisms such as bacteria and fungi to transform hazardous chemicals into less toxic or nontoxic substances. Such biological transformation is more attractive than direct chemical or physical treatment. Microorganisms directly degrade contaminants rather than merely transferring them from one medium to another, employ metabolic degradation pathways and can be used in situ to minimize disturbance of the cleanup site. Hence, microorganisms can be effective, economical and non-disruptive tools for eliminating hazardous chemicals (Rončević *et al*., 2005; Maletić *et al*., 2009).

Bioremediation is suggested for treating contaminated soil sites because of its low cost and ability to convert contaminants to harmless end products. The rates of uptake and mineralization of many organic compounds by a microbial population depends on the concentration of the compound (Rahman *et al*., 2002a) High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. It is a technology for removing pollutants from the environment thus restoring the original natural surroundings and preventing further pollution (Sasikumar and Papinazath, 2003). However, most of the physical and chemical methods employed, despite the high cost, do not always ensure that the contaminants are completely removed (Ajao *et al*., 2013).

The objectives of the study include: (1) Screening the strains of *Pseudomonas putida* and *Bacillus megaterium* isolated from a petroleum refinery effluent for their capacity to degrade diesel and spent engine oil (2) monitoring hydrocarbon degradation by the selected strains using Hydrocarbon Utilizing Bacterial (HUB) count and percentage degradation.

Materials and Methods

Collection of samples

The effluent sample was collected in a sterile amber-coloured glass bottle (500 mL), from Kaduna Refinery and Petrochemical Company (KRPC), located in Kaduna State, North Western Nigeria. The petroleum products were both collected in Zaria, Kaduna State, Nigeria. Diesel (1litre) was purchased from a petrol filling station, while spent engine oil was collected from the service section of a petrol filling station. It was collected directly from the vehicle during oil change into a clean 1litre plastic bottle. The samples were transported to the Environmental Microbiology laboratory in the Department of Microbiology, Ahmadu Bello University Zaria for analysis.

Enrichment and Isolation of hydrocarbon-degrading Pseudomonas putida and Bacillus megaterium

The mineral salts medium (MSM) which was used throughout this study consist of the following composition per litre: $Na_2PO_4.7H_2O$ (64g), KH_2PO_4 (15g), NaCl (2.5g), NH_4Cl (5.0g), $MgSO_4$ (0.12g), $CaCl_2$ (5.5g). The salts were dissolved in a litre of distilled water and it was adjusted to a pH of 7.0. The preparation was sterilized by autoclaving at 121ºC for 15minutes (Musa *et al*., 2015).

Two hundred milliliters (200mL) of the prepared MSM medium contained in a 500ml Erlenmeyer flask was inoculated with 1% (v/v) of the effluent sample. The flask was incubated for 7 days at room temperature on a rotary shaker. A ten-fold dilution of the suspension was conducted and aliquots (0.1mL) of dilutions 10^{-3} and 10^{-4} was aseptically inoculated onto the surface of freshly prepared cetrimide agar (selective for *Pseudomonas* species) and nutrient agar (prepared according to manufacturer's instructions) using spread plate method. The inoculated media were incubated at 37ºC for 24 hours, and the resulting colonies were examined for size, shape, margin consistency and pigmentation. Colonies suspected to be *Pseudomonas* spp. will typically be green in colour on cetrimide agar. Discrete colonies were then aseptically sub-cultured unto freshly prepared nutrient agar based on their colonial morphology, after 24-hour incubation, the pure isolates were subsequently sub-cultured unto freshly prepared nutrient agar slants (Manal, 2011; Musa *et al*., 2015). The isolates were primarily identified based on Gram's reaction and biochemical tests, and subjected to phenotypic tests using Microgen kits; (Bioproducts, U.K) Bacillus-ID kit for *Bacillus megaterium*, while the GNA and GNB Enterobacteriacae-ID kit was used for *Pseudomonas putida*.

Screening strains of Pseudomonas putida and Bacillus megaterium for their hydrocarbon-degrading ability

Fifty millilitre (50mL) of MSM prepared as previously described was dispensed into five (5) 100mL Erlenmeyer flasks and autoclaved at 121°C at 15atm for 15 minutes. The inoculum for each strain was standardized using McFarland standard 6,

corresponding to $1.8x10^9$ CFU/mL and introduced into the sterile MSM medium at a concentration of 10% (v/v) and diesel was added at 0.5% (v/v). Two additional flasks served as control containing uninoculated MSM and uninoculated MSM + diesel, respectively. The flasks were incubated at ambient temperature on an orbital shaker for seven days. Absorbance readings (at 540nm) were recorded on the first and the seventh day of incubation respectively; increase in absorbance values was used as a measure of increase in bacterial growth and their corresponding ability to degrade diesel. The same set-up was repeated for spent engine oil (Atta, 2009). The strain of each species with the highest optical density based on the absorbance values was selected for biodegradation experiments.

Biodegradation studies

The most efficient strains in the screening test were selected for biodegradation studies. One hundred milliliters (100mL) of MSM prepared as previously described was dispensed into four (4) 250mL Erlenmeyer flasks and the selected strains of *Pseudomonas putida, Bacillus megaterium* (McFarland standard 6 was used to standardise the inoculum size) were added individually and as a consortium to the sterile MSM medium at 10% (v/v) and enriched with 0.5% (v/v) diesel or spent engine oil as sole source of carbon. An additional flask which served as the control contained only MSM and diesel. The treatments in the flasks were incubated on a rotary shaker at 150rpm at ambient temperature for a period of eighteen days and at three days' intervals, the ability of the isolates to degrade hydrocarbons was assessed by determining the hydrocarbon utilizing bacterial (HUB) count and percentage hydrocarbon degradation (using the optical density of the broth culture) (Okerentugba and Ezeronye, 2003).

Hydrocarbon utilising bacterial (HUB) count

An aliquot (1mL) of the broth culture from each flask was introduced into sterile bottles containing 9 mL of sterile distilled water, then a ten-fold dilution was carried out up to dilution 10^{-5}, and then 0.1mL of dilution 10^5 was aseptically inoculated on the surface of nutrient agar using the spread plate method and incubated at 37˚C for 24 hours, the colonies obtained were counted.

Optical density

Five milliliter (5mL) of the broth culture was aseptically transferred from each flask into test tubes, and the residual hydrocarbons extracted by adding 5mL dichloromethane (DCM) and centrifuged at 5000 rpm for five minutes. The resulting supernatant is read at a wavelength of 250nm using a UV-Visible spectrophotometer [21]. The residual hydrocarbon was calculated after determining the amount of hydrocarbon from a prepared standard using known amounts of hydrocarbon; using the formula below:

$$\text{Percentage degradation} = \left[\frac{\text{RESIDUAL HYDROCARBON } \mathbf{control} - \text{RESIDUAL HYDROCARBON } \mathbf{treatment}}{\text{RESIDUAL HYDROCARBON } \mathbf{control}}\right] \times 100$$ (Manal, 2011).

Results and Discussion

Three strains of *Pseudomonas putida* and seven strains of *Bacillus megaterium* (Table 1) were isolated from the refinery effluent sample; and were tested for their ability to degrade various hydrocarbons using diesel and spent engine oil as the sole source of carbon (Table 1). It was determined by taking their absorbance readings on day 1 and day 7 respectively. The inoculated media showed turbidity after the period of incubation, indicating the ability of each strain to utilize the hydrocarbons, but to varying degrees. Increase in optical density was related with the bacterial growth, which also shows the extent of degradation of the hydrocarbons. Highest percentage degradation of both diesel and spent engine oil for each species was observed in the strains, *Pseudomonas putida* C15a and *Bacillus megaterium* N9; as such, they were selected for the biodegradation experiments.

Table 1: Absorbance values of *Pseudomonas putida* and *Bacillus megaterium* during hydrocarbon (spent engine oil and diesel) utilization.

Isolate code	Identity	Percentage degradation (%)	
		Spent engine oil	Diesel
C2b	*Pseudomonas putida*	56.64	57.74
C11b	*Pseudomonas putida*	-37.85	-27.45
C15a	*Pseudomonas putida*	70.86	69.08
N8a	*Bacillus megaterium*	1.50	-18.8
N8b	*Bacillus megaterium*	44.88	3.1
N17b	*Bacillus megaterium*	9.64	12.5
N16a	*Bacillus megaterium*	-35.55	- 4.1
N9b	*Bacillus megaterium*	48.45	-22.8
N10b	*Bacillus megaterium*	22.73	-19.4
N9a	*Bacillus megaterium*	50.54	48.4

The highest Hydrocarbon Utilizing Bacterial Count (HUB) observed in the treatments for the degradation of diesel (Figure 1) was observed at day 15 for the treatment inoculated with *Pseudomonas putida* (1.85 x 10^7CFU/mL) followed by *Bacillus megaterium* (1.35 x 10^7CFU/mL) while the consortium had the lowest population density (6.1 x 10^6 CFU/mL). A decline in growth was observed in all the treatments by the end of the experiment (day 18).

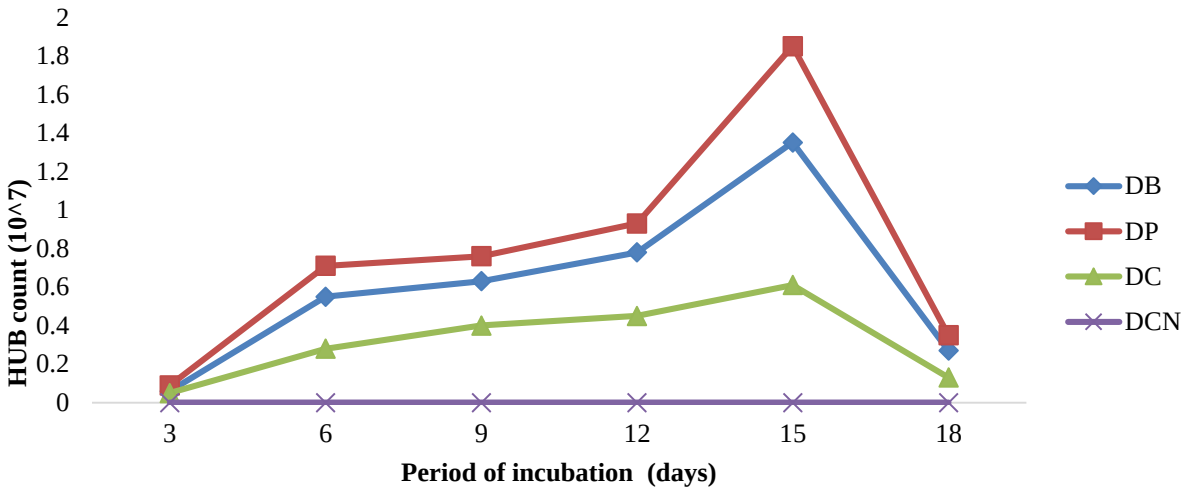

Fig. 1: Hydrocarbon utilizing bacteria count (HUB) of *Pseudomonas putida* and *Bacillus megatarium* during degradation of diesel

Key:
DB: Treatment containing only *Bacillus megatarium* and diesel
DP: Treatment containing only *P*seudomonas *putida* and diesel
DC: Treatment containing the consortium and diesel

The hydrocarbon utilizing bacterial count of the strains observed during the biodegradation of spent engine oil (Figure 2), revealed that the treatment inoculated with *Pseudomonas putida* had the highest value of 1.35 x 10^7CFU/mL followed closely by the treatment inoculated with *Bacillus megaterium* (1.05 x 10^7CFU/mL) and 5.6 x 10^6CFU/mL for the consortium on day 15. A decline in population density was observed in all the treatments by the end of the experiment (day 18).

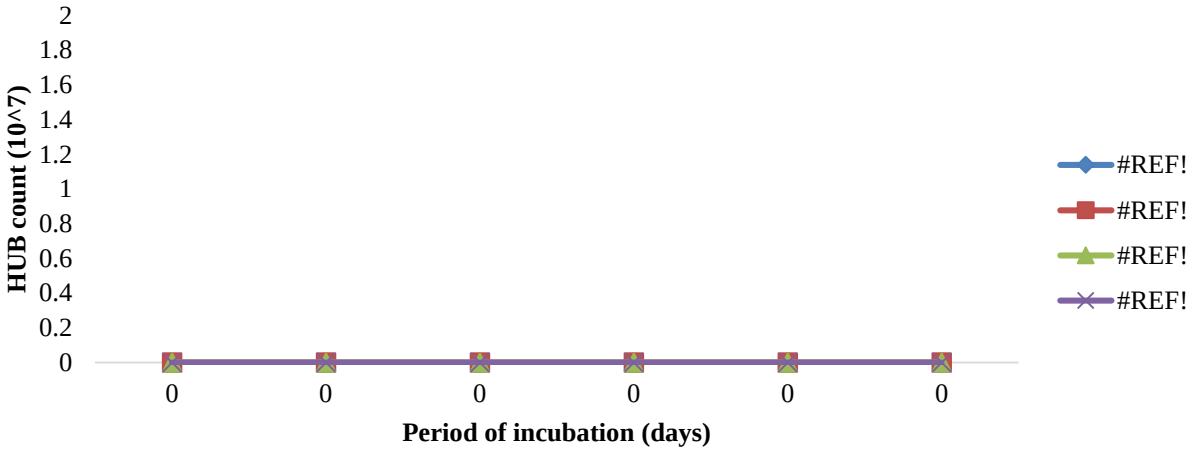

Fig. 2: Hydrocarbon utilizing bacterial count (HUB) of *Pseudomonas putida* and *Bacillus megatarium* during degradation of spent engine oil

Key:

SP: Treatment containing only *Pseudomonas putida* and spent engine oil,
SB: Treatment containing only *Bacillus megatarium* and spent engine oil
SC: Treatment containing the consortium and spent engine oil

The percentage degradation of diesel by *Bacillus megaterium*, *Pseudomonas putida* and their consortium (Figure 3) showed that the removal of the hydrocarbons increases with the period of incubation during the experimental period. The highest amount of hydrocarbon degraded was observed in the treatment inoculated with *Pseudomonas putida* (98.3%) followed by *Bacillus megaterium* (81.03%) while the consortium (68.97%) showed the least degradative capacity.

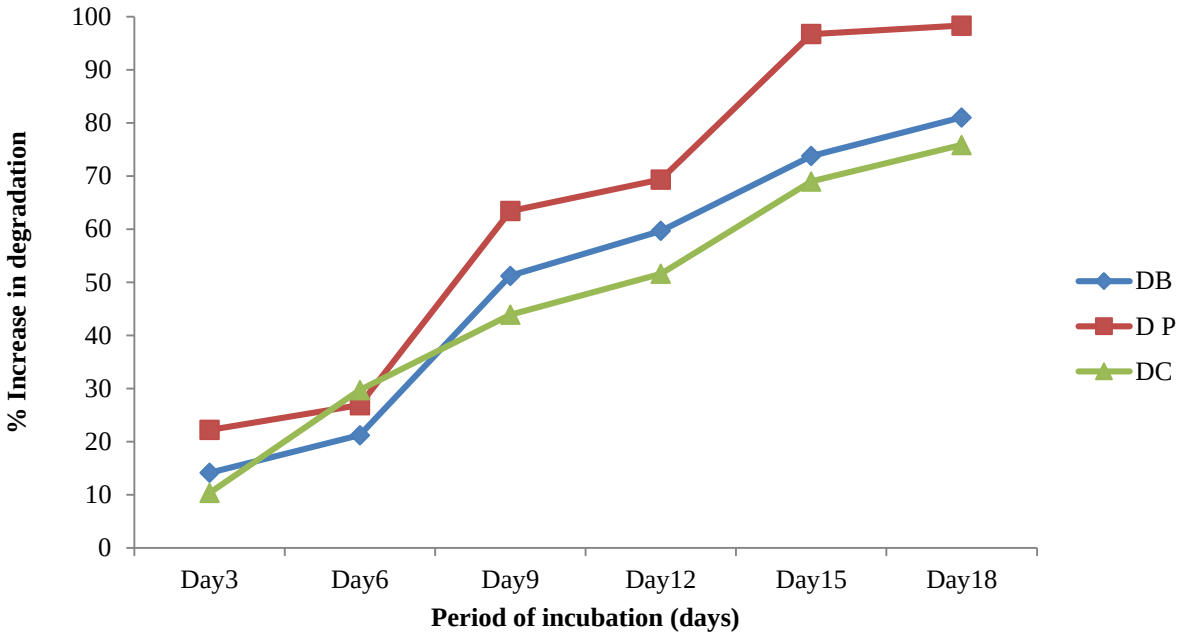

Fig. 3: Percentage degradation* of diesel by *Pseudomonas putida* and *Bacillus megaterium*

Key:
DB: Treatment containing *Bacillus megatarium* and diesel
DP: Treatment containing *Pseudomonas putida* and diesel
DC: Treatment containing the consortium and diesel
*Calculated based on the optical density

The relative degradation of spent engine oil (Figure 4) was relatively low compared to the degradation of diesel all through the period of incubation. *Pseudomonas putida* (75.03%) showed highest percentage amount of hydrocarbon degraded, while consortium (60.86%) showed the least capacity to degrade the hydrocarbons.

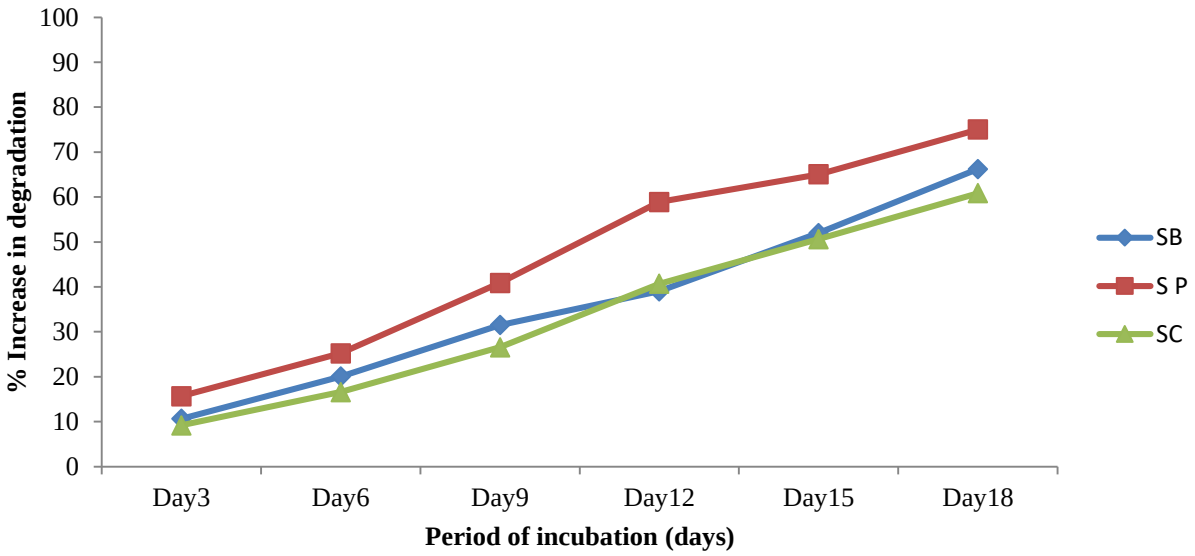

Fig. 4: Percentage degradation of spent engine oil by *Pseudomonas putida* and *Bacillus megaterium*

Key:
SP: Treatment containing only *Pseudomonas putida* and spent engine oil
SB: Treatment containing only *Bacillus megatarium* and spent engine oil
SC: Treatment containing the consortium and spent engine oil

A very pertinent factor in biodegradation of petroleum hydrocarbons is understanding the role played by the kind of bonds existing in their molecules. Diesel generally has more straight chain hydrocarbons than spent engine oil, thus making it potentially more susceptible to microbial enzymes than spent engine oil. *Pseudomonas putida* and *Bacillus megaterium* are known hydrocarbon degraders thus their presence in the effluent sample used in this study is plausible. During the period of authentication of the biodegradation of diesel and spent engine oil by the isolates (Table 1), *Pseudomonas putida* C15a (70.86% and 69.08%) and *Bacillus megaterium* N9a (50.54% and 48.4%) were observed to exhibit the highest potential for biodegradation because they had higher optical densities than the other strains. The growth of the isolates indicates the occurrence of enzyme induction which enabled growth in all the media due to prior exposure from the polluted sites, thus, enhancing their ability to degrade hydrocarbons. This is in agreement with previous reports (Okerentugba and Ezeronye, 2003; VanHamme *et al*., 2003; Idise *et al*., 2010). Increase in optical density was related with the bacterial growth, which also shows the extent of degradation of the hydrocarbons. The difference in the ability of the various strains of *Bacillus megaterium* and *Pseudomonas putida* to utilise diesel and spent engine oil may be attributed to better autochthonous adaptation with corresponding better developed enzyme system for hydrocarbon metabolism than other strains; and also the presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species (Darsa *et al*., 2014). Other researchers who isolated either *Bacillus* or *Pseudomonas* species or both from petroleum polluted sites have also reported them to be competent hydrocarbon degraders (Okerentugba and Ezeronye, 2003; Idise *et al*., 2010; Nwanyanwu and Abu, 2010; Sarma and Sarma, 2010; Ebrahimi *et al*., 2012; Riskuwa-Shehu and Ijah, 2016).

Different growth patterns were observed in all the treatments during degradation of the diesel and spent engine oil as shown in figures 2 and 3, respectively. On day 3 of the incubation period, there was little growth recorded for all the treatments probably because, the bacteria were in the lag phase of growth. However, an increase in population density was observed in all the treatments during the subsequent period of incubation before eventually a decline occurred. The decline in growth could be attributed to a depletion of carbon source which is the hydrocarbon, for energy and growth requirements. The highest growth rate was observed at day 15 for all the

treatments containing diesel (DB: 1.35 x 10^7CFU/mL; DP: 1.85 x 10^7CFU/mL; DC: 6.1 x 10^6 CFU/mL) (Table 2) and spent engine oil (SB: 1.05 x 10^7CFU/mL; SP: 1.35 x 10^7 CFU/mL; SC: 5.6 x 10^6 CFU/mL) (See Table 3). The control treatments for both hydrocarbons (DCN and SCN) showed no bacterial growth all through the period of biodegradation, these treatments were uninoculated as they only contained diesel and spent engine oil. The higher population density observed in the treatments containing diesel could be attributed to the nature of the bonds in its molecules. Diesel consists mainly of low molecular weight alkanes (about 75%) which are easily biodegradable by microbes, whereas spent engine oil consists mostly of polycyclic aromatic hydrocarbons (PAHs) (Wang *et al*., 2000) which are highly toxic and have tendency to be bioaccumulated (Shivendra and Hardik, 2014). Hence, the lower HUB count exhibited in the treatments containing spent engine oil probably reflects its lower content of saturated hydrocarbons and high aromatic content (Okerentugba and Ezeronye, 2003). The utilization of the hydrocarbons resulted in increase in the growth of the bacterial strains. The growth rate of the organisms seems to be directly proportional with the rate of oil degradation, suggesting that the breakdown of the hydrocarbons resulted in the provision of utilizable compounds required for their growth and multiplication (Ajao *et al*., 2003) also reported an increase in log number of cells with increase in degradation efficiency. A decrease in population density occurred at day 18 for the strains in all the treatments and this could be attributed to decrease in the amount of utilizable hydrocarbons consequently resulting in build-up of toxic substances such as metabolic products that may be unfavourable to the growth of the bacteria. The bacterial growth reached the stationary phase and moved into the death phase in almost all the cases. This shows that the bacterial isolates utilized and degraded the hydrocarbons (Okerentugba and Ezeronye, 2003). It was observed that the treatments increased in population with increase in the rate of degradation but decreased progressively as the incubation continued. Using one-way analysis of variance to compare hydrocarbon utilizing bacteria count, it was observed that all the treatment set containing diesel and spent engine was significantly different. This is likely due to the role the nature of the bonds in the hydrocarbons play in their breakdown by the bacterial strains, and consequently in their growth rate. Higher bacterial counts were observed in the treatments containing diesel due to its relatively simpler C-C bonds which are mostly linear.

From the findings, it was observed that the strains of *Pseudomonas putida* and *Bacillus megaterium* had varying hydrocarbon degradation capacity for diesel and spent engine oil. However, *Pseudomonas putida* showed a more superior ability to degrade both diesel and spent engine oil than *Bacillus megaterium* and the consortium. This may be due to its catabolic genes encoding for the utilization of the hydrocarbons for its growth (Marchal *et al*., 2003). Moreover, owing to the presence of these catabolic enzymes, bacterial strains are extremely well equipped to make adaptive changes for their survival in adverse or highly contaminated environments (Heinonsalo *et al*., 2000). This is in agreement with the findings of Nwanyawu and Abu (2010); Sathisshkumar *et al*. (2008) and Vinothini *et al*. (2015) who working independently observed that *Pseudomonas* species had higher hydrocarbon degradation efficiency than *Bacillus* species.

The consortium exhibited less capacity to degrade hydrocarbons in both samples than the individual organisms. This may be due to the antagonistic effects of their individual enzyme systems which could reduce their effectiveness (Manal, 2011); it could also be due to their competing for the same catalytic site. This finding agrees with the work of Singh and Lin (2008) and Manal (2011) who reported higher hydrocarbon degradation in the individual cultures than the consortia, but disagrees with the findings of Juhasz *et al.* (2000); Rahman et al. (2002b); Mukred et al. (2008) and Sathishkumer et al. (2008), Zhang et al. (2010) who reported higher hydrocarbon biodegradation by mixed cultures than the pure cultures of individual strains.

The higher rate of hydrocarbon degradation observed in diesel compared to spent engine oil may be due to the type of hydrocarbons that constitute the samples. This agrees with the work of Amund and Akaqngbou (1993) who showed that crude oil fraction with lower amount of saturated hydrocarbons were more resistant to microbial degradation than the fraction containing higher amount of saturated hydrocarbons. Mukred et al. (2008) also showed that short and medium chain alkanes undergo higher degradation than the longer chain alkanes.

The selected strains achieved considerable amount of hydrocarbon degradation as the percentage degradation ranged from 75.86 - 98.3% for diesel and 60.8- 75.03% for spent engine oil at the end of the experiment. The high rate of biodegradation observed is attributable to the expression of hydrocarbon degrading genes by the strains, another critical factor is the availability of nutrients which inevitably led to the highest removal of the hydrocarbons during the log phase of growth of the bacteria. This implies that the

stage of growth of the microbe plays an important role in the clean up or removal of oil from the environment (Darsa et al., 2014). This is in agreement with the findings of Rahman et al. (2002a) who reported increase in the rate of biodegradation of crude oil, as the concentration of oil reduces and recorded 78% oil degradation after incubation of samples for 20 days. One-way analysis of variance revealed that there is significant difference in the rate of percentage degradation of both samples. This could possibly be due to the fact that using the strains individually or in consortium substantially affects the rate of reduction in the concentration of the hydrocarbons. Thus, the mechanism of breaking down the compounds differs between the strains probably due to the difference in their enzymatic capabilities and the nature of the hydrocarbons.

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Conclusion

Pseudomonas putida and *Bacillus megaterium* identified in this study have an inherent ability to degrade petroleum hydrocarbons. Thus, they could be effectively utilized in the bioremediation of soils contaminated with hydrocarbons. The individual strains performed hydrocarbon degradation better than the consortium, thus it's important to include functional genomics in biodegradation studies in order to fully study the role of the enzymatic machinery of hydrocarbon degrading bacteria.

Conflict of Interest: The authors declare that there are no conflict of interests.

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