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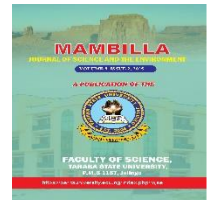

ASSESSMENT ON BIOAUGUMENTATION AND HYDROCARBON UTILIZATION OF A CAR REPAIR SHOP POLLUTED SOIL USING *Bacillus subtilis*

[1] John, E.A, *[2]Stephen, E., [3]Otorkpa, O.J., [2]Fredrick, F.U.

[1]Department of Microbiology, Prince Abubakar Audu University Anyigba, Kogi-Nigeria.
[2]Department of Biological Sciences, Taraba State University, Jalingo-Nigeria.
[3]Department of Public health, Faculty of Health Sciences, National Open University of Nigeria.

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ABSTRACT

The potential of *Bacillus subtilis* in bioaugumentation of car repair shop oil impacted soil was studied for 28 days. Three kilograms (3000g) of oil rich soil were collected in two different perforated plastic bowls labelled as car repair shop soil (CRS) and car repair shop soil inoculated with *Bacillus subtilis* (CRSB). CRSB was inoculated with *B. subtilis* obtained from known stock culture. Total viable bacteria (TB) counts was determined by dilution and plate count methods. For *Bacillus subtilis* counts, 10g of the soil was heated at $80^{o}C$ for an hour before dilution and plating. Bacterial isolates that grew were characterized and identified by morphological and biochemical tests. The pH, organic carbon, organic matter content, nitrate and phosphate were determined using standard procedures. Hydrocarbon degradation was determined weekly by gravimetric method. The scope of total bacteria (TB) and *B. subtilis* counts were 8.0×10^3-2.2×10^4cfu/g and 2.0×10^3-4.8×10^4cfu/g respectively. The peak bacterial population was recorded after 21 days of assessment. Species of *Staphylococcus, Proteus, Streptococcus* and *Escherichia coli* were among the organisms isolated from the soil. The pH ranged from 6.12±0.15–6.48± 0.17. The organic carbon, organic matter content, nitrate and phosphate were higher in CRS compared to CRSB. There were no significant differences in values obtained for pH, nitrate, phosphate and moisture at 0.05 probability limits while significant differences were observed in organic carbon and organic matter contents of the soils. This study revealed that *B. subtilis* in synergy with other hydrophilic organisms can be considered for bioremediation of oil polluted soil.

1. Introduction

Soil, a known ecosystem receives industrial and household wastes (Udom & Nuga, 2011). Soil pollution occasion by derivatives of hydrocarbon was regarded to be a major concern of countries involved in crude oil exploration (Stephen *et al*., 2021a). The report of Obazuaye & Obueh (2014), shed light on the global problem affecting both petroleum rich and importing countries. This stem from increase over the years in the population of vehicles and attendant hydrocarbon products channeled to lands used for farming purposes (Obazuaye & Obueh, 2014).

According to Obazuaye *et al*. (2017), used oil is a common soil pollutant in most farming communities in Nigeria. Okwute *et al*. (2020) are of the opinion that hydrocarbon exploration and products account significantly for alteration in soil microcosm and nutrient status. Chandran e*t al.* (2019), are of the view that these alterations in the environment due to hydrocarbon enrichment can result in low soil moisture, nitrate, phosphate and oxygen level eventually limiting the microbial activity in the soil (Bamidele & Igiri, 2011; Debojit *et al*., 2011).

Stephen & Egene (2012) reported that some forms of remediation have become imperative in reducing level of pollution in soils. Biological and physical approaches can be deployed in the remediation of hydrocarbon enriched soil. Physicochemical approaches are expensive and very effective compared to biological methods (Yusuf *et al.*, 2024). As a result of the cost of deploying physicochemical method utilizing great energy and chemical reagents, use of bacteria and fungi with the ability to mineralize hydrocarbon and persistent substances has attracted more interest (Stephen *et al*., 2014a). One of such utilization of microbes in the field is known as bioaugumentation.

* Corresponding author. Tel.: +2348036347036
E-mail address: psychsea07@gmail.com

Bioaugumentation incorporates normal flora or microflora with modified/altered genes with innate ability to break down pollutants (Yusuf *et al.,* 2024). In the argument of Yusuf *et al.* (2024), native microflora is best suited for bioaugumentation. Sharma (2012) is of the opinion that bioremediation is a less expensive method with minimal energy compared to either physical or chemical method employed over the years in mopping up organic waste that may be hazardous.

Abandoned car repair shop soil may not be utilized for farming activities as a result of varied hydrocarbon derivatives previously incorporated into the soil over time (Stephen *et al*., 2015). Used lube oil, greases, diesel oil over time alters the composition of autochthonous microflora, soil color, pH, moisture and other physicochemical properties of the soil (Babaee *et al.*, 2018). This alteration necessitated the cleaning of the soil using *Bacillus subtilis.* This organism features prominently in bioremediation studies (Stephen & Egene, 2012, Stephen *et al*., 2014a and Okwute *et al*., 2020).

2. Materials and Methods

2.1 Sample Collection

Hydrocarbon enriched soil was obtained from a car repair shop by first gate of Prince Abubakar Audu University (PAAU) Anyigba, Kogi State. Soil samples from a depth of 0-50 mm deep was obtained in four distinct locations from the car repair shop and were properly homogenized. The enriched soil weighing 6000g was evenly divided and placed in two plastic bowls (3000g each) perforated on all sides and taken for analysis. The bowls were labelled as CRS (car repair shop soil) and CRSB (car repair shop soil inoculated with *Bacillus subtilis*). Bowl CRS served as the control for the experiment.

2.2 Preparation and inoculation of *Bacillus subtilis*

Preparation and inoculation of *Bacillus subtilis* in the car repair shop soil was carried out as described by Stephen *et al.* (2014b). *Bacillus subtilis* was obtained from the Microbiology laboratory, Prince Abubakar Audu University stock culture and grown in 1000mls of peptone broth in conical flask at 37 ºC for 24hrs after which it was introduced into CRSB.

2.3 Sample analysis

The selection of soil sample for analysis commenced at day 0 before introduction of *B. subtilis* to determine the microbial population of the soil as described by Stephen *et al.* (2014b). Afterwards, sampling continued weekly for a span of four (4) weeks to assess the bacterial basis of the soil.

2.4 Bacterial Count and Identification

The total bacteria (TB) counts were appraised by aseptically plating a dilution of 10^3 soil samples on Nutrient Agar (N.A) plates. *Bacillus subtilis* was recovered by heating 10 g of the soil at 80 ºC for 1 hr in a hot air oven (Wen *et al*., 2022). The bacterial plates were incubated at 37 ºC for 24 to 72 hrs. Isolates that grew were characterized and identified by morphological and biochemical tests.

2.5 Physico-chemical properties of car repair shop soil

Physico-chemical properties such as pH, Phosphorus were determined as described by Onyeonwu (2000). Organic carbon, organic matter content and moisture were determined as outlined by Ibitoye (2006). Nitrate was determined using the micro Kjeldahl method as described by Association of official Analytical Chemist, AOAC (2005) and Ibitoye (2006).

2.6 Hydrocarbon degradation

Hydrocarbon degradation was determined using the gravimetric method as described by Abioye *et al*. (2013). Soil samples from the car repair shop oil impacted soil were analyzed at days seven (7), fourteen (14), twenty-one (21) and twenty-eight (28).

2.7 Statistical analysis

Descriptive statistics and T-test was performed using procedure of statistical package for social sciences (SPSS) version 16 (2007). Experimental precision achieved was reported at $p \leq 0.05$ level.

3. Results and Discussion

The count of total bacteria (TB) and *B. subtilis* sourced from car repair shop enriched soil undergoing bioaugumentation is shown in Figure 1. The scope of TB as well as *B. subtilis* counts were 8.0×10^3-2.2×10^4 cfu/g and 2.0×10^3-4.8×10^4 cfu/g respectively. *B. subtilis* had higher population at day 21, while the total soil bacteria count peaked at day 14. There were no significant differences in the bacterial counts at 0.05 probability limits ($p \leq 0.05$).

The TBC as well as *B. subtilis* increase was proportionate with time. This may be occasioned by the favourable adaptation and exploitation of oily compounds in the car repair shop polluted soil by *Bacillus subtilis* and other hydrocarbon metabolizing organisms isolated in the course of the study. This is in line with a report by Yusuf *et al.* (2024). They opine that *B. subtilis* could grow rapidly and are able to tolerate adverse conditions in an ecosystem. *B.*

subtilis as well as TBC nosedive after 21 days. This could be ascribed to depletion in nutrients present in the soil within the scope of the study or the production of toxic metabolites as reported by Abdulsalam & Omale (2009). Similar observation was reported by Yusuf *et al*. (2024). These authors reported decline in the population of *Pseudomonas aeruginosa* and *Bacillus subtilis* used in bio-augmentation of diesel polluted soil with time.

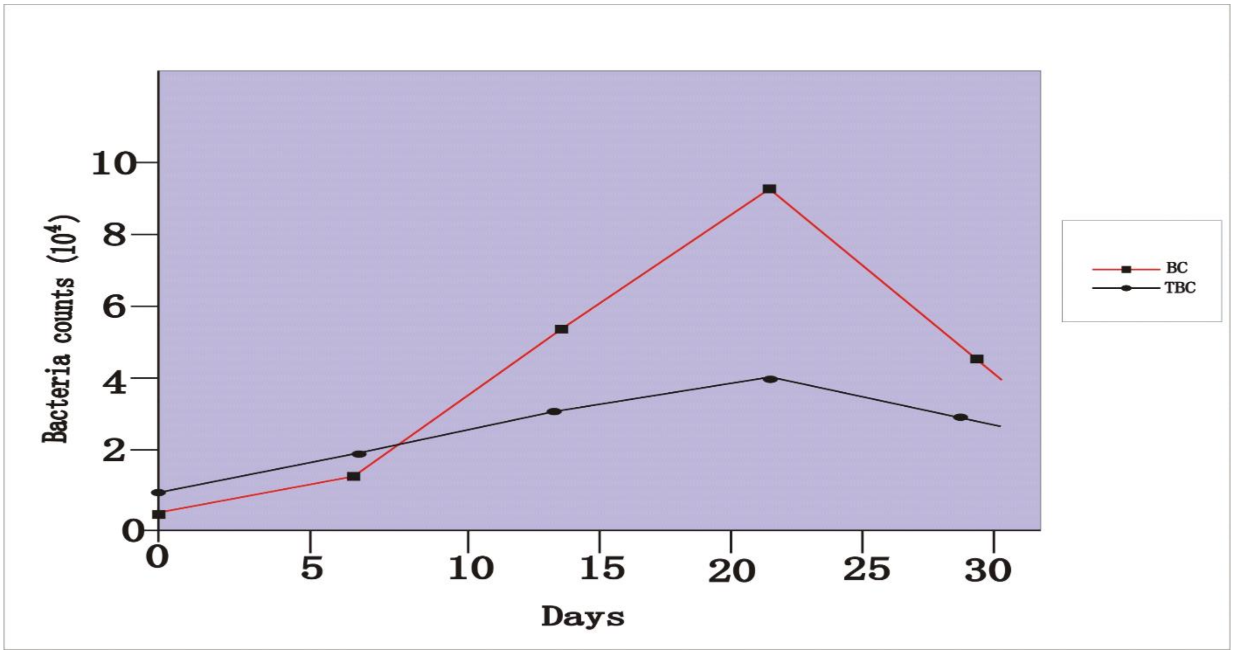

Fig 1: Total viable counts obtained from the car repair shop undergoing bioaugumentation.
Keys: BC=*Bacillus* Count, TBC=Total Bacteria Count.

Table 1 shows the morphological and biochemical characteristics of the organisms isolated from the soil in the course of the study. The organisms isolated were *Staphylococcus* spp*., Proteus* spp., *Micrococcus* spp., *Bacillus subtilis, Escherichia coli, and Streptococcus* spp. This is in agreement with the reports of Stephen *et al.* (2013, 2015) from car repair shop enriched soil treated with cowpea chaff and litter from poultry farm.

Table 1. Morphological and biochemical characteristics of bacteria isolates from car repair shop polluted soil

Codes	Colonial xtics	Gram stain	Cell shape	Spore	Catalase	Man	Glu	Suc	Lac	Coag	Isolates
A.	Smooth yellow	+	cocci	-	+	+	+	+	+	+	*Staphyloccus aureus*
B.	Milky white	-	rods	-	+	-	+	+	-	-	*Proteus* spp
C.	Small white opaque	+	cocci	-	+	-	-	+	-	-	*Micrococcus* spp
D.	Cream color, rough edges, opaque	+	rods	+	+	+	+	+	-	-	*Bacillus subtilis*
E.	Creamy white, flat surfaces	-	rods	-	+	-	+	+	+	-	*Escherichia coli*
F.	Milky white, small round colony	+	cocci	-	-	-	+	+	+	-	*Streptococci* spp

xtics**:** characteristics, **+:** positive, - : negative, Man: mannitol, Glu: glucose, Suc: sucrose, Lac: lactose, Coag: coagulase

Table 2 shows the findings of the physicochemical properties of the soil sample. The pH ranged from 6.12±0.15 to 6.48±0.17. The highest pH was observed in CRS while the lowest pH was observed in CRSB. There was no significant difference in the pH observed in CRS and CRSB at 95% confidence interval. The low pH observed in CRSB may be attributed to biodegradation of the hydrocarbons present in the car repair shop polluted soil (Jones & Smith, 2017). Jones & Smith (2017), are of the opinion that decomposition of organic matter impacts on pH level of soil undergoing biodegradation.

Organic carbon ranged from 1.40±0.26 to 3.20±0.23. A higher carbon content was observed in CRS compared to CRSB. Similar trend was observed in the values of organic matter content i.e CRS > CRSB. Significant differences were observed in the organic carbon and organic matter contents of CRS and CRSB at 0.05% probability limits. The low values of organic carbon and organic matter observed in CRSB compared to CRS is consistent with report of Smith *et al.* (2019) and Ramos-Miras *et al.* (2019). These researchers in their separate findings, reported lower values in organic carbon and organic matter contents in soil undergoing bioremediation.

The nitrate contents of CRS and CRSB ranged from 0.41±0.15 mg/kg to 0.75±0.20 mg/kg. A higher nitrate content was observed in CRS compared to CRSB. There was no significant difference in the nitrate contents observed in CRS and CRSB at 0.05 probability level. The low nitrate content observed in CRSB is an indication of active utilization of nitrates by microbes present in the soil. The findings in this study is in contrast to the reports of Johnson *et al.* (2018). These authors reported elevated nitrates in polluted soil undergoing bio-stimulation.

The pattern of phosphate concentration was CRS > CRSB. There was no significant difference in the phosphate concentration in CRS and CRSB at 5% probability level. The low level of phosphate observed in CRSB may be an indication of phosphate utilization by soil microbes. This is consistent with earlier reports by Krasner *et al*. (2009). These authors were of the opinion that soil undergoing active biodegradation has reduced phosphate and nitrate levels due to their utilization during the biodegradation process.

Moisture content ranged from 3.71±0.75 % to 6.20±4.42 % in all soil samples. The highest moisture content was observed in CRSB. There was no significant difference in the moisture content in CRS and CRSB. According to Rodriguez *et al.* (2017), water-holding capacity of soil increases in the presence of organic manure.

Table 2: Physicochemical qualities of Mechanic Workshop Polluted soil undergoing bioremediation (M+SE) and control plot

Parameters	CRS	CRSB
pH	6.48 ± 0.17^{a}	$6.12\pm.0.15^{a}$
Organic carbon (%)	3.20 ± 0.23^{a}	1.20 ± 0.46^{b}
Organic matter content (%)	5.42 ± 0.38^{a}	0.62 ± 0.03^{b}
Nitrate (mg/kg)	0.70 ± 0.06^{a}	0.41 ± 0.15^{a}
Phosphate (mg/kg)	16.35 ± 1.70^{a}	14.04 ± 3.70^{a}
Moisture content (%)	3.71 ± 0.75^{a}	6.20 ± 4.42^{a}

a,b: means denoted by different superscripts along the same row are significantly ($p<0.05$) different. Values are means of three replicates. CRS: Car repair shop polluted soil, CRSB: Car repair shop polluted soil augmented with *B. subtilis*.

Hydrocarbon degradation of car repair shop oil impacted soil by *B. subtilis* increased with time. Figure 2 shows the amount of oil degraded after 28 days. There was a continuous increase in the amount of oil degraded from day 0 till it peaked at day 21. This also coincide with the highest bacterial population observed in the course of the study. The increase in hydrocarbon degradation in this study is in line with the observation of Yusuf *et al*. (2024). They reported increase in utilization of diesel with time in soil undergoing bioaugumentation and ascribed it to synergy between the introduced organisms and normal micro-flora in the soil.

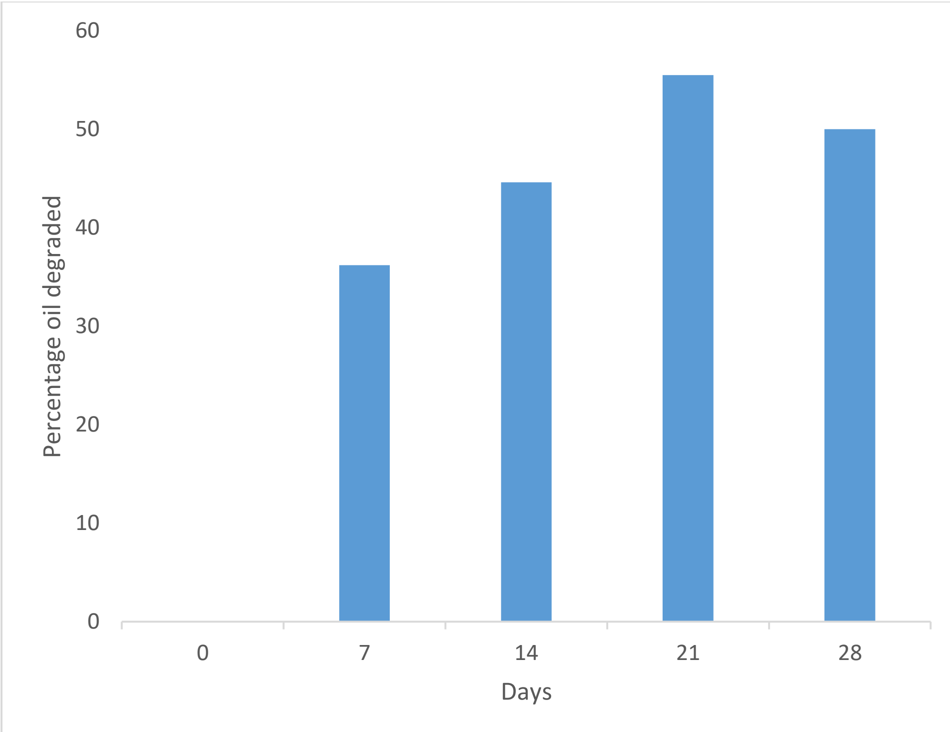

Fig 2. Hydrocarbon degradation by *B.subtilis* in car repair shop soil.

4. Conclusion

This assessment revealed that *Bacillus subtilis* had the capability to grow in oil rich car repair shop soil as well as degrade hydrocarbon present in the car repair shop oil impacted soil. This study also projects *B. subtilis* as a potential microbe that can be used to reclaim oil enriched soil.

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