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Hydrocarbon degradation potential by soil bacteria using vapour-phase and spectrophotometric methods

***Azuwike C.O[1]., Ahumibe, N.C[1]., Mgbemena, I.C[2]., Nwanaforo, M[2] and Braide, W[3]**

[1]Biotechnology Department, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria
[2]Department of Biology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria
[3]Department of Microbiology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria
*Correspondence: *azuwikec@gmail.com*

Abstract

Crude and refined oil contamination is inevitable is oil producing communities. The attendant effects of environmental degradation and low crop yield had remain a burden to mankind. Soils from oil polluted areas were assessed microbiologically using vapour phase and spectrophotometric methods. This study assessed bacteria with capabilities of utilizing diesel, toluene and kerosene as sole source of carbon for growth. Culturing and sub-culturing was done in nutrient agar and Bushnell Haas Agar (BHA).*Micrococcus luteus*, *Enterococcus fecalis*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* were the predominant microorganisms isolated from the samples locations. The standard plate counts for each source of sample include FA1 (2.80×10^6), FA2 (1.50×10^6), MW1 (3.80×10^6), MW2 (1.7×10^6), FS1 (1.22×10^7), and FS2 (1.30×10^3). Spectrophotometric reading showed visible growth/turbidity in tubes labeled MW1bT, MW1bK, MW1bdD, FS2bD, and FS2bT. The growth of bacteria was observed from visible increase of turbidity and was enumerate by CFU/ml. Among isolates, *Bacillus subtilis* exhibited relatively higher ability to grow on the three hydrocarbons. Microorganisms have metabolic capabilities to utilize the carbon compounds and degrade them to innocuous by-products. These abilities can be targeted for bioremediation purposes.

Keywords: spectrophotometric, vapour phase, hydrocarbon degradation, bacteria

Introduction

Environmental pollution resulting from crude oil pollution and other major oil exploration and exploitation activities are prevalent especially in oil-producing countries where efficient remediation measures have not been fully practiced. Petroleum resources have contributed enormously to the global energy demand and economic development of oil producing nations, especially, Nigeria, for the past fifty five years (Ite *et al*., 2016). Inadvertent discharges of petroleum hydrocarbons and chemical derived waste streams associated with petroleum exploration and production have caused environmental pollution, adverse environmental and/or human health problems (Sudakin *et al.*, 2011; Olawoyin *et al.*, 2012; Bahadar *et al*., 2014; Wu *et al.*, 2012;Asghar*et al*., 2016; Wickliffe *et al.*, 2014; Kponee *et al.*, 2015; Ezekwe and Edoghotu, 2015; Briggs and Briggs, 2018), negative impacts on the terrestrial ecosystems (Clinton *et al.*, 2009; Linden and Palsson., 2013; Sharma *et al*., 2015), detrimental impacts on regional

economy, socio economic problems and degradation in oil producing host communities in the Niger Delta region(Nduka and Orisakwe, 2010; Ordinioha and Brisibe., 2013; Ite *et al.*, 2013; Ite *et al*., 2016; Bahadar *et al.*, 2014; Obida *et al*., 2018).

Leaks and accidental spills occur regularly during the exploration, production, refining, transport, sabotage, illegal refining, bunkering and storage of petroleum and petroleum products (Nduka and Orisakwe, 2010). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution. (Head *et al*., 2006; Yakimov *et al.*, 2007; Jahangeer and Kumar, 2013; Mahjoubi *et al.*, 2013; Nriagu *et al.*, 2016; Okwechime, 2018).

The technology commonly used for the soil remediation such as*in-situ* burning, incineration, thermal conversion, landfilling and pyrolysis are expensive and can lead to incomplete decomposition of contaminants(Malviya and Chaudhary, 2006; Leonard and Stegemann, 2010; Ferrarese *et al.*, 2008; Lam and Chase, 2012;Hu *et al.*, 2013). However, the major drawbacks of these methods are that, there is always residual oil damage to the ecosystem and disposal of contaminated soil and water.

Diverse microorganisms (strains of bacteria, fungi, yeast, algae etc.) have been reported to have the capacity to degrade the hydrocarbon pollutants. While fungi and algae degrade hydrocarbon aerobically, bacteria and archae are capable of both aerobic and anaerobic degradation (Throne-Holst *et al.*, 2007; Vidali, 2001; Katsivela *et al.*, 2003; Philip and Atlas, 2005; Whelan *et al.*, 2015; Dias *et al.*, 2015; Gomez and Sartaj, 2013; Eweje,2006;George-Okafor *et al*., 2009;Hamza *et al.*, 2010; Ghosal *et al.*, 2010; Sharma*et al*., 2015). *Acinetobacter* species was found to be the most dominant strain capable of utilizing hydrocarbons in marine sediments in a study carried out by Mahjoubi *et al.* (2013).

This study reports on the comparative study of hydrocarbon degradation by vapour phase and spectrophotometric methods.

Materials and Methods

Description of study area

The study was domiciled within Ihiagwa, Umuchima and Federal University of Technology (FUTO) communities in Owerri West Local Government Area of Imo State, Nigeria. The communities are located 12 km south of the capital city of Owerri (N 5° 27' 39" and E 6° 55' 23").

Collection of soil samples

Soil sample was randomly collected from six (6) different sites within the Ihiagwa, Umuchima and FUTO community. Soil samples collected were categorized in two (2) different forms, as oil contaminated soil samples and uncontaminated soil samples.

The contaminated soil samples were collected from vehicle mechanic workshops and two gas stations in Ihiagwa and FUTO community while the uncontaminated was collected from FUTO farm and St. Thomas Aquinas Catholic Chaplaincy (STACC) farm.

Fig 1: Soil sample from uncontaminated FUTO farm land.

The soil samples were collected from the sub-soil (0 – 15cm) with a soil augur. Four soil samples labelled MW1, MW2, FS1, and FS2 were contaminated with hydrocarbons (Kerosene. Toluene, Diesel). Two samples labelled $FA1_C$ and $FA2_C$ from farm land were used as control (Fig 1).

Fig 3: Vapour phase inoculation

Microbiological analysis of soil samples

Ten grams of soil samples (contaminated and uncontaminated) were serially diluted in 90 ml of sterile distilled water. An aliquot portion (0.1 ml) from dilution $10^5$was inoculated on pre-sterilized surface dried nutrient agar medium and uniformly spread to obtain discrete and countable colonies (Cheesbrough, 2000).Equal volume of the 10^3 was inoculated into Bushnelli Haas agar(BHA) medium supplement with crude oil (vapour phase method). The plates inoculated with the suspension from the dilutions were incubated at room temperature for 24-48 h for total heterotrophic bacteria and 7-14 day for hydrocarbon degrading bacteria.

Air- drying of soil samples

The soil samples were air – dried for 7 days at ambient temperature on a polyethylene mat (Fig 2). This was done until the soil became crispy and then sieved to obtain fine powder.

Fig 2:Air-drying of soil samples

Vapour phase inoculation method

Vapour phase inoculation method was used for Bushnelli Haas Agar (BHA). It was done by placing a sterile filter paper over the lid cover of the petri-dish and the filter paper soaked with crude oil until it was saturated (Fig 3).

Enumeration and characterization of bacterial isolates

Colonies were counted after incubated and pure cultures (Fig 4) characterized colonially, microscopically and biochemically using standard methods (Cheesbrough, 2000; Beishir, 1987).

Fig 4: Pure cultures of bacterial isolates

Preparation of mineral salt medium (MSM)

Utilization of hydrocarbon components by bacterial isolates obtained from BHA was done be growing organisms on mineral salt medium prepared by dissolving 1.8 g K_2HPO , 4.0 g NH_4CI, 0.2 g $MgSO_4$.$7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4$. $7H_2O$ in 1 L of distilled water. The pH was adjusted to 6.9 and the medium dispensed in 100 ml portion into a 250 ml conical flask and sterilized by autoclaved at 121°C for 15 min (Sharma, 2000).

Incorporation of hydrocarbons with mineral salt medium

One milliliter (1 ml) each of sterile hydrocarbons (kerosene, diesel and toluene) was added into the sterile mineral salt medium and votexed to mix properly for 30 secs. Equal volume (10 ml) of hydrocarbon degrading bacteria were inoculated into the mineral salt medium and incubated at ambient temperature for 7 days on a shaker.

Spectrophotometric Reading

The turbidity of the solution after incubation was read on a spectrophotometer at a wavelength of 420 nm following the principle of absorbance and transmission of light as an indication of the amount of viable cells present and the hydrocarbon degraded. The results were recorded in triplicates and the average taken (Fig 5).

Fig 5: Spectrophotometric reading

Results

Bacteria enumeration, purification and identification

Heterotrophic bacteria counts from soil samples ranged between 1.30×10^3- $2.80\times10^{6.}$ Counts obtained from farm was higher than those from hydrocarbon contaminated soils on the general purpose medium (Table 1). Four species of bacteria, namely, *Enterococcus, Micrococcus, Staphylococcus* and *Bacillus* were isolated from the soil samples a shown in Table 1.

The isolates were further identified by their ability to utilize carbohydrates (sugars) and exhibited different biochemical characteristics is shown in Table 2.

Table 1: Colonial and microscopic characteristics of bacterial isolates on Nutrient Agar

	Sample code	Colony counts (Cfu/g)	Colony code	Colonial characteristics	Gram Reaction	Probable identity
1	FA1	2.80×10^6	FA1a	Small circular yellow colonies	Gram positive cocci predominantly in tetrads	*Micrococcus* sp
			FA1b	Pinpoint circular cream colonies	Gram positive cocci chains	*Enterococcus* sp
			FA1c	Creamy Irregular, raised and opaque colonies	Gram Positive Bacilli in short Chains	*Bacillus subtilis*
2	FA2	1.50×10^6	FA2a	Creamy Irregular, flat and opaque colonies	Gram Positive Bacilli in short Chains	*Bacillus* sp
			FA2b	Creamy Irregular, raised and opaque colonies	Gram Positive Bacilli in short Chains	*Bacillus subtilis*
3	MW1	3.80×10^6	MW1a	white regular and opaque colonies	Gram Positive Bacilli in short Chains	*Bacillus* sp
			MW1b	Creamy Irregular, raised and opaque colonies	Gram Positive Bacilli in short Chains	*Bacillus subtilis*
			MW1c	Small circular yellow colonies	Gram positive cocci predominantly in tetrads	*Micrococcus* sp
4	MW2	1.70×10^6	MW2a	Circular, moist and shiny low convex golden yellow colonies	Gram positive cocci predominantly in clusters, few in pairs and tetrads	*Staphylococcus* sp
			MW2b	Creamy Irregular, flat and opague colonies	Gram Positive Bacilli in short Chains	*Bacillus* sp
5	FS1	1.22×10^3	FS1a	Small circular yellow colonies	Gram positive cocci predominantly in tetrads	*Micrococcus* sp
			FS1b	Creamy Irregular, raised and opague colonies	Gram Positive Bacilli in short Chains	*Bacillus subtilis*
			FS1c	Creamy Irregular, flat and opague colonies	Gram Positive Bacilli in short Chains	*Bacillus* sp
6	FS2	1.30×10^3	FS2a	Creamy Irregular, flat and opague colonies	Gram Positive Bacilli in short Chains	*Bacillus* sp
			FS2b	Creamy Irregular, raised and opague colonies	Gram Positive Bacilli in short Chains	*Bacillus subtilis*

KEY: FA1 (sample from STACC farm), FA2 (sample from FUTO farm), FS1(sample from filling station), FS2(sample from 2nd filling station), MW1(sample from mechanic workshop 1), and MW2(sample from mechanic workshop 2)

Table 2: Biochemical and Carbohydrate Fermentation of Bacterial isolates

Colony code	Mot	Spo	Cat	Oxi	Coag	In	MR	VP	Cit	Ure	NO_3	H_2S	Suc	Lac	Mal	Mann	Glu	Identity of isolates
FA1a	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	*Micrococcus luteus*
FA1b	-	-	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	*Enterococcus faecalis*
FA1c	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus subtilis*
FA2a	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus cereus*
FA2b	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus subtilis*
MW1a	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus cereus*
MW1b	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus subtilis*
MW1c	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	*Micrococcus luteus*
MW2a	-	-	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+	*Staphylococcus aureus*
MW2b	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus cereus*
FS1a	-	-	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	*Micrococcus* sp
FS1b	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	*Micrococcus luteus*
FS1c	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus* sp
FS2a	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus cereus*
FS2b	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	*Bacillus subtilis*

Catalase test (Cat),Oxidase test (Oxi), Coagulase test (Coag), Indole test (In), Methyl Red (MR) and Voges Prokauer (VP), Urease test (Ure), Citrates test (Cit), Motility test (Mot), Glucose (Glu), Sucrose (Suc), Lactose (Lac), Maltose (Mal), Mannitol (Mann), Sporulation (Spo), Nitrate Reduction test (NO_3), Hydrogen Sulphide production test (H_2S).

Figure 6 shows the spectrophotometric reading of the hydrocarbon utilization by bacteria on mineral salt medium. The level of turbidity determines the growth of the organisms and the utilization of the hydrocarbon components. *Bacillus* and *Micrococcus* species performed better that *Enterococcus* and *Staphylococcus* species.

Table 3 shows the HUB index of soil bacteria. This was obtained by calculating the percentage of total heterotrophic bacteria and total hydrocarbon utilizing bacteria. Soils obtained from hydrocarbon impacted environment showed higher HUB index (Table 3)

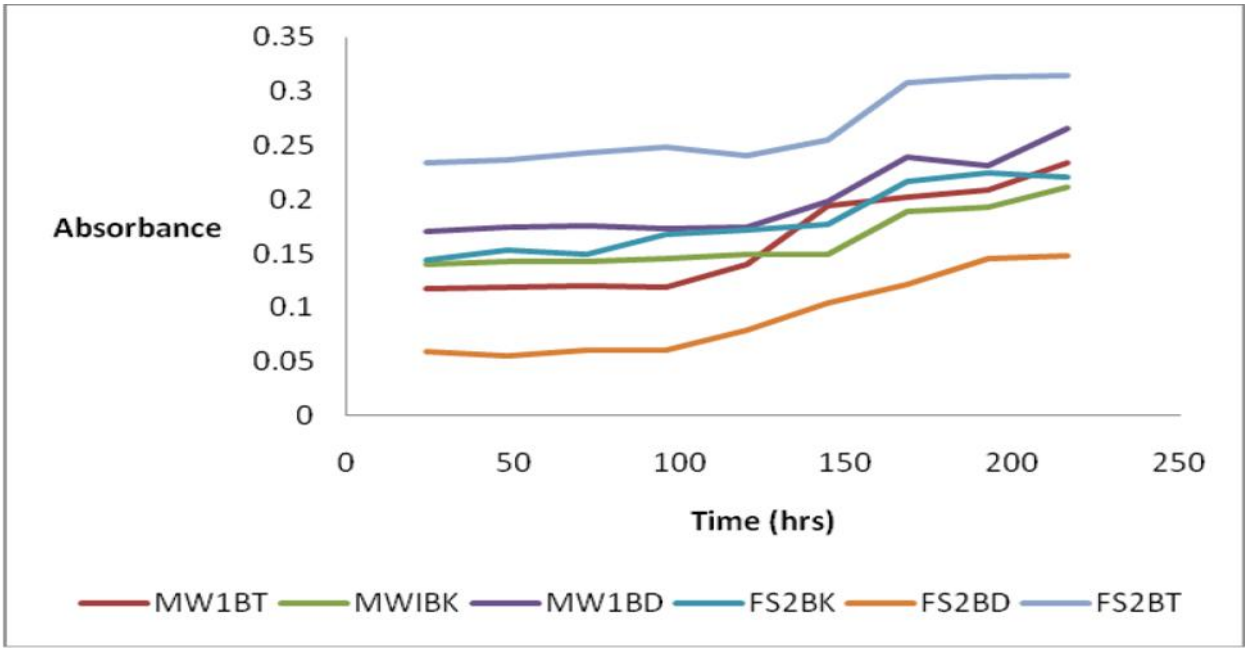

Fig 6: Spectrophotometric results (absorbance) for each isolates. The colours represents the isolates.

Table 3: HUB Index of soil samples

ISOLATES	THBC	HUB	HUB INDEX
FA1	2.80×10^{6}	1.20×10^{5}	4.29%
FA2	1.50×10^{6}	1.70×10^{4}	1.13%
FS2	3.8×10^{6}	2.24×10^{6}	58.95%
MW2	1.7×10^{6}	2.08×10^{5}	12.24%
FS1	1.2×10^{7}	2.10×10^{6}	17.21%
MW1	1.3×10^{5}	3.40×10^{4}	26.15%

THBC; total heterotrophic bacterial counts; HUB, Hydrocarbon Utilizing Bacteria; HUB index, the percentage of HUB by THB.

Discussion

Petroleum products are a major source of energy for industries and daily life. Increased oil explorations in the East and West African region and the anticipated oil spillages that occur during routine operations and transportation have raised concerns of environmental pollution. The use of bioremediation as opposed to physicochemical methods to conquer this problem is emerging as a more efficient, economical and effective strategy. Bacteria in particular have been identified as the most active agents in mitigation of petroleum oil pollution (Throne-Holst *et al.*, 2007; Vidali, 2001; Katsivela *et al.*, 2003; Philip and Atlas, 2005; Mahjoubi *et al.*, 2013; Ebakota *et al*., 2017). Thus, attempts to isolate hydrocarbon degrading microorganisms from different environments have led to isolation of a wide variety of potential bacterial candidates amenable to multiple biotechnological applications including bioremediation. Results obtained have shown that *Bacillus* species actually degrade crude oil in contaminated soil from petroleum stations and mechanic workshops.

Studies conducted by Panda *et al*. (2013) concluded that *Pseudomonas aeruginosa* had shown 49.93% of diesel oil degradation. Ebakota *et al*. (2017) reported that soil samples contaminated with hydrocarbons examined from different auto-mechanic repair workshop harboured bacteria of possible biodegradation importance. *Pseudomonas, Bacillus, Staphylococcus* and *Streptococcus* species were able to utilize and/or degrade spent engine oil as their source of energy (Arulazhagan *et al.*, 2010; Ebakota *et al*., 2017).

Bacillus subtilis demonstrated higher potential hydrocarbon degradation. Microorganisms involved in the cleanup of oil contaminated sites have been well documented (Throne-Holst *et al.*, 2007; Vidali, 2001; Katsivela *et al.*, 2003; Philip and Atlas, 2005).

Bacterial counts from soils contaminated with hydrocarbon is higher (3.8×10^6 Cfu/g – 1.7×10^7 Cfu/g) compared to uncontaminated farm land soil. The higher HUB index obtained from contaminated soil further confirmed this claim. The hydrocarbon utilizing bacteria index shows the percentage of hydrocarbon degrading organisms in the soil. For soil samples from farm land(FA1) and (FA2), the HUB Index is 1.13%.The HUB index for mechanic workshop 1 and 2 is 58.95% and 12.24% respectively, whereas, HUB index for filling stations 1 and 2 is 17.21% and 26.15% respectively. Results shows that the samples from virgin farm lands used as control recorded low HUB index, indicating sparsely distribution of hydrocarbon degrading bacteria.

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