

SCREENING OF HYDROCARBONOCLASTIC BACTERIA USING REDOX INDICATOR 2, 6-DICHLOROPHENOL INDOPHENOL

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ABSTRACT

Petroleum products are used in different forms in auto-mechanic workshops every day. Spent motor oil disposed-off improperly contains potentially toxic substances which can seep into the water tables and contaminate ground water. This study involved isolation and screening of bacterial species capable of utilizing hydrocarbons from three auto-mechanic workshops in Abeokuta, Ogun State. Total Heterotrophic Bacterial Counts ranged from 1.03×10^6 to 2.81×10^6 CFU g⁻¹. Total Oil Degrading Bacterial Counts varied between 4.0×10^5 and 2.01×10^6 CFU g⁻¹ while Surface Active Bacterial Counts were from 1.2×10^4 to 2.76×10^5 CFU g⁻¹. Twenty-four bacteria species capable of utilizing petroleum as a carbon source were isolated from various contaminated soils using enrichment technique. Isolated bacteria include: *Bacillus* spp., *Pseudomonas aeruginosa*, *Micrococcus* spp., *Proteus mirabilis*, *Proteus vulgaris* and *Enterobacter* spp. Redox indicator 2, 6-dichlorophenol indophenol (DCPIP) was used to screen for efficient hydrocarbon (Kerosene, Premium Motor Spirit, and Engine oil) degradation by bacteria. Degradation efficiency was measured by optical density at 600 nm. *Micrococcus* spp., *Proteus mirabilis*, and *Pseudomonas aeruginosa* were found to be the best isolates growing on majority of hydrocarbons due to their high utilization value when growing on the hydrocarbons tested.

Keywords: Auto-mechanic workshop, Petroleum, Degradation, 2, 6-dichlorophenol indophenol, Optical density.

1. Introduction

Petroleum based fuels are one of the most prevalent pollutants, particularly in developing countries (Joshi and Pandey, 2011). The widespread nature of petroleum products and their use is strongly associated with anthropogenic discharge of hydrocarbons into the environment (Bidoia *et al.*, 2010). Environmental pollution arising from petroleum leakages in storage tanks, spillage during transportation of petroleum products, deliberate discharge of petroleum products and various industrial processes is hazardous to soil and water ecosystems (Geetha *et al.*, 2013). This also results in huge disturbances of the abiotic and biotic components of the ecosystem (Okoh, 2006).

A major concern for petroleum hydrocarbon pollution is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs), asphaltenes and many branched compounds with twenty or more carbon atoms (Bidoia *et al.*, 2010). The processes of removal of hydrocarbon pollutants from the environment involve physical, chemical and biological methods. Physical and chemical processes which include dispersion, dilution, sorption and volatilization requires heavy machinery and the environmental consequences of this pollutant removal often result in huge air pollution (Matsumiya and Kubo, 2007). These processes have limited effectiveness and are usually expensive (Vyas *et al.*, 2013). The biological method is an extremely effective way of recovering oil contaminated areas among several other cleaning-up techniques (Bhupathiraju *et al.*, 2002). It is believed to be non-invasive technology (April *et al.*, 2000), less expensive, efficient and do not introduce additional chemicals to the environment (Geetha *et al.*, 2013).

Bioremediation processes have been found to be an efficient method for remediation of petroleum by-products, pesticides and other potential harmful chemical (Castro-Gutierrez *et al.*, 2012). Okoh (2006) described bioremediation as a method that can be employed to achieve effective detoxification and volume reduction of petroleum hydrocarbon pollution.

Petroleum products such as engine oil, petrol and diesel are used in different forms in mechanic workshops every day. These products tend to harden and change the colour of the soil, which may have untold health hazard on the technicians and artisans (Antai, 1990). Spent motor oil disposed-off improperly contains potentially toxic substances which can seep into the water tables and contaminate ground water (Onuoha *et al.*, 2011). The vast range of substrates and metabolites present in petroleum impacted soils provides an environment for the development of complex microbial community (Butler and Mason, 1997). The occurrence of metabolically active bacterial populations in areas polluted with hydrocarbons suggests that these organisms are able to utilize hydrocarbons as their energy and carbon source (Salleh *et al.*, 2003). These microorganisms can degrade a wide range of target constituents present in oil and a number of them had been reported (Barathi and Vasudevan, 2001). However limited information is available on quantitative reduction, oxidation or degradation of various hydrocarbon substrates by bacteria.

A number of techniques have evolved for screening of hydrocarbon degrading microorganisms. These include use of liquid medium with hydrocarbons, oil containing mineral agar plates, measurement of turbidity in microtiter plates, oxygen consumption, most probable techniques and sheen screen technique (Desai and Vyas, 2006). However all these methods are either laborious, time consuming, expensive or not reliable (Desai and Vyas, 2006). 2, 6-Dichlorophenol Indophenol (DCPIP) is a qualitative and quantitative redox indicator. Its principle is based on oxidation of carbon source in which electrons are transferred to electron acceptors such as oxygen, nitrate and sulfate (Bidoia *et al.*, 2010). Colour change as a result of utilization of the substrate from blue (oxidized) to colorless (reduced) (Hanson *et al.*, 1993). Although a number of microorganisms have utilized alternate electron acceptors such as nitrate, manganese (IV), iron (III), sulfate and carbon dioxide (Heider *et al.*, 1999; Grishchenkov *et al.*, 2000; Boopathy, 2002; Massias *et al.*, 2003). The DCPIP technique is a rapid, simple and low cost procedure for evaluating capability of different microorganisms to degrade different oil (Mariano *et al.*, 2008).

This study surveyed hydrocarbonoclastic bacteria present in hydrocarbon contaminated soil and determined qualitatively and quantitatively the oxidation or degradative ability of the isolates on selected hydrocarbon.

2. Materials and Methods

2.1. Sample collection

Soil samples contaminated with various hydrocarbons were collected from Camp, Ajebo and Oke-mosan mechanic workshops in Abeokuta, Ogun State. Samples were taken at the depth of 10-20 cm using surface sterilized soil auger. Unpolluted soil samples (control samples) were collected from the campus of Federal

University of Agriculture, Abeokuta (FUNAAB). Soil samples were stored in sterile plastic bags and labeled appropriately. They were stored at 4 °C until used.

2.2. Total Heterotrophic Bacterial Counts (THBC)

Total Heterotrophic Bacterial Counts was carried out as described by Rahman *et al.*, (2002). Six-fold serial dilution was carried out on 1 g of each soil sample using sterile distilled water. Aliquot (0.1 ml) of the diluents was dispensed into sterile Petri dish aseptically. It was then plated out on Plate Count Agar (LAB M, UK) using pour plate method. The plates were incubated at 37 °C for 24 h after which the colonies were counted. This was carried out in replicates.

2.3. Total Hydrocarbon Utilizing Bacterial Counts (THUBC)

Total Hydrocarbon Utilizing Bacterial Counts was carried out on Mineral Salt Medium (MSM) agar as described by Balogun and Fagade (2010). The MSM consist of Basal Salt Medium (BSM) and trace element solution. The BSM consist of (g l^{-1}): K_2HPO_4 , 1.8; KH_2PO_4 , 1.2; NH_4Cl , 4.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl , 0.1; yeast extract, 0.1 and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05. Trace elements solution contained (g l^{-1}): H_3BO_3 , 0.1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.04 at pH of 6.5. Kerosene was used as the carbon source and 2% agar was added to solidify the medium. The basal salt medium and the trace elements solution were sterilized separately with 10 ml of the trace elements solution added aseptically to the sterilized basal salt medium to make it up to a litre. Then, 0.1 ml of the serially diluted sample was plated out using the pour plate method. The plates were incubated at 37 °C for 5 days. The colonies were counted and sub-cultured to obtain pure colonies. This was carried out in replicates.

2.4. Surface Active Bacterial Counts (SABC)

Surface active-agents producing bacterial count was carried out on blood agar. The agar contained 5% defibrinated rabbit blood and nutrient agar (LAB M, UK). Then, 0.1 ml of the appropriate diluents was dispensed into sterile Petri dish aseptically. This was plated out on blood agar as described by Tabatabaee *et al.*, (2005) using pour plate method. The plates were then incubated at 37 °C for 48 h after which the colonies were counted. This was carried out in replicates.

Bacterial isolates were identified on the basis of cultural and biochemical characteristics according to the Bergey's Manual of Determinative Bacteriology. The isolates were subjected to Gram staining, oxidase, catalase, motility, urease, citrate Voges-Proskauer and indole tests, glucose, sucrose and lactose fermentation.

2.5. Screening test for the utilization of Hydrocarbons

Bacterial isolates were tested for their potential to utilize Kerosene, Engine oil and Premium Motor Spirit as carbon source respectively (Bidoia *et al.*, 2010). Pure strain was inoculated into 7.5 ml of MSM incorporated with 50 μl of each hydrocarbon substrate. Then, 40 μl of 2, 6-Dichlorophenol Indophenol (DCPIP) was added and incubated at 37 °C for 60 hrs. Samples were taken for analysis at 12, 24, 36, 48 and 60 h. The DCPIP was prepared by dissolving 1 g of the DCPIP powder in 1 litre of sterile distilled water (Mariano *et al.*, 2008). Absorbance of the medium at 600 nm was measured at intervals of 24 h for 5 days using a digital colorimeter (Jenway 6051, UK).

3. Results

Total heterotrophic bacterial count of soil samples from the oil impacted (automechanic villages) showed that Ajebo mechanic village generally had the highest THBC. The THBC ranged from 1.03×10^6 to 2.81×10^6 CFU g^{-1} in contaminated soils while in unpolluted soil it was 1.72×10^6 CFU g^{-1} . Generally, the TODBC is higher in

contaminated soil compared to the control soil sample. The TODBC varied between 4.0×10^5 and 2.01×10^6 CFU g⁻¹ while SABC ranged from 1.2×10^4 CFU g⁻¹ to 2.76×10^5 CFU g⁻¹ (Table 1).

Table 1. Bacterial counts from each sampling location

Sampling station	Coordinate		Code	THBC ($\times 10^6$ CFU g ⁻¹)	TODBC ($\times 10^6$ CFU g ⁻¹)	SABC ($\times 10^5$ CFU g ⁻¹)
Camp Mechanic Workshop	07°22.849'N	03°43.572'E	AN	2.47±0.4 ⁱ	1.06±0.4 ^g	1.03±0.2 ^j
	07°22.888'N	03°43.566'E	AS	1.83±0.2 ^c	0.93±0.7 ^f	0.64±0.4 ^h
	07°18.781'N	03°43.354'E	AE	1.03±0.5 ^a	0.47±0.5 ^c	2.76±0.2 ⁱ
	07°22.859'N	03°43.629'E	AW	2.04±0.4 ^d	0.76±0.3 ^e	0.61±0.7 ^g
Ajebo Mechanic Workshop	07°19.648'N	03°43.421'E	BN	2.62±0.5 ^j	1.52±0.8 ^l	0.92±0.5 ⁱ
	07°18.67'N	03°42.56'E	BS	2.81±1.0 ^k	1.30±0.6 ^k	0.54±0.5 ^e
	07°18.619'N	03°42.556'E	BE	2.32±0.3 ^g	0.40±0.6 ^k	0.12±0.5 ^a
Oke-Mosan Mechanic Workshop	07°18.714'N	03°42.421'E	BW	2.16±0.3 ^e	1.16±0.7 ⁱ	0.13±0.3 ^a
	07°18.735'N	03°42.539'E	CN	2.38±0.4 ^h	1.25±0.4 ^l	1.75±0.6 ^k
	07°18.67'N	03°46.562'E	CS	2.65±1.0 ^j	2.01±0.6 ^m	0.34±0.0 ^d
	07°19.13'N	03°43.722'E	CE	2.27±0.6 ^f	1.08±0.2 ^h	0.59±0.5 ^f
FUNAAB Control soil	07°22.844'N	03°43.618'E	D	1.72±0.3 ^b	0.43±0.5 ^b	0.21±0.8 ^c

Values are means of replicate readings ± standard error of means

Mean values with the same letter within the column are not significantly different at $p < 0.05$

THBC: Total Heterotrophic Bacterial Count

TODBC: Total Oil Degrading Bacterial Count

SABC: Surface Active Bacterial Count

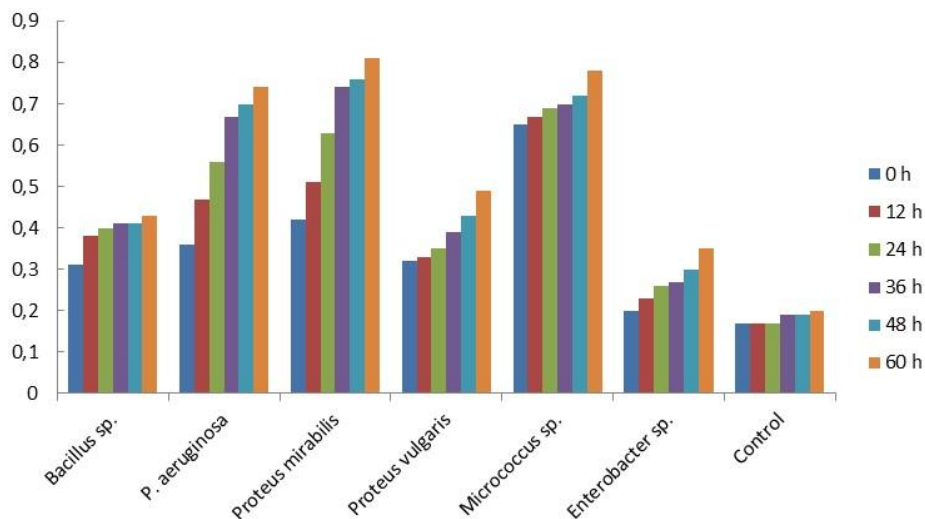


Figure 1. Utilization of kerosene by bacterial isolates

Twenty-four bacterial isolates were obtained from all the samples. They include: *Bacillus* spp., *Pseudomonas aeruginosa*, *Micrococcus* spp., *Proteus mirabilis*, *Proteus vulgaris* and *Enterobacter* spp. *Proteus mirabilis* had the highest utilization of 0.81 on kerosene after 60 hours while *Enterobacter* spp. had the lowest value of 0.35 (Fig.1).

Micrococcus spp. had the highest utilization value of 0.88 after 60 hours; *Pseudomonas aeruginosa* had 0.83 while *Enterobacter* spp. had the lowest utilization value of 0.34 on premium motor spirit (Fig. 2).

Figure 3 showed that *Micrococcus* spp. had the utilization value of 0.82, *Proteus mirabilis* had 0.80, *Pseudomonas aeruginosa* had 0.75 and *Enterobacter* spp. had 0.09 on engine oil after 60 hours.

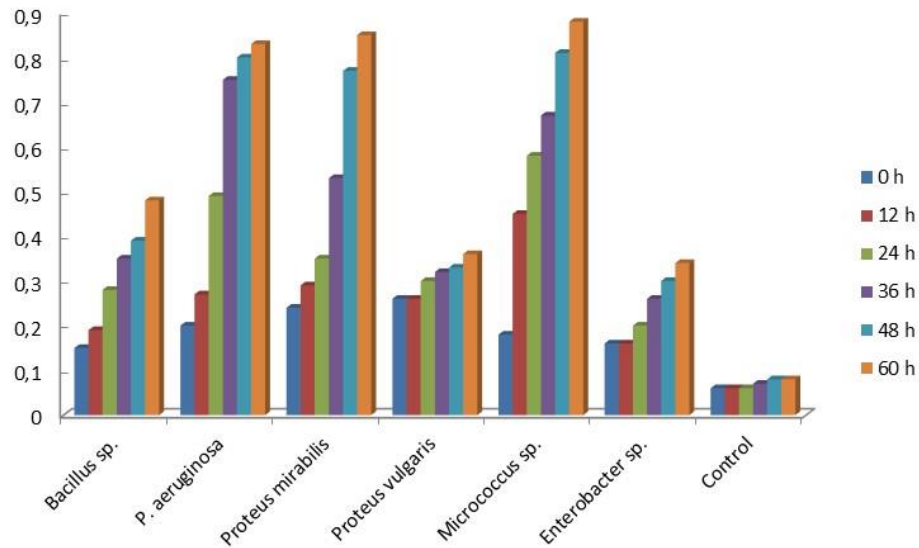


Figure 2. Utilization of premium motor spirit by bacterial isolates

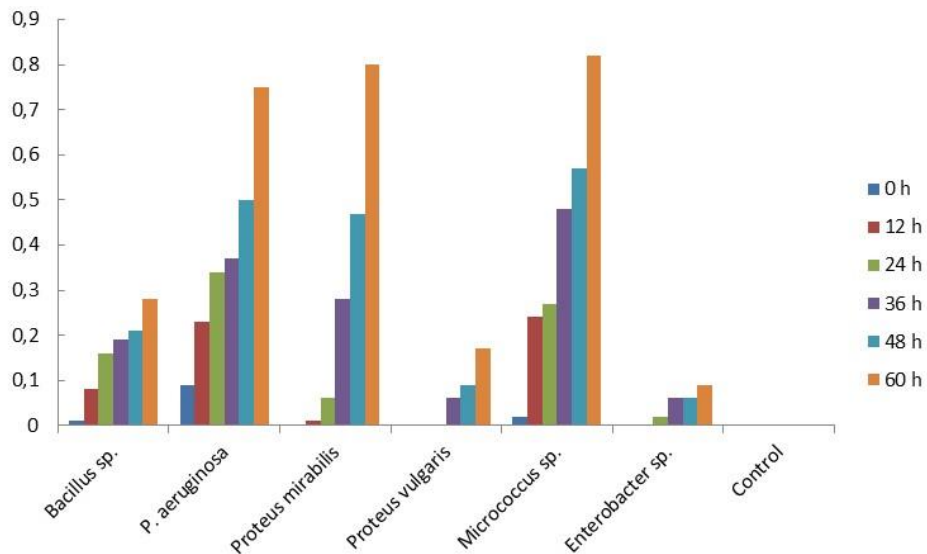


Figure 3. Utilization of engine oil by bacterial isolates

4. Discussion

Microorganisms are diverse and are capable of utilizing contaminants as energy and carbon source to survive in natural environment (Singh *et. al.*, 2010). Elimination of wide ranges of pollutants from the natural

environment is required to enhance a sustainable development of the ecosystem with low ecological impacts (Selvakumar *et al.*, 2014). Microorganisms play a major role in the removal of contaminants taking advantage of their versatile catabolic activity to degrade or convert such compound to harmless substances.

All sampling location showed high population of bacterial counts. This showed that these sites contain pollutants that serve as valuable source of carbon and energy for the bacterial isolates. This agrees with the work of Youssef *et al.*, (2010) who reported that the levels of hydrocarbon present in a contaminated site represent a nutrient rich environment where less-recalcitrant organic carbon may be limiting.

Enumeration of hydrocarbon degrading bacteria in all the sites showed that all natural ecosystems contain hydrocarbon degrading bacteria that can metabolize some components of oil even if those ecosystems have never been exposed to oil or its products (Syvokiene and Micheniene, 2004). It is also an important measure of the potential for bacterial degradation of oil contaminated sites which also assess the level of oil pollution that has taken place (Varjani *et al.*, 2013).

The results of TODBC from all contaminated sites were higher than the control soil sample. This agreed with the work of Piehler *et al.* (2002); Syvokiene and Micheniene (2004) and Youssef *et al.*, (2010) who reported that the hydrocarbon polluted sites have greater bacterial abundance and a large proportion of bacteria capable of hydrocarbon degradation than unpolluted soils.

Petroleum degrading isolates were identified as *Bacillus*, *Proteus* and *Pseudomonas*, *Micrococcus* and *Enterobacter* spp. Ability of the isolates to degrade the hydrocarbon was confirmed by the colour change from blue to colourless. This is due to reduction of the indicator by the oxidized product of hydrocarbon degradation which supports the facts that the isolates are potential hydrocarbon oxidizers (Selvakumar *et al.*, 2014). *Pseudomonas* and *Bacillus* have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites (Atlas 1992; Okoh and Trejo-Hernandez, 2006). Species of *Pseudomonas*, *Bacillus*, *Micrococcus* and *Proteus* isolated from hydrocarbon contaminated site have been found by several authors to utilize hydrocarbon through oxidation of DCPIP (Roy *et al.*, 2002; Joshi and Pandey, 2011; Patil *et al.*, 2013; Adegbola *et al.*, 2014). The oxidation of DCPIP supports the facts that the isolates were potential hydrocarbon degraders. Absorbance at a wavelength of 600 nm was monitored for the organisms because a peak in absorbance was observed at 600 nm as reported by Yoshida *et al.* (2001).

It is believed that individual organism could only metabolize limited range of hydrocarbon substrates (Adebusoye *et al.*, 2007). *Proteus mirabilis*, *Micrococcus* spp. and *Pseudomonas aeruginosa* were able to utilize the entire hydrocarbon tested. Compared to other isolates *Micrococcus* spp. showed better potential in utilizing premium motor spirit and engine oil than *Proteus mirabilis* and *Pseudomonas aeruginosa*. *Proteus mirabilis* had a preference for kerosene compared to premium motor spirit and engine oil. Premium motor spirit was better utilized by all the organisms than kerosene and engine oil. The rate and extent of colour change of DCPIP from blue to colourless with time revealed the potential of the isolates to utilize the hydrocarbon tested as a carbon source in the presence of DCPIP (Patil *et al.*, 2012).

It is evident from this study that *P. aeruginosa* and *Proteus mirabilis* did not exhibit any lag phase in the screening culture medium. This can be attributed to the expression of hydrocarbon catalyzing enzymes constitutive of their genetic makeup (Olajide and Ogbeifun, 2010). This finding agrees with the study of Okerentugba and Ezeronye (2003) who reported that microorganisms growing on crude oil did not exhibit any lag phase. Based on oil utilization capacity, *P. aeruginosa* is the most active hydrocarbon utilizer in crude oil (Prakash and Irfan, 2011). Previous observations have identified the *Pseudomonas* genus as the most efficient among hydrocarbon degrading microorganisms (Banat *et al.*, 2000; Saadoun, 2002). The results also showed that *Proteus mirabilis* is a highly adapted bacterium with great potential to degrade hydrocarbons

Isolation and screening of microorganisms for their efficiency in utilization of hydrocarbons before field trials is important in bioremediation process and the development of efficient techniques is an important tool in recommending different approaches for bioremediation of hydrocarbon polluted areas (Varjani *et al.*, 2013).

5. Conclusion

This study revealed a quantitative and qualitative evaluation of potentials of hydrocarbonoclastic bacteria degradation of hydrocarbon. Thereby giving a measureable ability of these groups of bacterial possible use in hydrocarbon impacted soil remediation.

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