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Isolation of Hydrocarbon Utilizing Bacteria from Petro-Chemical Polluted Soil in Bauchi Metropolis, Nigeria.

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Abstract

The aim of the study was to isolate Hydrocarbon Utilizing Bacteria from Petro- chemical polluted soil. A total of twenty-five soil samples were collected from five different mechanic workshop in Bauchi metropolis. The samples were bulked together to form one composite soil sample. The samples were examined for temperature, pH and moisture content. In this present study 13 bacteria genera were isolated by enrichment culture technique using nutrient agar, and utilization of hydrocarbon by the isolates were investigated on agar agar supplemented with 0.2ml refined petroleum products (Petrol, engine oil, diesel and kerosene). The isolated bacteria were characterized by morphological and biochemical test. Total hetrotrophic bacteria count ranged from 1.59 x 10^4 to 8.5 x 10^4 cfu/ml and total hydrocarbon utilizing bacteria count ranged from 1.0 x 10^4 to 7.0 x 10^4 cfu/ml, the bacteria isolates found in all sample sites were mainly staphylococcus sp, Arthrobacter, flavobacterium and Alcaligens faecalis. The highest degradation performance was observed on micrococcus sp, pseudomonas sp and Bacillus sp and the lowest degradation performance was observed on staphylococcus sp, flavobacterium sp, and corynebacterium. The temperature values obtained from different petrochemical polluted soil during this investigation fall within mesophilic range and the pH of each soil sample tends from slightly acidic towards neutrality.

***Keywords**: Petro-chemical, Hydrocarbon, Bacteria and Degradation.*

Introduction

The discovery of petroleum resulted in tremendous changes on the globe. It speeds up civilization through mechanization and industrialization. In addition to the good improvement attributed to petroleum, it has ushered in environmental contamination, wreaking havoc on the biotic and abiotic components of the ecosystem (Mi-ilion *et al,* 2009). Chemical compounds that enters the ecosystem as a result of human activities build up in soil and water reservoirs. As a result, soil could be thought of as a long-term repository of environmental toxins from which these substances reach terrestrial food chain and subsurface water. The ability of soil microorganisms to multiply swiftly even in unfavorable environmental conditions indicated a high level of receptivity to either positive or negative effects such as those caused by contaminants (Adriaens *et al,* 2001). Several researchers have reported that soil contamination with crude oil can cause shift in microbial populations (Ellis *et al,* 2001). Microorganisms that can utilized petroleum oil and hydrocarbon for growth, nutrition and metabolic activities are abundant in oil contaminated environments. Microorganisms in the soil play critical role in the survival of the terrestrial ecosystem. They are important in the detoxification of toxins such as heavy metals and radionuclides, as well as the recycling of elements (such as carbon, nitrogen, Sulphur and phosphorous) and cleanup of oil spillage (Makut and Ishaya, 2010). Rise in crude oil mining activities as well as poor maintenance of oil pipelines and transportation vessels. As a result, crude oil and its products are released into the land and aquatic habitats. Despite the fact that some microorganisms have hydrocarbon classics capability (Romanus, et al; 2015). Extremely heigh quantities of crude oil pollution in the environment may have hampered the expression of the characteristics, resulting in an imbalanced carbon-nitrogen (C; N) ratio. Oil spills cause this disequilibrium which is harmful to microorganisms.

MATERIAL AND METHODS

Sampling Sites/Location

Soil samples (about 200g each) were collected from petro-chemical polluted Soils mainly from Bauchi State, metropolis. A scoop was used to remove debris of organic particles from the surface of the soil. Surface soil (0 to 10 cm deep) was collected from each site at random with sterile spatulas. Altogether 25 soil samples were collected from different sites in Bauchi metropolis. The temperature of each soil sample was taken, specific sites included are Gwallameji mechanic workshop and Yankari garage. Dass moto-car workshop, Yelwa mechanic workshop and Dan Gombe mechanic workshop. These samples were used as the source of petroleum-degrading microorganisms. The age of contamination of these sites varied from 3-25 years.

Determination of physiochemical properties of soil.

The following physiochemical parameters of each contaminated soil samples were analyzed, moisture, pH and temperature of each soil sample was determined.

Sterilization of glassware and media

Petri dishes, test tubes, flasks, etc. were sterilized in hot air oven at 160^0c 180^0c for 2 to3 hours. Before they were put in the oven they were washed, dried and packed in aluminum foil.

Media was sterilized by autoclave at 121^0c for 15minutes.

Enumeration of total heterotrophic bacterial count

The total heterotrophic bacterial count was enumerated using the pour plate method on nutrient agar. Soil suspension were prepared by 10-fold serial dilutions with one gram of soil and 0.1ml of 10 and 10^6 dilutions was spread on the plates in triplicates, and incubated at 30^0c for 24 to 48 hours. The coliform forming units (CFU) of the bacterial were counted after incubation. According to Jayanthi, (2015).

Enumeration of Hydrocarbon Utilizing Bacteria

Hydrocarbon utilizing bacteria in the sample were enumerated by viable count method using the surface spreading technique on nutrient agar. Soil suspension

were prepared by 10-fold serial dilution with 1g 0f soil and 0.1ml of 10^{-4} and 10^{-6} dilutions was spread onto nutrient agar plates in triplicates. After inoculations of the nutrient agar plates with the samples, a sterile filter paper (Whatman No.1), impregnated with crude oil, was aseptically placed onto the inside of the lid (cover) of petri dishes and sealed with a masking tape. The filter paper saturated with crude oil served as a sole carbon and energy source for growth of the microorganism on the surface through a vapours pressure phase transfer. The plates were incubated in an inverted position at room temperature for seven days, after which the average counts from triplicates plates were counted and recorded.

Utilization of Petroleum Products by isolates

A loop full of isolates on nutrient agar were picked and inoculated on agar - agar plates in triplicates 0.2ml of each test substrate (petrol, engine oil, diesel, kerosene) was measured and spread aseptically on the surface of the agar-agar and plates were inverted and incubated at 35^0c for 24 __ 48 hours and the utilization of each test substrates were estimated visually, according to (Nada, 2007).

Morphological characterization for bacterial identification

Morphological examination was done by phase contrast microscopy and other methods according to (M. nada 2007) and Bergeys Manuel of systematic Bacteriology (1984) as follows.

Gram staining

For each bacterial isolate a heat fixed smear from 24 hours old cultures was prepared, stained with crystal violet solution for 1-2 minutes, rinsed rapidly with water and iodine solution was added and the smear was blot dried. The slide was washed with 95% ethanol for 15 seconds, rinsed with tap water and stained with slaframine for 20 seconds. The slide was air dried and examined under microscope.

Motility

Tubes of motility medium were prepared by soaking the gelatin in the water for 30 minutes and other ingredients were added and sterilized at 115c for 20 min. The medium was stabbed with the isolate to a depth of about 5cm. and incubated

at the 37c. Motile organisms migrated through the medium which became turbid: growth of non-motile organism was confined to the stab.

Biochemical tests for bacterial identification

Generally, biochemical test in addition to Gram's reactions are important in the identification of bacteria. This is because some bacteria species have similar morphological cultural or even staining reaction, which make exhaustive biochemical test important in bacteria identification.

Catalase test

The catalase test was carried out as described by Oguile *et al.,* (2001). Two drops of hydrogen peroxide (3%) was placed on a clean grease-free glass slide. Using a clean glass rod, the test organism was transferred to one of the drop and other used as the control. Formation of bubble or effervescence on the slide indicates a positive result.

Oxidase test

The method used was the wet filter paper described by Oguile *et al.,* (2001). This is used to differentiate bacteria that can produce oxidase enzyme from non-oxidase producing type. Oxidase catalases the electron transport between the bacteria electron donor in the bacteria and a redox dye (tetraethyl p-phenylenediaminedihydrochloride) which is reduced to deep purple color. 1% tetraethyl p-phenylenediaminedihydrochloride was dissolved in 1 litre of distilled water. These constitute the oxidase reagent. A small portion of the isolates was smeared on part of a filter paper strip. The reaction was observed for about 10 seconds. A positive reaction was indicated by an intense deep-purple coloration within 5-10seconds.

Coagulase test

The coagulase test was carried out as described by Oguile *et al.,* (2001). A drop of normal saline was placed on both ends of a clean microscopic slide. A colony of the test organisms was emulsified in each of the drops to make two thick suspensions. Then a drop of plasma was added to one of the suspensions and mixed thoroughly and gently and observed for clumping within some seconds. This method is to detect blood coagulase.

Indole test

The indole test was done as described by Oguile *et al.,* (2001). The indole test was employed to determine the ability of an isolate to breakdown the amino acid tryptophan with the enzyme tryptophanase to liberate indole. A test tube of 1% peptone water was inoculated with the test organism and incubated for 48 hours at 37^0C. then 0.5ml of Kovac's reagent (prepared by dissolving 5g p. dimethylamino-ben-zaldehyde in 75ml of amylalcohol and 25ml concentrated hydrochloric acid) was added to the test tube and was shaken gently. Indole positive indicated a reddish concentric ring while negative result indicated a brown concentric ring at the upper layers of the medium.

Sugar fermentation test

The isolates were further characterized by their ability to ferment a number of sugars such as glucose, lactose and sucrose. The basal medium used for the test was peptone water. The medium was prepared by dissolving 15g of the peptone water in a liter of distilled water according to the manufacturer's specification. 30ml of phenol red (indicator) and 2.5g of sodium chloride (NaCl) were added to 970ml of the peptone water. Sodium hydroxide (NaOH) was added to adjust the pH and get a full reddish color. 300ml of each of the peptone water was transferred to three different conical flasks designated for a particular sugar. The solution in each of the conical flask were dispensed into test tubes containing inverted Durham tubes, stoppered with cotton wool and wrapped with an aluminum foil. The test tube was inoculated with test organism and incubated for 24 hours at 37^0C. acid production was indicated by change in color of the indicator to yellow, and also gas production was observed in Durham tubes.

Urease test

Urea base of 2.4g was added to 95ml of distilled water and 10ml of phenol red was also added and autoclaved for 15 minutes at 121^0C. It was then allowed to cool to 50^0C and 5 ml was dispensed aseptically, into each of the test tubes. The test isolates were inoculated into the test tubes with a wire loop. Change in color to intense red-pink indicated positive result while negative result shows no color change.

RESULT

The table below represents the mean count of total heterotrophic and hydrocarbon utilizing bacterial from each soil sample.

Table 1: Bacteria counts of petro-chemical polluted soil from different sample sites

Sample site	Mean Values of Bacteria (CFU/ml)	
	Total Heterotrophic Bacterial(THB)	**Total Hydrocarbon Utilizing Bacteria (THUB)**
A	**1.59×10^4**	**1.0×10^4**
B	**6.6×10^4**	**2.5×10^4**
C	**8.5×10^4**	**7.2×10^4**
D	**7.4×10^4**	**2.0×10^4**
E	**5.2×10^4**	**5.2×10^4**

A = Gwallameji mechanic site; B = Yelwa mechanic site, C = Dass park mechanic site; D = Yankari mechanic site; E = Dan Gombe mechanic site.

The table above shows variation in the total heterotrophic bacteria count and hydrocarbon utilizing bacteria count from each soil sample.

Table 2: Distribution of Bacterial Isolates According to sample sites

Isolates	**Sampling sites**				
	A	**B**	**C**	**D**	**E**
Micrococcus sp	**-**	+	+	+	+
Staphylococcus sp	+	+	+	+	+
E. coli	+	+	+	**-**	**-**
Bacillus sp	**-**	+	+	+	+
Arthrobacter sp	+	+	+	+	+

Flavobacterium sp	+	+	+	+	+
Achromobacter sp	+	-	+	+	-
Vibrio sp	+	+	-	-	-
Alcaligens faecalis	+	+	+	+	+
Norcadia sp	+	+	-	-	-
Streptococcus sp	+	-	-	-	-
Pseudomonas sp	-	+	+	+	+
Corynebacterium sp	-	+	+	+	+

Key: Present (+), Absent (-)

A = Gwallameji mechanic site; B = Yelwa mechanic site, C = Dass park mechanic site; D = Yankari mechanic site; E = Dan Gombe mechanic site.
The above table shows a variation in the distribution of bacterial isolates from each soil sample sites.

Table 3: Distribution of Bacterial Isolates according to utilization of petroleum product

Isolates				
	B	**C**	**D**	**E**
Micrococcus sp	+	+	+	+
Pseudomonas sp	+	+	+	+
Bacillus sp	+	+	+	+
Flavobacterium sp	+	+	-	-

Corynebacterium sp	+	-	-	-
Staphylococcus sp	+	+	-	-

Key: (+) = Positive utilization, (-) = No utilization

The above table indicated that half of bacterial species have the ability to utilize the commonly used petroleum products in the sampling sites. But the lowest degradation ability was observed on *Corynebacterium sp,* which utilized only petrol then followed by *Staphylococcus sp* and *Flavobacterium sp.*

Table 4: Physiochemical parameters that determine survival of the bacteria in the soil sample

Physiochemical parameters			
Sample site	**Temperature (^{0}C)**	**Moisture (%)**	**pH**
A	**33**	**71.2**	**6.9**
B	**35**	**66.1**	**6.7**
C	**32**	**67.0**	**6.0**
D	**34**	**70.0**	**6.5**
E	**38**	**66.4**	**6.6**

The above table indicated that degradation of petroleum hydrocarbons occur at a wide range of temperature, moisture, and pH tending towards neutrality.

DISCUSSION

Detection of hydrocarbon utilizing bacteria from petrochemical polluted soil was investigated. The result of bacterial count (Table 1) shows that Gwallameji mechanic site had the lowest total heterotrophic bacterial count of 1.59 x 104 (cfu/ml) and lowest hydrocarbon utilizing bacterial count of 1.0 x 104 (cfu/ml) followed by Dass park mechanic sites which had total hetrotrophic bacterial count of 8.5 x 104 (cfu/ml). and Yankari mechanic sites had total hetrotrophic bacterial count of 7.4 x 104 (cfu/ml). and hydrocarbon utilizing count of 2.0 x 104 (cfu/ml). Gwallameji mechanic sites had existed for 3 year, Yelwa and Dass park mechanic site had existed for over 25 years. And Dan Gombe had being in existence for over 10 years. Analysis of variance, for the total heterotrophic

bacteria count, and hydrocarbon utilizing bacteria revealed that the counts between sample sites were significantly different (P<0.05). this difference in bacteria count of each sample site may be due to organic contents, accumulation of resistance component such as cyclo-alkane, and also may be due to rate of contamination of each sites. Eze and Okpokwasili, (2919; Baldwing, 2007). Distribution of bacterial isolate according to sample sites shows (table3) that bacterial *genera* ***isolated*** during this study were 13 *genera*. The bacterial isolates found in all sample sites were mainly *Staphylococcus sp, Arthrobacter, Flavobacterium, and Alcaligensfaecalis* were found in all sample sites. However, Micrococcus sp, Bacillus sp, Pseudomons and Corynebacterium sp were also present in all sample site with the exception of Gwallameji. Vibrio sp and Norcadia sp were present in Gwallameji and Yelwa only while Streptococcus sp was only found in Yelwa sample site. Thses organisms have been previously implicated with petroleum product degration (Umealoikwa, 2003, Zang et al., 2006). However, the variation in the distribution of this bacterial isolates may be attributed to seasonal differences or more petrochemical activities, as well as increased atmospheric content of the soil and in the environment (Eze and Okpowasili, 2010). Distribution of bacterial isolates according to utilization of refined petroleum product in (table 4) shows thar half of bacterial species have the ability to utilized the commonly used petroleum products in the sampling sites. But the highest degradation performance was observed on Micrococcus sp, pseudomonas sp, and Bacillus sp. This is in accordance to the findings of Nada, (2007) and 2013). While, the lowest degradation performance was observed on staphylococcus sp, Flavorbacterium sp, and Corynebacterium. These bacterial species have been previously implicated with refined petroleum products degradation (Makut and Ishaya, 2010). It was clear from the result obtained that all sites harboured hydrocarbon utilizing bacterial, although there was variations in the utilization of refined petroleum products by the isolates. Yasmeen, (2015) and Zulfa, et al, (2016) reported that variation in the utilization of petroleum hydrocarbon by soil microbes is attributed to various enzymes encoded in theire plasmid. This investigation took into consideration factors affecting survival of these microbes in the soil. These factors (table 5) shows that biodegration of petroleum hydrocarbon occur at a wide range of temperature, moisture and pH. The temperature values obtained from different petrochemical polluted soils during this investigation fall within mesophilic range. This means that the temperature of different petrochemical polluted soils support mesophlic organisms. Temperature plays very important roles in biodegrading of petroleum hydrocarbons, firstly by it direct effect on the chemistry of the pollutant and secondly its effect on the physiology and diversity of microbial (Okoh, 2006). Venosa and Zhu (2003) also reported that ambient temperature

of an environment affects both the properties of spilled oil and the activity or population of microorganisms. The pH of each petrochemical polluted soils sample tends from slightly acidic towards neutrality. Neutral pH, enabled biodegradation activity of bacterial in soils. Obire, et al; (2002). Moisture content also plays vital role in utilization of refined petroleum hydrocarbon by soil microbes. Efficient utilization of petroleum hydrocarbon occurs from 50% to 80% moisture contents (Nada, 2007).

Conclusion

This study revealed that discharge pollutants from a place where petrochemical activities take place is considering one of the critical problems to the environment, due to negative impact it had on the health and ecosystem. Currently, the biological control to remove hazards from environment is successful process due to it being a safe way to enhance a healthy environment in particular with low cost, technique and wide public acceptance to cleaning up contaminated sites. Based on previous studies, some bacteria and fungi species have the ability to degrade crude oil in contaminated soil. The data contained in this study shows that some bacterial species were capable of degrading the crude oil in varying degrees. The higher crude oil biodegradation efficiency was exhibited by Micrococcus sp, pseudomonas sp, Bacillus sp and Staphylococcus sp compared to other species, nevertheless bacterial species isolated from contaminated soil can be exploited in the bioremediation of crude oil to remove petroleum hydrocarbon from contaminated environments as well as their consortium culture has promising potential in bioremediation of poly nuclear aromatic hydrocarbon polluted soil. They could also be utilized in bioremediation of used engine oil, diesel and petroleum contaminated soil.

Recommendation

It is here recommended.

a. To promote further research in the determination of total organic carbon, total nitrogen and oxygen of petro-chemica polluted soil.
b. To apply our bacteria isolates on industrial scale.
c. To establish a culture collection centre of crude oil degrading microorganisms.

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