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Original Research

Isolation and characterization of gram negative hydrocarbonoclastic bacteria and fungi strains from non-contaminated, premium motor spirit contaminated and refuse environment

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Abstract
The availability of bacteria and fungi with hydrocarbonoclastic potential are of major concern to the environmentalist. Therefor this work is aimed at isolating and characterizing of organism that are able to grow in a contaminated environment without amendment. Soil samples were collected from three different sites (non-contaminated, petrol contaminated and refuse contaminated) around Nsukka environs and were used for the isolation using different differential medium. The morphological characteristics of the isolates were identified by Gram staining, biochemical reactions and motility test as well. *Pseudomonas putida*, *Acinetobacter buamanii* and *Proteus vulgaris* were found to grow in all the soil samples, but there growth density were found to be more in the soil polluted with the Premium Motor Spirit. Their ability to grow well on the contaminated soil shows that they effectively broke down the hydrocarbon skeleton of Premium Motor Spirit into metabolites which served as a rich source of carbon and nitrogen for their metabolism and growth hence can effectively be employed in bioremediation of crude oil contaminated sites.

INTRODUCTION

A variety of technologies are currently available to treat soil contaminated with hazardous material, including excavation and containment in secured landfills, vapour extraction, stabilization and solidification, soil flushing, oil washing, solvent extraction, thermal desorption, vitrification and incineration [1].

Many of these technologies, however, are either costly or do not result in compute destruction of contamination. On the other hand, biological treatment "bioremediation" appears to be among the most promising methods for dealing with a wide range of organic contaminants particularly petroleum hydrocarbons.

Bioremediation is a means of cleaning up contaminated environments by exploiting the diverse metabolic abilities of microorganisms to convert contaminants to harmless products by mineralization, generation of carbon (iv) oxide and water, or by conversion into microbial biomass [2]. It is based on the premise that naturally occurring bacteria in an impacted environment develop the means to degrade or tolerate the presence of organic contaminants (chemicals) by using them as an energy source to promote their own growth. The ability to degrade petroleum hydrocarbons is not only restricted to a few microbial genera; a diverse group of bacteria and fungi have been shown to have this ability. Based on the number of published reports, the most important hydrocarbon degrading fungi in both marine and soil environments is in the orders Mucorales and Monilales as well as in the genera Aspergillus and Penicillium species (order Eurotailes). Other species in many fungal genera known to metabolize hydrocarbons and or thrive in

crude-oil contaminated sites include Acromonium, Aspergillus Cindida, Clasdosporium, Mucor, Penicillium, Saccharomyces, Trichoderma Trichosporon etc. [3]. Although developed countries have commercially produced fungal cultures for oil cleanup, this is less common in underdeveloped countries. One region subject to frequent detrimental oil spills, and lacking access to commercially produced cleanup strains is the Niger Delta. This region may benefit from development of indigenous fungi for bioremediation. It is recommended that the crude oil which cannot be recovered after a spill should therefore be subjected to bioremediation using fungi and fungal products such as enzymes [4]. In addition to the demonstration of the treatment efficacy, it is necessary to demonstrate that bioremediation does not produce any toxic intermediate products and to avoid undesired environmental and ecological effects [5, 6].

There is a great deal of interest in the use of genetically modified or engineered microorganisms (GEM) to enhance oil degradation, particularly the degradation of high molecular weight polyaromatic compounds and alkane hydrocarbons. Microorganisms with enhanced capabilities to degrade particularly aromatic hydrocarbons and their derivatives have already been developed [7, 8]. Although technologies based on these concepts hold promise for improved bioreactor performance, experience gained from bioaugmentation tests suggest that the use of GEMs will be ineffective without development of techniques to improve their survival in the face of competition from indigenous microbial population [9]. Convenient, economical and effective methods of tracking engineered microorganisms have been developed to enable the examination of their survival, transport and ecological impact when released in new environments [10, 11].

By nature, petroleum is a heterogeneous and water-insoluble substrate that presents special problems to microorganisms. Consequently, microbes that grow on crude oil or other hydrocarbon sources, as of necessity, have developed special traits to overcome the solubility problems. An oil-degrading microbe has been defined by its possession of three special characteristics that endows it with the ability to proliferate on hydrocarbon substrates [12] as listed below.

1. An efficient hydrocarbon uptake system (special receptor sites for binding hydrocarbons and/or production of unique chemicals that assist in the emulsification and transport of hydrocarbons into the cell).

2. Possession of group-specific oxygenases (i.e., genetic potential of the micro-organism to produce enzymes that will introduce molecular oxygen into the hydrocarbon and generate intermediates that subsequently enter the common energy-yielding catabolic pathways).

3. Inducer specificity (i.e. the positive response of the micro-organism to petroleum and its constituents in inducing the first two systems). Hydrocarbon-utilising micro-organisms act mainly at the oil-water interface. Microscopic observations have shown that these organisms grow over the entire surface of an oil droplet. Increased surface area provided by the dispersion of oil in the water phase should therefore accelerate biodegradation. Two general biological strategies have been identified as responsible for contact between bacterial cells and water-insoluble hydrocarbons:

1. Specific adhesion to hydrocarbon substrates;

2. Emulsification of the hydrocarbon.

These strategies are made apparent by the fact that the first step in hydrocarbon degradation involves membrane-bound oxygenase enzymes making it essential for microbes to come into direct contact with hydrocarbon substrates. The ability of microbes to adhere to hydrocarbons is inherent in their possession of surface features such as outer membranes, fimbriae, surface proteins and lipids that are collectively known as "hydrophobins". This work is aimed at comparing the possible hydrocarbonoclastic bacteria and fungi that exist in a Premium motor spirit (PMS) contaminated site, and comparing it with their presence in both uncontaminated and refuse dump with a view of studying their ubiquitous nature in the environment. Considering the fact that crude oil pollution and take place in any place and time.

MATERIALS AND METHODS

Soil Sample Collection

Crude oil contaminated soil samples, non-contaminated soil samples and refuse soil samples were collected from different locations around the Nsukka Community, Enugu State, Nigeria.

Five hundred grammes of soil was collected from different sites such as; Petrol filling station around Nsukka Community, Botanical village, University of Nigeria, Nsukka and Refuse dump site around Nsukka Community respectively.

The soil sample collections were done with hand trowel. A dept of 4-6cm was made on each of the site of collection of the samples. A sterile container was used to hold each of the soil samples. Each of the samples was labeled based on the area of collection.

Preparation of soil for isolation

The soil samples were taken to the laboratory in labeled

polythene bags. They were dried under gentle heat in the oven, crushed using mortar and pestle and then sieved using a sieve of mesh size 2.0mm. One (1) gram of each soil sample were collected under sterile condition and poured into separate conical flasks which were already containing 100ml of sterile distilled water, and then the conical flasks were covered using aluminum foil. The content of the conical flask was mixed thoroughly by shaking for 10 minutes. After shaking, the mixture was allowed to stand for 5mins so that the liquid portion of the mixture can separate properly from the solid portion. The upper liquid portion then was obtained by decantation after which a 10fold serial dilution was carried out by adding 1ml of the soil extract to 9ml of distilled water in a conical flask, and shaking properly to enhance mixing after the mouth part of the conical flask had been covered using an aluminum foil. Four concentration s were gotten from the serial dilutions and each of these four dilutions was used in identification of the microbial species.

Preparation of isolation media

The work bench and the surrounding air were sterilized using disinfectant and flame respectively before the commencement for the preparation of the isolation media. The media prepared include; Nutrient agar, Mac-Conkey agar, Sabourand Dextrose agar, KIA (Kligler Iron Agar) and Blood agar.

Nutrient agar

It was prepared by weighing out 28g of Nutrient agar (NA) into 1000ml (1litre) of distilled water in a conical flask. The mixture was allowed to stand for 10mins after which it was shaken properly to ensure homogeneity. The conical flask containing the mixture was covered with cotton wool wrapped with foil and then autoclaved at 121°C and 15Ib PSI for 20 mins. After autoclaving, the mixture was allowed to cool to a temperature of 42-45°C before pouring into sterile Petri dishes and allowed to set.

Blood agar.

It was prepared by weighing out 28g of Blood agar (NA) into 1000ml (1 litre) of distilled water in a conical flask. The mixture was allowed to stand for 10mins after which it was shaken properly to ensure homogeneity. The conical flask containing the mixture was covered with cotton wool wrapped with foil and then autoclaved at 121°C and 15Ib PSI for 20 mins. After autoclaving, the mixture was allowed to cool to a temperature of 42-45^{o}c. Then 2ml of freshly collected blood was added to each 20ml of nutrient agar in small bottles under aseptic condition. The mixtures were mixed properly by shaking and they were immediately distributed in sterile Petri-dishes (20ml per plate) and allowed to solidify. The surfaces of the medium were heated briefly with a bunsen burner before covering, so as to avoid contamination.

Mac-Conkey agar.

It was prepared by weighing out 52g of Mac-Conkey agar solute into 1000ml (1 litre) of distilled water in a conical flask. The mixture was allowed to stand for 10mins after which it was shaken properly to ensure homogeneity. The conical flask containing the mixture was covered with cotton wool wrapped with foil and then autoclaved at 121^{0} c and 15Ib PSI for 20 mins. After autoclaving, the mixture was allowed to cool to a temperature of 42-45^{o}c before pouring into sterile Petri dishes and allowed to set. 20ml of the agar was poured into each plate and allowed to gel.

Sabourand dextrose agar

It was prepared by weighing out 62g of Sabourand dextrose agar into 1000ml (1 litre) of distilled water in a conical flask. The mixture was allowed to stand for 10mins after which it was shaken properly to ensure homogeneity. The conical flask containing the mixture was covered with cotton wool wrapped with foil and then autoclaved at 121^{0} c and 15Ib PSI for 20 mins. After autoclaving, the mixture was allowed to cool to a temperature of 42-45^{o}c before pouring into sterile Petri dishes and allowed to set.

Kiegler Iron Agar (Slant medium).

It was prepared by weighing out 49g of Kiegler iron agar solute in 1000ml (1 litre) of distilled water in a conical flask. The mixture was allowed to stand for 10mins after which it was shaken properly to ensure homogeneity. The conical flask containing the mixture was covered with cotton wool wrapped with foil and then autoclaved at 121^{0} c and 15Ib PSI for 20 mins. After autoclaving, the mixture was allowed to cool to a temperature of 42-45^{o}C before pouring into sterile Petri dishes and allowed to set. Afterwards the test-tubes containing the media were kept in a slant position and allowed to set.

Culturing of the organisms.

Micro pipette was used to collect 0.1ml of the four dilutions gotten from each soil solution. The diluted soil solutions was inoculated on the surface of the prepared nutrient agar plates which were labeled P1,P2,P3and P4 for the petrol contaminated soil, R1,R2,R3 and R4 for refuse contaminated soil and then N1, N2, N3 and N4 for the normal soil. The soil solutions were streaked unto the surface of the agar plates using a sterilized wire loop. Then the plates were incubated for 24hours at 37^{o}C except for the Sabourand agar plates which were incubated at 28^{o}c for 72 hours.

After incubation, different microbial colonies grew on

the surface of the agar plates. The microbial colonies were isolated separately and characterized using features such as the morphology of the colonies formed (shape, size, consistency, colour, texture, and elevation), Gram staining and Microscopy. Further characterization was carried out using biochemical tests such as coagulase test, catalase test, glucose fermentation test, methyl red test, indole test, oxidase test, and motility test.

Phenotypic characterization

Discrete colonies grew on all the streaked agar plates labeled P1,P2,P3and P4 for the petrol contaminated soil, R1,R2,R3and R4 for refuse contaminated soil and N1,N2,N3nd N4 for the non-contaminated soil.

Macroscopy

The morphological characteristics of the growth on each agar was closely observed and recorded using a microscope.

Microscopy (gram reaction)

This staining was done to know the organisms which retained or decolorized acetone alcohol. Based on this reaction, organisms are classified into Gram-positive and Gram-negative.

Procedure:

On a clean glass slide, a smear of the organism was made with a drop of normal saline. The slide was allowed to air-dry and then passed three times through the flame to fix. The smear was covered (stained) with crystal violet and allowed to stand for 30 seconds and then rinsed with distilled water. Smear on the slide was covered with lugols iodine and left to stand for 30 seconds. The iodine was washed with water and decolourised with acetone-alcohol and washed off with water immediately. The smear was then counter stained with safranin for 20 seconds and washed off with water and then allowed to dry. The slide is then observed with a microscope using x100 objective with oil immersion.

This procedure was performed on each of the microbial colonies collected from P1, P2, P3 and P4, R1, R2, R3 and R4, and N1, N2, N3and N4 respectively. Based on microscopy, the bacteria present in each soil sample were grouped into Gram positive and Gram negative.

- Gram positive bacteria are those which retain the primary stain (crystal violet) after been treated with acetone. They have a purple coloration when viewed under the microscope.
- Gram negative bacteria are those bacteria species which take up the secondary stain (safranin) after the decolourization step. They appear pink when viewed with the microscope.

Catalase test.

This test is carried out to find out if any of the organisms present can synthesize the catalase enzyme; an enzyme that breakdown hydrogen peroxide (H_2O_2) to H_2O and O_2. The presence of water bubbles in the microbial smear is a positive result.

Procedure.

A smear of the microbial colony was made on a clean glass slide using normal saline solution. Then a drop of hydrogen peroxide was placed on the smear and mixed using a sterile wire loop. Then the presences of water bubbles was watched out for and noted.

Oxidase test.

This test classifies organisms into oxidase positive and oxidase negative. The oxidase reagent is colourless when freshly prepared but rapidly oxidized to purple coloured derivative by organisms that produce both peroxidase and hydrogen peroxide to detect C-type cytochrome.

Procedure:

A filter paper was moistened with a few drops of 1% tetramethyl-p-phenylenediamine dihydrochloride (a dye). A smear of the isolate is made on the filter paper and observed for colour change. For oxidase negative organisms, no colour change will be observed. For oxidase positive aerobic organisms, a colour change (grey to purple or violet) is seen if they possess c-type cytochrome.

Coagulase test.

This test is carried out to identify bacteria species which can synthesize the enzyme-coagulase in the presence of fresh plasma. Coagulase is an enzyme which catalyzes the conversion of fibrinogen to fibrin. A positive result is indicated by the presences of blood clumps in the microbial smear.

Procedure:

A microbial smear was made on a clean glass slide using normal saline solution, and then a drop of fresh plasma was added to the microbial smear. These were mixed using a sterilized wire loop and allowed to stand for 30seconds.Then the smear was observed for blood clumps.

Nitrate utilization test

This test is carried out to detect the presences of bacteria species which can utilize nitrate (NO_3) to nitrite (N_2) or another nitrogenous compound.

$$NO_3 \rightarrow NO_2 \rightarrow NH_3 \text{ or } N_2$$

Procedure:

Nitrate broth is prepared by dissolving 9g of the nitrate broth powder in 1litre of distilled water. Autoclaved at 121^{o} C for 15mins.Then using a sterile wire loop the microbial colony was collected from the agar medium and inoculated into the nitrate broth. Cover the tube and incubate at 35^{o}C-37^{o}C for 48hrs. After incubation, 10-15 drop each of alpha-napthylamine and sulfanilic acids are added. A positive result is indicated by a red colour change.

Fermentation test

This test is carried out to test for the presence of organisms that can ferment different sugars such as glucose, galactose, lactose, etc with or without gas production.

Procedure:

This test was carried out by culturing the organisms in the KIA slant medium by inoculating the organisms into the medium using a sterile straight wire loop and incubating it overnight .The test is carried out to identify organisms which can produce acid and gas from sugars indicated by the presences of cracks in the gel and decolourisation of the medium from a yellow colouration to either pink, black or dark green. KIA contains the sugars lactose, Glucose and Dextrose.

Test for fungi.

The presence of fungi species is tested for by using the reagent- Acetophenol cotton blue.

Procedure:

A drop of the reagent was placed on four different sterile glass slides, then four different fungi colonies were mixed with the reagent on the slide. The smears were covered using cover slide after which they were viewed under a light microscope using the x10 objective lens.

RESULTS

After the growth of the organisms, the macroscopic examination was carried and the result in table 1 showed the morphological characteristics of the colonies isolated from PMS contaminated site Refuse dump site and a non-contaminated site on different media. Seven different colonies were isolated from PMS contaminated soil and were labeled P1 – P7; twelve different colonies were isolated from Refuse dumps soil and were labeled R1 – R12 and seven different colonies were isolated from Refuse dumps soil and were labeled N1 – N7.

Table 1. Morphological characteristics of isolates from the different sites.

Isolates	Nutrient agar	Mac-conkey agar	Blood agar
P1	Yellowish tinge smooth colonies	Pale coloured colonies	Small mucoid colonies
P2	Large grey glass appearing colony	Pale colonies	Haemolytic large grey colonies
P3	Yellow to brown pigmented colonies	Pale colonies	Haemolytic colonies
P4		Pink colonies	Large mucoid colonies
P5		Pink colonies	Haemolytic
P6		Small white pale colony	Small white colony
P7			Anaerobic, single zone haemolysis.
R1	Yellowish tinge smooth colonies	Pale coloured colonies	Small mucoid colonies
R2	Blue green pigment, colony is flat and smooth	Flat, dry pale colour colonies	Dark-green pigmented colonies
R3	Large grey glass appearing colony	Pale colonies	Haemolytic large grey colonies
R4	Yellow to brown pigmented colonies	Pale colonies	Haemolytic colonies
R5		Pink colonies	Large mucoid colonies
R6	Convex, red pigment colony	Pale calonies	Less mucoid colony
R7		Pink colonies	Haemolytic
R8	Swarming individual colonies	Pale individual colonies	Swarning
R9		Pink colonies	Large mucoid colonies
R10		Non lactose fermenting colonies	Tiny colony
R11			Anaerobic, single zone haemolysis.
R12			Anaerobic, double zone haemolysis. Large grayish-yellow colonies slightly opaque.
N1	Large grey, glass appearing colonies	Pale coloured colonies	Haemolytic large grey colonies
N2	Yellow to brown pigmented colonies	Pale coloured colonies	Haemolytic colonies
N3		Pink coloured colonies	Large mucoid colonies
N4	Convex, red-pigmented colony	Pale coloured colonies	Less mucoid colony
N5	Swarming/individual colonies	Pale/individual colonies	Swarming on B.A
N6		Pink coloured colonies	Large mucoid colonies
N7			Anaerobic, single zone haemolysis.

Biochemical Characteristics

The biochemical characteristics of the isolates were determined and table 2 shows the gram staining, motility test, catalase test, oxidase and coagulase test of the isolated organisms from the three environment.

From the result in table 2, none of the isolated showed coagulate activity and only isolates P1, P6, R1 and R2 showed oxidase activity.

Sugar Fermentation and Other Tests

Sugar fermentation is also a basis for the characterization of the isolated organism. Table 3: shows the fermentation characteristics of the organisms isolated from the three environment.

The result showed that the isolate P6 which is aerobic did not ferment any of the sugar neither did it produce acid or gas.

Table 2. Biochemical characteristics of isolates from the different site

Isolates	Gram staining	Motility test	Catalase test	Oxidase test	Coagulase test
P1	-	+	+	+	-
P2	+	+	+	-	-
P3	+	+	+	-	-
P4	-	-	+	-	-
P5	-	+		-	-
P6	+	-	+	+	-
P7	+	+	-	-	-
R1	-	+	+	+	-
R2	-	+	+	+	-
R3	+	+	+	-	-
R4	+	+	+	-	-
R5	-	+	+	-	-
R6	-	+	+	-	-
R7	-	-	+	-	-
R8	-	+		-	-
R9	-	+	+	-	-
R10	+	-	-	-	-
R11	+	+	-	-	-
R12	+	-	-	-	-
N1	+	+	+	-	
N2	+	+	+	-	
N3	-	+	+	-	
N4	-	+	+	-	
N5	-	+		-	
N6	-	+	+	-	
N7	+	+	-	-	

Fungi Characterization

Table 42. Morphological characteristics of fungi isolated from refuse contaminated site

Isolates	Characteristics	Possible fungi present
A1	Wolly, shade of yellow or yellow-brown oigmentation	*Aspergillus flavus*
A2	White-bright yellowish colony with bright yellow to orange on reverse	*Microsporum canis*
A3	Abundant spindle- shape macroconidia	*Microsporum gypseum*
A4	Black, underside is yellow	*Aspergillus niger*
B1	Black , underside is yellow	*Aspergillus niger*
B2	Granules with shades of green, green-grey	*Aspergillus fumigatus*
B3	Brown to yellow brown	*Aspergillus flavus*

DISCUSSION

The isolates from petrol contaminated soil P2 ,P3,P6 and P7 have the ability of retaining the purple colour of crystal violet (primary stain) during decolourisation with the acetone alcohol; thus are Gram positive while the other isolates (P1, P4 and P5) lost the purple colour of crystal violet during decolourisation, hence are referred to as Gram negative bacteria. The isolates from refuse contaminated soil (R3, R4, R10, R11 and R12) also have the ability of retaining the purple colour of crystal violet (primary stain) during decolourisation with acetone alcohol and are as well referred to as Gram positive while the other isolates (R1, R2, R5, R6, R7, R8, R9) from the same soil sample lost the purple colour of crystal violet during decolourization , hence are referred to as Gram negative bacteria.

The isolates from normal soil (N1, N2, N3) also have the ability of retaining the purple colour of crystal violet (primary stain) during decolourisation with acetone and are referred to as Gram positive bacteria while N3,N4,N5,N6 lost the purple colour of crystal violet (primary stain) during decolorisation hence are referred to as Gram negative bacteria. From the physical and biochemical test conducted on the normal soil, the result indicates that N3, N4, N5 and N6 (Gram negative) could be *Enterobacter aerugenes, Serratia marcesceus, Proteus vulgari* and *Hafnia alvei.* The isolates; *Enterobacter aerugenes, Serratia marcesceus* and *Hafnia alvei* were present in the normal soil but absent in the petrol contaminated soil. This could be attributed to the fact that they were not able to use the hydrocarbon components of petroleum as carbon and nitrogen source and so could not survive or thrive in the contaminated soil.

The physical and biochemical test conducted on petrol contaminated soil indicates that P1, P4 and P5 (gram negative) could be *Pseudomonas putida, Acinetobacter buamanii and Proteus vulgaris*. Their ability on grow on the petrol contaminated soil shows that they were able to utilize the hydrocarbon present in petrol as a source of carbon for survival by degrading it into utilizable metabolites. The bacteria species *Pseudomonas putida* and *Acinetobacter buamanii* grew exclusively on the petrol contaminated soil and this shows that they effectively broke down the hydrocarbon skeleton of petrol into metabolites which served as a rich source of carbon and nitrogen for their metabolism and growth.

In the biochemical and physical test conducted on refuse contaminated soil, the results indicates that R1, R2, R5, R6, R7, R8 and R9 (Gram negative) could be *Pseudomonas putida*, *Pseudomonas aeroginosa*, *Enterobacter aerogenes*, *Serratia marcesceus*, *Acinetobacter buamanii*, *Proteus vulgaris* and *Hafnia alvei.* More Gram negative bacteria's were found in the refuse contaminated soil when compared with petrol contaminated soil and the normal soil, this suggest that most microorganism flourish very well in the refuge because of the readily availability of nutrients as a result of vast composition of the refuge soil. This result the growth of a large number of diseases pathogens that plagues the environment.

Fewer isolates found in petrol contaminated soil suggest that only few bacteria can thrive well in such areas and these few bacteria have the ability to degrade the hydrocarbon of the petrol oil in the soil, e.g. *Acinetobacter buamanii* isolated from petrol contaminated soil has also been shown by Throne-Holst *et al*, [13] who also asserted that Acinetobacter sp. have the capability of utilizing n-alkanes of chain length C10–C40 as a sole source of carbon [13]. These isolates when tested for oxygen utilization were found to be aerobic, indicating that their ability to degrade hydrocarbon solely depends on the availability of oxygen as reported by Van Hamme *et al*, [14] that the presence and concentration of oxygen is the rate limiting parameter in the biodegradation and catabolism of hydrocarbons by bacteria and fungi [14]. Those isolate which were not found in petrol contaminated soil lack degrading ability because of the absence of the enzymes oxygenases and peroxidases that catalyzes the in cooperation of oxygen for degradation to take place.

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