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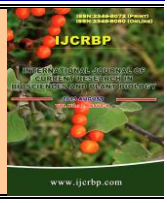


Original Research Article

Utilization of Hydrocarbons by Bacteria Isolated from Oil-Contaminated and Uncontaminated Soil in South-East Nigeria

C.C. Ezemba[1]*, C.C. Ekwealor[1], I.A. Ekwealor[1], C. A. Ozokpo[1], C. E. Chukwujekwu[2], V. N. Anakwenze[1], G. C. Anaukwu[1] and E.J. Archibong[1]

[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025, Awka, Anambra State, Nigeria
[2]Department of Microbiology, University of Benin, Edo State, Nigeria

**Corresponding author.*

Abstract

It is well known that a good many bacteria can utilize hydrocarbons as a sole carbon source. We have as yet little information on how to isolate hydrocarbon utilizing bacteria by using the ordinary culture technique using solid media, due to its strenuous method involved, however, as to what extent or the rate of dissimilation of hydrocarbons by bacterial. Above all, the rate of utilization from hydrocarbons has not been reported. We have isolated many strains of microorganisms from soil samples by enrichment techniques in a medium containing hydrocarbon, in order to screen hydrocarbon utilizing bacteria. Hydrocarbon utilizing bacteria were isolated from oil-contaminated soil. A total of 140 hydrocarbon utilizing bacteria were isolated and classified into 9 genera based in their physiological, morphological and biochemical characteristics. These genera include: *Pseudomonas, Klebsiella, Arthrobacter, Microbacterium, Proteus, Alcaligena, Beijerinkia, Bacillus* and *Micrococcus*. In the course of the study, the highest organism isolated was recovered from spent oil.

Keywords

Culture medium
Lysine
Microorganism
Oil contaminated soi

Introduction

Hydrocarbons are basically compound of hydrogen and oxygen and are ubiquitous class of natural compounds that are the major constituent of petroleum (83-87% carbon and 11 to 15% hydrocarbon). Hydrocarbons are divided into two chemical classes: open chain or aliphatic compounds and ring or aromatic compounds (Alloway and Ayres, 1993). Petroleum industry, discharge a great deal of hydrocarbon (Ogri, 2001). In Nigeria, the Niger Delta area is the center of petroleum exploration and development activities. Since hydrocarbons are natural products as well as pollutants, it is not surprising that hydrocarbon-oxidizing bacteria are widely distributed in nature (Okoh, 2006). Industrial chemicals such as hydrocarbons have been released into the soil environment as a result of mechanical failure, incineration practices, corrosion, leakage, accidental spillage and improper disposal practices (Morgan and Watkinson, 1989; Cerniglia, 1992).

The discharge of hydrocarbons on the soil may result in selective increase in hydrocarbon- utilizing microbes (Ventateswaran, et al., 1995. However, no single microorganism possesses the enzymatic capability to degrade all or even most of compounds in petroleum mixture. More rapid rates of degradation occur when there is a mixed microbial community than can be accomplished by single species (Saber and Crawford, 1985).

Microbial species exposed to hydrocarbon get adapted, showing selective enrichment. They also exhibit genetic change resulting in high proportion of hydrocarbon utilizing bacteria. The exposure equally results in plasmids encoding hydrocarbon catabolic genes (Lealy and Colwell, 1990). The factors limiting the utilization of hydrocarbon by bacterial species include: temperature, nutrient, pH, oxygen level, nature of the hydrocarbon, moisture (Atlas, 1981).

The ability to isolate high number of certain hydrocarbon utilizing bacteria from an environment is usually taken as evidence that those bacteria are active degraders of that environment. A good number of bacteria capable of utilizing hydrocarbon have been documented (Antai and Mgbomo, 1989; Lealy and Colwell, 1990). The hydrocarbon utilizing bacteria frequently isolated include: *Micrococcus, Pseudomonas, Acinetobacter, Brevibacterium, Arthrobacter, Flavobacterium, Vibreo* and *Achromobacter*.

Also the ability of microorganisms to utilize hydrocarbons as a sole carbon source has been recognized for many years (Yamada et al., 1963; Ezemba et al., 2014). Bacteria have evolved regulatory mechanisms that ensure the production of the enzymes that attack hydrocarbon when exposed. Some of these bacteria have developed effective system for responding to various hydrocarbons. The essential genes of bacteria are carried on chromosomes. However genes encoding enzymes needed for the utilization of substances like hydrocarbons may be carried on plasmids. Chukwujekwu (2000) found that plasmids implicated in the catabolism of hydrocarbons plasmids were involve during catabolism of octane, toluene and naphthalene (Chakrabarty et al., 1973; Dunn and Gunsalus, 1973).

In this study, the isolation method of hydrocarbon utilizing microorganisms from soil samples, the results of the screening of hydrocarbon utilizing bacteria and the rate of utilization by microorganisms are reported.

Materials and methods

Collection of soil samples

Oil contaminated soil samples were collected in sterile screw capped bottles from

- Nigerian National Petroleum Corporation (NNPC) refinery at Warri, Delta state.
- Nigerian National Petroleum Corporation (NNPC) refinery Port Harcourt, Rivers State.
- Mechanical workshop, Heritage Street, Omagba Onitsha, Anambra state.
- Mechanical workshop, Awka road, Onitsha Anambra state.

While uncontaminated soil samples were collected in sterile screw capped bottles from

- Refuse dump site, Awka road, Onitsha Anambra state.
- Forest situated at Mgbudu Ichida, Anambra state.

All the samples were collected at the soil depth of 2-8cm. Both kinds of samples were obtained in South-eastern part of Nigeria.

Collection of hydrocarbons

Kerosene, gasoline, motor oil, were obtained from Liquid gold filling station, along Enugu-Onitsha express road and spent oil from a mechanic workshops at Awka road, in Onitsha, Anambra State. Crude oil was obtained from (NNPC) refineries in Warri and Port-Harcourt.

Enumeration of total heterotrophic hydrocarbon utilizing bacteria in oil-contaminated soil and uncontaminated soil samples

Total heterotrophic bacterial counts in the oil contaminated soil and uncontaminated soil samples were monitored by plating in duplicates appropriate dilutions of the oil-contaminated and uncontaminated soil samples unto nutrient agar. One gram of both oil contaminated and uncontaminated soil sample was serially diluted in ten- folds in 10ml sterile distilled water in a sterile 100ml beaker. 0.1ml of each dilutions of soil sample was inoculated into sterile plates of nutrient agar using "spread plate method". The plates were incubated at 30^{o}C for 24h. Plates yielding 30-300 colonies were enumerated.

Isolation and screening hydrocarbon utilizers

Enrichment of hydrocarbon- degrading bacteria was done in the basal medium (Ward and Brock, 1976), consisting of: NaCl, 0.4g; NH_4Cl, 0.5g; $MgSO_4.7H2O$, 0.5g; KH_2PO_4, 0.05g, distilled H_2O, I L and $NaHPO_4.7H_2O$, 0.05g. Two grams of soil sample was serially diluted in ten- folds in sterile distilled water. One drop of hydrocarbon and 0.1ml of 10^{-5} dilutions of soil sample were introduced into 10ml of sterilized basal medium.

The tube was incubated at 30°C for 7 days. Purity test of the first stock culture were carried out by the ordinary culture technique using basal media mentioned above, which 2% agar was added. The carbon source consisted of 0.5ml of the hydrocarbons added to a sterile filter paper secured in the lids of 70 by 15mm-diameter sterile Petri dishes under aseptic condition. The dishes was then inverted and incubated at 30°C for 4 days. The pure isolates were transferred onto Nutrient agar (Oxoid) slants and stored at 4°C for further studies.

Occurrence of hydrocarbon utilizing microorganisms

By the procedure above mentioned, 140 strains of bacteria, as shown in Table I, were isolated from 10 kinds of samples in which varieties of soil from different origins and varying hydrocarbons were included. The bacteria isolated were those observed to grow and utilize kerosene, gasoline, motor oil, spent oil and crude oil as sole carbon and energy sources.

Characterization of hydrocarbon utilizers

The isolated organisms were characterized (Taxonomic studies) following the methods described by Buchanan and Gibbon (1974). Biochemical tests performed for the characterization of the isolate include: Growth on Nutrient agar, Growth on Yeast Extract-Peptone Glucose Agar, Growth on MacConkey agar, Gram staining, Spore staining. Catalase reaction, Motility test, Citrate Nitrate reduction test, Urease test, Indole test, Methyl Red (MR) test, Voges Proskauer test, Triple sugar iron test, Starch hydrolysis, Sodium chloride tolerance, Oxidase test, L-Tyrosine utilization, Tween 80 hydrolysis, Phenlyalanine test and Sugar fermentation. The following sugars were tested: Glucose, Lactose and Mannitol.

Morphological test

Cultures of the isolate inoculated on nutrient agar plates were observed morphologically for 24-48h incubation.

Growth on MacConkey agar

The isolate were point inoculated on MacConkey agar medium and the plates incubated at 30°C for 24-48h. The colonies with pink colour indicate lactose fermenters while colourless colonies are non-lactose fermenters.

Gram reaction

The isolate was gram stained and gram reaction observed under oil immersion lens (100×). Purple colour indicates Gram positive bacteria while red colour indicates Gram negative bacteria.

Catalase test

About 3 to 5 drops of 3% hydrogen peroxide were added to a thick suspension of the isolate on a clean slide and observed for effervescence. Presence of effervescences indicates a positive test.

Motility test

A 24h culture of the isolate in peptone broth was used for motility test. Five drops of the isolates was placed on a cover slip and the concave shaped slide smeared with Vaseline at the edge of the concavity, gently on the coverslip. This slide was carefully inverted and the drop on the coverslip observed under high power objective lens (40×).

Citrate test

The Isolate was incubated on Simmon Citrate agar slant for 24-96h at 30°C. Colour change from green to blue indicates a positive result.

Nitrate reduction test

The isolate G_1 inoculated in nitrate broth and incubated at 30°C for 96h. About 5 drops each of a solution of Sulphanilic acid dissolved in acetic acid and 5 drops of a solutin of alpha-napthylamine dissolved in ethanol were added to the broth culture in a test tube. The development of red colour indicated in 10 to 20min

indicates a positive test. Confirmation of a negative result is indicated by the observation of a red colour change after the addition of zinc dust to an earlier colourless broth culture.

Methyl red

About 5 drops of methyl red solution was added to 2ml of a five- day old culture of the isolates inoculated in glucose-phosphate broth. Red colour indicates a positive test while yellow colour indicates negative text.

Voges-Proskauer test

About 3ml of 5% alpha-naphthol dissolved in absolute ethanol and 1ml of 40% potassium hydroxide was added to 5ml of a five-day old culture of the isolate in glucose-phosphate medium. Bright pink or red colour indicates a positive reaction while yellow colour indicates a negative reaction.

Triple sugar iron (TSI) agar Test

A triple sugar iron agar slant with butt was streaked and stab inoculated respectively with the isolates. Colour changes to red, orange or black indicate alkaline reaction, acidic reaction, or hydrogen sulphide production respectively.

Starch hydrolysis test

Isolate G_1 was point inoculated on nutrient agar plates containing 2% starch. The plates were incubated at 30^{o}C and observation after 24h for a clear zone around the colonies when flooded with iodine solution. Colourless zones around the growth of isolate indicate a positive result while blue colour even around growth of the isolate indicates negative result.

Tyrosine utilization

Tyrosine agar plate was pointed inoculated with the isolate and incubated at 30^{o}C for 14 days. Positive test was indicated by a clear zone around the colony.

Tween 80 hydrolysis test

The isolate was inoculated on Tween 80 agar plates and incubated at 30^{o}C for fourteen days. An opaque halo of precipitaties around the growth of the isolate indicated hydrolysis.

Phenylalanine- deaminase test

Isolate G_1 was streaked on phenylalanine agar slant and incubated at 30^{o}C for 24h. About 5 drops of freshly prepared 10% ferric chloride solution was used to flood the slant. Development of a green colour within 5 minutes indicates a positive reaction while no colour change indicates a negative reaction.

Sugar fermentation test

The isolate G_1 was inoculated into 1% solution of different sugars (Rhamnose, Dulcitol, Maltose, Galactose, Mannitol, Lactose, Xylose, Glucose and Saccharose) in peptone water and incubated for 24-48h at 30^{o}C. Bromothymol blue was used as an indicator and a colour change from green to yellow indicates positive test.

Results and discussion

In the search for microorganism from oil-contaminated and uncontaminated areas that are hydrocarbon utilizers, one hundred and forty bacteria were isolated from basal medium, with their ability to grow on hydrocarbon. Enumeration of total bacteria in oil contaminated soil and uncontaminated soil samples (Table 1).

Table 1. Enumeration of hydrocarbon utilizing bacteria.

Soil samples	Kerosene utilizing bacteria	Gasoline(petrol) utilizing bacteria	Motor oil utilizing bacteria	Spent oil utilizing bacteria	Crude oil utilizing bacteria
Oil-contaminated soil	4.0×10^{10}	3.0×10^{10}	3.0×10^{5}	4.0×10^{10}	2.0×10^{10}
Uncontaminated soil	3.0×10^{6}	2.0×10^{6}	2.2×10^{5}	3.5×10^{10}	1.0×10^{6}

By the procedure above mentioned, 140 strains of bacteria, as shown in Table 1, were isolated from 10 kinds of samples in which varieties of soil from different origins and varying hydrocarbons were included. Those results show that hydrocarbon utilizing microorganisms are not always abundant in oil soaked soil around oil

wells and refineries, but they also occur in refuse dumps sites and forest. That is, hydrocarbon utilizers appear to be quite commonly present in oil-contaminated soil and uncontaminated soil. This work is supported by the work of Yamada et al. (1963), who isolated one hundred and sixty seven isolates among which one hundred and twenty seven were bacteria from both contaminated and uncontaminated areas. These organisms were recovered from soil and can be isolated from aquatic, terrestrial environment and if the area is polluted with hydrocarbons. The strains were tested for purity, one hundred and four isolates were found to utilize hydrocarbon as a source of carbon on solid agar medium. Hydrocarbon utilizers recovered were both Gram positive and Gram negative bacteria.

Fig. 1: Total heterotrophic hydrocarbon utilizing bacteria in the oil-contaminated and uncontaminated soil.

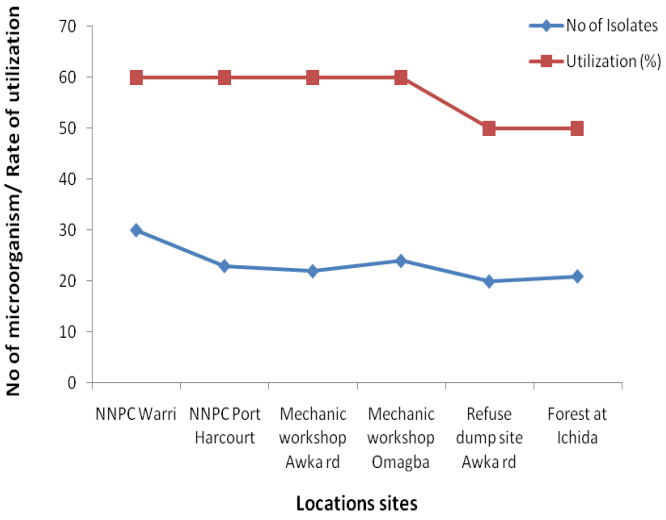
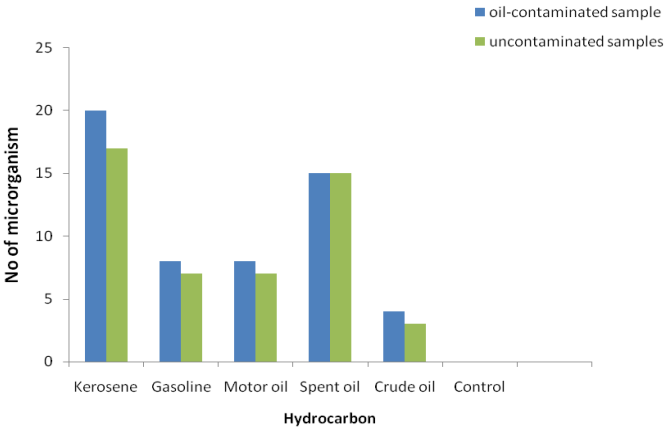


Fig. 1 shows that most bacterial species are capable of utilizing hydrocarbon can be isolated from contaminated and uncontaminated source. The ability to isolate microorganisms that are capable of utilizing hydrocarbons as a sole carbon source is in line with the work of Austine et al. (1977) that hydrocarbon degrading bacteria have the ability of utilizing hydrocarbon as a sole of carbon source and can be isolated from different site. Ezemba, (2008), also isolated one hundred and four hydrocarbon utilizers from both oil- contaminated and uncontaminated soil in south-east of Nigeria. This result is in line with the work of Saber and Grawford, (1985) who noted that population of hydrocarbon degraders are less than 1% of the total microorganism in unpolluted environments but increases from 1% to 10% in environment exposed to petroleum pollutants.

For us to isolate a quite number of bacteria might be attributed to the use of selective media containing hydrocarbons during isolation of hydrocarbon utilizing bacteria. Although, many microorganisms can utilize petroleum hydrocarbons, no single microorganism possesses the enzymatic capacity to degrade all or even most of the compounds in petroleum mixture.

Fig. 2: Isolation of hydrocarbon utilizing bacteria from oil-contaminated and uncontaminated soil.



As presented in Fig. 2, all bacteria were able to utilize the hydrocarbons tested as a source of carbon. This result shows that most bacterial species are capable of utilizing hydrocarbon, and also as a carbon source. Kerosene and spent oil were the most highly occurring hydrocarbon assimilated (Fig. 2). Although kerosene and spent oil were the most highly occurring hydrocarbon utilized as the carbon and energy source during enrichment process, other different compounds could also be utilized. In addition to bacteria have the ability of utilize hydrocarbon as a sole of carbon source is in line with the work of Ezemba et al. (2014). This goes to say that aliphatic components are utilized in preference to aromatic hydrocarbon components.

The inherent capacity of these bacteria organisms to assimilate petroleum hydrocarbon and it products is supported by the work of Okpokawasili and James, (1995). These workers isolated *Nocardia alkanoglutinousa* from soil, capable of utilizing various carbon sources like kerosene, crude oil, animal & vegetable fat and fatty acids. Also Guzik et al. (2009) and Gutlerrez et al. (2012) isolated a Gram-negative bacterium identified as *Stenotraphomonas maltophila* and *Porticoccus hydrcarbonoclasticus* by enrichment of the medium with different aromatic hydrocarbons substrates as sole carbon and energy source respectively.

Table 2. Morphological and biochemical properties of bacterial isolates.

Morphological characteristics	Gram reaction	Catalase	Motility	Glucose	Maltose	Mannose	Ducitil	Rhamnose	Mannitol	Xylose	Galactose	Lactose	Growth on MacConckey	Starch hydrolysis	Simon citrate	Nitrate reduction	Phenyl-alanine	TSl/slope	H_2O_2	Tween 80	Tyrosine	Methyl Red	Vogues Proskauer	Most Probable organism
Circular and pigmented of yellow)	+ve Cocci	+	-	+	-	+	+	-	+	-	-	+	-	+	-	+	-	A/AIK	+	-	+	-	-	*Micrococcus* species
Pigmented (Yellow) circular opaque Glistering.	+ve Rods	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	A/AIK	+	+	+	–	–	*Microbacterium* species
Pigmented (Green) circular opaque and flat	-ve Rods	+	+	+	_	+	+	-	+	-	+	+	-	-	-	+	-	A/AIK	+	-	+	-	+	*Pseudomonas* species
Serrated edge flat	-ve Rods	+	-	+	+	+	-	+	+	+	-	+	-	-	+	+	-	A/AIK	+	+	+	-	+	*Klebsiella* species
Irregular and raised	+ve Rods	+	-	+	+	+	+	+	+	-	+	-	-	+	-	-	+	-	+	-	+	-	-	*Arthrobacter* species
Creamy flat and entire	-ve Rods in cluster	+	+	+	+	-	-	V	-	-	-	-	-	-	+	+	+	A/AIK	-	-	+	+	-	*Bacillius* species
Wavy and entire	-ve Rods in cluster	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	+	AG/A	+	+	+	-	-	*Proteus* species
Circular an flat	-ve Rods in cluster	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	NIL/ AIK	+	-	+	+	-	*Alcaligena* species
Giant colonies with smooth folded surface	-ve Rods in cluster	+	+	+	-	-	-	+	+	-	-	+	-	-	+	+	-	A/AIK	-	+	-	-	-	*Beijerinkia* species

\+ = Positive ; Alk = Alkaline
\- = Negative; AG = Acid and Gas

The ability for this hydrocarbon utilizers to use hydrocarbon as a carbon source in Fig. 1 agrees with the work of Sen and Chatterjee (1983), who isolated *Arthrobacter globiformis* from Burdwan (Indian) soil that was able to utilize hydrocarbon (gas oil) as carbon source. This finding is in support with the work of Wantanabe et al. (1975) reported utilization of n-paraffins containing carbon preferably 13-18 atom or kerosene by Genus *Pseudomonas* and *Achromobacter* as a source of carbon. Yamada, et al. (1963), also observed different strains of hydrocarbon utilizing organisms using hydrocarbon such as kerosene, ligroin and liquid paraffin was the carbon source in a culture medium. This report agrees with the works of (Sen and Chatterjee, 1983; Sen, 1985; Watanabe, et al., 1975; Yamada, et al., 1963; Ezemba, 2008; Roseberg, 2014; Ezemba et al. 2014), they reported utilization of hydrocarbon as a source of carbon by hydrocarbon utilizers.

Statistical analysis of the result shows that there is significant difference between various hydrocarbon and soil samples.

The strains were tested for purity, one hundred and four isolates were found to utilize hydrocarbon as a source of carbon on solid agar medium, and they were among 9 genera (Table 2). Hydrocarbon utilizers recovered were both gram positive and gram negative bacteria.

Based on the characteristic features and with reference to Buchanan and Gibbon (1974), Bergeys's manual of determinative bacteriology, 9 different genera were chosen as representative organisms from the 140 isolated (Table 2). The physiological, morphological and biochemical properties of the isolates are presented in Table 1. Based on the characteristics features observed the organism identified include; *Pseudomonas, Microbacterium, Proteus, Alcaligena, Beijerinkia, Bacillus, Micrococcus, Flavobacterium* and *Corynebacterium.*

The ability of these bacteria to be predominantly Gram-negative is contrary to Austin et al. (1997) who isolated predominantly gram-negative hydrocarbon utilizing bacteria from soil and aquatic environment. The isolation of *Bacillus* species is in agreement with the work of Okpokwasili and Okorie (1988), who found that *Bacillus* species could also be one of the predominant Gram +ve organism found in oil polluted areas. However, Dixon, (1994) also reported that species of *Micrococcus*, *Pseudomonas* and other bacteria helped to clean up water where repeated spillages of both light and medium crude oil occurred. The ability of these bacteria to utilize the hydrocarbon suggests that it has a role to play in bioremediation.

Conclusion

The methodology was able to isolate hydrocarbon utilizing bacteria from oil contaminated soil, normal soil and forest using enrichment basal medium. This result shows that most bacterial species are capable of utilizing hydrocarbon, and also as a carbon source. Also interest in the microbial biodegradation of pollutant has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments (Diaz, 2008; Kooukkou, 2011). we have tried to establish that the main degraders of petroleum hydrocarbons are bacteria, can be obtain from oil-contaminated and uncontaminated sites and it is very likely that by improving the strains of these bacterial species, higher yields will be obtained and when these microorganisms are produced in large scale, they would be used for environmental remediation and biodegradation, hence restoring our land and water for safe use.

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