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Ex-Situ Assessment of Degradative Potentials of *Bacillus* Species Isolated from Diesel-Polluted Site Using Spectrophotometric Method

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ABSTRACT

Bioremediation is one of the current approaches in environmental microbiology or environmental biotechnology that has been exercised for the reduction removal of hydrocarbon pollutants. Microorganisms, typically bacteria that have particular metabolic capacities, are essential for the biodegradation of hydrocarbon pollutants. This study was undertaken to assess *ex situ* degradative potentials of *Bacillus* species isolated from diesel-polluted site using spectrophotometric method. Soil sediments were collected from different points at diesel contaminated site located at Chukwuemeka Odumegwu Ojukwu University power zone. The samples were analyzed for the presence of hydrocarbon degrading bacteria using a modified mineral basal medium. The bacterial isolates were characterized based on their cultural characteristics, microscopy, and biochemical characteristics. The hydrocarbon adaptation utilization potentials of the bacterial isolates were evaluated using spectrophotometric method. The biodegradative potentials of the bacterial isolates were evaluated using hydrocarbon supplemented modified mineral basal medium and spectrophotometer. The Gram positive bacteria isolated were *Bacillus* species. The optical diameter of the adapted bacterial isolates showed that the isolates adapted to the hydrocarbon medium while the biodegradative potentials of the isolates showed that the hydrocarbon was biodegraded as revealed in the weight loss, which increased with time. The study has shown that *Bacillus* species are good hydrocarbon utilizing bacteria, which can be optimized in bioremediation of hydrocarbon-polluted site.

Keywords: Bacteria, Biodegradation, Pollutants, Engines

INTRODUCTION

Microorganisms play a vital role in the biodegradation (bioremediation) of hydrocarbon pollutants in a polluted milieu. Hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), benzene, kerosene, and diesel are important organic pollutants and inputs for different industries, vehicles, and household activities as a source of energy. Among these, diesel is massively used for engine, fuel, and industrial applications. It is one of the products of petroleum compounds formed during fractional distillation of crude oil and is composed of a mixture of carbon chains between 9 and 25 carbon atoms that may include both aromatic and aliphatic hydrocarbon component (Jayanthi, and Hemashenpagam, 2015; Vinothini *et al.,* 2016).

These hydrocarbon components can be discharged into the environment (groundwater, soil, and air) from different sources (point and nonpoint), such as garages, gas station services, chemical and petrochemical industries, agricultural waste, automobile exhaust spillage of petroleum, run-off asphalt pavements, vehicular emission, and combustion of fossil fuel. This phenomenon may happen intentionally or accidentally mainly from anthropogenic activities because of urbanization, industrialization, and civilization and, to some extent, by natural disasters. (Sihag *et al*., 2014; Prathyusha *et al*., 2016).

As a result, hydrocarbon-derived pollutants are immuno-toxicant, mutagenic, and carcinogenic to humans and animals and affect natural ecosystem functioning in many ways there are different methods of mitigating hydrocarbon pollutions. These include mechanical, chemical, and biological approaches. The first two aforementioned means of mitigation of pollutants need high operational costs and are prone to secondary pollution that necessitates integrated pollution management to reduce and/or remove the toxic pollutants from the environment (Prathyusha *et al*., 2016).

On the other hand, the biological method (bioremediation) is a promising technology that is prominent, eco-friendly, cost-effective, efficient, and easily applicable for the treatment of hydrocarbon-contaminated environments, but it necessitates a long time for complete degradation of pollutants. This approach mainly relies on two main techniques, namely, bio-augmentation and bio-stimulation (Ashikodi and Abu, 2019). The aim of this study is to assess an *ex situ* degradative potentials of *Bacillus* species isolated from diesel-polluted site using spectrophotometric method.

MATERIALS AND METHODS

Study Area

The study was conducted at Umuoma Uli, Ihiala Local Government Area, Anambra State. Uli is a village located between latitudes 5.47°N and 5.783°N and longitude 6.52°E and 6.87°E on the South eastern part of Nigeria. Uli extends westward to the confluence of the rivers of Atammiri and Eyinja, and across Usham lake down to the lower Niger region. Uli has rainforest vegetation with two seasonal climatic conditions: rainy season and dry season, which is characterized by the harmattan between December and February. Uli is characterized by double maxima of rainfall with a light drop in either July or August known as dry spell or August break. The annual total rainfall is about 1,600 mm with a relative humidity of 80 % at dawn.

Sample Collection

The soil surface was carefully scrapped out using sterile spoon. Soil auger was used to a plough depth of 15 cm in the sampling sites (diesel-contaminated sites at COOU), and soil sample was drawn (up to 10 samples) from each sampling unit into a sterile tray. The samples were thoroughly mixed and foreign materials such as roots, stones, pebbles and gravels were carefully removed. The soil sample was then reduced to half by quartering the sample. Quartering was carried out by dividing the soil sample into four equal parts and the two opposite quarters were discarded and the remaining two quarters were mixed. The process was repeated for the rest of soil samples used for this study. The samples were carefully labeled and then kept in a disinfected cooler, to maintain its temperature and stability of the number of the isolates. The samples were transported to the laboratory for analysis within 2 h.

Sterilization of materials

As stated in prescott *et al.* (2005), conical flasks (pyrex), prepared media and other plastic materials were sterilized by autoclaving at 121℃ for 15 minutes at a pressure of 15 psi. Glass wares such as pipettes, glass spreader, petri dishes, measuring cylinder, and other glass materials were sterilized in the laboratory hot air oven at a temperature of 160 ℃ for 1hr before use.

Isolation of Palm Oil Degrading Bacteria from the Samples

Serial dilution

A ten-fold serial dilution of the samples was carried out by adding 1g or 1ml respectively of sediment and

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water samples aseptically in test tubes containing 9ml of 0.85% of physiological saline solution labeled 10^{-1} to 10^{-10} dilution factors with the aid of a sterile pipette in a repeated manner. With another sterile pipette, 0.1 aliquots of the appropriate dilutions (dilutions that produce colony counts between 30 – 300 colonies) were spread plated on the surfaces of the solidified media in triplicates with the aid of a glass spreader. Precisely, 10^{-3} dilutions were spread plated. The spreader was sterilized after each successive spreading by dipping it in 70% ethanol and then passing it through flame of a Bunsen burner (Kafilzadeh *et al*., 2012).

Enrichment, culturing and isolation of hydrocarbon degraders

The palm oil degraders were isolated from sediments of the three sampling sites using modified mineral basal agar (4g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.1g $MgSO_4$, 1.8g K_2HPO_4, 0.1g $FeSO_4$, 0.1g NaCl, 0.2ml $CaCl_2$, 15g Agar agar and distilled water 1000ml at pH 7±0.2) enriched with xylene, anthracene and pyrene as hole carbon and energy source. The medium was sterilized by autoclaving at 121ºC at a pressure of 15psi for 15 minutes. Thereafter, 0.2 ml acetone solution containing 0.1% w/v of the selected hydrocarbons (xylene, anthracene and pyrene) were aseptically pipetted and uniformly spread on the agar surface of the pre-dried petri dish plates. The acetone was allowed to evaporate under sterile condition before inoculating with 0.1ml of diluted sediment and water samples. The inoculated plates were sealed using adhesive tape and foil to prevent contamination and photolysis and later placed in black polythene bags, and then incubated in the dark at 28 ± 0.2 ℃ for 24 – 48h (John and Okpokwasili, 2012).

Purification and maintenance of cultures

Colonies that developed on hydrocarbon-coated plates were replicated onto fresh hydrocarbon-coated agar plates and incubated for 14 days. Isolates that grew on these plates were selected as xylene, anthracene and pyrene degraders and sub-cultured on Bjou bottles where they are preserved at 4℃ in refrigerator (John *et al*., 2012).

Cultural and Morphological Characteristics

After sub-culturing and incubation, culturing morphological properties such as shape, elevation, margin, optic, texture, colour, size and surface characteristics of the selected bacterial strains were observed and noted (Prescott *et al*., 2005).

Gram staining

This technique divides bacteria into Gram positive and Gram negative groups. A smear of the isolates was made on a clean dry grease free slide, using a sterile wire loop. The smear was air dried and heat fixed by passing over flame quickly three times. It was then covered with 0.5% crystal violet solution for 1minute and rinsed with distilled water. The slide was flooded with 1% Gram's iodine (which served as a mordant that fixes the dye inside the cell). The iodine was washed off after one minute and 95% ethanol was used to decolorize the smear for 15 seconds. The smear was counter stained with 0.1% safranin dye solution for one minute. It was then washed off and the slide air-dried, and observed under the microscope using oil immersion objective lens after placing a drop of oil immersion. Gram positive and negative reactions were indicated by purple and red colors respectively (Cheesbrough, 2010).

Spore staining

According to the method of HPA (2007), smears of the isolates were prepared and fixed on a slide. The underside was vapor heated and flooded with 5% malachite green solution. Heating would continue until visible water condensate forms under the slide with evaporation at the top. It was washed using distilled water. Smears were counter stained with 0.5% safranin solution for 10 seconds. Slides were washed, dried and observed under oil immersion objective lens after pacing a drop of immersion oil. A green space within the cells would indicate the presence of spores.

Biochemical characteristics

Catalase test

As stated in cheesbrough (2010), the test identifies organisms that produce the enzyme catalase. A drop of 30% freshly prepared hydrogen peroxide (3ml H_2O_2 in 7ml H_20) was placed on a clean slide and loopful of isolate was transferred into it and emulsified. The appearance of gas bubbles indicates positive reaction. The reagent was shaken before the test to expel any dissolved oxygen and avoid false positive result.

Indole test

As stated in cheesbrough (2010), the tryptone-broth was prepared and 5ml was dispensed into each test tube and sterilized. The isolates were inoculated into the test tube and incubated at 28℃ for 48h. After incubation, 5 drops of Kovac's reagent (4 – p – dimethyl – aninobenzaldehyde) were added to the tubes, shaken gently and allowed to settle. A red coloration in alcohol dye indicates a positive result for the reaction.

Motility test

A directional and purposefully movement of the organisms demonstrate motility. Nutrient broth was supplemented with 0.2% agar, dispensed into test tubes and sterilized by autoclaving at 121℃ and 15 psi for 15minutes. The inoculated test tubes were incubated for 24h. Diffused growth, which spreads throughout the medium, indicates motility. Non-motile organisms grew along the line of inoculation (Cheesbrough, 2010).

Citrate test

As stated in Prescott *et al.* (2005), the test was used to determine organisms that could utilize citrate as a sole-carbon source for metabolism. Slant of Simmon's citrate agar were prepared according to manufacturer's instructions. The slants were inoculated by streaking over the surface with a loopful of an 18h old culture and incubated at 37℃ for 48h. Positive results were indicated by the growth on agar and a change in color from green to blue and absence of color change indicates negative results.

Starch hydrolysis test

To determine the abilities of the isolates to hydrolyze starch, 50 µl of liquid cultures of each isolates were dropped on starch – based solid medium containing per litre, 3g meat extract, 10g starch and 15g agar (Cheesebrough, 2010).

Gelatin test

As stated by Prescott *et al.*(2005), gelatin agar medium was composed of 40g/l of gelatin, 30g/l of tryptic soy broth and 100ml of distilled water. A small inocula of the isolates was stabbed to about three-quarter of the way to the bottom of a tube of deep agar with the inoculating needle. The separate stab tubes for each of isolates were incubated at 37℃ for 24 – 48h. The incubated stab and the un-inoculated control tubes were placed into the refrigerator for approximately 30 minutes. The inoculated stab tubes were compared with the control by tapping the tubes gently.

Hydrogen sulfide production test

As stated in Prescott *et al.* (2005), The test determines that ability of organisms to reduce sulfur compounds. Triple sugar ion agar slants were prepared and each isolates were inoculated into test tubes by streaking the inocula across the top of the slants and stabbing the slant tubes to the bottom. Tubes were incubated at 28℃ for 24h. Positive result is indicated by the formation of black color coupled with displacement of the agar slant and red to yellow color observation.

Sugar fermentation

As stated in Prescott *et al*. (2005), the test determines the ability of the isolates to ferment glucose, sucrose, lactose, mannitol, maltose, xylose, arabinose and saccharose and also ability to produce gas. The fermentation medium contained 1% peptone water and 5 drops of 0.2% bromothymol blue indicator solution. Then 9ml of the medium was dispensed into clean dry test tubes in which Durham tubes been dropped (inverted and without air space) and sterilized by autoclaving at 121℃ and 15psi for 15 minutes. 1ml of the sterile 5% test sugar solution was added to medium and inoculated with a loopful of the test organisms and incubated at 30℃ for 24h. A change in color of the medium (from blue to yellow) was recorded as positive reaction, while presence of gas in Durham tubes indicates gas production.

Oxidase test

As stated in Prescott *et al*.(2005), the test identifies any organism that produces the enzyme oxidase. A loopful of isolates was transferred into pieces of Whatman No.1 filter paper, impregnated with a solution of freshly prepared oxidase test reagent (N,N,N',N' tetra-methyl-phenylene diamine) and smeared. Oxidation of the phenylene diamine in the reagent to dark purple or blue color within 10 seconds indicates a positive result.

Casein hydrolysis test

The casein hydrolysis was observed by zones of clearing after 24h of incubation. For this purpose, 50 µl liquid cultures of each isolates were dropped on casein-based solid medium containing (per litre) 10 g casein and 15g agar. After 24h of incubation, the inhibition zones were determined (Cheesbrough, 2010).

Hydrocarbon Adaptation Utilization Test

In order to screen and select the best and strongest degrading bacterial isolate, different organisms were tested by growing 5ml of each desired isolates in large test tubes containing 25 ml of the modified mineral basal medium with 1ml of xylene, anthracene and pyrene hydrocarbons which were dissolved in acetone and added to each tube autoclaving. Thereafter, the test tubes were incubated at room temperature (28±2°C) for five days. Bacteria that started growing fast with high turbidity in the vicinity of the medium containing aromatic compounds measured at 600nm using a UV – VIS spectrophotometer (Astell, UV – Vis Grating, 752W) were selected as the candidate of xylene, anthracene and pyrene degrading bacteria. Cultures without increase in turbidity over initial optical density (OD) and uninoculated control were scored as no growth (-) while cultures with increased turbidity significantly greater than the control were scored as growth (+) (John *et al*., 2012).

Degradation Assay

By adopting the methods of Bennet *et al*. (2012) and John and Okpokwasili (2012), as modified in this study, the degradation rates of bacterial isolates were determined using hydrocarbon supplemented modified mineral basal medium (4g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.1g $MgSO_4$, 1.8g K_2HPO_4, 0.1g $FeSO_4$, 0.1g NaCl, 0.2ml $CaCl_2$, 15g Agar agar and distilled water 1000ml at pH 7±0.2). Precisely, 1ml of 48 h old cultures of each organisms were introduced into 28 sterile 200ml capacity conical flasks (7 sets of 4 flasks) containing 100ml of sterile modified mineral basal medium supplemented with 1 ml of xylene or 1ml of anthracene or 1g of pyrene, respectively as source of carbon at 24℃ for 24 days. During incubation, representative samples from the three days sets of flasks were withdrawn at intervals of 0, 4, 8, 12, 16, 20 and 24 days and the residual hydrocarbons were determined spectrophotometrically using ethyl acetate as the extraction solvent. For each sample, 5 ml ethyl acetate was added and vigorously shaken manually. The organic and aqueous layers from media were separated by centrifugation at 5000rpm for 20 minutes. The aqueous layers were discarded while the organic layers were analyzed with UV – VIS spectrophotometer at 240 nm wavelength (Astell UV – Vis Grating, 752 W). The percentages of biodegradation of the hydrocarbons were determined as follows:

% degradation = $\frac{a-b}{a} \times \frac{100}{1}$

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Where a = the absorbance of the medium before incubation; b is the maximum absorbance of the medium after each 4^{th} day of the incubation period.

Statistical Analysis

The difference in the absorbance of the bacterial isolates was determined using students'‘t’ test and values of P that exceeded 0.05 ($P > 0.05$) were considered not significant**.**

RESULTS

Characterization of Bacterial Isolates in Impacted Soil

The result of characterization of the bacterial isolates in the impacted soil is presented in Table 1. The result revealed that the bacterial isolate Y appeared cream white on Nutrient agar while isolate Y appeared fussy white on the same agar. The edge, elevation, surface, optical nature, and size of the isolates appeared irregular, flat, rough, opaque, and large, respectively. Both isolates were Gram positive, motile, rods, and spore formers. The biochemical characteristics of the isolates revealed that the isolate X was positive to catalase, citrate, oxidase, gelatin, casein, starch, glucose, maltose, and xylitol while isolate Y isolate was positive to catalase, citrate, gelatin, casein, starch, glucose, maltose, maltose, and dulcitol.

Table 1: Cultural and morphological characteristics of the bacterial isolates

Parameter	Isolate X	Isolate Y
Appearance on Nutrient Agar	Cream white	Fussy white
Edge	Irregular	Irregular
Elevation	Flat	Flat
Surface	Rough	Rough
Optical Nature	Opaque	Opaque
Size	Large	Large
Gram Reaction	Positive	Positive
Cell Morphology	Rods	Rods
Motility	Positive	Positive
Endospore	Present	Present
Bacterium	*Bacillus* species	*Bacillus* species

Table 2: Biochemical characterization of the bacterial isolates

Parameter	Isolate X	Isolate Y
Catalase	+	+
Citrate	+	+
Indole	–	–

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Oxidase	+	_
H_2S	_	_
Gelatin	+	+
Casein	+	+
Starch	+	+
Glucose	+	+
Maltose	+	+
Dulcitol	+/_	+
Inositol	+/_	+/_
Xylitol	+	+/_
Bacterium	*Bacillus* sp.	*Bacillus* sp.

Optical Diameter of the Adapted Isolates and Weight Loss of hydrocarbon During Degradation

The result of optical diameter of the adapted isolates is presented in Table 3. The result revealed that at day 0, the bacterial isolates recorded the least optical diameter of 0.0012 and 0.0037, respectively. Meanwhile, as the day progresses, the optical diameter increased but the increment was not significant. Meanwhile, isolate Y showed higher optical diameter as the day increased. Similarly, the weight loss of hydrocarbon during degradation is presented in Table 4. The result revealed that at day 0, there was zero weight loss. Also, the weight loss during degradation increases as the day increases with the highest weight loss recorded at day 4 of degradation.

Table 3: Optical diameter of the adapted isolates

Day	*Bacillus* X	*Bacillus* Y
O	0.0012	0.0037
1	0.0182	0.0317
2	0.0492	0.0844
3	0.1020	0.1860
4	0.2030	0.3100

Table 4: Weight loss of the hydrocarbon during degradation

Day	Weight (%) *Bacillus* X	*Bacillus* Y
0	0.00	0.00
1	0.58	0.71
2	0.97	1.81

3	2.33	2.88
4	3.87	4.55

DISCUSSION

Environmental contamination has threatened the ecosystem in all ramifications. Various contaminants basically hydrocarbons alter the normal structure of soil and vital soil microorganisms that aid in nutrient recycling and soil fertility. The characteristic features of the bacteria isolated from the diesel impacted soil sediment in this study corroborate with the report of several researchers (Macaulay, 015; Zafra *et al.,* 2016; Vinothini *et al.,* 2016) who evaluated potentials of certain bacterial species in degrading hydrocarbons. In this study, *Bacillus* species was isolated while Kawo and Bacha (2016) isolated *Bacillus* species and *Micrococcus* species as crude oil degraders. The ability of the bacterial isolates to utilize various sugars and sugar alcohol such as starch, glucose, maltose, dulcitol, inositol, and xylitol could be attributed to their high degradative potentials. This observation agrees with the findings of several researchers (Sihag *et al*., 2014; Prathyusha *et al*., 2016; Ashikodi and Abu, 2019) who documented that *Bacillus* species utilize breakdown carbohydrate as source of carbon and energy for metabolism. The increase in the optical diameter of the bacterial isolates as the degradation day increases could be attributed to their ability to utilize the products of degradation as source of energy and carbon for optimum proliferation. This observation corresponds to the report of Kawo and Bacha (2016) who investigated degradative potentials of *Bacillus* species and *Micrococcus* species and recorded a high number of the isolates at the highest day of degradation. The loss in weight of the hydrocarbon as the degradation day increases could be attributed to biodegradation of the bacterial isolates where the major components are utilized as source of energy and carbon. This observation is in line with the reports of several researchers (Jayanthi, and Hemashenpagam, 2015; Prathyusha *et al*., 2016; Vinothini *et al.*, 2016) who evaluated biodegradative potentials of certain bacterial species.

CONCLUSION

This study has investigated the ex-situ assessment of biodegradtive potential of bacteria isolated from diesel contaminated sites and revealed that *Bacillus* species had ability to degrade hydrocarbon as energy and carbon sources. The study also showed that biodegradation increases with time, and there is always reduction in the weight of hydrocarbon as biodegradation increases. Therefore, *Bacillus* species can be optimized for environmental sanitation of hydrocarbon-polluted soil for optimum productivity.

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