Microbial Populations of an Agricultural Soil Polluted With Crude Oil

ABSTRACT:

Hydrocarbon pollution of the soil has become a public health and environmental concern. It impacts on soil microbial diversity and population. In this study, the microbial population of crude oil polluted soil and unpolluted were determined. The potential for the indigenous organisms to utilize hydrocarbon as source of carbon was also determined. Soil sample was obtained from an agricultural field and polluted with defined amount of crude oil for 12 weeks. The microbial population in the polluted soil and unpolluted soil were estimated and characterized following standard microbiological methods. The heterotrophic bacterial and fungal counts in the unpolluted and polluted soil were 6.5 x 10^4CFU/g and 4.7 x 10^4 CFU/g, and 6.3 x10^4 CFU/g and 3.5 x10^4 CFU/grespectively. Total hydrocarbon utilizing bacterial and fungal counts in the polluted soil were 4.6 x 10^4CFU/g and 2.8 x 10^4 CFU/g. The genera of bacteria and fungi identified in the soil samples were *Bacillus*, *Enterococcus*, *Micrococcus*, *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Serratia*, *Proteus*, *Klebsiella*, *Arthrobacter*, *Aspergillus*, *Candida*, *Penicillium*, *Fusarium*, *Trichoderma*, *Cladosporium*and*Hyphopichia. IThe preliminary screening for hydrocarbon utilization shows that Bacillus cereus, Bacillus subtilis, Aspergillus niger* and *Hypopichia burtonii* were excellent hydrocarbon utilizers.

Keyword: Hydrocarbon, degradation, crude oil, bacterial count, Fungi count

INTRODUCTION:

Soils are complex mixtures of minerals, water, air, organic matter and countless organisms that are the decaying remains of once-living things (Agarry *et al*., 2013). The Natural Resource Conservation Service (NRCS) defines soil as a natural body comprised of solids (minerals and organic matter), liquid, and gases that occurs on the land surface, occupies space, and is characterized by one or both of the following: horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or the ability to support rooted plants in a natural environment(Schoonover *et al.*, 2015; Enerijiofi and Ekhaise, 2022).Soil plays a vital role in sustaining life on the planet and alsoa

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habitat for many microorganisms. Nearly all of the food that humans consume, except for what is harvested from marine environments, is grown in the Earth's soils (Agarry and Oghenejoboh, 2015). Soil is responsible for nutrient cycling, plant growth, gas exchange, carbon storage, and waste disposal (Kalev et al., 2018; Enerijiofi and Ekhaise, 2019).

The contamination of the soil by frequent crude oil spillages renders the soil toxic and agriculturally unproductive. The oil reduces the soil's fertility to the extent that most of the essential nutrients are no longer available to plants and for crop utilization (Akarator and Molindo, 2016). Crude oil as a pollutant, decreases the nitrogen and phosphorus contents of the soil and promotes excessive hydrocarbon which affects soil enzymatic activities, due to the inability of soil microbes to degrade the excess hydrocarbons(Bento et al., 2018). The contamination of soil by crude oil and petroleum products has become a serious problem that represents a global concern for its potential consequences on ecosystem and human health (Agarry *et al*., 2019; Chemlal *et al*., 2013; Enerijiofi *et al*., 2020). Crude oil contaminations on land affect the physicochemical properties of the soil which causes deleterious effects on plant germination and growth. Soil properties such as; soil texture, moisture content, pH and bulk density are degraded. This leads to poor root and leaf development, which consequently affects plant growth and yield (Ikhajiabge *et al*., 2021).Contaminated land can present a risk to human health, the immediate ecosystem, and the environment. Oil spills cause soil pollution and eventual environmental degradation (Agarry, 2018; Chikere and Ekwuabu, 2014). Leakages and accidental spill commonly occur during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Chemlal *et al*., 2013).

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However, crude oil-polluted soil can remediated by the actions of indigenous microorganisms with inherent ability to utilize hydrocarbon as carbon source. Microorganisms are capable of degrading a wide range of hydrocarbon molecules in the environment, especially the lithosphere, where they attack and digest petroleum hydrocarbons; this thus forms the emergence of the field of bioremediation (Ajuzie *et al*., 2015; Onuorah *et al*., 2018). Indigenous microorganisms in hydrocarbon polluted sites have been reported to be excellent hydrocarbon utilizers and can be employed for the reclamation of polluted environment. The present study aimed at identifying the microbial populations in a crude oil-polluted and unpolluted soil, and to determine their potential for hydrocarbon utilization.

II Materials and Methods

A:Collection of samples

Agricultural soil sample (unpolluted soil) wasobtained from an agricultural field in Nnamdi Azikiwe University, Awka, Nigeria in a sterile sample bag using a sterile hand trowel and diggingat 15cm depth inthe soil. The sampleconveyed to the laboratory for analysis.The crude oil used in the experiment was obtained from a Petrochemical Company situated at Eleme Oil field, Eleme Local Government Area in Port Harcourt, Rivers state of Nigeria.

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B: Sample preparation

One hundred gram of the soil sample was polluted with 100 ml of crude oil and evenly mixed to obtain a homogenous mixture. The mixture was allowed to stand under room temperature for 12 weeks, during whichthere was periodic turningof the soil for aeration.

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C. Isolation of microorganisms from the soil samples

The method described by Onuorah et al. (2018) was used for the isolation of bacterial and fungal organisms from the polluted and unpolluted soil samples. Tenfoldserial dilutionswere carried out using 1g each of the samples in 10 ml of sterile distilled water. 1ml of the solvent was introduced into test tubes containing 9ml of sterile water and serially diluted from 10^{-1} to 10^{-10}.

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Enumeration of Total Heterotrophic Bacteria

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From the serially diluted samples, one milliliter of 10^{-4} was inoculated onto sterile nutrient agar plate by pour plating technique in triplicate.The plates were incubated at 25°C and colonies that developed after 24 hours were counted in the triplicate plates, and mean values recorded.and used to calculate the colony forming unit per gram (CFU/g).Distinct colonies were subcultured and stored at 4^{0}C for further identification.

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Enumeration of Total Fungi

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From theserially diluted samples, one milliliter of 10^{-4} was inoculated onto sterile sabouraud dextrose agar platefortified with chloramphenicol to suppress bacterial growth by pour plating

Comment [K17]: Wrong way to begin a sentence. Please recast technique in triplicate.The plates were incubated at 25°C and colonies that developed after 72 hours were counted in the triplicate plates, and mean values recorded. and used to calculate the colony forming unit per gram (CFU/g).The colonies that developed were thereafter sub-cultured to obtain pure cultures. The pure cultures obtained were transferred into agar slants for identification.

D. Isolation of Hydrocarbon-utilizing Bacteria and Fungi from the Soil

The method described by Olukunle (2013) was used. The crude oil-polluted soil sample was diluted tenfold at a series of 10^{-1} to 10^{-10}. An aliquot of 0.5ml from 10^{-4} was inoculated using pour plate method into a mineral salt agar for bacteriaand sabouruad dextrose agar fortified with chloramphenicol for fungi. A whatman filter paper impregnated with crude oil was placed on the inside cover of the petri dish, covered and incubated for 24h - 48h at 25°C in an inverted position for bacterial isolates and 72h at 25°C for fungi isolates. The crude oil-impregnated filter paper supplied hydrocarbons by vapour phase transfer to the inoculums.The test was done in triplicates. Developed colonies on the culture plates were counted and mean values were used to calculate the colony forming unit per gram (cfu/g).

E) Identification of bacterial and fungal Isolates

The identification of bacteria was done based on morphological characteristics and biochemical tests.Morphological characteristics observed for each bacteria colony after 24-48 hours of growth include colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation. Biochemical tests carried out for the identification of bacterial isolates were Gram stain test, Mortility test, Catalase test, Coagulase test, Citrate test, Indole test, Oxidase test, Methyl red test, Spore stain test, Voges proskauer test, Glucose fermentation, Sucrose fermentation, Lactose fermentation, Mannitol fermentation and the result compared with bergey's Manual of determinative bacteriology (Frank *et al.*, 2012).

The fungal isolates were identified based on morphological and cultural characteristics, lactophenol cotton blue stain and slide culture test.

Lactophenol Cotton blue staining technique: A drop of Lactophenol cotton blue solution was placed on a clean grease free slide. A sterilized straight wire was used to transfer, tease and

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Slide Culture technique: A sterile Sabouraud dextrose agar medium was prepared and inoculated with a mold isolate. A cover slip was inserted beside the mold and incubated in an inverted position for 4-7 days. The cover slip was placed on a slide containing a drop of lactophenol blue and was examined with x10 and x40 microscope objectives for the presence of fungal features. Fungal isolates were identified following the Scheme of Oyeleke and Manga, (2008).

F) Preliminary Screening for Hydrocarbon degradation by the Microbial Isolates from the soil samples

The method described by Ramaraj (1995) was used to screen the bacterial and fungal isolates for hydrocarbon degradation. The microorganisms isolated from the crude oil-polluted soil sample were used for the test.Mineral salt medium (MSM) supplemented with 1% crude oil was prepared and sterilized in autoclave at 121˚C for 15 minutes. A loopful of 24hr old culture of the isolate was inoculated into 50 ml MSM in 100 ml Erlenmeyer flask. The flask was incubated in a rotary shaker for 14 days. Triplicate flasks were used andan uninoculated flask served as control. Bacteria and fungi growth in the medium were indicated byturbidity and clarity respectively.

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III RESULTS:

The heterotrophic bacterial and fungal counts of the crude oil-polluted and unpolluted soil samples are shown in Table 1. The total heterotrophic bacteria and fungiare 6.5 x 10^4CFU/g and 4.7 x 10^4CFU/g respectively in the unpolluted soil and 6.3 x10^4 CFU/gand 3.5 x10^4 CFU/gin the polluted soil.

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Table 1: Heterotrophic bacterial and fungal counts in the soil samples

Sample	Count (CFU/g)

Unpolluted soil	
Total heterotrophic bacteria	6.5 x10^4
Total heterotrophic Fungi	4.7 x10^4
Crude oil-polluted soil	
Total heterotrophic bacteria	6.3 x10^4
Total heterotrophic Fungi	3.5 x10^4

The petroleum hydrocarbon utilizing bacterial and fungal counts were estimated in the polluted soil using the vapour phase transfer method (Thijsse and VanderLinder, 2017). The bacterial colonies were identified to their species level using conventional microbiological biochemical tests as described by (Cheesebrough, 2006, Taiwo and Oso 2004) using the Bergey's Manual of Determinative Bacteriology.The total hydrocarbon utilizing bacteria and fungi are 4.6 x 10^4CFU/gand 2.8 x 10^4CFU/g respectively (Table 2).

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Table 2: Petroleum Hydrocarbon Utilizing Bacterial and Fungal Count

	Count (CFU/g)
Petroleum hydrocarbon utilization bacteria	4.6 x10^4
Petroleum hydrocarbon utilization fungi	2.8 x10^4

Table 3 shows the morphological and biochemical characteristics of the heterotrophic bacteria isolated from the unpolluted soil,and the organisms isolated are*Bacillus cereus*, *Enterococcus feacalis*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marcescens*and*Bacillus subtilis.* Table 4 shows the morphological and biochemical characteristics of hydrocarbon utilizing bacteria isolated from the crude oil polluted soil. The organism isolated are *Bacillus cereus*, *Pseudomonas aeruginosa*, *Serratia mascescens*, *Bacillus subtilis*, *Micrococcus luteus*, *Proteus vulgaris*, *Klebsiella aerogenes* and *Arthrobacter oxydans.*

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Table 3: Morphological and Biochemical characteristics of the heterotrophic bacteria isolated from the unpolluted soil

Comment [K40]: combine tables 3 and 4 to show the effect of hydrocarbon pollution on microbial counts

Isolates	Gram stain	Form	Spore stain	Motility	Catalase	Oxidase	Citrate	Indole	Methyl red	Voges Proskauer	Coagulase	Glucose	Sucrose	Lactose	Mannitol	Identity
A	+	Coccus	-	-	-	-	+	-	+	+	-	+	+	+	+	*Enterococcus feacalis*
B	+	Coccus	-	-	+	+	-	-	-	-	-	-	-	-	-	*Micrococcus luteus*
C	+	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	*Escherichia coli*
D	+	Rod	+	+	+	-	+	-	-	+	-	+	-	-	-	*Bacillus cereus*
E	-	Rod	-	+	+	+	+	-	-	-	-	-	-	-	-	*Pseudomonas aeruginosa*
F	+	Coccus	-	-	+	-	-	-	+	+	+	+	+	+	+	*Staphylococcus aureus*
G	-	Rod	-	-	+	-	+	-	-	+	-	+	+	-	+	*Serratia marcescens*
H	+	Rod	+	-	+	-	+	-	-	+	-	-	-	-	+	*Bacillus subtilis*

KEY: + = Positive, - = Negative

Table 4: Morphological and Biochemical Characteristics of Hydrocarbon-Utilizing Bacteria Isolated from the Crude Oil-Polluted Soil.

Isolates	Gram stain	Form	Spore stain	Motility	Catalase	Oxidase	Citrate	Indole	Methyl red	Voges Proskauer	Coagulase	Glucose	Sucrose	Lactose	Mannitol	Identity
A	+	Rod	+	+	+	-	+	-	-	+	-	+	-	-	-	*Bacillus cereus*
B	-	Rod	-	+	+	+	+	-	-	-	-	-	-	-	-	*Pseudomonas aeruginosa*
C	-	Rod	-	-	+	-	+	-	-	+	-	+	+	-	+	*Serratia marcescens*
D	+	Rod	+	-	+	-	+	-	-	+	-	-	-	-	+	*Bacillus subtilis*
E	+	Coccus	-	-	+	+	-	-	-	-	-	-	-	-	-	*Micrococcus luteus*
F	-	Rod	-	+	+	-	-	+	+	-	-	+	+	-	-	*Proteus vulgaris*
G	-	Rod	-	-	+	-	-	+	+	+	-	+	+	+	+	*Klebsiella aerogenes*
H	-	Rod	-	-	+	-	+	+	+	-	-	-	-	-	-	*Arthrobacter oxydans*

KEY: + = Positive, - = Negative

The colonial and microscopic features of the heterotrophic fungi isolated from the unpolluted soil are shown in Table 5. The organisms isolated are*Aspergillus niger*, *Candida utilis*, *Penicillium expansum*, *Fusarium oxysporium*, *Trichoderma herbarum* and *Cladosporium resinae*.Table 6 shows the colonial and microscopic features of the hydrocarbon utilizing fungi isolated from crude oil polluted soil. The isolates are*Aspergillus niger*, *Penicillium expansium*, *Hyphopichia burtonii*, *Candida utilis*, *Fusarium oxysporium*,*Cladosporium resinae* and *Candida tropicalis.*

Table 5: Colonial and microscopic features of the heterotrophic fungi isolated from the unpolluted soil

Comment [K41]: combine tables 5 and 6 to show the effect of hydrocarbon pollution on microbial counts

Isolate	Colonial Characteristics	Microscopic Characteristics	Identity
1	Colonies were initially white but quickly formed black conidia. The reverse was yellow-grey.	Hyphae were septate.Conidiophores were long,smooth-walled,hyaline and contained globose vessicle each covered completely with biseriate phialides.Conidia were globose,dark and rough-walled.Conidia heads were large,globose,dark brownand biseriate.	*Aspergillus niger*
2	Colonies are cream coloured,oval,smooth,glaborous,dull,soft and yeast-like in appearance with a yeasty odour.	Sub-spherical budding yeast like cells called blastospores present, terminal chlamydoconidia are absent. Ferment glucose and maltose. Does not ferment lactose.	*Candida utilis*
3	Dull green mycelia with whitish lining, were seen.	Non-ceonocytic (septate)hyphae were seen.	*Penicillium expansium*
4	Whitish to pink cottony mycelium were seen. The reverse was dark purple.	Conidiophores were short with single lateral momophialides, Microconidia were in fusiforms and pointed at the tip.	*Fusarium oxysporium*
5	Colonies were slow growing, dark-green with black edges and powdery. The reverse was greenish brown	Conidiophores were erect, unbranched and dark, shield smooth and occured in long branching chains.	*Trichoderma herbarum*
6	Colonies were slow growing, dark-green with black edges and powdery. The reverse was greenish brown	Conidiophores were erect, unbranched and dark, shield smooth and occured in long branching chains.	*Cladosporium resinae*

Table 6: Colonial and microscopic features of the hydrocarbon utilizingfungi isolated from the crude oil-polluted soil.

Isolate	**Colonial Characteristics**	**Microscopic Characteristics**	**Identity**
1	Colonies were initially white but quickly formed black conidia. The reverse was yellow-grey.	Hyphae were septate.Conidiophores were long,smooth-walled,hyalineand contained globose vessicle each covered completely with biseriate phialides.Conidia were globose,dark and rough-walled.Conidia heads were large,globose,dark brownand biseriate.	*Aspergillus niger*
2	Dull green mycelia with whitish lining, were seen.	Non-ceonocytic (septate)hyphae were seen.	*Penicillium expansium*
3	Colonies are tannish white and butyrous to hyphal, ascopores formed are relesaed by ascus,cell are short,ellipsoidal or elongate and may form pseudohyphae and true hyphae.	Yeast like conidia borne laterally and terminally on short denticles along the parental, septate true hyphae.	*Hyphopichia burtonii*
4	Colonies are cream coloured,oval,smooth,glaborous,dull,soft and yeast-like in appearance with a yeasty odour.	Sub-spherical budding yeast like cells called blastospores present, terminal chlamydoconidia are absent. Ferment glucose and maltose.does not ferment lactose.	*Candida utilis*
5	Whitish to pink cottony mycelium were seen. The reverse was dark purple.	Conidiophores were short with single lateral monophialides, Microconidia were in fusiforms and pointed at the tip.	*Fusarium oxysporium*
6	Colonies were slow growing, dark-green with black edges and powdery. The reverse was greenish brown	Conidiophores were errect, unbranched and dark, shield smooth and occured in long branching chains.	*Cladosporium resinae*
7	Colonies are cream coloured,oval,smooth,glaborous,dull,soft and yeast-like in appearance with a yeasty odour.	Sub-spherical budding yeast like cells called blastospores present at both terminal chlamydoconidia are absent. Ferment glucose	*Candida tropicalis*

	and maltose, does not ferment sucrose.	

Table 7 shows the result of the screening test for hydrocarbon utilization by the microbial isolates from the crude oil polluted soil. *Bacillus cereus*,*Aspergillus niger*, *Hyphopichia burtoni*and*Bacillus subtilis*had heavy turbidity (+++), *Candida tropicalis*had moderate turbidity (++) while *Candida utilis*and *Penicillium expansium* had minimal turbidity (+).

Table 7: Screening characteristics for hydrocarbon utilization by the microbial isolates from the crude oil polluted soil.

Micro organism	Hydrocarbon utilization
Bacillus cereus	+++
Candida utilis	+
Hyphopichia burtonii	+++
Penicillium expansium	+
Aspergillus niger	+++
Bacillus subtilis	+++
Candida tropicalis	++

+ Minimal turbidity ++ Moderate turbidity +++ Heavy turbidity

DISCUSSION

This estimated and characterized themicrobial population in a crude oil polluted and unpolluted soil. The total viable count of bacteria fromcontaminated soil with used hydrocarbon was impressive showing the presence of some bacteria and fungi that utilizes hydrocarbon which makes bioremediation effective. Crude oil impaction caused decreased values of all the bacterial and fungal population as seen in Table 2. The total heterotrophic bacterial and fungal count in the unpolluted soil were 6.3 x 10^4CFU/g and 3.5 x 10^4CFU/g (Table 1), while that of hydrocarbon utilizing bacteria and fungi were 4.6 x 10^4CFU/g and 2.8 x 10^4CFU/g respectively.Morphological and biochemical characteristics of the heterotrophic bacteria isolated from the unpolluted soil are shown on Table 3, the organism isolated were *Bacillus cereus, Enterococcus feacalis. Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens and Bacillus subtilis* this agrees with the findings of Udebuani *et al*., (2012).

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Table 4 shows the morphological and biochemical characteristics of hydrocarbon utilizing bacteria isolated from the crude oil polluted soil. The organisms isolated were *Bacillus cereus*, *Bacillus subtilis, Proteus vulgaris, Klebsiella aerogens, Athrobacter oxydans, Psuedomonas aeruginosa, Serratia marcesens and Micrococcus luteus.* The hydrocarbon utilizing bacteria isolated conforms to the finding of Chikere *et al.*, (2011), in their assessment of bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria. This finding again revealed that there is a high population of active indigenous hydrocarbon utilizing bacteria which can be monitored and enhanced to bring about bioremediation. This agrees with work of Chikere and Ekwuabu (2014) in their study of the culture-dependent characterization of hydrocarbon utilizing bacteria in the selected crude oil-impacted sites in Bodo, Ogoniland, Nigeria. Again Omotayo *et al.*(2011), isolated similar organism in their work on the degradation of aviation fuel by microorganisms isolated from tropical polluted soils. Also Yakubu and Bello (2007) isolated similar organisms in their work on the biodegradation of Lagoma crude oil using pig dung.

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Table 5 shows the colonial and microscopic features of hydrocarbon-utilizing moulds isolated from the pristine soil. The isolates were *Aspergillus niger, Cladosporium resinea, Fusarium oxysporium and penicillium expansium.* Chikere and Azubike (2014) in their work of characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water, isolated similar organisms.

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Table 6 shows the colonial and microscopic features of the hydrocarbon utilizing fungi isolated from crude oil polluted soil. The isolates were *Aspergillus niger, Hyphopichia burtonii penicillium expansium, Cladosporium resinea, Fusarium oxysporium, Candida tropicalis and Candida utilis,*. Omotayo *et al.* (2011), in their work of the degradation of aviation fuel by microorganisms isolated from tropical polluted soils, isolated *Aspergillus, Penicillium* and *Candida*,while Stephen and Panneerselvam (2014) in their work on the study of the prevailing fungi on the hydrocarbon polluted soil, isolated similar organism, however, the fungal isolates in the crude oil-polluted soil differ with the result of this study. This could be as a result of environmental conditions like pH and percentage of mineral nutrients in the soil.

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Table 7 shows the result of the screening test for hydrocarbon utilization by the microbial isolates from the crude oil polluted soil before amendment with Livestock manure. *Bacillus cereus and Hypopichia burtonii* produced heavy turbidity (+++). *Aspergillus niger had* moderate turbidity (++) while *Penicillium expansium* and *Candida utilis* had minimal growth (+).This result corroborates with the result of Chikere and Ekwuabu (2014) in their study on molecular characterization of autochthonous hydrocarbon utilizing bacteria in oil-polluted sites at Bodo Community, Ogoni land, Niger Delta, Nigeria. The authors reported that *Aspergillus* and *penicillium sp* produced heavy turbidity. Also, Yakubu and Bello, 2007.On the biodegradation of Lagoma crude oil using pig dung, reported that *Bacillus* sp produced heavy growth, *Micrococcus* moderate growth while *Proteus* had minimal growth.

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CONCLUSION

The result obtained in this study has revealed that, petroleum hydrocarbon degrading bacteria and fungi isolated from the work supports other people's findings. The isolates such as *Bacillus cereus*, *Bacillus subtilis*,*Hypopichia butornii*, which are the excellent hydrocarbon utilizers, can be explored for use as hydrocarbon degraders in bioremediation.

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