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Research Article

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Molecular Identification of Hydrocarbon Degrading Bacteria Isolated from Contaminated Soil of Automobile Mechanic Workshop in Lapai, Niger State

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ABSTRACT

Hydrocarbon contamination is one of the central environmental problems today as a result of petrochemical industries' related activities. Petroleum hydrocarbons are the organic contaminants of key interest because of their complex structure, wide spread, toxicity and persistence. Accumulation of these pollutants in plants and animal tissues has been reported to cause death or mutation. Hence this study focused on molecular identification of hydrocarbon degrading bacteria isolated from hydrocarbon contaminated soil of automobile workshop in Lapai, Niger State, Nigeria. Isolation was carried out on Mineral Salt Media (MSM) containing 1% used engine oil as carbon source. The isolates hydrocarbon degradative activities were determined using turbidimetric analysis. The isolate with fastest growth was characterized based on morphology, biochemical test and subsequent molecular identification using 16S rRNA gene sequence analysis. Six bacteria strains; FS1, FS2, FS3, ES1, ES2 and ES3 that utilized 1% used engine oil as sole carbon and energy were isolated. Bacteria ES2 effectively utilized the used engine oil with the highest Optical density (OD) of 0.96 at 600 nm on the 10th day. Result from the biochemical test, blasting and phylogenetic analysis showed that bacteria ES2 belongs to Lysinibacillus specie with 89% maximum identity to this specie. These bacteria can be used to effectively bio remediate hydrocarbon polluted environment.

Keywords: *Hydrocarbon, Contaminated soil, Molecular Identification, Lysinibacillus.*

INTRODUCTION

Environmental contamination by hydrocarbon is becoming rampant possibly as a result of the substantial dependence on petroleum as the main source of energy across the globe, population expansion, rapid industrial development and total negligence for the environmental wellbeing (Abioye, 2011). Petroleum hydrocarbons are the organic contaminants of key interest because of their complex structure, widespread, toxicity and persistence.

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The most popular among the Petroleum hydrocarbons are aliphatic, cycloaliphatic and branched alkanes, and also polycyclic and monocyclic aromatic hydrocarbons (Chirwa & Bezza, 2015).

Illegal dumping, accidental spill as well as negligent managements of petroleum hydrocarbons at mechanic workshops have emerged as the major source of environmental contamination due to mostly amorphous practice of automobile repair services (Ebakota et al., 2017). Regardless of the point of contamination, petroleum products may get to lakes, groundwater and water passages. In the food chain, Petroleum hydrocarbon can bio accumulate and disrupt physiological or biochemical activities of various living things, resulting in carcinogenesis, mutagenesis and impairment of the genetic material, some organs and reproductive capacity respectively (Onwurah et al., 2007).

Although there are various conventional techniques in the remediation of petroleum waste, however, these techniques are expensive, ineffective, energy intensive and not environmental friendly. Bioremediation has been reported as the most efficient, cost-effective, a natural technique that completely mineralize the pollutant and does not damage the ecosystem (Perelo, 2010). It is accomplished with the aid of various set of microbes, predominantly the native bacteria in the soil. The fastest and full degradation of most organic pollutants is achieved under aerobic conditions. Fritsche and Hofrichter (Fritsche & Hofrichter, 2000) reported that aerobic degradation of hydrocarbon involve the following major principle: (1) the accessibility of the chemicals by the microorganisms. For instance, biosurfactants production is required for the degradation of hydrocarbons since they are insoluble in water. (2) The first intracellular attack of the pollutants in which oxygenases and peroxidases activate and incorporate oxygen which is the key enzymatic reaction. (3) Conversion of the organic pollutant stage by stage into peripheral degradation pathways into central intermediary metabolism intermediates like Krebs cycle. (4) Cell biomass biosynthesis from the central precursor metabolites such as acetyl- CoA, pyruvate, and succinate.

A huge number of hydrocarbon degrading bacteria have previously been isolated all over the world, table 1 shows some petro1eum hydrocarbon degrading bacteria isolated in some parts of Nigeria. Owing to the toxicity of petroleum hydrocarbons to all living organisms, isolation and identification of various indigenous microorganisms capable of degrading these contaminants is of great interest. In this study, bacteria capable of utilizing used engine oil as carbon and energy source were isolated from soil of automobile workshop in Lapai, Niger States. This organism can be used as an agent to bio-remediate the environment.

Table 1: Common bacteria invo1ved in petro1eum hydrocarbon biodegradation

Substrate	Bacteria	Reference
Crude oil	*Pseudomonas aeruginosa*, *Bacillus cereus, Dyadobacter koreensis, Campylobacter hominis and Micrococcus luteus*	Olowomofe *et al.*, 2018
Spent engine oil	*Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp.	Ebakota *et al.*, 2017
Diesel, kerosene and petrol	*Enterobacter aerogenes, Pseudomonas aeroginosa*, *Aerococcus viridian, Clostridium sporogenes, Staphylococcus aureus, Lactobacillus acidophilus, Micrococcus luteus, Streptococcus faecalis and Bacillus sp.*	Boboye *et al.*, 2010
Crude oil	*Bacillus, Acinetobacter, Micrococcus and Pseudomonas.*	Ijah *et al.*, 2008
Bonny Light (A) and Bonny Medium (B) crude oil	*Bacillus spp, Micrococcus spp* and *Proteus spp.*	Okerentugba and Ezeronye, 2003.

MATERIALS AND METHODS

Sample collection

The petroleum hydrocarbon contaminated soil sample was collected from three different automobile workshops in Lapai Niger State, Nigeria. The samples were mixed and refrigerated at 4ºC before the experiment.

Growth media preparation

The Mineral Salt Medium (MSM) was prepared according to the method of Boboye et al. (2010). 1.8 gram of K_2HPO_4, 4 gram of NH_4CL, 0.2 gram of $MgSO_4.7H_20$, 0.1 gram of NaCl, 0.01 gram of $Na_2S0_4.7H_20$, 20 gram of agar was dissolved in 1 litre of distilled water. The mixture was autoclaved at 121 ℃ for 15 minutes. The medium pH was adjusted to 7.2 and supplemented with 1% used engine oil as carbon source. The medium was poured into petri dishes, allowed to solidified and stored.

Isolation of hydrocarbon degrading bacteria

1g of the soil sample was suspended in test tube containing 9ml of sterile distilled water. The mixture was vortex for 1 minute and allowed to settle down. 1 mL of the supernatant was spread onto Mineral Salt Medium plate and incubated at 37˚C for 14 days. The prominent bacterial colonies observed were sub cultured. Pure culture obtained from this procedure was stored in slants (15.0g/l nutrient agar).

Hydrocarbon degradative activity screening

The isolates hydrocarbon degradative activities were determined using turbidimetric analysis. Each isolate was inoculated into 15 ml Mineral salt broth (MSB) with 1% used engine oil as carbon source. The inoculums were incubated at room temperature for 15 days. Measurement of the bacteria degradative activities were carried out by taking the OD at 600nm using a UV spectrophotometer from 1 to 15 days at regular intervals of 2 days against MSB as blank (Meenakshisundaram, & Bharathiraja, 2014). The bacteria that showed fastest growth was further characterized.

Biochemical Test

To characterize the isolates biochemically, the following tests: Citrate, Catalase, Coagulase, Methyl red, Mannitol and Sugar fermentation test were performed using Bergey's manual.

DNA Extraction and 16S rRNA Gene Amplification

The bacterial DNA was extracted using Bioneer Corps K-3032 Genomic DNA Extraction Kit. After the extraction, polymerase chain reaction (PCR) was done to amplify the bacterial 16S rDNA gene using bacterial universal primers. The PCR was accomplished after 30 cycles. Initial denaturation was at 94°C for 5min, followed by cooling, then denaturation at 94°C for 1 min. Annealing was at 55°C for 1 min, then final extension at 72°C for 10 min. Purification and sequencing of the amplicon was done by DNA Laboratory Kaduna, Nigeria.

Phylogenetic analysis

The BLASTn analysis was performed in National Centre for Biotechnology information to ascertain the genetic relationships of the bacteria with others in the Gene Bank from NCBI[4]. The sequences closest to the bacterial sequence were aligned using the Clustal W program in MEGA 6 software. Phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbor – joining method (Saitou, & Nei, 1987).

RESULTS

Isolation and characterisation of hydrocarbon degrading bacteria

Within the 14 days of incubation, bacterial strains with distinct abilities to use petroleum hydrocarbon (1% used engine oil) as sole carbon and energy source were isolated from hydrocarbon contaminated soil collected from three different mechanic workshop in Lapai. Six prominent isolates, designated as FS1, FS2, FS3, ES1, ES2 and ES3 were selected for bio degra dative activity screening.

Hydrocarbon degradative activity

The hydrocarbon degradative activities of the six isolates were evaluated in MSM broth with 1% used engine oil as carbon and energy source (figure 2). It revealed that isolate ES2 achieved the highest optimum degradation rate of 0.96 at A600nm compared to FS1 (0.89), FS2 (0.89), FS3 (0.81), ES1 (0.70), and ES3 (0.29) respectively. Table 2 display the isolates morphological and biochemical characteristics. Considering the rapid growth of isolate ES2 as compared to the other isolates, the bacteria was chosen for subsequent molecular identification. ES2

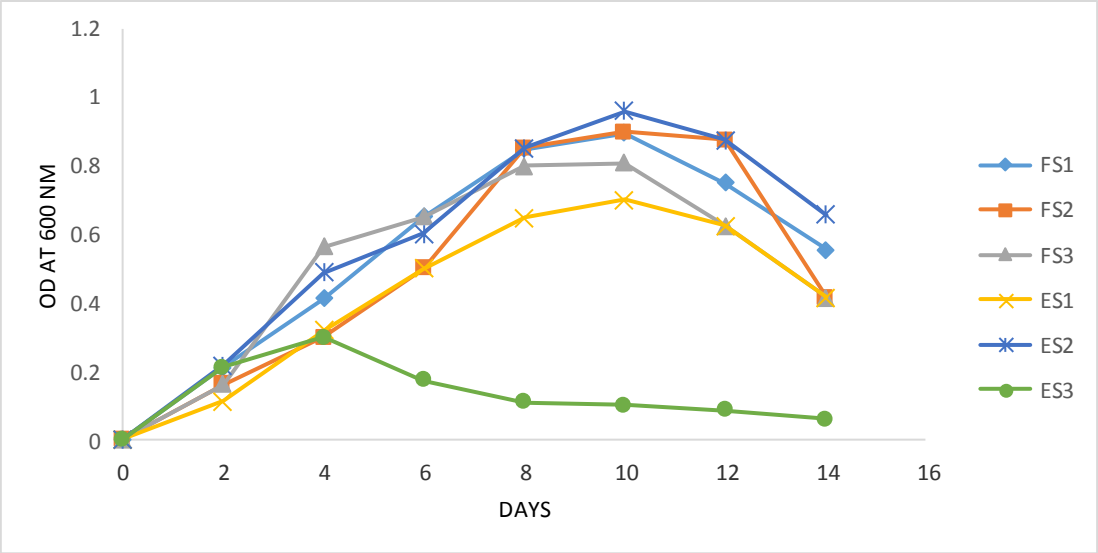

Fig. 1: Growth profile of the isolates showing their degradative activity in MSM broth with 1% used engine oil

Table 2: Morphological and biochemical characteristics of the isolates.

Parameters	FS1	FS2	FS3	ES1	ES2	ES3
Cell shape	*Bacillus*	*S.cocci*	*Cocci*	*S.cocci*	*Bacillus*	*Bacillus*
Pigmentation	White	white	White	White	White	White
Gram staining	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Coagulase	_	_	_	_	_	_
Citrate	+	+	+	+	+	+
Mannitol	+	_	_	_	_	_
Glucose	G	G	_	_	_	G
Lactose	G	G	_	_	G&A	G&A
Fructose	G	G&A	_	_	G	_

(+): Positive, (-): Negative, (G): Gas, (A): Acid

Identification of ES2 by DNA Analysis

The 16S rRNA gene sequence of bacteria ES2 was obtained from the PCR product (figure 3) which showed 1500 base pairs. The sequence was BLAST in NCBI to compare it with the strains in the Genbank database (Table 3). From the blasting result, bacteria ES2 belong to *Lysinibacillus species* with 89% identity to this specie. The biochemical tests (Table 2) and MEGA7 phylogenetic analysis (Fig. 4) verify this result.

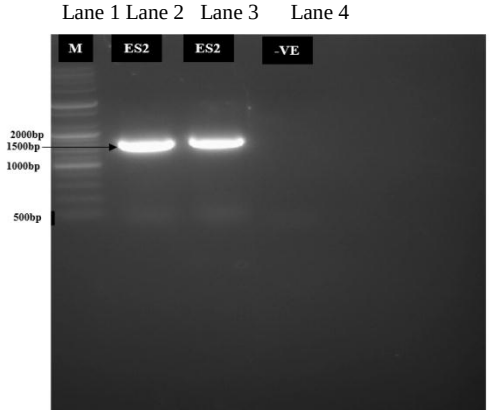

Fig. 2: PCR amplification of 16S rRNA gene on an agarose gel. Lane 1, DNA ladder, Lane 2 & 3 ES2 amplified 16S rRNA gene, Lane 4, control without primers.

Table 3: The NCBI database BLASTn result

Accession number	Description	Max Identity (%)
MH934930.1	*Lysinibacillus pakistanensis strain 18JY7-14*	89%
KX129780.1	*Lysinibacillus macroides strain ACOG1aDiu*	89%
KX028864.1	*Lysinibacillus sp. VKM B-751*	89%
KF261599.1	*Lysinibacillus fusiformis strain AGE1*	88%
HF585362.1	*Lysinibacillus xylanilyticus*	88%
JN638744.1	*Lysinibacillus sp. DWSR27*	88%

Phylogenetic tree

In this study, isolate ES2 Neighbor-Joining phylogeny tree was constructed with sequences from the NCBI BLAST result. MEGA6 software was used and about seven sequences were selected together with ES2 sequence. Figure 3 shows the phylogeny tree result, having ES2 among *Lysinibacillus species*.

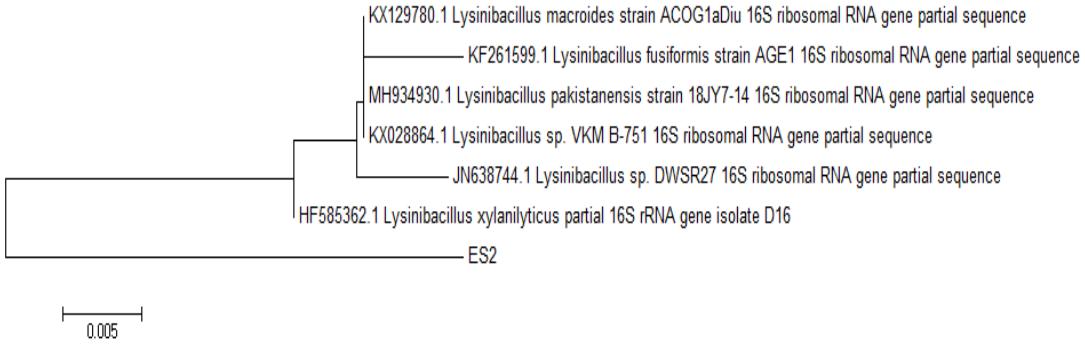

Fig. 3: Phylogenetic tree displaying evolutionary relationship of bacteria ES2 with other bacteria in the gene bank

DISCUSSION

So far, a number of bacteria that were able to use petroleum hydrocarbon as carbon and energy source have been isolated successfully (Table 1). This present study focused on molecular identification of hydrocarbon degrading bacteria isolated from hydrocarbon contaminated soil of automobile work shop in lapai, Niger State. Six bacteria strains; FS1, FS2, FS3, ES1, ES2 and ES3 that utilized 1% used engine oil as sole carbon and energy were isolated. FS1, FS2, FS3, ES1, and ES2 effectively utilized the used engine oil (figure 2). Bacteria ES2 has the highest OD of 0.96 on the 10^{th} day then FS1 with 0.89, FS2 with 0.89, FS3 with 0.81 and ES1 with 0.70. Bacteria ES3 was the only isolate with very low OD of 0.29 at the 4^{th} day. Though the degradation rate differs, all the bacteria isolates exhibited increase in optical density due to their capability to break down and use the engine oil in the media as carbon and energy source for their growth. As the organisms utilize the substrate, there was increment in their growth until the optimum growth was reached. The isolation of organisms from hydrocarbon contaminated environments indicates that the organisms exist and have developed tactics of adjusting to the environment and or use the contaminants for their growth (Ebakota et al., 2017), whereas microorganisms that cannot live in this environment are removed by the critical state caused by the contaminant (Boboye et al., 2010).

From the blasting result, bacterium ES2 belongs to *Lysinibacillus genus* with 89% similarity to this genus (table 3). Lysinibacillus; lysine bacillus, meaning Lys–Asp type of peptidoglycan found in the cell wall. Lysinibacillus genus belongs to the *Bacillaceae*familia with different species such as *Lysinibacillus fusiformis*, *Lysinibacillus boronitolerans Lysinibacillus fusiformis*

etcetera. These organisms are rod-shaped cells, motile, form spherical or ellipsoidal endospores, catalase, and oxidase test positive whereas beta galactosidase and nitrate reduction are negative (Ahmed et al., 2007).

Biodegradation of petroleum hydrocarbon by *Lysinibacillus* and other bacteria from related genera have previously been reported (Chirwa & Bezza, 2015, Ebakota et al., 2017, Meenakshisundaram & Bharathiraja, 2014, Ojo, 2006). These organisms have been identified as the efficient biosurfactant producer for the degradation of petroleum hydrocarbon (Najafi et al., 2011, Rodrigues et al). *Lysinibacillus* is a spore forming bacteria; which are highly resistant to physical and chemical influences and could be the reason of their survival in different contaminated environment such as oil polluted soil.

CONCLUSION

In this study, some isolates were obtained from hydrocarbon contaminated soil of automobile workshops in Lapai using used engine oil as substrate. Among the bacteria isolates, isolate ES2 has the highest degradation rate which was identified molecularly as *Lysinibacillus.* This bacteria can be used to effectively bio remediate hydrocarbon polluted environment. So far, this is the first *Lysinibacillus* obtained from this particular environment with this unique characteristic of petroleum hydrocarbon degradation. Hence, bio surfactant production by this organism needs to be assessed.

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