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Biosurfactant Producing Bacteria Associated with Oil Polluted Soils of some Auto Mechanic Workshops in Akure, Ondo State, (Southwest) Nigeria

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Abstract

Background: Auto mechanic shops account for a sizable fraction of hydrocarbon leaks that go unnoticed most of the time. This study looked into the biosurfactant-producing activity of hydrocarbon using bacteria isolated from five auto mechanic shops in Akure.

Methods: The bacteria were isolated using standard microbiological procedures. The isolates' oil degrading activity was evaluated on Bushnell Haas medium enhanced with 2% petroleum hydrocarbons (petrol, diesel, crude oil and kerosene). Biosurfactant activity was determined using the drop collapse test, oil spread assay, foaming property, and emulsification activity (E24). Biosurfactant-producing isolates were identified using molecular and morphological techniques, as well as biochemical and biochemical assays. Using universal primers, the isolates' hypervariable 16SrRNA region was amplified and sequenced.

Result: *Bacillus paramycoides* strain OFOa and *Clostridium punense* strain OFOb were discovered as the two (2) bacterial isolates with high biodegradation and biosurfactant generating activities in this study. Their sequences had since been submitted to GenBank, with accession numbers MN700654 and MN700653, respectively.

Conclusion: As bio-emulsifiers and biosurfactant makers, the strains of these two (2) bacteria identified in this investigation could be used in bioremediation of hydrocarbon-polluted locations.

Introduction

Petroleum hydrocarbons are the world's most widely used primary energy and fuel source. They are widely used as primary energy sources in business and everyday life all over the world [1,2]. Oil spills are a persistent and re-emerging environmental problem caused by petrochemical companies and other human-caused activities [3,4]. Accidental oil spills occur during activities [5], use and maintenance of automobiles, and operation of power plants. For decades, oil spills have been a source of environmental menace [6].

Microbiological biodegradation is an important natural pathway for pollutant degradation [7]. Only the growth and proliferation of hydrocarbon degraders are supported by enrichment medium supplemented with oil as an energy and carbon source. As a result, the test strains' degradative ability can be determined by cultivating isolates in the presence of hydrocarbon contaminants [8,9,10]. Researchers have identified bacteria from the genera *Bacillus, Pseudomonas*, *Alcaligenes* and, most recently, *Ochrobactrum anthropi* for the decomposition of hydrocarbon. *Aspergillus* and *Penicillium* are two fungi that have been linked to hydrocarbon breakdown. *Staphylococcus* sp., *Pseudomonas* sp., and *Streptococcus* sp. were identified from automotive workplaces by Ebakota et al. [11] and these bacteria were found to be involved in hydrocarbon degradation in these workplaces. Complex interactions are involved in microbial hydrocarbon breakdown [12]. Several microbes that use oil as a carbon source, such as *Rhodococcus* sp. and *Pseudomonas* sp., create lipoprotein or glycolipid biosurfactants [5]. Many hydrocarbon degraders create biosurfactant, which disperses hydrocarbon and makes it biologically available for metabolism. *Bacillus* spp., *Pseudomonas* spp., and *Ochrobactrum anthropi* are examples of such microbial species.

When spent motor oil was being biodegraded, novel strains of *Citrobacter freundii* HM-2 and *Ochrobactrum anthropi* HM-1 isolated from oil contaminated soil were able to produce biosurfactant, according to [13]. As a result, the surface area of the hydrophobic water-insoluble substrates and bioavailability increased which helped bacteria grow and enhanced the rate of bioremediation [14], making them more effective than artificial surfactants [15]. Molecular identification using 16S rRNA sequencing is a reliable technique of bacterial identification [16]. Hydrocarbon degraders share phylogenetic relatedness because they have the gene for degradation [17]. The primary focus of this work is to identify bacteria capable of degrading hydrocarbon and making biosurfactants, using auto mechanic workshops in Akure, Ondo state (South West), Nigeria as sampling sites.

Methods

Soil sample collection

Soil samples were taken with a scoop at a depth of 2-3 cm from auto mechanic workplaces in Mechanic villages near Ondo Road (7°15'18.1"N 5°11'17.1"E) and AKAD area (7°17'47.7"N 5°09'22.8"E), Akure, Ondo State (Southwest), Nigeria. They were carried to the laboratory in sterile cellophane bags, where they were stored at a temperature of 4°C.

Isolation of Bacteria

Bacterial isolation was done on Nutrient agar, which was made by dissolving 28 grams of Nutrient agar powder in 1000 mL of distilled water according to the manufacturer's instructions. In an autoclave (Astell, England), the dissolved medium was sterilized at 121°C for 15 minutes. The molten fluid was cooled to 40°C before being aseptically poured onto sterile Petri dishes with 1mL of serially diluted soil samples. The plates were allowed to harden before being incubated for 24 hours at 37°C in an incubator. After 24 hours, the plates were harvested. Using the streak culture pattern, distinct colonies were sub-cultured on fresh agar plates until pure cultures were achieved. At 4°C, pure cultures were maintained on agar slants.

Screening for hydrocarbon degradation potential of isolates

The preparation of Bushnell Haas medium used in this study was according to [18]. BHA is made up of 0.20 g MgSO4, 0.02 g CaCl2, 1.00 g KH2PO4, 1.00 g K2HPO4, 1.00 g NH4NO3, 0.05 g FeCl3, and 20 g agar-agar diluted in 1000 mL distilled water and sterilized in an autoclave (Astell, England) at 121°C for 15 minutes. The hydrocarbons (petrol, diesel, kerosene, and crude oil) were sterilized using membrane filter (0.22 μm) before adding to the sterilized medium. The isolates were thereafter, tested for hydrocarbon degradation using 2% of each sterilized hydrocarbons.

Screening and synthesis of biosurfactant

The surface-active property of bacterial isolates grown in Bushnell Haas medium (BHM) supplemented with 2% hydrocarbon was utilized to screen them for biosurfactant production.

Drop collapse test

Crude oil was utilized to create a thin film on a grease-free slide in this experiment. One drop of cell-free soup was dropped onto the thin oil layer to test if it collapsed and spread or was rejected by the oily surface. If the biosurfactant's lipophilic moiety is present in the cell-free broth, it will most likely interact with the oil surface [19].

Oil spread assay

The oil spread assay was performed following the guidelines in [19]. In a clean glass petri dish, ten microliters (10μl) of crude oil were mixed with forty milliliters (40 ml) of distilled water to create a thin layer. After that, the culture supernatant was gently poured into the oil's core, providing a yield of 10 microliters (μl). The amount of oil displaced and cleared by the supernatant was calculated.

Foaming activity

This is used to assess whether a biosurfactant synthesized in the medium has foaming properties. The isolates were inoculated and incubated over the night in nutrient broth. To get cell-free broth, the overnight broth culture was centrifuged to obtain supernatant. Two milliliters (2ml) of the supernatant was added in a test tube and violently mixed for 2 minutes before looking for steady foam formation. Broth without cells was employed for control. The following formula was used to calculate the percentage foam formation:
Foaming (%) = (height of the foam layer/total height) x 100 [18].

Emulsification activity (E_{24})

The emulsification activity was tested by adding 2ml of hydrocarbon (kerosene) to the same volume of culture supernatant, mixing for 2 minutes with a vortex, and letting it sit for 24 hours. The proportion of the height of emulsified layer (in millimeters) divided by the entire height of the liquid column was used to calculate the emulsification activity [13].

Identification of isolates

Individual colonies were identified with the help of visual and biochemical criteria using Bergey's Manual of Determinative Bacteriology as well as 16S rRNA sequencing.

Molecular Characterization of Bacteria Isolates

Genomic DNA was extracted using the Nexttec™ 1-Step DNA Isolation Kit for Bacteria. A ThermoScientific Nanodrop 2000c was used to analyze the quantity and purity of extracted DNA, and a 1% agarose gel was used to assess the genomic DNA's integrity. Forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and Reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify the bacterial 16S rRNA gene. Each PCR reaction was carried out in a 25 μl container containing 12.5 μl of One Taq® 2X Master mix with TAE buffer (New England Biolabs), 7.5 μl of nuclease-free water, 0.5 mM forward and reverse primers, and 4 μl of DNA template.

The following were the cycling conditions: Initial denaturation at 94°C for 30 seconds, then 40 cycles of denaturing at 94°C for 30 seconds, annealing at 49.5°C for 60 seconds, extension at 68°C for 120 seconds; final extension at 68°C for 5 minutes, hold at 4°C. The PCR products, amplified were separated on a 2 percent agarose gel and seen under UV light after being stained with 5μl ethidium bromide. Inqaba Biotec performed the 16S rRNA gene sequencing (South Africa). Sequencer 5.4.6, build 46289, was used to edit and assemble the sequence. The NCBI-BLASTn tool was used to compare sequences. ClustalW in MEGA 7 was used to align the sequences. Phylogenetic analysis was performed using the Tamura-Nei method's neighbour-joining (N-J) technique with 1000 bootstrap replications.

Data Analysis

The numerical data obtained were subjected to Analysis of Variance (ANOVA) and Duncan's New Multiple Range Test using Statistical Packages for Social Sciences (SPSS) 23.0 version (IBM). Differences in mean values were considered significant at $P \leq 0.05$.

Results

Morphology and biochemical characteristics of isolates

The two bacteria were tentatively identified as *Clostridium* sp. and *Bacillus* sp., They both appeared creamish on nutrient agar, they were rod shaped and Gram positive. *Clostridium* sp. was catalase negative while *Bacillus* sp. was catalase positive. *Clostridium* sp. could not hydrolyse starch while *Bacillus* sp. was able to hydrolyze starch.

Hydrocarbon Degradation Potential

Bacillus sp., *Clostridium* sp. demonstrated the ability to grow in the presence of all hydrocarbon oils used (Petrol, Diesel, Kerosene and Crude oil).

Biosurfactant activity

The two tests isolates demonstrated drop collapse, oil spread, foaming and emulsification activity as presented on table 1.

Isolate	Oil spread (mm)	E_{24} (%)	Drop collapse	Foaming activity(%)
Bacillus sp.	22.00	60.00	+	80
Clostridium sp.	19.00	34.78	+	60
Control	0.00	0.00	-	0

Legend
E24 = Emulsification activity
- = Negative
+ = Positive

Table 1: Tests to confirm the bacterial potential to produce biosurfactant .

Molecular characteristics of biosurfactant producing isolates

Plate 1 presents the prominent bands of genomic DNA extracted from the isolates as visualized under UV light after electrophoretic separation on 1% agarose gel stained with ethidium bromide while Table 2 presents the information on the molecular identity of bacteria

capable of producing biosurfactant. *Bacillus paramycoides* OFOa genomic DNA concentration and purity were 234.8 ng/l and 1.81, respectively, whereas *Clostridium punense* OFOb genomic DNA concentration and purity were 234 ng/l and 1.88, respectively (Table 3). Plate 2 shows the bands of 16S rRNA gene amplicons separated on 2% agarose. The amplicons were about 1500 base pairs relative to the molecular ladder. Molecular characteristics of the bacteria are presented on table 2. From the phylogenetic tree constructed, Strain A (*Bacillus paramycoides* strain OFOa) clustered with other *Bacillus cereus* groups (Figure 1). Strain B (*Clostridium punense* strain OFOb) identified in this study had close similarity with other *Clostridia* and soil bacteria (Figure 2). The percentage of trees in which associated taxa cluster is shown next to the branches of each tree.

Isolate Code	16SrRNA Nucleotide Sequences (bp)	Closest Homology	Strain Name	NCBI-GenBank Accession Number
A	619	*Bacillus paramycoides* MCCC 1A04098	*Bacillus paramycoides* strain OFOa	MN700654
B	1372	*Clostridium punense* BLPYG-8	*Clostridium punense* strain OFOb	MN700653

Table 2: The identities of isolates based on 16Sr RNA sequencing.

Isolates	Concentration ng/µL	Absorbance (A_{260}/A_{280})
Bacillus paramycoides OFOa	234.8 ng/µl	1.81
Clostridium punense OFOb	234.8 ng/µl	1.88

Table 3: Concentration and purity of isolated genomic DNA of bacterial isolates.

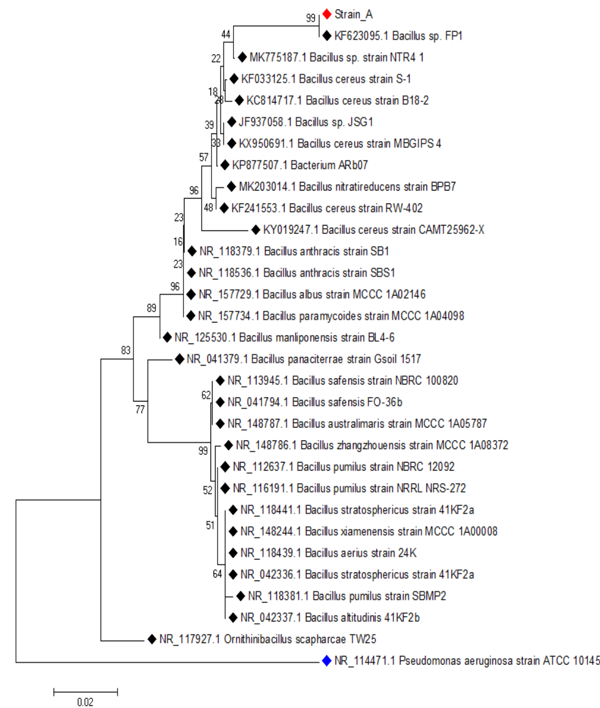

Figure 1: Dendogram of *Bacillus paramycoides* strain OFO

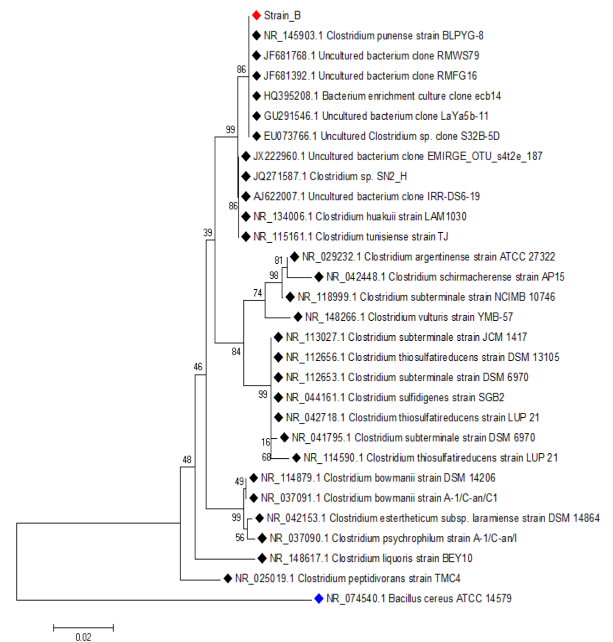

Figure 2: Dendogram of *Clostridium punense* strain OFOb

Discussion

The ecological system balance is negatively affected by oil spills [20]. However, the ubiquity of microorganisms make them flourish in a wide range of environmental circumstances, which is being explored for pollution biodegradation [21]. Complimentary relationship exists between different types of microorganisms and the occurence of hydrocarbons in oil-contaminated locations [22]. In a polluted environment with hydrocarbons, the microbial population has evolved to use the hydrocarbons for metabolic processes and growth [23]. In the Bushnell Haas medium, hydrocarbons were employed as carbon and energy sources. The bacterial isolates are likely to have the enzymatic ability to use the oils [24] through various metabolic pathways. The existence of hydrocarbon utilizers in an environment indicates that native microorganisms capable of decomposing the contaminant are present [25].

From their surface-active qualities, B. paramycoides and C. punense strains showed the ability to produce biosurfactants. Biosurfactant is important in bacterial breakdown of hydrophobic aromatic hydrocarbons [26]. Bacillus paramycoides had better biosurfactant emulsification (E24 = 60%) and foaming activity (80%) than other bacteria. The biodegradation of hydrocarbons is expected to be aided by oil-degrading bacteria with high emulsification activity [27]. It was also discovered that the rate of growth and the action of biosurfactants are linked. Biosurfactants are extracellularly generated and incorporated into the culture broth to aid in the transfer of insoluble

substrates across cell membranes, hence enhancing microbial growth [28]. The synthesis of biosurfactant in the growing medium followed by continuous hydrocarbon breakdown indicates that the bacterial strains were able to use hydrocarbon compounds as biosurfactant substrates [27]. The biosurfactants demonstrated a high emulsification index and oil displacement ability. The diameter of the clear zone was also a good indicator of biosurfactant activity. Rhamnolipids are biosurfactants generated by species of *Bacillus*. Rhamnolipids enhances bioremediation process by making available weathered oil from soil matrices for degradation by microbes [29]. There is a relationship between oil degrading capacity of bacterial isolates (measured by their ability to grow only on the hydrocarbon) and biosurfactant activity (measured by surface-active characteristics) expressed in the medium supplemented with hydrocarbon . This is consistent with the findings of [5]. The existence of microorganisms with metabolic capacities is a necessary condition for pollution biodegradation. Microbes' ability to create biosurfactant is often enhanced by their development on extremely insoluble hydrocarbons as their sole carbon source [30]. Biosurfactants have a physiological effect in enhancing the bioavailability of hydrophobic molecules, hence bring about the uptake of carbon sources in the supplemented medium.

Bacillus, *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, and *Ochrobactrum* have all been found to produce different types of biosurfactants [13,26,30]. It should be noted, however, that this is the first study to identify *Clostridium punense* as a biosurfactant producer. *Clostridium punense* was originally observed and picked from the feces of a healthy 56-year-old human male by Lanjekar et al. in 2015.

The most successful method of detecting hydrocarbon degrading species appears to be a molecular approach. The purity and concentration of genomic DNA collected and used in this investigation demonstrated that it was free of impurities and proteins that could obstruct the downstream applications like PCR and DNA sequencing. Pure DNA is defined as having an A260/A280 ratio of less than 1.8 [32]. The PCR amplicons obtained are around 1.5 Kb in size, which corresponds to the conserved 16S rRNA gene size [33]. In bacteria, this refers to the localized sequence of a variable region. The 16SrRNA gene is a conserved area in bacteria that is widely used to identify bacteria. *Bacillus paramycoides* and *Clostridium punense* strains were identified as the microorganisms responsible for biosurfactant synthesis in this investigation. Members of the *Bacillus* genera are linked to breaking down of hydrocarbon and synthesising biosurfactant, whereas, the *Clostridium* strain obtained in this investigation is not linked to any of these processes.

The phylogenetic tree (figure 1) shows that strain A is closely related to *Bacillus paramycoides* belonging to the *B. cereus* group. This is obvious as Strain A clusters with *Bacillus cereus* and some other Bacillus strains. Strain B clusters closely with *Clostridium punense* BLPYG-8 and shows relatedness with other soil Clostridia and uncultured bacteria strains. The fraction of trees with connected taxa clustered next to tree branches indicates the degree of relatedness. The phylogenetic analysis also showed that there is a relationship between genera *Bacillus* and *Pseudomonas* which are known for versatility in carbon utilization, hydrocarbon degradation, as well as biosurfactant production. This could mean that they have particular gene(s) in common that encodes for these abilities [24].

In the soil of an auto mechanic's workshop, biosurfactant-producing strains were discovered. The little-known bacterial strains can digest hydrocarbons and could be employed in bioremediation. The biosurfactant manufacturing process should be improved to provide a larger quantity of biosurfactant. Genetic engineering can increase the ability of hydrocarbon-degrading bacteria to degrade hydrocarbons and produce biosurfactants. Appropriate oil waste management processes should be designed to reduce random discharge of oil waste in the auto service business.

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Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Oluwatoyin Folake Olukunle (OFO) and Bukola Rukayat Oyelere (BRO) contributed to the conception and design of the study. All the authors Oluwatoyin Folake Olukunle (OFO) Bukola Rukayat Oyelere (BRO) and Tope Salam Fadipe (TSF) participated in the material preparation, data collection, and analysis. BRO wrote the first draft of the manuscript while all authors read, modified and approved the final manuscript.

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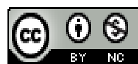

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