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PROFILING OF HYDROCARBON DEGRADING BACTERIA SPECIES ISOLATED FROM HYDROCARBON-POLLUTED WASTEWATER

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Abstract

Oil exploration and extraction wastewater is composed of organic hydrocarbons like aromatic and aliphatic hydrocarbons such that oil spillage and contamination of water pose great risk to humans and the ecosystem. This research is aimed at profiling indigenous hydrocarbon-utilizing microorganisms isolated from hydrocarbon polluted wastewater. Microorganisms indigenous to refinery effluents were isolated using nutrient agar and kings B medium and identified by standard morphological and biochemical tests, and confirmed by molecular characterisation. The isolates were screened for hydrocarbon degradation potential using Bushnell-Haas medium incorporated with 1% crude oil. The isolates identified were species of *Pseudomonas, Enterobacter, Bacillus, Corynebacterium, Proteus, Staphylococcus, Streptococcus, Achromobacter and Escherichia. Pseudomonas* was the most prevalent (20.9 %) while*Streptococcus* was the least (3.6%). The 16S rRNA of some of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Proteus, Escherichia, Pseudomonas, Bacillus* and *Enterobacter* sp. and revealed a closely relatedness to the *Proteus columbae, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Enterobacter mori.* Of the isolates, *Enterobacter* sp., *Bacillus* sp., *Pseudomonas* sp., were shown to have great potential in degrading petroleum hydrocarbons when grown on Bushnell Haas medium. Compared to the other isolates, *Pseudomonas* sp. had the best growth in Bushnell Haas medium. These microorganisms native to the hydrocarbon polluted wastewater could be employed in the clean-up of hydrocarbon polluted wastewater.

Keywords: Wastewater, Degradation, Hydrocarbon, Petroleum, Microorganisms.

Introduction

Globally, there is increase in water utilization in oil production industries and for anthropogenic purposes (Hamidi *et. al.*, 2023) which results in generation of large volume of wastewater. In the developing world, wastewater is mostly channeled to water bodies. Wastewater from Warri Refining and Petrochemical Company is frequently being discharged into Ifie river. This effluent however, is composed of organic hydrocarbons like aromatic and aliphatic hydrocarbons (PAH and AHC) which are relatively classified as high priority pollutants (Suman *et al*., 2016; Abdel-Shafy and Mansour, 2018). PAH and AHC pollutants have been reported by diverse investigators to be ubiquitous, found equally in aquatic and terrestrial ecosystems as well as in the atmosphere (Adeniji *et al*., 2019).

PAHs being recalcitrant persist as complex components in diverse environment (Abdel-Shafy and Mansour, 2016, Kuppusamy *et al*. 2017, Li *et al*. 2020) and are toxic to aquatic organisms when disposed in aquatic environments (Lee and Vu, 2010). Hydrocarbons are ever-present pollutants in our surroundings that are mostly released from anthropogenic activities and during incomplete

combustion or via industrial processes and are characterized by high mutagenic and carcinogenic potential (Lawal, 2017).

Hydrocarbons being ubiquitous (Zelinkova and Wenzl, 2015; Hamidi *et al*, 2023) reach water bodies mainly through petroleum spills, industrial effluents, runoffs, dry and wet deposition, combustion of fossil fuels and leaching from creosote-impregnated wood. Exposure to high levels of hydrocarbon has been shown to result in immunosuppressive reactions, oxidative stress, carcinogenic presentations (IARC, 2021), teratogenic and mutagenic effects which may lead to poor development of fetal lungs, brain, heart, bladder, and other important internal organs (Duan *et al*., 2016) as well as skin cancer. It is important therefore that wastewater must be treated effectively before its discharge into any environment such as aquatic ecosystem (Hamidi *et al.,* 2023). The capacity to breakdown these hydrocarbon substrates is displayed by a broad range of bacterial and fungal species (Al-Hawash *et al*., 2018, Daccò *et al*., 2020). The most significant hydrocarbon-degrading bacteria in both marine and soil habitats include Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia, Pseudomonas spp. and the Coryneforms with Pseudomonas, Micrococcus, and Nocardia spp., members of the family Enterobacteriaceae, actinomycetes, and coryneforms making up 95% of the isolates. Among the fungi, Aureobasidium, Candida, Rhodotorula, and Sporobolomyces spp. are the most frequent marine isolates and Trichoderma while Mortierella spp. are the most frequent soil isolates (Daccò *et al*., 2020) while Aspergillus and Penicillium sp make up the alga sp. In the marine environment however, bacteria are typically believed to constitute the primary hydrocarbon-degrading constituent of the microbial community.

Prior exposure of a microbial population to hydrocarbons, either from human sources such as accidental oil spills, petroleum exploration and transportation operations, and waste oil disposal, or from natural sources such as seeps and plant-derived hydrocarbons (Kuppusamy *et al*., 2020) leads to adaptation which is crucial in determining how swiftly hydrocarbon inputs may be biodegraded (Ivshina *et al*., 2017). Adaptation of microbial communities to hydrocarbons increases the rates of transformation of hydrocarbons (Muhammad *et al*., 2024). A study of mineralization of [14C]hexadecane and degradation of Il-alkane combinations by bacteria in different surface water and sand samples, researchers found that locations With heavier hydrocarbon loads had higher hydrocarbon-oxidizing activity (Dell'Anno *et al*., 2021). Exposure of freshwater sediments to a synthetic oil was also found to enhance the rate of polyaromatic hydrocarbon (PAH) mineralization (Yemele *et al*., 2024). Numerous investigations have demonstrated that certain bacterial populations in contact with hydrocarbons quickly transit to bacterial species able to breakdown and use hydrocarbon compounds as sources of carbon and energy (hydrocarbonoclastic bacteria) (Ruiz *et al*., 2021). Biodegradation of hydrocarbon comprises adherence to the substrate and formation of molecules such as biosurfactants/bioemulsionants, biopolymers, solvents, gases, and acids for making them bioavailable (Varjani, 2017).

Microbial degradation promises a feasible method for the cleanup of these pollutants in oily wastewater (Li *et al*. 2019). The method is faster, convenient and ecofriendly. Methods such as bioaugmentation have been evaluated for the potential of microbes to detoxify petroleum pollutant in wastewater (Karishma *et al.,* 2024). Bacteria indigenous to the polluted wastewater have greater potential since they are already adapted to the environment (Chukuka *et al.*, 2023; Chukuka *et al.*, 2024). In this process, petroleum hydrocarbons are utilized by

microorganisms as their energy source and thus converts it to non- hazardous compounds.
This research work therefore was done to ascertain indigenous microorganisms isolated from hydrocarbon polluted wastewater capable of utilizing hydrocarbon for growth.

Methodology

Collection of Samples

Crude oil used for the research purpose was obtained from Warri Refining and Petrochemical Company Limited, Nigeria while the wastewater was collected at the Ifie discharge terminal of the refinery. The crude oil for the evaluation of degradability was collected using 5L Welchester bottles initially sterilized by autoclaving. These were delivered in ice chests directly to the Microbiology Laboratory of Delta State University, Abraka and kept in the refrigerator at 4^{o}C for further analyses.

Isolation and Enumeration of microorganisms (bacteria) from wastewater/effluent

A 10-fold serial dilution of the samples was made and 1ml of diluted sample was plated out on Kings B-medium (Proteose peptone 20g, Glycerol 10ml, K_2HPO_4 1.5g, $MgSO_4.7H_2O$ 1.5g, Agar 1.5g, Distilled water 1L, 15g agar, pH 7.2), Nutrient agar and J-medium (Tryptone 5g, Yeast extract 15g, K_2PO_4 3g, Glucose 2g, Agar 20g, Distilled water 1litre, pH 7.4) for the isolation of bacteria species. The plates were incubated at 25^{o}C ±2 for 24hours. For the purpose of enumeration, 1ml of each dilution was plated out on the same media and enumerated after 24hours of incubation.

Phenotypic and biochemical identification of isolates

This was done in line with the method described by Cheesebrough, (2006).

Pure culture of the isolates were Gram-stained after 24 hours of incubation. The morphological characteristics (cell and colonial morphology) were noted. Biochemical tests such as catalase, oxidase, indole, citrate, hydrogen sulphide, lactase, glucose and motility tests were carried out.

Molecular Identification isolates

The DNA extraction, quantification, amplification and sequencing were done using the method of Sadhana *et al.*, 2021.

Bacterial DNA extraction

Extraction was done using a ZR fungi/ bacteria DNA mini prep extraction kit supplied by Inqaba South Africa. Pure colonies of the bacterial isolate were transferred into 200 microliters of isotonic buffer in a ZR Bashing Bead Lysis tubes, 750 microliter of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube were centrifuged at 10,000x*g* for 1 minute.

Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer were added to the filtrate in the collection tubes bringing the final volume to 1600 microliter, 800 microliter was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 minute followed by the addition of 500 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microliter centrifuge tube, 100

microliter of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* microliter for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

16S rRNA Amplification

The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Screening for Hydrocarbon Degrading Potentials of microorganisms.

Pure isolates were cultured in Bushnell-Haas media supplemented with crude oil and incubated at 25ºC. Results were read after 24 hours at intervals of 3days for a period of 21 days.

Results and Discussion

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Table 1: Identification chart of bacteria isolated from crude oil effluent

	Identification tests															Probable identity
Isolates	**Shape**	**Gram reaction**	**Catalase**	**Oxidase**	**Indole**	**Simon**	**H_2S**	**Urease**	**VP**	**MR**	**Acid**	**Gas**	**Lactose**	**Glucose**	**Motility**	
1	rod	-	+	+	-	+	-	+	-	-	+	-	-	-	+	*Pseudomonas* sp
2	rod	+	+	-	-	-	-	-	+	-	+	+	-	+	+	*Bacillus* sp
3	rod	-	+	-	-	+	-	+	+	+	+	+	+	-	-	*Enterobacter* sp
4	rod	-	+	-	+	-	-	-	-	+	+	+	+	+	+	*E. coli*
5	rod	-	+	-	+	-	-	+	-	+	+	-	-	+	+	*Proteus* sp
6	cocci	+	+	-	-	-	-	-	+	+	+	-	-	+	-	*Staphylococcus* sp
7	cocci	+	-	-	-	-	-	-	-	+	+	-	+	-	-	*Streptococcus* sp
8	cocci	+	+	-	-	-	-	+	-	-	+	-	-	-	-	*Micrococcus* sp
9	rod	+	+	-	-	-	-	-	-	-	+	-	-	+	-	*Corynebacterium* sp
10	Rod	-	-	+	-	+	-	+	-	-	+	-	-	+	+	*Achromobacter* sp

+ = Positive - = Negative

Table 2: Occurrence of Bacteria in crude oil effluent

Microorganisms	**No of isolates (cfu/ml)**	**Frequency (%)**
Pseudomonas sp	23	20.9
Bacillus sp	20	18.2
Enterobacter sp	15	13.6
E.coli sp	7	6.4
Proteus sp	7	6.4
Staphylococcus sp	11	10
Streptococcus sp	4	3.6
Micrococcus sp	12	10.9
Corynebacterium sp	6	5.5
Achromobacter sp	5	4.5

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Table 3: Qualitative assessment of growth of isolates in crude oil enhanced BH- medium

Isolates	Incubation period (days)						
	3	6	9	12	15	18	21
Pseudomonas sp	-	-	+	++	+++	+++	+++
Bacillus sp	-	-	-	++	+++	+++	++
Enterobacter sp	-	-	-	+	+++	+++	++
E.coli	-	-	-	-	-	-	-
Proteus sp	-	-	-	-	-	-	-
Staphylococcus sp	-	-	-	+	+	+	-
Streptococcus sp	-	-	-	+	+	+	+
Micrococcus sp	-	-	-	+	++	++	+
Corynebacterium sp	-	-	-	+	+	+	+
Achromobacter sp	-	-	-	+	+	+	+

- : No growth, +: Scanty growth , ++: Moderate growth, +++: Heavy growth.

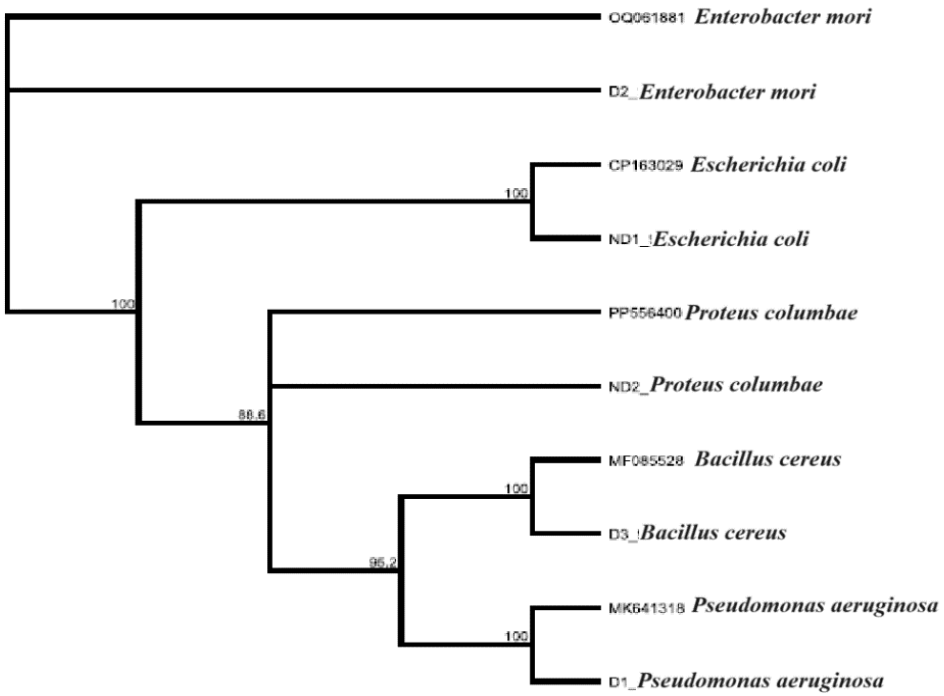

Fig 1: Phylogenetic tree showing the evolutionary relationship between the bacterial isolates

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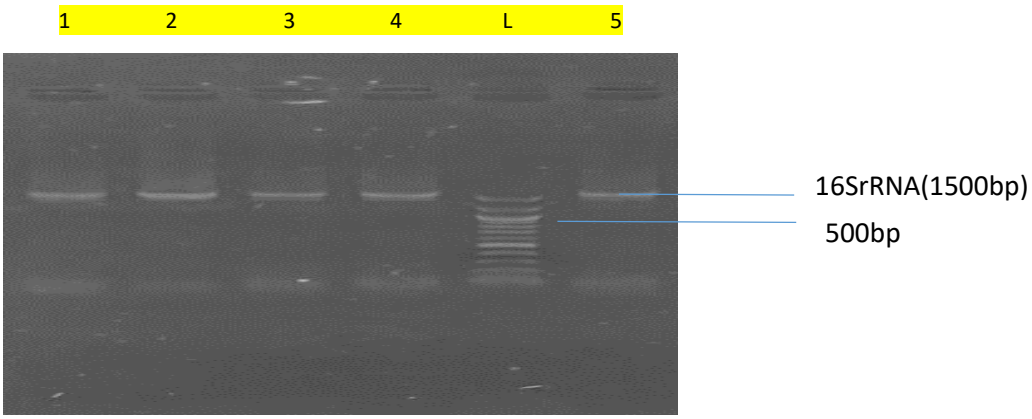

Plate 1: Agarose gel electrophoresis showing the amplified 16srRNA. Lanes 1-5 represent the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder.

The microbial load of hydrocarbon polluted wastewater as recorded in table 1 shows contamination of the sample with ten bacterial species namely *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Micrococcus* sp., *Acromobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Escherichia coli, Proteus* sp. *and Corynebacterium* sp. This conforms with the work of Madan *et al.* (2023), who reported the isolation of *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Acinetobacter* sp., *Corynebacterium* sp from oil spill. The wastewater sample recorded highest percentage of *Pseudomonas* sp (20.9%) followed by *Bacillus* sp *(*18.2%*)* and *Enterobacter* sp *(13.6%)* while *Achromobacter* sp is the least having an occurrence of (4.5%). These organisms could have been introduced into the effluent through the water used in the refining process or via the open well used to store wastewater prior to its discharge into Ifie river or by ineffective treatment of the effluent. The identity of five of the species, *Pseudomonas, Enterobacter, Bacillus, Proteus* and *Escherichia* were confirmed following molecular characterization. The 16S rRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method as shown in Fig.1. were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Proteus, Escherichia, Pseudomonas, Bacillus* and *Enterobacter* sp. and revealed a closely relatedness to the *Proteus columbae, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Enterobacter mori.* Agarose gel electrophoresis shows amplified 16srRNA. Lanes 1-5 represent the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder. Qualitative assessment of growth of the isolates in crude-oil enhanced Bushnell-Haas medium indicated that *Pseudomonas, Bacillus* and *Enterobacter* sp. were able to utilize hydrocarbon for growth whereas *Proteus* sp. and *E.coli* showed no growth following 21 days of incubation. *Pseudomonas species* showed moderate growth after 12days and this growth increased thereafter making it the best degrader of hydrocarbon followed by *Bacillus* and *Enterobacter* sp. The findings showed that *Pseudomonas, Bacillus* and *Enterobacter* sp efficiently utilized crude oil as their only source of carbon for growth. The organisms that did not show growth were unable to use hydrocarbon as the source of carbon since the mineral medium was carbon deficient. This conforms with the work of Madan *et al.,* 2023 who reported that small chain hydrocarbons are utilised by genetically diverse groups like *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Acinetobacter* sp., *Corynebacterium* sp *and* Chukuka *et al.,* 2023, who reported that

Pseudomonas and *Bacillus* have high potential in hydrocarbon degradation. Prior exposure of these bacteria to the hydrocarbon component of wastewater may have conferred adaptation to the environment through the presence of hydrocarbon degradative enzymes. According to Santisi *et al.*, (2015), the use of microorganisms in clean-up process is gaining more ground because of the low environmental impact.

Conclusion

This study records a high-level hydrocarbon utilizing microorganisms which could be as a result of the presence of organic constituents in the wastewater which the organisms utilize as their sole carbon source and also the presence of moisture for enhanced growth. This study also reveals that these microorganisms native to the hydrocarbon polluted wastewater are better adapted, hence, could be employed in the clean up of hydrocarbon polluted wastewater.

Recommendation

i. That the use of microorganisms could serve as a sustainable and environmental friendly strategy for the clean up of oily wastewater.

ii. that organisms native to petroleum polluted water should be harnessed in the biotreatment of hydrocarbon polluted environment.

iii. this process should be scaled-up to meet commercial demands for clean-up of hydrocarbon polluted wastewater.

Conflict of Interest

This research work was done without any conflict of interest

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