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Isolation and Genomic Identification of Potential Hydrocarbonoclastic Bacteria and Fungi in Orashi River, Rivers State, Nigeria

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ABSTRACT

The research study aimed at isolation and genomic identification of potential crude oil utilizing bacterial and fungal in water and sediment of Orashi River, in the Niger Delta region impacted with petroleum hydrocarbon pollutants. Potential crude oil utilizing bacteria and fungi were cultivated using spread plate technique on mineral salt medium (MSM) with bonny light crude oil as the carbon source. Cultured samples were incubated for 14days and 10 days for bacteria and fungi respectively. Extraction of the DNA of pure cultured crude oil Utilizing bacteria and Fungi was done using ZR fungal bacterial DNA Prep extraction kit and the extracted DNA was subjected to polymerase chain reaction (PCR). Amplicons was sequenced using Big Dye Terminator Kit. 16S rRNA and Internal transcribed spacer (ITS) sequences were downloaded into the database NCBI using BLASTN algorithm. Results of Total heterotrophic bacteria count ranged from $2.25\pm2.48\times10^6$ - $3.46\pm3.88\times10^6$ CFU/ml and potential crude oil utilizing bacteria were not recovered from the surface water. In the sediment, Total heterotrophic bacteria count range from $1.76\pm1.75\times10^6$ - $2.29\pm2.41\times10^6$ CFU/g while potential crude oil utilizing bacteria ranged from $1.50\pm2.12\times10^2$ - $2.10\pm1.27\times10^3$CFU/g. Total fungi count ranged from $1.16\pm1.19\times10^3$ - $6.5\pm0.64\times10^3$CFU/ml and potential crude oil utilizing fungi ranged from $8.1\pm0.84\times10^2$ - $1.80\pm1.70\times10^3$CFU/ml in surface water. While Total fungi count ranged from $1.20\pm1.13\times10^3$ - $2.30\pm2.40\times10^3$CFU/g and potential crude oil utilizing fungi ranged from $1.30\pm0.99\times10^2$ - $1.20\pm1.13\times10^3$CFU/g in sediment. Query sequences submitted to GenBank all showed 100% relatedness to the isolates in the database. And were assigned Accession numbers as *Serratia marcescens* PV642521, *Priestia flexa* PV642520 and *Pseudomonas aeruginosa* PV642519 (hydrocarbonoclastic bacteria); *Aspergillus flavus* PV642586, *Penicillium citrinum* PV642585 and *Aspergillus fumigatus* PV642584 (hydrocarbonoclastic fungi). These autochthonous bacteria and fungi with the potential to utilize crude oil can be used as inoculum in the clean – up of crude oil polluted environment.

Keywords: Orashi River, Polluted Environment, Potential Hydrocarbonoclastic Bacteria, Fungi, *Priestia flexa*, GenBank,

Introduction

The high demand of oil has caused an extensive coastal oil production and transportation of oil products in the remote and difficult terrains (Akomah & Abu, 2018). According to the International Energy Agency (IEA) (2022), the global oil demand is rising by 5.5 million barrel per day and 3.3 million barrel per day in 2021 and 2022 respectively. The oil sheen noticed on the surface water of Orashi River is an indication that the River is burden with various anthropocene such as accidental or overflow of oil during transportation, oil wastewater from artisanal refinery, runoff from oil on land and pipeline leakage which have had a deleterious effect on aquatic life and human health due to their carcinogenic properties (Abdel-Shafy & Mansour, 2016; Neff *et al*., 2005). There is an urgent need for efficient and environmentally safe approach to mitigate the impact of oil pollution on the environment and bioremediation technology which involves the use of microorganisms such as fungi and bacteria with the potential to degrade and use hydrocarbons as a sole carbon source is an environmentally friendly and cost-effective technique (Adam *et al.,* 2015**;** Kumar et al., 2011**;** Nagtode *et al,* 2003). Additionally, bioremediation technique will not generate other pollutants in the process of degrading hydrocarbons in the environment. The release of hydrocarbons in the environment promotes the growth of these hydrocarbonoclastic bacteria and fungi and these consortia become adapted, exhibiting selective enrichment and genetic change (Dirisu, 2015).

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The aim of the study is isolation and genomic identification of potential hydrocarbonoclastic bacteria and fungi consortia from Orashi River, River State, Nigeria.

Materials and Methods

Description of Study Area

Orashi River is located in the lower Niger Delta basin and is a fresh water ecosystem. Various sampling locations along the Orashi River are: Odual axis, Emughan axis, Ogbema axis axis and Mbiama axis. Coordinates and map of these locations is described in Sokolo *et al*., (2025).

Sample Collection

Water samples were collected using sterile amber bottles from 10 – 15cm below surface water. The bottle was positioned facing the direction of flow of the water and its cap was open under the water, filled and fastened while still under the water (USEPA, 1991).

Sediment samples were collected using Van Veen grab (Manufactured by Royal Eijelkamp) and emptid into a stainless bowl and with the aid of a sterilized spatula, the sediment was introduced into amber bottles, sealed and properly labeled. All samples were kept in ice pack and immediately transported to the laboratory for analysis.

Preparation of sediment Sample for Analysis

Sediment was homogenized using ceramic mortar and pestle cleansed with 100% ethanol and air dried. After homogenization, the sediment was sieved with 2mm mesh sieve in order to remove the debris (Seiyaboh *et al*., 2016) and air dried.

Enumeration of Total Heterotrophic Bacteria and Total Fungi Population

Enumeration of total heterotrophic bacteria and Total Fungi Population was carried out by tenfold serial dilution (Harrigan & McCance, 1990) with normal saline. Prior to dilution, one gram (1g) of the sediment and one milliliter (1ml) of the surface water was measured and transferred into test tubes containing9ml of normal saline and a dilution of 10^{-3} and 10^{-4} were used.

An aliquot (0.1ml) from the dilutions were inoculated into nutrient agar plate for bacterial count and sabouraud dextrose agar plate for fungi count. Inoculation of the plated was done using spread plate method (APHA, 1998) and the plates were covered and incubated at 37^{o}C for 24 hours for bacteria count and 30^{o}C for 3 - 5 days for fungi count.

Enumeration of Culturable Potential Crude Oil Utilizing Fungi and Bacteria

Potential crude oil utilizing bacterial and fungal populations was enumerated using Colony Forming Unit per millilitre (CFU/ml) for surface water and Colony Forming Unit per gram (CFU/g) for sediment. After a tenfold serial dilution (Harrigan & McCance, 1990) of the surface water and sediment sample, spread plate method was used on a mineral salt medium (MSM) as modified by Institute of Pollution Study (1990). The MSM is composed of 0.5g of K_2HPO_4, 0.3g of $MgSO_4$. $7H_2O$, 0.3g of $Nacl_2$, 0.2g of $MnSO_4.H_2O$, 0.02g of $FeSO_4.6H_2O$, 0.03g of $NaNO_3$, 0.3g of $ZnCl_2$ and 15g of Agar into 1 liter of distilled water. Crude oil was supplied to the medium using incorporation method. The plates were incubated at 37^{o}C for 14 days for bacteria culture and room temperature of 30^{O}C for 10 days for fungi culture when discrete colony was seen. Potential crude oil (hydrocarbonoclastic) utilizing bacterial and fungal isolates obtained were enumerated and subculture into nutrient agar for bacteria isolates and Sabouraud dextrose agar (SDA) for fungi isolates to obtain pure culture and the isolates were stored in nutrient slant and SDA slant bottle at 4^{O}C for further analysis.

Molecular Identification of Isolates

DNA Extraction

Genomic DNA of potential crude oil utilizing bacterial and fungal isolates was extracted using using ZR fungal/bacterial DNA prep kit supplied by Inquaba South Africa. Pure cultured potential crude oil utilizing bacterial and fungal was suspended in 200µl isotonic buffer in a ZR bashing bead lysing tube, thereafter, 750µl lysing solution was added to the tube and its cap was fastened and tube was securely fitted in a cell disruptor with a 2ml holder assembly and processed at a maximum speed for 5 minutes. The ZR bashing bead was further centrifuged at 10,000xg for 1 minute.

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This process lysed the cell membrane, thereafter, 400µl of the supernatant in the tube was transferred into ZYMO-spin IV spin filter in a collection tube and centrifuged at 7,000xg for 1 minute. DNA binding buffer of 1,200µl was added to the collection tube bringing the final volume to 1,600µl and 800µl from the final volume was transferred into ZYMO- spin 11C column in a collection tube and centrifuged at 10,000xg for 1 minute. The flow through was discarded from the collection tube and the remaining volume was transferred to the same ZYMO-spin and spun at 10,000xg for 1 minute. DNA pre-wash buffer of 200µl was added to the ZYMO-spin 11C in a new collection tube and spun at 10,000xg for 1 minute, followed by the addition of 500µl fungal/bacterial DNA wash buffer and centrifuged at 10,000xg for 1 minute. The ZYMO-spin 11C column was transferred to a clean 1.5µl centrifuge tube and 100µl DNA elution buffer was added to the column matrix and centrifuged at 10,000xg for 30 seconds to elute the DNA. The spin column has a positively charge ion and the DNA is negatively charged, it is trapped in the matrix of the spin column. The ultra – pure DNA extracted was properly labelled and stored at -20ºC. The extracted DNA samples were quantified using Nanodrop 1000 spectrophotometer.

Polymerase Chain Reaction (PCR) Amplification

Polymerase Chain Reaction (PCR) amplication of 16S rRNA gene conserve for bacteria were amplified using forward and reverse (27F: 5'-AGAGTTTGATCMTGG CTCAG-3’ and 1492R: 5'-CGGTTACCTTGTTACG ACTT-3') primers and Internal Transcribed Spacer (ITS) gene that is conserved to all fungal were amplified using forward and reverse (ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3) primers, synthesized by Inqaba South Africa. The double strength mix comprises of: Taq polymerse – which is a heat- stable DNA polymerase extracted from thermophile bacteria known as *Thermus aquaticus*. Its function is to automate the repetitive step in amplifying the DNA; Magnesium Chloride serves as a cofactor which enhances enzymatic activities of DNA polymerase thus boosting DNA amplification; Deoxynucleoside Triphosphate (DNTP) serves as a building block for the synthesis of new DNA strand. As a result, it turns a single strand of DNA into two; Buffer which provide the Nuclease free water which serves as the diluent to the reaction; Primers (forward and reverse) which are short oligonucleotide that pair with the gene of interest (extracted DNA). The final volume of the master mix in the tube was 25µl and the tube containing the master mix was inserted into the thermal block of the thermal cycler. The PCR conditions were as follow: Initial denaturation temperature was 95ºC for 4 minutes; Denaturation temperature was 95ºC for 30 minutes; Annealing temperature was 52ºC for 3 minutes and holding temperature at 4ºC.

Agarose Gel Electrophoresis, Sequencing and Phylogenic Analysis

Amplicons were subjected to 1% agarose gel electrophoresis to ensure that 16s RNA gene and ITS gene bands were within 1500bp and 500 – 700bp respectively when compared to DNA ladder. The electrophoresis gel was set at 120 voltage for 15 minutes. Thereafter, the gel was placed on a UV transilluminator (Ce bro *et al*., 2008) in order for band to be visualized and captured. Sequences of 16S rRNA gene and ITS gene was generated using Big Dye Terminator kit. The sequence conditions was 32 cycle at 96ºC for 10 seconds, 55ºC for 5 seconds and 60ºC for 4 minutes.

Sequences of 16S rRNA and ITS gene generated were edited edited using bioinformatics algorithm and these sequences were downloaded into the datebase of National Centre for Biotechnology information (NCBI) using Basic Alignment Search Tool – Nucleotide (BLASTN). Sequences were aligned using ClustalX and evolutionary history was inferred using Neighbour – Joining method in MEGA 6.0 (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985).

Results

Total heterotrophic bacteria and crude oil utilizing bacteria count in surface water and sediment of Orashi River is shown in Table 1. The result revealed that total heterotrophic bacteria count in surface water ranged from $2.25\pm2.48\times10^6$ - $3.46\pm3.88\times10^6$ CFU/ml while potential crude oil utilizing bacteria were not recovered from the surface water. However, in the sediment total heterotrophic bacteria ranged from $1.76\pm1.75\times10^6$ - $2.29\pm2.41\times10^6$ CFU/g while potential crude oil utilizing bacteria ranged from $1.50\pm2.12\times10^2$ - $2.10\pm1.27\times10^3$CFU/g.

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Table 1: Population of total heterotrophic bacteria and total crude oil utilizing bacteria in surface water and sediment samples from various locations along Orashi River

Locations	Surface Water		Sediment	
	Total heterotrophic bacteria (CFU/ml)	Total crude oil utilizing bacteria (CFU/ml)	Total heterotrophic bacteria (CFU/g)	Total crude oil utilizing bacteria (CFU/g)
Odual	$2.25 \pm 2.48 \times 10^6$	0.0	$1.38 \pm 1.44 \times 10^6$	$3.0 \pm 4.24 \times 10^2$
Emughan	$2.73 \pm 2.93 \times 10^6$	0.0	$1.76 \pm 1.75 \times 10^6$	$8.5 \pm 0.21 \times 10^2$
Ogbema	$3.46 \pm 3.88 \times 10^6$	0.0	$2.29 \pm 2.41 \times 10^6$	$2.10 \pm 1.27 \times 10^3$
Mbiama	$3.13 \pm 2.81 \times 10^6$	0.0	$2.12 \pm 2.11 \times 10^6$	$1.50 \pm 2.12 \times 10^2$

Total fungi and crude oil utilizing fungi count is presented in Table 2. While the Genomic identification of potential Crude oil utilizing fungi and bacteria using polymerase chain reaction (PCR)and the result of the agarose gel electrophoresis result is seen in Plate 1 and Plate 2.

Table 2: Population of total fungi and total crude oil utilizing fungi in surface water and sediment samples from various locations along Orashi River

Locations	Surface Water		Sediment	
	Total fungi (CFU/ml)	Total crude oil utilizing fungi (CFU/ml)	Total fungi (CFU/g)	Total crude oil utilizing Fungi (CFU/g)
Odual	$6.5 \pm 0.64 \times 10^3$	$6.5 \pm 0.50 \times 10^2$	$2.30 \pm 2.40 \times 10^3$	$6.50 \pm 0.50 \times 10^2$
Emughan	$1.16 \pm 1.19 \times 10^3$	$1.20 \pm 1.13 \times 10^3$	$1.20 \pm 1.13 \times 10^3$	$1.75 \pm 1.77 \times 10^2$
Ogbema	$2.77 \pm 3.01 \times 10^4$	$1.80 \pm 1.70 \times 10^3$	$1.35 \pm 0.92 \times 10^3$	$1.30 \pm 0.99 \times 10^2$
Mbiama	$5.40 \pm 5.09 \times 10^3$	$8.1 \pm 0.84 \times 10^2$	$1.75 \pm 1.77 \times 10^3$	$1.20 \pm 1.13 \times 10^3$

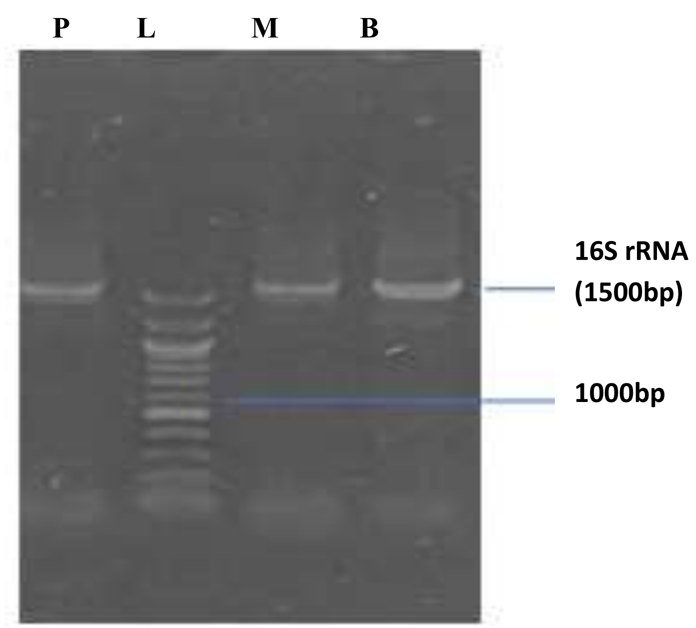

Plate 1: Agarose Gel Electrophoresis Showing the Amplified 16S rRNA of Hydrocarbonoclastic Bacteria Isolates. Lanes P, M and B Represent the Amplified 16S rRNA at 1500bp of *Serratia marcescens, Priestia flexa* and *Pseudomonas aeruginosa* respectively While Lane L Represents the 1000bp DNA Ladder

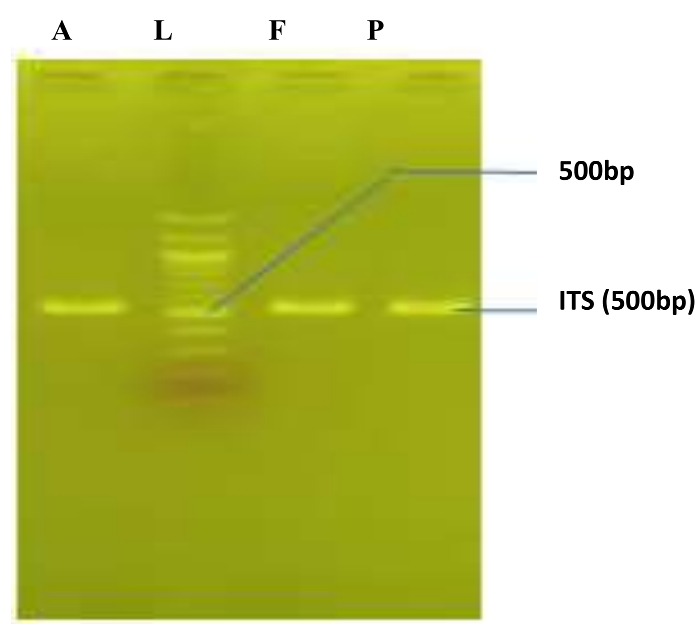

Plate 2: Agarose Gel Electrophoresis Showing the Amplified ITS fragment of Hydrocarbonoclastic Fungal Isolates. Lane A, F and P Represents the ITS at 500bp of *Aspergillus flavus, Aspergillus fumigatus* and *Penicillium citrinum* Respectively While Lane L Represents the 1000bp DNA Ladder

The result in Table 2 reveals that the total fungi count in surface water ranged from $1.16 \pm 1.19 \times 10^3$ - $6.5 \pm 0.64 \times 10^3$CFU/ml while potential crude oil utilizing fungi in surface water ranged from $8.1 \pm 0.84 \times 10^2$ - $1.80 \pm 1.70 \times 10^3$CFU/ml. Conversely, in the sediment, total fungi count ranged from $1.20 \pm 1.13 \times 10^3$ - $2.30 \pm 2.40 \times 10^3$CFU/g and potential crude oil utilizing fungi ranged from $1.30 \pm 0.99 \times 10^2$ - $1.20 \pm 1.13 \times 10^3$CFU/g.

The agarose gel electrophoresis result as shown in Plate 1 and Plate 2, indicates that the Targeted gene were amplified. Furthermore, the sequences were subjected to NCBI database and their evolutionary relatedness with that on the database was drawn as seen in Fig 1 & 2. Sequences of 16s rRNA and ITS gene were sent to Genbank and their accession numbers is revealed in Table 3 and Table 4.

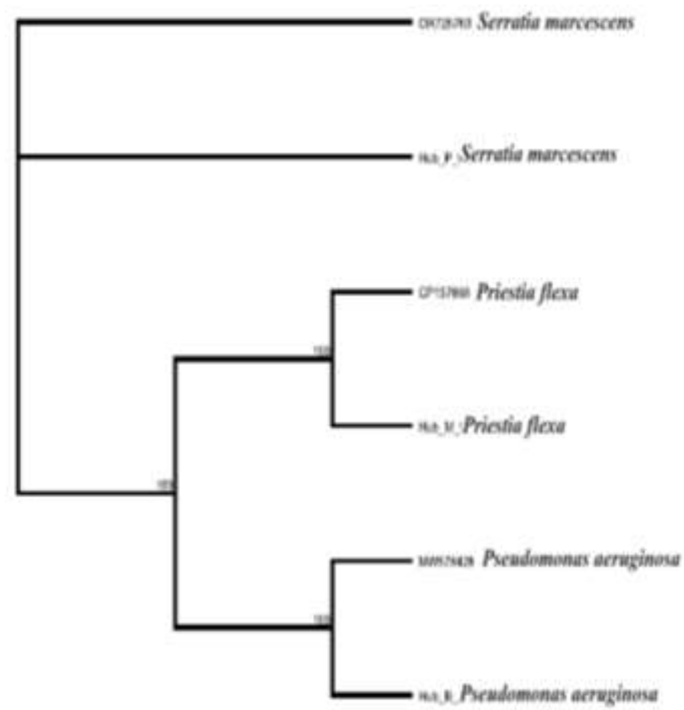

Fig. 1: Phylogenetic Tree Showing the Evolutionary Distance Between Potential Hydrocarbonoclastic Bacterial Isolates harvested from Orashi River

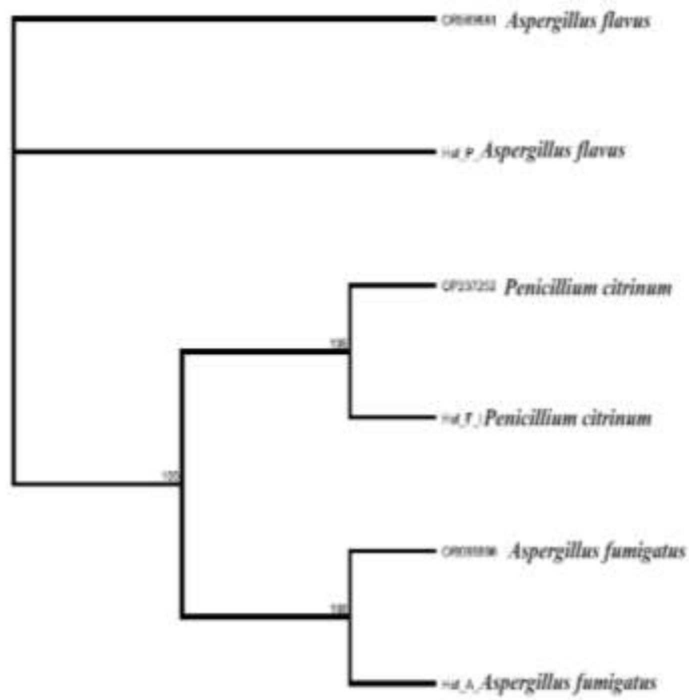

Fig. 2: Phylogenetic tree showing the evolutionary distance between potential hydrocarbonoclastic fungal isolates harvested from Orashi River

Table 3: Potential crude oil utilizing bacterial isolates and their GenBank relatives and accession numbers

Submission Number	Isolates Code	Query Isolates	Genbank Closest Organism	Percentage Relatedness	Genbank Accession Number
SUB15322247	Hub_P_907R_H10_22.ab1	*Serratia marcescens*	*Serratia marcescens* OR725763	100	PV642521
SUB15322247	Hub_M_908 R_F07_16.ab1	*Priestia flexa*	*Priestia flexa* CP157868	100	PV642520
SUB15322247	Hub_B_907-R_B08_05.ab1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* MW578428	100	PV642519

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Table 4: Potential Crude Oil UtilizingFungal Isolates and its GenBank Accession Numbers

Submission Number	Isolates Code	Query Isolates	Genbank Closest Organism	Percentage Relatedness	Genbank Accession Number
SUB15322292	Huf_P_ITS1_C10_07.ab1	*Aspergillus flavus*	*Aspergillus flavus* OR569681	100	PV642586
SUB15322292	Huf_F_ITS1_A10_01.ab1	*Penicillium citrinum*	*Penicillium citrinum* OR237252	100	PV642585
SUB15322292	Huf_A_ITS1_B10_04.ab1	*Aspergillus fumigatus*	*Aspergillus fumigatus* OR095908	100	PV642584

Discussion

This study has revealed the presence of crude oil utilizing bacteria and crude oil utilizing fungi in the surface water and sediment in some stations of Orashi River. The Ogbema station recorded high total heterotrophic bacteria and total crude oil utilizing bacteria count in surface water and sediment of Orashi River because the station sampling point was in close proximity to the Ogbema jetty were various anthropogenic activities are carried out. The bacteria diversity or total heterotrophic bacterial count in surface water which ranged from $2.25\pm2.48\times10^6$ - $3.46\pm3.88\times10^6$CFU/ml were high which could be as a result of human activity such as waste and wastewater effluent, oil wastewater from artisanal refinery and runoff from terrestrial environment. The high total heterotrophic bacterial count recorded is in consonance with Frank-Ogu *et al*., (2023) that reported high bacteria diversity which ranged from 1.00×10^6 – 9.70×10^6 CFU/ml from Otamiri River, Nigeria. Total fungi and crude oil utilizing fungi count (Table 2), in the surface water of Orashi River was high in Ogbema station compared to the other stations. The River from the Odual and Emughan stations recorded low fungi count compared to Ogbema station because both Rivers flows and collide to the direction of Ogbema station. The results of the total fungi and total crude oil utilizing fungi count were below the total fungi and crude oil utilizing fungi count reported by Entim & Antai (2007) which were $5.55\pm0.25\times10^5$ and $3.17\pm0.21\times10^4$ respectively from freshwater in Cross Rivers State, Nigeria. The middle reach of Orashi River that is Mbiama station recorded high total crude oil utilizing fungi than the tail end of Orashi stations which comprises of Odual, Emughan and Ogbema stations of this study.

Potential crude oil utilizing bacterial (Fig 1 and Table 3) isolated from Orashi River were *Serratia marcescens* with accession number PV642521; *Priestia flexa* with accession number PV642520 and *Pseudomonas aeruginosa* with accession number PV642519. *Priestia flexa* and *Serratia marcescens* were reported as hydrocarbon utilizing bacteria harvested from Eleme soil (Chunwafor *et al.,* 2024). *Pseudomonas aeruginosa* is a metabolically highly versatile organism inhibiting numerous ecological niches and are frequently isolated from hydrocarbon – contaminated environment (Norman *et al*., 2022; Cai *et al*., 2015). Potential crude oil utilizing fungi (Figure 1 and Table 4) isolated from Orashi River were *Penicillium citrinum* with accession number PV642585; *Aspergillus fumigatus* with accession number PV642584 and *Aspergillus flavus* with accession number PV642586.

These three hydrocarbonoclastic fungi harvested from Orashi River were reported has having the potential to utilize Phenanthrene as a source of carbon (Sokolo et al., 2025) and *Penicillium citrinum* were also isolated in Ebubu – Ejamah soil as hydrocarbon utilizing fungi (Sokolo *et al*., 2018). *Aspergillus flavus* and *Penicillium citrinum* are also reported as crude oil utilizing fungi (Barnes *et al*., 2018).

Petroleum hydrocarbon is a heterogeneous compound (Obire, 2018) and requires mixed microbial communities with the potential to degrade petroleum hydrocarbons (Thakur, 2012; Sokolo *et al.,* 2020). According to Dirisu (2015), bacteria are secondary invaders while fungi initially attack petroleum hydrocarbons due to their mycelial and penetrate the soluble fractions thus increasing the surface area making it available for bacterial to attack.

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Conclusion

The study reports potential crude oil utilizing bacteria such as *Priestia flexa*, *Serratia marcescens* and *Pseudomonas aeruginosa* and potential crude oil utilizing fungi from Orashi River which includes *Penicillium citrinum*, *Aspergillus fumigatus* and *Aspergillus flavus.* These autochthonous microorganisms can be harnessed and used as inoculum in the clean-up crude oil polluted environment.

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