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MOLECULAR CHARACTERIZATION AND TOLERANCE POTENTIAL OF CULTURABLE CRUDE OIL-DEGRADING MICROBES IN SANTA-BARBARA RIVER, BAYELSA STATE, NIGERIA

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ABSTRACT: *There has been chronic contamination of the surrounding aquatic and terrestrial environments in the Niger Delta region of Nigeria due to the enormous activities of crude oil exploration. This study aimed to characterize and assess the crude oil tolerance potential of indigenous microbes from crude oil spills in Santa Barbara River, Bayelsa State, Nigeria, that could serve as potential consortia for bioremediation of the crude oil spills. Total crude oil hydrocarbon-utilizing bacteria count (THUBC) and total crude oil hydrocarbon-utilizing fungi count (THUFC) in the samples were determined with a culture-dependent spread plate technique. Bacterial and fungal isolates were characterised using standard phenotypic and 16S/Internal Transcribed Spacer region rRNA gene sequencing techniques, respectively. The tolerance of autochthonous bacterial isolates to different concentrations of crude oil was subsequently determined. THUBC and THUFC in crude oil-polluted water samples ranged from 1.88 log_{10} CFU/ml to 2.74 log_{10} CFU/ml and from 0.00 log_{10} CFU/ml to 1.70 log_{10} CFU/ml, respectively. Representative strains of Proteus mirabilis, Bacillus subtilis, Pseudomonas aeruginosa, Micrococcus luteus and Aspergillus flavus isolates obtained from crude oil-polluted water samples were deposited in the GenBank (NCBI) under accession numbers OQ969924, OQ969951, OQ969987, OQ970009 and OQ975908. Proteus mirabilis, Pseudomonas aeruginosa and Aspergillus flavus demonstrated the most significant tolerance to crude oil pollutants (minimum crude oil inhibitory concentrations (MIC) = 80%) followed by Bacillus subtilis and Micrococcus luteus (MIC= 40%). The findings from this study are pointers to the potential role of the microbial isolates as bioremediation consortia to remediate the polluted Santa Barbara River.*

KEYWORDS: Crude oil spills, Bioremediation, *Proteus mirabilis*, *Aspergillus flavus*, Microbial tolerance, Internal Transcribed Spacer region rRNA gene.

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INTRODUCTION

Nigeria is placed at the eleventh position in the ranking of the largest crude oil producers in the world because of its estimated 35.2 billion barrels of crude oil reserve [1]. In 1956, crude oil was first discovered in commercial quantities in Nigeria, thereby signaling the start of oil exploration in Nigeria. Crude oil accounts for 70% of the total revenue accrued to Nigeria [2, 3, 4]. Most of the oil exploration in Nigeria is done in the Niger Delta region, which accounts for the country's oil reserves [5].

The National Oil Spill Detection and Response Agency (NOSDRA), established in 2006, is the Nigerian agency responsible for monitoring and responding to oil spills in Nigeria. However, acquiring spill data is difficult unless it has been published in scholarly journals or research reports to which authors could obtain access [6].

The spilt oil is reported to deeply penetrate some ecosystems, like the breeding areas for fish and other aquatic animals, and it also destroys some native plant species [7]. Some crude oil hydrocarbons have high lipophilicity, enhancing their bioaccumulating activity in the aquatic ecosystem; hence, the spills' soil and groundwater quality are constantly impacted by the spills [8]. Mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of crude oil hydrocarbons in freshwater and marine environments [9, 10]. Commonly used consortia of microbes for bioremediation, which are often identified by molecular characterizations of 16S/ITS rRNA genes, include *Achromobacter, Acinetobacter*, *Pseudomonas, Candida, Cryptococcus, Aspergillus* and *Cladosporium* [11]. This research aims to characterize and assess the crude oil-tolerance potential of indigenous microbes isolated from crude oil spills in Santa Barbara River, Bayelsa State, Nigeria, that could serve as potential consortia for bioremediation of the crude oil spills.

MATERIALS AND METHODS

Description of the Study Area

Santa Barbara River, geographical coordinates are Latitude 4.3358, 4^{o} 20. 89" North and Longitude 6.6022, 6^{o} 36. 81" East. It is situated in Nembe Local Government Area of Bayelsa State, Nigeria, lying south of the Brass Creek and East of the Odiame Creek. Crude oil spills from a blowout Nigerian National Petroleum Corporation and AITEO Exploration and Production Company Limited OML-29-WELL-1 which occurred on the 5th of November 2021 emptied over two million barrels of oil and gas into the Santa Barbara River. The spills subsequently spread into creeks and waterways in Kula and neighbouring communities such as Aberebiya, Arapakama, Angbakiri, Kalawo kiri, New camp, Inemaboko, Robert kiri, Ofoinama and Belama. It was halted on 8th December 2021. The Santa Barbara River flows into the Atlantic Ocean, and many communities bordering the river are rural.

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Study Design

Santa Barbara River was visited four times between May and August 2022 to collect crude oil-polluted water samples. On each visit, oil-polluted water samples were randomly collected in triplicate by scooping into calibrated sterile 30 ml containers to a depth of 15 cm. Sampling was at five different points along the downstream station of the Santa Barbara River over a distance of 500 to 800 meters away from the drilling facilities of Santa Barbara flow Station (OML-29). Overall, 60 oil-polluted water samples were collected from 20 sampling points along the Santa Barbara River. A total of 12 control water samples were collected from sampling points along the upstream station of the Santa Barbara River over a distance of over 1000 meters away from the drilling facilities where there are minimum influences of crude oil spillage. The water samples were assigned to four independent treatment groups using the iterative Wei's Urn randomization model [12]. The treatments that were performed were the total crude oil hydrocarbon-utilizing bacteria count (THUBC), total viable bacteria count (TVBC), total crude oil hydrocarbon-utilizing fungi count (THUFC) and total viable fungi count (TVFC).

Enumeration of Crude Oil Hydrocarbon-utilizing and Heterotrophic Microbes

THUBC, TVBC, THUFC and TVFC were performed according to previously described methods [13]. The THUBC, TVBC, THUFC and TVFC were determined with the spread plate technique. 100 µl of each of the dilutions were spread onto Tryptic Soy agar Petri plates in duplicate for isolation and enumeration of total viable bacteria, as well as onto Bushnell Haas agar Petri plates in duplicate amended with 1% sterile crude oil (BHC) for isolation and enumeration of crude oil hydrocarbon-utilizing bacteria.

For THUFC, 100 µl of each dilution was spread on Bushnell Haas agar medium amended with 1% sterile crude oil, 4% glucose and 0.005% chloramphenicol. TVFC was done by spreading 100 µl of each dilution of the samples on Sabouraud Dextrose agar (SDA) plate amended with chloramphenicol to suppress bacterial growth. The TSA and BHC plates were incubated for 48 hours at 35^{o}C for bacterial growth while SDA and BHCG Petri dishes were incubated at room temperature for up to 7 days. Enumeration of the colonies were expressed as bacterial (THUBC and TVBC) or fungal (THUFC and TVFC) colony-forming units per millilitre (CFU/ml) of the water sample.

Characterization of the Microbial Isolates

Genus-level and species-level identification were performed using standard phenotypic and molecular techniques respectively [14, 15, 16, 17, 18, 19].

Phenotypic Identification of the Microbial Isolates

The phenotypic tests used in the identification of the bacterial isolates included Gram-staining, coagulase, catalase, oxidase, citrate, indole, Voges Proskauer, methyl red tests, as well as sugar (lactose and mannitol) fermentation tests [14]. Mycological examination of the fungal phenotypes was based on colonial morphology and microscopic characterization [15].

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Molecular Identification of the Microbial Isolates

Molecular identification employed techniques that involved polymerase chain reaction (PCR) of template DNA extracted from the bacterial and fungal isolates. This was followed by sequencing of the partial 16S and ITS rRNA gene amplicons obtained from the bacterial and fungal isolates, respectively. Universal 16S rRNA bacterial primers [27F-AGAGTTTGATCMTGGCTCAG; 1492R GGTTACCTTGTTACGACTT] often employed for bacterial taxonomy were used to determine the presence of 16S rRNA gene, while ITS rRNA gene primers (ITS1-F- CTT GGT CAT TTA GAG GAA GTA A; ITS4- TCC TCC GCT TAT TGA TAT GC) were used to detect the presence of ITS rRNA gene. Amplification of 16S rRNA gene was done in a GeneAmp PCR system 9700 (Applied Biosystems, United States) with the following cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles, with each cycle consisting of denaturation at 94°C for 45 seconds, annealing at 55^{o}C for 60 seconds, extension at 72^{o}C for 120 seconds, and a final extension at 72°C for 300 seconds. Amplification ITS rRNA gene was performed in a GeneAmp PCR system 9700 (Applied Biosystems, United States) with the following cycling conditions: initial denaturation at 95^{o}C for 5 minutes followed by 30 cycles of denaturation at 95^{o}C for 1 minute, annealing for 30 seconds at 55°C, extension for 1 min at 72 °C, and a final extension at 72 °C for 7 minutes. DNA sequencing of the amplicons was performed with the dideoxy-chain termination method [16]. Taxonomic classification of the microbial isolates was confirmed by a comparison of the experimentally determined nucleotide sequence against the sequence database (rRNA_typestrains/prokaryotic_16S_ribosomal_RNA). The sequence comparison was performed with the BLASTN 2.8.0 + program [National Center for Biotechnology Information (NCBI)]. The sequences of the identified microbial species were subsequently deposited in the NCBI GenBank database under specific accession numbers.

Phylogenetic Analysis

Multiple sequence alignments were implemented with the MUSCLE algorithm in MEGA software, version 6 [20]. Phylogenetic trees were constructed using the neighbour-joining algorithm in MEGA software. The statistical significance of the clusters in the trees was estimated by bootstrap iterations (1000 replications).

Evaluation of the Crude Oil Tolerance

The microbial isolates were inoculated into Bushnell Hass agar plates containing various concentrations of crude oil, ranging from 5% to 80% v/v crude oil, using the spread plate technique. The plates were incubated at 35^{o}C for 24 hours for bacteria, and 3–7 days for fungi. After incubation, Petri plates were examined for growth. Tolerance was measured by observing the minimum inhibitory concentration (MIC) of the crude oil on the microbial isolates.

Statistical Analysis

The discrete THUBC, TVBC, THUFC and TVFC were logarithmically transformed into continuous variables. Descriptive statistics of THUBC, TVBC, THUFC and TVFC were done with NCSS ver. 12 data analysis software. Shapiro–Wilk normality test, Levene test of homogeneity and non-parametric Mann-Whitney U (Wilcoxon rank-sum) test were also performed with NCSS ver. 12 data analysis software.

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RESULTS

Table 1 shows the THUBC, TVBC, THUFC and TVFC in the samples. The THUBC in the crude oil-polluted water samples ranged from 1.88 $\log_{10}$ CFU/ml to 2.74 $\log_{10}$ CFU/ml, with a mean count of 2.39 ± 1.25 CFU/ml. The TVBC in the crude oil-polluted water were between 2.04 $\log_{10}$ CFU/ml and 2.78 $\log_{10}$ CFU/ml, with a mean count of 2.47 ± 1.26 CFU/ml. Those of the control samples ranged from 2.60 $\log_{10}$ CFU/ml to 2.91 $\log_{10}$ CFU/ml for THUBC and from 3.02 $\log_{10}$ CFU/ml to 3.42 $\log_{10}$ CFU/ml for TVBC, respectively, with mean counts of 2.71 ± 1.65 CFU/ml for THUBC and 3.24 ± 2.14 CFU/ml for TVBC. The THUFC in the crude oil-polluted water samples were between 0.00 $\log_{10}$ CFU/ml and 1.70 $\log_{10}$ CFU/ml, with a mean count of 1.18 ± 0.27 CFU/ml. The TVFC in the crude oil-polluted water samples ranged from 1.00 $\log_{10}$ CFU/ml to 1.85 $\log_{10}$ CFU/ml, with a mean count of 1.53 ± 0.37 CFU/ml. Those of the control samples ranged from 2.54 $\log_{10}$ CFU/ml to 2.85 $\log_{10}$ CFU/ml and from 3.00 $\log_{10}$ CFU/ml to 3.31 $\log_{10}$ CFU/ml, respectively, with mean counts of 2.66 ± 1.52 CFU/ml and 3.15 ± 2.04 CFU/ml for THUFC and TVFC, respectively.

The non-parametric Mann-Whitney U (Wilcoxon rank-sum) test showed that there was a significant difference ($p < 0.05$) between THUBC and TVBC in the oil-polluted water samples, indicating that the counts of all viable bacteria were more than those of only the crude oil hydrocarbon-utilizing bacteria. Similarly, THUFC in the oil-polluted water samples was significantly different from the TVFC, also revealing that counts of all fungi in the oil-polluted water samples was higher than those of only the crude oil hydrocarbon-utilizing fungi. When THUBC and TVBC were compared to THUFC and TVFC, a significant difference ($p < 0.05$) was observed, indicating that the bacterial organisms were more in the crude oil-polluted water samples than the fungal organisms. The non-parametric Mann Whitney U (Wilcoxon rank-sum) test also showed that there were significant differences ($p < 0.05$) between THUBC, TVBC, THUFC and TVFC obtained from the oil-polluted water samples and those of the control samples, indicating that bacteria and fungi were more in the control samples when compared to those present in the oil-polluted water samples.

Phenotypic and Molecular Characterizations of the Microbial Isolates

Table 2 shows the characterization of the bacterial isolates obtained from the water samples. *Proteus*, *Micrococcus*, *Bacillus* and *Pseudomonas* were the main bacterial genera that were identified in the crude oil polluted-water samples, while *Bacillus*, *Pseudomonas* and *Proteus* were the main genera that were identified in the control samples. The 16S rRNA gene analysis revealed the speciation of the representative isolated genera in the oil-polluted water samples to be *Proteus mirabilis*, *Micrococcus luteus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, and in the control samples as *B. subtilis*, *P. aeruginosa* and *P. mirabilis*. Representative *P. mirabilis* strain BLESSING 101, *B. subtilis* strain BLESSING 102, *P. aeruginosa* strain BLESSING 103 and *M. luteus* strain BLESSING 104 isolated from the crude oil-polluted samples have been deposited in the United States NCBI GenBank under accession numbers OQ969924, OQ969951, OQ969987 and OQ970009, respectively. Representative *P. mirabilis* strain BLESSING 105, *P. aeruginosa* strain BLESSING 106 and *B. subtilis* strain BLESSING 107 isolated from the control samples have also been deposited in the GenBank under accession numbers OQ974182, OQ970066 and OQ970109, respectively.

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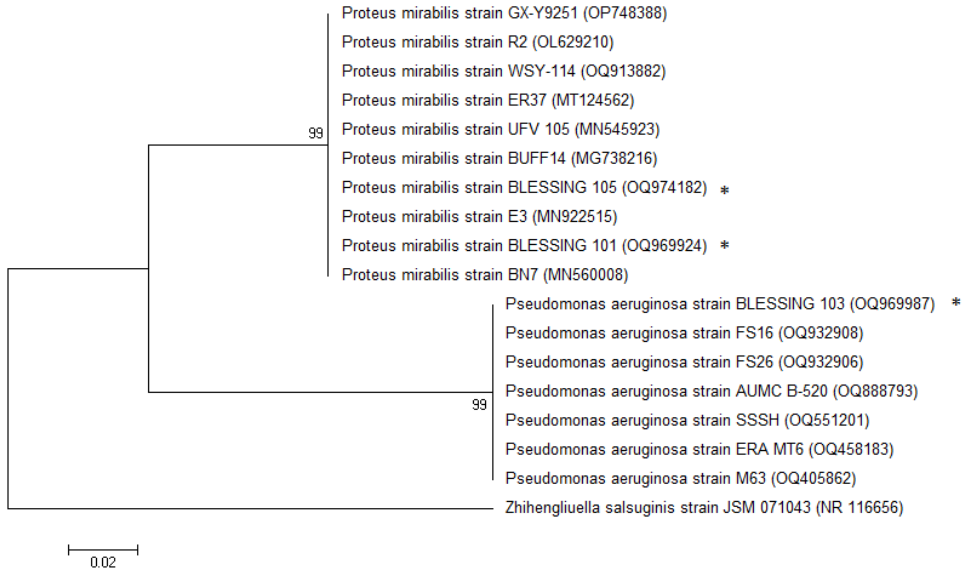
Figure 1: Neighbour-joining algorithm used to construct a phylogenetic tree emphasizing bacterial strains within the Phylum Proteobacteria



*** is used to indicate *P. mirabilis* and *P. aeruginosa* strains isolated from water samples collected from the crude oil-polluted Santa Barbara River. GenBank accession numbers of all the strains used to implement the phylogenetic tree are indicated in parenthesis. The tree was rooted on midpoint and only bootstrap values that were above 50 % are displayed on branches.**

Phenotypic and Molecular Characterization of the Fungal Isolates

Table 3 below shows the characterization carried out on fungal isolates obtained from the water samples. ITS rRNA gene analysis showed that *A. flavus* was the main fungal species that was identified in the crude oil polluted-water samples, while *A. niger* and *A. flavus* were the main species that were isolated from the control samples. Representative *A. flavus* strain BLESSING 108 isolated from the crude oil-polluted water samples, as well as *A. flavus* strain BLESSING 109 and *A. niger* strain BLESSING 110 isolated from the control samples, have been deposited in the United States GenBank under accession numbers OQ975908, OQ975909 and OQ975940, respectively. Gel electrophoresis of amplified ITS rRNA genes obtained from fungal species isolated from water samples collected from the crude oil-polluted Santa Barbara River is shown in Figure 1 above.

Phylogeny

Phylogenetic trees highlighting the evolutionary relatedness of bacterial/fungal strains from water samples collected from the crude oil-polluted Santa Barbara River and reference strains isolated from other environmental sources in the world are shown in Figures 2–5. Partial 16S and ITS rRNA gene sequences were selected from the present study and the United States NCBI GenBank database to construct phylogenetic trees using the neighbor-joining algorithm.

In the phylum Proteobacteria phylogeny (Figure 2), *P. mirabilis* strain BLESSING 101 and *P. mirabilis* strain BLESSING 105 were found in the same cluster with reference *P. mirabilis*

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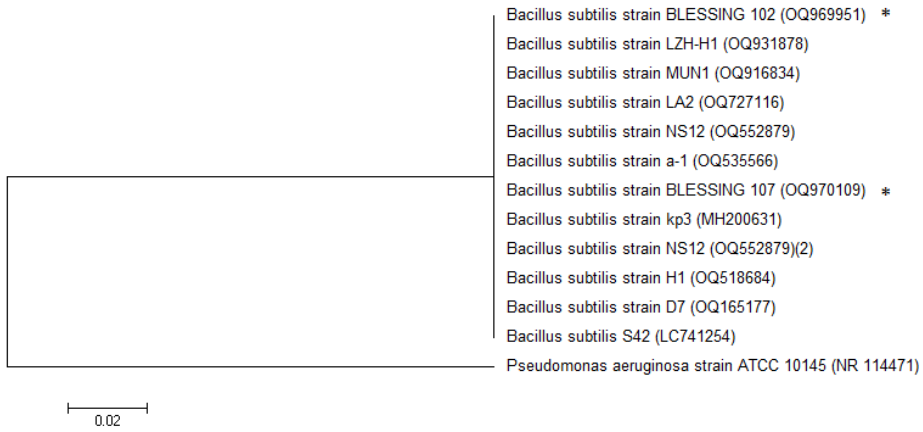
strains used to construct the phylogenetic tree, and with a 99% likelihood that these strains shared a common ancestry.

As observed in the phylum Firmicutes phylogeny (Figure 3), there was a 100% likelihood that the clustered *B. subtilis* strain BLESSING 102, *B. subtilis* strain BLESSING 107 and the reference strains originated from a common ancestor.

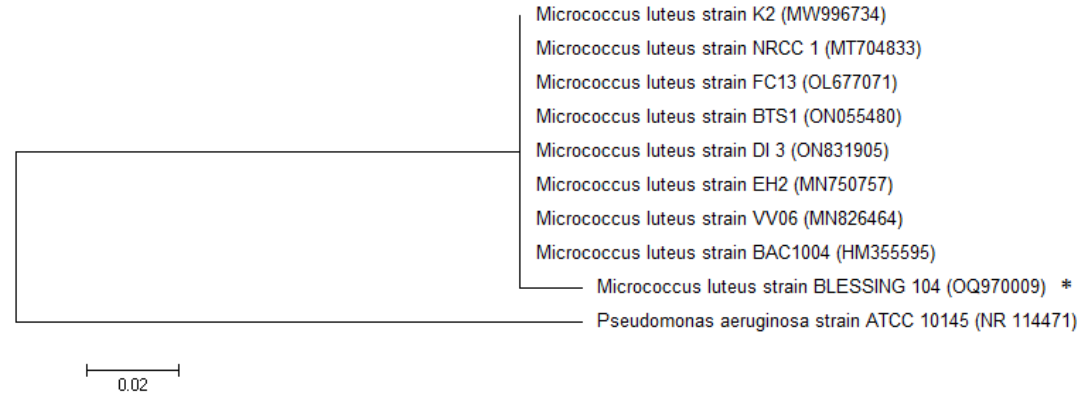
Figure 2: Phylogenetic tree highlighting bacterial strains of the Phylum Proteobacteria constructed with the neighbour-joining algorithm



*** is used to indicate *B. subtilis* strains isolated from water samples collected from the crude oil-polluted Santa Barbara River. GenBank accession numbers of all the strains used to implement the phylogenetic tree are indicated in parenthesis. The tree was rooted on midpoint and only bootstrap values that were above 50 % are displayed on branches.**

In the phylum Actinobacteria phylogeny (Figure 4), the *M. luteus* strain BLESSING 104 isolated from the crude oil-polluted Santa Barbara River appeared to have slightly diverged from the reference *M. luteus* strains used to construct the phylogenetic tree.

Figure 3: Utilizing the neighbor-joining algorithm, this figure shows a phylogenetic tree highlighting bacterial strains belonging to the Phylum Firmicutes



*** is used to indicate *M. luteus* strains isolated from water samples collected from the crude oil-polluted Santa Barbara River. GenBank accession numbers of all the strains used to implement the phylogenetic tree are indicated in parenthesis. The tree was rooted on midpoint and only bootstrap values that were above 50 % are displayed on branches.**

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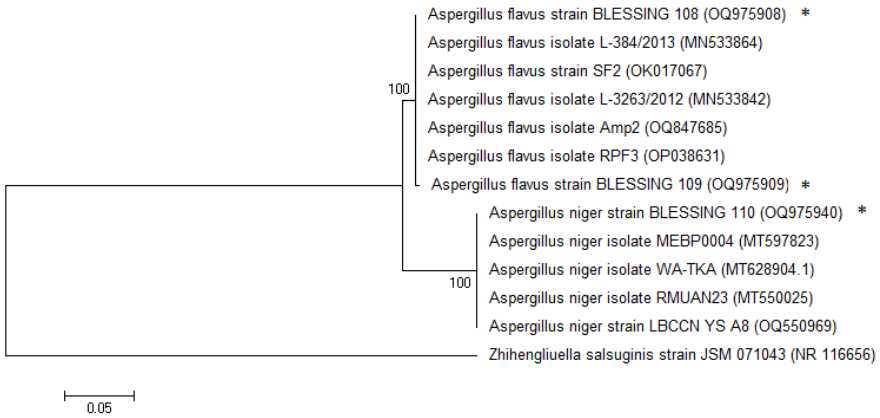
In the phylum Ascomycota phylogeny (Figure 5), *A. flavus* strain BLESSING 108 and *A. flavus* strain BLESSING 109 shared the same cluster with reference *A. flavus* strains used to construct the phylogenetic tree and with 100% likelihood that these strains evolved from a common ancestor.

Figure 4: Phylogenetic tree showcasing the fungal strains of the Phylum Ascomycota constructed with the neighbour-joining algorithm



*** is used to indicate *A. flavus* and *A. niger* strains isolated from water samples collected from the crude oil-polluted Santa Barbara River. GenBank accession numbers of all the strains used to implement the phylogenetic tree are indicated in parenthesis. The tree was rooted on midpoint and only bootstrap values that were above 50 % are displayed on branches.**

Crude Oil Tolerance Potential of the Microbes

The crude oil tolerance potential of the bacterial and fungal isolates obtained from the crude oil-polluted water samples and the control samples is shown in Table 4. The minimum inhibitory concentration (MIC) referred to the least concentration of crude oil that inhibited visible growth of all the colonies of a microbial species tested. *P. mirabilis* and *P. aeroginosa* isolates obtained from the crude oil-polluted water samples had the highest MIC (MIC = 80%) while *B. subtilis* and *M. luteus* had the lowest MIC (40%). For the control samples, *P. mirabilis* had the highest MIC of 40% while the lowest MIC of 20% was exhibited by *B. subtilis* and *P. aeroginosa*. *A. flavus* isolated from the crude oil-polluted water samples had an MIC of 80%. For the control samples, *A. niger* and *A. flavus* had MIC of 40% each.

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DISCUSSION

It is an acknowledged fact that environmental pollution issues associated with petroleum exploration and production subsist in the Niger Delta area of Nigeria. The task over the quality of crude oil-polluted waters applies not just to the water itself but also to the risk of diffusion of toxic substances into other ecosystems [21-23]. Crude oil pollution is presently considered a great risk to the health of living things in the environment, including humans. Although people are becoming increasingly worried about the toxic effects of oil pollution on humans and animals in affected areas, the fierce toxic brunt of petroleum hydrocarbons on affected microbial communities is frequently overlooked [24]. Microorganisms are omnipresent, and when faced with severe conditions, some still find a way of thriving in the environment through several means of adaptation, such as mutation in membrane permeability, switch of metabolic pathways and spore development [25, 26].

In the present study, the mean THUBC and THUFC in the crude oil-polluted water samples were 2.39 ± 1.25 CFU/ml and 1.18 ± 0.27 CFU/ml, respectively (Table 1). The total crude oil hydrocarbon-utilizing bacteria and fungi counts obtained from the present study were significantly lower than those reported by Udomessien *et al*. [27] who worked with polluted water samples from the Iko River situated within the petroleum belt of the Niger Delta, Nigeria, but were relatively similar to those in the study of Ogbonna *et al.* [28] who carried out an analysis of water samples from crude oil-impacted surface water in Bodo/Bonny River, Nigeria. As shown in Table 1, the ability to isolate elevated numbers of certain oil-degrading microorganisms from the crude oil-polluted environment is often taken as proof that these microorganisms are the vigorous degraders of that environment, with the crude oil tolerance test and Gas Chromatography-Mass Spectrometry (GC-MS) confirming the ability of these microorganisms to degrade or utilize crude oil hydrocarbons [29, 30]. In the recent past, the applications of microbes to deal with environmental pollutants have become an auspicious technology because of its economical and eco-friendly nature [31]. However, extremely elevated levels of petroleum hydrocarbons firmly obstruct microbial growth, resulting in meagre biodegradation efficiency and even death of the microbes [32, 33].

P. mirabilis, *M. luteus*, *B. subtilis* and *P. aeruginosa* were the bacterial species that were isolated from the crude oil-polluted water samples (Table 2). In agreement with the present study, Olajide and Adeloye [34] isolated *P. mirabilis* from crude oil-polluted water in the Bonny community, Niger Delta, Nigeria. Ramoutar *et al*. [35] reported that indigenous bacteria isolated from Pitch Lake at La Brea in Trinidad were capable of degrading petroleum hydrocarbons. *A. flavus* was the main fungal species that was isolated in the crude oil polluted water samples examined in this study (Table 3). In the study of Ogbonna *et al.* [28], *A. flavus* was also isolated from crude oil-impacted surface water in Bodo/Bonny River, Nigeria. Ramoutar *et al*. [35] isolated *Aspergillus*, *Fusarium*, *Penicillium*, *Curvularia* and *Mucorales* that were able to break down polycyclic aromatic hydrocarbons in crude oil-polluted Pitch Lake, Trinidad. A great number of scientists have disclosed that mixed populations of bacteria and fungi with wide-ranging enzymatic capacities are needed to break down complex mixtures of hydrocarbons such as crude oil in freshwater and marine environments, according to Khairul Anuar *et al*. [36]. Fungi are particularly superior candidates for remediation because they have long-range transportation systems since the movement of hyphae is not limited by a hydrophobic environment, thereby surpassing air-water interfaces and air-filled pores in the soil where they aid in the transport of extra-hyphal fungi that would otherwise be limited by

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this physical barrier. Fungal hyphae can operate in anoxic conditions, which are often the case with thick oil slicks and can condone fierce environments, especially due to the xero- and osmo-tolerant nature of numerous fungi which can survive in a pH range of 1–9 and at temperatures of 5–60ºC. Unlike bacteria, numerous fungi have extracellular enzymes that can break down hydrocarbons and are capable of digesting recalcitrant hydrocarbons [37, 38]. Extracellular enzymatic alteration of recalcitrant compounds by fungi, followed by bacterial degradation of the ensuing intermediates generated through fungal action, may play a part in the combinatorial strategy for the breakdown of recalcitrant hydrocarbons [11, 39].

P. mirabilis, *P. aeroginosa* and *A. flavus* isolates obtained from the crude oil-polluted water samples had the highest MIC [MIC = 80%] (Table 4). Similar findings by Nkanang *et al.* [40] also revealed that *Proteus* species were more tolerant to high levels of crude oil hydrocarbons. However, the MIC of *A. flavus* in this study was at variance with MIC of 20% reported for *A. flavus* species reported in the study by Al-Zaban *et al.* [41]. The awareness of the potential of microbial isolates to break down hydrocarbons is bound to increase the likelihood of developing more vigorous microbial strains that could be of relevance in the bioremediation of petroleum-contaminated environments. Microbes are vital agents in petroleum degradation, and they work as degraders of spilt oil in the environment [42]. There is a need to maintain the use of natural cleanup of crude oil-polluted environments, especially by suitable microbial consortia, because it will decrease chemical deposits in the environment and enhance climate sustainability [43].

CONCLUSION

Isolation of microbes with 40–80% crude oil tolerance in the crude-oil polluted water samples, as revealed in this study, is a pointer to their potential role as bioremediation consortia to remediate the polluted Santa Barbara River, thereby recovering the polluted river of the crude oil contaminants and ultimately restoring the aquatic ecosystem.

Ethics Approval and consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Availability of Data and Materials

The dataset from the study are available to the corresponding author upon request.

Poster Presentation at British Ecological Society Annual Conference

A poster version of this manuscript was presented at the British Ecological Society Annual Conference on the 13th of December, 2023.

Competing Interests

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Authors have declared that they have no competing interests.

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Table 1: Microbial counts in water samples collected from Santa Barbara River

Microbial counts							
Mean THUBC		Mean TVBC		Mean THUFC		Mean TVFC	
Oil-polluted water N = 60 Log_{10} CFU/ml	Control N = 12 Log_{10} CFU/ml	Oil-polluted water N = 60 Log_{10} CFU/ml	Control N = 12 Log_{10} CFU/ml	Oil-polluted water N = 60 Log_{10} CFU/ml	Control N = 12 Log_{10} CFU/ml	Oil-polluted water N = 60 Log_{10} CFU/ml	Control N = 12 Log_{10} CFU/ml
2.39 ± 1.25	2.71 ± 1.65	2.47 ± 1.26	3.24 ± 2.14	1.18 ± 0.27	2.66 ± 1.52	1.53 ± 0.37	3.15 ± 2.04

THUBC: total crude oil hydrocarbon-utilizing bacteria count; TVBC: total viable bacteria count; THUFC: total crude oil hydrocarbon-utilizing fungi count; TVFC: total viable fungi count; N: number of samples examined; Mean counts presented as mean ± standard error of the mean.

Table 2: Characterization of bacterial isolates obtained from the Santa Barbara River

Water samples examined	Representative bacterial colonies	Colonial and morphological characteristics		Biochemical characterizations									Molecular analysis		Identified bacteria	Prevalence of identified bacteria	
		Growth on the agar Petri plates	Gram staining	CO	CA	OX	IN	MR	VP	CI	LA	MA	16S homology	16S identity		F	%
Oil-polluted sample	1	Mucoid colonies	Negative rods	NP	+	-	-	+	-	+	-	-	99 – 100%	100%	*Proteus mirabilis*	598/720	83.06
	2	Mucoid colonies	Positive cocci	-	+	+	-	-	-	-	V	V	95 - 99%	95 - 98%	*Micrococcus luteus*	58/720	8.06
	3	Mucoid colonies	Positive rods	NP	+	V	NP	NP	NP	+	V	+	98 - 100%	97 - 98%	*Bacillus subtilis*	36/720	5.00
	4	Mucoid colonies	Negative rods	NP	+	+	-	-	-	+	-	+	96 - 98%	95 - 98%	*Pseudomonas aeruginosa*	28/720	3.89
Control sample	1	Mucoid colonies	Positive rods	NP	+	V	NP	NP	NP	+	V	+	99 - 100%	99 - 100%	*Bacillus subtilis*	79/144	54.86
	2	Mucoid colonies	Negative rods	NP	+	V	NP	NP	NP	+	V	+	96 - 98%	95 - 97%	*Pseudomonas aeruginosa*	36/144	25.00
	3	Mucoid colonies	Negative rods	NP	+	-	-	+	-	+	-	-	98 - 100%	96 - 98%	*Proteus mirabilis*	29/144	20.14

CO: Coagulase test. CA: Catalase test. OX: Oxidase test. CI: Citrate test. IN: Indole test. MR: Methyl red test. VP: Voges Proskauer test. LA: Lactose fermentation test. MA: Mannitol fermentation test. +: Positive results. -: Negative results. F: Fractional prevalence. %: Percentage prevalence. V: Variable reaction. NP: Not performed.

Table 3: Characterization of fungal isolates obtained from the Santa Barbara River

Water samples examined	Representative isolates	Morphology on Petri plates	Microscopy	Molecular analysis		Identified fungi	Prevalence of identified fungi	
				ITS homology	ITS identity		F	P (%)
Oil-polluted sample	1	White velvety colonies on surface of agar plates that turn yellowish-green	Conidiophores arising from septate hyphae with central vesicles that were completely filled with conidia.	98 - 100%	97 - 99%	*Aspergillus flavus*	209/209	100.00
Control sample	1	White colonies on surface of agar plates that turn black	Conidiophores arising from septate hyphae with central vesicles that were completely filled with conidia.	98 - 99%	97 - 99%	*Aspergillus niger*	86/144	59.72
	2	White velvety colonies on surface of agar plates that turn yellowish-green	Conidiophores arising from septate hyphae with central vesicles that were completely filled with conidia.	99 - 100%	97 - 100%	*Aspergillus flavus*	58/144	40.28

F: Fractional prevalence. P: Percentage prevalence

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Table 4: Tolerance potential of isolated microbes to varying concentrations of crude oil

Water samples examined	Identified microbial species tested	Varying concentrations of the crude oil examined									
		5%		10%		20%		40%		80%	
		F	N %	F	N %	F	N %	F	N %	F	N %
Oil-polluted water	*Proteus mirabilis*	598/598	100.00	598/598	100.00	598/598	100.00	574/598	95.99	0/598	0.00
	Micrococcus luteus	58/58	100.00	39/58	70.91	6/58	10.35	0/58	0.00	0/58	0.00
	Bacillus subtilis	36/36	100.00	23/36	63.89	3/36	8.33	0/36	0.00	0/36	0.00
	Pseudomonas aeruginosa	28/28	100.00	22/28	78.75	21/28	75.00	5/28	17.86	0/28	0.00
Control sample	*Bacillus subtilis*	79/79	100.00	31/79	39.24	0/79	0.00	0/79	0.00	0/79	0.00
	Pseudomonas aeruginosa	36/36	100.00	5/36	13.89	0/36	0.00	0/36	0.00	0/36	0.00
	Proteus mirabilis	15/15	100.00	15/15	100.00	8/15	53.33	0/15	0.00	0/15	0.00
Oil-polluted water	*Aspergillus flavus*	209/209	100.00	146/209	69.86	73/209	34.93	12/209	5.74	0/209	0.00
Control sample	*Aspergillus niger*	86/86	100.00	38/86	44.19	22/86	25.58	0/86	0.00	0/86	0.00
	Aspergillus flavus	58/58	100.00	33/58	56.90	18/58	31.04	0/58	0.00	0/58	0.00

F: Fractional prevalence of microbial isolates that showed growth in the varying concentrations of the crude oil. N: percentage prevalence of microbial isolates that showed growth in the varying concentrations of the crude oil.

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