

Original Research Article

Culture based isolation and characterization of Endophytic Bacteria isolated from the roots and leaves of Mangrove plants *Avicennia germinans* (Black mangrove), *Acrostichum aureum* (golden leather fern) and *Rhizophora mangle* (Red mangrove).

Abstract

Aim: The aim of this study is to isolate and characterize the culturable endophytic bacteria from the roots and leaves of three different mangrove plants found growing in same environment.

Study Design: This study employs experimental design, statistical analysis of the data and interpretation.

Place of Study: The roots and leaves of three mangrove plants; *Rhizophora mangle*, *Avicennia germinans* and *Acrostichum aureum* were gotten from a waterfront location in old Port Harcourt township of Rivers State, situated at Longitude 4° 45.18'N and Latitude 7° 05.35' E

Methodology:

Using standard Microbiological techniques, the roots and leaves were treated and endophytic bacteria isolated and subjected to morphological and biochemical tests.

Results:

From the roots, the hydrocarbon utilizing bacteria counts ranged from 7.0×10^3 cfu/g – 1.6×10^4 cfu/g, while the that of the leaves ranged from 3.0×10^5 cfu/g – 8.0×10^5 cfu/g, the nitrogen fixing bacteria counts for roots ranged from 4.5×10^3 cfu/g – 1.0×10^4 cfu/g and that of the leaves ranged from 3.0×10^5 cfu/g – 6.0×10^5 cfu/g, nitrifying bacteria counts for the roots ranged from 5.0×10^3 cfu/g – 7.0×10^3 cfu/g while the counts from the leaves ranged from 3.5×10^5 cfu/g - 5.0×10^5 cfu/g. Out of the 24 isolates, 14 were from the roots, while 10 were from the leaves. Percentage of occurrence of the isolates was in this order *Bacillus* sp. 33% > *Staphylococcus* sp. 21% > *Klebsiella* sp. 13% > *Pseudomonas* sp. 9% > *Micrococcus* sp. 8% > *Nitrobacter* sp. 8% > *Nitrosomonas* sp. 4% > *Azotobacter* sp. 4%. This reveals that *Bacillus* sp. occurred most in the plant samples. The result also revealed that the Red Mangrove Roots had the highest number of organisms. The endophytic bacteria isolated were further subjected to morphological and biochemical identification and they were identified as: *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Klebsiella* sp., *Azotobacter* sp., *Nitrobacter* sp. and *Nitrosomonas* sp. Five of the isolates showed a high potential to degrade crude oil in the following order *Pseudomonas* sp. (H2) > *Bacillus* sp. (BA) > *Klebsiella* sp. (SB) > *Bacillus* sp. (TG) > *Pseudomonas* sp. (H1).

Conclusion:

From the results, the mangrove roots and leaves contained high numbers of active indigenous bacteria most of which are known to use up crude oil as their carbon source. The mangrove roots had higher number of endophytic bacteria than the leaves.

Keywords: Endophytic bacteria; mangrove roots; nitrogen fixation; leaves

Introduction

Nigeria has the largest mangrove forest in Africa and the third-largest in the world after India and Indonesia [1]. Mangroves are found in nine states out of the 36 states of Nigeria namely Abia, Akwa-Ibom, Bayelsa, Cross River, Delta, Edo, Imo, Ondo, and Rivers States, and they are generally referred to as the Niger Delta (James *et al.*, 2013). Notwithstanding approximately 80% of Niger Delta mangrove forest has its vegetation spread evenly in only three states: Bayelsa, Rivers and Delta States. The largest stretch of about 30 to 40 kilometers of mangroves can be found in the Niger Delta region [1]. There are over hundred species of mangroves globally, but in the Niger Delta region the species commonly found are red (*Rhizophora* species), black (*Avicennia germinans*), white (*Laguncularia racemosa*), and golden leather fern (*Acrostichum aureum*) mangroves [2].

Mangrove ecosystem is one of the richest ecosystem in the world as it offers diverse services and benefits both to humans and the ecosystem where it is found [3]. They serve as a link between terrestrial and marine ecosystem [4]. Mangrove forests improve coastal water quality and provide shelter to species of fish, crab, shrimp, and mollusks. However, if these forests are compromised by

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Culture on Nutritive Media and Characterization of Some Endophytic Bacteria Isolated from the Roots and Leaves of Mangrove Plants: *Avicennia germinans*, *Acrostichum aureum* and *Rhizophora mangle*

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From the roots, the hydrocarbon utilizing bacteria counts ranged from 7.0×10^3 cfu/g – 1.6×10^4 cfu/g, while the that of the leaves ranged from 3.0×10^5 cfu/g – 8.0×10^5 cfu/g. The nitrogen fixing bacteria counts for roots ranged from 4.5×10^3 cfu/g – 1.0×10^4 cfu/g and that of the leaves ranged from 3.0×10^5 cfu/g – 6.0×10^5 cfu/g. Nitrifying bacteria counts for the roots ranged from 5.0×10^3 cfu/g – 7.0×10^3 cfu/g, while the counts from the leaves ranged from 3.5×10^5 cfu/g - 5.0×10^5 cfu/g.

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oil spills, they can no longer shield coastlines, provide habitat, or feed organisms living among their roots and branches [5]. Once there is a spill, because oil rarely moves, the oil often coats the vegetation penetrating the soil. Mangroves can suffer lethal and sublethal effects when exposed to oil, the mangrove leaves can stunt or deform and branches can defoliate or die back [5]. And because these mangroves receive their oxygen through the lenticels on the exposed roots once the root is damaged or coated with oil, respiratory capabilities of the plant will suffer, which could cause them to suffocate or die [5].

Due to illegal bunkering activities in the Niger Delta region, oil spills have become a case of concern as the mangrove ecosystem is extremely susceptible to oil spills. Today, mangrove forests and swamps are among the most threatened habitats in the Niger Delta. Crude oil that has been released via spillage into the Niger Delta environment within the last 50 years has been estimated to be about 13 million barrels or more, making it to the list of top five world's most adversely damaged environment by petroleum [6]. Most of these spills are not cleaned or not thoroughly cleaned thereby creating wastelands in some of these areas.

Various groups of bacteria are found in the mangrove ecosystem [7] and there they perform several activities which include photosynthesis, nitrogen fixation, and methanogenesis [8]. Endophytic bacteria can be defined as those that can be isolated from healthy, superficially disinfected plant tissues and are not known to cause any damage to the host plant rather are beneficial to them [9][10]. Bacterial endophytes play an important role in the metabolic activities of their host plants, as they produce important chemical compounds that can trigger several biochemical pathways in the host plant [3]. These organisms get protection and nutrients from their host plant, while providing enzymes, antibiotics, alkaloids and other metabolites that enables the plant to tolerate unfavourable environmental conditions, such as pollution [10]. Endophytic organisms also provides nutrients by nitrogen fixation and have potential to enhance the removal or reduce pollutants like benzene, pesticide, toluene, ethylbenzene and phosphorus solubilization [11].

2. MATERIALS AND METHODS

Sample Collection

The three mangrove plant samples (roots and leaves) were collected from the Bonny Jetty waterfront (Okrika) in Old Port Harcourt Township, Rivers State, Nigeria. The location is situated at Longitude $4^{\circ} 45' 10.13976''$ N and Latitude $7^{\circ} 14' 13.012''$ E at Bonny water front. The roots and leaves collected were separately placed in sterile polyethylene bags, transported aseptically first to the Department of Plant Science & Biotechnology Laboratory to be identified before being transferred to the Microbiology Laboratory, all of Rivers State University, Port Harcourt for immediate processing.



Fig 1: *Rhizophora mangle* – Red Mangrove leaves and roots

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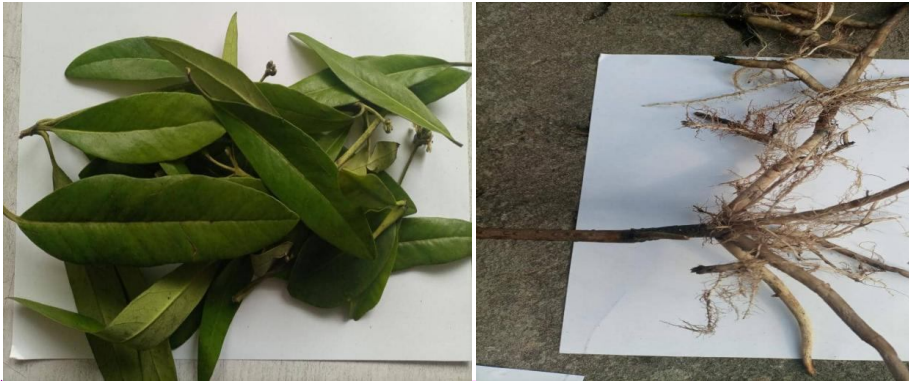


Fig 2.: *Acrostichum aureum* – golden leather fern leaves and roots

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Fig 3.: *Avicennia germinans* – Black Mangrove leaves and roots

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Isolation of Organisms

The organisms were isolated from the roots and leaves of the mangrove plants. They were treated to obtain only the endophytic bacteria using the following processes. First, the plants were washed under running water thoroughly to remove surface adhering debris. Then, they were cut into small pieces washed in sterile distilled water for 5minutes, surface-sterilized with 70% ethanol for 1 minute, 3% sodium hypochlorite (NaOCl solution) for 3minutes and then rinsed 6 times in sterile distilled water in different containers. Afterwards, they were grounded separately with a sterile mortar and pestle to make plant slurries. Serial dilution of 1g of the plant slurry was used for dilution (up to 10^{-4}). An aliquot of 0.1ml of the several dilutions were inoculated aseptically unto the properly dried media; Burk's N-Free Agar, Winogradsky Agar (for both *Nitrosomonas* and *Nitrobacter*), Mineral Salt Agar and Nutrient Agar. Using vapour phase transfer method, filter paper was dipped into crude oil and placed on the cover of the plate containing Mineral Salt Agar. The inoculated plates were incubated at 37°C for 24hours to 7days. After incubation, bacterial colonies were differentiated and counted based on their morphological characteristics. Individual colonies were picked randomly and sub-cultured by streaking them onto nutrient Agar plates using the streak plate technique and incubated at 37°C. Pure cultures were stored in slants and refrigerated at 4°C until required for use.

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The isolates were screened to determine those that had the ability to degrade crude oil in contaminated water.

Characteristics and identification of bacterial isolates

The bacterial isolates were characterized on the basis of their colonial morphology, use of selective media, cultural and biochemical characteristics. References were made to Bergey's Manual of Determinative Bacteriology [12] for identification of bacteria. Pure cultures of bacteria were each subcultured onto freshly prepared Nutrient Agar plates, incubated at 37°C for 24hours and these served as pure stock cultures for the morphological features such as shape, size, colour, edge,

texture and elevation of the colony, motility and gram stain which were observed visually with hand lens and the the various biochemical test which includes; gram reaction, motility, methyl red, Voges Proskauer, catalase, oxidase, indole, citrate utilization and sugar fermentation tests, starch hydrolysis, Nitrate and hydrogen sulphide test.

Results and Discussion

Isolation of the test Organisms

The three plant samples used were identified in the Department of Plant Science & Biotechnology Laboratory as: *Avicennia germinans* (Black mangrove), (*Acrostichum aureum* (Golden leather fern mangrove) and *Rhizophora mangle* (Red mangrove). The results of the enumeration of the roots and leaves of the three different plant samples used for this study are presented in Table 1.

Table 1. Total Bacterial Counts from Mangrove Plants Samples

Sample Code (CFU/g)	Total Heterotrophic Bacteria (cfu/g)	Hydrocarbon Utilizing Bacteria (cfu/g)	Nitrogen Fixing Bacteria (cfu/g)	Nitrifying Bacteria (cfu/g)
BMR	$1.9 \times 10^5 \pm 0.007$	$7.0 \times 10^3 \pm 0.007$	$1.0 \times 10^4 \pm 0.007$	$7.0 \times 10^3 \pm 0.21$
BML	$1.2 \times 10^5 \pm 0.07$	$3.0 \times 10^3 \pm 0.07$	$6.0 \times 10^3 \pm 0.70$	$3.5 \times 10^3 \pm 0.007$
RMR	$3.1 \times 10^5 \pm 0.007$	$1.6 \times 10^4 \pm 0.00$	$7.0 \times 10^3 \pm 0.007$	$7.0 \times 10^3 \pm 0.006$
RML	$2.0 \times 10^5 \pm 0.00$	$8.0 \times 10^3 \pm 0.00$	$3.0 \pm 10^3 \pm 0.00$	$5.0 \times 10^3 \pm 0.14$
GLFR	$2.7 \times 10^5 \pm 0.007$	$1.2 \times 10^4 \pm 0.007$	$4.5 \times 10^3 \pm 0.007$	$6.0 \times 10^3 \pm 0.007$
GLFL	$2.2 \times 10^5 \pm 0.07$	$5.0 \times 10^3 \pm 0.07$	$5.0 \times 10^3 \pm 0.07$	$5.0 \times 10^3 \pm 0.007$

Key: BMR – Black Mangrove Roots, BML – Black Mangrove Leaves, RMR – Red Mangrove Roots, RML – Red Mangrove Leaves, GLR – White Mangrove Roots, GLL – White Mangrove Leaves

Results of Total Heterotrophic Bacterial mean counts for Black Mangrove Roots was 1.9×10^5 CFU/g, Golden leather fern roots had a mean count of 2.7×10^5 CFU/g while the Red Mangrove Roots had a mean count of 3.1×10^5 CFU/g. For the leaves, Black Mangrove was 1.2×10^5 CFU/g, Golden leather fern had a mean count of 2.2×10^5 CFU/g while the Red Mangrove had a mean count of 2.0×10^5 CFU/g.

Results of average Hydrocarbon Utilizing Bacterial counts for roots ranged from 7.0×10^3 CFU/g to 1.6×10^4 CFU/g with the sample Red Mangrove recording the highest counts and the Black Mangrove recording the lowest counts. While for the leaves, the counts ranged from 3.0×10^3 CFU/g to 8.0×10^3 CFU/g with the sample Black Mangrove recording the lowest count and the Red Mangrove recording the highest bacterial counts.

For the isolation of Nitrogen fixing bacteria, the Burks N-Free medium was used, the results of average Bacterial counts for roots ranged from 4.5×10^3 CFU/g to 1.0×10^4 CFU/g with the sample Black Mangrove recording the highest counts and the Golden Leather Fern recording the lowest counts. While for the leaves, the counts ranged from 3.0×10^3 CFU/g to 6.0×10^3 CFU/g with the sample Red Mangrove recording the lowest counts and the Black Mangrove recording the highest bacterial counts.

For the isolation of nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) the Winogradsky medium was used, the results of average Bacterial counts for roots ranged from 6.0×10^3 CFU/g to 7.0×10^3 CFU/g

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with the sample Black Mangrove and Red Mangrove recording the highest counts and the Golden Leather Fern recording the lowest counts. While for the leaves, the counts ranged from 3.5×10^3 CFU/g to 5.0×10^3 CFU/g with the sample Black Mangrove recording the lowest count and the Red Mangrove recording the highest bacterial counts.

UNDER PEER REVIEW

ISOLATES	Gram Stain	Shape	Colour	Elevation	Translucent	CAT	OXI	CIT	MOT	IND	MR	VP	GLU	LAC	SUC	MAN	STH	NITRATE	H ₂ S	PROBABLE ORGANISMS
H1	-ve	Rod	Yellow	Flat	Opaque	+	+	+	+	-	-	-	-	-	-	-	-	+	-	<i>Pseudomonas</i> sp
H2	-ve	Rod	Yellow	Flat	Opaque	+	+	+	+	-	-	-	-	-	-	-	-	+	-	<i>Pseudomonas</i> sp
H3	+ve	Bacilli	Cream	Raised	Opaque	+	-	+	-	-	+	+	A	-	-	-	-	-	-	<i>Micrococcus</i> sp
H5	-ve	Rod	White	Raised	Translucent	+	+	-	+	+	-	+	AG	+	AG	A	-	+	+	<i>Azotobacter</i> sp
T A	+ve	Cocci	Yellow	Smooth	Translucent	+	+	-	-	-	+	+	A	-	AG	A	+	+	-	<i>Staphylococcus</i> sp
T B	+ve	Cocci	Yellow	Smooth	Translucent	+	+	+	-	-	+	-	A	-	A	A	-	+	-	<i>Staphylococcus</i> sp
T C	+ve	Rod	Cream	Raised	Opaque	+	+	-	-	-	-	+	AG	-	AG	A	+	+	-	<i>Micrococcus</i> sp
T D	+ve	Rod	Cream	Raised	Opaque	+	+	-	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
T E	+ve	Cocci	Yellow	Smooth	Translucent	+	-	+	-	-	+	+	AG	-	-	A	-	+	-	<i>Staphylococcus</i> sp
T F	+ve	Cocci	Yellow	Smooth	Translucent	+	+	-	-	-	+	+	A	-	AG	A	+	+	-	<i>Staphylococcus</i> sp
T G	+ve	Rods	Cream	Raised	Opaque	+	+	-	-	-	-	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
KA	+ve	Cocci	Yellow	Smooth	Translucent	+	-	-	-	-	+	-	A	-	A	-	+	+	-	<i>Staphylococcus</i> sp
KB	+ve	Rods	Cream	Raised	Opaque	+	-	-	+	-	+	+	AG	A	AG	A	+	+	-	<i>Bacillus</i> sp
KC	+ve	Rod	Cream	Raised	Opaque	+	+	-	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
KD	-ve	Rod	Cream	Raised	Translucent	+	+	+	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
KE	-ve	Rod	Cream	Flat	Opaque	+	+	-	+	-	-	-	A	-	A	-	-	-	+	<i>Nitrosomonas</i> sp
BA	-ve	Rod	Cream	Raised	Translucent	+	+	+	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
BB	+ve	Cocci	Gray	Flat	Opaque	+	+	-	+	-	-	-	-	-	-	-	-	-	-	<i>Nitrobacter</i> sp
BC	-ve	Rod	Gray	Flat	Opaque	+	+	-	-	-	-	-	AG	-	-	-	-	-	-	<i>Nitrobacter</i> sp
BD	-ve	Rod	Cream	Flat	Opaque	+	-	+	+	-	-	+	A	-	A	A	-	+	-	<i>Klebsiella</i> sp
BE	-ve	Rod	Cream	Raised	Translucent	+	+	+	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp
SA	-ve	Rod	Cream	Flat	Opaque	+	-	+	+	-	-	+	A	-	A	A	-	+	-	<i>Klebsiella</i> sp
SB	-ve	Rod	Cream	Flat	Opaque	+	-	+	+	-	+	+	A	-	A	A	-	+	-	<i>Klebsiella</i> sp
SC	-ve	Rod	Cream	Raised	Translucent	+	+	+	+	-	+	+	AG	-	AG	A	+	+	-	<i>Bacillus</i> sp

Table 2. Morphology and Biochemical Characteristics of the Bacterial Isolates

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Characterization and identification of bacterial isolates

The results of morphological and biochemical characteristics of the bacterial isolates are presented in Table 2. From the morphological features such as shape, size, colour, elevation, etc the colony of the bacterial isolates were identified macroscopically and under a hand lens and from the biochemical characteristics such as motility test, gram stain, catalase test, oxidase test, indole test, methyl red test, Voges-proskauer test, sugar fermentation test, starch hydrolysis and citrate utilization test the following probable organisms identified include; *Pseudomonas* sp, *Micrococcus* sp, *Nitrobacter* sp, *Nitrosomonas* sp, *Staphylococcus* sp, *Azotobacter* sp, *Klebsiella* sp and *Bacillus* sp.

Table 3. Total number of Isolates from each Pplant Ssample

Isolates	BML	BMR	RML	RMR	GLFL	GLFR	SUM
<i>Pseudomonas</i> sp	-	2	1	1	1	1	4
<i>Staphylococcus</i> sp	2	1	1	2	1	1	8
<i>Klebsiella</i> sp	-	1	-	1	-	2	4
<i>Micrococcus</i> sp	-	-	1	1	1	-	3
<i>Bacillus</i> sp	2	1	2	3	2	1	11
<i>Nitrosomonas</i> sp	-	-	-	2	-	1	3
<i>Nitrobacter</i> sp	-	2	-	2	-	-	4
<i>Azotobacter</i> sp	1	-	-	-	-	-	1

Key: BMR – Black Mangrove Roots, BML – Black Mangrove Leaves, RMR – Red Mangrove Roots, RML – Red Mangrove Leaves, GLR – White Mangrove Roots, GLL – White Mangrove Leaves

The various organisms isolated from the roots and leaves of the three plant samples are found in Table 3. *Bacillus* sp had the highest occurrence followed by *Staphylococcus* sp the *Klebsiella* sp then *Pseudomonas*, *Micrococcus* and *Nitrobacter* sp while *Nitrosomonas* sp and *Azotobacter* sp was least. Also the table shows where the various isolates where isolated from *Azotobacter* sp and *Bacillus* sp were isolated from the Black Mangrove Leaves, *Staphylococcus* sp, *Bacillus* sp and *Nitrobacter* sp was isolated from the roots of the Black Mangrove, from the Red Mangrove leaves, *Staphylococcus* sp, *Micrococcus* and *Bacillus* sp was isolated, *Staphylococcus* sp, *Pseudomonas* sp, *Klebsiella* sp, *Nitrobacter* sp, *Nitrosomonas* and *Bacillus* sp were isolated from the roots of the Red Mangrove, for the Golden Leather Fern roots, the following were isolated, *Staphylococcus* sp, *Klebsiella* sp and *Bacillus* sp and for the Golden Leather Fern leaves, *Pseudomonas* sp, *Micrococcus* sp and *Bacillus* sp were isolated. Also the Red Mangrove roots had the highest count of organisms while the Black Mangrove Leaves had the lowest.

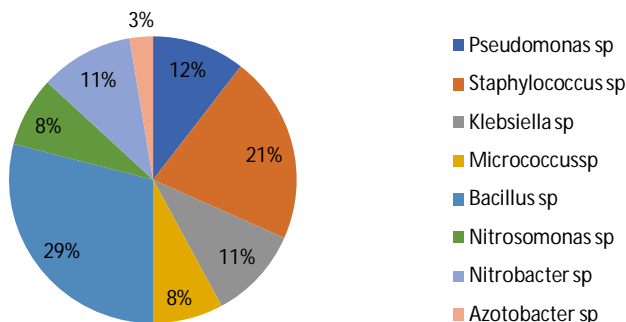
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Fig 4: Percentage Occurrence



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The results of the mangrove roots counted on all plates from each media, the Total Heterotrophic Bacterial (THB) counts from the roots was highest in the Red Mangrove followed by the Golden Leather Fern and the Black Mangrove roots recorded the lowest bacterial counts. For the hydrocarbon degraders in the roots, the bacterial counts were highest in the Red Mangrove (RM) as they had the highest growth of organisms followed by the Golden Leather Fern (GLF) while the Black Mangrove (BM) had the lowest counts of the hydrocarbon degraders. Results for the Nitrogen Fixing Bacteria showed that the roots of the Black Mangrove recorded the highest counts and that of the Golden Leather Fern recorded the lowest bacterial counts. For the Nitrifying Bacteria results, the roots of the Black Mangrove recorded the highest bacterial counts while the Golden Leather Fern roots revealed the lowest bacterial counts.

From the results for the mangrove leaves counted on all plates from each media, the Total Heterotrophic Bacterial (THB) counts were highest in the Golden Leather Fern followed by the Red Mangrove and the Black Mangrove contained the lowest counts of bacteria; for the Hydrocarbon Utilizing Bacteria (HUB), the leaves of the Red Mangrove recorded the highest bacterial counts while the Black Mangrove recorded the lowest counts; for the Nitrogen fixing bacteria, the leaves of the Black Mangrove revealed highest bacterial counts while the leaves of the Red Mangrove recorded the lowest count; lastly for the nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) the leaves of the Red Mangrove recorded highest bacterial counts and the Black Mangrove recorded the lowest count.

Important economic and environmental functions about endophytes isolated from mangrove plants have been reported by several researchers. The Red Mangrove had highest count of organisms this indicates that the red mangrove is well adapted to the environment and has the ability to support and aid the sustenance of varying species of bacteria [13].

Morphological and biochemical characteristics of the isolates were carried out to identify the probable organisms isolated from the mangrove plants. In the present study the plants yielded a total of 49 bacterial isolates (23 from the roots and 15 from the leaves). But based on visible morphological differences 24 isolates were selected for further study. They were studied further and the probable organisms identified include; 4 *Pseudomonas* sp, 3 *Micrococcus* sp, 4 *Nitrobacter* sp, 1 *Nitrosomonas* sp, 8 *Staphylococcus* sp, 1 *Azotobacter* sp, 4 *Klebsiella* sp and 11 *Bacillus* sp. Of the thirty eight isolates, 23 (*Pseudomonas* sp, *Klebsiella* sp, *Bacillus* sp, *Staphylococcus* sp, *Nitrobacter* sp, *Nitrosomonas* sp) were from the roots of the mangrove while 15 (*Bacillus* sp, *Staphylococcus* sp, *Pseudomonas* sp, *Micrococcus* sp, *Azotobacter* sp) were from the leaves.

From the results of the isolation done by Rahman et al, 2019, 8 genera of isolated bacteria consist of *Klebsiella*, *Pantoea*, 3 *Vibrio*, 2 *Enterobacter*, *Pseudomonas*, *Virgibacillus*, *Staphylococcus*, and 8 *Bacillus* isolates were isolated from the leaves of mangrove leaves. The results of this study were also different from Feliatra (2001) research. The research found 7 genera, namely *Neisseria*, *Plesiomonas*,

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Comment [E40]: Italic, please: *Klebsiella*, *Pantoea*, 3 *Vibrio*, 2 *Enterobacter*, *Pseudomonas*, *Virgibacillus*, *Staphylococcus*, and 8 *Bacillus*

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Yersinia, Corynebacterium, Bacillus, Staphylococcus and Acinobacter. While that of [16] resulted in 5 bacterial species from leaves and none from the roots of mangrove plants. Ntabo et al 2018 also reported isolating Forty-two bacterial isolates (twenty three from the leaves and nineteen from the roots) from the leaves and roots of six mangrove plants. From the leaves 8 *Bacillus* species, 4 *Streptomyces*, *Staphylococcus*, *Pseudochrobactrum*, *Klebsiella*, *Achromobacter*, *Alcaliigenes*, 3 *Myroides*, 2 *Serratia* and from the roots; 13 *Bacillus*, 2 *Myroides*, 2 *Streptomyces*, *Pseudomonas*, *Staphylococcus*. There appears to be significant variation in the number and types of indigenous bacteria isolated from diverse host plant species. These endophytes vary from one plant species to another and their diversity depends on the climatic condition of a particular region and age of the plant [17]. Several factors may explain these differences, including host specificity, geographical distribution, plant age, and tissue type [17].

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Several other researchers [18, 19] all reported isolation of several endophytes from mangrove plants which all have several enzymatic capabilities with mostly *Bacillus* sp. showing strong enzymatic production. Our data corroborate the results obtained by [20] Ando et al. (2001) who isolated a large number of *Bacillus* sp. from mangrove sediments in Japan and reported the possible ability of these isolates to degrade organic pollutant compounds by fermentation. Similarly, the endophytic strain *B. amyloliquefaciens* (RS261) is a biological agent isolated from the leaf of *R. stylosa* [21].

Endophytic bacteria often produce metabolites similar to those produced by their host plants. Many of these endophytes have shown ability to synthesise bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proven useful for drug discovery [22].

From this study, all identified isolates corresponded to genera commonly isolated and identified from the endophytes of plants. It was observed that all mangrove plants harbor bacterial endophytes with various enzymatic activities [17]. Based on the assessed microbial populations, the present study suggests that mangrove ecosystems harbour diverse functional microbial groups that may potentially be directed to biotechnological approaches for either ecological restoration or agricultural purposes [20] [21]. Some endophytic bacteria can be used as biofertilizers because they fix Nitrogen and this influences plant growth through the production of phytohormones, siderophores, they can induce systemic tolerance by producing 1-aminocyclopropane-1-carboxylase deaminase and also induce systemic resistance and antagonistic activities [19]. *Klebsiella pneumonia* was able to fix N₂ and produce indole acetic acid (IAA) hormone [23]. These Nitrogen-fixing endophytic bacteria have edge over its rhizospheric counterparts because, they make the fixed nitrogen available directly to the plants and they face less competition as they are sheltered inside plant tissues [19]. Endophytic bacteria are known to have several potential applications in medicine and in other sectors of biotechnology. This study when compared to the report by [17], it is evident that beneficial microorganisms that may play a significant role in the C, N, or P cycles are in the mangrove ecosystem, as well as potential degraders of petroleum hydrocarbon [24]. Abiye et al., 2022 from their research were able to prove that various endophytic bacteria (*Pseudomonas aeruginosa* (MN314747), *Brevibacillus brevis*, *Bacillus amyloliquefaciens*, *Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Nitrobacter* sp, *Staphylococcus aureus*) isolated from mangrove plants can be used to clean up oil spills in hydrocarbon polluted environment.

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From the results, it showed that the roots and leaves of the Mangrove had varying species of organisms, this indicates that the mangrove plants is well adapted to the environment and has the ability to support and aid the sustenance of varying species of bacteria. It has also been stated that the red mangrove can survive a wide range of environmental pressures, and therefore colonizes rapidly forming near monospecific stands that allows it outcompete native species [22].

Conclusion

The results revealed that there are high numbers of active indigenous bacteria in the roots, most of which are known to possess catabolic abilities to use up pollutants. The these mangrove roots may harbour bacterial genera that may play important role in nitrogen fixation, medicine and can also be enhanced to bring about bioremediation in polluted environment. This study has several culturable functional groups of

Comment [E47]: 4.CONCLUSION

bacteria that might be directed to further biotechnological approaches. Therefore, further studies should be carried out to determine the potential application of these isolates in bioremediation, growth promotion, enzyme production and generally in biotechnology.

References

1. Aju, P. C. and Aju, J. A. (2018). Mangrove Forests in Nigeria: Why their restoration, rehabilitation and conservation matters. *African Journal of Environment and Natural Science Research*. 5;(1) 505-524.
2. Numbere A. O and Camilo GR (2018). Structural characteristics, above-ground biomass and productivity of mangrove forest situated in areas with different levels of pollution in the Niger Delta, Nigeria. *African Journal of Ecology* 56(4):917-927.
3. Fadiji, A. E. and Babalola, O. O. (2020). Elucidating Mechanisms of Endophytes Used in Plant Protection and Other Bioactivities With Multifunctional Prospects. *Frontiers in Bioengineering and Biotechnology*. 8:467.
4. Ukoima, N. H and Umechuruba, C. I. (2009). Enumeration of fungi on some mangrove plants in Port Harcourt. *International Journal of Agriculture* 1(2); 126-129.
5. Wilson, M., Hale, C., Maung-Douglass, E., Partyka, M., Sempier, S., Skelton, T., & Swann, L. (2019). Impacts of oil on mangroves. GOMSG-G-19-010.
6. Kadafa, A. A (2012a). Environmental impacts of oil exploration and exploitation in the Niger Delta of Nigeria. *Global Journal of Science, Frontier Research Environmental Earth Science*, 12(3):1–11.
7. Holguin G, Vazquez P, Bashan Y (2001). The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. *Biology and Fertility of Soils*, 33: 265-278.
8. Das S, Lyla PS, Khan SA (2006). Spatial variation of aerobic culturable heterotrophic bacterial population in sediment of the Continental slope of western Bay of Bengal. *Indian Journal of Marine Sciences* 36: 51-58.
9. Costa, L. E. D., Queiroz, M. V., Borges, A. C., deMoraes, C. A. and deAraujo, E. F. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*). *Braz J Microbiol*. 2012 Oct-Dec; 43(4): 1562–1575.
10. Douglas, S. I., Nrior, R. R. and Somiari, A. A. (2021). Culture Based Isolation and Characterisation of Endophytic Bacteria from Mangrove (Rhizophora mangle) Roots. NSM Scientific Conference and AGM, Uniport 2021- Book of proceedings. Pp 13-14.
11. Gupta, R. M., Kale, P. S., Rathi, M. L. and Jadhav, N. N.(2015) Isolation, Characterization and Identification of Endophytic Bacteria by 16S rRNA partial Sequencing technology from Roots and Leaves of *Prosopis cineraria* Plant. *Asian Journal of Plant Sciences & research*. 5(6): 36 – 43.
12. Chen, W., Tang, Y. and Wu, X. (2012) Distribution of Culturable Endophytic Bacteria in Aquatic Plants and their Potential for Bioremediation in Polluted Water. *Journal of Aquatic Biology*, 15:88 – 110.
13. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, Williams and Wilkins, Baltimore, Maryland, USA; 151 – 157.
14. Rahman, S. A., Sukenda, S., Widanarni, W., Alimuddin, A. and Ekasari, J. (2019). Isolation and identification of endophytic bacteria from the mangrove leaves of *Avicennia marina* and evaluation of inhibition to bacterium causing ice-ice disease. *International Journal of the Bioflux Society* 12(3) 1-12
15. Feliatra, (2001). Isolation and identification of heterotrophic bacteria found in mangrove leaves (*Avicennia* spp. and *Sonneratia* spp.) from marine Dumai station area]. *Jurnal Natur Indonesia* 2:104-112.
16. Maulani, B. I. G., Rasmi, D. A. C. and Zulkifli, L (2019). Isolation and characterization of endophytic bacteria from mangrove *Rhizophora mucronata* Lam. and antibacterial activity test against some pathogenic bacteria. *Journal of Physics: Conference Series* 1402 (2019) 033038

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Inside the text, I found only.....22?

Right, there were some citations in another form, but, please, respect Instructions for authors. Please use the brackets.

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Comment [E50]: Please Respect Instructions for authors.
Please verify every reference.

Example:
Gulson BL, Mizon KJ, Korsch MJ, Howarth D. Non-orebody sources are significant contributors to blood lead of some children with low to moderate lead exposure in a major lead mining community. *Science of Total Environment*. 1996;181:223–230.

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17. Ntabo, R. M., Nyamache, A. K., Lwande, W., Kabii, J. and Nonoh, J. (2018). Enzymatic Activity of Endophytic Bacterial Isolates from Selected Mangrove Plants in Kenya. *The open Microbiology Journal* 12; 354-363
18. Thatoi H, Behera BC, Mishra RR, Dutta SK (2013) Biodiversity and biotechnological potential of microorganisms from mangrove ecosystems: a review. *Annals of Microbiology* 63: 1-19.
19. Dias ACF, Andreote FD, Dini-Andreote F, Lacava PT, Sá ALB, Melo IS, Azevedo JL, Araújo WL (2009) Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. *World Journal of Microbiology and Biotechnology*, 25: 1305-1311.
20. Ando Y, Mitsugi N, Yano K, Karube I (2001). Isolation of a bacterium from mangrove soil for degradation of sea sludge. *Applied Biochemistry and Biotechnology*, 95: 175-182.
21. Feng L, Ou X, He H, Hu H, Zhang X (2009). Control of *Capsicum phytophthora* blight by endophytic bacteria RS261 from mangrove. *Acta Phytopathologica Sinica*, 39: 333-336.
22. Gayathri, S., Durai, S., Radhakrishnan, M., Balagurunathan, R. and Kandasamy, K.. (2010). Bioprospecting potential of fast growing endophytic bacteria from leaves of mangrove and salt-marsh plant species. *Indian Journal of Biotechnology*. 9. 397-402.
23. Rosenblueth M., Martinez-Romero E., 2006 Bacterial endophytes and their interaction with hosts. *Molecular Plant-Microbe Interactions* 19(8):827-837.
24. Abiye, S. A. A., Ibietela, D. S., & Renner, N. R. (2022). Screening for Biodegradation Potential of Endophytic Bacteria Isolated from the Roots and Leaves of Mangrove Plants (*Avicennia germinans*) (Black Mangrove), *Acrostichum aureum* (Golden Leather Fern) and *Rhizophora mangle* (Red Mangrove). *Journal of Advances in Microbiology*, 22(7), 19-28.
25. Deivanai, S., Bindusara, A. S., Prabhakaran, G., & Bhore, S. J. (2014). Culturable bacterial endophytes isolated from Mangrove tree (*Rhizophora apiculata* Blume) enhance seedling growth in Rice. *Journal of natural science, biology, and medicine*, 5(2), 437-444.
26. Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczarski, D., Higley, P., Ishimaru, C. A., Arunakumari, A., Barletta, R. G. and Vidaver, A. K. (2020). Isolation and Characterization of Endophytic Colonizing Bacteria from Agronomic Crops and Prairie Plants. *Applied and Environmental Microbiology*. 68 (5) 145-164.
27. Kathiresan, K., Selvam, M.M., 2006. Evaluation of beneficial bacteria from mangrove soil. *Botanica Marina*. 49, 86-88.
28. Gupta, G., Panwar, J., Akhtar, M.S., Jha, P.N. (2012). Endophytic Nitrogen-Fixing Bacteria as Biofertilizer. In: Lichtfouse, E. (eds) Sustainable Agriculture Reviews. Sustainable Agriculture Reviews, vol 11. Springer
29. Ramírez-Elías, M. A., Ferrera-Cerratoa, R. Alarcóna, A., Almaraz, J. J., Ramírez-Valverde, G., de-Bashanc, L. E. Esparza-García, F. J. and García-Barradas, O. (2014). Identification of culturable microbial functional groups isolated from the rhizosphere of four species of mangroves and their biotechnological potential. *Applied Soil Ecology* 82 (2014) 1-10
30. Dias, A.C.F., Andreote, F.D., Dini Andreote, F., Lacava, P.T., Sá, A.L., Melo, I.S., Azevedo, J.L., Araújo, W.L., 2009. Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. *World Journal of Microbiology and Biotechnology*. 25, 1305-1311.
31. Savitri, W. D., Wirjaputra, M. V. and Hardjo, P. H. (2016). Isolation and Characterization of Endophytic Bacteria from the leaf explants of *Avicennia marina* (Forsk). *Proceeding Seminar Nasional Biodiversitas VI*
32. Nair, D. N. and Padmavathy, S. (2014). Impact of Endophytic Microorganisms on Plants, Environment and Humans. *The Scientific World Journal*. Vol 2014. Article ID 250693
33. Numbere, A. and Madiuke, E. (2021) Investigation of the anti-bacterial properties of mangrove fern, *Acrostichum aureum* in the Niger Delta, Nigeria. *African Journal of Biotechnology* 20(4):142-149
34. Oyeleka, S. B. and Manga, S. B. (2008). Essentials of Laboratory Practical in Microbiology. Pp. 20-33

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