Asian Journal of Environment & Ecology

Volume 23, Issue 8, Page 128-139, 2024; Article no.AJEE.119962
ISSN: 2456-690X

Molecular Assessment of Dominant Genus in Heavy Metal Contaminated Soil from Selected Dumpsites in Port Harcourt, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ajee/2024/v23i8589

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/119962

Original Research Article

Received: 24/05/2024
Accepted: 26/07/2024
Published: 08/08/2024

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Cite as: *Obaretin, Nelson N., Chidinma G. Daokoru-Olukole, Juliana O. Pondei, Emmanuel O. Fenibo, Philip O. Okerentugba, Herbert O. Stanley, and C.D. Onwukwe. 2024. "Molecular Assessment of Dominant Genus in Heavy Metal Contaminated Soil from Selected Dumpsites in Port Harcourt, Nigeria". Asian Journal of Environment & Ecology 23 (8):128-39. https://doi.org/10.9734/ajee/2024/v23i8589.*

ABSTRACT

Heavy metal contamination distorts and diminishes microbial diversity due to their inhibitory effects on bacteria, resulting in the elimination of susceptible bacteria and the proliferation of resistant strains, which eventually become dominant. Consequently, this study seeks to characterize the dominant bacteria (RCBBR_B37 and RCBBR_B38) isolated from soil at a dump site contaminated with heavy metals. DNA extraction from the isolates was performed using the Zymo Quick DNA Fungal/Bacterial Kit, and the purity of the extracted DNA was assessed using a NanoDrop 2000c spectrophotometer. Gel electrophoresis confirmed the presence of DNA bands, including a control band. Subsequently, PCR amplification using 27F and 1492R complementary primers was carried out on the extracted DNA. The PCR products underwent sequence analysis followed by BLAST search in the National Center for Biotechnology Information (NCBI) database. A phylogenetic tree was constructed based on the BLAST results. Isolate RCBBR_B37 exhibited a 98% similarity index to *Bacillus fungorum*, while RCBBR_B38 showed a 97% similarity index to *Bacillus paramycoides*. The prevailing presence of *Bacillus* in heavy metal contaminated dumpsite is a signature, representing the difference mechanisms of microbial interaction for heavy metals amelioration, including but not limited to bioaccumulation, sequestration, redox transformation, neutralization, metabolization, and detoxification.

Keywords: 27F/1492R primers; Bacillus spp.; detoxification; heavy metal contamination; molecular analysis.

1. INTRODUCTION

Dumpsites indeed represent substantial sources of heavy metal contamination [1]. When waste is deposited in dumpsites, diverse materials containing heavy metals such as lead, cadmium, mercury, arsenic, chromium, and others can seep into the soil and groundwater. Several factors contribute to the presence of heavy metals in dumpsites, including waste from industrial activities, electronic waste, household waste, and construction/demolition waste [2,3,4,5]. The existence of heavy metals in dumpsites presents notable environmental and health hazards. These metals can amass in the soil and water, impacting microbial diversity. Microorganisms exhibit various responses to heavy metal pollution, possessing mechanisms to either endure, accumulate, or detoxify heavy metals [6,7]. These reactions can carry significant implications for the environment, influencing nutrient cycling and the destiny of heavy metals in ecosystems.

Certain microorganisms possess inherent tolerance to specific heavy metals [8], enabling them to endure elevated metal concentrations in their surroundings without succumbing to toxicity. This tolerance may stem from genetic adaptations [9], the presence of particular cellular structures [10], or the existence of enzymes that shield against metal toxicity [11]. Some microorganisms can accumulate heavy metals within their cells or on their surfaces [12] through various mechanisms, including metal binding to cell wall components or sequestration within specialized compartments [13]. By sequestering heavy metals, microorganisms can diminish metal availability in the surrounding environment, potentially mitigating their toxicity. Certain microorganisms can catalyze chemical transformations of heavy metals via redox reactions. For instance, specific bacteria can convert toxic metal forms into less harmful or mobile forms through reduction, oxidation, or methylation processes [14], thereby affecting metal mobility and bioavailability in the environment. Microorganisms possess detoxification pathways that enable them to metabolize or neutralize heavy metals [15], often involving the production of metal-binding proteins or peptides and the synthesis of enzymes facilitating the conversion of toxic metals into less harmful forms. Examples include metallothioneins, which bind metals, and metal-reducing enzymes such as metallothionein, glutathione, and phytochelatins [16,17]. Moreover, heavy metal pollution can alter the composition and dynamics of microbial communities in affected environments. Some microorganisms may thrive in metal-contaminated habitats due to their ability to tolerate or utilize metals, while others may be inhibited or displaced [18]. Bacteria, in particular, have been extensively studied for their responses to heavy metal contamination.

Bacteria demonstrate diverse reactions to heavy metal contamination, capitalizing on their varied metabolic capacities and genetic flexibility. They can harbor genes responsible for metal resistance mechanisms, often facilitated by synergistic interactions [19], which may be situated on bacterial chromosomes, plasmids, or transposons. These genes encode proteins like metal efflux pumps, metal-binding proteins, and metal-detoxifying enzymes [20], aiding bacteria in their survival within metal-laden environments. Additional pathways for responding to heavy metal exposure include expelling heavy metals from their cells, sequestering them, undergoing redox reactions, forming biofilms as protective structures, adapting genetically to prolonged exposure, and engaging in synergistic interactions to enhance survival [21,22,23]. Commonly implicated bacteria in one or more of these responses include *Cupriavidus metallidurans*, *acidophilic* bacteria, *Stenotrophomonas maltophilia*, various *Arthrobacter*, *Pseudomonas*, *Corynebacterium*, and *Bacillus* species [24]. These bacteria, among others, play vital roles in microbial communities thriving in metal-contaminated environments.

This study was designed to characterize the dominant bacterial isolates retrieved from specific dump sites in Port Harcourt using a molecular approach. The findings reveal that *Bacillus* species dominate the dump sites under investigation.

2. METHODOLOGY

2.1 Subculturing of Dominant Bacteria

Bacterial isolates previously obtained from dumpsites contaminated with heavy metals in Port Harcourt were subcultured using the spread plate method. Nutrient Agar was inoculated in duplicate with 0.1 ml of a 10-3 dilution using a sterile glass spreader. All plates were properly labeled, inverted, and then incubated at 37 °C for 24 hours. Morphological features were utilized to identify the most prevalent bacterial isolate that also spread across the cultured plates examined.

2.2 Molecular Characterization of Isolates

The DNA extraction process utilized the Zymo Quick DNA Fungal/Bacterial Kit, following the kit's protocol with some adjustments. Initially, the bacterial culture was collected and transferred into a sterilized mortar. Next, 750 µl of bashing bead buffer was added to the sample and homogenized. The resulting homogenate was then transferred to a bashing bead tube and further homogenized using a benchtop vortex for 20 minutes. Subsequently, the bashing bead tube containing the homogenized sample underwent centrifugation in a refrigerated centrifuge at ≥10,000 x g for 1 minute. Following centrifugation, 400 µl of supernatant was transferred to a Zymo-Spin III-F Filter in a Collection tube and centrifuged at 8,000 x g for 1 minute, after which the Zymo-Spin III-F Filter was discarded. To the filtrate in the Collection Tube, 1,200 µl of Genomic Lysis Buffer was added and thoroughly mixed. From this mixture, 800 µl was transferred to a Zymo-Spin II-C Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow-through from the Collection Tube was then discarded, and this step was repeated. Next, 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin II-C Column and centrifuged at 10,000 x g for 1 minute. Following this, 500 µl of g-DNA Wash Buffer was added to the Zymo-Spin II-C Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin II-C Column was subsequently transferred to a clean 1.5 ml micro-centrifuge tube, and 100 µl (with a minimum of 35 µl for higher output) of DNA Elution Buffer was directly added to the column matrix. Finally, the tube was centrifuged at 10,000 x g for 30 seconds to elute the DNA.

2.3 Determination of DNA Concentration and Purity Using Nanodrop

The DNA concentration and purity were assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Purity was determined by calculating the ratio of ultraviolet (UV) light absorbance at 260 nm to that at 280 nm. The NanoDrop was connected to a computer system, and the sensor was cleaned using cotton wool soaked in 70% ethanol. To blank the system, one microliter (1 µl) of Elution buffer (the solution used to re-suspend the DNA) was dispensed directly into the NanoDrop sensor. Following this, DNA samples (1 µL each) were individually loaded onto the sensor according to Bunu et al. [25]. Prior to loading a new sample, the sensor was routinely wiped to prevent contamination. Duplicate measurements were taken for each sample using the NanoDrop.

2.4 Gel Electrophoresis

Gel electrophoresis was conducted using a 1.5% agarose gel prepared by dissolving 0.75g of

agarose powder in 50 ml of 1X Tris-Boric acid-EDTA (TBE) buffer in a measuring flask, followed by microwaving for 2 minutes until a clear solution was obtained [26]. Subsequently, 5 µL of EZ viewing dye (Blue Light) was added to the solution in a conical flask and then poured into the casting tray or gel holder. The comb was positioned within the casting tray, and the mixture was allowed to solidify at room temperature for 20 to 30 minutes. Following solidification, the gel electrophoresis unit was set up by placing the gel holder containing the gel onto the platform inside the gel tank, and 1X TBE buffer was poured into the tank until the gel was fully submerged. A molecular weight marker (1Kb DNA Ladder) was loaded into the first lane, while DNA samples were individually loaded into the wells created by the comb on the gel. Each DNA sample (5 µL) was mixed with 1 µL of 6X loading dye before loading onto the gel. Additionally, a control containing all components of the PCR reaction mixture except the template DNA was loaded. The electrophoresis apparatus was operated at 100 volts for 40 minutes. Following the completion of the run, DNA fragments were visualized using an ultraviolet (UV) transilluminator (Gel Documentation microDOCTM, Cleaver Scientific Ltd, UK).

2.5 Polymerase Chain Reaction (PCR)

The target region was amplified using OneTaq® Quick-Load® 2X Master Mix (New England Biolabs, USA, Catalogue No. M0486) in an Eppendorf Mastercycler (Nexus Gradient 230, Germany), following the conditions outlined in Table 1. The primer sets utilized consisted of the forward primer 27F-AGAGTTTGATCMTGGCTCAG and the reverse primer 1492R TACGGYTACCTTGTTACGACTT. Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; followed by 35 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. The thermal cycler was set to maintain the PCR mix at 4°C after completion. PCR products underwent purification using an enzymatic method (ExoSAP) as follows: An ExoSAP master mix was prepared by combining 50 µl of 20 U/µl Exonuclease I (Catalogue No. NEB M0293L) and 200 µl of 1 U/µl Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) in a 0.6 ml micro-centrifuge tube. The reaction mixture was then prepared by mixing 10 µl of the amplified PCR product with 2.5 µl of the ExoSAP mix from step 1. The resulting mix was incubated at 37°C for 15 minutes followed by 80°C for 15 minutes [27].

2.6 Sequence Analysis and Blasting

The PCR products underwent sequencing utilizing the Nimagen Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000, following the manufacturer's instructions as outlined at: Nimagen Sequencing Kit Instructions. Subsequently, the labeled products were purified using the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053), as detailed in the instructions available at: Zymo Research DNA Sequencing Clean-up Kit Instructions. The purified products were then injected into the Applied Biosystems ABI 3500XL Genetic Analyzer with a 50cm array, employing POP7 polymer, accessible at: Applied Biosystems ABI 3500XL Genetic Analyzer with POP7, and sequence data were acquired. The sequences obtained from the sequencer underwent base calling using Chromaslite. Subsequent sequence editing was performed using BioEdit software prior to conducting a Basic Local Alignment Search Tool (BLAST) search within the NCBI (National Center for Biotechnology Information) database, available at: NCBI BLAST. Similar sequences retrieved from the search were downloaded and aligned using ClustalW.

2.7 Phylogenetic Tree

The evolutionary lineage was deduced utilizing the Neighbor-Joining technique [28], with the optimal tree depicted. The percentage of replicate trees wherein the associated taxa formed clusters in the bootstrap test (500 replicates) is displayed adjacent to the branches [29]. The tree is drawn proportionally, with branch lengths reflecting the evolutionary distances used in constructing the phylogenetic tree. These distances were calculated using the Jukes-Cantor method [30] and are expressed in units of base substitutions per site. The evolutionary analysis was carried out using MEGA11 [31].

Table 1. Polymerase chain reaction cocktail components

Component	Volume of a 12.5 µL reaction
Template DNA	2 µL
10µM Forward Primer	0.25µL (10nM)
10µM Reverse Primer	0.25µL (10nM)
One Taq Quick Load 2X Master Mix with Standard Buffer	6.25µL
Nuclease free water	3.75 µL

3. RESULTS

3.1 Molecular Characterization of Bacterial Isolates

Table 2 presents the molecular characterization of bacterial isolates, with Fig. 1 showcasing the gDNA bands and Fig. 2 depicting the 16S rRNA PCR bands of the isolates. The results of the blasting are subsequently presented in Table 3 (Fasta sequences) and used in phylogenetic tree reconstruction (Fig. 3), while the outcomes of the similarity assignment check are presented in Table 4.

Table 2. NanoDrop spectrometry characteristics of the isolates

S/N	Sample	Conc. (ng/mL)	A260	A280	Purity (A260/A280)
1	RCBBR_37a	200.5	4.010	2.160	1.86
2	RCBBR_37b	208.5	4.170	2.239	1.86
3	RCBBR_38a	184.0	3.681	1.987	1.85
4	RCBBR_38b	184.2	3.685	1.989	1.85

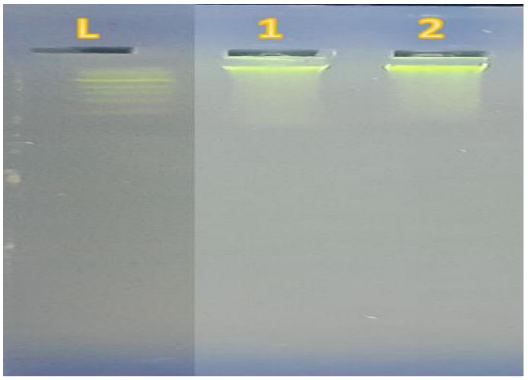


Fig. 1. gDNA bands from the isolates (Lane 1: RCBBR_37; Lane 2: RCBBR_38)

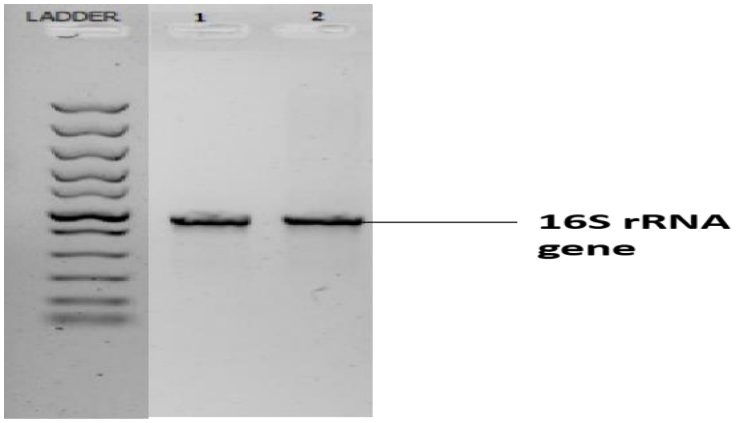
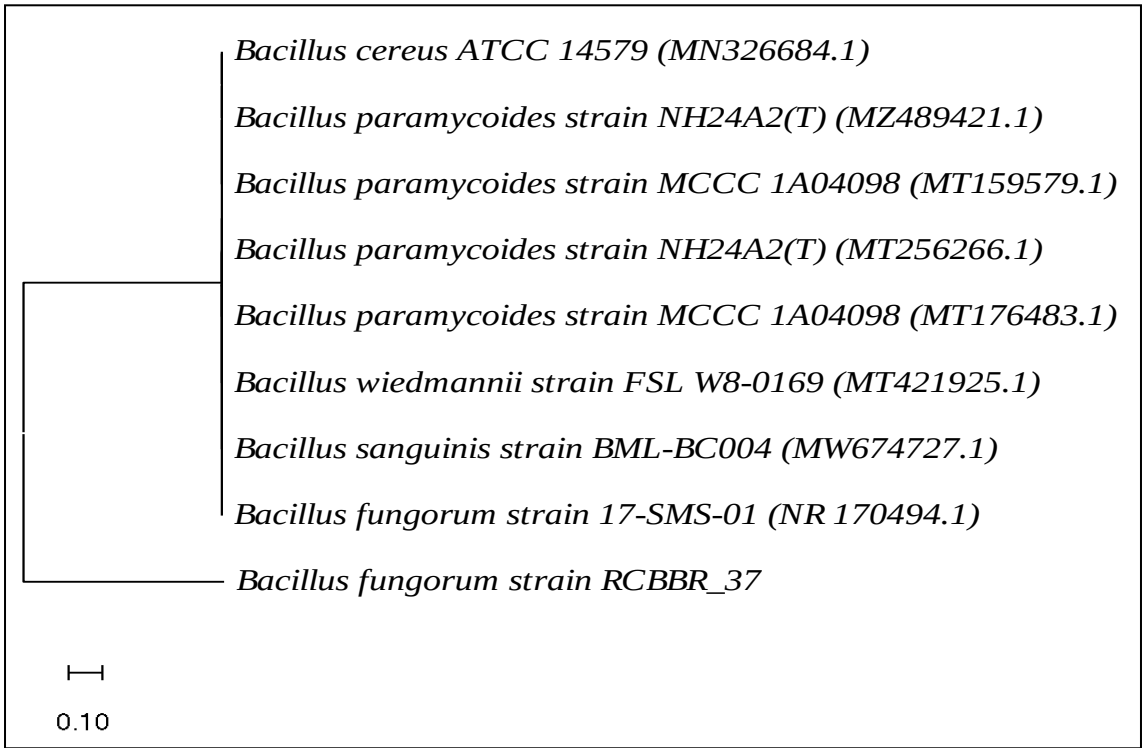
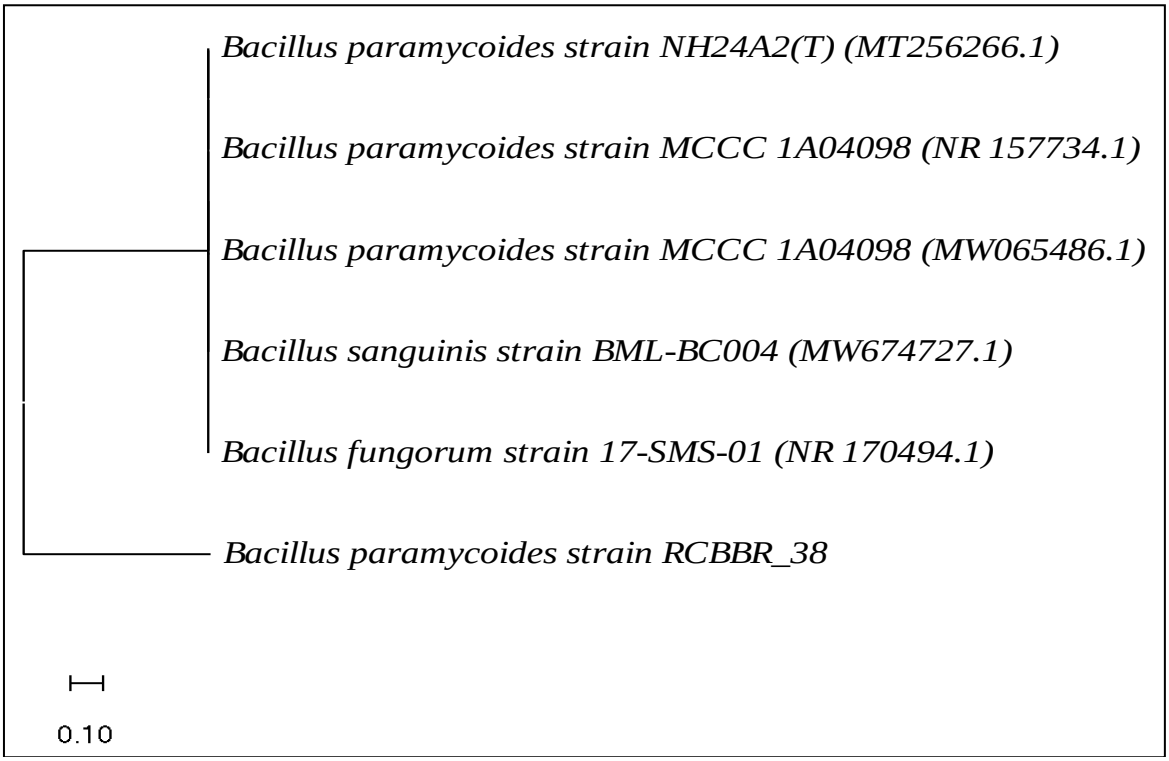


Fig. 2. 16S rRNA PCR bands from the isolates (1: RCBBR_37; Lane 2: RCBBR_38)



Isolate 1. RCBBR_B37



Isolate 2. RCBBR_B38

Fig. 3. Phylogenetic tree of the identified bacterial isolates using neighbor joining tree

Table 3. The sequencing reads of RCBBR_B37 and RCBBR_B38 isolates

Isolate code	Affiliated sequence
RCBBR_37	>RCBBR_37 GCTTAATGCGTTAACTKCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCG TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCA GTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCA TTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCA ATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAARAAACCACCTGCGCGC GCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGC ACGTAGTTAGCCGKGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGC ACTTGTTCTTCCCTAACAACARAGTTTTACRACCCGAAAGCCTTCATCACTCACGCGGCG TTGYTCCGTCAGAYTTTCGTCCATTGCGGAARATTCCCTACTGCTGCCTCCCGTAGGAGT CTGGGCCGTGTCTCAGTCCCAGKGKGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTT GCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGYCCATCCATAAGTGA CAGCCGAAGCCGCCTTTCAATTTCGAACCATGCRGTTCAAAATGTTATCCGGTATTAGCC CCGGTTTCCCGGAGTTATCCCARTCTTATGGGCAGGTTACCCACGKGTWACTCACCCGTC CGCCGCTAACTTCATARGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTATTAGG
RCBBR_38	>RCBBR_38 AATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTA CGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGT CAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTC ACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGA CCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTT TACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGT AGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACTT GTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGC TCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGG GCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCT TGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGC CGAAGCCGCCTTTCAATTTCGAACCATGCRGTTCAAAATGTTATCCGGTATTAGCCCCGG TTTCCCGGAGTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCC GCTAACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCATGTATTAGGCACGC CGCCAGCGTTCATCCTGAGC

Table 4. Identity of the bacterial isolates - strain, name of organism, closest GenBank, percentage identity and accession

S/N	Strain	Organism	Closest GenBank Match	Similarity (%)	Accesion No
1	RCBBR_37	*Bacillus fungorum*	*Bacillus fungorum* strain 17-SMS-01	98.09	OR105047
2	RCBBR_38	*Bacillus paramycoides*	*Bacillus paramycoides* strain NH24A2(T)	99.88	OR105048

4. DISCUSSION

This investigation focused on the molecular characterization of prevalent bacteria within a heavy metal-contaminated dump site. Molecular characterization methods provide distinct advantages over cultural approaches for studying microbial communities. While cultural methods are restricted to identifying only cultivable microbes, molecular techniques enable the thorough examination of both cultivable and non-cultivable microorganisms present in environmental samples. Techniques such as polymerase chain reaction (PCR) and high-throughput sequencing offer precise and sensitive evaluations of microbial diversity, allowing for unbiased detection of various taxa and the identification of rare and novel microbes that may play significant ecological roles [32]. Moreover, molecular methods facilitate the quantitative analysis of microbial communities, empowering researchers to assess changes in community structure, relative abundance, and gene expression levels over time or in response to environmental changes. When combined with functional assays, molecular techniques also permit the exploration of microbial metabolic capabilities and activities, offering insights into the functional potential of microbial communities. In summary, molecular characterization methods offer robust and comprehensive tools for comprehending microbial diversity, ecology, and function in natural environments, as well as providing more precise characterization of microorganisms.

Culture-dependent molecular characterization has been utilized to identify a significant number of bacteria present in heavy metal-polluted environments. Islam and Sar [33] employed this dual technique to investigate the bacterial community within uranium ore. Heavy metal-contaminated sites often harbor a diverse array of bacteria capable of surviving and thriving in these challenging conditions [34,35,36]. Among the common bacteria found in such environments are *Pseudomonas* spp., known for their versatility and ability to tolerate and detoxify metals through efflux pumps and metal-binding proteins, and *Bacillus* spp., which form resilient endospores and exhibit resistance to heavy metals. Alhasawi et al. [37] identified *P. fluorescens* as capable of tolerating and detoxifying heavy metals in contaminated media. Previously, Aguilar-Barajas et al. [38] reported on the role of *Pseudomonas* spp. in metal homeostasis *and* detoxification. *Acidithiobacillus* spp. are excellent in bioleaching metals from ores and can tolerate extreme pH and metal concentrations. Akinci et al. [39] confirmed the role of *Acidithiobacillus* spp. in heavy metal leaching in contaminated sediment, both in pure and mixed states. Alviz-Gazitua et al. [40] reported *Cupriavidus metallidurans* as a model for studying extreme resistance to heavy metals such as cadmium, zinc, and cobalt, while *Arthrobacter* spp. and *Stenotrophomonas maltophilia* possess mechanisms for detoxifying and tolerating heavy metal exposure [41,42]. *Rhodococcus* spp. also contribute, often playing roles in bioremediation efforts due to their metabolic versatility and ability to withstand metal toxicity [43]. *Bacillus* spp. form resilient endospores and exhibit resistance to heavy metals [44]. Collectively, these bacteria play critical roles in the ecology and biogeochemistry of heavy metal-contaminated environments, influencing metal cycling, remediation processes, and ecosystem dynamics.

Bacillus spp. demonstrate significant adaptive responses to heavy metal exposure, rendering them crucial contributors to mitigating metal pollution in contaminated environments. These bacteria utilize various mechanisms to cope with metal toxicity, including metal sequestration through cell surface structures and exopolysaccharides [45], enzymatic detoxification involving metallothioneins and metalloenzymes [46], and active efflux pump systems that expel metals from the cytoplasm [44]. Additionally, *Bacillus* species are proficient biofilm formers [47], which not only shield cells from metal toxicity but also facilitate interactions with metals, such as sequestration and

reduction. Maity et al. [48] isolated *Bacillus* strain GH-s29, exhibiting outstanding biofilm formation linked to multi-metal remediation potential. Through genetic adaptation, *Bacillus* spp. can acquire or upregulate genes encoding metal resistance determinants, augmenting their capacity to survive and thrive in metal-contaminated habitats [49] (Shin et al., 2012;) [50,51,52]. Their metabolic versatility and resilience position *Bacillus* species as promising candidates for bioremediation applications, wherein they can be employed to enhance the removal or immobilization of heavy metals from contaminated sites, thereby contributing to environmental remediation endeavors [53-56].

5. CONCLUSION

This research underscores the crucial role of molecular characterization techniques in elucidating microbial dominance within heavy metal-contaminated environments. Molecular methods offer distinct advantages over cultural characterization approaches, allowing for the comprehensive analysis of both cultured microbial taxa present in these challenging habitats. By utilizing techniques such as PCR, high-throughput sequencing, and functional assays, researchers can glean insights into microbial diversity, ecology, and functional potential in natural settings. The study also sheds light on the prevalence and adaptive mechanisms of bacteria, particularly *Bacillus* spp., in response to heavy metal exposure. It provides evidence of *Bacillus* species employing versatile strategies to cope with metal toxicity, including metal sequestration, enzymatic detoxification, and active efflux, thereby making them indispensable contributors to the mitigation of metal pollution in contaminated sites. The resilience and adaptability of *Bacillus* spp. position them as promising candidates for bioremediation applications, offering optimism for the restoration of heavy metal-contaminated environments and the preservation of ecosystem health.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ACKNOWLEDGEMENT

We extend our sincere appreciation to Prof. C.J. Ogugbue, Mr. and Mrs. Smart Obaretin, and Mr. and Mrs. Godspower Ogboms for their generous support in facilitating the dissemination of this research as a public document.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/119962