Bioremediation of heavy metals by some bacterial and fungal species

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Abstract:
Heavy-metal contaminants are increasingly becoming one of the most difficult challenges of contemporary agriculture due to their high toxicity and ability to accumulate in soils and crops. This presents potential threats to humans through food contamination, which could cause detrimental health effects. To overcome this concern, it is necessary to accelerate the pace of restoration of disturbed agricultural lands. Bioremediation is an effective treatment for agricultural soil pollution as it relies on the ability of microorganisms to remove pollutants. Hence, this study aimed to isolate and use bacterial and fungal species from oil-polluted soil samples to remediate heavy metal contaminants. Soil samples were collected from three locations in the B-dere community, Gokana, Rivers State. Physicochemical analysis, identification of bacterial and fungal isolates, selection of heavy metals degrading bacteria and fungi, percentage occurrence of isolates, and screening microbial isolates for heavy metals tolerance index were conducted. The results showed a physicochemical pH of 8.8 at 28.6 °C. Electrical conductivity was 1213 µs/cm, with total nitrogen and organic carbon contents of 14.6% and 9.22%, respectively. Eight bacterial isolates were identified in this study: *Nocardia* spp., *Bacillus cereus, Bacillus subtilis, Corynebacterium* spp, Clostridium spp. *Chromobacterium violaceum, Pseudomonas putida and Serratia marcescens. Pseudomonas aeruginosa* was the most prevalent bacterial isolate (29%). However, *Bacillus* sp had the highest tolerance index to the heavy metals studied. Five fungal cultures were identified from the oil polluted soil: *Penicillum sp, Aspergillus terreus, Aspergillus niger, Rhizopus sp.,* and *Fusarium sp* with *Aspergillus niger* being the most prevalent (34%). *Aspergillus* sp. had the highest tolerance index. The increase in population observed in both *Bacillus* sp and *Aspergillus* sp shows that they have the ability to remediate heavy metals such as lead, cadmium and nickel. Though heavy metals affected some soil microorganisms, which are important in soil fertility but *Bacillus* and *Aspergillus* spp have the ability to utilise and degrade lead, nickel and cadmium, particularly in contaminated areas.

Keywords: **Bioremediation, Degradation, Heavy metals, Microbial isolates, Soil pollution.**

1. Introduction

HEAVY metal pollution, a pressing environmental issue, originates from various anthropogenic activities including industrial processes, mining, and agricultural practices. Three distinct criteria are used to describe heavy metals: atomic number, chemical characteristics and density [1]. They belong to a vaguely defined group of elements that have metallic characteristics, such as actinides, lanthanides, transition metals, and certain metalloids. Lead, chromium, nickel, copper, cadmium, and zinc are among the non-biodegradable metals that build up in soil and water bodies despite their economic significance, posing serious threats to ecosystems and human health [2][3].

Point and nonpoint sources are the two categories into which water contamination falls [4]. Point sources of pollution are distinct, localized sources of water contamination that can happen when dangerous materials are released straight into bodies of water [1]. According to Medfu-Tarekegn et al. (2020), non-point source pollution affects water bodies through diffuse sources such as contaminated runoff from farms that enter rivers and the ocean [1]. It remains quite challenging to identify a single remedy to reduce nonpoint source pollution since it comes from a wide range of sources. Yet, agricultural, geogenic, atmospheric, pharmaceutical, industrial, and household effluents are identified sources of heavy metals present in the environment [5][6]. Refineries that process metal, power plants that burn coal, nuclear power plants, high-tension lines, petroleum, plastics, textiles, microelectronics, insecticides, wood preservation facilities, and paper mills are examples of industrial sources [7].

Heavy metal sources in arable lands in most countries include sewage sludge applications, smelting, mining, agrochemicals, livestock dung, and natural sources [8][9]. Through their roots, plants take up these heavy metals from the soil [10]. Heavy metals from airborne particles may also be absorbed by the leaves or stems of the plants [11][12]. Due to human interference and acceleration of nature's slow-moving geochemical cycle of metals, most soils in both rural and urban areas can build up one or more heavy metals above defined baseline levels sufficiently high to pose hazards to human well-being, plants, animals, and ecosystems [13].
Traditional remediation techniques, such as chemical treatment and physical removal, often prove to be costly, disruptive, and may generate secondary pollutants. Consequently, there is a growing demand for sustainable and eco-friendly approaches to mitigate heavy metal contamination. Bioremediation, a technology that employs microorganisms to degrade or detoxify pollutants, has emerged as a promising alternative. According to Kour et al. (2021), bioremediation is described as the process of converting organic or inorganic waste into generally harmless products through biological degradation or transformation [14]. Using the ecosystem's natural biological activity, this technology can eliminate or render a variety of

toxins harmless [2]. Bacteria and fungi, with their diverse metabolic capabilities, have demonstrated potential for bioremediation of heavy metals. These microorganisms can utilise various mechanisms, including biosorption, bioaccumulation, biotransformation [15], and biovolatilisation [16].

Several studies have highlighted the potential of microbial bioremediation for heavy metal removal [3][17][18][19]. A notable mention is the report of Medfu-Tarekegn *et al*. (2020), who showed the efficiency of *Aspergillus* sp. employed during the clearance of chromium in tannery wastewater [1]. Kumar *et al.* (2014) explored the biosorption of heavy metals such as copper, lead, and chromium by fungal and bacterial isolates [20]. Similarly, Abioye *et al*. (2018) recorded promising results in their investigation of the biosorption of Pb, Cr, and Cd in tannery effluent using *Bacillus megaterium*, *B. subtilis*, *Penicillium* sp and *Aspergillus niger* [17]. Bacteria are essential in soil fertility and organic matter decomposition, aiding the process of nutrient recycling [21]. Sadly, the increasing anthropogenic activities impact agricultural soils' productive qualities and fertility by reducing organic matter, depleting nutrients, and polluting them with polycyclic aromatic hydrocarbons, mineral fertilisers, pesticides, and heavy metals [22]. This is because industrialisation and rapid population growth increase the demand for food. Devastating economic losses and a sharp reduction in agricultural soil quality are the results of poor agricultural practices and management of land and water resources [23]. This circumstance made it imperative to increase the repair and decrease the degradation rates of disturbed agricultural areas. This assignment is one of the 17 sustainable development objectives established by the UN through 2030 [24].

The heavy-metal poisoning of agricultural fields is a significant worry for agrarian communities worldwide. Another cause of soil contamination by heavy metals is the ongoing use of fertilisers in farm operations. Heavy metals are extremely harmful substances that have a lengthy half-life in soil. Fertilisers, especially organic ones, incorporate Cd, Pb, Zn, and Cu into agricultural soil. However, arsenic and mercury may mix with the soil if a farming field follows an industrial facility [25]. Furthermore, bioaccumulation of heavy metals is a common occurrence. They have been shown to build up in crops, altering their biochemical and physiological processes [26]. If allowed to accumulate to a high concentration, they lower plant productivity and can cause plant tissues to necrotise [27]. Biomagnification aids the accumulation of heavy metals, which pose a major risk to human and animal health [28]. Mining companies, burning of industrial waste, oil and oil product spills, transportation emissions, and residential waste dumps are all examples of soil contamination, whether anthropogenic or technogenic [29]. When industrial waste is left unchecked, heavy metals tend to migrate to agricultural areas due to strong rains, microbiological activity, and natural erosion [30]. Another cause of heavy-metal contamination of soil is the ongoing and careless application of fertilisers in farming operations. For instance, a significant misuse of chemical fertilisers may be linked to elevated cadmium, copper, and arsenic levels. Phosphate ore, a crucial metal component of phosphate fertilisers, contains Cadmium (Cd) and arsenic (As). Agricultural soil pollution is also a result of pesticides and herbicides, as pesticides contain chromium, copper, lead, and nickel. Additionally, Bai et al. (2015) found a correlation between the cultivation time and greenhouse soils' Co, Zn, and Cu content [31]. Fertilisers most likely cause the accumulation of these contaminants. Both physicochemical and biological techniques can extract heavy metals from soil. Physical and chemical procedures include soil layer replacement, electrokinetic removal, thermal processing, soil washing, vitrification, and chemical treatment with lime, phosphate compounds, or organic compounds [32], each method having pros and cons.

Although physicochemical techniques are quick and effective, they are costly and time-consuming. They have the potential to alter markers of soil quality significantly over time. Accordingly, physicochemical approaches do not offer the best agricultural option [32]. Due to its affordability and environmental friendliness, biological treatment is a worthy substitute, with plants (phytoremediation) and microorganisms (bioremediation) being the foundation of biological techniques [33]. In recent years, bioremediation has garnered much scientific interest. Redox transformations, absorption, and modifications in the medium's reaction are the foundation for its mechanisms. According to Rahman et al. (2020), the most often used microbial processes for removing heavy metals include biosorption–bioaccumulation, biosurfactant synthesis, bioleaching, oxidation–reduction, biovolatilisation, and biomineralisation [34]. Although microorganisms can create defence mechanisms to fend off harm, most heavy metals damage microbial cell membranes. Thus, in restoring disturbed environments, microbes' ability to survive under the effect of heavy metals is crucial [35]. Proteobacteria are also observed to be resistant to elevated Zn and Pb concentrations [36]. Certain microbes can release extracellular polymeric materials, including proteins, lipids, and polysaccharides, when subjected to stress from heavy metals. In some parts of Nigeria, particularly the Niger Delta region, where substantial oil is extracted and industrialisation is growing, lacks commercially manufactured microbial cultures for oil clean-up, even though several wealthy countries do [37]. In essence, due to the overwhelming harm that heavy metal pollution does to ecosystems and human health, it has grown to be a serious environmental concern, with lead, cadmium, mercury, and arsenic among the metals that are frequently discharged into the environment as a result of mining operations, industrial processes, and agricultural practices. Considering the cost and adverse environmental effects of traditional remediation methods such as chemical treatments and physical removal, there is growing interest in investigating more economical and environmentally friendly alternatives, like bioremediation, which uses organisms' natural abilities to clean up contaminated sites in a sustainable and environmentally friendly manner. Hence, the current study sought to isolate microbial species that are capable of degrading heavy metals.

II. Materials And Methods

Sample collection and materials

Farmyard topsoil and subsoil (digging at a depth of 10cm) samples were obtained from three different farmlands of the B-

dere community, Gokana, Rivers State, Nigeria. These locations had different climatic and ecological conditions. About 500 g of soil samples were collected in sterile polythene bags. The soil samples collected appeared to have sandy loam properties at the top, with loamy sand subsoil, which appeared brown and well-drained. After being adequately homogenised, the samples were brought to the Microbiology Laboratory of the University of Port Harcourt for physicochemical and initial microbiological investigations.

Heavy metals, cadmium (Cd), arsenic (As), nickel (Ni), and lead (Pb) were sourced from local merchants in Port Harcourt, Rivers State. These heavy metals were prepared in 250 ml conical flasks. The choice of heavy metals used for the study was due to their widespread use in flower lawns and farming in the Niger Delta regions.

Physicochemical and heavy metal properties of the soil.

Before the addition of heavy metals, the physicochemical characteristics of freshly homogenised soil samples were assessed using standard procedures. These include the following: particle size, pH, chemical oxygen demand (COD), soil organic matter, total organic carbon (TOC), biological oxygen demand (BOD), organic carbon, total nitrogen, phosphorus, calcium, electrical conductivity, magnesium, sodium, potassium, and total exchangeable bases. The modified hydrometric method of [38] was used to determine the size of the soil particles. The dry oven method measured the moisture content [39]. The calibrated conductivity meter was used to determine the soil sample's electrical conductivity (EC). The percentage of organic matter and carbon in the soil was determined using Ataikiru & Ajuzieogu's (2023) methods, while potassium dichromate oxidation was used to determine the soil organic carbon (SOC). Soil organic matter (SOM) was calculated from SOC utilising the equation:

SOM (%) = SOC (%) * 1.724. (This equation assumes that organic matter contains approximately 58% carbon.)

As the titration ended, the indicator's violet colour turned green. The total nitrogen concentration was determined using the Kjeldahl method [40]. Organic carbon content was measured with the wet dichromate oxidation technique.

The total exchangeable bases were also determined according to standard procedures. Magnesium and calcium contents were also measured. Heavy metal analysis was done following the methods of [39] through AAS, PerkinElmer model 2130.

The characterisation of heavy metals was done through the Gas chromatography-mass spectroscopy (GC-MS) technique.

Microbiological analysis of the soil samples

Serial dilution was done by introducing 1 g of the soil sample into a 9 ml volume of sterile physiological saline. Aliquots of 0.1 ml dilutions were plated using appropriate media to enumerate microorganisms. The bacterium was isolated from the farm yard soil using nutrient agar (Sigma Aldrich, USA), while the fungus was isolated from the soil using chloramphenicol-amended potato dextrose agar (Merck, Germany). The soil samples were homogeneously mixed and then sieved with the use of a 2.0mm sieve to remove debris. One gram (1g) of the sieved soil was weighed into test tubes containing 9 ml sterile distilled water, and agitated for a minute, then serially diluted. Aliquots of 0.1 ml were spread on suitable media (as indicated above) with incubation at room temperature for 24 h for the fungus, and 37°C for 48 h for the bacterium. Discrete colonies of the most prolific (fastest growing) isolates were sub-cultured onto fresh medium for 3 – 4 cycles in order to obtain pure cultures. These fast-growing bacteria and fungi were preserved on slants for characterisation studies [41].

Characterisation for the bacterium was carried out using Gram's staining technique and standard biochemical tests including nitrate, indole, coagulase, starch hydrolysis, oxidase, catalase, citrate utilisation, sugar fermentation, urease, triple sugar iron agar test, methyl red and Voges-Proskauer tests. The isolate was identified based on the results from biochemical analysis using the scheme provided by [41]. Identification to the species level was done using the API® 20E kit and Microgen identification. Fungi were counted using potato dextrose agar (PDA) (Sigma Aldrich), which had chloramphenicol agar (0.1 g/L) and was selective only for fungal growth [42]. Ataikiru & Ajuzieogu's methods [39] were used to count heterotrophic bacteria on a plate count agar enriched with griseofulvin (50 µl/ml). Fungal identification entailed macroscopic and microscopic analysis using lactophenol blue stain.

Selection of heavy metal-degrading bacteria and fungi

The radial growth of identified fungi and bacteria on media mixed with the test heavy metals was examined. Single colonies of pure cultures of bacteria and fungi were picked and initially tested for their ability to degrade heavy metals.

For bacterial culture, 1 ml of a 24 h culture, grown in liquid media (nutrient broth), was inoculated into growth media (nutrient agar) containing the heavy metals in concentrations of 0, 100, 500, 1000, and 2000 µg/l, then incubated for 48 h at 37 °C. For fungal isolates, 1 ml of the spores of the fungal isolates was inoculated into potato dextrose agar containing the heavy metals in concentrations of 0, 100, 500, 1000, and 2000 µg/l, and incubated for 7 days at room temperature. Plants were covered with aluminium foil to prevent the visual deterioration of heavy metals. The radial growth of the metals was then photographed and documented. In comparison to control plates, the ability of bacteria and fungi to thrive on such media containing heavy metals was computed. Three iterations of this experiment were conducted (Pollution induced Community Tolerance, 88) [37].

Identification of Bacterial Isolates

The bacterial isolates were subcultured onto nutrient agar to obtain pure cultures for Gram reaction, microscopy, and biochemical features. The isolates were identified to the species level using the Microgen Kit. Standard microbiological techniques were used to determine the bacterial isolates. This involves using the API® 20E identification kit to identify Gram-negative bacteria.

Identification of fungal isolates

To obtain a pure culture, the fungal isolates were sub-cultured onto potato dextrose agar (PDA), and lactic acid was used to observe mounted cultures under a microscope. Each fungal

colony's morphological characteristics were assessed and recorded using the fungal atlas.

One (1) ml of 24h bacterial and fungal spore suspensions (each ml of fungi containing approximately 106 colony-forming individuals) was added to the media containing various concentrations of the heavy metals (500, 1000, 2000 ppm) and control (without heavy metals) for bioremediation activities. Sampling for bacterial isolates was conducted at intervals after 24 h, 48 h, and 168 h, while sampling for fungal isolates was done after 7 days, 21 days, and 28 days. COD and TOC analyses were performed on the filtrates after the samples were filtered using a 0.45 µm filter paper for bioremediation investigations. During this procedure, one drop of freshly made 1 N H_2SO_4 was introduced to the filtrates to stop the growth of fungal colonies. The assays were performed in triplicate.

Performance of the potential bioremediation activities of the tolerant organisms on heavy metals

Bioremediation activities on the heavy metal-tolerant bacteria and fungi were investigated based on COD and TOC. To begin with, COD and TOC values were measured in the enriched environment where the microorganisms were found. The entire experiments were carried out in triplicate, with the average value of replicates taken. For COD analysis in the study, the open reflux method outlined in standard method 5220B was employed; however, for TOC studies, the Shimadzu TOC-V device and the standard procedure with elevated temperature combustion method 5310A were preferred. The COD concentration was determined using the closed reflux colourimetric method for the DR860 HACH spectrophotometer COD tests. The measurement differences were fewer than 0.02, and the test values represent the average of three measurements. The strength of COD and TOC elimination was determined through Eq. (1) and Eq. (2), respectively [43].

COD Removal Efficiency (%) = $[(COD_{initial} - COD_{final})/COD_{initial}] \times 100$

TOC Removal Efficiency (%) = $[(TOC_{initial} - TOC_{final})/TOC_{initial}] \times 100$

Where:

$COD_{initial}$ and COD_{final} (mg/L) are the values of COD before and after the COD bioremediation process at time *t*, respectively. $TOC_{initial}$ and TOC_{final} (mg/L) are the values of initial TOC and TOC after the bioremediation process at time *t*, respectively.

Impact of heavy metals on bacterial and fungal growth

The effect of heavy metal degradation was determined by optical density (bacteria) and wet weight (fungi). Following bioremediation, the media turbidity's optical density (OD), a measure of bacterial growth, was measured to track the impact of heavy metal degradation on bacteria. Following fungal incubation, the fungal mycelia mats were removed, weighed with an electronic balance, and used to determine the mycelium's moist weight. The fungal growth parameter is calculated using the wet biomass of the fungal mycelium. The mean values were calculated and recorded from the duplicate values. The growth of the fungal isolates was calculated using the formula proposed by [44].

Analysis of biodegradation residues of heavy metals by HPLC

A modified [45] approach was used to remove and clean up heavy metal deposits in the bioremediated liquids. A total of 10 ml of 0.1% acidified MeCN was vortexed with 10 ml of the bioremediated liquid for one minute. Then, 4 g of MgSO4 (anhydrous) and 1 g of NaCl were vigorously combined for one minute. After that, tubes were filled with the internal standard triphenyl phosphate (TPP) solution and shaken for 30 seconds. Centrifugation was performed for 10 minutes at 1350 × g (Hermle Labortechnik GmbH, Siemensstr 25, D-78564 Wehingen, Germany). Exactly 150 mg of $MgSO_4$ (anhydrous) and 25 mg of PSA sorbent were manually combined with 1 ml of supernatant (acetonitrile) for 5 minutes, and the mixture was centrifuged for 5 minutes at 1350 × g. About 500 µl of each tube was filtered through 0.22-µm PTFE filters (Millipore, USA) into HPLC vials for GC–MS analysis.

With a mobile phase made up of ACN: trimethylamine (60:40, v/v) and a pH adjusted to 2.5 with orthophosphoric acid (85%), an HPLC analysis was carried out using an isocratic method to examine the biodegradation of heavy metals. The column was kept at room temperature for the entire 10-minute run time, and the flow rate was set at 1.0 ml/min. A wavelength of 226 nm was employed for detection, and the injection volume was 20 µL. Before chromatographic analysis, all samples were collected, filtered using 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium), and kept at 4 °C. A modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 and Smartline Manager 5000 (Berlin, Germany) was used to analyse fluoxetine. ClarityChrom® software was used to integrate and monitor the output signal. A Guard Column Xbridge-C18 column (4.6 × 200 mm, 5 µm particle size) and a reversed-phase Spherisorb C18 column (250 × 4.6 mm, 5 µm particle size) with silica technology were used to separate the chemicals. Both were acquired from Waters Corporation (Milford, MA, USA).

Statistical analysis

All values were presented as mean ± standard deviation, and the experiments were conducted in triplicate. Excel Data Analysis Tools were used to perform a one-way analysis of variance (ANOVA) (Single Factor) test on the pesticide degradation data. The p-value of 0.05, which was deemed statistically significant, was used in the statistical analyses.

III. RESULTS

The physicochemical characteristics of the oil-polluted soil sample are presented in Table 1. The results showed that the soil has pH of 8.8, a temperature of 28.6°C, an electrical conductivity of 1213 µS/cm, and total nitrogen and organic carbon of 14.60% and 9.22%, respectively. The result also showed the presence of sulphate (31.2 mg/L), nitrate (15.42 mg/ L), phosphate (8.20 mg L), chloride (13.31 mg/ L), copper (10.29 mg/ L), zinc (8.80 mg/ L) and manganese (2.92 mg/ L).

Table 1: Physicochemical characteristics of oil-polluted soil

Parameters	Mean ± SE
pH	8.8 ± 0.20
Temperature (°C)	28.6 ± 0.33
Electrical conductivity (mho/cm)	1213 ± 2.05
Total nitrogen %	14.60 ± 0.16
Total organic carbon (%)	9.22 ± 0.32
Sulphate (mg/ L)	31.2 ± 0.22
Nitrate (mg/ L)	15.42 ± 0.51
Phosphate (mg/L)	8.20 ± 0.02
Chloride (mg/L)	13.31 ± 0.68
Copper (mg/ L)	10.29 ± 0.49
Zinc (mg/ L)	8.80 ± 0.17
Manganese (mg/ L)	2.92 ± 0.56

Values are means of duplicates: ± Standard error of the mean

Table 2. shows the total bacterial and fungal counts from the oil-polluted soil. The result indicated that soil sample CS_{2b} had the highest heterotrophic bacterial count (30.0 cfu/g×10^4), followed by soil sample CS_{1b} (9.75 cfu/g×10^4), while soil sample FS1 had the lowest heterotrophic bacteria count (1.15 cfu/g×10^4). The result also showed that for fungal count, CS_2 is reported to have the highest fungal count (4.4 sfu/g× 10^3) while CS_{2b} is reported to have the lowest fungal count (0.5 sfu/g× 10^3). There is no fungal growth observed in the soil sample $CS_{1b.}$

Table 2: Total viable counts of bacterial and fungal isolates from oil-polluted soil

Soil Samples	Total Heterotrophic Bacteria (cfu/g×10^4)	Fungi (sfu/g× 10^3)
CS_{1a}	9.75 ± 0.22	0.95 ± 0.32
CS_{1b}	4.5 ± 0.33	NG
CS_2	6.05 ± 0.00	4.4 ± 0.35
CS_{2b}	30.0 ± 0.43	0.5 ± 0.02
FS1	1.15 ± 0.00	2.2 ± 0.15
FS2	9.50 ± 0.10	3. 0 ± 0.00

Counts represent means of triplicate samples ± standard error. Key: CS=; CS=; FS=; cfu/g= Colony forming units per gram; sfu=spore forming unit units per gram; NG= No growth

Morphological and biochemical characteristics were employed for the identification of isolates. The results of the Morphological and biochemical characteristics of bacterial isolates from oil-polluted soil are shown in Tables 3 and 4, respectively. Eight bacterial isolates were identified, five of which were Gram-positive bacteria, including *Nocardia* spp., *Bacillus cereus, Bacillus subtilis, Corynebacterium* spp., and *Clostridium* spp., and three Gram-negative bacteria (*Chromobacterium violaceum, Pseudomonas putida, Serratia marcescens)* were obtained from the polluted soil sample. However, ten bacterial genera/species were isolated from the oil-polluted soil sample (Table 4).

Table 3: Morphological characteristics of the bacterial isolates from the oil-polluted soil

ID	Color	Shape	Margin	Surface	Elevation	Opacity	Size (mm)	Samples
CS_110^2	Purple	Circular	Entire	S&S	Raised	Opaque	0.2	FM1
CS_110^2	Cream	Irregular	Serrated	R&D	Raised	Translucent	2.2	FM2
CS_110^2	Cream	Circular	Entire	R&D	Flat	Opaque	0.4	FM3
CS_210^2	Cream	Circular	Entire	R&D	Raised	Opaque	0.3	FM4
CS_210^2	Cream	Irregular	Serrated	R&D	Raised	Translucent	0.5	FM5
CS_210^2	Cream	Circular	Entire	R&D	Raised	Opaque	0.3	FM6
CS_210^2	Cream	Irregular	Serrated	S&S	Flat	Translucent	0.6	FM7
CS_210^4	Cream	Irregular	Serrated	S&S	Raised	Translucent	0.8	FM8
$FS10^4$	Cream	Irregular	Serrated	R&D	Raised	Opaque	0.3	FM9
$FS10^4$	Cream	Irregular	Serrated	R&D	Raised	Translucent	3.0	FM10

Table 4: Biochemical characteristics of the bacterial isolates

6	FM1	FM2	FM3	FM4	FM5	FM6	FM7	FM8	FM9	FM10
Catalase	+	+	+	+	+	+	+	+	-	+
Citrate	+	-	+	+	-	-	+	-	+	-
Glucose	+	-	+	+	+	+	+	-	+	-
Lactose	-	-	-	-	-	-	-	-	+	-
Indole	-	-	-	-	-	-	-	-	-	-
MR	-	-	+	+	-	+	-	-	-	-
VP	-	-	-	+	+	+	+	-	-	-
Motility	+	+	-	+	+	-	+	+	-	+
Starch	+	+	-	+	+	-	+	+	+	+
TSI	AA+	AA-	AA+	AB-	AB-	AB-	AB-	AA-	AB-	AA-
H2S	-	-	-	+	+	+	-	-	+	-
Gram stain	-	-	+	+	+	+	-	-	+	-
Possible genera	*Chromobacterium violaceum*	*Pseudomonas putida*	*Nocardia* spp.	*Bacillus cereus*	*Bacillus subtilis*	*Corynebacterium* spp.	*Serratia marcescens*	*Pseudomonas putida*	*Clostridium* spp.	*Pseudomonas putida*

Key: '+'=Positive; '-'=Negative

Table 5 shows the morphology and microscopic characteristics in identifying fungal cultures from the polluted cultures, five fungal cultures were identified as *Penicillium sp, Aspergillus terreus, Aspergillus niger, Rhizopus fusarium sp*.

Table 5: Morphology and microscopic characteristics identification of fungal isolates

Isolate code	Macroscopy	Microscopy	Probable fungi
FMF1	Cream non-cracked reverse, green powdery mycelia, white margin.	Long hyaline hyphae with brush-like conidiophores	*Penicillium* sp.
FMF2	Golden Brown non-cracked reverse, white margin, Brown mycelia with shiny crystals.	Conidiospores are long, hyaline, and typically bear a vesicle at the tip, which can be globose or ellipsoidal. The vesicle is covered with phialides. Conidia are one-celled, typically smooth, and hyaline, but can be pigmented.	*Aspergillus terreus*
FMF3	Cream cracked reverse, black powdery mycelia, cream margin.	Thick-walled hyphae are unbranched, bearing conidia. Presence of scattered black globose spores	*Aspergillus niger*
FMF4	Cream non-cracked reverse, white woolly mycelia with black spores	Presence of hyphae, sporangia, and spores	*Rhizopus* sp.
FMF5	Pink non-cracked reverse, white woolly mycelia	Presence of septate hyphae bearing conidia	*Fusarium* sp.
FMF6	Cream cracked reverse, white margin, greenish dense mycelia.	Presence of thin, long, branched septate conidiosphores bearing conidia	*Penicillium* sp.
FMF7	Yellowish non-cracked reverse, white margin, greenish mycelia with shinny crystals.	Presence of bluish round spores, thin hyphae	*Penicillium* sp.

Analysis of the occurrence of bacteria isolates from the oil-polluted soil reveals that *Pseudomonas putida* is reported as the highest occurring bacteria isolate with 29% occurrence, while *Nocardia sp*. is reported to be the lowest occurring bacteria isolate with 5% occurrence (Fig. 1). For Fungal cultures, *Aspergillus niger* is more prevalent in the oil polluted soil with 34% occurrence,

While *Rhizopus sp.* (22%), *Penicillum sp.* (19%), *Aspergillus terreus* and *Fusarium sp.*, have 13% occurrence each (Figure 2).

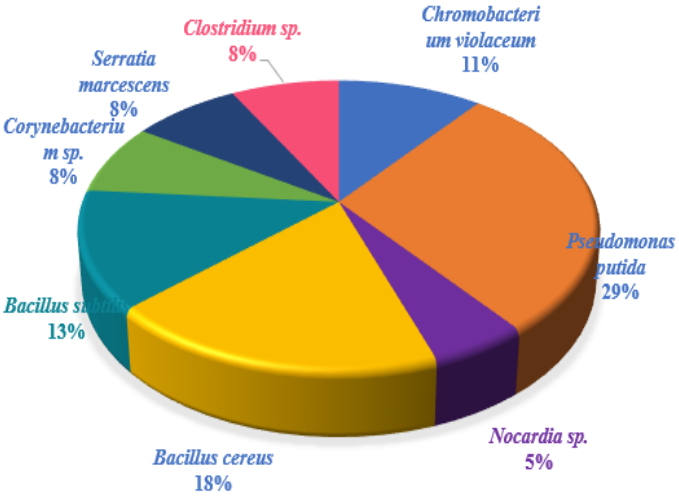

Figure 1: Prevalence of bacterial isolates from oil-polluted soil

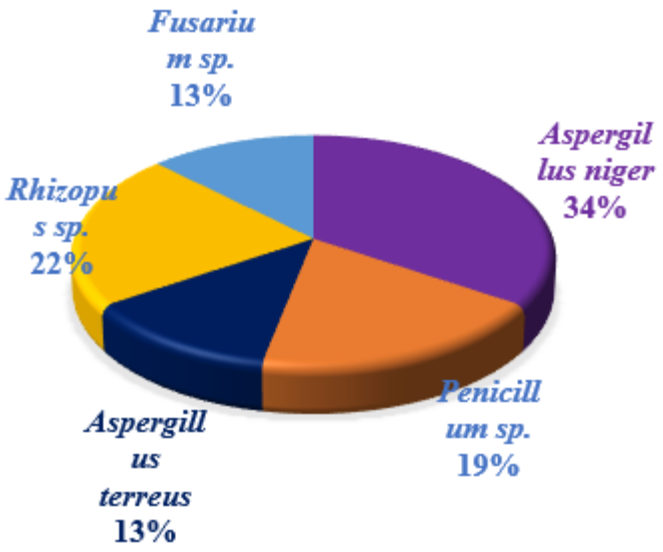

Figure 2: Prevalence of fungal isolates from oil-polluted soil

Table 6 shows microbial growth in the presence of heavy metals. The bacteria isolates, *Chromobacterium violaceum, Nocardia* spp., *Bacillus subtilis, Corynebacterium* spp., and *Clostridium* spp., were reported to have no growth in the presence of 100 ppm of Cadmium. *Bacillus cereus* showed moderate growth in Cadmium (100 ppm) compared to *Pseudomonas putida and Serratia marcescens*, which exhibited low growth. *Bacillus cereus* exhibits higher growth than *Chromobacterium violaceum, Pseudomonas putida, Nocardia* spp., and *Bacillus subtilis* in the presence of 100 ppm of Nickel, which exhibits moderate growth. In contrast, *Corynebacterium* spp., *Clostridium* spp., and *Serratia marcescens* exhibit low

growth in the presence of Nickel (100 ppm). Only bacterial isolates of the *Bacillus* genus (*Bacillus cereus and Bacillus subtilis*) exhibit moderate growth at 100 ppm of lead; other bacterial isolates exhibit low growth in lead (100 ppm). At the same time, *Clostridium* spp. was reported to have no growth.

Among the fungal cultures, only *Aspergillus niger* showed low growth at 100 ppm of Cadmium. In contrast, other fungal isolates, *Penicillium* sp., *Aspergillus terreus, Rhizopus* sp., and *Fusarium* sp., are reported to have no growth in the presence of 100 ppm of Cadmium. In the presence of 100 ppm of Nickel, *Aspergillus niger* show high growth compared to *Rhizopus* sp., *Fusarium* sp., which exhibited moderate growth while *Penicillium* sp., *Aspergillus terreus,* was reported to exhibit low growth. No growth was observed for *Aspergillus terreus* at 100ppm of lead while *Penicillium* sp., *Rhizopus* sp., *Fusarium* sp., show low growth and *Aspergillus niger* show moderate growth in the presence of Lead (100 ppm).

Table 6: Screening of microbial isolates for growth in the presence of test heavy metals

Code		Control	Growth in the presence of 100 ppm of heavy metals		
			Cadmium	Nickel	Lead
	Bacterial isolate	+++	-	++	+
FM1	*Chromobacterium violaceum*	+++	+	++	+
FM2	*Pseudomonas putida*	+++	-	++	+
FM3	*Nocardia* spp.	+++	++	+++	++
FM4	*Bacillus cereus*	+++	-	++	++
FM5	*Bacillus subtilis*	+++	-	+	+
FM6	*Corynebacterium* spp	+++	-	++	+
FM7	*Serratia marcescens*	+++	+	+	+
FM9	*Clostridium* spp.	+++	-	+	-
Fungal isolates					
FMF1	*Penicillium* sp.	+++	-	+	+
FMF2	*Aspergillus terreus*	+++	-	+	-
FMF3	*Aspergillus niger*	+++	+	+++	++
FMF4	*Rhizopus* sp.	+++	-	++	+
FMF5	*Fusarium* sp.	+++	-	++	+

'-' = No growth, '+' = low growth, '++'= moderate growth, '+++' = High growth

Figure 3 shows the tolerance index of the microbial isolates to the heavy metals used in this study. Bacillus *cereus* is reported to have higher tolerance for Cadmium, Nickel and Lead than other bacterial isolates, while *Clostridium* spp. have a low tolerance level for cadmium, nickel and lead. *Bacillus cereus* exhibits higher tolerance to cadmium when compared to lead and nickel. *Clostridium* sp have an equal level of tolerance to nickel and lead as to cadmium, and generally have low tolerance to the three heavy metals under study compared to other bacterial isolates. *Nocardia* and Corynebacterium spp showed low tolerance index to all three heavy metals studied. Other bacterial isolates showed higher tolerance to nickel when compared to lead and cadmium.

Figure 4 shows that the fungal cultures have relatively low tolerance to Cadmium. *Penicillium sp., Aspergillus terreus,* and *Fusarium sp* show no tolerance to Cadmium. However, they exhibit high tolerance for nickel (*Penicillium sp., Aspergillus niger*). *Aspergillus terreus* and *Rhizopus sp.* exhibit equal tolerance for both nickel and lead, with *Fusarium sp.* showing high tolerance for lead.

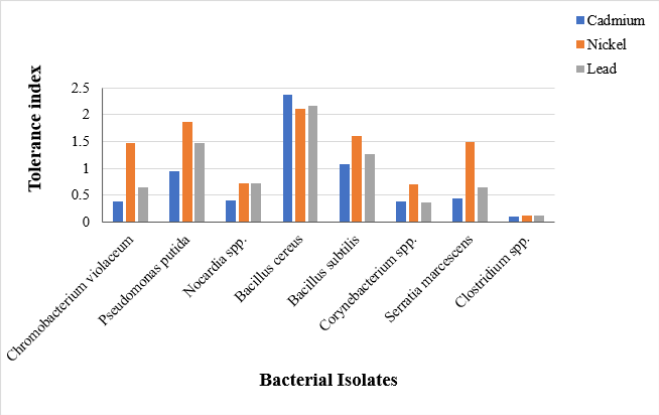

Figure 3: Heavy metals tolerance index of bacterial isolates

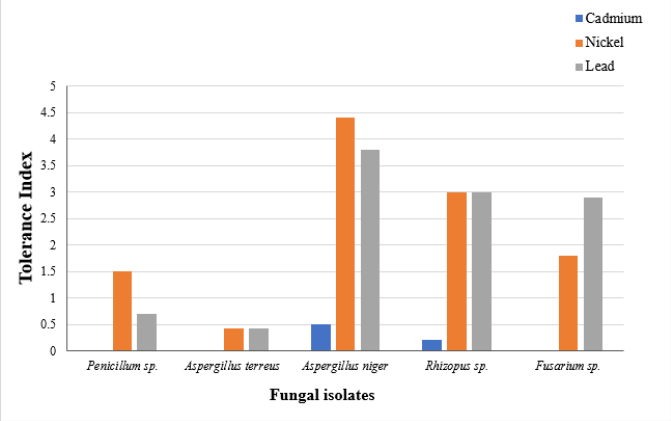

Figure 4: Heavy metals tolerance index of fungal isolates

Figure 5 shows the pattern in the biodegradation of heavy metals by *Bacillus cereus* at 24 hours. At 20 ppm and 40 ppm, respectively, *Bacillus cereus* is reported to record high growth, which increased at 48 hrs, but decreased after 168 hrs in cadmium-treated soil, but at 80 ppm, the growth rate of *Bacillus cereus* reduced in the 48 h and increased growth was observed at 168 hrs.

For nickel-contaminated soil, there was significant growth of *Bacillus cereus* observed. At 24 h, the growth of *Bacillus cereus* was reported to be highest in soil contaminated with 80 ppm of nickel, followed by growth observed at 20 ppm. While the least growth of *Bacillus cereus* was observed in 40 ppm concentration of nickel contamination, the growth of *Bacillus cereus* increased significantly among the different

concentrations of nickel contamination. At 40 ppm of nickel contamination, *Bacillus cereus* growth was observed at 24 h and increased significantly at 48 h; hence, maximum growth of *Bacillus cereus* was observed at 48 h, while the least bacterial growth at 48 h was recorded at 20 ppm concentration of nickel.

Bacillus cereus growth in lead-contaminated soil was observed to have the same growth pattern as observed in cadmium and nickel-polluted soil, however, the difference in the growth between 24 h and 48 h is very minute compared to that observed in the other treatments.

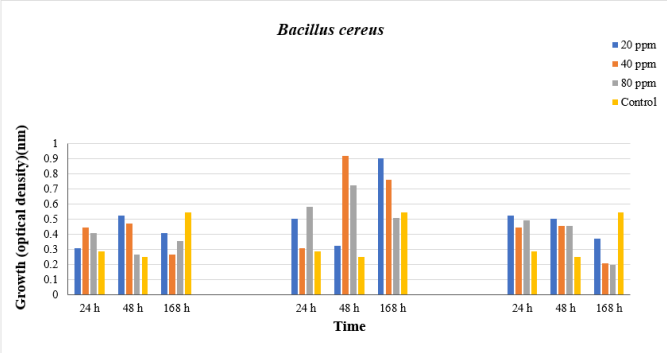

Figure 5: Biodegradation of heavy metals by *Bacillus cereus*

No fungal(*Aspergillus niger*) growth in cadmium-contaminated soil, while for nickel-contaminated soil, the highest fungal growth of *Aspergillus niger* was observed in 40 ppm nickel contamination, while the least growth was observed at 80 ppm contamination. *Aspergillus niger* growth in lead-contaminated soil decreased with increase in concentration of the lead in the soil, At 20 ppm contamination, the highest growth of *Aspergillus niger* was observed while the least growth of *Aspergillus niger* was observed at concentration of 80 ppm as shown in Figure 6.

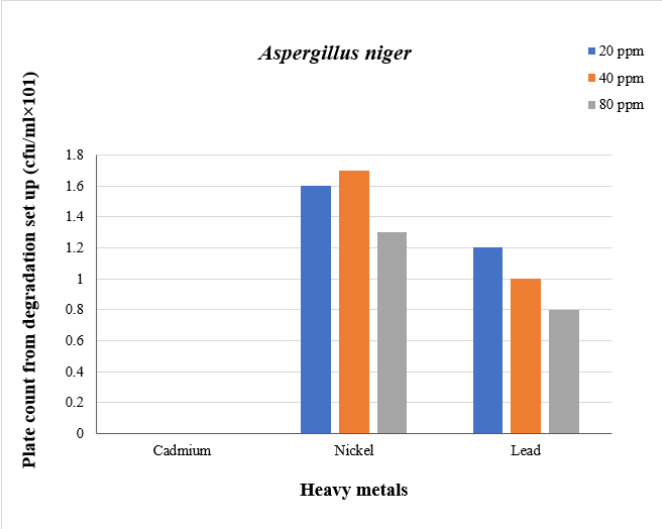

Figure 6: Biodegradation of heavy metals by *Aspergillus niger*

III. Discussion

Various techniques, such as filtration, ion exchange, and chemical precipitation, have been used to remediate soil contaminated with heavy metals in economically developed regions [46]. However, these techniques are reported to be expensive, and they exhibit other harmful and toxic effects in the environment [47]. Microorganisms possess the capability for the remediation of poisonous pollutants without being environmentally harmful [48]. It has been proposed that agents used for bioremediation purposes include bacteria [49], fungi [50], macroalgae, and microalgae [51]. These physicochemical parameters in this study were similar to the work of [52]. These conditions were favourable for microbial remediation.

Eight bacteria isolates were identified, five of which are Gram-positive bacteria, which include *Nocardia* spp., *Bacillus cereus, Bacillus subtilis, Corynebacterium* spp., and *Clostridium* spp., and three non-duplicate Gram-negative bacteria (*Chromobacterium violaceum, Pseudomonas putida, Serratia marcescens)* were obtained from the polluted soil sample. The most predominant bacterial isolates are of the genera *Pseudomonas* (29%) and *Bacillus* (31%), which agrees with the results of [53]. The identification of bacterial isolates revealed a predominance of Gram-negative bacteria such as *Pseudomonas putida and* Gram-positive bacteria, including *Bacillus subtilis* and *Bacillus cereus.* Among the fungal cultures, *Aspergillus niger* was the most prevalent. The findings of this study agree with another study that *Pseudomonas* and *Bacillus* genera are implicated as the most prevalent bacteria that degrade hydrocarbons and heavy metals [53].

The tolerance index results further supported the potential of *Bacillus cereus* and *Aspergillus niger* for bioremediation. *Bacillus cereus* showed high tolerance to all three heavy metals, while *Aspergillus niger* showed high tolerance to nickel and lead. These results are crucial as they highlight the resilience and adaptability of these microorganisms in soil contaminated with heavy metals. This shows the ability of some bacterial species to degrade and accumulate heavy metals [54][55].

This study reports that *Bacillus cereus* degrades heavy metals better than other bacterial isolates studied. The ability of bacteria to absorb or degrade heavy metals is time and concentration-dependent [54]. The biodegradation analysis revealed that *Bacillus cereus* was effective in degrading cadmium, nickel, and lead at various concentrations and times. The growth patterns of *Bacillus cereus* in contaminated soils indicated its bioremediation potential.

This study reports the absence of *Aspergillus niger* growth in cadmium contaminated soil, this could mean be as a result of cadmium toxixity to microorganisms [54]. While in Nickel contaminated soil, the highest growth of *Aspergillus niger* was observed in 40 ppm while the least growth was observed in 80 ppm contamination. Fungal growth in lead-contaminated soil decreases with an increase in the concentration of lead. This pattern was also observed in nickel-contaminated soil, as reported by [56], where the addition of increasing amounts of heavy metals led to a decrease in the number of fungal spores.

Aspergillus niger demonstrated effective biodegradation of nickel and lead, although could not degrade cadmium.

IV. CONCLUSION

Bioremediation provides a technique for cleaning up pollution by enhancing the natural biodegradation processes without exhibiting harmful and toxic environmental effects, unlike conventional techniques such as filtration, ion exchange, and chemical precipitation. This study highlights the potential of utilising microbes especially *Bacillus cereus* and *Aspergillus niger* for the bioremediation of heavy metal-contaminated soils. These microorganisms' high tolerance and biodegradation capabilities make them suitable alternatives for bioremediation.

These microorganisms' high tolerance and biodegradation capabilities make them suitable alternatives for bioremediation. This underscores the relatively untapped potential of microorganisms for the bioremediation of soils contaminated with heavy metals such as cadmium, nickel, and lead. Nonetheless, there should be optimisation of physicochemical parameters of the soil (pH, temperature, and organic carbon content) to enhance microbial activity and efficiency in bioremediation processes. Field trials should be conducted to validate laboratory findings and assess the practical applicability of these microbes in real-world scenarios. This would help refine bioremediation techniques and tackle challenges encountered in larger-scale applications.

DISCLOSURE OF CONFLICT OF INTEREST

No conflict of interest to be disclosed.

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