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EFFECT OF GROWTH MEDIA ON HEAVY METAL TOLERANT BACTERIA ISOLATED FROM OWODE-ONIRIN IRON SCRAP MARKET IN NIGERIA

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ABSTRACT

Heavy metals often form complexes with the organic components of most growth media resulting in decrease metal availability to bacteria cells. Hence, this study assessed different growth media used in studying heavy metal tolerance in bacteria vis elucidating the impact of heavy metals on the biochemical traits of heavy-metal tolerant bacteria. Standard cultural techniques were used to recover heavy metals (cadmium, lead and nickel) tolerant bacteria from Owode-Onirin metal-laden soil. The isolates were presumptively identified using their biochemical characteristics and further authenticated using 16S rRNA gene sequence analysis. Three different heavy metals supplemented media (nutrient broth, low-phosphate broth and Luria-Bertani broth) were used to determine the growth and physiological effect of the metals on the selected isolates. The nine isolates obtained from the soil samples tolerated 3 mM of cadmium, lead and nickel. The isolates recorded least growth in the low phosphate broth compared to the other two media because it prevented complexation of the metals in the medium. The order of bacterial growth in the media is low phosphate broth < nutrient broth < Luria-Bertani broth. Also, an increase in metal concentration resulted in loss of oxidase, catalase and pigmentation ability in some of the isolates. This study therefore recommends the use of a minimal nutrient medium such as low phosphate broth in evaluating heavy metal tolerance in bacteria.

Keywords: Bacterial-pigmentation, Heavy metal, Complexation, Precipitation, Low phosphate broth.

INTRODUCTION

The presence of heavy metals in the environment, stemming from industrial activities, agriculture and other human sources, poses significant threats to the ecosystem and human well-being. Excessive levels of all heavy metals often impact the environment negatively (Mishra *et al.,* 2019). Metal and metalloid element with an atomic density greater than that of water or more than 4 g/cm^3 is generally referred to as heavy metal (Abdulrahman, 2022). Toxic heavy metals such as mercury, chromium, and cadmium lack any biological function, yet they are recognized for their toxic, mutagenic, and carcinogenic effects on humans and other organisms, particularly those metals identified on the priority pollutant list (Rehman *et al*., 2018). Heavy metals are natural components of the earth's crust and remain a recalcitrant environmental issue due to their non-biodegradable nature (Fashola *et al*., 2016). Heavy metals can enter the environment through either natural processes or anthropogenic activities. Considerably, they enter the human system via air, food, and water and subsequent bioaccumulation over time (Clark, 2018). The main anthropogenic sources of heavy metal in the environment include processes such as mining, industrial activities, agricultural practices and so on (Yoro and Michael**,** 2020). Nonetheless, in certain instances, remnants of metals remain in the environment long after mining operations. Heavy metals in agricultural soils can contaminate aquatic environments via runoff into surface water or leaching into groundwater (Huang *et al*., 2016).

Studies have shown that heavy metals affect bacterial growth, as indicated by lowering of growth rate and generation time (Sivan *et al*., 2015; Khan and Khan, 2021). Metal uptake by bacteria is a complex process that is influenced by the chemistry of metal ions, bacteria surface features, cell physiology, and physicochemical influences from the environment, such as pH, temperature, and metal concentration (Priyadarshanee and Das, 2021). The uptake of heavy metals and metalloids by bacterial cells often results in the development of metal resistance factors on the genetic elements of the organism thus enabling them to be regarded as metal-tolerant bacteria (Mosa *et al*., 2016). Heavy metals in extremely high

concentrations exert their deleterious effects on the biochemical attributes of the metal-tolerant bacteria.

Ngwewa (2022), emphasized the importance of fully understanding the effects of heavy metal toxicity on living organisms, particularly bacteria, as they are typically the first-line organisms to be exposed to toxicity of heavy metals in the environment. However, to grasp the impact of heavy metals on bacteria, it is necessary to use suitable substrate (growth medium) that would not form complex with the heavy metals thus increasing the concentration of heavy metals available for uptake by the bacterial cells. Most reports on tolerance of heavy metals by bacteria from different environments are determined using general bacteriological agar (Issazadeh *et al.*, 2013; Fashola *et al.*, 2020). This choice of such growth medium is usually premised on the ability of general bacteriological agar to support the growth of wide array of bacteria (Rathnayake *et al.*, 2013). However, this class of growth medium often results in high heavy metal tolerance levels by the bacteria grown in those media (Nath *et al.*, 2019). The complexation of positively charged metal ions to the free organic components and poor stability constants of heavy metal-organic complexes may be responsible for the overestimation of heavy metal tolerance levels in bacteria when subjected to general bacteriological agar (Rathnayake *et al.*, 2013). The high phosphate content of such media can also lead to precipitation of heavy metal ions thus reducing the bioavailability and toxicity of the metal ions to the bacteria. Thus, determining phosphate levels in the growth medium is essential to optimize heavy metal bioavailability and uptake in tolerance experiments.

Kumar *et al.* (2013), used two different growth media (Mueller-Hinton broth and low phosphate broth) to comparatively determine the effect of uranium and other metals on the growth of *Serratia marcescens* PKRS1 and *Pseudomonas ficuserectae* PKSR11. He later concluded that low phosphate medium provides more authentic results in studying metal toxicity because it contains limited source of carbon and insignificant phosphate level, which prevents metal precipitation in the medium, thus allowing bioavailability of metals during logarithmic growth phase as compared to complex media such as Mueller-Hinton medium. Hence, this study focused on accessing the best suitable growth medium in studying heavy metal toxicity in bacterial cells vis elucidating the impact of heavy metals on some biochemical traits of heavy-metal tolerant bacteria.

MATERIALS AND METHODS

Sample site location and sampling

Soil samples were collected from Owode-Onirin metal-laden environment, a vast steel market situated at Kosofe Local Government Area of Lagos, Nigeria. The Owode-Onirin steel market is a location for trading metallic sheets and dumping of metal-containing wastes. This site has been in existence since 1979. The sampling site is located at latitude 6.673750 N and longitude 3.369455 E as shown in Figure 1. Surface soil samples were collected from three different points at a depth of 10-12 cm after clearing the debris using a sterile hand trowel and pooled together to form a composite sample representative of the site. The samples were transported on ice to the Lagos State University Microbiology laboratory and stored in a refrigerator at 4°C until it was analyzed. Samples for physicochemical parameters were stored in room temperature prior to analyses.

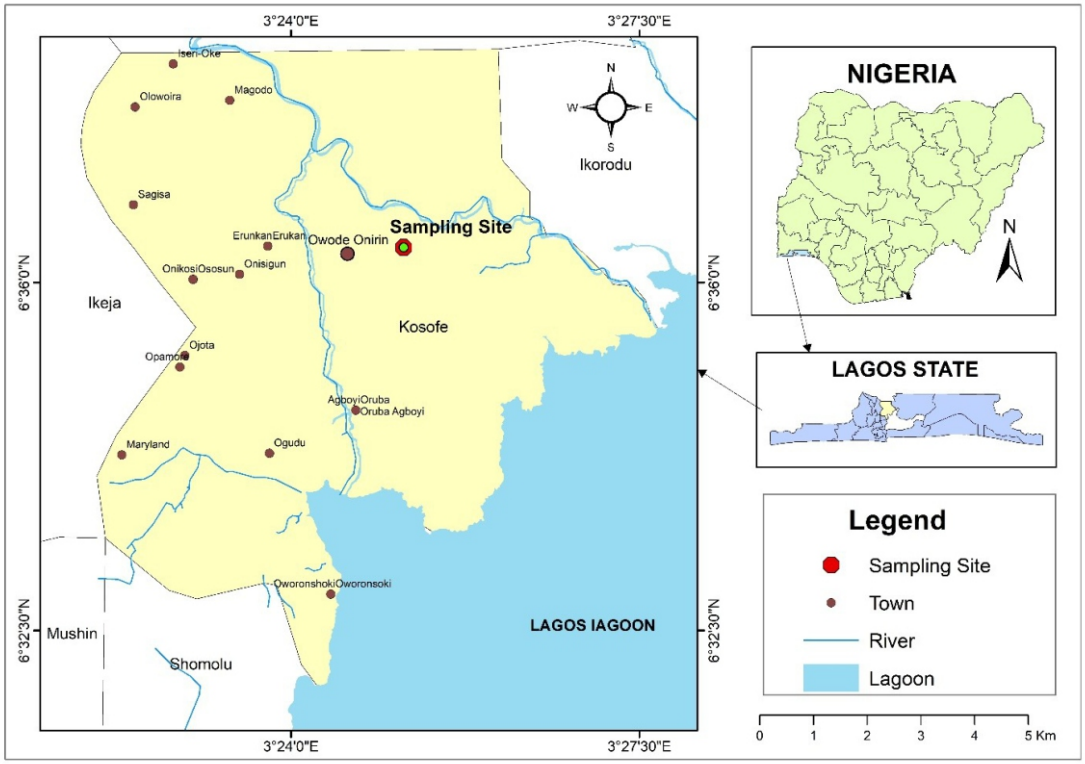

Figure 1: Geographic information of sampling site at Owode-Onirin, Lagos.

Physicochemical parameters and heavy metal contents determination

The pH of the soil sample was determined using tabletop Adwa pH 94 meter (AD1040 pH/ mV Szeged, Hungary). Other parameters such as moisture content, total phosphorus content, total potassium content, total sodium content, total nitrogen content, organic carbon content, electrical conductivity and cation exchange capacity were determined using standard analytical procedures (AOAC, 1999). Moisture content was determined by drying the sample at 105°C until it reaches a constant weight, then calculating the difference in weight before and after drying. This difference represents the moisture lost, which is expressed as a percentage of the original sample weight. The total phosphorus content in the soil was determined using the Bray-1 method, where phosphorus was extracted with Bray-1 solution, followed by colorimetric analysis at 650 nm after adding colour reagents and ascorbic acid. Total nitrogen content was measured by the Macro Kjeldahl method, involving digestion of the soil sample with concentrated sulfuric acid and mercury catalyst, followed by distillation and titration of the distillate with standard acid. Organic carbon content was quantified using the Walkley-Black method, where the soil was treated with potassium dichromate and the resulting organic carbon measured spectrophotometrically at 600 nm. Electrical conductivity of the soil was measured using an Extech Digital Meter after calibration with standard solutions, to assess the soil's salinity. The concentration of heavy metals present in the soil sample was determined using a hot plate digester according to the standard procedure of USEPA, (2006). The sample volume was adjusted to 50 ml using distilled water, and each metal concentration was measured using Model 210 VGP of the Buck Scientific atomic adsorption spectrophotometer (AAS) series with air-acetylene gas mixture as oxidant upon digestion.

Microbial enumeration and isolation of heavy metal-tolerant bacteria

For enumeration of microbial population, the sample stock solution was prepared by weighing 1 g of the sample then suspending them into 9 ml of normal saline water in a test tube, gently shaken and serially diluted into various dilution factors. Subsequently, an aliquot (0.1 ml) of the appropriate dilutions (10^{-5}, 10^{-6}, and 10^{-7}) of the serially diluted sample was inoculated on sterile

nutrient agar and potato dextrose agar plates then incubated at 25±2°C for 18-24 hours and 25±2°C for 5 days. The resultant colonies were then counted and estimated using 220V microbial colony tester counter.

Metal-tolerant bacteria were isolated using already serially diluted sample as described above and inoculated on 0.5 mM of metal (cadmium, lead and nickel) supplemented Luria-Bertani (LB) medium. Analytically graded metal salts ($CdCl_2$, $NiCl_2$ and $Pb(NO_3)_2$) were diluted in deionized water and filtered using 0.22 μm, Millipore Nucleopore, Pleasanton, CA, USA before adding to the growth medium. Inoculated plates were incubated at 25±2°C for 18-24 hours and distinct bacterial colonies were purified and sub-cultured on LB agar plates supplemented with 0.5 mM of the tested heavy metal. Bacterial strains were maintained on LB agar slants at 4°C.

Biochemical characterization of the heavy metal tolerant bacteria

The probable identity of the isolates was determined using various biochemical tests such as: Gram reaction, oxidase, nitrate reduction, citrate utilization, catalase test, Voges-Proskauer test, indole, motility, oxidase, nitrate reduction, starch hydrolysis, urease and sugar fermentation test. Pure cultures of the isolates were identified according to the Bergey's manual of Determinative Bacteriology.

16S rRNA-based identification

The identity of the isolates was confirmed using their 16S rRNA gene sequencing. Genomic DNA of the isolates were extracted from overnight-grown cultures using the Quick-DNA Bacterial Miniprep Kit (Zymo Research, USA). Polymerase chain reaction amplification of 16S rRNA gene was achieved using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') 1492R (5'-CGGTTACCTTACGACTT-3'). The amplicons were purified using the Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050 and were used to determine the nucleic acid sequence of the isolates. The identity of the isolates was determined using the Basic Local Alignment Search Tool (BLAST) by comparing with available bacterial nucleic acid sequences from the National Centre for Biotechnology Information database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree and sequence alignment were done using Molecular Evolutionary Genetics Analysis software version 11.

Determination of heavy metal tolerance activity on different growth media

The maximum tolerance concentration of Ni, Pb and Cd by the selected isolates was determined by broth dilution method as described by Wiegand *et al.*, (2008). Graded concentrations of the heavy metals were introduced into each medium used (nutrient broth, low-phosphate broth and Luria-Bertani broth) with 0.5 mM as the starting concentration for each metal. A 0.5 McFarland standard of the isolates was prepared by picking pure cultures of the isolates and emulsified in deionized water. The resulting mixture was compared with the 0.5 McFarland standard solution previously prepared. The inoculum was then introduced into the metal-supplemented growth medium. The test tubes were kept at room temperature (25±2°C) and were observed for growth for 72 hours, readings were taken at 24 hours interval using spectrophotometer Surgispec-SM23. The concentration was subsequently raised to 1 mM, 2 mM, and 3 mM after growth was observed at the initial concentration.

Determination of heavy metal toxicity on selected bacteria biochemical traits

Heavy metals are known to affect some enzymatic activities on bacterial cells. Based on this, some initial positive traits of the isolates previously determined such as catalase, oxidase and pigmentation were re-determined in the presence of Cd, Pb and Ni to evaluate the effect of metals on these traits.

RESULTS

Physicochemical characteristics, heavy metal profile and microbial count of soil sample

The physicochemical properties, heavy metal profile and microbial count of the soil sample are shown in Table 1. The values obtained from the soil sample revealed a neutral pH of 7.44. High electrical conductivity and low nitrogen content was detected in the soil sample with value of 85.40 μS/cm and 0.37% respectively. The heavy metal

index of the soil sample recorded significant level in contamination of lead (1385.68 $mgkg^{-1}$), nickel (44.56 $mgkg^{-1}$), cadmium (51.74 $mgkg^{-1}$), copper (14437.93 $mgkg^{-1}$) and zinc (1562.77 $mgkg^{-1}$). Additionally, the microbial load of the soil sample showed a total heterotrophic bacterial count and total heterotrophic fungal count of 8.4×10^7 cfu/g and 5.5×10^4 cfu/g respectively.

Table 1: Physicochemical properties, heavy metal profile and microbial count of soil sample.

Parameter	Value
pH	7.44
Moisture content (%)	6.32
Total phosphorus ($mgkg^{-1}$)	1795.33
Total potassium ($mgkg^{-1}$)	1224.23
Total sodium ($mgkg^{-1}$)	1743.86
Nitrogen content (%)	0.37
Total organic carbon (%)	6.18
Electrical conductivity (µS/cm)	85.40
Cation exchange capacity (Cmol/kg)	5.77
Lead ($mgkg^{-1}$)	1385.68
Cadmium ($mgkg^{-1}$)	51.74
Nickel ($mgkg^{-1}$)	44.56
Cobalt ($mgkg^{-1}$)	0.00
Calcium ($mgkg^{-1}$)	4011.63
Manganese ($mgkg^{-1}$)	13.22
Magnesium ($mgkg^{-1}$)	1016.72
Copper ($mgkg^{-1}$)	14437.93
Zinc ($mgkg^{-1}$)	1562.77
Iron ($mgkg^{-1}$)	31767.14
THB (cfu/g)	8.4×10^7
THF (cfu/g)	5.5×10^4

Key: THB= Total Heterotrophic Bacteria, THF = Total Heterotrophic Fungi

Isolation and biochemical characteristics of heavy metal tolerant bacteria

A total of nine (9) bacterial isolates were recovered from the soil sample. The isolates were able to tolerate 0.5 mM of cadmium, lead and nickel as a selective and isolation pressure. The heavy metal tolerant isolates displayed varying biochemical characteristics as shown in Table 2. These isolates were coded as OW1 - OW9 which indicate their source of isolation (Owode-Onirin).

Table 2: Biochemical characteristics of the heavy metal tolerant isolates.

Code	Colour	Gram reaction	Cellular morphology	Catalase test	Oxidase test	Indole test	Motility test	Methyl red test	Voges Proskauer test	Citrate utilization	Urease test	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	Spore test	NO_3 reduction	Coagulase test	Glucose	Sucrose	Xylose	Lactose	Raffinose	Fructose	Mannitol	Arabinitol	Sorbitol	Maltose	Galactose	Trehalose	Probable identity
OW 1	Cream	-	Rods	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	*Raoutella terrigena*
OW 2	Cream	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	*Enterobacter amnigenus*
OW 3	Cream	+	Rods	+	+	-	+	-	+	+	+	+	+	-	+	+	-	+	+	-	+	-	+	-	-	-	+	-	+	*Bacillus laterosporus*
OW 4	Cream	-	Rods	+	+	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+	+	*Acinetobacter mallei*
OW 5	Yellow	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	*Aeromonas hydrophila*
OW 6	Green	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	*Pseudomonas alcaligenes*
OW 7	Cream	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	*Bacillus megaterium*
OW 8	Yellow	+	Rods	+	+	-	+	-	-	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	*Bacillus cereus*
OW 9	Brown	-	Rods	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	*Enterobacter cloacae*

16S rRNA-based identification of heavy metal tolerant bacteria

The heavy metal-tolerant bacteria identified belong to different strains of *Alcaligenes faecalis*, *Bacillus cereus,* and two species of *Providencia* (*Providencia rettgeri and Providencia manganoxydans*). Percentage query cover and percentage identity ranged between 98% to 100% and 86.72% to 99.74% respectively and assigned accession numbers are shown in Table 3. Closely related sequences were downloaded from the NCBI GenBank and compared in relation to their phylogenetic relatedness as shown in Figure 2.

Table 3: Identification of sequences of the isolates from GenBank of NCBI.

Isolate code	BLAST hit	Percentage query cover	Percentage identity	Sequence length	Accession number
OW1	*Providencia rettgeri*	99	98.87	1512	PP239577
OW2	*Alcaligenes faecalis*	99	99.53	1506	PP239568
OW3	*Bacillus cereus*	100	99.74	1523	PP239569
OW4	*Providencia manganoxydans*	99	98.87	1514	PP239570
OW5	*Alcaligenes faecalis*	99	94.74	1500	PP239571
OW6	*Alcaligenes faecalis*	99	99.73	880	PP239567
OW7	*Alcaligenes faecalis*	99	86.72	1500	PP239573
OW8	*Lysinibacillus fusiformis*	98	97.05	1501	PP239572
OW9	*Alcaligenes faecalis*	99	99.87	1506	PP239583

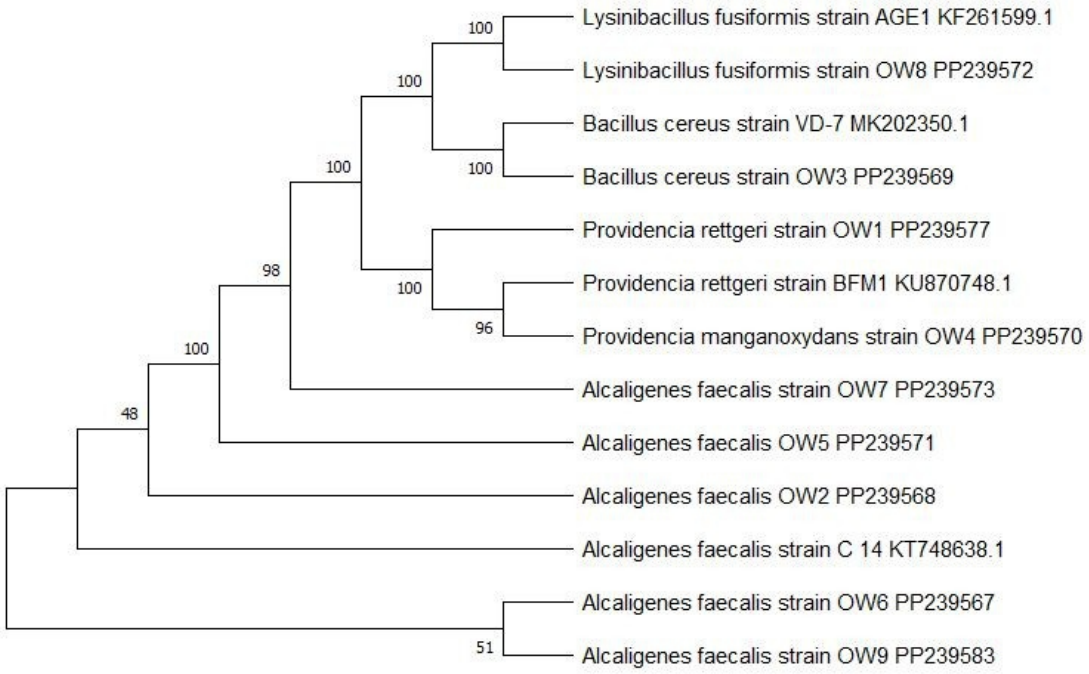

Figure 2: Phylogenetic tree based on neighbor joining analysis of 16S rRNA gene sequences of heavy metal tolerant bacteria obtained from Owode-Onirin metal laden soil and related bacterial species retrieved from NCBI database. The 16S rRNA gene sequence of *Alcaligenes faecalis* strain C 14KT748638.1 was used to outgroup the tree (bootstrap values were run at 1000 replications) (isolates with accession numbers PP239567, PP239568, PP239569, PP239570, PP239571, PP239572, PP239573, PP239577 and PP239583 are from this study).

Effect of growth media on heavy metal tolerance activity

The isolates were incubated for 72-hours in three media (low phosphate broth, Luria-Bertani broth and nutrient broth) supplemented with different heavy metals (cadmium, lead and nickel) as a growth limiting factor. The growth kinetics of these isolates at wavelength of 600 nm are shown in Figure 3 - 5. The result obtained indicated that low phosphate broth displays low optical density for the isolates when grown at same condition.

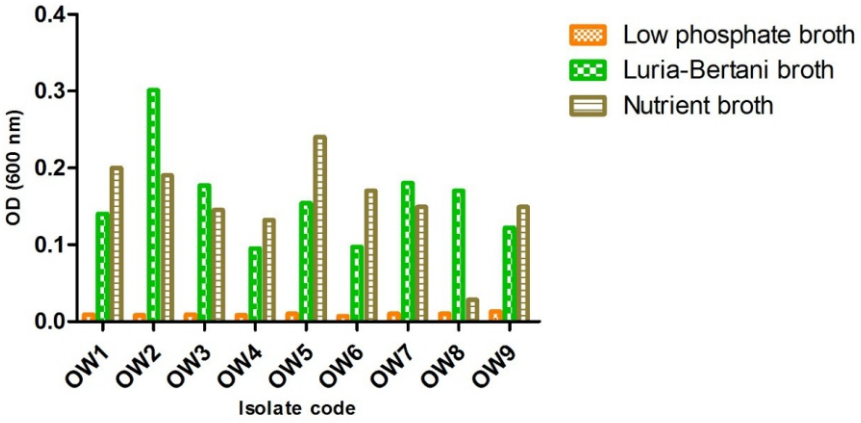

Figure 3: Growth kinetics of isolates in cadmium-supplemented (3 mM) growth media after 72-hours of incubation (OW1: *Providencia rettgeri,* OW2: *Alcaligenes faecalis,* OW3: *Bacillus cereus,* OW4: *Providencia manganoxydans*, OW5: *Alcaligenes faecalis,* OW6: *Alcaligenes faecalis,* OW7: *Alcaligenes faecalis,* OW8: *Lysinibacillus fusiformis,* OW9: *Alcaligenes faecalis*).

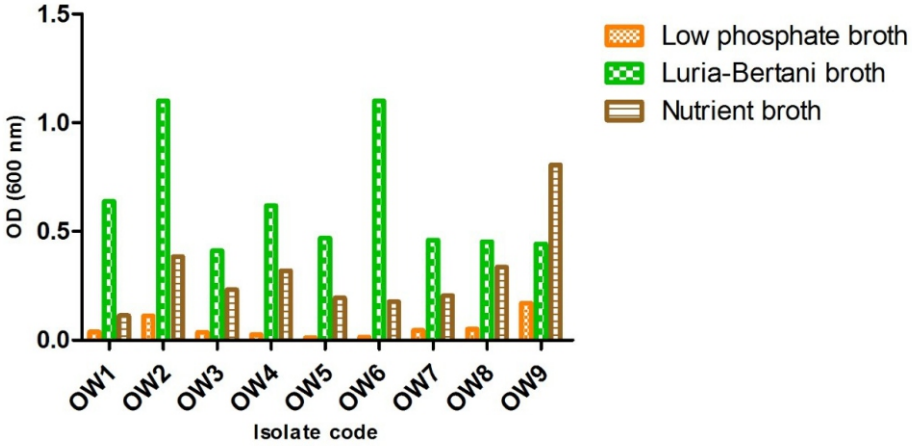

Figure 4: Growth kinetics of isolates in lead-supplemented (3 mM) growth media after 72-hours of incubation (OW1: *Providencia rettgeri,* OW2: *Alcaligenes faecalis,* OW3: *Bacillus cereus,* OW4: *Providencia manganoxydans*, OW5: *Alcaligenes faecalis,* OW6: *Alcaligenes faecalis,* OW7: *Alcaligenes faecalis,* OW8: *Lysinibacillus fusiformis,* OW9: *Alcaligenes faecalis*).

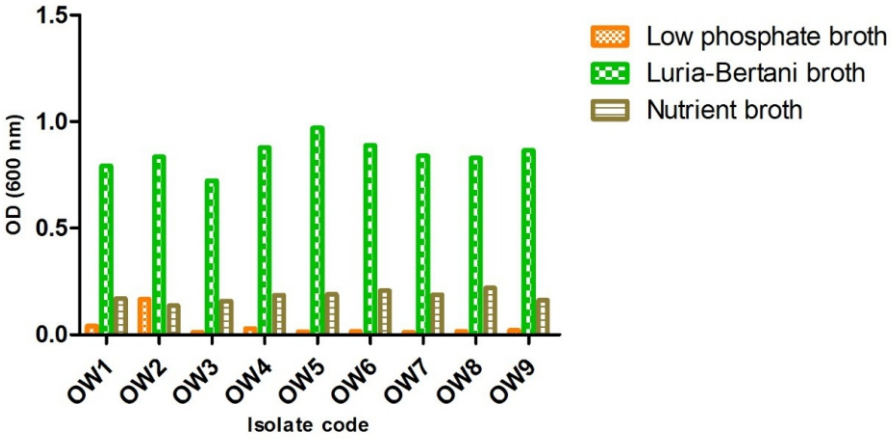

Figure 5: Growth kinetics of isolates in nickel-supplemented (3 mM) growth media after 72-hours of incubation (OW1: *Providencia rettgeri,* OW2: *Alcaligenes faecalis,* OW3: *Bacillus cereus,* OW4: *Providencia manganoxydans*, OW5: *Alcaligenes faecalis,* OW6: *Alcaligenes faecalis,* OW7: *Alcaligenes faecalis,* OW8: *Lysinibacillus fusiformis,* OW9: *Alcaligenes faecalis*).

Effect of cadmium, lead and nickel on catalase production potential of the isolates

All the isolates produce catalase enzyme however, heavy metals induce physiological changes in the ability of the isolates to produce the enzyme. Table 4 shows the effect of the heavy metals on the catalase production potential of the isolates. The ability to produce catalase was retained by all isolates in the presence of 2 mM of cadmium with exception to OW3. At an increased concentration of 3 mM, most of the isolates still produced catalase. However, more physiological changes were observed for OW3, leading to its inability to produce catalase in the presence of lead, nickel and cadmium. Additionally, OW8 lost its catalase production ability in 3 mM of cadmium and lead, while OW1 lost its ability to produce catalase in the presence of 3 mM of cadmium.

Table 4: Effect of cadmium, lead, and nickel on catalase production of the isolates.

Isolate code	16S rRNA identity	Control	Cd 2mM	Pb 2Mm	Ni 2mM	Cd 3mM	Pb 3mM	Ni 3mM
OW1	*Providencia rettgeri*	+	+	+	+	-	+	+
OW2	*Alcaligenes faecalis*	+	+	+	+	+	+	+
OW3	*Bacillus cereus*	+	+	-	-	-	-	-
OW4	*Providencia manganoxydans*	+	+	+	+	+	+	+
OW5	*Alcaligenes faecalis*	+	+	+	+	+	+	+
OW6	*Alcaligenes faecalis*	+	+	+	+	+	+	+
OW7	*Alcaligenes faecalis*	+	+	+	+	+	+	+
OW8	*Lysinibacillus fusiformis*	+	+	-	+	-	-	+
OW9	*Alcaligenes faecalis*	+	+	+	+	+	+	+

Key: Control: non-metal supplemented medium, (+): positive, (-): negative

Effect of cadmium, lead and nickel on oxidase production potential of the isolates

Table 5 shows the effect of the heavy metals on the oxidase production potential of the isolates. All the isolates are capable of producing oxidase enzyme with the exception of OW9. The heavy metals induce observable change in the ability of the isolates to produce oxidase. The oxidase production ability of the isolates was retained more in the presence of 2 mM of lead however, this was reduced at increased concentration of the heavy metals.

Table 5: Effect of cadmium, lead and nickel on oxidase production of the isolates.

Isolate code	16S rRNA identity	Control	Cd 2 mM	Pb 2 mM	Ni 2 mM	Cd 3 mM	Pb 3 mM	Ni 3 mM
OW1	*Providencia rettgeri*	+	+	+	-	+	+	-
OW2	*Alcaligenes faecalis*	+	-	+	+	-	-	+
OW3	*Bacillus cereus*	+	+	-	+	+	-	-
OW4	*Providencia manganoxydans*	+	-	+	+	-	+	-
OW5	*Alcaligenes faecalis*	+	-	+	-	-	-	-
OW6	*Alcaligenes faecalis*	+	+	-	+	-	+	+
OW7	*Alcaligenes faecalis*	+	-	+	+	-	+	+
OW8	*Lysinibacillus fusiformis*	+	+	+	-	+	-	-
OW9	*Alcaligenes faecalis*	+	-	-	-	-	-	-

Key: Control: non-metal supplemented medium, (+): positive, (-): negative

Effect of cadmium, lead and nickel on bacterial pigmentation

Table 6 shows the physiological effect of cadmium, lead and nickel on selected pigmented isolates. Isolate OW5 retained its ability to produce pigment in the presence of cadmium however, lost its pigmentation ability in the presence of lead and increased concentration of nickel. Similarly, isolate OW6 retained its ability to produce green pigment in the presence of cadmium (2 mM) but, lost its pigmentation ability in the presence of other metals and increased concentration of cadmium.

Table 6: Effect of cadmium, lead and nickel on the pigmentation ability of the isolates.

Isolate code	16S rRNA identity	Initial pigment	Cd 2 mM	Pb 2 mM	Ni 2 mM	Cd 3 mM	Pb 3 mM	Ni 3 mM
OW5	*Alcaligenes faecalis*	Yellow	+	-	+	+	-	-
OW6	*Alcaligenes faecalis*	Green	+	-	-	-	-	-
OW9	*Alcaligenes faecalis*	Brown	-	+	-	-	+	-

Key: (+): initial pigment retained, (-): pigment lost

DISCUSSION

The continued persistence of heavy metals within the soil environment can have adverse effect on the growth of microorganisms, plants and animals. Commercial activities of an environment can influence the physicochemistry of an ecosystem (Fashola *et al.*, 2020). In this study, most results from the soil physicochemical characteristics exceed the tolerable standards provided by Nigeria's Federal Environmental Protection Agency (FEPA), (2003). The soil pH is within the acceptable range (6.5–8.5), but elevated total phosphorus concentrations can affect processes such as phosphorus cycling, biological activity, soil formation, heavy metal behavior, and erosion. The heavy metal profile of the soil (Table 1) revealed high level of lead, cadmium and nickel pollution when compared to the FEPA, (2003) standards of 420 $mgkg^{-1}$, 50 $mgkg^{-1}$ and 30 $mgkg^{-1}$ respectively. This level of pollution is greatly influenced by the deposition of metallic waste and sales of metal scraps within the sample location.

Heavy metals tolerance study has been conducted for decades, with special interest in understanding the level of tolerance, mechanisms, and its effect on the acquisition of antimicrobial resistance. However, the common trend in all these studies is the prominent use of rich media for bacterial isolation and tolerance assays. In a much earlier study by Rayner and Sadler (1989), it was established that rich media such as Luria-Bertani favored growth of *Escherichia coli* under cadmium stress, while minimal media did not. Other recent

but limited works have also highlighted these differential effects of culture media on heavy metals tolerance in bacterial isolates (Ansari *et al.*, 2016; Onuoha *et al.*, 2016; Mitra *et al.*, 2021). This study provides further evidence of disparity in bacterial tolerance to metals in varying media conditions and how these changes affect their physiological properties.

This present study examined the applicability of three different growth media (low phosphate broth, Luria-Bertani broth and nutrient broth) in determining heavy metal (cadmium, lead and nickel) tolerance and toxicity in heavy metal tolerant bacteria. The optical densities of the isolates showed significant tolerance to the tested metals up to 3 mM. It was observed that the cell densities obtained when grown in Luria-Bertani broth were relatively higher when compared to nutrient broth and low in phosphate broth. Luria-Bertani broth is a rich non-defined liquid media, particularly the presence of yeast extracts whose chemical composition has not been fully established. Visual observation during media preparation with metal supplementation showed precipitation in Luria-Bertani broth, while clear precipitates of metals were observed in other media especially low phosphate medium. Variations in optical densities were observed even after similar conditions were maintained in all growth media. This is possible based on the theory proposed by Kumar *et al.* (2018), that the minimum inhibitory concentration (MIC) value of metal supplemented media is dependent on the type of growth media used. Due to factors such as metal availability, complexation and diffusion, higher optical density value is expected in rich media compared to minimal media (Yilmaz, 2003; Kumar *et al.*, 2011). The higher optical density values displayed in Luria-Bertani broth and nutrient broth may also be attributed to the non-uniform availability of metals in the media due to metals affinity for precipitation in rich media. The clear metal precipitate observed in low phosphate broth may be due to the presence of low carbon and negligible phosphate (Kumar *et al.*, 2018). These findings align with El-Baz *et al.* (2015) results, where bacterial tolerance to heavy metals was tested using Duxbury agar and nutrient agar. Their study demonstrated that Duxbury agar had minimal impact on metal bioavailability, leading to its selection as a suitable medium for selecting heavy metal-tolerant bacteria. The study conducted by Kumar *et al.* (2013), also explored the MIC of metals for *S. marcescens* PKRS1 and *P. ficuserectae* PKRS11 using low phosphate and Mueller-Hinton media. Interestingly, they observed a higher MIC in Mueller-Hinton medium compared to low phosphate medium. Therefore, to study heavy metal-bacteria interactions accurately, minimal media like low phosphate broth should be used, as it has negligible phosphate, reducing metal precipitation and increasing their availability to bacteria. Heavy metals when present in high amounts in the environment can lead to loss or change in certain traits of organisms (Priya *et al.*, 2022). Effect of cadmium, lead and nickel was tested on biochemical traits such as catalase, oxidase and pigmentation of the isolates to determine whether the heavy metals induce physiological changes in the heavy metal tolerant bacteria, without interfering with their growth. Isolates OW2, OW4, OW5, OW6 and OW7 can produce catalase even when exposed to heavy metals like cadmium, lead and nickel at 3mM. The enzyme catalase helps protect bacteria by breaking down hydrogen peroxide, which is toxic to cells. However, isolate OW3 may not have been able to tolerate cadmium, lead, and nickel, potentially leading to hydrogen peroxide buildup, which is harmful at the cellular level. It is possible that the presence of oxidase enzymes in OW3 could be detected using tetramethyl-p-phenylenediamine dihydrochloride, which acts as an artificial electron acceptor in place of oxygen. This test would yield a positive result if the bacteria possess cytochrome C (Eleftheriadis *et al.*, 2016). Isolate OW1 retained its oxidase production ability in the presence of cadmium and lead however, physiological change in oxidase production was observed when exposed to nickel. Also, the concentration of the heavy metals became important as isolate OW2 had physiological changes in oxidase production at 3 mM concentrations of cadmium, lead and nickel. This study also shows the loss of pigmentation ability in the presence of cadmium, lead and nickel in isolate OW5, OW6 and OW9. Our finding agrees with the report of Lima e Silva *et al.* (2012), that reported the loss of catalase, oxidase and bacterial pigmentation in both *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia*

coli ATCC 25922 under increased concentration of mercury and gold. Heavy metals, even in trace amounts, can prevent bacteria from producing their characteristics color. Depending on their bioavailability and absorbed dose, heavy metals can have negative impact on bacterial cells (Fashola *et al.*, 2023).

Therefore, culture media have significant impacts on the ability of bacteria to tolerate heavy metals. This is because the composition of the growth medium can influence the metabolic activity of bacteria, which in turn can affect their ability to tolerate heavy metal (Wang *et al.*, 2020). Most general bacteriological media contain compounds such as peptone, tryptone, yeast extract, meat extract, inorganic salts, and other undefined additives that can chelate or bind heavy metals, reducing their bioavailability and making them less toxic to bacteria (Aljerf and Al Masri, 2018). In identifying the organisms, there is a change in the identity obtained from biochemical characterization of the isolates after the sequencing of the 16S rRNA gene of the isolates. This is not surprising because, 16S rRNA gene sequencing provides a more accurate result than biochemical characterization due to its increased sensitivity and specificity. This study showed the presence of heavy metal tolerant *Alcaligenes* spp, *Providencia* spp, and *Bacillus cereus.* These bacteria could survive at the sample sites, where their interaction with heavy metals in the soil could be used for metal remediation. Several reports have shown bioremediation potential of *Alcaligenes faecalis, Alcaligenes aqualitis, Bacillus cereus* as well as *Providencia* spp in removal and/or reduction in mobility and bioavailability of heavy metals in contaminated environment (Lade *et al.*, 2015; Saini *et al.,* 2018; Abou-Aly *et al.,* 2021; Haouas *et al.,* 2021).

CONCLUSION

Heavy metal-tolerant bacteria traits can be greatly influenced by type of culture media. The heavy metal-tolerant bacterial isolates under investigation showed noticeable physiological changes in pigmentation, growth dynamics and enzyme activities when exposed to cadmium, lead and nickel. However, the type of culture media employed has a major influence on these results. This study demonstrated that minimal nutrient medium (low phosphate broth) is the most suitable medium to study bacteria tolerance to heavy metals in a contaminated environment. Utilization of such medium will greatly reduce the false-positive interpretations in bacteria tolerance to heavy metals experiment.

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CONFLICT OF INTEREST

The authors declared they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

F.M.O. conceived and designed the study, contributed to data analysis tools, analysis of data and manuscript writing. A.O.S. and A.K.O. contributed to data analysis and manuscript writing. L.D.A., O.M.E., B.Z.A., A.O.A., O.O.A. and S.E.E. performed data collection. All authors approved the final copy of the manuscript.

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