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ORIGINAL RESEARCH ARTICLE

Isolation and Assessment of Metal-tolerant Bacteria and their Potential for Heavy Metal Removal

Bilyaminu, I. A.[*1]; Shamsudeen, S.[1]; Yahya, S.[1]; Lawal, N.[2] and Mudassiru, S.[3]
[1]Department of Microbiology, Bayero University Kano, Kano, Nigeria
[2]Department of Biology, Ahmadu Bello University Zaria, Kaduna, Nigeria
[3]Department of Microbiology, Sokoto State University, Sokoto, Nigeria

ABSTRACT
Heavy metal contamination is one of the major global ecological concerns; it is commonly found to contaminate soil, sediments, and wastewater, where they remain persistent and become toxic to many species exceeding certain threshold concentrations. Bacteria resistant to heavy metals can be used for detoxification and prevent further deterioration of contaminated sites. Soil samples were collected from two different auto-mechanic workshops: one located at Kofar Ruwa Market (main activity here is car battery charging), and the other one located at Unguwa Uku Motor park (main activities here include welding and soldering), and also a control site at Ecological Study Area of Bayero University, Kano, Nigeria (this area is free from any human activities). The physicochemical parameters of the soil samples were initially determined. Bacterial enumeration and isolation were conducted. Ten-fold dilutions of the soil samples were made in which an aliquot was plated in nutrient agar amended with nystatin (0.5-1 µg/ml) to suppress fungal growth. The bacterial isolates were screened for chromium, lead, Zinc, Copper, and Iron resistance by plate diffusion method. The maximum tolerable concentration of the isolates was measured in terms of O.D. at 595 nm, and optimization of temperature and pH was carried out. Atomic absorption spectrophotometric analysis was carried out for the removal of chromium, lead, Zinc, Copper, and Iron by the isolates confirmed by Scanning electron microscopy (SEM). Of the 7 isolated bacteria, 4 bacterial isolates with maximum tolerance trends for Cr, Pb, Zn, Cu, and Fe were selected and identified as *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae*. The highest removal percentage was observed at 72 and 96 hours of incubation using AAS analysis, and the efficiency of removal of the five heavy metals in decreasing order was *P. aeruginosa* (97.5% Cu, 76.5% Pb, 72.6% Fe, 72.2% Zn, 50.2% Cr) >*E.coli* (93.8% Cu, 65% Pb, 64.2% Fe, 64.5% Zn, 19.4% Cr) >*K. pneumonia* (75.7% Cu, 49.3% Pb, 49.3% Fe, 27.1% Zn, 34.9% Cr) >*S. aureus* (55.5% Cu, 57.5% Pb, 19.9% Fe, 64.5% Zn, 17.3% Cr). Scanning electron microscopy (SEM) showed changes in the surface morphology of all four bacterial isolates after metal treatment. These results suggest that all four identified heavy metal-tolerant bacteria can be useful for the bioremediation of soil environments contaminated with heavy metals.

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INTRODUCTION
Heavy metals are metallic elements with a large atomic weight and density. They exist abundantly and are essential for many industrial and technological processes (Singh *et al.*, 2023). However, their excessive use and improper disposal have resulted in widespread environmental contamination, seriously threatening human health and the ecosystem (Velusamy *et al.*, 2021). Microorganisms deal with heavy metals through various mechanisms, including adsorption, accumulation, oxidation, reduction, volatilization, and precipitation. Understanding these mechanisms is critical for developing effective bioremediation strategies to mitigate the adverse effects of heavy metal pollution (Ayangbenro and Babalola, 2017). More recently, the definition has been broadened to include naturally occurring elements with atomic number greater than 20 (Ali and Khan, 2018; Ali *et al.*, 2019).

Heavy metal-tolerant bacteria are found in various environments, including contaminated soils, sediments, wastewater, and mine tailings (Du *et al.*, 2023). These bacteria exhibit diverse metal tolerance capabilities, enabling them to survive and thrive in high metal

Correspondence: Bilyaminu, I. A. Department of Microbiology, Bayero University Kano, Kano, Nigeria. ✉ idrisbilyaminu1@gmail.com.
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concentrations (Forsyth *et al.*, 2018). The variety of heavy metal-tolerant bacteria is reflected in their taxonomic diversity, encompassing various genera such as *Pseudomonas*, *Bacillus*, *Cupriavidus,* and *Rhizobium* (Bhende *et al.*, 2022). Bacterial resistance to heavy metals is a complex phenomenon involving a variety of passive and active mechanisms. Understanding the mechanisms, sources, factors, and implications of bacterial resistance to heavy metals is critical for developing effective strategies for the remediation of heavy metal-contaminated sites and mitigating the impact of heavy metal pollution on the environment and human health. (Fardami *et al.*, 2023).

Heavy metal contamination is possible in the Kano metropolis, considering the rampant activities and improper disposal of heavy metal-containing material handled in mechanic workshop clusters. These workshops are widely located in areas such as Kofar Ruwa market, Unguwa Uku motor park, and Gadan Kaya/Tal'udu, among others. These places are for repairs and servicing of motor vehicles and other machinery.

Conventional methods to remediate heavy metals contaminated sites are excavation and solidification/stabilization, these technologies are suitable to control contamination but not permanently remove heavy metals (Bahi *et al.*, 2012). However, they have some disadvantages: cost-effectiveness limitations, generation of hazardous by-products or inefficiency. On the other hand, biological methods potentially solve these drawbacks since they are easy to operate and do not produce secondary pollution (Su, 2014). Heavy metals with relatively high density are toxic at low concentrations (Iram *et al.*, 2013).

While conventional remediation methods are costly and environmentally unsustainable, bacterial bioremediation offers a promising alternative. This study investigates the potential of metal-tolerant bacteria from mechanic workshop soils to remove heavy metals through biosorption and bioaccumulation. The specific objectives include;

(1)- To determine the physicochemical characteristics of the contaminated workshop soil,

(2)- To isolate and identify heavy metal-tolerant bacteria from mechanic workshops,

(3)- To determine tolerant limits to different concentrations of heavy metals of the isolates and

(4)- To determine the effect of pH and temperature on metal removal potentials.

MATERIALS AND METHODS

Sample site

Three sampling sites were selected for this study, consisting of two different workshops: one located at Kofar Ruwa Market (12.0272°N, 8.4974°E), where car battery charging is the main activity, and the other one located at Unguwa Uku Motor park (11.966°N, 8.5639°E) which are involved in welding and soldering, and also a control site at Ecological Study Area of Bayero University, Kano (11.9818°N, 8.4801°E) (this area is free from any human activities). All the sampling locations were within the Kano metropolis.

Sample Collection

Soil samples were collected randomly using the quadrant sampling technique and homogenized from five spots. The soil samples were collected 0-10 cm below the soil surface using a soil auger, put into sterile polyethylene bags, and transported to the Microbiology Research Laboratory, BUK, Nigeria. The soil samples were stored in a refrigerator at 40C until they were analysed.

Physico-chemical Analysis of the Soil Samples

Determination of Hydrogen-ion Concentration (pH)

Twenty (20) grams of soil sample was air dried after sieving through a 2-mm mesh size and transferred into a 50ml-beaker to which 20ml of distilled water was added. The soil suspension was stirred several times for 30 minutes using a glass rod. The suspension was allowed to stand for 30 minutes. The electrode of the pH meter (Jenway 3051 model) was then inserted into the partly settled suspension and measured the pH (Omotayo *et al.*, 2012).

Determination of Total Organic Carbon (TOC)

(Blank titer – Actual titer) x 0.3 x M x F

Weight (g) of air-dried soil

The soil sample was grinned and passed through 0.5mm sieve, and 1.00g soil was placed in a 250ml conical flask containing 10ml of 1N $K_2Cr_2O_7$ solution. The flask was swirled gently to dispense the soil. A quantity (20ml) of concentrated H_2SO_4 was added immediately and swirled gently until the soil and reagents were mixed. The contents were then swirled more vigorously for a minute. The flask was rotated again and allowed to stand on a sheet of asbestos for 30 minutes, after which 100ml of distilled water was added and cooled. The suspension was filtered, and 5ml of O-phosphoric acid was added, after which 3 drops of the o-phenanthroline indicator were added and the contents titrated against 0.5N ferrous sulphate on a white background. As the end point approached, the solution turned to a greenish cast and changed to dark green. The ferrous sulphate was added in drops until the color changed sharply from blue to red (maroon color) in reflected light against the white background. Blank determination in the same way was made but without soil to standardize the dichromate (Mclean, 1965). The result was calculated according to the following formula:

$$\% \text{ Organic carbon (air-dried soil} = \frac{(\text{Blank titer} - \text{Actual titer}) \times 0.3 \times M \times F}{\text{Weight (g) of air-dried soil}}$$

Where: M = Concentration of $FeSO_4$ and F=Correction factor = 1.33

Determination of Moisture Content

Five (5) grams of soil sample were transferred into a pre-weighed can box with a tight-fitting lid. The can box containing the moist soil sample was weighed immediately and placed with its lid off in the drying oven at a temperature of 105 ^{0}C (to dry the soil to constant weight) for 24hr after which the set up was removed from the oven and immediately covered with its lids, cooled in dessicator and weighed again (Luyafor *et al*., 1990). The moisture content of the soil sample was determined using the following formula:

$$\text{Moisture content (\%)} = \frac{W1 - W3\ X100}{W3 - W1\ (g)}$$

Where:

W_1 = Weight of empty soil can box (g).
W_2 = Weight of can box + moist soil (g).
W_3 = Weight of can box + oven dry soil (g).

Determination of heavy metal concentrations

About 3g sieved soil samples were digested with a mixture of 10 ml concentrated hydrochloric acid (HCl) and 3.5 ml concentrated nitric acid (HNO_3). The mixtures were left overnight without heating under the switch-on fume cupboard and heated for 2 hours to 140oC the next day. Distilled water was added to cool the digested sample, filtered with filter paper, and topped up to 100 ml with distilled water. The heavy metals Cu, Cr, Zn, Pb and Fe concentration in the top soil samples were analyzed using an AAS (Najib *et al*., 2012).

Microbiological Analysis

Total Heterotrophic Bacterial Count

Samples were enumerated by making ten-fold dilutions of the soil samples from 101 to 105. Aliquot 0.1ml of the 10^{-3} dilution was transferred and plated in nutrient agar amended with nystatin (0.5-1 µg/ml) to isolate bacteria (Adams *et al*., 2014). The plates were prepared and inoculated in duplicates. The inoculated nutrient agar plates were incubated at 37°C for 24 hours. After incubation, the colonies of the isolates were counted and expressed in CFU/g.; isolated colonies were then further purified by repeated sub-culturing.

Assessment of heavy metal resistance potential

Heavy metal resistance was determined by the plate diffusion method. 1.0 mM of metal solution (Pb, Zn,Cr, Cu and Fe) was prepared to isolate metal-resistant bacteria. Nutrient Agar media plates were swabbed with an overnight culture of each bacterial isolate, and in each plate, wells were prepared with a sterile corkborer. Fifty microliter (50µl) of appropriate heavy metal solution was poured into each well, and plates were incubated at 37^{0}C for 24 hours. After incubation, the zone of inhibition was then measured. A zone smaller than 1mm was then scored as a heavy metal-tolerant strain (Hassen *et al*., 1998).

Identification of selected heavy metal-tolerant bacterial isolates

Bacterial isolates that exhibited the heavy metal-tolerant potential were then identified according to Bergey's manual of systemic bacteriology and other biochemical tests (Chesebrough, 2003).

Gram's staining

After the smear was prepared, the slide was flooded with crystal violet and allowed to stand for one minute. It was washed off with tap water and then flooded with Gram's iodine (a mordant) and left for one minute, after which the smear was washed off with tap water again decolorized with alcohol (95%) until no more color washed off. This was the most critical step; care was taken so as not to over-decolorize the smear, as many Gram-positive organisms may lose the violet stain easily and thus appear to be Gram-negative after they are counterstained. The smear was washed off with tap water, and safranin (counter stain) was applied and allowed to stand for 30 seconds. The smear was washed off with tap water, drained, and blotted gently with bibulous paper. The slide was air-dried thoroughly before being examined under the microscope. Gram-positive organisms retained the primary stain, while Gram-negative ones took up the secondary (counter) stain (Chesebrough, 2003; Todar *et al*., 2005).

Catalase Test

This was carried out by introducing 2.0ml of hydrogen peroxide into a clean, grease-free glass tube. With the edge of a sterile glass rod, a colony of organisms was picked and introduced onto the hydrogen peroxide on the slide. Bubbles indicated a positive reaction, while the absence indicated a negative reaction (Bhattacharya *et al*., 2002).

Urease Test

Urea agar medium was inoculated with culture and incubated at 37°C for 48 hours. After incubation, the change of colour from light orange to pink showed a positive result (Kummerer, 2004).

Oxidase Test

A few drops of 1% aqueous solution of tetramethyl-p-phenylenediamine hydrochloride were added to a loopful of bacterial culture on a clean slide. The slide was observed for the development of purple colour within 5 seconds, and the result was taken (Chesebrouhg, 2003).

Citrate Utilization Test

This was carried out by inoculating the test organism in a test tube containing Simon's citrate medium, which was incubated for 24 hours. The development of deep-blue

colour after the incubation period indicated a positive result (Udeani *et al*., 2009).

MR-VP Test

A quantity (5.0 ml) of MR-VP broth was inoculated with the test organism and incubated for 72 hours at 37°C, after which 1.0 ml of the broth was transferred into a small test tube. Three drops of methyl-red solution were added. Red colour development on the addition of the indicator signified a positive methyl red test, while yellow colour signified a negative test. Five drops of 4% potassium hydroxide (KOH) were added to the rest of the broth in the original tube, followed by fifteen (15) drops of 5% -naphthol in ethanol. No colour change indicated a VP negative test (Dubey, 2002).

Indole Test

One percent tryptophan broth in a test tube was inoculated with a bacterial colony and incubated at 37 °C for 48 hours. Then, 1.0 ml of chloroform was added to the broth. The test tube was shaken gently, then 2 ml of Kovac's reagent was added and shaken gently and allowed to stand for twenty (20) minutes. The formation of red colouration at the top layer indicated a positive test, while yellow colouration indicated a negative test (Udeani *et al*., 2009).

Determination of maximum tolerable concentrations of heavy metals on nutrient broth medium

Heavy metal ion resistance was studied using the metal ions' maximum tolerable concentrations (MTCs) in Nutrient Broth (NB) media. The metals Cr, Cu Pb, Zn, and Fe were used as $K_2Cr_2O_7$, $CuSO_4$, $Pb(NO_3)_3$, $ZnSO_4$, and $FeSO_4$, respectively. Stock solutions (1M) were prepared by dissolving metal salts in distilled water. Each stock solution was sterilized by filtration and stored at 40C. All plastic and glassware used were acid-washed in 2 $NHNO_3$ and thoroughly rinsed several times with deionized water before use to avoid metal contamination. A volume of 0.1 ml of overnight broth culture (OD600 nm=0.8) of the isolate was inoculated in 10 ml sterile NB containing 1, 2, 5, 10, 15, and 20 mM of Pb, Cr, Zn, Cu, and Fe, each. The inoculated culture was incubated at 30°C for 48 hours in addition to negative control (culture media containing the same concentration of metals without inoculation) and blank (culture media neither inoculated with bacteria nor heavy metal addition). After 48 hours, bacterial growth was measured as optical density values at a wavelength of 600nm using a Spectrophotometer (Spectrumlab 752s). Experiments were carried out in triplicates.

Optimization of growth parameters (temperature and pH)

The optimal growth conditions with reference to pH and temperature were studied. For studying the effect of pH, 0.1 ml of overnight broth culture (OD600 nm=0.8) of the isolate was inoculated into NB medium with different pH values of 5, 6, 7, 8, and 9 using 1.0 mol/l NaOH or 1.0 mol/l HCl and incubated at 30°C for 24h under shaking conditions (120 rpm/min). The effect of temperature was studied by inoculating bacteria into NB medium and incubating at different temperatures of 25, 30, 35, 37, and 40°C for 24 h under shaking conditions (120 rpm/min). All experiments were performed separately in the presence of all five heavy metals. Also, positive control (only medium inoculated with the bacteria) and negative control (only heavy metal-containing medium) were used. Bacterial growth was measured as optical density at 600 nm using a Spectrophotometer.

Determination of heavy metal removal by bacteria

Liquid culture (overnight) was preincubated in 100 ml of metal deficient NB until it reached mid-log phase, and 1 ml bacterial sample (OD600 nm=0.8) was transferred into 100 ml NB supplemented with heavy metal ions (Cr, Pb, Zn, Cu, and Fe) in 250 ml Erlenmeyer flasks. The culture was incubated at optimum conditions of each isolate for 96 hours in a shaking condition (150rpm). A control flask without bacterial biomass was running simultaneously with the experimental flasks. An aliquot of 5 ml sample was withdrawn daily (24-hour intervals) from each flask. The samples were centrifuged for 10 min at 6000 rpm using a Sigma-Aldrich, Germany centrifuge, and the supernatant was used for residual metal analysis using an atomic absorption spectrophotometer (AAS). The amount of metal ions removed by the bacterial strain was determined by the difference between the initial and residual concentrations (Xiao-xi, 2009). All experiments were performed in triplicates, and the average values were determined.

Scanning electron microscopy

Samples of bacterial suspensions from the experiment of heavy metal removal and control were analyzed by scanning electron microscopy (SEM). Prepared samples were placed with carbon tape on the holder (stub). To increase the electron conduction and to improve the quality of micrographs, a conductive layer of gold was made with a portable SC7620'Mini' sputter coater, and the outer morphology of the bacterial cells was examined.

Statistical analysis

The data was analyzed by calculating mean ±SD, and analysis of variance (ANOVA) was performed using Microsoft Office Excel 2007. P value was calculated to observe the level of significance. Results showing P value less than 0.05 were considered significant ($P < 0.05$).

RESULTS

Physico-chemical properties of the soil samples

Different physicochemical characteristics of the soil, such as pH, temperature, organic matter, moisture content, and heavy metal concentration, were determined for both soil samples from three different locations (Kofar Ruwa, Unguwa Uku, and BUK site). The soil samples obtained from Kofar Ruwa and Unguwa Uku were found to be

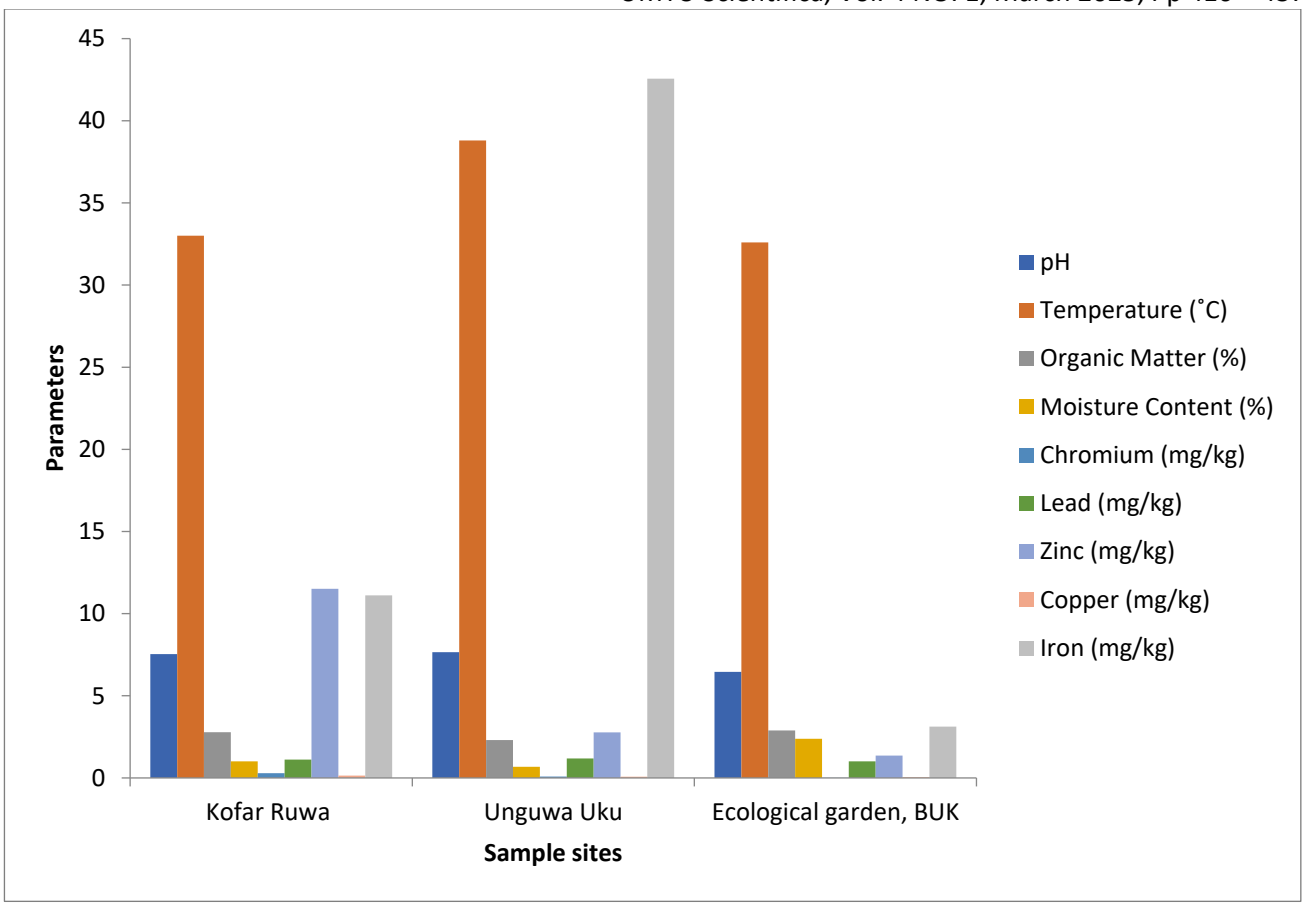

Figure 1: Physico-chemical properties of the soil samples at Kofar Ruwa, Unguwa Uku, and Ecological garden BUK.

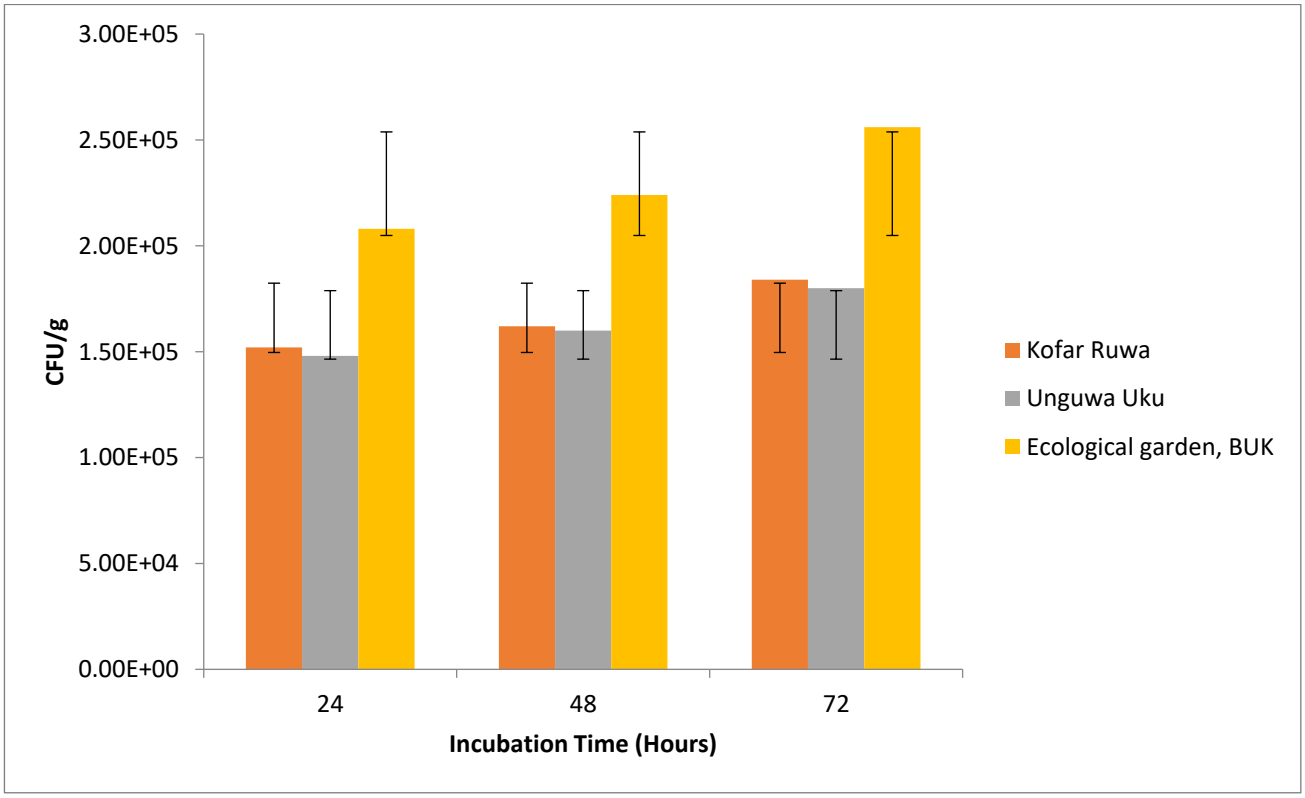

Figure 2: Heterotrophic bacterial counts of the soil samples after 3 days of incubation at 30˚C.

more neutral (pH 7.4 and pH 7.6), respectively, than those from the BUK site (pH 6.4). Both soil samples' moisture content and organic matter were lesser than the BUK site (2.4%). The temperature of the BUK site (32.6˚C) is lesser

compared to that of Kofar ruwa (33.0˚C) and Unguwa Uku (38.8˚C). The concentration of Lead, Chromium, Zinc, Copper, and Iron were determined with the help of Atomic Absorption Spectroscopy. The data reveals that Zinc is maximum with a concentration of 11.51 mg/kg in Kofar Ruwa, while Iron is maximum from Unguwa Uku and BUK (42.56 mg/kg and 3.12 mg/kg respectively). The data is shown in Figure 1.

Heterotrophic bacterial counts of the soil samples

In this study, the heterotrophic bacterial counts of the soil samples obtained from the BUK site after 24, 48, and 72hrs of incubation were 2.08 x 10^5cfu/g, 2.24x 10^5cfu/g, and 2.56 x 10^5cfu/g respectively. These values were comparatively higher than those of soil samples obtained from Kofar ruwa (1.48 x 10^5cfu/g, 1.60 x10^5cfu/g and 1.80 x 10^5cfu/g) and Unguwa Uku (1.52 x 10^5cfu/g, 1.62 x10^5cfu/g and 1.84 x 10^5cfu/g) (Figure 2). The standard deviation of the mean was also determined. The data is shown in Figure 2.

Screening of heavy metal tolerant bacteria by plate diffusion method

In the plate diffusion method, zone formation results signify the organisms' ability as heavy metal-resistant or sensitive strains to heavy metals. Heavy metal-resistant strains show no inhibition of growth at a concentration of heavy metals, whereas heavy metal-sensitive strains show inhibition of growth (S3, S5, and S6) at a concentration of heavy metals. Based on this concept, S1, S2, S4, and S7 were identified as efficient strains that were resistant to (Pb, Cr, Zn, Fe), (Pb, Cr, Zn, Fe, Cu), (Pb, Cu, Zn, Fe) and (Pb, Cr, Zn, Fe, Cu) respectively (Figure 3). The identified efficient strains were selected for further studies.

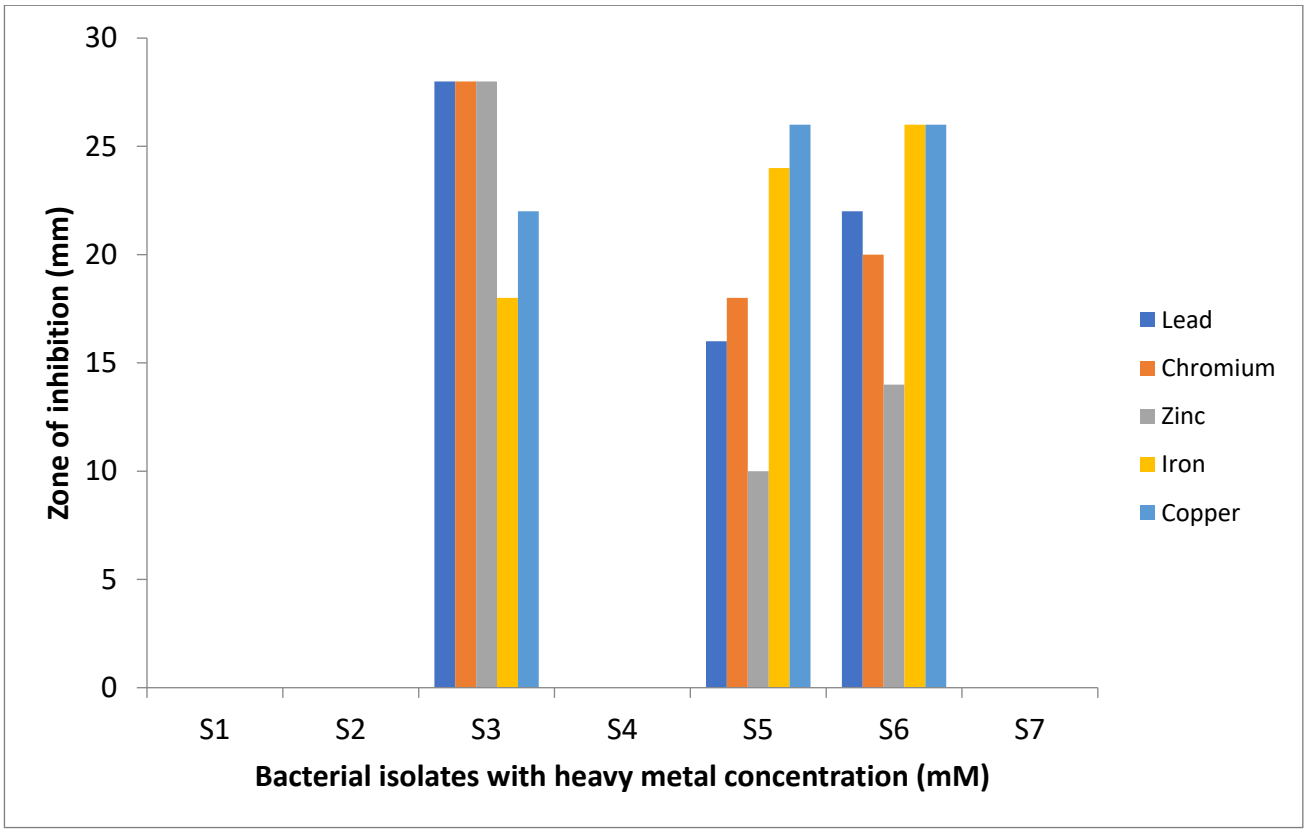

Figure 3: Growth of bacteria with heavy metals concentration by plate diffusion method.

Table 1: The biochemical characterization of isolated tolerant bacteria from the soil samples

Test	Strain S2	Strain S7	Strain S4	Strain S1
Gram-staining	GN, straight rods	GN, rod-shaped	GP, round-shaped	GN, rod-shaped
Catalase	+	+	+	+
Indole	+	-	-	-
Oxidase	-	-	-	-
Methyl red	+	-	-	+
Voges Proskauar	-	+	-	+
Citrate	-	+	-	+
Urease	-	-	-	+
TSI	-	-	-	+
Coagulase	-	-	+	-
Strains	***Escherichia coli***	***Pseudomonas aeroginosa***	***S. aureus***	***Klebsiella pneumoniae***

Key: **GN**: Gram negative; **GP**: Gram positive; **+:** Positive and **–**: Negative result

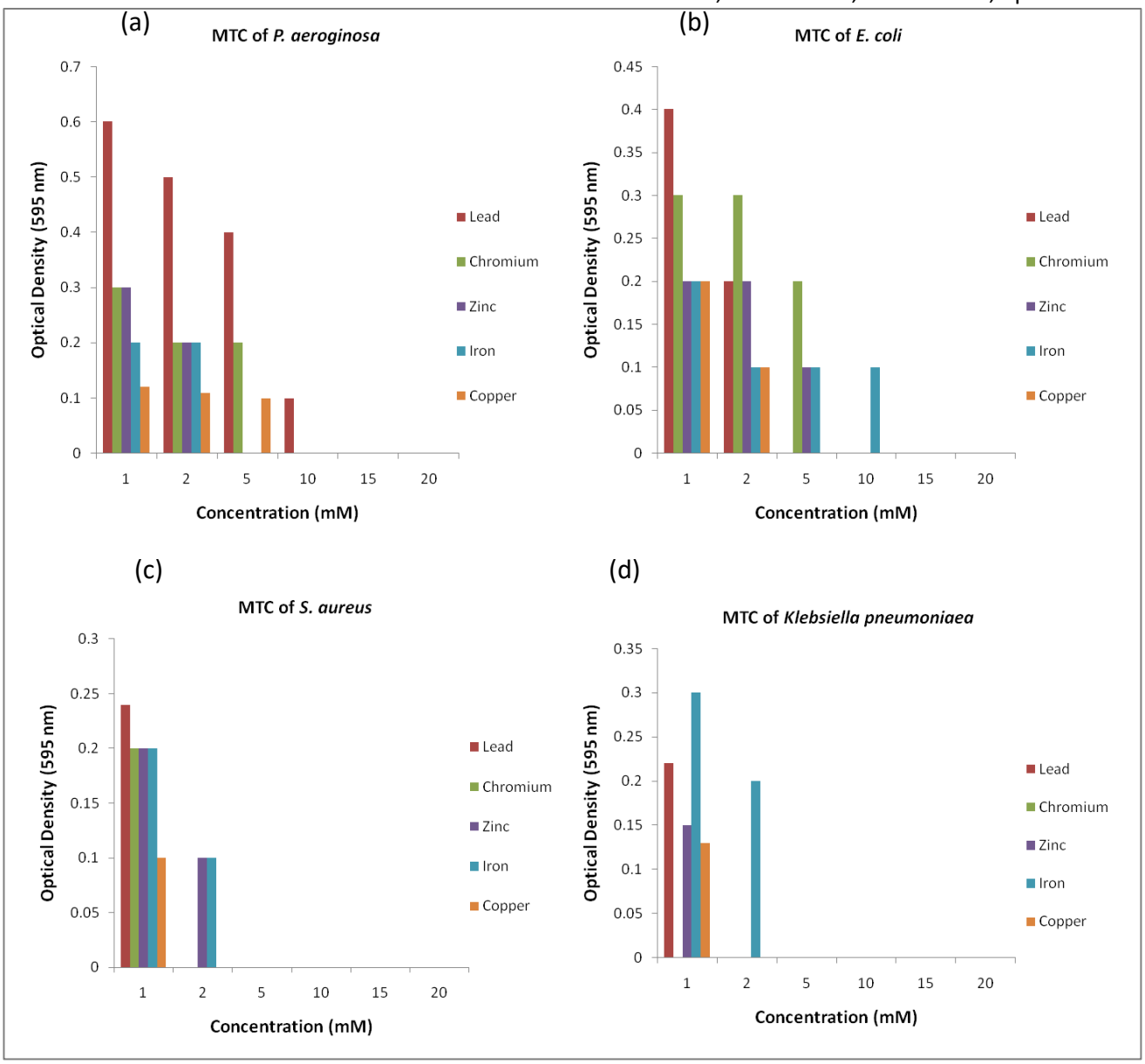

Figure 4: Maximum tolerable concentration of all the four bacterial isolates (a-d) on different concentration of heavy metals compared to control (0.82).

Biochemical characterization of the bacterial strains

The biochemical characterization of isolated tolerant bacteria is given in Table 1. The organisms identified include *Escherichia coli, Pseudomonas aerugiosa, Staphylococcus aureus, and Klebsiella pneumoniae*. The identified organisms were used to remove heavy metals from contaminated soil.

Maximum Tolerable Concentration (MTC) of heavy metals by bacterial isolates

Heavy metal tolerance test by *Pseudomonas aeroginosa* showed maximum tolerance to Pb (10 mM) and minimum tolerance to Fe (2mM), Cu (5mM), Zn (2mM) and Cr (5mM) (Figure 4). *E. coli* showed maximum tolerance to Fe (10mM) and minimum tolerance to Cr (5mM), Zn (5mM), Pb (2mM) and Cu (2mM) (Figure 4).*S. aureus* showed maximum tolerance to Zn (2mM) and Fe (2mM) and minimum tolerance to Cr (1mM), Cu (1mM), and Pb (1mM) (Figure 4). *Klebsiella pneumoniae* showed maximum tolerance to Fe (2mM) and minimum tolerance to Zn (1mM), Pb (1mM), and Cu (1mM) (Figure 4). No Significance difference was observed between the heavy metals tolerant test of all four bacterial isolates at 5% level of significance ($P>0.05$), but it was evident from the results that there is significance difference ($P < 0.05$) in the rate of growth of the different bacterial isolates.

Determination of optimal pH

Each metal tolerant isolate was studied for its optimum pH requirement based on the maximum OD observed in the presence of heavy metals. A pH range of 6 to 7 was found to favour the growth of *Pseudomonas aeruginosa*, *E. coli*, *S. aureus,* and *Klebsiella pneumoniae* in the presence of heavy metals, while low pH and high pH inhibit their growth (Figure 5). Significance difference was observed between the pH ranges of all four bacterial isolates at 5% level of significance ($P<0.05$).

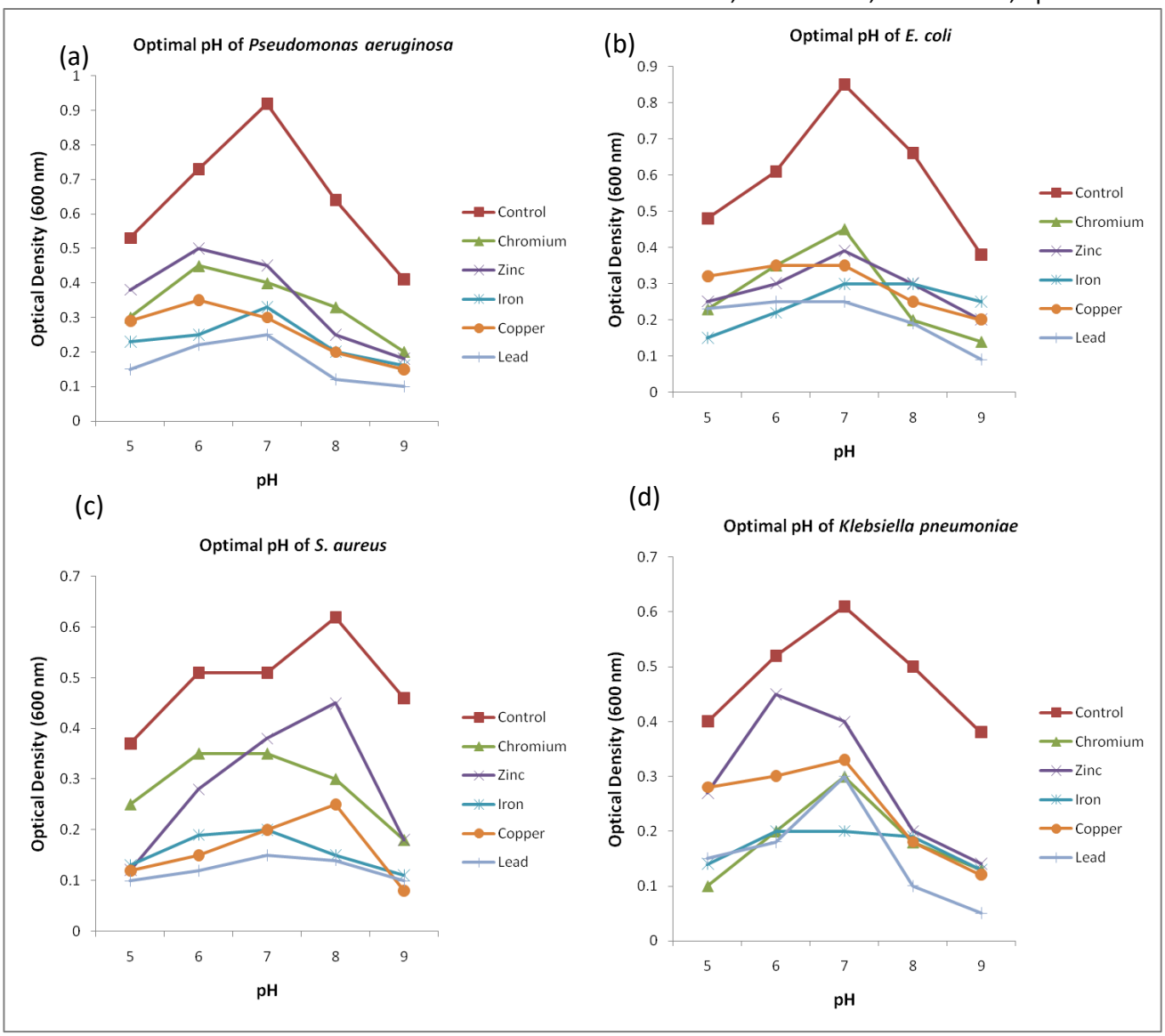

Figure 5: Microbial growth of the isolates on the different heavy metals at varying pH levels (a-d).

Determination of optimal Temperature

Each metal tolerant isolate was also studied for its optimum temperature requirement based on the maximum OD observed in the presence of heavy metals. A temperature range of 35^{0}C to 37^{0}C was found to favour the growth of *Pseudomonas aeruginosa*, *E. coli*, *S. aureus,* and *Klebsiella pneumoniae* in the presence of heavy metals, while low temperature and high temperature inhibited their growth (Figure 6). Significance difference was observed between the temperature ranges of all four bacterial isolates at 5% level of significance ($P<0.05$) (Appendix I. c).

Removal of heavy metals by bacteria

The residual heavy metal concentration was determined by the use of an atomic absorption spectrophotometer (AAS). Zinc concentration was observed reduced to 72.2% in the presence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* (36.9%), *E. coli* (64.5%), and *Klebsiella pneumonia*e (27.1%). Chromium concentration was reduced to 50.2% by *Pseudomonas aeruginosa*, *Staphylococcus aureus* (17.3%), *E.coli* (19.4%), and *Klebsiella pneumonia* (34.9%). Pseudomonas aeruginosa reduced lead concentration to 76.5%, *Staphylococcus aureus* (57.5%), *E. coli* (65%), and *Klebsiella pneumonia* (49.8%). Copper concentration was reduced by *Pseudomonas aeruginosa* (97.5%), *Staphylococcus aureus* (55.5%), *E. coli* (93.8%), and *Klebsiella pneumoniae* (75.7%). Pseudomonas aeruginosa reduced iron concentration to (72.6%), *Staphylococcus aureus* (19.9%), *E. coli* (64.2%), *and Klebsiella pneumoniae* by (49.3%). The percentages of removal by all four bacterial isolates are shown in Table 2. All bacterial isolates observed a significant difference on metal removal ($P<0.05$).

Scanning Electron Microscopy (SEM).

The SEM analysis of all four bacteria before and after exposure to heavy metals, showed a significant alteration in the morphology of the bacterial cells and was shown in Figure 7 (e, f, g, h). The SEM micrograph of the metal-free and metal-loaded of all four bacteria visibly showed

that the metal particles were disintegrated and adsorbed on to the surface of the bacterial cells. This resulted in an alteration in the cell-surface morphology of the bacterial cells, clearly visible in the SEM micrographs. Scanning electron microscopy showed changes in cell growth patterns (size and shape) and in exopolysaccharide production in response to metal exposure (Figure 7).

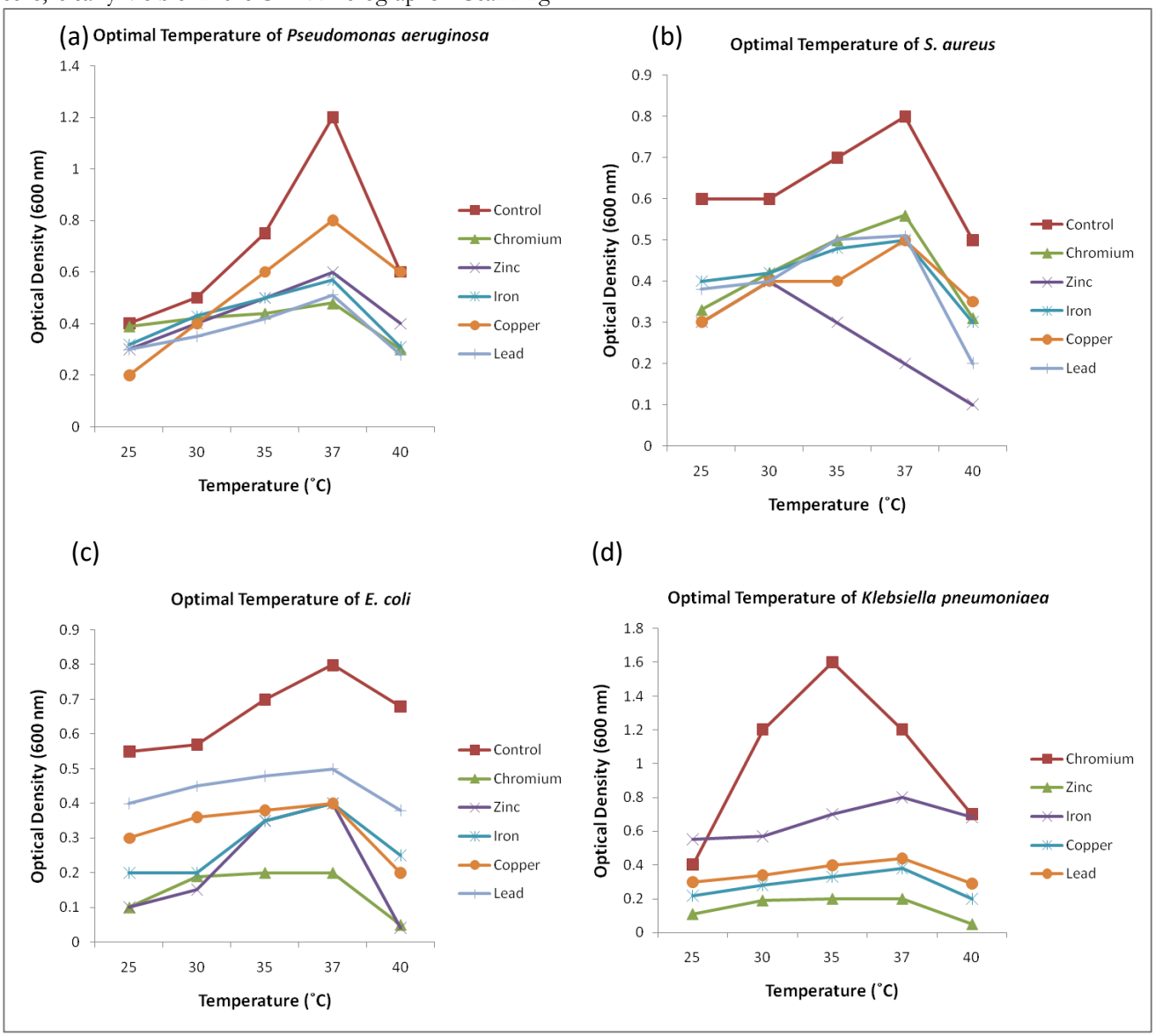

Figure 6: Microbial growth of the isolates on the different heavy metals at varying temperature conditions (a-d).

DISCUSSION

Metals and other physicochemical parameters play an important role in developing metal tolerance in indigenous bacteria of particular sites (Shi *et al.,* 2013). There is an increasing interest and it seems that it is the current emphasis to isolate and identify some indigenous heavy metal tolerant bacteria and their possible use for the bioremediation of heavy metal polluted/contaminated areas (Abuzar *et al.,* 2017).

In this study, a total of four heavy metal-resistant bacteria were isolated from soil samples collected from the Kofar Ruwa, Unguwa Uku, and BUK sites in Kano, Nigeria. The bacteria were selected in the presence of Zn^{2+}, Cr^{3+}, Pb^{2+}, Cu^{2+}, and Fe^{3+} at concentrations of up to 2mM. Based on their cultural, morphological, and biochemical characteristics, the isolates were putatively identified as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Klebsiella pneumoniae. Similar findings of the occurrence of heavy metal resistant bacteria in contaminated soil samples have been reported (Olukoya *et al.,* 1997; Fagade and Adetutu, 1999; Nwaugon *et al.,* 2008; Oyetibo *et al.,* 2010; Owolabi and Hekeu, 2014). Olukoya *et al.* (1997) isolated *Pseudomona s*and *Streptococcus* spp. showing resistance to Cr and Pb from alagoon area. Oyetibo *et al.* (2010) found *Corynebacterium* and *Pseudomonas* spp. with resistance to Cr and Cd from soil samples from an industrial estate in Lagos, Nigeria. Nwaugo and collaborators (2008) identified *Pseudomonas* spp. resistant to Pb from soil samples collected from a mining pit. Owolabi and Hekeu (2014) isolated *Aeromonas* spp., *Arthrobacter* spp., *Corynebacterium* sp., *Pseudomonas* spp.,

and *Streptococcus* spp. showing resistance to lead, cadmium, and chromium from soil samples collected from Lagos and Ota, Nigeria. Likewise, this work conformed to Enimie *et al.* (2016), who isolated five bacterial isolates with maximum tolerance trend for cadmium, chromium, Nickel, and Zinc, which were identified as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli, Proteus vulgar is and Klebsiella pneumoniae* from petroleum refinery effluent.

Table 2: Removal of heavy metals by bacteria

Organism	Heavy Metals	Initial concentration (mg/L)	24 Hours (%)	48 Hours (%)	72 Hours (%)	96 Hours (%)
E. coli	Zinc	0.100	0.0705 (29.5)	0.0453 (54.7)	0.0390 (61.0)	0.0355 (64.5)
	Chromium	0.100	0.0825 (17.5)	0.0820 (18.0)	0.0809 (19.1)	0.0806 (19.4)
	Lead	0.100	0.0763 (23.0)	0.0485 (51.0)	0.0424 (57.0)	0.0342 (65.0)
	Iron	0.100	0.0375 (62.5)	0.0362 (63.8)	0.0360 (64.0)	0.0358 (64.2)
	Copper	0.100	0.0201 (79.9)	0.0124 (87.6)	0.0068 (93.2)	0.0062 (93.8)
P. aeroginosa	Zinc	0.100	0.0706 (29.4)	0.0498 (50.2)	0.0357 (64.3)	0.0278 (72.2)
	Chromium	0.100	0.0734 (26.6)	0.0626 (37.4)	0.0626 (37.4)	0.0498 (50.2)
	Lead	0.100	0.0754 (24.6)	0.0501 (49.9)	0.0341 (65.9)	0.0235 (76.5)
	Iron	0.100	0.0299 (70.1)	0.0282 (71.8)	0.0276 (72.4)	0.0274 (72.6)
	Copper	0.100	0.0097 (90.3)	0.0079 (92.1)	0.0075 (92.5)	0.0025 (97.5)
S. aureus	Zinc	0.100	0.0872 (12.0)	0.0767 (23.3)	0.0702 (29.8)	0.0631 (36.9)
	Chromium	0.100	0.0884 (11.6)	0.0862 (13.8)	0.0848 (15.2)	0.0827 (17.3)
	Lead	0.100	0.0663 (33.7)	0.0486 (51.4)	0.0435 (56.5)	0.0425 (57.5)
	Iron	0.100	0.0822 (17.8)	0.0816 (18.4)	0.0808 (19.2)	0.0801 (19.9)
	Copper	0.100	0.0703 (29.7)	0.0599 (40.1)	0.0506 (49.4)	0.0445 (55.5)
K. pneumoniae	Zinc	0.100	0.0985 (1.5)	0.0958 (4.2)	0.0844 (15.6)	0.0729 (27.1)
	Chromium	0.100	0.0815 (18.5)	0.0725 (27.5)	0.0719 (28.1)	0.0651 (34.9)
	Lead	0.100	0.0627 (37.3)	0.0546 (45.4)	0.0505 (49.5)	0.0502 (49.8)
	Iron	0.100	0.0552 (44.8)	0.0534 (46.6)	0.0519 (48.1)	0.0507 (49.3)
	Copper	0.100	0.0795 (20.5)	0.0647 (35.3)	0.0427 (57.3)	0.0243 (75.7)

The maximum tolerable concentration (MTC) of heavy metals was designated as the highest concentration of heavy metals that allowed growth after 24 hours (Schmiatt and Schlege, 1994). The MTC value for Pb (10mM) and Fe (10mM) in this study was higher than that obtained by Fagade and Adetutu (1999) and Sanuth *et al.* (2010), which ranged between 2 and 2.5 mM but similar to the findings of Owolabi and Hekeu (2014). However, the MTC values for Cr (5 mM), Cu (5 mM), and Zn (5 mM) are much lower than those reported by Oyetibo and others (2010), where the MTC for Cr was 17 mM. These studies propose variability in the potency of bacteria towards heavy metals to which they are resistant. This variation in response might be due to the difference in resistance mechanisms (AbouZeid *et al.,* 2009). Toxicity testing in liquid medium facilitates a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates (Hassen *et al.,* 1998). Liquid medium

toxicity testing differs from toxicity testing on solid medium, where the conditions of diffusion, complexation, and availability of metals differ from those in solid medium (Hassen *et al.,* 1998; Saad *et al.,* 2015).

The bacterial resistance to heavy metal ions was affected not only by the surface properties of the organism but also by environmental conditions like temperature and pH. The bacterial strains were able to grow at a wide range of temperatures, with high growth rates (Figure 6). The range of growth temperatures helped to describe heavy metal-resistant bacterial strains as a potential agent for use in bioremediation processes under a wide range of temperatures. This is an important aspect, considering temperature control may not be possible during some bioremediation processes (Nascimento and Chartone-Souza, 2003).

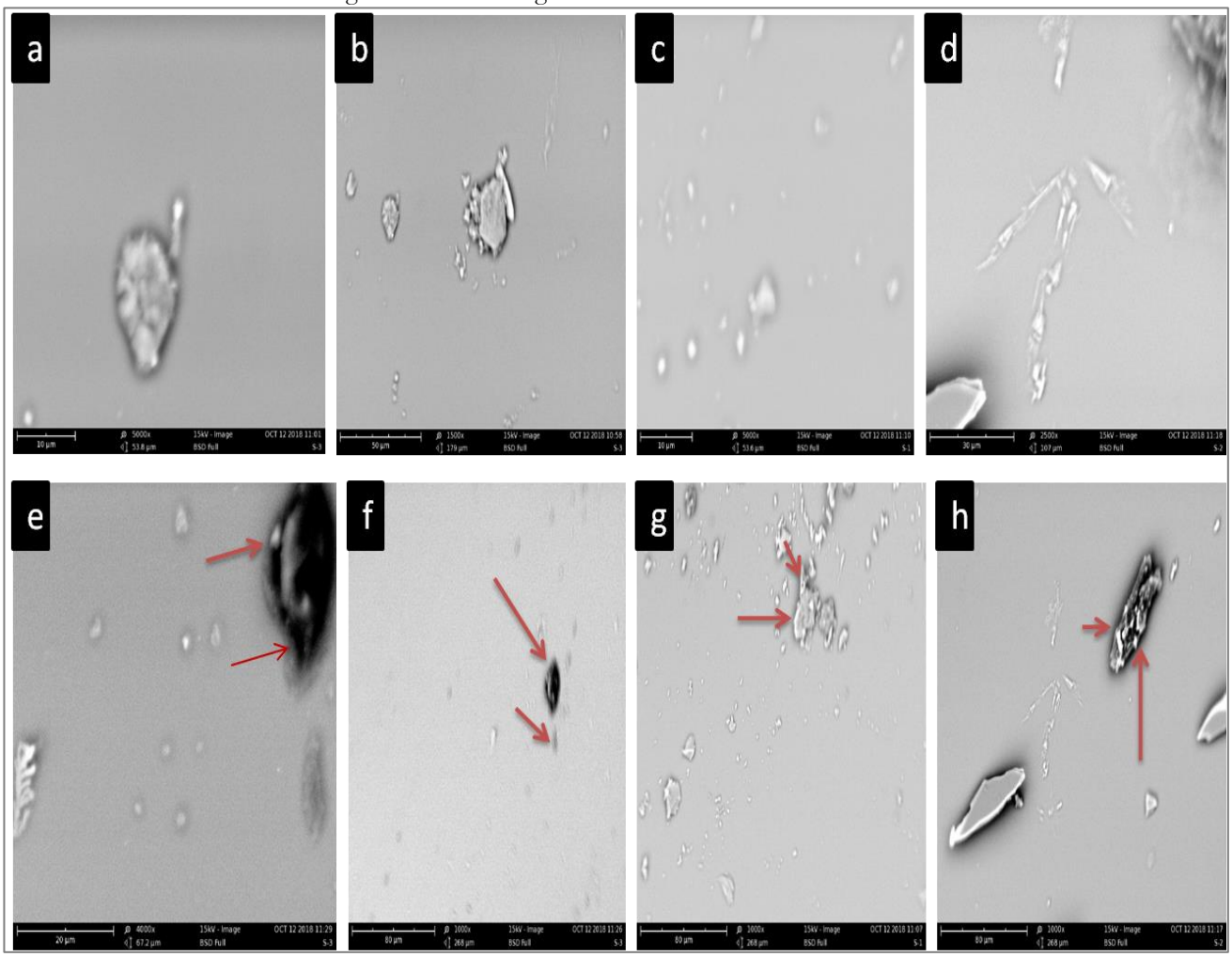

Figure 7: SEM images of all the bacteria; a,b,c, and d are the control before exposure to heavy metal for *P. aeruginosa, E. coli, S. aureus,* and *K. pneumonia*e respectively, likewise for e, f, g, and f after exposure to heavy metal.

The pH value is one of the main factors affecting the growth of heavy metal-resistant bacteria (Lopez *et al.,* 2000; Pardo *et al.,* 2003; Saad *et al.,* 2015). pH plays a critical role in microbial metal resistance and uptake by influencing the metal speciation solution chemistry, and surface properties of bacterial cells. pH was evaluated as it affects the number of cellular surface sites available to bind cations and metal speciation (Yan and Viraragh, 2003). Results indicated that the optimum pH for all the bacterial strains was 7.0 using the controlled medium (no metal), whereas the optimum pH with Pb, Cr, Zn, Fe, and Cu was around 6 and 7 (Figure 5). These results agreed with those of Congeevaram *et al.* (2007), who found that pH optimal for growth and bioaccumulation of Cr^{6+} and Ni^{2+} by the heavy metal-resistant bacteria *Micrococcus* spp. was pH 7. The selected metal-resistant strain showed that different pH values only slightly affected their growth. Therefore, it is clear that the growth of all the bacterial isolates is not inhibited with different pH values, making them a strong candidate for future application in metal bioremoval. In a similar research on physicochemical and molecular characterization of heavy metal–tolerant bacteria isolated from soil of mining sites in Nigeria, results of physical and chemical characteristics showed mean pH values and percentage organic carbon to range from 7.1 to 8.2 and 0.18 to 1.12%, respectively with statistical significance between sampling sites ($P \leq 0.05$). Similarly, cation exchange capacity, electrical conductivity, moisture, total nitrogen, and carbon/nitrogen ratio (C:N) in the soil ranged between 1.52 to 3.57 cmol/kg, 0.15 to

0.32 ds/m, 0.14 to 0.82%, 0.10 to 0.28%, and 1.7 to 4.8 respectively. The highest heavy metal concentration of 59.01 ppm was recorded in soils obtained from site 3. The enumeration of viable aerobic bacteria recorded the highest mean count of 4.5 × 106 cfu/g observed at site 2 with statistical significance ($P \leq 0.05$) between the sampled soils. *Alcaligenes faecalis* strain UBI, *Aeromonas* sp. strain UBI, *Aeromonas sobria*, and *Leptothrix ginsengisoli,* which make up 11.2% of total identified bacteria, were able to grow in higher amended concentrations of heavy metals. The evolutionary relationship showed the four heavy metal–tolerant bacteria identified belonged to the phylum Proteobacteria of class Betaproteobacteria in the order Burkholderiales. Heavy metal biosorption by the bacteria showed *Alcaligenes faecalis* strain UBI having the highest uptake capacity of 73.5% for Cu (Ibrahim, U.B *et al.*, 2021).

The heavy metal reduction by each bacterial isolate revealed *P. aeruginosa* as the best heavy metal-tolerant bacteria, followed by *E. coli*, *K. pneumoniae,* and *S. aureus* (Figure 5). Similar findings of heavy metals reduction by bacteria have been reported (Usman *et al.,* 2012; Momba and Ikonga, 2013; Enimie *et al.,* 2016). According to Usman *et al.* (2012), *Pseudomonas* spp. has the ability to degrade heavy metals present in industrial effluent. Momba and Ikonga (2013), in their research, observed that *Pseudomonas putida* showed removal rates of 100% for Thalium, 96% for Lead, 83% for Vanadium, 71%for Cobalt, 57% for Nickel, 49% for Copper and 45% for Manganese. Enimie *et al.* (2016), *Pseudomonas aeruginosa* (Cd (100%), Cr (23.1%), Ni (64.3%) & Zn (53.9%) yielded high values for the reduction of heavy metals in the refinery effluent when compared to *Staphylococcus aureus, E.coli, Proteus vulgaris,* and *Klebsiella pneumonia.* In general, *P. aeruginosa* showed the highest efficacy for heavy metal reduction. This is because the isolate is a potent heavy metal tolerant strain capable of growing in high concentrations of Zn, Cu, Fe, and Cr (Garima *et al.,* 2015). The efficacy of removal of the five heavy metals in decreasing order was *P. aeruginosa*>*E. coli*>*K. pneumonia*>*S. aureus.* The high bioremoval rate observed in *P. aeruginosa* might be attributed to the possession of versatile metabolic activities for which the isolate was employed to take a variety of substances.

The results of SEM agreed with the observations of Suriya *et al.* (2013). The result was also similar to the findings of Srivastava and Thakur (2007) in their study of the assessment of morphological changes as a result of chromium accumulation within the bacterial strain, *Acinetobacter* sp. Using SEM pictures; they claimed that chromium was uniformly bound on the cell wall surface of the bacteria. Morphological transformation due to exposure of the cell to the heavy metals was also evident (Figure 7). The SEM studies revealed that before heavy metal exposure, the cells appeared to be plump with smooth surfaces in a loosely bound form, but after interaction with the metals, precipitates in the form of round globules and amorphous substances aggregated all over the cell surfaces of all the bacterial isolates; this is similar to the reports made by Chatterjee *et al.,* (2011). The morphological changes due to nickel stress were explored by SEM analysis, which revealed an increase in the size of the cells and possible secretion of extrapolymeric substance after exposure of *Sinorhizobium* sp., BEL5B strain to 3 mM nickel (Jobby *et al.,* 2015). Similar morphological changes like increased size were also demonstrated in phototropic bacteria after exposure to metalloid oxyanions as a protection strategy for facing contaminated environments (Nepple *et al.,* 1999; Duraisamy and Thatheyus, 2018). Helmann *et al.* (2007) showed that binding metal ions can limit the effect of metals on cells to exopolysaccharide, and the cells can survive the metal stress along with normal metabolic activities. Increased exopolysaccharide production by bacterial strains with the presence and increase in heavy metal concentrations in culture medium was reported by Chien *et al.* (2013). Nevertheless, no difference in exopolysaccharide production was observed between all four strains with the highest and lowest metal removal rates. This confirmed that greater removal may be linked to these exopolysaccharides' composition and their metal adsorption capacity.

CONCLUSION

This study isolated Cr3+, Pb2+Zn2+ Fe3+, and Cu2+ resistant bacteria from three different sampling sites. The four isolates were identified as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus,* and they all showed various levels of maximum tolerance to different concentrations of lead, chromium, Zinc, Copper, and Iron. The efficacy of removal of the five heavy metals in decreasing order was *P. aeruginosa*>*E.coli*>*K. pneumoniae*>*S. aureus.* Overall, *Pseudomonas aeruginosa* had the highest percentage reduction of heavy metal analyzed by AAS in the study. Thus, *Pseudomonas aeruginosa* is more effective for bioremediation of heavy metals. SEM revealed visible morphological changes in cells due to heavy metal stress, indicating that all four bacterial isolates could transform heavy metal ions by depositing them as mineral crystals, which may play a role in the removal of heavy metals in soils.

RECOMMENDATIONS

It is recommended that:

1. All four bacterial isolates should serve as potential candidates to detoxify heavy metal-contaminated soil within natural environments in Kano State.
2. The studies assessing the potential ability of the selected isolates to remove heavy metals from contaminated soils and their plasmid profiles should be evaluated.

Further research should be conducted to determine the mechanisms by which the bacterial isolates reduce heavy metals.

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SUPPLEMENTARY MATERIALS

Supplementary I: Statistical analysis

a) Optimal pH

pH	*P. aeruginosa*	*E. coli*	*S. aureus*	*K. pneumoniae*
5	0.313333	0.276667	0.181667	0.223333
6	0.416667	0.346667	0.266667	0.308333
7	0.441667	0.431667	0.298333	0.356667
8	0.29	0.316667	0.318333	0.225
9	0.2	0.21	0.185	0.158333

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
5	4	0.995	0.24875	0.003366
6	4	1.338333	0.334583	0.004062
7	4	1.528333	0.382083	0.004556
8	4	1.15	0.2875	0.001905
9	4	0.753333	0.188333	0.000506
Pseudomonas aeruginosa	5	1.661667	0.332333	0.009683
E. coli	5	1.581667	0.316333	0.006776
S. aureus	5	1.25	0.25	0.004044
K. pneumoniae	5	1.271667	0.254333	0.006106

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.089983	4	0.022496	16.40603	0.000083	3.259167
Columns	0.026727	3	0.008909	6.497341	0.007362	3.490295
Error	0.016454	12	0.001371			
Total	0.133164	19				

b) Optimal Temperature

Temperature	*P. aeruginosa*	*S. aureus*	*E. coli*	*K. pneumoniae*
25	0.318333333	0.385	0.275	0.286667
30	0.416666667	0.44	0.32	0.478333
35	0.535	0.48	0.41	0.596667
37	0.693333333	0.511667	0.45	0.591667
40	0.415	0.293333	0.266667	0.37

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
25	4	1.265	0.31625	0.002436
30	4	1.655	0.41375	0.004553
35	4	2.021667	0.505417	0.006317
37	4	2.246667	0.561667	0.011069
40	4	1.345	0.33625	0.004675
Pseudomonas aeruginosa	5	2.378333	0.475667	0.020704
S. aureus	5	2.11	0.422	0.007413
E. coli	5	1.721667	0.344333	0.006727
K. pneumoniae	5	2.323333	0.464667	0.018596

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.179841667	4	0.04496	15.90607	9.65E-05	3.259167
Columns	0.053227778	3	0.017743	6.276963	0.008318	3.490295
Error	0.033919444	12	0.002827			
Total	0.266988889	19				

c) Maximum Tolerable Concentration (MTC)of heavy metals by bacterial isolates

Organisms	Lead	Chromium	Zinc	Iron	Copper
Pseudomonas aeroginosa	0.266667	0.116667	0.083333	0.066667	0.055
E. coli	0.09	0.0875	0.072222	0.066667	0.05
S. aureus	0.04	0.033333	0.05	0.05	0.016667
Klebsiella pneumoniea	0.036667	0	0.025	0.083333	0.021667

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Pseudomonas aeroginosa	5	0.588333	0.117667	0.007477
E. coli	5	0.366389	0.073278	0.000267
S. aureus	5	0.19	0.038	0.000192
Klebsiella pneumoniea	5	0.166667	0.033333	0.000957
Lead	4	0.433333	0.108333	0.011737
Chromium	4	0.2375	0.059375	0.002759
Zinc	4	0.230556	0.057639	0.000666
Iron	4	0.266667	0.066667	0.000185
Copper	4	0.143333	0.035833	0.000379

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.022864	3	0.007621	3.761699	0.040985	3.490295
Columns	0.011262	4	0.002815	1.389644	0.295438	3.259167
Error	0.024312	12	0.002026			
Total	0.058438	19				

d) Removal of heavy metals by bacteria

Time (Hours)	*E. coli*	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*
24	29.5	12	1.5	29.4
48	54.7	23.3	4.2	50.2
72	61	29.8	15.6	64.3
96	64.5	36.9	27.1	72.2

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
24	4	72.4	18.1	190.14
48	4	132.4	33.1	563.4067
72	4	170.7	42.675	567.4225
96	4	200.7	50.175	466.3292
E. coli	4	209.7	52.425	250.0225
S. aureus	4	102	25.5	111.8467
K. pneumoniae	4	48.4	12.1	137.34
P. aeruginosa	4	216.1	54.025	352.3092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2297.223	3	765.7408	26.78118	0.000081	3.862548
Columns	5104.563	3	1701.521	59.50934	0.000003	3.862548
Error	257.3325	9	28.5925			
Total	7659.118	15				

Supplementary II: Physico-chemical properties of the soil samples at Kofar Ruwa, Anguwa Uku and Ecological garden BUK.

Parameter	**Kofar Ruwa**	**Anguwa Uku**	**Ecological garden, BUK**
pH	7.54	7.65	6.46
Temperature (˚C)	33.00	38.80	32.60
Organic Matter (%)	2.785	2.304	2.889
Moisture Content (%)	1.013	0.675	2.389
Chromium (mg/kg)	0.286	0.0853	0.0006
Lead (mg/kg)	1.12	1.19	1.01
Zinc (mg/kg)	11.5102	2.7757	1.3583
Copper (mg/kg)	0.139	0.079	0.047
Iron (mg/kg)	11.11	42.56	3.12

Supplementary III: Heterotrophic bacterial counts (CFU/g) of the soil samples after 3 days of incubation at 30˚C

Sample sites	**Incubation time (hours)**		
	24	**48**	**72**
Kofar Ruwa	152000±4000	162000±5291	184000±8718
Anguwa Uku	148000±3606	160000±4583	180000±4359
Ecological garden, BUK	208000±4359	224000±5292	256000±7211

Values are means ± standard deviation

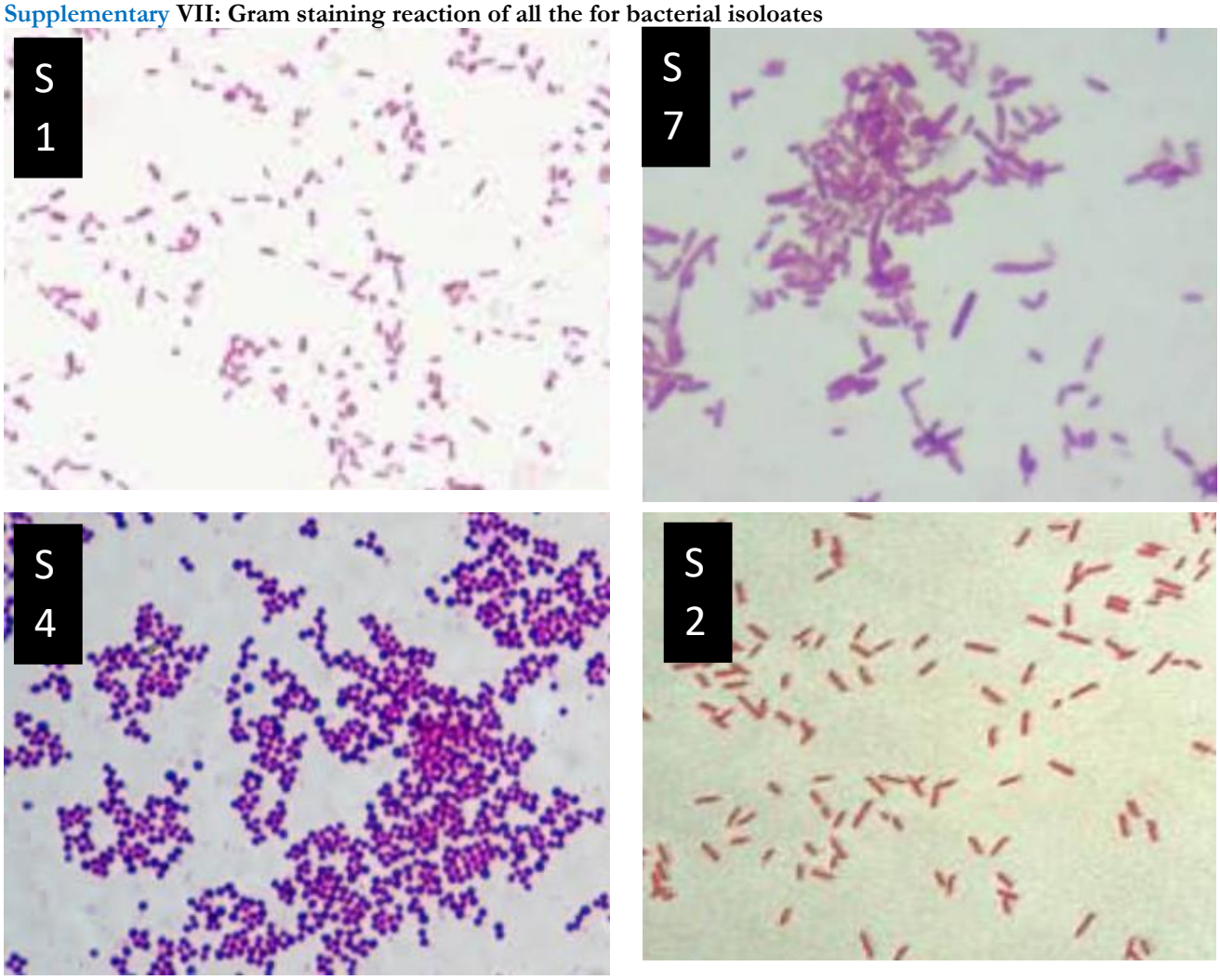
Supplementary IV: Microbial growth of the isolates on the different heavy metals controlled by varying pH condition.

Organisms	pH	Control	Chromium	Zinc	Iron	Copper	Lead
P. aeruginosa	5	0.53	0.30	0.38	0.23	0.29	0.15
	6	0.73	0.45	0.50	0.25	0.35	0.22
	7	0.92	0.40	0.45	0.33	0.30	0.25
	8	0.64	0.33	0.25	0.20	0.20	0.12
	9	0.41	0.20	0.18	0.16	0.15	0.10
E. coli	pH	Control	Chromium	Zinc	Iron	Copper	Lead
	5	0.48	0.23	0.25	0.15	0.32	0.23
	6	0.61	0.35	0.30	0.22	0.35	0.25
	7	0.85	0.45	0.39	0.30	0.35	0.25
	8	0.66	0.20	0.30	0.30	0.25	0.19
	9	0.38	0.14	0.20	0.25	0.20	0.09
S. aureus	pH	Control	Chromium	Zinc	Iron	Copper	Lead
	5	0.37	0.25	0.12	0.13	0.12	0.10
	6	0.51	0.35	0.28	0.19	0.15	0.12
	7	0.51	0.35	0.38	0.20	0.20	0.15
	8	0.62	0.30	0.45	0.15	0.25	0.14
	9	0.46	0.18	0.18	0.11	0.08	0.10
K. pneumoniae	pH	Control	Chromium	Zinc	Iron	Copper	Lead
	5	0.40	0.10	0.27	0.14	0.28	0.15
	6	0.52	0.20	0.45	0.20	0.30	0.18
	7	0.61	0.30	0.40	0.20	0.33	0.30
	8	0.50	0.18	0.20	0.19	0.18	0.10
	9	0.38	0.13	0.14	0.13	0.12	0.05

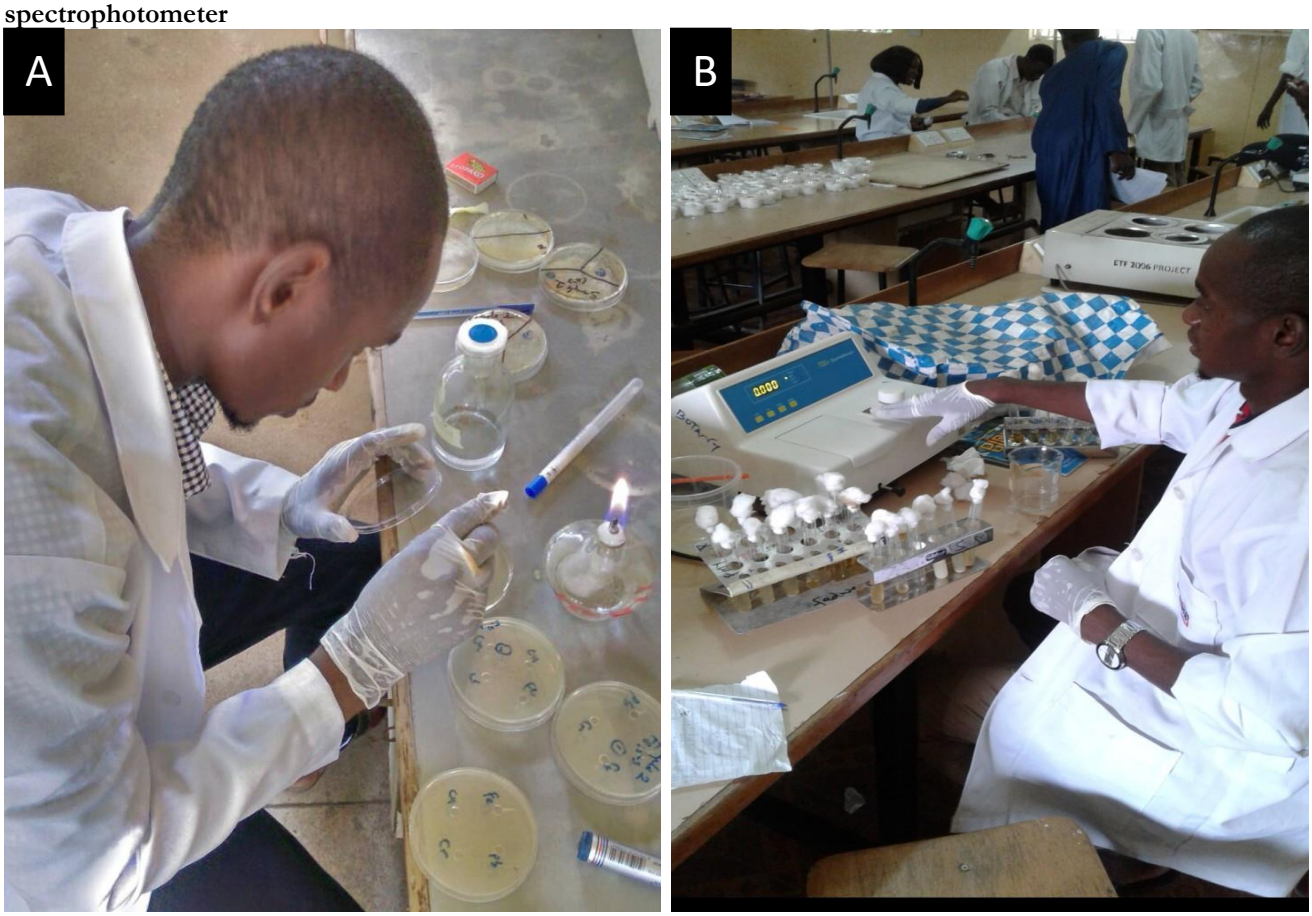
Supplementary VI: Microbial growth of the isolates on the different heavy metals controlled by varying temperature condition.

Organisms	Temp (°C)	Control	Chromium	Zinc	Iron	Copper	Lead
P. aeruginosa	25	0.40	0.39	0.30	0.32	0.20	0.30
	30	0.50	0.42	0.40	0.43	0.40	0.35
	35	0.75	0.44	0.50	0.50	0.60	0.42
	37	1.20	0.48	0.60	0.57	0.80	0.51
	40	0.60	0.30	0.40	0.31	0.60	0.28
E. coli	Temp (°C)	Control	Chromium	Zinc	Iron	Copper	Lead
	25	0.55	0.10	0.10	0.20	0.30	0.40
	30	0.57	0.19	0.15	0.20	0.36	0.45
	35	0.70	0.20	0.35	0.35	0.38	0.48
	37	0.80	0.20	0.40	0.40	0.40	0.50
	40	0.68	0.05	0.04	0.25	0.20	0.38
S. aureus	Temp (°C)	Control	Chromium	Zinc	Iron	Copper	Lead
	25	0.60	0.33	0.30	0.40	0.30	0.38
	30	0.60	0.42	0.40	0.42	0.40	0.40
	35	0.70	0.50	0.30	0.48	0.40	0.50
	37	0.80	0.56	0.20	0.50	0.50	0.51
	40	0.50	0.31	0.10	0.30	0.35	0.20
K. pneumoniae	Temp (°C)	Control	Chromium	Zinc	Iron	Copper	Lead
	25	0.40	0.11	0.55	0.22	0.30	0.14
	30	1.20	0.19	0.57	0.28	0.34	0.29
	35	1.60	0.20	0.70	0.33	0.40	0.35
	37	1.20	0.20	0.80	0.38	0.44	0.53
	40	0.70	0.05	0.68	0.20	0.29	0.30

Supplementary **VII: Gram staining reaction of all the for bacterial isoloates**



Supplementary VIII: Making wells on nutrient agar plates and, B: taking O.D values (absorbance) using spectrophotometer



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Supplementary IX: An overnight pure culture of all the four metal tolerant bacterial isolates