The Microbe 3 (2024) 100087

Contents lists available at ScienceDirect

The Microbe

journal homepage: www.sciencedirect.com/journal/the-microbe

Bioremediation potential of *Bacillus* sp. and *Paenebacillus* sp. novel lead-resistant isolates: Identification, characterization, and optimization studies

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ARTICLE INFO

Keywords:
Heavy metals
Lead
Isolation
Lead-tolerant
Characterization
Bioremediation

ABSTRACT

Environmental pollution by heavy metals and other toxic substances has increasingly become a global health concern. Lead is a hazardous environmental contaminant that is been released to the surface causing deleterious health effects to man. Various strategies have been employed such as excavation, precipitation, and vitrification to remove lead from polluted sites. However, bioremediation of heavy metal contaminated sites has served as a cost-effective and ecofriendly tool for detoxifying and restoration of ecological entities. In this study, we reported the isolation and optimization of two novel lead-resistant isolates from active goldmine contaminated site of Anka, Zamfara state Nigeria. Results of Atomic Absorption Spectroscopy (AAS) showed higher lead concentration from the sampled sites ($p<0.05$) than the USEPA standard (400 mg/Kg). *Paenebacillus* sp. strain BUK_BCH_BTE 3 (MT160418) and *Bacillus* sp. strain BUK_BCH_BTE 4 (MT160452) were identified based on the 16 S rRNA partial gene sequencing of the locally isolated microorganisms. Characterization of the two isolates revealed optimum growth at 37 °C, a pH of 7.0 over 48 hrs while utilizing inoculum volumes of 100 μL each. The two isolates utilized optimum sucrose concentrations at 10–20 g/L, while 5 g/L nitrogen source and 5 g/L of urea were optimum for *Paenebacillus* sp. as well as *Bacillus* sp. respectively. An optimal lead concentration of 1000 g/L was obtained by both isolates. Furthermore, the two isolates tolerated up to 3000 mg/L lead nitrate. Mercury, a toxic heavy metal, was noticed to hinder the growth of both isolates significantly at $p<0.05$. Hence, the locally isolated *Paenebacillus* sp. and *Bacillus* sp. could be used as promising tools for bioremediation of lead-contaminated environment.

1. Introduction

Mining is an artificial process that involves digging and removal of rocks from the ground. This in turn leads to extraction of metal ores-that can be an important cause of environmental degradation (Dukda and Adriano, 1997). The release of metal contaminants has increased because of anthropogenic processes (Akhtar et al., 2013a). These metal contaminants are the cause of specific toxicity symptoms, harmful effects on both human and animal health as well as polluting the environment. The accumulation of these heavy metals reaching a toxic level may damage the ecosystem. Such heavy metals include mercury, lead, cadmium, and chromium (Yakasai et al., 2019; Ghosh et al., 2019). Lead is found in ore deposit and has no known positive biological function and causes serious environmental and health hazards (Buba, 2016). Lead is a major toxin that is well-known on a global scale and has several dangerous impacts on the environment, human health, and animal

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https://doi.org/10.1016/j.microb.2024.100087
Received 17 December 2023; Received in revised form 3 May 2024; Accepted 9 May 2024
Available online 13 May 2024

health (Buba, 2016). An unusual lead poisoning pandemic in Zamfara State in northern Nigeria was addressed between 2010 and 2013 by coordinating environmental and health initiatives. Lead poisoning is a serious public health issue in Zamfara due to its high frequency, morbidity, and mortality (>400 children) (Medecins Sans Frontieres MSF, 2012). Inhalation of lead dust and hand-to-mouth contact are the two main ways that kids get exposed to lead. Metals like mercury, lead, and arsenic have hazardous effects on the kidneys and nervous systems; they have been shown to cause symptoms including anemia, weakness, migraines, and abdominal cramps. Prolonged contact with such heavy metals can irreversibly harm organelles (Babandi et al., 2024; Meignanalakshmi et al., 2018).

To protect the ecological system from these harmful contaminants several physical and chemical methods have been employed. Such conventional methods of remediation include soil excavation, soil covering, encapsulation, ion exchange and vitrification. These conventional methods are widely used due to short remediation time and simple operations (Maroof et al., 2018; Li et al., 2023). However, their limitations include high cost and inefficiency (Yakasai et al., 2019; Chai et al., 2021). Production of other toxic metabolites is a possible consequence of conventional methods of remediation (Ghosh et al., 2019). Interestingly, the use of microbiological agents for removal or detoxification of both organic and inorganic contaminants is an alternative tool for remediation because it is cost effective and ecofriendly (Hussein et al., 2019).

Several research have been done to determine the biodegradation potential of heavy metals by either bacteria or fungi isolated from metal contaminated sites because of anthropogenic activities. Research conducted by authors (Singh and Vaishya, 2017) showed the bioremediation potential of consortia developed from municipal wastewater isolates. The consortia were able to reduce lead to 84.33 % when incubated at 37 °C for 72 hours. Bioremediation is a technological process that utilizes living organisms to eradicate or neutralize pollutants from contaminated sites. The mechanism of bioremediation includes reduction, detoxification, degradation, mineralization, or transformation of toxic pollutants to less toxic/hazardous forms (Dukda and Adriano, 1997; Yakasai et al., 2019). Bioremediation is an economical and eco-friendly technique that is less expensive. This process of remediation is achieved by utilizing the right microbe in an appropriate manner with its specific environmental conditions (Singh and Vaishya, 2017; Akhtar et al., 2013b).

Several studies have centered on isolating microorganisms with the potential to tolerate lead and their bioremediation potential as innovative approaches rather than the inefficient conventional methods. The aim of this study is not only to isolate but also to characterize an indigenous microorganism for lead-tolerance, which could be used as an effective tool for the heavy metal remediation.

2. Materials and methods

2.1. Chemicals and equipment

All the chemicals employed in this research work are of the analytical grade. A class II biosafety cupboard was utilized for studies involving microorganisms. The flowchart for the experimental methodology is shown in Fig. 1 below.

2.2. Description and sampling of soil

Soil samples from lead-contaminated areas of Zamfara state, Northwestern Nigeria were collected at a depth of about 10 cm beneath the topsoil, in the month of June, 2018. The sampling sites were (i) Abare, (ii) Bagega, (iii) Dareta, and (iv) Waramu. Samples obtained from Gusau and Kano state were used as control I and control II respectively. A soil auger was used for sampling; the sample was kept in an aseptic container and brought to the laboratory for analysis. All plastic and glassware used in the study were sterilized with 2 mM HNO_3 and rinsed with deionized water before use.

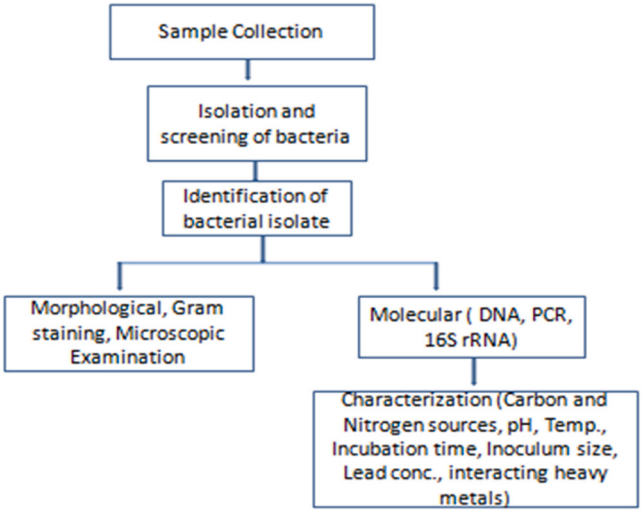

Fig. 1. Flowchart showing the experimental methodology.

2.2.1. Assay of lead levels

The soil samples were estimated for lead content utilizing a calibrated AAS instrument (Black et al., 1983). The sample was first digested by double- acid (HCl and HNO_3 in a ratio of 3:1) extraction to determine the overall content of heavy metals. A crucible containing precisely 1 g of each soil sample was heated in a furnace for two hours at 500 °C. The cooled sample was then put into a conical flask with 20 ml of the acid mixture and heated with a heating mantle until a brown fume turned white. The digested soil was filtered and topped up to 50 ml mark in a sterile container. The samples were further determined for lead level using an AAS machine (Greenberg et al., 1985; Atuanya and Oseghe, 2016).

2.2.2. Isolation and identification of lead resistant bacteria

The collected soil samples were utilized to isolate and identify bacterial strains. A serial dilution of the soil sample was performed by utilizing 5 g of each soil sample in 45 ml of double distilled water separately (Sapale et al., 2015). 100 μL of the serially diluted soil sample (10^{-4}) was spread onto LB Agar plates enriched with 500 mg/L lead nitrate and incubated for 24 hours at 37 °C. Individual distinct colonies were sub-cultured prior to isolation and further incubated at 37 °C for 24 hours. The purified bacterial isolates were further identified based on morphological characteristics and Gram staining test using the manual of ref (Bergey and John, 2000).

2.2.3. Screening of bacterial isolates for lead tolerance

Bacterial tolerance to lead was determined by utilizing lead nitrate at different concentrations (500, 700, 900, 1100, 1300, 1500, and 3000 mg/L), incorporated into LB media separately and incubated at 37 °C over a period of 24 hours. After the necessary incubation, the petri dishes were examined for developed colonies; organisms unable to withstand greater lead concentrations failed to develop, and the isolates that were able to condone as much as 3000 mg/L were chosen for further work.

2.3. Genomic DNA extraction

The screened bacterial cultures that could tolerate 3000 mg/L lead nitrate were used for genomic DNA extraction. This was done in accordance with QIAGEN DNA extraction kit manual. The extracted DNA was utilized for PCR reaction.

2.3.1. Polymerase chain reaction (PCR)

The 16 S rRNA gene was amplified by polymerase chain reaction (PCR), which is achieved using the general-purpose bacterial primers1447-F (AGAGTTGATCCTGGCTCAG) and 1492-R (GGTTACCTTGTTACGACTT) using KapaTaq DNA polymerase. The reaction mixture was prepared based on modified method of (Bergey and John, 2000). Amplification of the desired genes was carried out using standard conditions. The size of the PCR amplification product was estimated using agarose gel electrophoresis. The PCR product was loaded into the precast wells in the gel and a current was applied. Purified PCR products were further amplified unidirectional with 16 S primer using Big DyeTM Terminator Ready Reaction Mix (ABI) and used for sequencing.

2.3.2. Phylogenetic analysis

The sequences obtained were blasted using the NCBI for identification. BIOEDIT version 7.2.5 was used for alignment of the sequences, and blasted on the NCBI website (Hefnawy et al., 2017), further analysis was done by downloading and using all the closely related species. Alignment of sequences was done using the software Mega version 7.0, alongside phylogenetic tree construction using neighbor-joining methods (Birniwa et al., 2022a). Sequences were then submitted to NCBI to receive the accession number.

2.4. Characterization of the lead-tolerant isolates

A selective media-Mineral salt media (MSM) (pH 0 f 7.0) was used to characterize the lead-tolerant bacterium using one factor at a time (OFAT) to define the factors that influence the growth of the bacterium (Ogunnusi and Oyetunyi, 2017). The composition of the media was prepared based on modified method of (Zainith et al., 2019).

2.4.1. Screening of carbon source

MSM was supplemented with four energy sources (glucose, fructose, sucrose, and lactose) separately and incorporated with 500 mg/L lead nitrate. Bacterial culture (100 μL) was separately inoculated in the media and incubated at 37 °C for 24 hours to select the best energy source required to produce lead resistant bacterium. After the required incubation, bacterial production was determined by measuring optical density (OD) at 600 nm. The best carbon sources obtained were also optimized at different concentrations (2.5, 5, 10, 20, 40, and 80 g/L) to determine the optimum concentration of the carbon sources for the isolates (Sapale et al., 2015).

2.4.2. Screening of nitrogen source

Aside from carbon source, nitrogen is also an essential parameter for bacterial growth. Various nitrogen sources were used to determine the best that supports the development of lead tolerant isolates.100 μL of the bacterial culture was inoculated in MSM media supplemented with 500 mg/L lead nitrate alongside the nitrogen sources and incubated at 37 °C for 24 hours. After incubation, the mixtures were estimated for growth by spectrophotometric measurement at 600 nm. The most suitable source of nitrogen was then optimized at different concentrations (1.25–40 g/L) (Sapale et al., 2015).

2.4.3. Screening for optimum pH

The initial pH of the MSM (50 ml) supplemented with 500 mg/L lead nitrate along with the optimum conditions observed varied from 3 to 8. Bacterial aliquot (100 μL) was separately inoculated and incubated at 37 °C over a period of 24 hours to determine the most suitable pH needed for the growth of the lead resistant isolates. The culture was estimated for growth by measuring the optical density at 600 nm. The pH was adjusted using 1 M HCl and 1 M NaOH.

2.4.4. Effect of temperature on lead-tolerant isolates

MSM (50 ml) supplemented with lead nitrate (500 mg/L) alongside the optimum conditions observed above was used for the incubation of bacterial culture (100 μL) at varying temperature between 25 and 50 °C for both isolates. The mixtures were incubated at 37 °C over the period of 24 hours. The bacterial culture was determined for growth by spectrophotometric measurement at an O.D. of 600 nm.

2.4.5. Effect of lead nitrate concentration on the lead-tolerant isolates

The bacterial isolates were examined for their ability to tolerate lead by nurturing them on MSM containing various volumes of lead nitrate (500, 1000, 2000, and 4000 mg/L). The bacterial isolates (100 μL) were inoculated and incubated at pH 7.0 and 37 °C for 24 hours alongside the best conditions observed above. The spectrophotometer was used to estimate the growth of the bacterial cultures at an O.D. of 600 nm.

2.4.6. Effect of incubation time

50 ml of MSM incorporated with 500 mg/L lead nitrate and the two isolates (100 μL each) were incubated separately with optimum conditions observed above, at a temperature of 37 °Cover time (6–96 hours). Growth of the bacterial culture was estimated by measuring their optical densities at 600 nm using a spectrophotometre.

2.4.7. Effect of inoculum size

To identify the ideal inoculums volume for the lead tolerant isolates, the inoculums volume was varied between 25 and 400 μL. The bacterial cultures (100 μL) each was inoculated into the MSM media (50 ml) alongside the optimum conditions observed above and incubated at 37 °C over 48 hours. Bacterial growth was estimated by measuring absorbance at an O.D. of 600 nm (Muhammad et al., 2023; Harun et al., 2023).

2.5. Impact of other interrelated heavy metals

The ability of the isolates to grow amongst co-contaminants at 1 ppm was evaluated (Sapale et al., 2015). The bacterial cell was incubated at 37 °C over time (6, 24, 30, 48, 54, 72, 78 and 96 hrs) in MSM broth incorporated with 1000 mg/L lead nitrate augmented with 1 ppm solution of various metal salts (zinc chloride, copper (ii) chloride, mercury (ii) chloride, manganese (ii) chloride pentahydrate, nickel chloride, selenium chloride, cobalt chloride, copper (ii) chloride, tin chloride, lithium chloride potassium dichromate). Bacterial growth was estimated by measuring absorbance at an O.D. of 600 nm.

2.6. Statistical analysis

GraphPad Instat was used for data analysis. Tukey was used for comparison between groups.

3. Results and discussion

3.1. Detection of soil lead levels

Table 1 shows the soil lead levels of the various sites of sampling.

Table 1
AAS analysis of the soil samples with their respective soil lead levels.

Sampling sites	Soil lead levels (mg/kg)
Abare	738.430 ± 186.781^{a}
Bagega	$641.368 \pm 135.699^{b,c}$
Dareta	522.646 ± 140.288^{d}
Waramu	731.869 ± 40.755^{e}
Gusau (control i)	$960.591 \pm 113.584^{b,d,}$
Kano (control ii)	$225.126 \pm 219.676^{a,c,e,}$
USEPA standard	400

Each value represents the mean ± S.D of the results of five different analyses (n=5). Mean with the same superscripts are significantly different (ANOVA, Tukey's test, $P < 0.05$).

Significantly high soil lead levels ($p<0.05$) were observed in Abare, Bagega, Dareta, Waramu as well as Gusau (control I), above the United States Environmental Protection Agency (USEPA) standard (400 mg/Kg) (Table1). However, 225.126 mg/Kg lead concentration of Kano (Control II) was within the permissible lead levels by USEPA.

Lead is a non-essential heavy metal that is toxic even at low concentrations (Harun et al., 2023). Lead has no biological role and causes irreversible health effects to man (Buba, 2016; Getso et al., 2014). Hence the need to remediate lead rich environment. Result from Table 1 showed high concentration of lead 522.646–738.430 (mg/Kg) in all the test soils of Anka. This, however, could be as a result of the illegal gold ore mining and processing activities occurring in the said places. Research conducted by (Tijjani et al., 2019) showed high lead concentration (1153± 165 mg/kg) in the mining soil of Bagega, which is above the soil lead concentration permissible limit (WHO, 420 ppm). The significantly higher ($p<0.05$) soil lead level seen in Gusau (control I) could be as a result of sampling the soil by the roadside. A study by (OGUTUCU et al., 2021) showed high concentrations of lead from 45 soil samples collected by the roadside above the permissible limit and could be because of accumulation of lead from motor exhaust. Urban cities are more exposed to lead pollution at roadsides due to massive industrialization; this in turn may result in either acute or chronic health effects (OGUTUCU et al., 2021).

Lead leaching into the environment as a result of anthropogenic activities like illegal gold ore mining accumulates into the system. It (lead) mimics and displaces essential metals like calcium and alters its biological activity. Also, it leads to cellular damage by generating reactive oxygen species and eventually oxidative stress (Saez-Nieto et al., 2017). It is difficult to reduce the entrance of heavy metals into the body, as these heavy metals form stable structures in soil, which makes it difficult for conventional methods of remediation to remove them (Wróbel et al., 2023). Hence the need to assess soil lead levels for appropriate remediation strategies.

3.2. Isolation, morphological identification, and screening of lead-tolerant bacteria

Six microorganisms (A, C, C_c, D, E and F) were isolated and screened for lead tolerance and growth, of which isolates A, D, E and F tolerated only concentrations between 500 and 1500 mg/L lead nitrate. Interestingly, isolates C and C_c tolerated up to 3000 mg/L of the heavy metal (Table 2), hence selected for morphological and molecular identification. Morphological identification of the lead-tolerant isolates indicated isolate C to be a gram positive *Paenibacillus* sp., while isolate C_c was morphologically identified as a gram-negative Bacillus sp. *Paenibacillus* sp. was reported to be isolated from environmental sample in Spain (Saez-Nieto et al., 2017). Also, (Felson, 2023) reported Gram-negative *Bacillus* sp. to naturally inhabit soil (Felson, 2023) and can be pathogenic when found in a contaminated environment causing infections that may develop into clinical cases. *Bacillus* sp. has been reported by Wróbel et al., (Wróbel et al., 2023) to have the unique trait of spore-forming under extreme conditions. The unique structure makes it tolerate extreme environmental conditions like high temperature, drought, humidity, and radiation. Zamfara state, Nigeria has been known for extreme weather conditions, which could be a significant figure for existence of *Bacillus* sp. in the environment.

Table 2
Screening for lead tolerance by bacterial isolates over 24 hours of incubation period.

Isolate	500	700	900	1100 (mg/L)	1300	1500	3000
A	+	+	+	-	-	-	-
C	+	+	+	+	+	+	+
C_c	+	+	+	+	+	+	+
D	+	+	+	+	+	+	-
E	+	+	-	-	-	-	-
F	+	-	-	-	-	-	-

Key: + = Growth, - = No growth

3.3. Molecular identification and phylogenetic analysis

The16S rRNA gene sequences of the bacterial isolates were compared with related gene sequences from the Gene Bank database on Blast server available at NCBI. The results obtained showed less than 50 % similarity for isolates C and Cc. Species names are accompanied by the accession numbers of their 16 s rRNA sequences. The numbers at branching points are nodes representing bootstrap values, based on 1000 re-samplings. The analysis involved 25 nucleotide sequences and a total of 878 positions for isolate C, while 26 nucleotide sequences with a total of 937 locations was used for isolate C_c in the final dataset. *Serratiamarcescens* was used as the out group. For this reason, isolate C was conditionally designated as *Paenebacillus* sp. strain BUK_BCH_BTE 3 (MT160418) (see Fig. 2). While *Bacillus* sp. strain BUK_BCH_BTE 4 was the identification for isolate Cc. The accession number for the sequences in the GeneBank is MT160452 (C_c).

Several bacterial strains have been reported to be isolated from heavy metal contaminated sites, thanks to several mechanisms they exert to reduce or tolerate the toxicity of the heavy metals (Henao and Ghneim-Herrera, 2021). Authors (Saeed et al., 2020) reported the ability of bacterial species to resist toxic heavy metals due to their genetically determined systems. Research was conducted in Nigeria in the year 1997 by (Halmi et al., 2016), it revealed some heavy metal tolerant isolates from 8 selected sites in Lagos Lagoon amongst which *Bacillus* sp. was included. To date, several bacterial species have been isolated around the world that could tolerate heavy metals. Interestingly, this research isolated indigenous *Bacillus* sp. and *Paenibacillus* sp. that could withstand as much as 3000 mg/L lead nitrate.

3.4. Optimization of lead tolerant bacteria

3.4.1. Screening of energy (carbon) source

Fig. 3a shows the outcome of the four energy sources required for the growth and effective metabolic activities of isolates (C and Cc). Sucrose was observed as the leading energy source that aids the development of the isolates after incubation at 37 °C over 24 hours with significant difference ($p<0.05$), in the order sucrose>lactose>fructose>glucose. These isolates were shown to grow best at a sucrose concentration between 10 and 20 g/L (Fig. 3b). However, a drastic reduction in bacterial growth was noticed after sucrose concentration of 20 mg/L.

Sucrose is a simple, abundant, and inexpensive carbon source hence might be the reason why the isolates utilized it as the best carbon source for their growth (Fig. 3a). This however does not go in line with the research of (Sapale et al., 2015), where lead resistant *Bacillus* sp. and *Proteus* sp. were reported to utilize glucose as the most preferred energy source. Sucrose was similarly report as the preferred energy source by a molybdenum-reducing bacteria isolated from Egypt (Saeed et al., 2020). The work of (Halmi et al., 2016) reported *Serratia* sp. strain MIE2 to utilize sucrose as the sole energy source for growth on molybdenum reduction. The usage of simple assimilable carbon sources by lead-tolerant isolates indicates that lead reduction is a growth-related process.

Carbon source concentration is a critical factor for bacterial growth. Inhibition of *Bacillus atrophaeus* and *Acinetobacter nectaris* growth due to high concentration of sucrose was reported by (Hestrin et al., 1956). Sucrose inhibits growth by creating an environment of low water activity that interferes with bacterial growth (Anguluri et al., 2022). A study by authors (Harun et al., 2023) reported utilization of low concentration of sucrose by *Bacilluscereus* strain BUK_BCH_BTE2isolated from a Nigerian soil. Bioremediation utilizes simple and inexpensive compounds for decontamination/detoxification processes. This makes the isolates to be competent potential tools that could be utilized in remediating lead polluted environment.

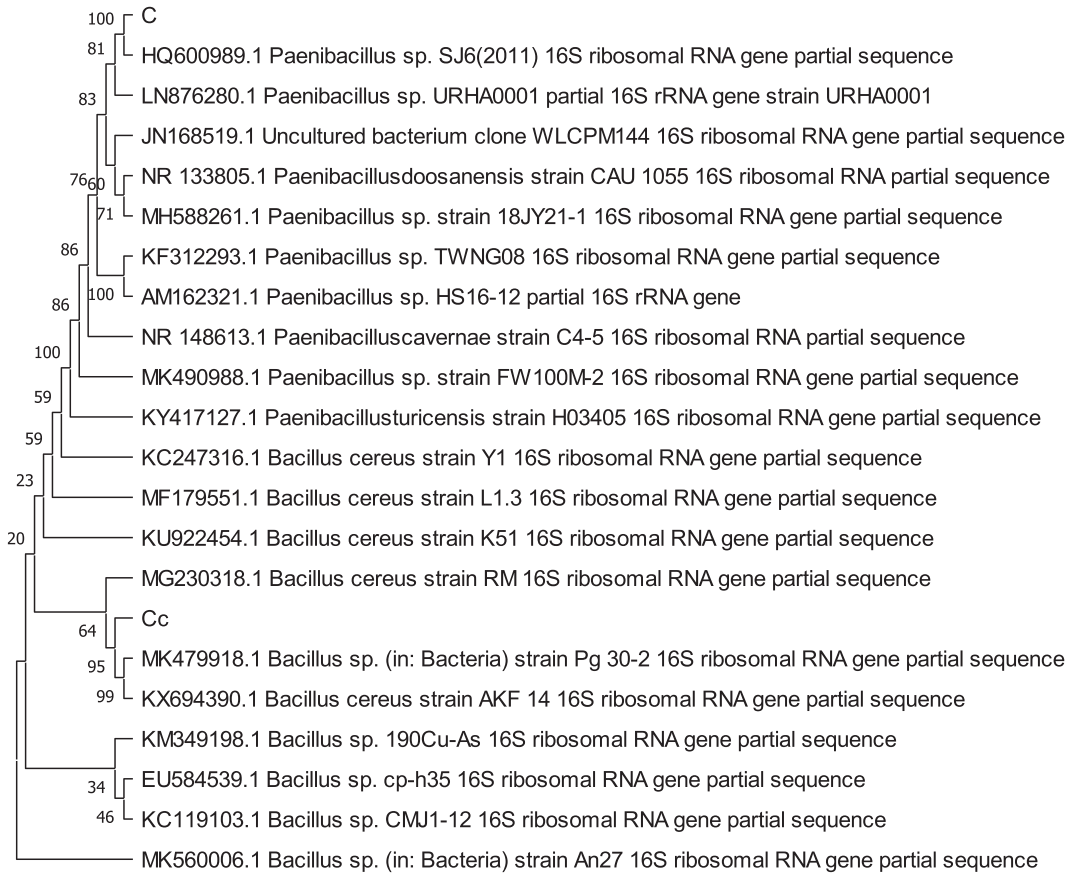

Fig. 2. Cladogram (neighbor-joining method) indicating the genetic relationship between strain C and referenced related microorganisms based on 16 S rRNA sequences.

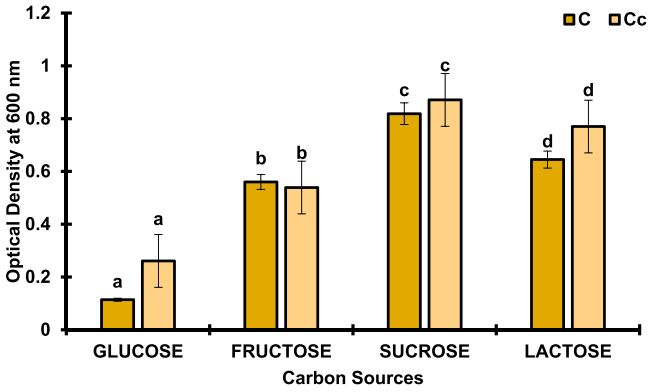

Fig. 3a. Result of carbon sources on the growth of isolates C and Cc after 24 hours of incubation. Data represent mean ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

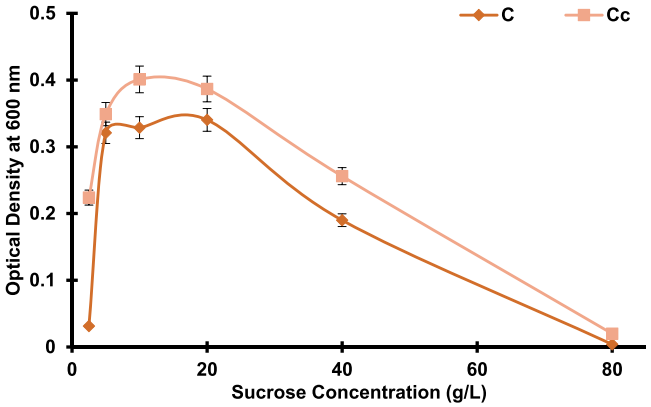

Fig. 3b. Result of sucrose concentrations on the growth of isolate C and Cc after 24 hours of incubation. Data represent means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

3.4.2. *Screening for nitrogen sources*

Fig. 4a and Fig. 4b shows the influence of nitrogen source and nitrogen source concentrations on the development of the lead resistant *Bacillus* sp. and *Paenebacillus* sp. The findings of this study demonstrated that glycine, valine, urea, ammonium chloride and ammonium sulfate were the nitrogen sources that supported the growth of isolates C and Cc, with ammonium sulfate being the most desirable source of nitrogen for *Paenebacillus* sp. and urea the most effective nitrogen growth source for *Bacillus* sp. (Fig. 4a). It could be seen that Isolate C grew best at a value of 5 g/L ammonium sulphate (Fig. 4b), while isolate Cc attained optimum growth at 5 g/L urea. Nitrogen is an essential nutrient which is required for nucleotide synthesis in the cell. It is the most important compound to produce amino acids and aids in protein synthesis (Gillespie, 2018). Most reports on lead tolerant microorganisms did not discuss the utilization of nitrogen (Atuanya and Oseghe, 2016; Sapale et al., 2015; Ogunnusi and Oyetunyi, 2017; Saini et al., 2013; Kalita and Joshi, 2017; Murthy and Bali, 2011). However, the utilization of both ammonium sulphate and urea, where both are cheap nitrogen sources indicated the competency of both isolates for future bioremediation works. Nitrogen sources support bacterial rapid growth and high cell yields (Chai and Adnan, 2018). Fig. 4b shows a drastic decline in bacterial growth at a concentration above 5 g/L, this might be because at lower

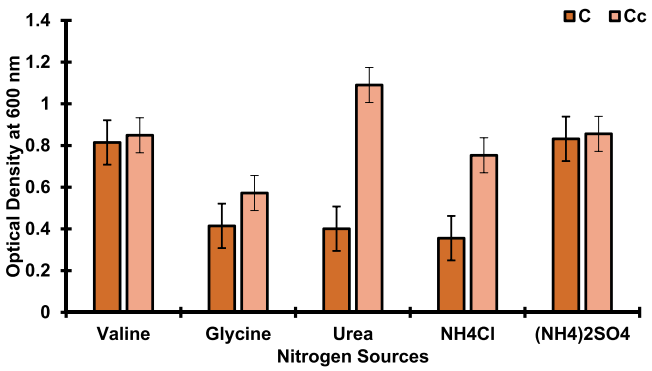

Fig. 4a. Result of nitrogen sources on the growth of isolate C and Cc after 24 hours of incubation. Data represent means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

concentration, reaction rate increases linearly with increase in substrate concentration, while high substrate concentrations inhibit and may even distort the metabolism of microorganism (Harun et al., 2023; Edwards, 1970).

3.4.3. *Screening of initial pH*

To identify the pH that promotes the development of the bacteria at best, impact of initial pH on the growth of lead-tolerant bacteria was assessed (Fig. 5). Growth of both isolates (C and Cc) was more effective at a pH of 7.0 at 37 °C over a period of 24 hours ($p<0.05$). Bacterial production was not observed at pH of 3.0 for organism C, while isolate Cc started growth from pH of 4.0. Optimum growth pH is the most favorable pH for the growth of an organism (Harun et al., 2023). For a favorable reaction, the H^+ concentration of a system must be optimum. The desired pH of 7.0 observed in this study (Fig. 5) is in conjunction with the findings of (Sapale et al., 2015), where lead-tolerant *Bacillus* sp. and *Proteus* sp. isolated from India grew best at a pH of 7.0.This also goes in line with the molybdenum reducing bacteria *Bacillus sonorensis* strain Pharon3 (MK078035) isolated from Egypt, where it grew best at a pH of 7.2, a near neutral pH (Yakasai et al., 2019). However, this study is not in accordance with the research of (Atuanya and Oseghe, 2016) which stated an acidic pH, while (Saini et al., 2013) reported a pH of 5.0, (Huang et al., 2019) reported a pH of 3.0 on molybdenum reduction and (Shamsedini et al., 2015) reported a pH of 11.0 for atrazine degradation. Most bacteria are neutrophiles, they are extremely sensitive to even little changes in the hydrogen ion concentration in their environment, which could be the reason why they favor neutral pH conditions. This may explain why isolates C and Cc had an ideal pH of 7.0. Change in pH affects the rate of biological reactions. Extreme pH levels can disrupt the structure of protein, leading to its inactivation and denaturation. This affects virtually all cellular processes. Low pH in addition can lead to morphological changes in cells, causing a decrease in cell fluidity (Muhammad et al., 2023; Harun et al., 2023). Research conducted by Heidari and Panico (Heidari and Panico, 2020) revealed that at a more acidic condition, biosorption of heavy metals is more effective, consequently resulting to toxicity of the heavy metals to the microbial biomass, thus hindering the growth of the organism. Less growth of both isolates (C and Cc) at acidic condition indicates more biosorption of the heavy metal (Pb) at low pH. A reason why more precipitate was noticed (result not shown) in the incubation bottle. This could be an indication of bioprecipitation mechanism of reaction imbibed by both isolates (Wróbel et al., 2023; Heidari and Panico, 2020). Interestingly, bacteria that is capable of precipitating lead to lead phosphate was isolated and identified as B. *thuringiensis* 016 (Chen et al., 2015). Thus, pH is a critical parameter for binding metals to cell wall of bacteria, while growth is optimal at neutral pH because metals are less soluble at that pH range, hence less toxic to the microbial systems and obviously less biosorption at that pH (Heidari and Panico, 2020; Chen et al., 2015).

3.4.4. *Effect of temperature*

Fig. 6 reveals the influence of different temperatures on the production of isolates C and Cc. The isolated growth was found to attain optimum temperature of 37 °C after 24 hours incubation. A significant decrease in growth was noticed. The optimal temperature of 37 °C for both isolates gotten in this study (Fig. 6) is in line with the research of (Ogunnusi and Oyetunyi, 2017) that reported *Aspergillus niger* isolated from metal scrap dump site of Ibadan Nigeria to grow optimally at 37 °C, (Muhammad et al., 2023) also reported an optimum growth temperature

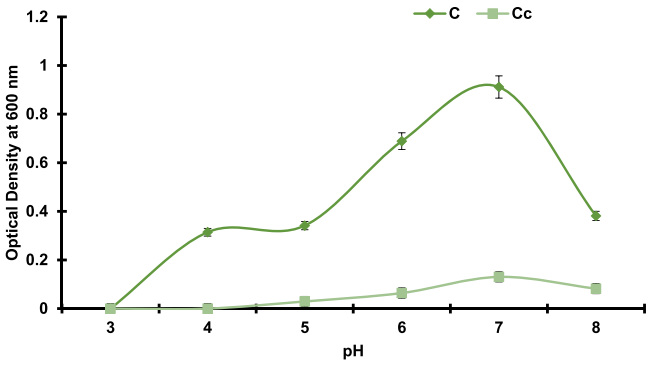

Fig. 5. Effect of initial pH on the growth of isolates C and Cc after 24 hours of incubation. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

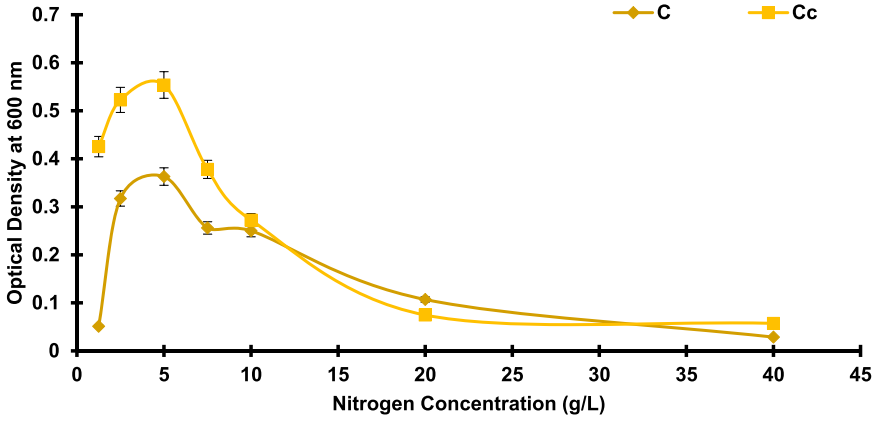

Fig. 4b. Result of nitrogen source concentrations on the growth of isolates C and Cc after 24 hours of incubation. Data represent mean ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

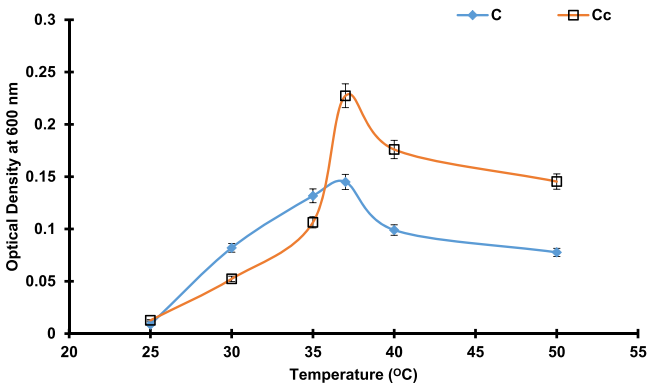

Fig. 6. Effect of temperature on the growth of isolates C and Cc after 24 hours of incubation. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

between 35 and 40 °C. Similar result was reported by (Yakasai, 2017) on molybdenum reduction where *Serratia* sp. strain HMY3 isolated from a Nigerian soil grew optimally at a temperature of 35°C. In contrast to this study, a lower temperature of 25 °C was obtained by author (Halmi et al., 2013) using *Klebsiella oxytoca* strain DRY14. Whereas ref (Birniwa et al., 2023a). and ref (Shukor et al., 2010). reported higher temperatures of 46.1 °C and 40 °C respectively on molybdenum reduction. Temperature has an influence on growth of microbial entities as well as biosorption of heavy by microorganisms. Generally, moderate temperature favors the tolerance capacity of microbes to toxic metals (Birniwa et al., 2023b). Extreme temperature on the other hand, harms the DNA and the membrane structure and lead to protein denaturation (Lawal et al., 2021). Low temperature leads to thickening of cell membrane fluid, hence reducing the mobility of required nutrients into the cell (Birniwa et al., 2022b). Temperature as a critical parameter for heavy metal uptake influences microbial metabolism as well as enzymatic activities mostly at temperature between 30 and 40 °C (Heidari and Panico, 2020). Finally, increase in temperature leads to an increase in metals' solubility and thus, improving accessibility of metals to microbial systems (Heidari and Panico, 2020).

3.4.5. *Effect of lead nitrate concentrations on the lead resistant bacteria*

The lead tolerant isolates (C and Cc) were observed to have the best growth pattern at 1000 mg/L of lead nitrate (Fig. 7). Development of the isolates (C and Cc) rises linearly at 500 mg/L lead nitrate, reaching its peak at 1000 mg/L lead nitrate and significantly declined after 2000 mg/L ($p<0.05$). Perhaps no significant development ($p<0.05$) was seen in both isolates at 4000 mg/L lead nitrate. The continuous boost in growth of both isolates at a value between 500 and 1000 mg/L lead nitrate could denote that low concentration of this toxic metal has no significant toxic effect on the bacterial growth (Fig. 7). Usually, at low concentration of substrate, there will be a significantly linear increase in bacterial growth as well metabolic activities and subsequent increase in heavy metal uptake with increasing substrate concentration. But as lead nitrate concentration increases higher, the reaction becomes saturated with the toxic metal and eventually decreases the metabolic activity of the bacterial isolates (Muhammad et al., 2023; Harun et al., 2023).This study reported maximum tolerable lead nitrate concentration of up to 3000 mg/L (Fig. 7), which deviates from previous works on lead tolerant isolates that could tolerate less concentration of the non-essential inorganic pollutant-lead (Atuanya and Oseghe, 2016; Sapale et al., 2015; Saini et al., 2013; Kalita and Joshi, 2017; Damodaran et al., 2011). Hence, such a lead tolerant isolate could be utilized by making use of their inherent biological mechanisms to detoxify lead, which is a hazardous contaminated, from a lead polluted environment.

3.4.6. *Effect of incubation time*

Time is an essential criterion that may affect bacterial growth. Little incubation time may lead to lower bacterial counts, while bacterial culture left for too long leads to utilization of almost all nutrients needed for the organisms' growth and eventual decline of microbial biosorption process (Heidari and Panico, 2020). This may lead to excretion of toxic substances and eventual death of the entire bacterial population (Harun et al., 2023). The effect of incubation time on *Paenibacillus* sp. and *Bacillus* sp. in MSM is presented in Fig. 8. A continuous rise in bacterial growth was observed from a period of 6 hours to 48 hours where the isolates attained their optimum growth significantly ($p<0.05$). A significant decrease in growth could be noticed after 48 hours of incubation ($p<0.05$). This result is in contrast with the research of Ashrafi et al (Ashrafi et al., 2022). where long-term contamination of *Microbacterium oxydans* strain CM3 and CM7 with Pb (II) increased the growth rate of the organisms. Less biosorption of the heavy metal lead was also reported at long-term contamination using the CM3 and CM7 strains.

3.4.7. *Effect of inoculums size*

The result of this findings depicted in Fig. 9a and Fig. 9b demonstrates a significant linear increase in growth from 25 µL to 100 µL where it attained optimum growth after 96 hours of incubation at 37 °C in both isolates (C and Cc) ($P<0.05$). However, a decline in growth was observed between 100 µL and 400 µL ($p<0.05$). The decrease in bacterial growth (Fig. 9a and Fig. 9b) after exceeding 100 µL inoculums size could be associated with speedy rise in the cell mass utilizing the finite quantity of nutrition, which promotes competition and eventually

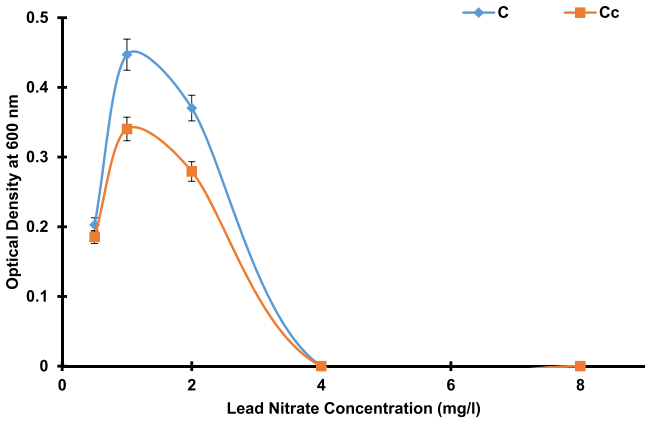

Fig. 7. Result for lead nitrate concentrations on the growth of isolates C and Cc after 24 hours of incubation. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

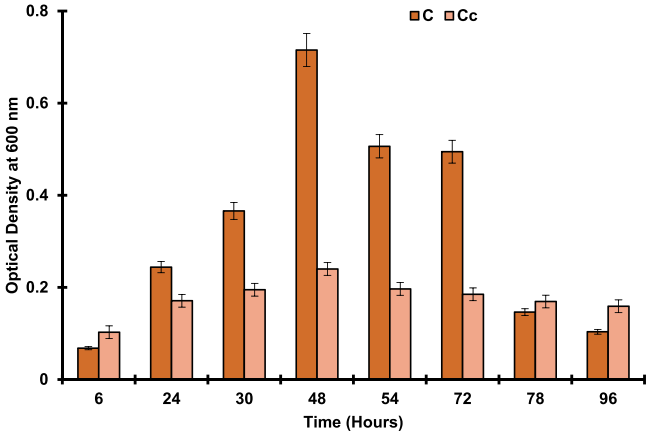

Fig. 8. Incubation time impacts on the growth of isolates C and Cc over time. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

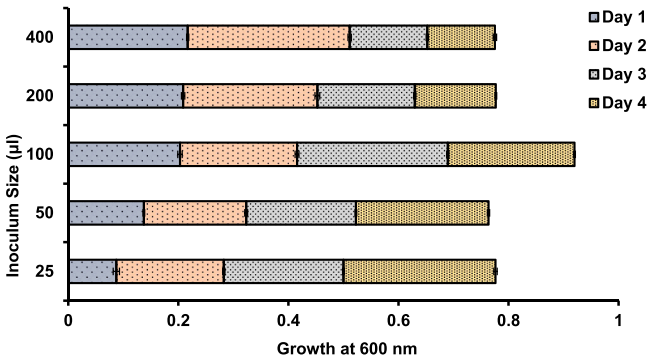

Fig. 9a. Effect of inoculums size on the growth of isolate C after incubation. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

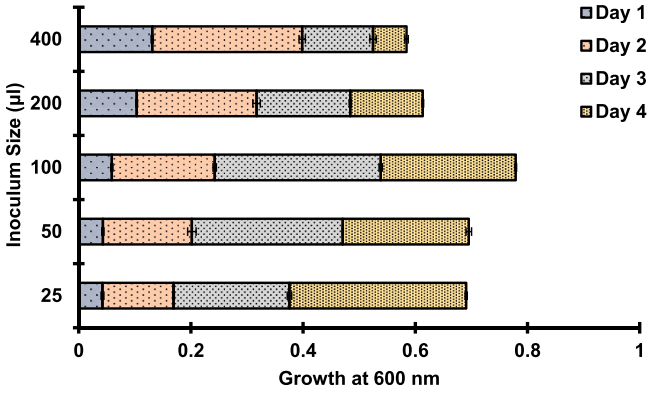

Fig. 9b. Effect of inoculums size on the growth of isolate Cc after incubation. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

results in the death of less capable cells. Inoculums size can enhance or alter bacterial growth (Harun et al., 2023). A study conducted by (Muhammad et al., 2023) contrasts with this present work, where the author observed a high inoculums size of 600 µL by an atrazine-degrading bacteria. In line with this study is the report of (Zhao et al., 2017), where 100 µL was the best inoculums, size observed for atrazine degrading bacteria.

3.4.8. Effect of other interacting heavy metals

The effect of various interacting heavy metals on the growth of lead tolerant isolates C and Cc is shown in Fig. 10. Growth of the isolates (C and Cc) in the existence of 1 ppm other occurring metals have different effects. Cobalt, copper, potassium, nickel, and tin promotes the growth of the isolates, with potassium being the most significant growth influencer ($p<0.05$). Alternatively, mercury has an almost complete inhibitory effect on the growth of both isolates. The order of inhibition was mercury>manganese>selenium>zinc>lithium following 48 hours of incubation at 37 °C.

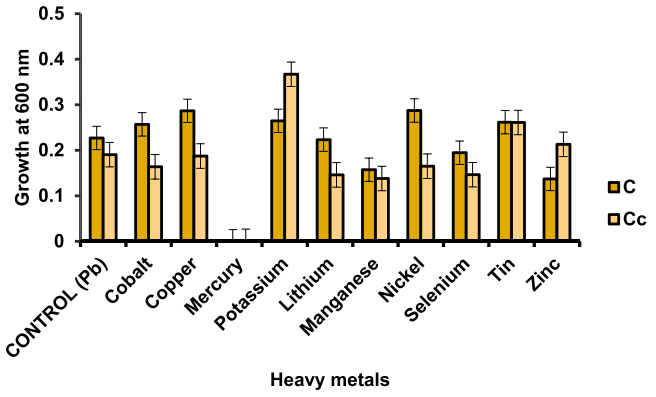

Fig. 10. Effect of other metals (1 ppm) on the growth of isolates C and Cc on MSM media at 37 °C over 48 hours. Data is presented as means ± SD of triplicate determinations (ANOVA, Tukey's test, $P<0.05$).

Heavy metals (HM) often occur as co-contaminants with other compounds in polluted environments. It poses toxic effects to both fauna and flora even at low level of exposure, and this could be related to their high density (compared to water) (Tchounwou et al., 2012). HM has been proven to be the major threat to human health. HM enters the circulation via the gastrointestinal tract, skin or through inhalation, this may lead to membrane and DNA damage and may hinder protein function and enzymatic activity (Abdulmalik et al., 2023). Mercury was found to have a significant impact on delaying the production of the organism. This might be because mercury is highly poisonous, which inhibits growth by decreasing metabolism and lengthening the lag period in the lead-tolerant isolate. This is in line with the findings of (Yakasai et al., 2019), which discovered that, the metals; mercury, copper, and silver impede molybdenum reduction. However, some metals like potassium, selenium, copper, and zinc are essential, needed for normal biological functions, but are needed in minute concentrations, as high concentration pose toxicity and are dangerous. Toxicity of the essential metal can lower energy levels and may damage the functioning of vital organs like the brain, lungs, kidney, and liver (Jaishankar et al., 2014).

Mining is an anthropogenic activity that is of environmental concern, which leads to the exposure of waste materials containing metal-rich sulfides. These inorganic pollutants can bind to nonmetallic elements of cellular macromolecules exerting toxic effects (Fashola et al., 2016). Lead is non-biodegradable and cannot be broken down in the biological system (Harun et al., 2023). Instead, it bioaccumulates in the body and causes biological and physiological complications affecting the cellular organelles, causing DNA damage that may even lead to carcinogenesis (Briffa et al., 2020). Hence, the use of living organisms is the most effective way to remediate the heavy metal polluted environment to have a green domain/territory fit for the survival of both fauna and flora.

4. Conclusion

Bacteria with the ability to condone as much as 3000 mg/L lead nitrate were isolated from lead polluted soil of Zamfara state Nigeria and identified as *Paeniballus* sp. strain BUK-BCH_BTE 3 and Bacillus sp. strain BUK_BCH_BTE 4 with the accession number MT160418 and MT160452 respectively. The best condition for the growth of the isolates were found to be sucrose concentration of 10–20 g/L, 5 g/L ammonium sulphate for the former and 5 g/L urea for the latter. Both isolates developed excellently at a pH of 7.0 over 48 hours of incubation with the most appropriate lead nitrate concentration of 1000 mg/L. The mining region has a substantial concentration of lead based on atomic absorption spectroscopy (AAS). This finding has provided basic information on the characteristics and growth requirement of lead-tolerant isolates obtained from the gold mine polluted soil of Zamfara that could be exploited for the remediation of lead contaminated sites.

CRediT authorship contribution statement

Hafeez Muhammad Yakasai: Writing – review & editing, Methodology, Conceptualization, Resources, Supervision. **Fatima Abdullahi Harun:** Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Shehu Usman:** Resources, Data curation. **Ahmad Hussaini Jagaba:** Writing – review & editing, Resources, Conceptualization.

Hassan Abba Umar: Data curation. **Mohd Yunus Shukor:** Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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