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Hydrolases secreting, heavy metal resistant halophilic bacteria isolated from metal dumpsites

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Abstract. *Mgbodile CF, Otutu U, Onuoha S, Eze U, Ugwuoji T, Nnabuife O, Nwagu TNT. 2022. Hydrolases secreting, heavy metal-resistant halophilic bacteria isolated from metal dumpsites. Asian J Trop Biotechnol 19: 11-19.* Microorganisms with the potential to accumulate heavy metals are currently under investigation for application in environmental detoxification. The current study aimed to isolate heavy metal tolerant bacteria from metal dump sites. The soil samples were sourced from two metal dumpsites in Eastern Nigeria. The pH and metal content of the samples were evaluated. Microorganisms were screened for tolerance of lead, copper, silver and chromium. Isolated strains were characterized and identified by molecular techniques. All isolates grew in 1000 ppm Pb, Cu and Cr. The optimal temperature for their growth was 37°C. While isolates B and C grew optimally at 12.5% NaCl, the growth rate increased for isolate A (i.e., 7.5%>12.5%>15%). The three isolates produced extracellular protease, and inulinase isolates B and C produced amylase, while isolating A produced xylanase. Isolate A, B, and C were identified as *Pseudomonas asiatica*, *Sphingobacterium caeni*, and *Burkholderia cenocepacia*. The bacteria were resistant to a wide range of antibiotics, including ampicillin (30 mcg/disc), ceporex (10 mcg/disc), and streptomycin (30 mcg/disc). These results indicate that the bacterial strains have the potential as sources of inoculants for the bioremediation of heavy metal contaminated environments and application in various industrial processes where metal-resistant organisms and their hydrolases are required.

Keywords: *Burkholderia* sp., environmental remediation, heavy metal tolerance, *Pseudomonas* sp., *Sphingobacterium* sp.

INTRODUCTION

Heavy metal contamination is a major environmental problem due to its toxicity (Juhaeti et al. 2004; Gurave et al. 2015; Irawati et al. 2021). Urbanization and industrialization have resulted in several activities that interact with or utilize various heavy metals in one form or another; a typical example is mining (Gautam et al. 2016; Irawati et al. 2017). As a result, high concentrations of heavy metals, including silver, copper, cadmium, zinc, iron, manganese, nickel, and lead, pollute the environment. The current widespread contamination of the air, surface waters, ground waters, sediments, and soil with heavy metals constitute a serious threat to all living organisms; since most metals are highly toxic and not easily degraded, unlike organic molecules, they indefinitely persist in the environment (Zulkali et al. 2006). Cadmium is a non-essential metal, not involved in any known biological process. However, it is fast accumulating in our environment due to its wide application in many industrial processes, including the pulp and paper industry, the production of copper alloys, electroplating, alkaline batteries, fertilizer, metal mining, and refining. Unfortunately, low cadmium concentrations (about 0.001-0.1 mg/L) are harmful to organisms, and accumulation causes much damage to plants leading to poor productivity (Chellaiah 2018). The environmental stress caused by these heavy metals generally decreases soil bacterial diversity and activity, leading to a reduction in the total microbial biomass, reduced numbers of specific populations, and a shift in the microbial community (Zulkali et al. 2006)

Cleaning metal-contaminated sites are required to avoid environmental degradation and preserve human health. Standard methods for environmental clean-up involve physicochemical methods, including electrodialysis, ion exchange, ultrafiltration, and precipitation (Hashim et al. 2011). Owing to the high cost, low efficacy, and environmentally unfriendly nature of most of these methods, alternative techniques are currently being researched and adopted (Wang and Chen et al. 2009). The application of bacterial biomass in environmental clean-up has received great attention in recent years, especially due to its cost-effectiveness and eco-friendly nature (Wang and Chen 2009). The response of the soil microbial population to heavy metal contamination provides a relevant model for environmental studies. Different microorganisms display various reactions to toxic metals that confer them with a range of metal tolerance (Chihomvu et al. 2014). Many studies have shown that these heavy metals affect the microbial population differently, including morphology, growth, density, and biochemical activities (Kathiravan et al. 2011; Abd et al. 2013). Microbial response to toxic metals depends on the microbial strain and the type of metal implicated (Mohamed and Abor-Amer 2012). Environments heavily contaminated with heavy metals harbor organisms that can deal with pollution, including

prokaryotes and eukaryotes. The metal resistance and ability to convert the toxic metals to less harmful states make them potentially useful in bioremediation and other industrial applications (Gurave et al. 2015).

This study aimed to isolate and characterize bacterial isolates from soil from heavy metal dump sites and evaluate their potential for heavy metal resistance, sugar and salt tolerance, and enzymatic activities.

MATERIALS AND METHODS

Sample collection

Soil samples from the surface layer (0-20 cm) were collected from two metal dumpsites in Enugu and Anambra state, both in the South-Eastern part of Nigeria. The soil samples were placed into labeled plastic polyethene bags, quickly transported to the laboratory for microbiological analysis and stored at 4ºC till further use. A small portion of the sample was pulverized with a pestle in a sterile mortar, air-dried and sieved with a 2-mm metal screen, and then kept aside for physical and chemical analysis (Jiang et al. 2017).

Determination of soil pH

According to Aka and Babalola (2017), the soil samples were analyzed for pH. First, the soil sample (5 g) was suspended in 12.5 mL of distilled water and allowed to stand for 30 min. Then, pH was recorded using a calibrated glass electrode pH meter (Apera Instruments, LLC).

Total metal (Pb, Cd, and Cu) concentration in soil

To determine the metals present in the soil samples, the samples were first digested with a microwave digester per the standard method by Aka and Babalola (2017). Afterward, the samples' volume was adjusted to 50 ml each with distilled water, and the concentrations of Pb, Cd, and Cu in the sample were measured using the Atomic Absorption Spectrophotometer, AA-7000 (Shimadzu Scientific Instruments).

Isolation and screening for heavy metal-resistant bacteria

Soil sample (1 g) was suspended in an enrichment medium comprising 10 mL of sterilized peptone water containing 500 ppm metal salt (chloride of Cd, Pb, Hg, or Cu) and agitated continuously at 24+2ºC for 18 h (Singh et al. 2010). After 18 h, 0.1 mL of each incubated sample was placed on Luria-Bertani (LB) agar plates by the spread plate method. The plates were incubated at 24+2ºC for 2-3 days and observed for visible colonies.

In order to isolate strains tolerant to high concentrations of the heavy metals, distinct colonies were subcultured (streak method) onto LB agar plates containing increasing concentrations of the heavy metal salts (500-1000 ppm). Isolates displaying the greatest resistance to the metals were selected for further studies.

Determination of the cellular and biochemical properties of the isolates

The colonies grown on LB plates were analyzed morphologically and then Gram-stained to observe under a light microscope using oil immersion at 100 x to 400x. Biochemical tests were carried out, including sugar utilization, urease and indole production, nitrate reduction, catalase and oxidase detection tests (Khosro et al. 2013).

Temperature tolerance of the heavy metal-resistant bacterial strains

The isolates (20 µL of 12-hour culture) were inoculated into 20 mL of sterilized LB broth containing 500 ppm of Pb, Cd, or Cu chloride and incubated at different temperatures (10ºC, 25ºC, 37ºC, and 40ºC) for 48 h to determine the effect of temperature on the bacterial growth. After incubation, each sample's optical density (OD) was measured at 600 nm using a UV spectrophotometer.

Glucose tolerance of the heavy metal-resistant bacterial strains

The glucose tolerance of the bacterial isolates was determined according to Aka and Babalola (2017). LB broth (20 mL) containing increasing concentrations of glucose (8%, 12%, 16%, and 24%) were inoculated with 100 µL (OD = 0.5) of 12-hour bacterial cultures and incubated at 37ºC for 24 h. The optical density was determined at 600 nm using a UV spectrophotometer.

Screening for the ability to produce selected enzymes

The microbial isolates were screened to produce hydrolytic enzymes by using a modification of the method by Veras et al. (2018). Agar plates containing casein, starch, cellulose, xylan, Tween 80 and inulin were used to screen for protease, amylase, cellulase, xylanase, lipase and inulinase, respectively. First, a loopful of the organism was streaked on the agar plate and incubated for 48-72 h. Afterward, the plates were flooded with 0.2 g/L potassium iodide for 5 min and observed for the presence of clear zones around bacteria colonies.

Determination of antibiotic resistance

According to Kirby Bauer's methods, the antibiotic resistance of our isolates was determined by a susceptibility test (Bauer et al. 1966). The isolates were enriched in nutrient broth for 24 h and swabbed aseptically onto freshly prepared Muller Hinton agar plates (Neethu et al. 2015). Standard antibiotic-impregnated discs were then placed on the plates and incubated for 24h at 37ºC. The diameter of the inhibition zone was measured, and results were recorded in terms of Resistant (R), Susceptible (S), or Intermediate (I), using the standard antibiotic disc chart.

Molecular identification and phylogenetic analysis

The DNA extraction of the bacterial isolates was performed using the ZR Bacterial DNA Miniprep Kit. The polymerase chain reaction (PCR) was performed on the extracted DNA samples using universal bacterial primers 27F (5′ AGA GTT TGA TCM TGGCTC AG 3′) and 1525R (5′ AAG GAG GTG WTC CAR CCG CA 3′)

(Chouari et al. 2005). The PCR cycle started with an initial denaturation step at 94ºC for 5 min, followed by 36 cycles of denaturation at 94ºC for 30 sec. Annealing was at 55ºC for 30 sec and elongation at 72ºC for 45 sec, followed by a final elongation step at 72ºC for 7 min, and cooling to 4ºC. DNA amplification was evaluated by electrophoresis of 10 µL of each PCR product in 1% (w/v) agarose gel as described by Abor-Amer et al. (2014). The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems, while the sequencing kit used was the Big Dye terminator v3.1 cycle sequencing kit. The genetic analysis was conducted using the Bio-Edit and MEGA 6 software. Bioinformatics analysis of the sequencing results was performed using the blastn algorithm (Altschul et al. 1997), at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis was done using the neighbor-joining method in the MEGA-X software (Kumar et al. 2018). The Neighbor-Joining method was applied to determine the evolutionary history and the Kimura 2-parameter method for the distances (Kimura 1980).

RESULTS AND DISCUSSION

Determination of soil pH and total Pb, Cd, and Cu concentration

The soil pH of samples was recorded as 8.2 and 6.7, collected from Abagana and Enugu, respectively. The Atomic Absorption Spectrophotometric (AAS) analysis of the soil samples revealed that the order of concentration of Cu, Pb, and Cd was 6.42, 3.00, and 0.12 ppm, respectively, for the Abagana soil sample, and 0.50, 0.39 and 0.09 ppm, respectively for Enugu soil sample (Figure 1). It was observed that the Abagana soil sample had relatively higher levels of heavy metals than the Enugu soil sample. While Cu was the highest contaminating metal (among those evaluated) in the Abagana soil sample, Pb was the highest contaminating metal in the Enugu sample. Heavy metal contamination in soils is one of the world's major environmental problems, posing significant risks to the ecosystems and the general public (Sheng et al. 2008). Several environments around us are battling heavy contamination, and environmental pollution is fast spreading worldwide as industrialization increases (Gautam et al. 2016). Copper, chromium, cadmium, and lead are the most commonly implicated in these heavy metals (Mendez et al. 2008). Though traces of heavy metals serve as essential co-factors to some enzymatic reactions, at high concentrations, these metals may become toxic to living organisms due to the inhibition of metabolic reactions (Hussein et al. 2004).

Screening for heavy metal tolerant microorganisms

Three isolates were obtained based on the ability to grow on 1000 pm of salts containing Cu^{2+}, Pb^{2+}, and Cd^{2+}. One isolate (Isolate A) was obtained from the Abagana soil sample, while two isolates (Isolate B and Isolate C) were from the Enugu soil samples. The pH of the soil samples varied from slightly acidic to slightly alkaline, depending on the source. The soil pH is an important parameter that strongly affects the behavior of metal ions present in a terrestrial environment. It also affects the soil solubility and ability to form chelates with other soil constituents (Aka and Babalola 2017). All the isolates could not grow in the presence of Hg^{2+} (result not shown). Heavy metal pollution of soil changes its microbial communities (Pacwa-Plociniczak et al. 2018). The metal tolerance of the isolated bacteria was in the order of Cd>Pb> Cu>Hg, Pb> Cd>Cu>Hg, and Pb>Cu>Cd> Hg for isolates A, B, and C, respectively. Table 1 shows the result of the isolates' growth rate under different concentrations (500, 600, and 1000 ppm) of the heavy metal salts (Cu, Pb, and Cd). Very good growth was recorded for isolates A when grown on 500 ppm of all the metal salts tested. Though very good growth was observed when isolate C was grown on 500 ppm Pb^{2+} and Cu^{2+}, the same was not the case on Cd^{2+}. Isolates A, B, and C had good growth on 1000 ppm Pb^{2+} but were inhibited by 1000 ppm Cu^{2+}. Interestingly, isolate A had very good growth on 1000 ppm Cd^{2+}, while isolates B and C had poor growth.

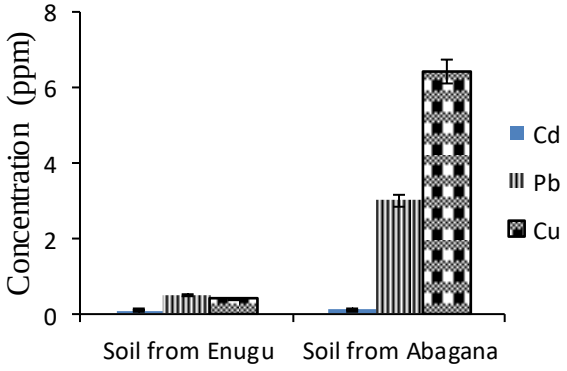

Figure 1. The concentration of heavy metals in the soil sample

Table 1. The growth rate of the heavy metal resistant bacteria in varying concentrations of the metal salts

Isolate	Conc. (ppm)								
	Pb^{2+}			Cu^{2+}			Cd^{2+}		
	500	600	1000	500	600	1000	500	600	1000
A	+++	++	++	+++	++	+	+++	+++	+++
B	++	++	++	+++	++	+	++	++	+
C	+++	+++	++	+++	++	+	++	++	+

Note: +++: very good, ++: good, + poor, - : no growth

When the isolates were subjected to increasing concentrations of heavy metal, it was discovered that the time required for colony formation was longer, suggesting that the organisms had to constantly adjust their cellular environments to accommodate the heavy metal stress. Microbial colonies' sizes also varied; for instance, in a lower concentration, such as 500 ppm, the colonies appeared robust and profuse, while in higher concentration (1000 ppm), the colony sizes were much smaller. The isolates thrived in 500 ppm of Pb^{2+}, which may be due to the high concentration of Pb^{2+} in the soil samples. High amounts of heavy metals in the soil confer resistance to the soil microbial population (Mohamed and Abor-Amer 2012). The Pb concentration for Anambra and Enugu soil was 3.0 ppm and 0.50 ppm, respectively. Though lead is not a micro-nutrient, higher concentrations (600 and 1000 ppm) did not significantly inhibit the growth of the bacterial strains, although the colony sizes were slightly reduced. A low concentration of Cu^{2+} (500 ppm) strongly promoted the bacterial strains' growth, which could be attributed to Cu^{2+} being a micro-nutrient required by living organisms in minute concentrations (Gurave et al. 2015). Another responsible factor may be the concentration of Cu^{2+} in the soil samples (a concentration as high as 6.40 ppm for Anambra soil and 0.39 for Enugu soil). It was, therefore, not surprising that the isolates could thrive in the copper-containing medium (Raja et al. 2009). However, with increasing Cu^{2+} concentrations, a decline in bacteria growth was observed; at 1000 ppm, growth was very poor. Though a micro-element for some metabolic activities, at high concentration, it could play inhibitory roles to organisms that previously promoted growth in minute concentrations (Abd et al. 2013). Although the soil samples did not contain higher cadmium concentrations than copper or lead, the isolates were relatively tolerant to the Cd^{2+} even at 1000 ppm. Pacwa-Plociniczak et al. (2018) isolated a Cd-resistant *Sphingomonas* sp. from a long-term heavy metal polluted soil; and concluded from their observations that Cd-resistant bacteria are common in long-term polluted soils. Of particular interest is *Pseudomonas asiatica*, which displayed a notable growth trend in cadmium supplemented media maintain a relatively same density of growth at all the concentrations investigated. Thus *P. asiatica* strain A could be said to be 'cadmium loving' loosely speaking. Ahirwar et al. (2016) isolated a Cd^{2+} and Pb^{2+} resistant *Pseudomonas spp*. among other isolates from an industrial soil in Central India. Similar to our work, Hg was the most toxic of the metals investigated. Ahirwar et al. (2016) attributed the high Pb^{2+}/Cd^{2+} resistance of isolated Pseudomonas strain to the high concentration of these metals in the soil from which these strains were isolated.

Characterization of the isolates

The physicochemical characteristics of the selected isolates are shown in Table 2. The isolates showed a similar form (circular), elevation (convex), and margin (entire) but different colors when growing on the agar containing the different metals. The isolates were Gram-negative, in line with findings that Gram-negative bacteria dominate metal-contaminated soil (Frostgard et al. 1993; Sulowicz et al. 2011; Pacwa-Plociniczak et al. 2018). They were catalase-positive and negative for the indole test and methyl red. Isolates A, B, and C were able to utilize glucose and sucrose but unable to utilize dulcitol. While isolate C showed weak growth in mannose and lactose, isolates A and B could not utilize these sugars.

The isolates' ability to secrete industrially relevant enzymes such as protease, amylase, cellulase, xylanase, and inulinase was evaluated. The enzyme assay result showed the bacterial strains varying abilities to secrete extracellular hydrolases when growing on a solid medium. While all the isolates could secrete extracellular proteases and inulinase, only B and C could synthesize amylase and cellulase. Isolates A and C were xylanase producers, while B was negative for xylanase production (Table 2).

The effect of temperature on the growth of the isolates on different is shown in Figure 2. All the isolates grew poorly at 10ºC irrespective of the metal present, except for isolate B grown in a medium containing Pb^{2+}. Optimum growth rates for all our isolates were generally recorded between 25-37ºC, regardless of the heavy metal present. A drastic drop in growth occurred when the temperature increased to 45ºC. There was no remarkable difference when isolate B incubated in a medium containing Pb^{2+} was grown over the temperatures of 10, 25, and 37ºC. There was a rapid decline in growth following a temperature increase to 45ºC. Growth characteristics, metabolism, and the microorganisms' cell membrane are altered due to temperature variations (Kathiravan et al. 2011). The result shows that the isolated bacterial strains were mesophilic, which aligns with the sample source's environmental temperature and climatic condition.

Considering the presence of varying solutes in the environment and their effects on microbial growth and metabolite production, we investigated the effect of low to a high concentrations of salt (NaCl) and glucose on the heavy metal-resistant strains. The isolates exhibited varying levels of NaCl tolerance. Isolate A grew with increasing concentration of NaCl (10% > 12.5% > 15.0%). Isolates B and C grew optimally in the presence of 12.5% NaCl concentration. A further increase in NaCl concentration to 15% led to a decrease in microbial growth (Figure 3). As seen in Figure 4, all the isolates grew at glucose concentrations in the range of 8-24 %; however, the highest growth at 8% glucose concentration was observed for isolates B and C. As expected, the varying concentration of these agents influenced the bacterial growth. Isolate A grew exponentially in the presence of 10-15% salt concentration, indicating its moderate or extreme halophile nature (Singh et al. 2019; Irwin 2020).

Molecular identification

The size of the fragments produced from the PCR amplification of the 16S rRNA gene of each bacterium was determined. The variable sized DNA fragments of the bacterial isolates can be seen in Figure 4. The PCR products were sequenced and matched to the available sequences in the NCBI database. Table 3 indicates that isolates A was *P. asiatica* strain A (NCBI Accession

number MW304002). Isolate B was identified as *Sphingobacterium caeni* strain B (NCBI accession number MW304003), while isolate C was identified as *Burkholderia cenocepacia* strain C (NCBI accession number MW304004). The evolutionary history of bacterial strains was inferred using the Neighbor-Joining method. Figure 5 shows the phylogenetic relatedness of *P. asiatica* strain A, *S. caeni* strain B and *B. cenocepacia* strain C to other closely related strains. It is established that the soil harbors varying species of *Pseudomonas* (Noura et al. 2009), and *Burkholderia* (Hall et al. 2015). *Sphingobacterium* species have been isolated from the soil and activated sludge (Sun et al. 2013; Feng et al. 2014; Cheng et al. 2019). There are available reports of moderately halophilic *Pseudomonas* species isolated from saline and hypersaline environments such as brined foods and water bodies (Korcan et al. 2015; Patel and Saraf 2015). However, *P. asiatica* is a novel species isolated from a stool sample from a hospitalized patient in Japan (Tohya et al. 2019). More recently, *P. asiatica* C1 capable of synthesizing coenzyme B12 while growing aerobically on glucose was isolated from activated sludge in Korea's waste treatment plant (Nguyen et al. 2021). This was the first report to isolate a heavy metal tolerant halophilic *P. asiatica* from the soil. Potential applications of halophiles include producing compatible solutes, biopolymers (polyhydroxyalkanoates and polysaccharides) and enzymes. Halophiles are also employed in biodegradation and remediation, enhanced oil recovery, cancer detection, and drug screening (Waditee-Sirisattha et al. 2016). Feng et al. (2014) reported that *Sphingobacterium paludis* sp. nov and *S. caeni* sp. nov. did not grow in NaCl concentrations above 5% (w/v). In comparison, the *S. caeni* strain A isolated in the current study grew in 15% salt concentration while the optimum concentration required for growth was 12.5% (w/v). Several *Burkholderia* species are halotolerant and help ameliorate plant salt stress (Arora et al. 2020). Recently reported cadmium, nickel, and mercury tolerant *B. cenocepacia* isolated from plant environments (Torres et al. 2019). *Burkholderia* species are mainly endophytes with heavy metal tolerant abilities used in plant growth promotion and environmental remediation (Jiang et al. 2008; Liu et al. 2019). Inoculation of soil with heavy metal-resistant bacteria enhances the expression of metal stress-related genes in plants with a consequent increase in plant growth and yield (Liu et al. 2018).

Table 2. Cultural, morphological and biochemical properties of the heavy metal-resistant bacterial isolates

Characteristics	Isolates		
	A	B	C
Morphology			
Form	Circular	Circular	Circular
Elevation	Convex	Convex	Convex
Margin	Entire	Entire	Entire
Colony colour when grown on			
Pb^{2+}	Brown	Silvery	Light
Cd^{2+}	Creamy	Whitish	Silvery white
Cu^{2+}	Whitish	Yellowish	Yellowish
Biochemical characteristics			
Gram stain	-	-	-
Catalase	+	+	+
Indole	-	-	-
Nitrate reduction	+	-	+
Methyl red	-	-	-
Citrate	+	+	-
Utilization of carbohydrate			
Glucose	+	+	+
Sucrose	+	+weak	+
Dulcitol	-	-	-
Mannose	-	-	+weak
Lactose	-	-	+weak
Production of some hydrolases			
Protease	+	+	+
Amylase	-	+	++
Cellulase	-	+	+
Xylanase	+	-	+
Inulinase	+	+	+

Note: ++ : very good; + : good; +weak : poor; - : negative

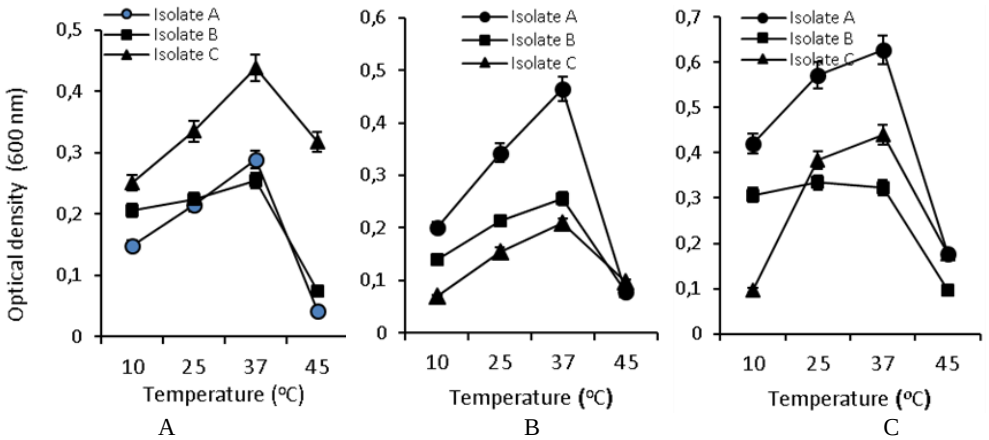

Figure 2. Growth of the isolate at varying temperatures in the presence of (A) Cu^{2+}, (B) Cd^{2+}, and (C) Pb^{2+} salts. Luria Bertani (LB) broth was supplemented with 500 ppm of the salt

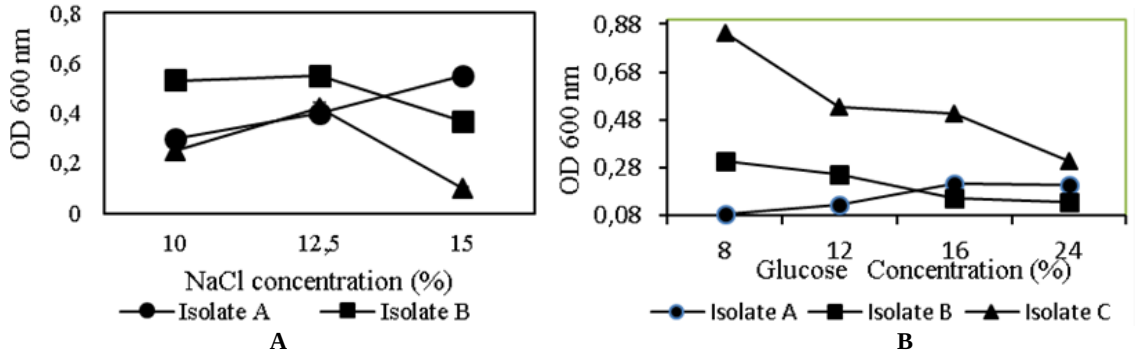

Figure 3. Growth of the metal-resistant bacteria under different NaCl (A) and glucose (B) concentrations

Table 3. Bacterial strains identified by 16s rRNA sequencing

Isolate	Phylum	Nearest phylogenetic neighbour (Accession no.)	Affiliation (Accession no.)	Similarity (%)
A	Proteobacteria	*Pseudomonas asiatica* strain CWC NVN (MK836042)	*Pseudomonas asiatica* strain A (MW304002)	100.0
B	Actinobacteria	*Sphingobacterium caeni* strain LH6 (MG786758)	*Sphingobacterium caeni* strain B (MW304003)	94.0
C	Proteobacteria	*Burkholderia cenocepacia* strain IST439 (LR798194)	*Burkholderia cenocepacia* strain C **(**MW304004)	97.4

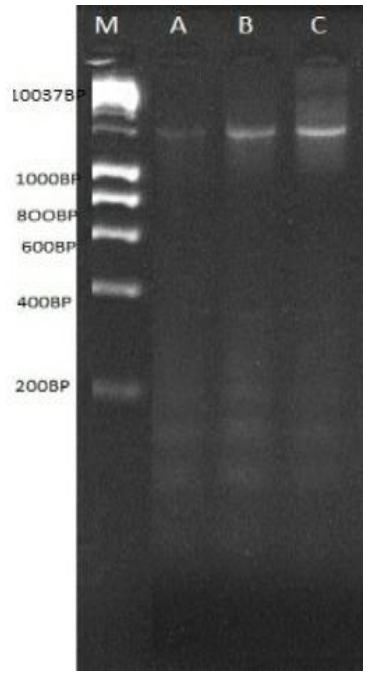

Figure 4. The agarose gel shows bands of the DNA fragments. Lane M represents the molecular ladder, while lanes A, B, and C represent the bands formed by the DNA of the isolates. These fall between 1000-10037 base pairs

Recall that the isolated bacterial strains produced some industrially relevant enzymes. Only 2% of the microbial population has been successfully investigated as enzyme sources (Demain 2000). The ability of soil microorganisms to synthesize enzymes aid in the biodegradation of environmental polymers and pollutants in the soil (Sun et al. 2014). A microorganism's ability to produce specific enzymes facilitates its heavy metal resistance ability (Thavamani et al. 2015). Cellulase is a cell wall degrading enzyme, and the extracellular secretion helps facilitate plant colonization by endophytes (Verma et al. 2001; Ma et al. 2015). Enzyme activities also assist in these organisms' heavy metal resistance ability and subsequently enhance the detoxification of heavy metals in contaminated soils (Thavamani et al. 2015). *Sphingobacterium athyrii* from decaying fern, produced both cellulase and xylanase (Cheng et al. 2019); however, *S. caeni* strain B did not secrete xylanase. Of special interest are the enzymes produced by the halophilic *P. asiatica* strain A. Halophilic bacteria are important sources of novel hydrolytic enzymes and are often extremozymes (Moreno et al. 2013; Munawar and Engel 2013). Typically, extremozymes tolerate extreme conditions, e.g., high salt concentrations, high temperature, and organic solvents. The *P. asiatica* is a recently proposed species of *Pseudomonas*, which before this study has only been isolated in Japan and Myanmar (Tohya et al. 2019). Our work is the first account of a halophilic metal-resistant strain. Both halophilic/halotolerant, osmophilic and enzyme-producing render our isolates potentially useful in a wide range of biological processes.

Based on the current findings, the isolated bacterial strains can serve as candidates for bioremediation, biodegradation and plant growth in metal-polluted soils or saline environments. However, the extent of their capabilities can only be determined following well-designed detailed studies with the respective bacterial strains. In addition, there is also a need to study the capacity of these strains to produce halotolerant or halophilic hydrolases, which are useful for various industrial processes.

Bacterial antibiotic resistance

All three bacterial strains were resistant to ampicillin and ceporex (Table 4). Notably, the *B. cenocepacia* exhibited resistance against all ten antibiotics tested, while *S. caeni* was the most susceptible. Burkholderia species are significantly antibiotic resistant (Rhodes and Schweizer 2016). The *P. asiatica* and *S. caeni* strains were susceptible to tarivid, reflacine, augmentin, and ciproflox. Multiple antibiotic resistance in bacterial strains may be related to the soil heavy metals, the isolate's genetic makeup, and the factors influencing gene transfer between bacteria (Rhodes and Schweizer 2016; Sinegani and Younessi 2017). Only *P. asiatica* was susceptible to streptomycin; meanwhile, it showed intermediate activity to nalidixic acid, gentamycin, and septrin. The antibiotic resistance was further represented as single-resistance, co-resistance, and multiple resistance to capture the resistance to one, two or more antibiotics, respectively (Figure 6). It was hypothesized that exposing bacteria to heavy metal increases antibiotic tolerance (Sharma et al. 2000; Verma et al. 2001; Samanta et al. 2012).

Table 4. Antibiotic sensitivity of the heavy metal-resistant bacterial isolates

Drug (mcg)	*P. asiatica*	*S. caeni*	*B. cenocepacia*
Ampicillin (30)	R	R	R
Ceporex (10)	R	R	R
Tarivid (10)	S	S	R
Nalidixic acid (30)	I	S	R
Reflacine (10)	S	S	R
Gentamycin (10)	I	S	R
Augmentin (30)	S	S	R
Ciproflox (10)	S	S	R
Streptomycin (30)	S	R	R
Septrin (30)	I	S	R

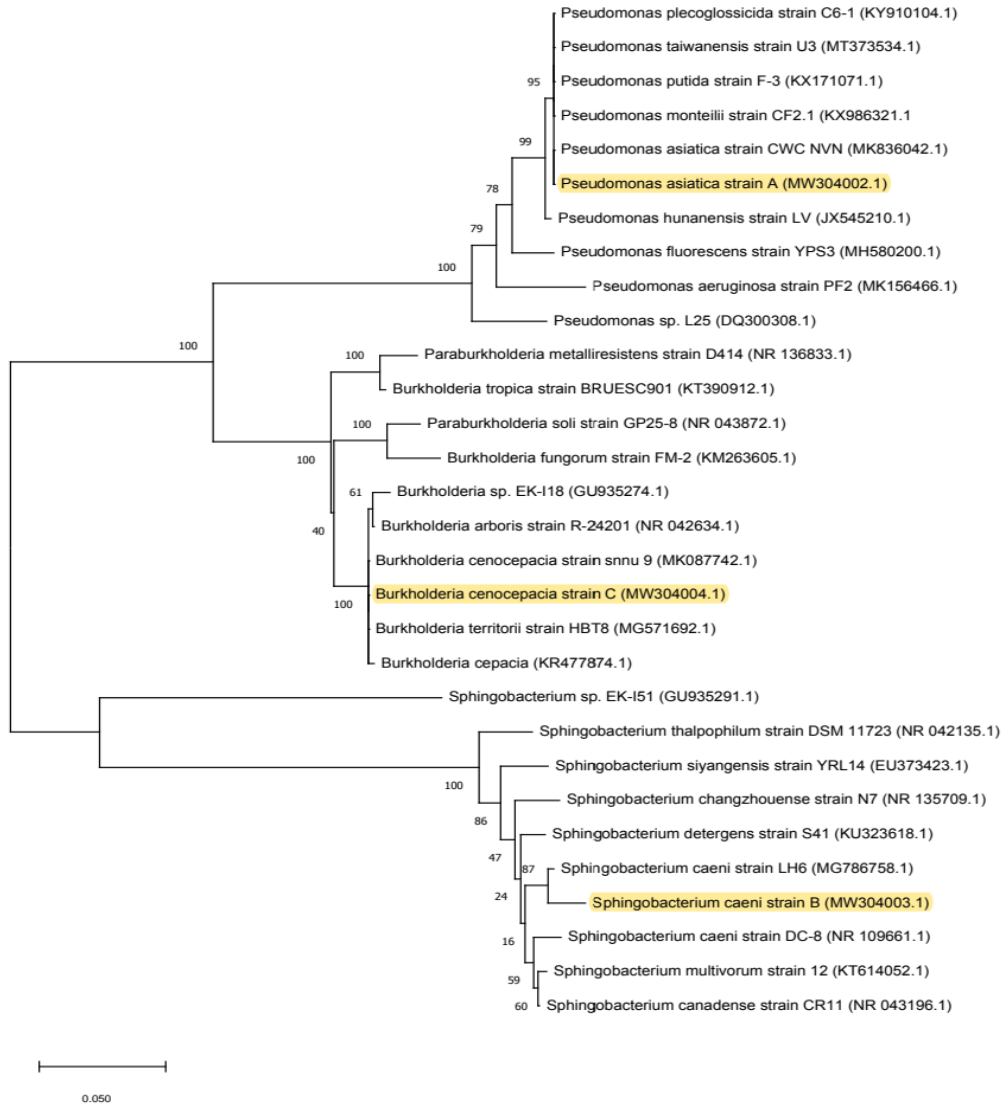

Figure 5. Phylogenetic relationships of the heavy metal-resistant isolates (*Pseudomonas asiatica* strain A, *Sphingobacterium caeni* strain B, *Burkholderia cenocepacia strain C*) to other closely related species

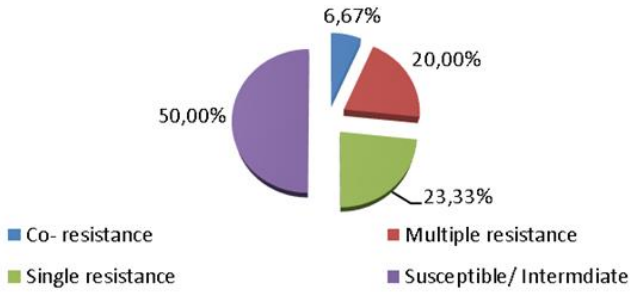

Figure 6. The percentage of antibiotic resistance of the bacterial strains. Letters in parenthesis indicate the sensitivity of the isolates to the antibiotic, and are represented as R: Resistant, I: Intermediate, and S: Susceptible

In conclusion, *P. asiatica* strain A, *S. caeni* strain B, and *B. cenocepacia* strain C were isolated from soils obtained from the metal dumpsite. The isolated bacteria thrived in high concentrations of Cu, Cd and Pb. The *P. asiatica* was the most resistant to the tested metals. The bacterial strains were halotolerant/ halophilic and osmotolerant and exhibited wide degrees of resistance to different antibiotics. The *B. cenocepacia* was resistant to all the antibiotics tested. The properties of the isolated bacteria strains imply their potential as bioremediation agents and possible candidates for producing halotolerant and osmotolerant hydrolases such as cellulase, xylanase, amylases and proteases, enzymes that currently dominate the world enzyme market. Further studies are required to ascertain the scope of these strains for the biotechnological processes.

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