Research article

Halotolerant and metalotolerant bacteria strains with heavy metals biorestoration possibilities isolated from Uburu Salt Lake, Southeastern, Nigeria

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ABSTRACT

Environmental contaminations by heavy metals are currently an increasing public health concern globally. One key challenge of these toxic metals is the extremely difficulties involved in their detoxification from the environment and effluents because of their non-degradability. An efficient biologic agent with potentials of remediating these toxic metals may ease these ever-increasing problems. We reported toxic metals tolerance and bioremediation potentials of novel bacteria sp. Strains USL2S, USL4W and USL5W isolated from Uburu salt lake, Ebonyi State, Nigeria. The phenotypic characteristics and the 16S rRNA gene analyses revealed that USL2S strain belongs to the genus Klebsiella, whereas USL4W and USL5W strains belong to the genus Pseudomonas. The bacteria isolates grew well in media containing 5–15 % of sodium chloride. The bacteria isolate showed capacity to tolerate 50.0 mM Hg^{+2} and Pb^{+2}, 17.0, 12.50 and 4.0mM Ni^{+2}, Cd^{+2}, and Zn^{+2} respectively in solid media. Pseudomonas putida A4W Strain also tolerated 16.0 mM Cu^{+2}, while Klebsiella sp. Strain USL2S, Pseudomonas putida USL5W Strain tolerated 4.0 mM each. AAS analyses showed 85, 95, and 95 % Hg; 97.13, 98.89, and 97.55 % Pb; 73.33, 77.42 and 69.72 Cd; 88.06, 99.54, and 97.91 % Ni; 100, 100 and 83.62% Cu; 42.30, 84.52 and 98.80 % Zn removal from media broth incorporated with the tested metals by Klebsiella sp. USL2S, Pseudomonas sp. Strain USL5W and Pseudomonas sp. USL4W respectively. We therefore recommend these novel moderately halophilic and metal tolerant isolates as possible biologic agents for effective bioremediation of mercury, lead, cadmium, nickel, copper and zinc in contaminated environments and effluents.

1. Introduction

Past and present industrial and ore mining activities have left large areas in many developing countries to become contaminated with both organic (crude oil) and inorganic (heavy metals) pollutants. Heavy metals, also known as toxic metals are natural elements that are widely distributed in the environment because of their multiple applications. Currently, heavy metal pollution is one of the main universal environmental issues and industrial wastes, ore mining, atmospheric deposition are some of the anthropogenic sources. As their name suggest, they present a risk for human health and the environment as they are teratogens, carcinogens and mutagens Sanjeeda et al., 2020); especially when they get accumulated in water bodies available for domestic purpose above the permissible limit (Lenntech, 2017). Heavy metals fouled water, impedes with the health and growth of crops, reducing their worth and marketability (Augusto-Costa and Pereira-Duta, 2001). It is very difficult to reclaim environments polluted with heavy metals because of their non-biodegradability nature. Proven ploys used for reclamation are high-priced and their effectiveness may not be certain (Abdel-Razik et al., 2020). Consequently, bio-restoration is a suggested novel alternative for heavy metal detoxification from the environment. It is cheap, proficient, and environmentally friendly and has a high public approval. Bacteria possess several mechanisms for bioremediation of heavy metals, such as absorption

of heavy metals into bacterial cell, and transformation of toxic metal into less toxic form or degradation (Jin et al., 2018). Several studies have demonstrated the potentials of various microorganisms in reclamation of media polluted with assorted heavy metals (Divakar et al., 2018; Babavaliana et al., 2013; Debajit and Josh, 2017; Igiri et al., 2019; Akcay and Kaya, 2019; Afzal et al., 2017, Sanjeeda et al., 2020).

Hypersaline lakes with salt level span at/or close to fullness are extreme environment which however sustain amazingly great microbial cell densities and are biologically very lively ecosystem (Rohban et al., 2009). Hypersaline environments shelter a substantial diversity of extremely salt-loving archaea in addition to halophilic and halotolerant bacteria.

Ebonyi State, southeast Nigeria has several high-salt environments and one of them is Uburu salt lake. Interestingly, the residents and some salt making companies currently use the lake brine for salt production besides using the water for other domestic purposes. Elevated heavy metals have been reported on the salt lake (Akubugwo et al., 2007; Okoyeh and Egboka, 2003). Ogbanshi et al. (2016) reported significant level of polycyclic aromatic hydrocarbons in the salt lake. However, there is currently no scientific investigation on the microbial diversity of this lake, thus the potential usefulness of this microbial community in biotechnology remains unknown. We therefore screened, isolated and characterized metal resistant halophilic and/halotolerant bacteria of the salt lake and evaluated their heavy metals bioremediation potentials. The investigation provides valuable information for the application of the isolated bacteria into heavy metal bioremediation technology for the restoration of heavy metal and salt contaminated effluents and environments.

2. Materials and methods

2.1. Materials

Atomic Absorption Spectrophotometer (AAS) (Hitachi Polarized Zeeman AAS, Z-8200, Japan), ZR fungal/bacterial DNA miniprep (Manufactured by Zymo research cat number: D6005). All media (Nutrient agar, mannitol salt agar, MacConkey agar, centrimide, Mueller-Hinton agar, Cysteine Lactose Electrolyte Deficient (CLED) agar, Eosin-Methylene Blue agar, Nutrient broth are of Titan Biotech, India and all chemical ($CuSO_4.5H_2O$, $NiSO_4.7H_2O$, $ZnSO_4.7H_2O$, $HgCl_2$, $Pb(NO_3)_2$, $CdSO_4.8H_2O$) used were of analytical grade. Molecular analysis was carried out in Bioinformatics services, Queen Elizabeth road, Ibadan, Nigeria.

2.2. Sample collection

Ten samples each of brine and sediment were collected from L1 (6° 2′ 52.107″ N 7° 44′ 50.115″ E), L2 (6° 2′ 58.462″ N 7° 44′ 36.407″ E), L3 (6° 2′ 53.727″ N 7° 44′ 39.647″ E), L4 (6° 2′ 47.994″ N 7° 44′ 30.55″ E) and L5 (6° 2′ 42.636″ N 7° 44′ 39.273″ E) locations at Uburu salt lake, Ebonyi State Nigeria as shown in Figure 1. The samples were transported in ice to the Microbiology laboratory complex of Ebonyi State University, Abakaliki within 2 h of collection for analysis.

2.3. Isolation and screening of toxic metals tolerant bacteria

Test tubes were filled with 9 mL of distilled water each and sterilized in an autoclave set at 15 psi pressure and 121 °C for 15 min. One gram (1) of the sediment samples and 1 mL of the brine samples were consecutively diluted into tenfold and then 0.1 mL of the solution in the second dilution factor was collected and spread across nutrient agar medium plates incorporated with each heavy metal (200 mg/L): mercury, lead, cadmium, nickel, copper and zinc. The cultures were incubated at 37 °C for 24 h and thereafter the colony forming units (CFU) were counted. Pure cultures were maintained by repeated sub-culturing on fresh nutrient agar for further identification (Oaikhena et al., 2016).

2.4. Isolates tolerance to salt (NaCl)

The isolates were tested for their salt tolerance by growth in Mueller hinton agar plates impregnated with varied doses of sodium chloride (NaCl): 5, 10, 15, 20 and 25 %. The NaCl incorporated plates were inoculated with freshly grown culture of the isolates and incubated at 37 °C for 5 days. Growth of the isolates were determined visually as positive or negative.

2.5. Identification of bacteria isolates

The obvious salt and heavy metals tolerant isolates were typified morphologically (gram staining), biochemically (citrate utilization, catalase, oxidase, indole, MR-VP) and molecularly using standard methods.

2.5.1. Gram staining

A sterile wire loop was used to aseptically pick a colony of each test organism and was placed on the slides and air dried. The smear was first covered with crystal violet for 30–60seconds. The stained slides were washed off with tap water. The smear on the slide was covered with lugol's iodine for 30 s and was washed off with clean tap water. The smears were rapidly decolorized with acetone-alcohol and were immediately washed with clean tap water. The stained slides were counter stained with Safranin for 60 s and washed off with clean water. The slides were air-dried and the under surface blotted. Oil immersion was added to the stained slide and examined using X100 objective.

2.5.2. Biochemical tests

2.5.2.1. Oxidase test. A piece of filter paper was placed in a clean Petri dish and 2–3 drops of freshly prepared oxidase reagent (tetramethylene diamine) was added on it. With the aid of glass rod, a colony of the tested organism was removed and smeared on the filter paper. This was observed for blue-purple colour appearance after few seconds. Catalase test: A drop of 3% hydrogen peroxide solution was place on a clean, grease-free glass slide; sterile wire loop was used to pick the test organisms and dip into the hydrogen peroxide and observe immediately for bubble formation.

2.5.2.2. Citrate utilization. A 5 ml of Simmon citrate agar was sterilized by autoclaving at 121 °C for 15 min at 15 psi and allowed to cool to 45 °C (placed to form a slant direction 3cm each) before inoculation of the isolate 5 mm into the bottom of the tube. After inoculation the tubes were incubated at 37 °C for 24 h. Growth with blue colony indicated positive test (Bergey's Manual of Systematic Bacteriology, 1986).

2.5.2.3. Indole test. A dense suspension of the test organism in 72 h nutrient broth culture was added with 2–3 drops of Kovac's reagent and observed for red surface layer appearance within 3 min which indicates indole positive and yellow surface layer shows negative result.

2.5.2.4. Mehtyl red test. Exactly 5 ml of MR-VP broth was poured into the various test tubes each. The test tubes were corked properly and sterilized by autoclaving at 121 °C for 15 min at 15 psi. They were allowed to cool at 40 °C and thereafter the test isolates were inoculated into the test tubes and incubated at 37 °C for 48 h. After incubation, 3 drops of methyl red reagent was added to the test tube and observed for colour change from straw yellow to red.

2.5.3. Molecular

Genomic DNA was extracted from pure colonies using Zr Fungal/Bacteria DNA Minirep (Zymo research D6005). 16S rRNA genes were amplified by a reverse transcriptase polymerase chain reaction (RT-PCR) using primers: 27F: AGAGTTTGATCMTGGCTCAG; 1525R:
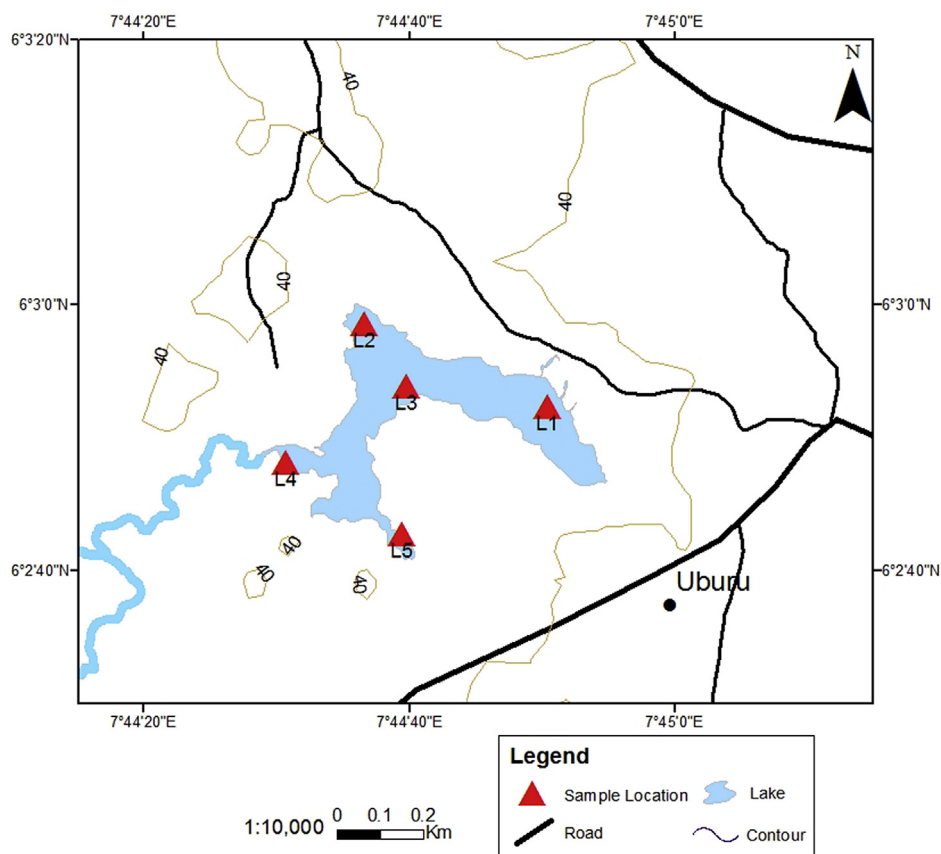
Figure 1. Map of Uburu salt lake showing samples collection locations.

AAGGAGGTGWTCCARCCGCA of the gene under the following reaction conditions. Initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 45 s. Followed by a final elongation step at 72 °C for 7 min and hold temperature at 10 °C. Partially sequenced amplified 16S rDNA fragment was compared with other gene sequences in Gen bank and aligned with gene sequence of the isolates (Bezza and Chirwa, 2015). One major disadvantage of Polymerase chain reaction in the detection of bacteria is false positive results. For this reason, reverse transcriptase polymerase chain reaction was used in this study.

2.6. Determination of minimum inhibitory doses of the isolates to the heavy metals

Nutrient agar was prepared according to the manufacturers specifications. It was autoclaved, put into Petri dishes and allowed to cool. Using sterile swab, the halotolerant isolates were inoculated by streaking all over the nutrient agar contained in the petri dishes ensuring that every space is streaked with the inoculum. Procedure was repeated for each petri dish and allowed to stand. Using a hole borer, 5 holes numbered 1–5 were made on each of the petri dishes containing the nutrient agar, each hole representing a dose of each heavy metal (12.5, 25 and 50mMol for Hg^{+2}, Pb^{+2}, Cd^{+2}, and 16mMol for Ni^{+2}, Zn^{+2} and Cu^{+2}) and were labeled accordingly. Using a micropipette, 3 drops of each concentration of heavy metal were taken and introduced into its corresponding hole on the petri dish. They were incubated at 37 °C for 24 h (Orji et al., 2021).

2.7. Evaluation of toxic metals removal by the isolates

Triplicate Erlenmeyer flasks with nutrient broth enriched with 10 % NaCl and 1700 mg/L were set up for each of the tested metals: mercury, lead, cadmium, Nickel, copper and zinc were prepared. Two (2) mL of 12

h old of the test isolates culture containing 0.5 McFarland Scale ($\sim$1.5 $\times$ 10^8 cell/mL) was aseptically transferred to the flasks. It was properly covered and incubated at room temperature with constant shaking at 200 rpm. One milliliter sub-samples were collected after 24, 48 and 72 h, centrifuged at 10,000 g for 15 min at 24 °C and supernatants filtered through sterilized 0.22 μm membrane filters. Filtrates were diluted 10-fold with 10% HNO3 for determination of the heavy metals from the test media using the Atomic Absorption Spectrophotometer (AAS). The percentage bio-removal of each metal was calculated based on its initial quantity (I_o) using the equation below.

% Biosorption removal = Io-I$_f$/I$_o$ where I_o = Initial concentration of metal in the solution before cultured with tested organism (mg/L), I_f = Final concentration of metal in the solution after cultured with tested organism (mg/L) (Gourdon et al., 1990).

2.8. Statistical analysis

Each test was performed in triplicates and the results were analyzed using the statistical package, SPSS 20.0. The averages were weighed against each other with one-way analysis of variance (ANOVA) and multivariate Post Hoc Duncan tests were used to examine the statistical significance of the mean difference.

3. Results

3.1. Isolation and identification of toxic metals tolerant bacteria

Three unique halotolerant and metal tolerant aerobic bacteria strains isolated from brine and sediment samples of Uburu salt lake, Ebonyi state, Nigeria are presented in Table 1. They were all gram negative, rod shaped, cetrimide, oxidase and catalase positive. Based on the criteria of Bergey's Manual of Determinative Bacteriology, the isolates were

Table 1. Morphological and Biochemical Characteristics of the isolates USL2S, USL4W and USL5W Isolated from Uburu Salt Lake, Ebonyi State, Nigeria.

Test	USL2S	USL4W	USL5W
Cell morphology	rod	rod	rods
Gram's reaction	-	-	-
Colour	Pink	orange	orange
Indole	-	+	-
Citrate	+	-	+
Methyl red	-	+	-
Cetrimide	+	+	+
Oxidase	-	+	+
Catalase	+	+	+
Ornithine decarboxylase	-	+	-
Glucose	+	+	-
Lactose	+	-	-
Identification	*Klebsiella sp.*	*Pseudomonas sp.*	*Pseudomonas sp.*

(+) = positive (-) = negative.

identified as *Klebsiella pneumoniae* (USL2S) and *Pseudomonas putida* (USL4W and USL5W). The identification of the isolates was confirmed by 16S rDNA sequence analyses Figure 2. The 16S rDNA sequence of USL2S was 99.52% similar to that of *Klebsiella Pneumoniae*. The analyses of 16S rDNA revealed that stain USL4W had 71.31% identity similarity to *Pseudomonas putida* strain Huaian_84_2 (NCBI accession number: MN314422), while USL5W had 82.28 % identity similarity to *Pseudomonas putida* osibote 28 (NCBI accession number: LC349899). From their identity similarities, these two strains USL5W and A4W might be considered as novel strains of putida. The Coded names, USL2S, USL4W and UL5W of these strains were coined using the number of the isolates (2, 4 and 5), type of the sample (Water (W) and sediment (S) and place of isolation (Uburu salt lake (USL).

3.2. Salt tolerance of the isolates

The salt tolerance of the isolates USL2S, USL4W and USL5W Isolated from Uburu Salt Lake, Ebonyi State, Nigeria is presented in Table 2. All the isolates were able to grow optimally on 5–15% (w/v) of nutrient media.
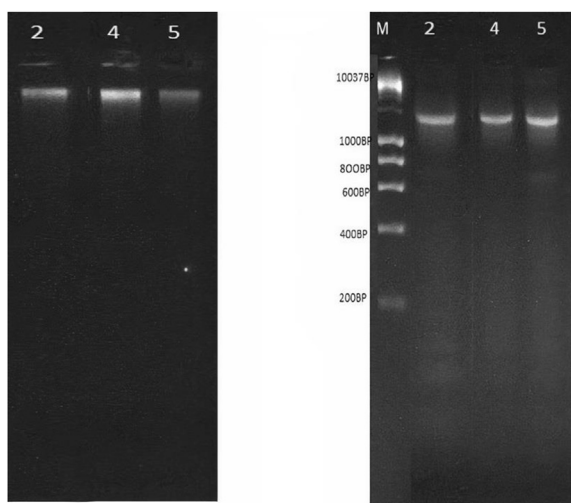

Figure 2. DNA products and PCR amplification of Isolate: A is the DNA products and B is the gel (Agarose) electrophoresis of Polymerase chain reaction (PCR) products of 16S rRNA gene isolates of *Klebsiella pneumoniae* (USL2S) (lane 2) and *Pseudomonas putida* (USL4W and USL5W) (lane 3 and 4), and Marker (M) is represented (Lane 1). The full-length gels are presented in Supplementary Figures 1 and 2.

3.3. Minimum inhibitory dose toxic metals

The susceptibilities of the isolates against the heavy metals: mercury (Hg), cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn) and lead (Pb) is presented on Table 3. The isolates showed extreme tolerance to Hg^{+2}, Pb^{+2} and Ni^{+2}. The minimum inhibitory concentrations (MICs) for the aforementioned metal ions were more than 50, 50 and 17 mM respectively (1000 mg/100 ml). The tolerance of the isolates to the tested metals is in this order: Hg ~ Pb > Ni > Zn > Cu > Cd. Cadmium was the most toxic among the tested metals.

3.4. Removal of toxic metals from culture broth model

The heavy metal removal potentials of *Klebsiella pneumoniae* USL2S strain, *Pseudomonas putida* USL4W strain *and Pseudomonas putida* USL5W strain are presented in Figure 3. Our results revealed that the removal of Hg, Zn and Ni by *P. putida* strains USL4W and USL5W after 24h of incubation were significantly (P < 0.05) higher than that of USL2S Figure 3. The observed differences among the species in the removal of Pb and Cd were not significant (P > 0.05) after 24h. The results showed that greater proportions of the metals were removed from the culture broth by the isolates after 24h of incubation. The percentage of Hg and Ni removed by the *P. putida* strains were also significantly higher than that of *K. pneumonia* strain USL2S. The trend of Cd removal was as follows: USL5W > USL4W > USL2S. *Pseudomonas putida* USL4W *strain* removed 69–98% of the tested metals after 72 h Figure 3. Similarly, Strain USL5W followed the same trend.

4. Discussion

Bio-restoration has been recognized as a sustainable approach for the removal of toxic metals from polluted environments. There is currently a growing interest in the scientific community for the separation and identification of some heavy metal tolerant bacteria and their potential exploitation for the bioremediation of heavy metals polluted environments. We describe for the first time, salt and multi-metals (Hg, Pb, Cd, Ni, Cu and Zn) tolerances and toxic metal removal potentials of *Klebsiella pneumoniae USL2S, Pseudomonas putida* USL4W and *Pseudomonas putida* USL5W isolated from Uburu salt Lake in Ebonyi State, Nigeria. The bacteria isolates showed a profound tolerance capacity to high quantities of the tested heavy metals in solid media with potent bioremediation potentials.

Our isolates *Klebsiella pneumoniae* USL2S, *Pseudomnonas putida* strains (USL4W and USL5W) are moderate halophiles as they demonstrated maximum growth on 5–15% (w/v) salt enriched nutrient media. Since these bacterial strains could grow in a relatively wide range of salinity, it

Table 2. Salt (NaCl) tolerance of the Isolates USL2S, USL4W and USL5W Isolated from Uburu Salt Lake, Ebonyi State, Nigeria.

Isolates	Concentrations NaCl(%)	Growth (hour)			
		24	48	72	96
USL2S	5	+	+	+	+
	10	+	+	+	+
	15	-	-	+	+
	20	-	-	+	+
	25	-	-	+	+
USL4W	5	+	+	+	+
	10	+	+	+	+
	15	-	-	+	+
	20	-	-	+	+
	25	-	-	+	+
USL5W	5	+	+	+	+
	10	-	+	+	+
	15	-	-	+	+
	20	-	-	+	+
	25	-	-	+	+

+ = Growth; - = Negative.

becomes obvious that they could be used as agents for cleaning up environments of varying salinity. They are deemed as a good source of biocatalysts and are fast gaining popularity among the environmental biotechnologists as the cost effective means to clean up polluted environments (Rajesh et al., 2014).

The agar well diffusion method remains acceptable approach for the assessment of metal toxicity to microorganisms although it has been noted that metals and several constituents of solid nutrient media may interact, thereby making interpretation of results complicated. In polluted soil and sewage sludge where situations of dispersion, complexation and availability of metals were not the same from those observed in liquid media, this method could be practical in the evaluation of metal toxicity.

In this study, *P. putida* USL4W and USL5W exhibited high tolerance capacity of 50.0 mM for Hg^{+2} and Pb^{+2}, 17.0, 12.50 and 4.0mM for Ni^{+2}, Cd^{+2}, and Zn^{+2} respectively in on agar plates. *Pseudomonas* putida USL4W also tolerated 16.0mM Cu^{+2}, while *Pseudomonas putida* USL5W Strain tolerated 4.0 mM. In relation to previous reports on the metal tolerance of *P. putida* strains, inhibitory values of 10, 9, 3, 1.25, and 1.0 Mm for zinc, cadmium, nickel, copper and lead ions respectively for *P. putida* HMR37 (Ali and Harshada, 2016); 5, 0.5, 0.25 mM for Zn, Ni, Cu in that order for *Pseudomonas putida* MU 139 (Ceylan and Ugur,2012); 4.34, 1.4 mM for Cd and Zn for *Pseudomonas* sp. MB8 (Tamiru et al., 2014); 7.5 mM Cd for *P. putida* strain YR8 (Zhang et al., 2014); 2.43, 1.26, 1.2, 1.03 mM respectively for Zn, Ni, Cu and Cd for *Pseudomonas putida* KNU12 (Hussein and Joo, 2013), these halotolerant *P. putida* strains (USL4W and USL5W) exhibited more tolerance to heavy metals.

Cell wall structures, presence of antioxidant enzymes and environmental factors may have contributed to the high metal tolerance exhibited by the isolates. Biochemically, the catalase enzyme produced by these isolates might have provided protective mechanism against oxidative stress caused by heavy metals. Our result revealed that mercury and lead were least toxic to the isolates. This was not expected as the metals are reported to be most toxic to micro-organisms. The results agree with the report of Sowmya et al. (2014). The high level of tolerance of the isolates against mercury and lead could have been as a result of bio-methylation. Bacteria possess capacity to change toxic metal ions into their respective non-toxic forms using ATPase-dependent reduction pathways, which results in increasing their probabilities of existence in the natural habitats. Metallothionein like proteins and plasmid-encoded energy dependent metal efflux systems involving ATPases and

chemiosmotic proton pumps have been linked to cadmium tolerance in *Pseudomonas putida*. Some bacteria can combine detoxification and production of chelating agents that bound metals and reduce their toxicity (Kavamura and Esposito, 2010). It has been reported that *Pseudomonas putida* encodes an unanticipated ability to withstand toxic metals and metalloids (Canovas et al., 2003). Strains of this species were also earlier reported to have two czc chemiosmotic and four Zn/Cd/Pb efflux transporters (Leedjarv et al., 1996).

Klebsiella pneumoniae isolated by Daware et al. (2015) tolerated 16, 1.6 and 32 mM of nickel, mercury and copper respectively. Nevertheless, a direct correlation of our results with previous studies is difficult, since assorted biotic and abiotic indices vastly influence the bacteria.

The removal of mercury (Hg^{+2}), lead (Pb^{+2}), cadmium (Cd^{+2}), nickel (Ni^{+2}), copper (Cu^{+2}) and zinc (Zn^{+2}) separately from media broth enriched with the respective toxic metals at initial doses of 1700 mg/l by *Klebsiella pneumoniae* USL2S, *Pseudomnonas putida* strains (USL4W and USL5W) at pH 5 and different contact time are shown in Figures 3–5 respectively. The bio-sorption and/or bioaccumulation assays were done in a buffered media because pH is one of the vital indices in metal uptake efficiency (Pardo et al., 2003). It has been noted that low pH causes reduction of negative charge intensity by protonation of carboxyl, phosphate and amino functional groups. Similarly, alkaline pH can bring about reduction of bio-sorption by secondary hydrolysis, speciation and precipitation (Khalid et al., 2011). For instance it has been reported that at pH 8.0, cadmium precipitates from solution as $Cd(OH)_2$, $Cd(OH)_3$ which makes bio-sorption impossible. Areco et al. (2012) reported that at pH 6–7, Zn would precipitate out. Lead also form insoluble complexes in

Table 3. Toxic metals tolerance of isolates USL2S, USL4W and USL5W isolated from Uburu salt lake, Ebonyi state, Nigeria.

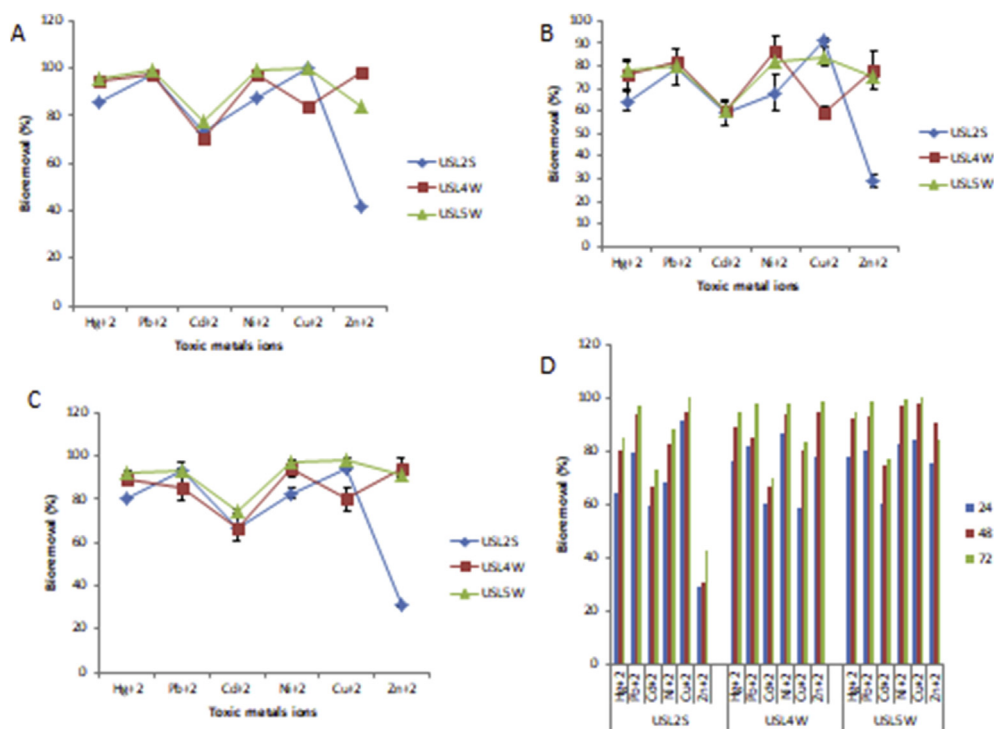| Metals	Concentrations (mg/l)	Zone of Inhibition (mm)		
		USL2S	USL4W	USL5W
Lead	10000	-	-	-
	5000	-	-	-
	2500	-	-	-
	1250	-	-	-
	625	-	-	-
Mercury	10000	-	-	-
	5000	-	-	-
	2500	-	-	-
	1250	-	-	-
	625	-	-	-
Cadmium	10000	25	20	23
	5000	15	16	13
	2500	-	-	-
	1250	-	-	-
	625	-	-	-
Nickel	10000	-	-	-
	5000	-	-	-
	2500	-	-	-
	1250	-	-	-
	625	-	-	-
Copper	10000	-	35	35
	5000	-	20	26
	2500	-	29	
	1250	-		
	625			
Zinc	10000	15	17	16
	5000	14	15	14
	2500	-	-	-
	1250	-	-	-
	625	-	-	-

(-) = No inhibition.

Figure 3. Toxic metals removal of the isolates: USL2S, USL4W and USL5W from heavy metal enriched nutrient broth media. A, B, C represent metals removal after 24, 48 and 72h respectively, while D shows the performance of each isolate.

alkaline pH, which may permeate the cell wall and block metabolic activity (Kathiravan et al., 2011).

The Hg, Pb, Cd, Ni, Cu, and Zn levels were decreased from an original concentration of 1700 mg/l to 85, 41.65, 566.61, 226.61, 278.54 and 20.40 mg/l in that order by USL4W and to 514.76, 18.87, 944.52, 299.88, 0, and 59.16 mg/l by A5W after 72h incubation at room temperature. Isolates USL2S removed 85, 97.13, 73.33, 88.06, and 30.77% of Hg, Pb, Cd, Ni, Cu, and Zn respectively from the growth medium. In general, all test organisms showed a gradual increase in heavy metal removal over the exposure period. Nevertheless, higher heavy metal removal efficiencies were noted with *P. putida* strains than with *K. peumoniae* strain. Strains USL4W and USL5W removed 98.8 and 84.53% of Zn2+, while *K. pneumoniae* USL2S removed 42.3%. This difference might be due to different mechanism of biosorption and toxicity of the metals.

The initial concentration of 1700 mg/kg /metal solution was chosen based on the highest tolerance shown by the bacterial strains. Previous report by Kamika and Momba (2013) showed that *Pseudomonas putida* removed 71, 57, 45, 83, 96, 100, and 49% of Co, Ni, Mn, V, Pb, Ti and Cu respectively from wastewater having 1.53–24.32 mg/L. Pardo et al. (2003) reported that *Pseudomonas putida* was able to remove 80% Cd^{+2}, Pb^{+2}, Zn^{+2}, and Cu^{+2} from aqueous solution. In another study carried out by Clausen (2000), *Pseudomonas putida* removed 25% of copper from nutrient agar. Furthermore, *Pseudomonas putida* also removed 70, 70, and 95% in that order of Zn, Cd and Hg in media having 10^{-8} M of the respective metals (Ledin et al., 1997). Compared to these previous studies, *Pseudomonas putida* species strains USL4W and USL5W seem to be more efficient for the bioremoval of thee metals. According to Xin et al. (2005) about 40–50% of Cu(II) and Zn(II) were actively taken up by *P. putida* CZ1, with the residues being passively bound to the bacterium. Cadmium-tolerant *P. putida* strain possessed the ability of intracellular sequestration of copper, cadmium, and zinc ions with the help of cysteine-rich low molecular weight proteins (Kuiper et al., 2003).

Klebsiealla planticola grew anaerobically at a Cd dose of 15 mM and precipitated CdS (Sharma et al., 2000). Aransiola *et al* (2017) reported that *K. edwardsii, K.* oxytoca, and *K. pneumoniae* strains isolated from diesel polluted soil significantly reduced Cadmium, copper and nickel. Several studies have revealed that mechanism of bio-sorption were convincingly associated to physicochemical interaction of metal in solution (Aksu et al., 2002; Lodeiro et al., 2006; Amini et al., 2008). It incorporates passive accretion action like surface adsorption, micro-precipitation, chelation, coordination and ion exchange (Lim et al., 2008).

Incubation period is one of the critical factors of the bio-sorption course for the reason that it links to bacterial life cycle and metal exposure function. Figure 3 depict the effect of gestation time on the bio-sorption of Hg, Pb, Cd, Ni, Cu and Zn by the isolates. We observed a profound diminution of the toxic metals residue in the supernatant during the first 24 h. This was followed by a gradual increase in reduction after 72 h of incubation. There was no variance in initial and final metal concentration of the control, which implied that there was no proof of extemporaneous heavy metals reduction. It however signaled that the main reduction mechanism was microbial metabolism.

5. Conclusion

We have shown that the isolates of *Klebsiella pneumoniae* (AUSL2S) and *Pseudomnonas putida* strains (USL4W and USL5W) demonstrated extreme tolerance to Pb, Hg, Cd, Cu, Ni and Zn. More so, bioremediation studies using batch experiments in a culture broth model unveiled that the isolates possessed potent bio-removal capacity for Pb, Hg, Cd, Cu, Zn, Ni respectively at extreme conditions of high salt levels and acidic pH, which is an innovation of other existing remediation processes. The isolates are moderately halophilic, metalophiles and could provide exceptional tool for mercury, lead, cadmium, nickel, copper and zinc bioremediation in any contaminated environment.

Declarations

Author contribution statement

Orji, O. U.: Conceived and designed the experiments; Wrote the paper.

Awoke, J. N., Aja, P. M.: Analyzed and interpreted the data.

Aloke, C., Obasi, O. D., Alum, E. U1.: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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