Atomic Spectroscopy

Article

Evaluation of metal resistance, uptake capacity and metal toxicity reduction by multimetal resistant bacteria as bioremediation agents for heavy metals

Eeman Alhammadi[1], Normala Halimoon[1], Zufarzaana Zulkeflee[1]and Wan Lutfi Wan Johari[1]
[1]Department of Environment, faculty of forestry and environment. Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

ABSTRACT: This paper is based on the removal of toxic heavy metals from polluted water bodies by effluent to halt and reduce pollution from being transported further into the environment. Biological removal method of these toxic heavy metals depending on metal-resistant bacteria are considered a more beneficial and inexpensive alternative that is of interest to be pursue. The capability of nine bacterial isolates isolated from electroplating effluent to tolerate and absorb toxic metals of zinc, chromium, copper, nickel individually and quaternary at high concentrations was evaluated when isolates were single and consortium. The highest concentration that bacteria were able to tolerate was used to determine the correlation between metal uptake and bacterial growth. Using the metal toxicity index B (L/mg) and its inverse (1/B, mg/L) on each individual bacterium, the toxicity limits and tolerance of metals were calculated. The isolates were more resistant and grew better at 100 mg/L, but growth was reduced and resistance declined at 300 mg/L, more toxicity and growth discontinued at 500mg/L for most metals. All isolates demonstrated ability to grow and uptake metals, but consortium exhibited perfect ability of metal uptake and resist compared to individual isolates. The consortium was able to flourish and compete within a united microbial community, which resulted in apparent increase in metal removal capability. The findings revealed no statistically significant correlation between metal uptake and growth. The isolates established the feasibility of employing them to remove heavy metals and support the development of an integrated system for treating metal pollutants bacterium-based.

Keywords: Heavy metals; multimetal resistant bacteria; bacterial growth; metal uptake; consortium; bioremediation; toxicity

INTRODUCTION

Heavy metals from various industrial activities are a major concern because of their toxicity to all living organisms, accelerated rates of movement and mobility in environment, and their influence on the ecosystem balance (Ali *et al*., 2019). Electroplating industry is one of these industries, as the wastewater from them always contains a high concentration of heavy metal ions such as Cr (VI), Cu^{2+} and Zn^{2+} and Ni^{2+} (Akbal and Camc, 2011). Heavy metals are stable elements with accumulating properties and non-biodegradability, which leads to great environmental concern over wastewater that discharge into water bodies (Qin *et al*., 2012; Peng *et al*., 2004). Detoxify heavy metals is necessary to control environmental pollution (Okolo and Olowolafe, 2016).

Bioremediation is used to disintegrate toxic metal substances into less toxic state using microbes (Aka and Babalola, 2016) or their enzymes to remove or neutralize the pollutants (Okoduwa *et al*., 2017). It is a safe, cost-effective method in reducing environmental pollutants (Hrynkiewicz and Baum, 2014). Species of bacteria have been isolated from contaminated areas with heavy metals, over the long term have the ability to resist and reduce heavy metals, this is due to exposure to metals creates tolerant microbial groups (Huang *et al*., 2016; Huang *et al*., 2017; El-Shanshoury *et al*., 2013). The metal toxicity resulting from competition with or replacing a functional metal or the oxidative stress or disrupting of cells components by form complex compounds can be counteracted by these species using precision resistance systems. Such as exportation or reduction via redox reactions, that guarantee them to survive and resist toxic metals (Chen *et al*., 2018; Xie *et al*., 2015; Losada *et al*., 2016). They able to survive well in high concentrations of heavy metals are of great beneficial as bioremediation agents because these species can achieve diverse transformation and immobilization processes (Ruttens *etal*., 2006; Gauatam *et al*.,2016).

Microbes in contaminated environments have adapted and become resistant to long term contamination. They have potentiality in bioremediation of high content of heavy metals in such environments during absorption mechanism of heavy metals (Issazadeh *et al*., 2013). The methods by which microbial cells absorb metal ions can take three forms; the first is the absorption of metal ions on the cell surface, second intracellular uptake of metal ions and third, chemical transformation of metal ions by microbial cells (Zimmerman, 2010). Among the diverse technique used for removal of metals biosorption which has been found to be highly qualified for detoxifying (Gauatam *et al*.,2016; Oremland *et al*.,1989). The microbial biomass has different absorptive abilities, which may differ among microbes (Igiri *et al*., 2018). However, determining the

absorption capacity of each microbial cell and its correlation with its growth rate can be useful in determining the quantity of biomass microbial and their efficiency as biosorbents. That is due to their ability to grow under controlled conditions and flexibility in environmental conditions (Srivastava *et al*., 2015).

The present study aims to determine the toxicity degrees and maximum resistance of these isolates and the absorptive capacity of high metals concentrations that can affect the survival of bacteria after exposure. The potential possibility of bacteria to grow and uptake the metals and then determining the correlation between them which may be needed in the application of these bacteria as bioremediation tools for metal pollution. The study findings can be used for further study on the efficacy of physiological or enzymatic agents that metal resistant bacteria use for bioremediation of metals.

MATERIAL AND METHODS

2.1 Bacteria and Culture Media

The bacterial isolates employed in the present study was isolated from electroplating industrial effluents, a total of 60 bacterial isolates were obtained initially from nutrient agar plates (Oxoid, Lab-Lemco Powder) incorporated with a concentration of (10 - 50) mg/L of copper, zinc, nickel and chromium as individual metals and as a quaternary metal solution at 37º C for 24h (Pandit R.J *et al*., 2013). Out of the 60 isolates, 23 isolates were selected on the basis of their morphological and cultural diversities. Among the 23 isolates, 9 isolates were able to grow at the highest concentration of 50 mg/L were chosen for further study. They were maintained on Nutrient Agar slants at 4°C for further use (Silva *et al* ., 2012). The isolates and accession numbers were identified and confirmed as *Microbacterium paraoxydans* (NR_025648.1), *Streptomyces werraensis* (NR_112390), *Microbacterium arabinogalactanolyticum* (NR_0449321) , *Staphylococcus haemolyticus* (NR_036956.1), *Bacillus paramycoides* (NR_1577341), *Bacillus megaterium*(NR_117473.1), *Sphingobacterium ginsenosidimutans* (NR_117473.1),*Kocuria rhizophila* (NR_026452.1) and *Sphingobacterium detergens* (NR_116238) based on 16S rDNA data. The isolates were given the following abbreviations respectively; (BMA-1), (ACM-2), (DMA-3 (STM-4), (BSM-5), (BME-6), (A6MA-7), (MIC-8) and (RMA-9).

2.2 Preparation of standardized cultures from bacterial isolates

Bacterial isolates were re-cultured into nutrient broth medium for 24hr in a rotary shaker at 150 rpm, pH 7.0 and temperature 37◦C, respectively. The cells harvested by centrifugation (4000 r/min, 10 min) were washed with sterile phosphate buffered saline twice to avoid any nutrient carryover. Washed cells were re-suspended in saline and the turbidity adjusted to give an optical density of 0.6 at 600 nm (Contains roughly $3x10^7$ cells/ml) (Sannasi *et al*,2010).

2.3.1 Preparation of metal stock solution

The stock solutions of chromium (Cr), copper (Cu), zinc (Zn) and nickel (Ni) were prepared in deionized water and sterilized by filter membrane (0.22 µm) and stored at 4°C. The salts used were potassium dichromate ($K_2Cr_2O_7$), copper sulphate ($CuSO_4.5H_2O$), zinc sulphate ($ZnSO_46H_2O$), and chloride nickel ($NiCl_2$. $6H_2O$). All working concentrations were obtained by diluting the stock solution (1000 mg/L) with deionized water. The solutions were then left for 30mins until complete dissolution occurred and sterilization was followed by membrane filtration (Odokuma and Akponah, 2010). The solutions were checked for their concentration using atomic absorption spectrophotometer before using (Shaaban *et al*., 2015).

2.3.2 Preparation of series of standard solutions of the tested metals

The working solutions of metals in this study were Zinc, Nickel, Copper and Chromium. Six Standared solutions were prepared from the stock solution (1000 µg/ml) for each metal. Quantitative analysis method carried out by preparing a series of standard solutions during a concentration range appropriate for the sample being analyzed. Standared solution were prepared from the stock solutions by pipetting 2, 4, 6,8,10 mL of 1000 µg/mL into 100-mL volumetric flasks and diluting with deionized water to the mark. Calibration standards were prepared with 24 dilutions of 100 µg/ml working solution. Calibration standards for all metals have a concentration working range of 20 to 100 µg / L.

2.4 Heavy metals resistance and metals toxicity

Aliquots 1000ul suspension of each the bacterial isolates (24 h old) and O.D= 0.6, were inoculated in 100ml nutrient broth medium containing (100, 300, 500 mg/L) of $CuSO_4$, $NiCl_2$, $K_2Cr_2O_7$, and $ZnSO_4$ individual and quaternary. The degree of resistance of bacterial isolates to heavy metals was estimated in the nutrient broth by measuring the optical density at 600 nm with controls which were consisted of a metal deficient medium inoculated with the bacterial isolates. The growth rate of isolates were expressed as a percentage of those obtained in untreated control which were considered 100% (Munees and Malik, 2012). The influence of metals on growth of bacterial isolates, also the potency of bacterial isolates to reduce the toxicity of metal can be quantified, as described by Duxbury through taking the natural logarithm (ln) of equation (Malakul *et al.,* 1998):

$$\mathbf{B} = -\frac{1}{C} In \frac{Y}{a}$$

Where: Y is the growth rate (OD600) of bacterial isolates at metal concentrations (mg/L).
C and ***a*** are the growth rate (OD600) of bacterial isolates without metal in the control.
B variable factor (inverse concentration; L/mg) is indicator of metal toxicity to bacterial isolates at metal concentrations
1/ B metal tolerance maximum (mg/L)

2.4 Bacterial growth rate and its correlation to uptake

Individual and consortium bacterial isolates (24 hr, OD 600 = 0.6) were inoculated in Nutrient broth supplemented 100mg/l of Cu, Zn, Ni and Cr individual and quaternary. Controls were employed metal free bacterial culture. The cultures were

incubated at 37°c on a shaker (200 rpm). The growth was monitored by measuring optical density the percentage of growth for each bacteria was estimated to control (Irawati *et al*., 2015).

2.5 Heavy metal uptake assay

All glassware washed with 0.1N HCl before and after each experiment to avoid binding of the metal to it (Wierzba and Lata, 2010). Aliquate1% of the isolates bacterial suspension inoculum (24hr old) having turbidity equal to (OD= 0.6) was inoculated individual and consortium into100 mg/L of sterilized separately and quaternary heavy metal (Zn, Cu, Cr, and Ni). Then, incubated at 150 rpm and 37 °C for 24 hours)(Sannasi *et al*,2010). Control media without heavy metals were also included for comparison, 10ml of each culture was centrifuged at 4,000 rpm for 20 min. The supernatants were separated and mixed with a double volume of concentrated HNO_3. Thereafter those mixtures were heated to 100°C on a Hotplate Stirrer to complete acid digestion until the final volume decrease and down to initial supernatant volume. The extract was filtered through a filter paper (Whatman 42) to remove any insoluble material and collected into a volumetric flask and then diluted (Marzan *et al.*, 2017). This extract of total reduction was analyzed by ICP-ME and the result is heavy metal compared with control to calculate heavy metal uptake capacity (%) as follows:

% of heavy metal utilized= Heavy metal utilized (ppm) / Heavy metal added to the Nutrient broth (ppm) x 100

Heavy metal utilized (ppm) = Heavy metal added to the Nutrient broth (ppm) — Heavy metal at the end of culture (ppm)

% absorption = ⌈I - F / I) x 100

Where: I = initial metal concentration, F = final metal concentration

2.6. Standard solutions of metals

Individual metals were prepared with varying concentrations in deionized water. The standard's absorption of metal solutions was measured by Atomic Absorption Spectroscopy (UV: Visible Spectrophotometer, Cecil CE10211000 Series) at wavelengths 324.8 nm, 213.9nm, 232.0 and 357.9 nm for copper, zinc, nickel and chromium, respectively. A standard curve was plotted from the uptake of standard metal solutions with concentration against absorption. The supernatant was analyzed for residual metal concentration in the bacterial treated and bacteria free control media. Similarly, the residual metal was also determined by intersecting the uptake of supernatant in the standard curve (Syed and Chinthala, 2015).

2.8 Statistical analysis

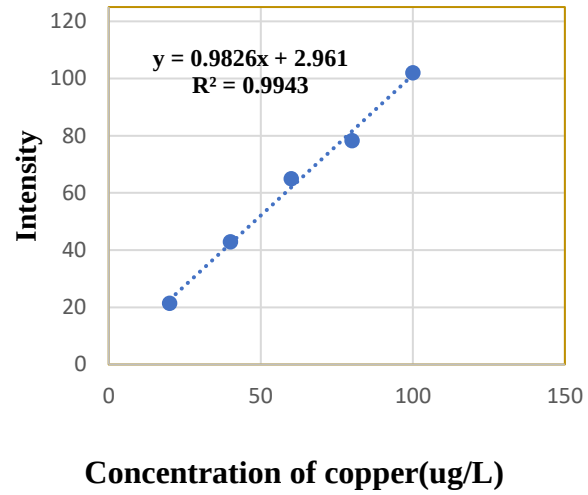
The data was analysed by calculating mean ± SE, analysis of variance (ANOVA), was performed by using SPSS, to assess the differences of bacterial growth and uptake among the heavy metals. This analysis followed by Tukey's Honest Significant Difference (HSD) to find means that are significantly different from each other. Also, correlation analysis was done for quantitative analysis and to confirm whether there is a relationship between bacterial growth and the absorption of heavy metals, Pearson correlation Coefficient analysis was applied to the data set.

RESULTS DISCUSSION

3.1 Preparation of calibration curves

Preparation of standard solutions for calibration curves was done to verify the concentration of solutions used in the experiments. Standard solutions of zinc, nickel, chromium, and copper were prepared from the 1000 μg/L. Calibration curve for the determination of metals in the unknown samples were built based in the known concentration of each metal and the measured intensity in the ICP-ME.

Measurements were carried out by recording three readings of intensity from each solution. The concentrations of working standard solutions, the observed intensity and values of correlation coefficients of the calibration graphs for the four metals are presented in Figures (1-4).



Figure(1): Calibration curve of copper

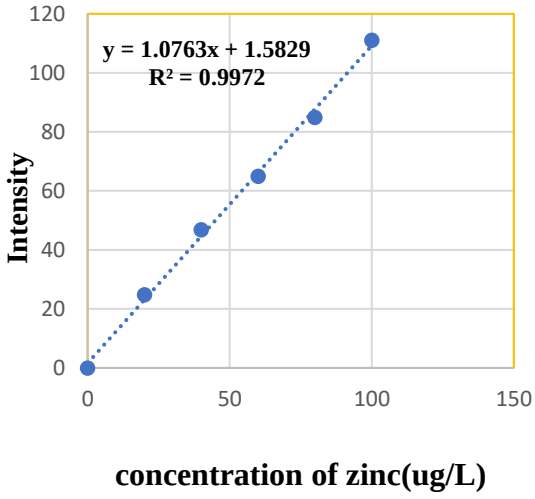
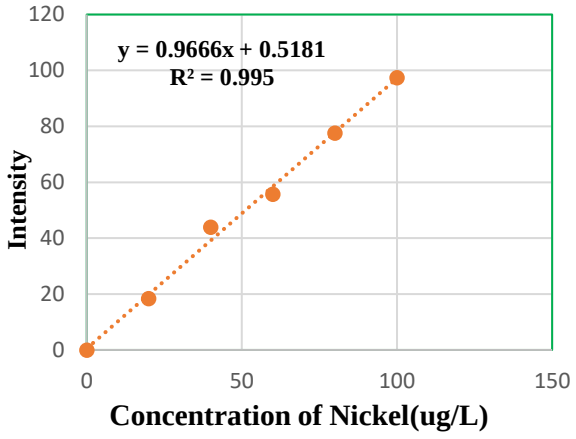
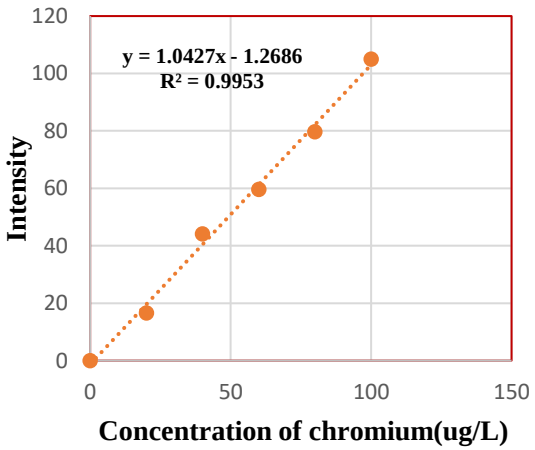


Figure (2): Calibration curve of zinc



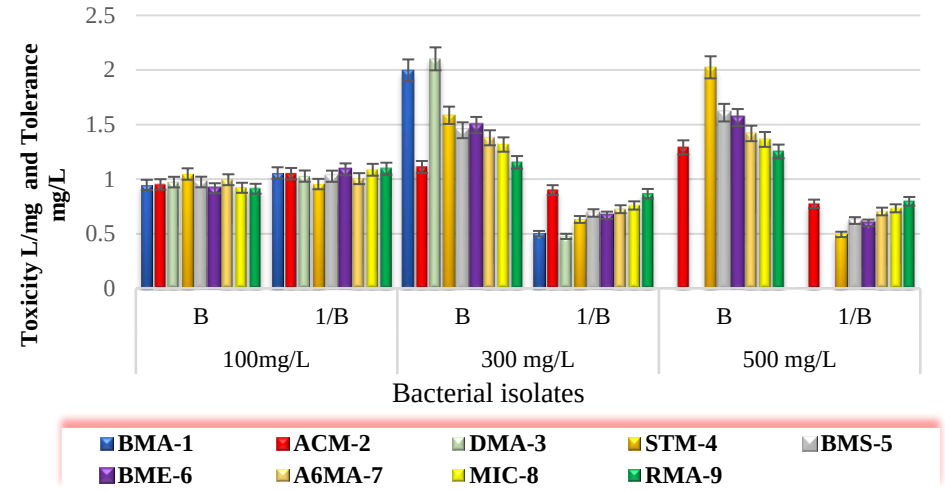
Figure(3): Calibration curves of Nickel



Figure(4): Calibration curves of chromium

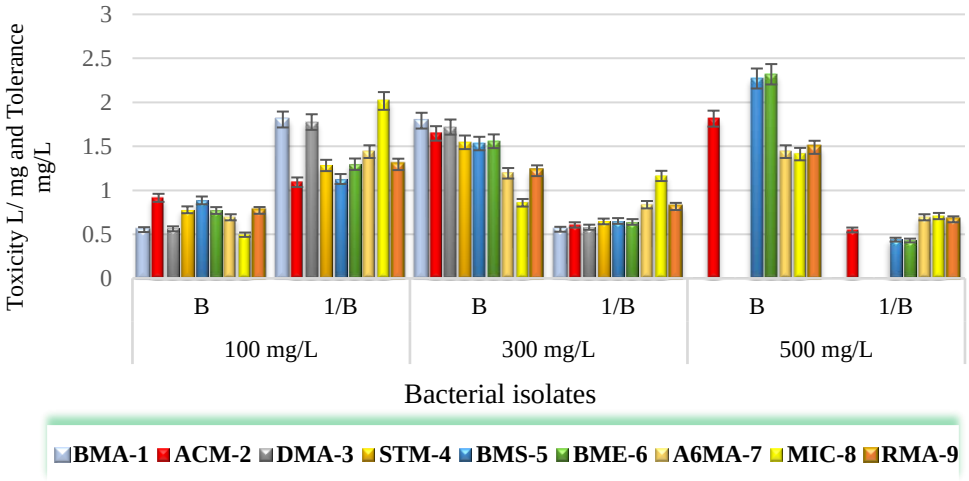
3.2 Toxicity level and tolerance limits

The results for tolerance and toxicity at high concentrations are shown in Figures 5-9. Heavy metal toxicity levels and tolerance limits were investigated using nine isolates; the highest B values indicated the highest toxicity. Toxicity values B are inversed to reflect the highest theoretical concentration of metal ions that bacteria can withstand (Sannasi *et al.*, 2010).



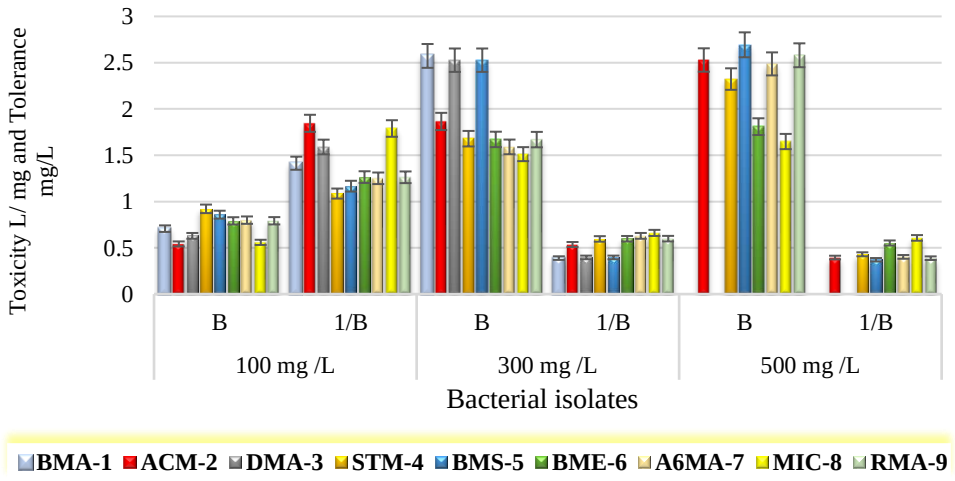
(Where : B= toxicity level, 1/B= tolerance limits)

Figure 5 : Toxicity Level and Tolerance Limits of Copper by Bacterial Isolates



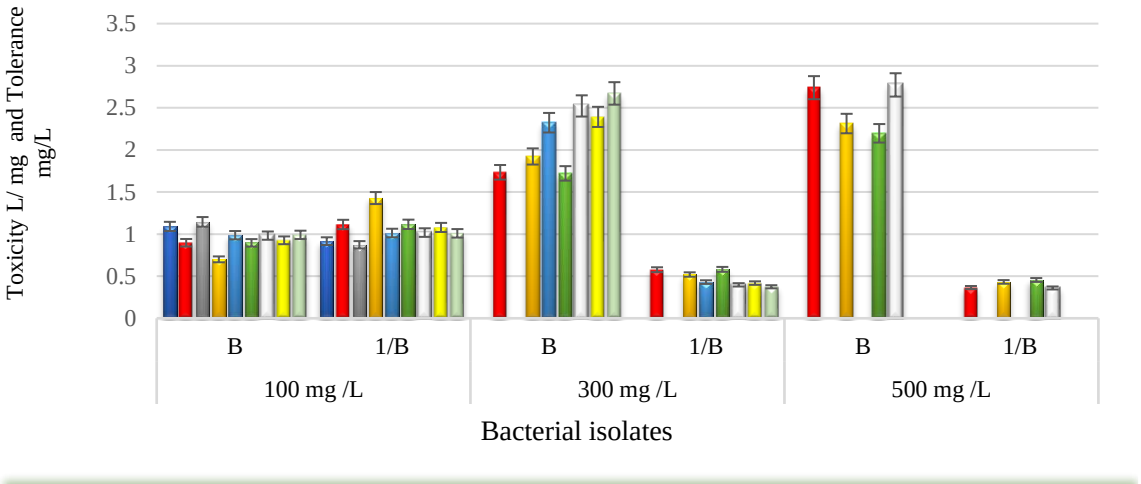
(Where : B= toxicity level, 1/B= tolerance limits)

Figure 6 : Toxicity Level and Tolerance Limits of Nickel by Bacterial Isolates



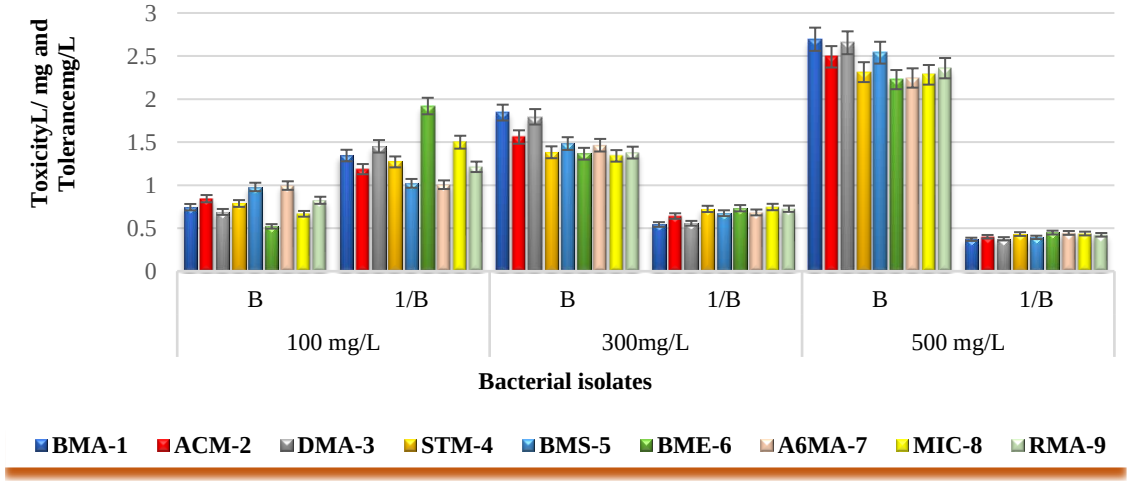
(Where: B= toxicity level, 1/B= tolerance limits)

Figure 7 : Toxicity Level and Tolerance Limits of Zinc by Bacterial Isolates



(Where: B= toxicity level, 1/B= tolerance limits)

Figure 8 : Toxicity Level and Tolerance Limits of Chromium by Bacterial Isolates



(Where : B= toxicity level, 1/B= tolerance limits)

Figure 9: Toxicity Level and Tolerance Limits of Quaternary by Bacterial Isolates

All isolates showed the ability to withstand all metals, especially at 100 mg/L. Resistance varied according to metal variation and concentration. The highest tolerance and lowest toxic effect at 100 mg/L were, respectively, (2.016 mg/L) Ni in *K rhizophila*, (1.845 mg/L) Zn in *Streptomyces werraensis*, (1.919 mg/L) quaternary in *Bacillus megaterium*, (1.428 mg/L) Cr in *S haemolytics* and (1.096 mg/L) Cu in *S detergens.* At 300 mg/L, *K. rhizophila* (1.164 mg/L) Ni, (0.746 mg/L) quaternary, (0.661 mg/L) Zn, and *S. werraensis* (0.900 mg/L) Cu were the most tolerant. At the highest metal concentration 500 mg/L *S detergens*, was more tolerant than others with (0.797 mg/L) Cu and (0.694 mg/L) Ni.

As for the toxicity, the least toxic was with quaternary; all isolates were able to grow in all tested concentrations, which show the multi-metal resistance abilities of these isolates, although the resistance gradually decreased and the cell density became relatively low. These results prove resistance capacity of isolated species from polluted sites as indicated previous research that in contaminated environments, various types of microbes make great efforts to ensure their growth, cell structure and vital processes under contaminated stress conditions are protected, so they have the ability to deal with pollution conditions using their own biological mechanisms (Baker and Banfield, 2003). Such as, *M paraoxydans*, *M arabinogalactanolyticum S haemolyticus* and *B paramycoides* isolates lose their ability to grow of 500 mg/L with all individual metals. However, isolates were more tolerant of 100 mg/L, but when metal concentrations increased to 500 mg/L, the rate of toxicity increased; as in the toxicity rates of *S. werraensis*, *B. megaterium*, and *S. ginsenosidmutans* that were, respectively, 2.529 mg/L for Zn, 2.319 mg/L for Ni, and 2.772 mg/L for Cr.

The important factor affecting the toxicity and levels of resistance in bacterial cells is the concentration of the metal. The previous results proved that the growth rate of bacteria is strongly influenced by the concentration of metal ions, which is often inversely proportional to the toxicity of heavy metals. This decrease in growth in response to metal stress differs with bacterial species (Mishra and Malik, 2013). It was found that different types of microbic cells respond differently to metallic stresses (Zhang *et al*., 2022). Furthermore, it was explained that bacterial tolerance to heavy metals varies depending on the type of bacteria and metals; thus, the efficacy of microbial remediation varies depending on the type of microbe, resistance,

nature, level, and synergistic toxicity of heavy metals (Kapahi and Sachdeva, 2019; De Sliva *et al*., 2012). The results confirmed that chemical mixtures present in aqueous environments can cause toxicity due to additive or synergistic effects between the components, or the adverse effects can be reduced via antagonistic reactions (Dondero *et al*., 2011). As a result, this fact can explain the previous results of experiment, which may be attributed to chemical processes such as those that occur in wastewater between metal mixtures and result in the metals being transformed to a more mobile and readily bioavailable form, or to other forms that are unavailable or complex.

Interactions between metals that are collectively can increase or reduce toxicity. The synergistic effect of multimetal, which could be due to the increases in the total metal ion concentration compared to the single metal ion systems, leads to a significant difference in concentration between the cell surface and the metal solution; the latter could be a strong driving force for further metal uptake in multimetal systems (Mishra and Malik, 2013). Mixing zinc, nickel, copper, and chromium, which have antagonistic effects on each other, reduced the chemical effects of these metals in the medium, according to the findings. In addition, it was found that the toxicity index indicated that the combined effect of metals like quaternary may have an antagonistic or synergistic effect, and the antagonistic effect of metals is possible (Nweke *et al*., 2017). Antagonistic effects that may be produced during metal interactions may cause a decrease in the uptake or solubility of another metal or lead to precipitation or the formation of complexes and the induction of physicochemical changes in an aqueous environment. As a result, the obtained results can be attributed to antagonistic effects between the tested metals, which resulted in a reduction in toxicity.

The results of the statistical analysis of tolerance and toxicity at 100 mg/L are presented in tables (1) and (2).

Table (1): Statistical data of tolerance limits (1/B) of single metals and quaternary by bacterial isolates

Metals Isolates	Copper	Zinc	Nickel	Chromium	Quaternary
BMA-1	0.519±0.528^{a*}	0.788±0.924^{a*}	0.601±0.730^{a*}	0.305±0.529^{a*}	0.725±0.519^{a*}
ACM-2	0.908±0.138^{b}	0.749±0.298^{b}	0.925±0.799^{b}	0.685±0.386^{b}	0.742±0.402^{b}
DMA-3	0.501±0.514^{c}	0.786±0.905^{c}	0.827±0.661^{c}	0.291±0.504^{c}	0.795±0.575^{c}
STM-4	0.693±0.237^{d}	0.643±0.641^{d}	0.704±0.341^{d}	0.793±0.551^{d}	0.809±0.425^{d}
BMS-5	0.779±0.217^{e}	0.740±0.352^{e}	0.644±0.451^{e}	0.481±0.508^{e}	0.696±0.314^{e}
BME-6	0.786±0.265^{f}	0.789±0.452^{f}	0.804±0.398^{f}	0.717±0.350^{f}	1.033±0.779^{f}
A6MA-7	0.811±0.168^{g}	0.990±0.396^{g}	0.760±0.439^{g}	0.591±0.369^{g}	0.711±0.281^{g}
MIC-8	0.859±0.197^{h}	1.296±0.663^{h}	0.890±0.667^{h}	0.499±0.544^{h}	0.894±0.546^{h}
RMA-9	0.920±0.156^{i}	0.928±0.326^{i}	0.749±0.456^{i}	0.461±0.510^{i}	0.787±0.399^{i}

* Values are mean ± SD of three concentrations (100,300,500) mg/L. Values with different superscript letters per column are statistically significant ($p<0.05$)

Table (2): Statistical data of toxicity level (B) of single metals and quaternary by bacterial isolates

Metals Isolates	Copper	Zinc	Nickel	Chromium	Quaternary
BMA-1	0.981±0.998^{a*}	0.782±0.917^{a*}	1.093±1.329^{a*}	0.363±0.629^{a*}	1.760±0.978^{a*}
ACM-2	1.118±0.169^{b}	1.459±0.479^{b}	1.645±1.011^{b}	1.790±0.922^{a}	1.631±0.826^{b}
DMA-3	1.025±1.051^{c}	0.760±0.876^{c}	1.052±1.316^{c}	0.381±0.661^{a}	1.712±0.985^{c}
STM-4	1.552±0.489^{d}	0.775±0.773^{d}	1.641±0.701^{d}	1.645±0.841^{a}	1.494±0.769^{d}
BMS-5	1.343±0.330^{e}	1.563±0.693^{e}	2.026±1.014^{e}	1.103±1.166^{a}	1.667±0.796^{e}
BME-6	1.325±0.335^{f}	1.549±0.774^{f}	1.423±0.552^{f}	1.604±0.658^{a}	1.370±0.852^{f}
A6MA-7	1.264±0.234^{g}	1.109±0.380^{g}	1.625±0.844^{g}	2.092±0.969^{a}	1.568±0.631^{g}
MIC-8	1.200±0.243^{h}	0.922±0.461^{h}	1.239±0.593^{h}	1.106±1.206^{a}	1.429±0.811^{h}
RMA-9	1.107±0.176^{i}	1.161±0.362^{i}	1.680±0.894^{i}	1.220± 1.350^{a}	1.520±0.777^{i}

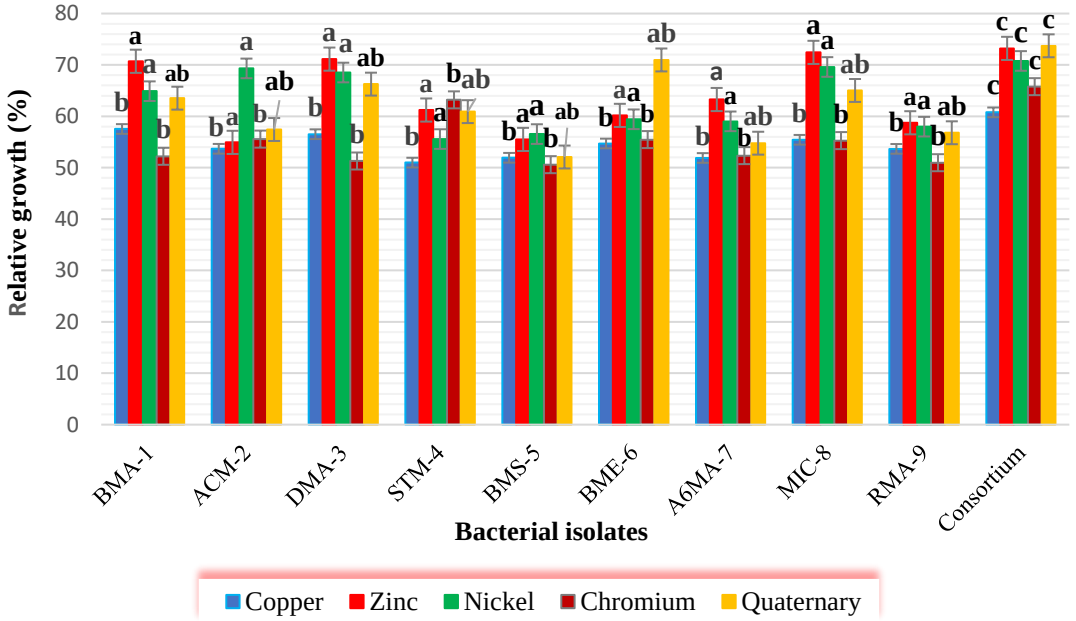
* Values are mean ± SD of three concentrations (100,300,500) mg/L. Values with different superscript letters per column are statistically significant ($p\leq0.05$)

The results of statistical analysis of tolerance and toxicity at 100mg/L in Appendix (G) and Appendix (H), revealed that the tolerance level was significantly different ($p \leq 0.05$) at 100 mg/L of Cu, Zn, Ni, Cr, and quaternary compared with the other concentrations in all bacterial isolates. While there is no significant difference ($P\geq0.05$) in the tolerance level of Cu, Zn, and Cr metals at 300 and 500 mg/L. However, there was a significant difference ($p\leq 0.05$) in the tolerance level for all nickel and quaternary concentrations (100 to 300mg/L).

For toxicity, there were significant differences ($p\leq 0.05$) between 100 mg/L and other concentrations of Cu, Zn, Ni, and quaternary, while there were no significant differences ($P\geq0.05$) in toxicity with all chromium concentrations and between 300 mg/L and 500 mg/L concentrations of Cu, Zn, and Ni. On the contrary. While, the concentrations of quaternary at 300 mg/L and 500 mg/L in addition to 100 were significantly different. Zinc tolerance was higher than Cr and Cu tolerance. The results may support the fact that Zn does not stimulate free radical production, and at high concentrations, it leads to low ROS compared to Cr and Cu, although it may increase oxidative stress (ROS) within the cell at high concentrations (Markowicz *et al.*, 2010; Joutey *et al.*, 2015). Cr (VI) is typically inhibitory of cell activity due to its cell membrane permeability, ability to interact with macromolecules (DNA and proteins), and solubility in cell water (Wang *et al.*, 2017).

3.3 comparison relative growth of isolates individually and consortium

The growth of single and consortium isolates on individual and quaternary metals at the highest initial concentration of metals (100 mg/L) was carried out for 24 hr. The results are shown in Figure 10.



*[a, b, c]Means on columns bearing different superscript letters are significantly different ($p \leq 0.05$)

Figure 10: The Percent of Growth of Bacterial Isolates Individually and Consortium at 100 mg/L Concentrations of Heavy Metals

In the case of individual metals, the results demonstrated that the growth of the isolates with Zn, Ni and quaternary was the best. While a consortium achieved the highest growth with individual and quaternary metals, and it was significantly higher compared to individual isolates, this was due to the biomass abundance and diversity that supported the symbiotic activity of the microbial community in the exploitation of any substance as food and improved absorption ability and detoxification for survival and proliferation (Matz, 2011; Chamy *et al*., 2015). These results are consistent with previous investigations that illustrated that bacteria have the ability to immobilize and remedy metal ions from the media and that consortia are able to thrive in metal-containing media and at the same time immobilize metal ions (Zhang *et al*., 2022; Sannasi *et al.*, 2010). Various researchers reported that bioremediation of pollutants using the microbial consortium is faster and more effective than individual species in the natural environment. Therefore, co-cultivation is better than single bacteria, as it can improve bioremediation as a result of the presence of different species that can play various functional roles for treating pollutants (Bhatt *et al*., 2021; Zhang *et al*., 2021; Varjani *et al*., 2021).

The individual bacterial isolates achieved good growth on the tested metals; the extents of growth of single bacterial isolates on individual metals were as follows: Zn (54.93–72.44%), Ni (55.57–69.60 %), Cu (50.99–57.53%), Cr (50.59–63.20%), and quaternary (52.08–70.97%). while it was found that the growth extents of a mixture of bacterial isolates or consortium were as follows: Cu (60.77%), Zn (73.22%), Ni (70.77%), Cr (65.77%), and quaternary (73.72%).

The statistical analysis of the relative growth of single bacteria and consortium on individual and quaternary metals revealed that growth differed significantly between individual metals Zn, Ni, and quaternary with Cu and Cr ($p \leq 0.05$). The significant difference between isolates was indicated by different superscript letters or lowercase (a, b and c) within columns.

3.4 Heavy metals uptake of isolates individually and consortium

The uptake capability of individual metals and quaternary by single and consortium isolates were investigated, all isolates showed the ability to uptake single metals and quaternary at 100 mg/L as shown Table (3).

Table (3): Metals Uptake in Bacterial Isolates Individually and Consortium

Metal Conc Isolates	Copper	Zinc	Chromium	Nickel	Quaternary
BMA-1	56.33^{a}	60.73^{b}	62.24^{a}	66.90^{b}	64.59^{b}
ACM-2	55.35^{a}	67.82^{b}	52.48^{a}	65.45^{b}	65.37^{b}
DMA-3	52.03^{a}	62.38^{b}	60.95^{a}	69.86^{b}	61.99^{b}
STM-4	60.23^{a}	69.93^{b}	62.29^{a}	71.91^{b}	72.48^{b}
BMS-5	56.76^{a}	61.85^{b}	58.48^{a}	69.60^{b}	67.91^{b}
BME-6	58.85^{a}	76.34^{b}	56.69^{a}	79.94^{b}	75.58^{b}

A6MA-7	56.98[a]	70.91[b]	62.82[a]	79.29[b]	77.84[b]
MIC-8	58.47[a]	75.37[b]	66.73[a]	79.47[b]	77.19[b]
RMA-9	51.62[a]	68.58[b]	60.72[a]	78.39[b]	75.98[b]
consortium	69.16[a]	82.29[b]	75.73[a]	85.46[b]	88.31[b]

*Values bearing different superscript letters are significantly different ($p \leq 0.05$)

The highest metal uptake was observed with Ni and Zn compared with Cu and Cr. At 100 mg/l, the metal uptake ranges of single bacterial isolates were Ni (65.45-79.94%), Zn (60.73-76.34%), Cu (51.62-60.23%), Cr (52.48-66.73%), and quaternary (61.99-77.84%). While were Ni (85.46%), Zn (82.29%), Cu (69.16%), Cr (75.73%), and quaternary (88.31%) with consortium. The results demonstrated that the consortium was able to flourish and compete within a united microbial community, which resulted in an apparent increase in metal removal capability and resistance. The obtained results showed that isolates individually and in consortium were able to achieve multimetal uptake and absorption of quaternary and single metals together at high metal concentrations of 100 mg/L. This supports the application of metal-resistant bacteria in single and consortium forms for bioremediation of heavy metals. It was confirmed that application of metal-resistant bacteria in single and consortium forms for remediation of heavy metals yielded effective results (Chamy *et al*., 2015; Igiri *et al*., 2018). It was shown that bacteria were able to absorb all types of metals because their cells can produce substances capable of adsorbing metal ions onto their cell walls, which they then transfer into the cells.

The statistical analysis of metal uptake illustrated that there were significant differences in metal uptake. The Cu uptake by bacterial isolates was significantly different ($p \leq 0.05$) from Zn, Ni, and quaternary, as well as the Cr uptake was significant different from Ni and quaternary. In contrast, there is no significant difference ($p \geq 0.05$) between Ni, and Zn and uptake of Cu uptake and Cr. The uptake of metals differed with the different types of metals, and this may be attributed to the need or biological importance of the metals for bacteria, which is different according to that.

All isolates werevable to absorb all tested metals. The isolates that achieved the highest multimetal uptake were *Kocuria rhizophila, Bacillus megaterium, Sphingobacterium ginsenosidimutans and S haemolyticus.* The process of absorption through living biomass includes adsorption on the cell wall and entry into the cytoplasm. Gram-positive bacteria like *B megaterium* and *K rhizophila* were achieved high uptake, followed by Gram-negative bacteria like *S ginsenosidimutans*. This was due to the different morphological and biological properties of each group, which affect the way the metal ions spread within the cell parts. It was found that in the cell walls of bacteria, functional groups are responsible for metal binding tasks, including carboxyl, phosphonate, amine, and hydroxyl groups. Therefore, the success of biosorption depends on the diversity of cell wall structures. Gram-positive bacteria have been shown to have a high adsorption capacity due to the thick peptidoglycan layer (Pham *et al*., 2022). It was reported that the structural characteristics of cell walls of gram-positive bacteria, such as higher thickness, anionic capacity, and presence of peptidoglycan, teichoic acid, and teicuronic acid, make them more absorbable than gram-negative bacteria. Extracellular polysaccharides found in cell walls are able to bind metals (Okoro *et al*., 2022). It is worth noting that in addition to the structural characteristics of bacterial cell walls, there are other factors, including the nature of heavy metal ions, the conditions of the growth medium, and the mechanisms used in the absorption, that affect the absorption of the metals by living biomass (Shamim, 2016).

3.5 Correlation Bacterial Growth and Uptake Rate of Selected Heavy Metals

The results of the correlation between bacterial growth and metal uptake are shown in Figure (11). Determining the absorption capacity of each microbial cell and its correlation with its growth rate can be useful in determining the quantity of microbial biomass and their efficiency as biosorbents.

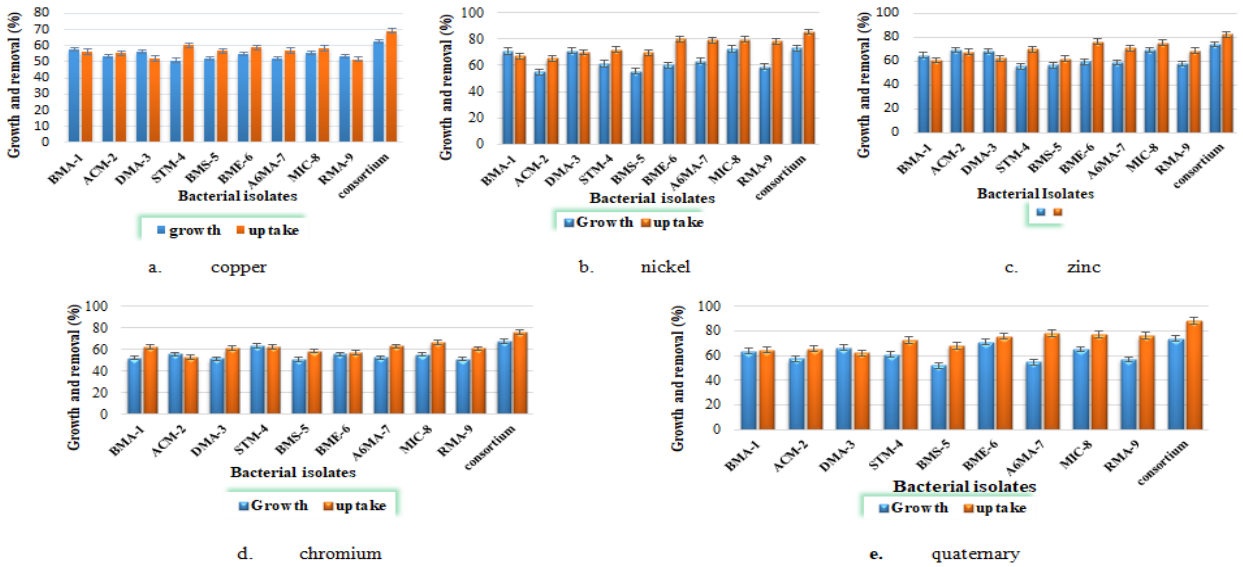


Figure 11: Growth Relative and Uptake of Bacterial Isolates at 100 mg/L of Different Metals (a-e)

The results of the statistical analysis showed that there was no statistically significant correlation between metal uptake and the growth of isolates ($p \geq 0.05$). There was no correlation between uptake and growth due to the high concentration of minerals in the growth medium of bacterial isolates; the uptake of metal ions may have been intended to alleviate or detoxify in metallic stress time more than doubling or creating new biomass. The isolates were able to absorb metals at a high concentration, primarily to reduce toxicity, even after that, the bacteria could use them for growth and biological activities.

3. CONCLUSION

The ability of bacterial isolates to grow, metals uptake and the levels of resistance were examined. The feasibility of use and effectiveness individual and consortium bacterial isolates were investigated. Bacterial isolates distinguished as multi metal resistant and able to grow at different heavy metals with well removal percentage. These characteristics were prominent in consortium compared to single isolates, may be encouraging for their use in the remediation of metal pollutant. These metal resistant bacteria are considered promising and important to take advantage of them for application in the field of bioremediation. Providing more information or data collection on their characteristics such as, resistance and absorption will lead to development bacteria-based biosorbents for the treatment of industrial waste and wastewater.

4. DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

References

1. Akbal, F., and Camc, S., "Copper, chromium and nickel removal from metal plating wastewater by electrocoagulation "Desalination, 269,(2011): 214-222.
2. Aka, R. J. N. and Babalola,O. O. “Effect of bacterial inoculation of strains of pseudomonas aeruginosa, alcaligenes feacalis and bacillus subtilis on germination, growth and heavy metal (Cd, Cr, and Ni) uptake of brassica juncea,” International Journal of Phytoremediation, vol. 18, no. 2, pp. 200–209, 2016.
3. Ali, H., Khan, E., Ilahi, I. "Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation", Journal of Chemistry, vol. 2019, Article ID 6730305, 14 pages, 2019.
4. Baker, B.J.and Banfield, J.F . (2003). Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44: 139–152.
5. Banerjee, A., Jhariya, M. K., Yadav, D. K., Raj, A. (2018) Micro-remediation of Metals: A New Frontier in Bioremediation. Handbook of Environmental Materials Management pp 1-36.
6. Chamy, R., Rosenkranz, F.and Soler, L. 2015. Biodegradation and Bioremediation of Polluted Systems-new advances and technologies. First puplished, December, 2015. Technology & Engineering , 2015 - 178 pages.
7. Chen, Y ., Jiang, Y., Huang, H., Mou, L ., Ru, J., Zhao, J.and Xiao, S.(2018).Long-term and high-863 concentration heavy-metal contamination stronglyinfluences the microbiome and functional 864 genes in Yellow River sediments. Science of the Total Environment 637–638 (2018) 1400–1412.
8. El-Shanshoury, A.El. R., Elsilk, S.E. and Ateya P. S. (2013). Uptake of some heavy metals by metal resistant Enterobacter sp. isolate from Egypt . Afr. J. Microbiol. Res. Vol. 7(23), pp. 2875-2884, 4 June, 2013.
9. Gaetke, L.M.and Chow, C.K. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189, 147, 2003.
10. Gautam, P. K, Gautam, R. K, Banerjee, S., Chattopadhyaya, M. C. and Pandey, J. D. Heavy metals in the environment: Fate, transport, toxicity and remediation technologies Chapter • February 2016.
11. Guine, V., Martins, J.M.F, Causse, B., Durand, A., Gaudet, J.P and Spadini, L. (2007) Effect of cultivation and experimental conditions on the surface reactivity of the metal-resistant bacteria Cupriavidus metallidurans CH34 to protons, cadmium and zinc. Chem Geol 236:266–280
12. Hernandez, A., Mellado, R.P. and Martinez, J.L., Metal accumulation and vanadium induced multidrug resistance by environmental isolates of E.coli and E. cloacae. Appl. Environ. Microbiol., 64: 4317- 4320 (1998).
13. Hrynkiewicz K., Baum C. “Application of microorganisms in bioremediation of environment from heavy metals,” Environmental Deterioration and Human Health: Natural and Anthropogenic Determinants, pp. 215–227, 2014.
14. Huang, H., Wu, K., Khan, A., Jiang, Y., Ling, Z.and Liu, et al., P. A novel Pseudomonas gessardii strain LZ-E simultaneously degrades naphthalene and reduces hexavalent chromium. Bioresour. Technol., 207 (2016), pp. 370-378
15. Huang, H., Tao, X., Jiang, Y., Khan, A., Wu,Q. and Yu, et al., X. The naphthalene catabolic protein NahG plays a key role in hexavalent chromium reduction in Pseudomonas brassicacearum LZ-4 Sci. Rep., 7 (2017), p. 9670
16. Igiri, B. E. , Okoduwa, S. I. R., Idoko, G. O., Akabuogu, E. P., Adeyi, A. O. and Ejiogu, I. K.(2018). Toxicity and Bioremediation of Heavy Metals Contaminated Ecosystem from Tannery Wastewater: A Review. Hindawi Journal of Toxicology Volume 2018, Article ID 2568038, 16 pages.
17. Irawati , W., Yuwono, T. , Soedarsono, J., Hartiko, H. The potency of copper-resistant bacteria cupriavidus sp. irc4 isolated from industrial wastewater treatment plant in rungkut-surabaya as a bioremediation agent for heavy metals. The 3rd International Conference on Biological Science 2013. ISSN 2413-0877 Volume 2 (2015) 375-381.

18. Issazadeh,. K., Jahanpour,. N., Pourghorbanali,. F., Raeisi,. G. Heavy metals resistance by bacterial strains. Annals of Biological Research. 2013;4(2):60-63.
19. Joutey, N.T., Sayel, H., Bahafid, W. and El Ghachtouli, N. Mechanisms of hexavalent chromium resistance and removal by microorganisms. Rev. Environ. Contam. Toxicol. 2015, 233, 45–69.
20. Losada, L., DebRoy, C., Radune, D., Kim, M., Sanka,R.and L. Brinkac, et al.Whole genome 1121 sequencing of diverse Shiga toxin-producing and non-producing Escherichia coli strains reveals 1122 a variety of virulence and novel antibiotic resistance plasmids Plasmid, 83 (2016), pp. 8-11
21. Malakul, P., Srinivasan, K.R. and Wang, H.Y., Metal toxicity reduction in naphthalene biodegradation by use of metal-chelating adsorbents. Appl. Environ. Microbiol., 64: 4610-4613 (1998).
22. Markowicz, A., Płociniczak, T., Piotrowska-Seget, Z.(2010). Response of Bacteria to Heavy Metals Measured as Changes in FAME Profiles. Polish J. of Environ. Stud. Vol. 19, No. 5 (2010), 957-965.
23. Marzan, L ., W., Hossain, M., Mina, S. A., Akter, Y. and Chowdhury, A.M. Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. The Egyptian Journal of Aquatic Research, Volume 43, Issue 1, March 2017, Pages 65-74.
24. Matz, C. Competition, communication, cooperation: molecular crosstalk in multi-species
25. biofilms. In: Flemming H-C, Wingender J, Szewzyk U, editors. Biofilm Highlights. London:
26. Springer-Verlag Berlin Heidelberg, 2011. pp. 29–40.
27. Mishra, A.and Malik, A.(2013). Recent Advances in Microbial Metal Bioaccumulation. Critical Reviews in Environmental Science and Technology, 43:1162–1222, 2013.
28. Munees, A., and Malik, A. 2012. Bioaccumulation of Heavy Metals by Zinc Resistant Bacteria Isolated from Agricultural Soils Irrigated with Wastewater. Bacteriology Journal, Volume 2 (1): 12-21, 2012.
29. Murthy,S.,Bali,G.and Sarangi,S.K. (2012). Biosorption of Lead by Bacillus cereus Isolated from Industrial Effluents. British Biotechnology Journal. 2(2): 73-84, 2012.
30. Nweke C.O., Mbachu, I.A.C, Opurum, C.C and Mbagwu, C.L. Toxicity of quaternary mixtures of metals to aquatic microbial community. International Research Journal of Environmental Sciences ISSN 2319–1414 Vol. 6(3), 30-37, March (2017).
31. Okoduwa, S. I. R., Igiri, B., Udeh, C. B., Edenta, . C. and Gauje, B. "Tannery effluent treatment by yeast species isolates from watermelon," Toxics, vol. 5, no. 1, p. 6, 2017.
32. Okolo, V. N., Olowolafe, E. A., Akawu,. I. and Okoduwa,S. I. R. "Effects of industrial effluents 581 on soil resource in challawa industrial area," Journal of Global Ecology and Environment, vol. 5, no. 1, p. 10, 2016.View.
33. Oremland, R. S., Maest, J. T. A. S., Presser, T. S., Miller, L. G., and Culbertson, C. W. Selenate reduction to elemental selenium by anaerobic bacteria in sediments and culture: biogeochemical significance of a novel sulfate-independent respiration, Appl. Environ. Microbiol., 55 (1989) 2333 – 2343.
34. Pandit ,R.J. , Patel, .B, Kunjadia ,P.D .and Nagee, A .(2013). Isolation, characterization and molecular identification of heavy metal resistant bacteria from industrial effluents, Amala-khadi- Ankleshwar, Gujarat. International Journal of Environmental Sciences Volume 3 No.5, 2013.
35. Peng, C., S. Song, Lu, S., López-Valdivieso, A. Electroplating wastewater treatment through chemical precipitation and electrodialysis. The European Journal of Mineral Processing and Environmental Protection Vol.4, No.3, 1303-0868, 2004, pp. 210-215.
36. Qin, B., Luo, H., Liu, G., Zhang, R., Chen, S., Hou, Y., and Luo, Y., "Nickel ion removal from wastewater using the microbial electrolysis cell "Bioresource technology, 121,(2012): 458-461.
37. Ruttens, J., Colpaert, M. Mench, J. Boisson, R. Carleer, J. Vangronsveld, Phytostabilization of a metal contaminated sandy soil. II. Influence of compost and/or inorganic metal immobilizing soil amendments on metal leaching, Environ. Pollut., 144 (2006) 533 – 539.
38. Sannasi, P. Salmijah, S. and Kader, J. Effect of heavy metals to bacterial culture and the removal of heavy metals from an industrial effluent. Biosci., Biotech. Res. Asia, Vol. 7(2), 543-557 (2010).
39. Shaaban, M. T. Ibrahim, H. A.H. Abouhend, A. S. and ElMoselhy, K. M. "Removal of Heavy Metals from Aqueous Solutions Using Multi-Metals and Antibiotics Resistant Bacterium Isolated from the Red Sea, Egypt." American Journal of Microbiological Research, vol. 3, no. 3 (2015): 93-106.
40. Shamim, S. Comparative analysis of metal resistance, accumulation and antioxidant
41. enzymes in Cupriavidus metallidruans CH34 and Pseudomonas putida mt2 during cadmium stress. Ph. D. thesis. Department of Microbiology and Molecular Genetics, University of the Punjab: Pakistan; 2016.
42. Silva,A., Carvalho,M.,Souza,S.,Dias,P.,Filho,R.,Saramago,C.,Bento,C.and Hofer ,E.(2012). Heavy metal tolerance (Cr, Ag AND Hg) in bacteria isolated from sewage. Braz J Microbiol. 2012 Oct-Dec; 43(4): 1620–1631.
43. Srivastava, S., Agrawal, S.B. & Mondal, M.K. A review on progress of heavy metal removal using adsorbents of microbial and plant origin. Environ Sci Pollut Res 22, 15386–15415 (2015).
44. Stams, A.J.M and Plugge, C.M. Electron transfer in syntrophic communities of anaerobic
45. bacteria and archaea. Nat Rev Microbiol. 2009;7:568–577.
46. Stohs, S.J.and Bagchi, D. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321, 1995.
47. Syed,S., Chinthala, P. (2015).Heavy Metal Detoxification by Different Bacillus Species Isolated from Solar Salterns. Scientifica (Cairo) 2015;2015:319760.
48. Vasudevan, V., Padmavathy, N., Tewari, S.C. and Dhingra, J. (2001) Biosorption of heavy metal ions. J Sci Ind Res 60(2):112–120
49. Wierzba, S., Lata, A.aBiosorption lead(II) and nikel(II) from an aqueous solution by bacterial
50. Biomass. Pol. J. Chem. Tech., Vol. 12, No. 3, 2010.

51. Xie, P., Hao, X., Herzberg, M., Luo,Y. Nies,D.H.and Wei, G. Genomic analyses of metal resistance 1340 genes in three plant growth promoting bacteria of legume plants in northwest mine tailings, 1341 China J. Environ. Sci., 27 (2015), pp. 179-187.
52. Zimmerman, R. Abiotic and microbial oxidation of laboratory-produced black carbon (Biochar), Environ. Sci. Technol., 44 (2010) 1295 – 1301.
53. Acknowledgements:
54. This study was sponsored by the Fundamental Research Grant Scheme (FRGS) [(KPT Reference Code: FRGS/1/2019/STG05/UPM/02/11) (Vote Number: 5540228) (Project Code: 01-01-19-2103FR)] awarded by the Ministry of Higher Education (MOHE), Malaysia.
55. Corresponding author
56. Correspondence to Eeman.A.Alhammadi, emanbensaeed@gmail.com
57. Ethics declarations
58. Ethical approvals
59. Since this study did not recruit any human and/or animal subjects, this section does not apply.
60. Consent to participate
61. None of the authors has any objection to participating in the study.
62. Consent for publication
63. None of the authors has any objection to publishing in the study.
64. Competing interests
65. The authors declare no competing interests.