BIOREMEDIATION OF HEAVY METALS CONTAMINATED SOIL BY USING INDIGENOUS METALLOTOLERANT BACTERIAL ISOLATES

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Abstract. The pollution caused by heavy metals is a major problem for our environment, as the high levels of exposure to these metals have harmful consequences for wildlife, vegetation, and human health. Even in trace amounts, several heavy metals, including lead, mercury, cadmium, zinc, arsenic, and nickel, not only possess carcinogenic properties but also have the capacity to cause genetic mutations. In this study, a total of 150 bacteria were isolated, out of which 25 were selected for secondary screening. After secondary screening, five strains were further processed based on their maximum tolerance level. The required indigenous metallotolerant bacterial strains were isolated and identified based on phenotypical and genotypical characterization. The phenotypical characteristics and topology of the phylogram confirm that the bacterial isolate 1LB is *Kingella sp.*, 2LB is *Listeria sp.*, 3LB is *Bacillus sp.*, 4LB is *Pseudomonas putida*, and 5LB is *Cupriavidus necator*. According to the result while using LB medium, all the bacterial isolates showed the highest tolerance level against different heavy metal concentrations, i.e., 1LB and 4LB bacterial isolates showed the highest tolerance rate against copper (Cu), while 2LB and 5LB bacterial isolates showed maximum tolerance against chromium (Cr), and 3LB bacterial isolates showed the highest tolerance rate against lead (Pb). Thus, the LB medium was used for the optimization of bioremediation purposes. For bioremediation of heavy metal-contaminated soil, the maximum removal efficiency was 83.80% of the 4LB bacterial strain for Pb, 90.49% of the 5LB bacterial strain for Cu, and 81.87% of the 1LB and 2LB bacterial strains for Cr in the YPG medium. Thus, the results indicate that indigenous heavy metal-tolerant bacterial strains of the area could be used for biological remediation of heavy-metal-contaminated soil, which is the most effective, economical, and environmentally friendly approach as an alternative to traditional methods.

Keywords: *Korangi Industrial Area, heavy metals contaminated soil, soil pollution, metallotolerant indigenous bacteria, bioremediation*

Introduction

One of the biggest problems affecting human society today, particularly in developing nations, is pollution. Despite being a result of human activities, it has a detrimental effect on resources and human settings. Pollution is considered a major

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problem for humans. Pollutants can cause immediate environmental harm or subtle disruptions to the slightly stable organic food chain that are only identified over extended periods (Naveed et al., 2023a, Naveed et al., 2023b; Zameer et al., 2023; Naggar et al., 2014). Almost any human action that impairs or slows down the quality of the environment is considered a form of pollution. Even though environmental pollution is nothing new, it continues to be one of the most significant problems in the world and a major source of illness and mortality. In 2015, it was estimated that 9 million premature deaths were caused by illnesses associated to pollution, which is more than three times the number of deaths caused by dengue fever, AIDS, and tuberculosis combined (Zhang and Batterman, 2013).

The average level of environmental pollution is higher in developing than in high-income nations, presumably as a result of the lack of resources, weak regulations, and ignorance of the sources of pollution. Humans probably face pollution every day without even realizing it, or perhaps our fast-moving lifestyles have made us resistant to it (Ukaogo et al., 2020). Unaware of the different sorts of pollution, humans perform activities that result in harmful byproducts in amounts and forms that the ecosystem is no longer able to compensate for without causing a complete system breakdown. For instance, inappropriate treatment of electronic wastes, burning of brush, dumping of household and agricultural waste into bodies of water, use of pesticides while collecting marine life and deforestation, they all contribute to severe air, land and water pollution. Additionally, human activity and its impact on the environment grow in tandem with human population density. In addition to people, other aquatic and terrestrial creatures are also affected by the consequences, including microbes, which tend to maintain the biogeochemical function necessary for ecosystem sustainability due to their number and diversity (McMichael, 2000). A number of variables, such as industrialization, urbanization, population expansion, exploration, and mining, as well as the transboundary migration of pollutants from developed to developing countries, are responsible for environmental degradation. Pollution has continued to be a global problem because of multilateral pollution. No country can afford to ignore pollution because it can be found in one country producing issues in another via many channels, principally air and water (Abdullah, 1995). A fundamental source of toxic metal pollution in the environment is the transfer of damaged electrical and electronic equipment over the boundary from industrialized to developing nations under the presence of closing the digital disparity (Chapman et al.). Activities include mining, deforestation, landfills, garbage dumps, industrial effluents, agricultural runoff, and electronic waste into water bodies. People frequently say that they are unaware of how human activity has disrupted the natural environment, and as a result, these activities continue even if they are known to cause major illnesses and even death. Some human activities that have been proven to be hazardous to the environment are nonetheless carried out in middle- and low-income countries, either as a result of weak laws, a lack of strict punishments, or a lack of concern for the effects of such practices on the environment and public health (Vesilind et al., 2013).

Microbial bioremediation has attracted interest on a global scale, probably as a result of the viability and environmental friendliness of this approach to environmental restoration. Pollution comes in many forms, but there are three primary types (Mia et al., 2019).

Although heavy metals are prevalent in our environment naturally as they are necessary for life, they can be hazardous when they accumulate in organisms. Some of

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the common heavy metals that pollute the environment include lead, nickel, copper, chromium, mercury, cadmium, and nickel (Hazrat et al., 2019; Hadi and Aziz, 2015). Most heavy metals, particularly those that are present in the dissolved form are frequently associated with hazardous issues and environmental contamination. The extreme occurrence of any of these heavy metals puts individuals in danger and interrupts many good characteristics of the environment. Additionally, removing these HM is more challenging because they are not biodegradable (Ali et al., 2013). Heavy metals have a substantial impact on food production, security, and health in addition to deteriorating the quality of the soil (Agarwal, 2009). However, some HM may accumulate in plant tissues to moderately abnormal forms without obvious negative consequences or decreased yields (Mohammed et al., 2011). Even in very small amounts, some HM is dangerous for plants. Plants cultivated in these highly contaminated conditions have physiological, biochemical, and metabolic changes that lead to metal buildup, decreased biomass production, and sluggish biomass development. There are several things to consider when analyzing the toxicity of heavy metals in plants (Huamain et al., 1999).

Soils naturally cover the surface of the ground and serve as the interface between solids (such as geological materials and biologically degraded matter), liquids (such as water), and gases (such as air in soil pores) (Liu et al., 2018). One distinguishing characteristic of soil is present in soil minerals (Dominati et al., 2010). 'Microbiome' main part of the soil ecosystem, affects several soil-related processes. Deeper soil layers, as a result, make up a component of the soil microbiome that has received less attention. The term "subsoils" is frequently used to describe these deeper layers, which are classified as soil underneath the top layer (Angst et al., 2018). In heavy metal-contaminated soil, only bacteria with the ability to tolerate and overcome the toxicity of heavy metals and convert them into detoxification byproducts can survive. This study aims to isolate strains with maximum bioremediation potential. Thus, the current study focused on the indigenous bacterial based biological remediation of heavy metals contaminated soil that could be used for in-situ or ex-situ bioremediation techniques.

Materials and methods

Sample collection and isolation

A sharp-edged plastic spatula was used to plough a layer of 0–20 cm soil from several locations spaced 10–20 meters apart in order to get a total of 15 soil samples from the Korangi Industrial Estate Karachi (*Fig. 1*). Following labeling and sterile plastic bag sealing, the samples were carefully moved to the Microbiology Research Laboratory for further processing. By using the serial dilution technique, indigenous metallotolerant bacteria were isolated from soil. For isolation procedure 9 mL of sterilized distilled water was used to suspend 1 gram of soil placed in various containers, and treated to a 15-min heat shock at 90°C monitored by cooling to room temperature. After that, the proper serial dilution was performed. A 0.1 mL sample was spread on nutrient agar plates and then incubated at 50°C for 24-48 h to produce normal bacterial colonies. Colonies with clearly different morphologies were further purified by sub culturing and streaking on nutrient agar. On freshly ready starch agar plates, the isolated pure organisms were chosen and cultured at 50°C for 48 h. After that, they were kept at 4°C for advance analysis (Khan et al., 2019).

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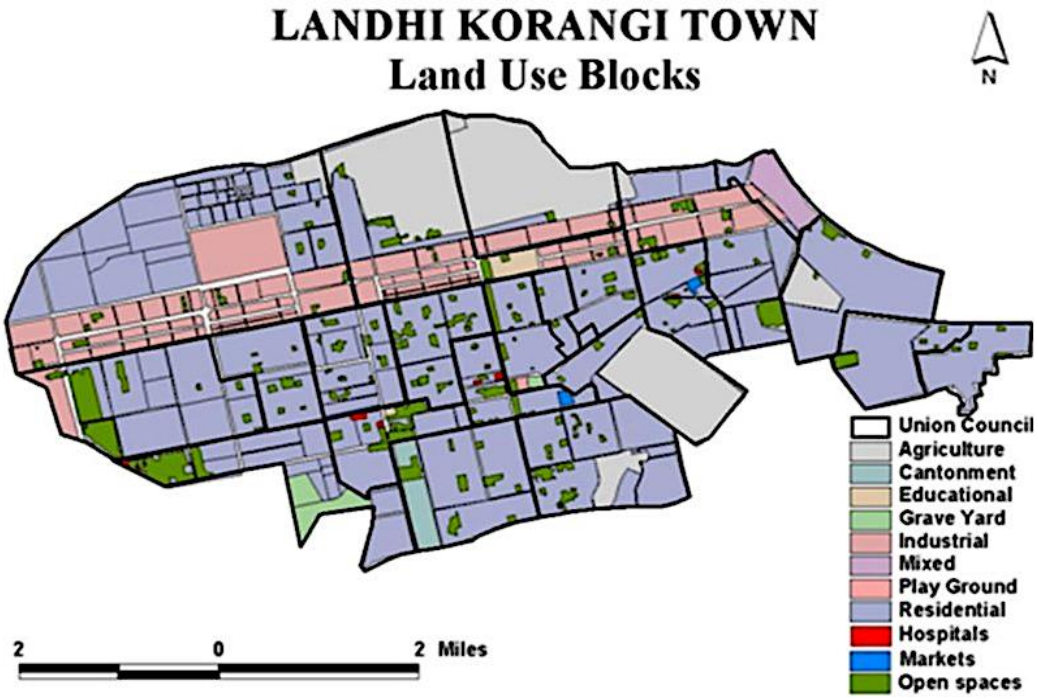

Figure 1. *Sample site from Korangi Industrial Estate Karachi (Khan et al., 2017)*

Identification of metallotolerant bacterial isolates

Morphological characterization

The different bacterial strains were identified by observing colony morphology, including color, shape, edge, elevation, and surface, as well as cell morphology, including shape, gram reaction, and organization were examined for identification of the selected bacterial strain by using different growth media, such as Nutrient Agar, MacConkey Agar, Luria-Bertani media (Agrawal and Agrawal, 2013).

Gram staining

All bacteria first take up the crystal violet dye, however the solvent eventually destroys the lipid layer of gram-negative bacteria. As the lipid layer becomes less effective, gram negatives lose their prominent stain. While inhibiting the spread of the violet-iodine combination and rendering bacteria colorless, solvent dehydrates and seals pores in gram-positive cell walls (Greenwood et al., 2012).

Biochemical characterization

The bacterial strain was tested biochemically in a variety of ways, including the catalase test, Coagulase Test, Citrate Agar Test, Indole Test, Kovac's Oxidase Test and Urease Test. Briefly described as follows:

Catalase test

Catalase, an enzyme that helps oxygen move from hydrogen peroxide (H_2O_2), is confirmed by this experiment. The catalase enzyme converts hydrogen peroxide into oxygen and water. The fast formation of oxygen bubbles when a little amount of inoculum

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is exposed to hydrogen peroxide serves as a sign that the enzyme is present. Lack of or insufficient bubble production is a sign of catalase insufficiency (Reiner, 2010).

Coagulase test

A protein that resembles an enzyme called coagulase can change fibrinogen into fibrin, which is what causes blood to clot. Both bound and free coagulase can be produced by *Staphylococcus aureus* (Sperber and Tatini, 1975). Direct interactions between the bacterial cell wall-bound coagulation enzyme (clumping factor) and fibrinogen occur. Fibrinogen precipitates on the *staphylococcal* cell when plasma is combined with a bacterial solution, causing the cells to cluster. There is no need of coagulase-reacting factor in this.

When plasma coagulase-reacting factor (CRP), a thrombin-derived or modified molecule, is activated, a coagulase-CRP complex is formed. This complex subsequently interacts with fibrinogen and in result fibrin clot is produce.

Citrate agar test

This experiment determines whether an organism can use citrate as a source of energy. The only sources of carbon and nitrogen in the media are citrate and inorganic ammonium salts ($NH_4H_2PO_4$). Bacteria that may flourish in this medium create citrate-permease, an enzyme that has the capacity to convert citrate into pyruvate. The metabolic cycle of the organism can then incorporate pyruvate to produce energy. When there is growth, citrate, a Krebs cycle intermediate, is utilized. As citrate is broken down by bacteria, the ammonium salts are changed into ammonia, increasing alkalinity. The medium's bromothymol blue indicator transforms from green to blue above pH 7.6, as a result of the pH change (MacWilliams, 2009).

Indole test

Indole test is performed to check out the capability of an organism to break tryptophan to produce indole (MacWilliams, 2012). Indole was detected using the Kovac's reagent, which involves para-dimethylaminobenzaldehyde, isoamyl alcohol, and concentrated hydrochloric acid in an acidic environment: In the presence of para-dimethylaminobenzaldehyde, indole interacts to produce the red dye rosindole.

Kovac's oxidase test

By catalyzing the transfer of electrons from donors to a redox dye tetramethyl-p-phenylene-diamine, the cytochrome. oxidase system, which is present in bacteria, indicates oxidase test. Cytochrome c is more easily oxidized because of oxidase enzyme. When the reagent comes into touch with cytochrome C, it turns blue or purple, which is present in the respiratory chains of oxidase-positive organisms (Chaudhary, 2019).

Urease test

When amino acids were decarboxylated, urea was produced. Ammonia and CO_2 was created during urea degradation. Phenol red, which transformed from a pale orange color at a pH of 6.8 to a magenta (pink) color at a pH of 8.1, was used to detect pH changes in solutions by indicating the presence of ammonia, which caused the solution

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to become more alkaline. In 24 h, urease-positive bacteria turned the medium pink. Negative organisms either generated yellow or no color change as a result of acid production (Brink, 2010).

Molecular characterization

DNA extraction

For DNA extraction, colonies were grown on NA media for 48 h at 28°C, and genomic DNA were extracted using a straightforward boiling/heating procedure. A loop full of a pure isolate was suspended in 500 mL DW, heated to 95° for 10 min, chilled on ice, and then centrifuged for 3 min at 13,000 g. The supernatant was kept frozen at 20°C until it was required (Izadiyan and Taghavi, 2020).

Polymerase chain reaction (PCR) assay

The internal transcribed spacer (ITS) from the isolated strains were amplified and sequenced using specific primers (*Table 1*) to determine the phylogenetic correlation between all of the isolated bacteria and different strains reported at the National Center for Biotechnology Information (NCBI) (Pederson et al., 1997). For amplification, a Perkin-Elmer GeneAmp PCR System 2400 PCR cycle was employed. The reaction mixture system contained 10 buffer, 2.5 ul, 1.25 U of Taq DNA polymerase, 0.8 mM of dNTP, 0.5 mM of either ITS-F or ITS-R, 1 l of DNA template, and 16 ul of water (for a total of 25 ul). Initial denaturation at 94°C for 10 min, preliminary denaturation at 94°C for 1 min, annealing for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min were all steps in the PCR amplification process. 32 cycles of PCR were run in total. A 1% agarose gel electrophoresis was used to separate the PCR products at the end (Adriko et al., 2014). Sequencing was done by Genescript Company in Nanjing, China, while gel bands with the required product were obtained using the TIANgel Midi Purification kit from TIAGEN BIOTECH in Beijing, China.

Sequencing and phylogenetic analysis

After sequencing the retrieved sequences were analyzed by using NCBI BLAST study (http://blastt.ncbi.nlm.nih.gov/Blast.cgi) to incorporate the sequences obtained previously for the development of the phylogenetic tree. Sequences having 98 percent similarity to currently existent sequences were considered to be the similar species. Furthermore, numerous alignments were done using Clustal X 1.83, and MrEGA 4.00 in order to construct the phylogenetics tree (Tamura et al., 2013).

Bioremediation potential analysis

Test for tolerance trend of bacteria isolates to various concentrations of heavy metal (MIC)

Each isolated strain of bacteria was added into a test tube with 10 ml of nutritional broth and left to develop for 24 h at room temperature. Twenty microliters (20 µl) of the bacterial isolates were each inoculated into a nutrient broth that included various concentrations of heavy metals; 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 8 mg/ml respectively for Cr, Pb and Zn while the concentrations for Cadmium were 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml and 1 mg/ml. A spectrophotometer was used to measure the inhibitory concentrations in comparison to a nutritional broth (blank) that contained the similar quantity of heavy metals (Pandit et al., 2013).

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Table 1. *Primers used for molecular identification*

Primer	Sequence 5′- 3′	Annealing temperatures
16S rRNA-F	5'AGA GTT TGA TCC TGG CTC AG 3'	55-60°C
16S rRNA-R	5'GGT TAC CTT GTT ACG ACTT 3'	55-60°C

Screening for heavy metal tolerant bacterial isolates

In order to screen the isolated bacterial isolates for heavy metals tolerance Six heavy metals: chromium (Cr), copper (Cu + 2) and lead (Pb + 2) were used in their salt structures as: CuSO4•5H2O and PbSO4 or $Pb(NO_3)_2$ and $K_2Cr_2O_7$ According to the method of Vijayadeep and Sastry (2014) heavy metals salt solutions (25, 50, 75, 100 µg/mL) were prepared in distilled water to obtain concentrations and sterilized by 0.2-µm pore-size Millipore sterile filters. The bacterial isolates were checked for the heavy metals' tolerance using the agar well diffusion method as reported by Collins et al. (1985) in sterile NAM plates. On incubation of plates at 35-37°C for 72 h, the inhibition zones (the distance between the end of the zone and the edge of the well, mm) were measured. Isolates showing a clear zone of 1 mm or less was considered as resistance (R) isolate according to Rani and Moreira (2010). All the bacterial isolates were further screened by streaking on NA medium containing different concentration of each heavy metals separately. Streaking of bacterial isolates on normal NA medium reserved as control (normal growth) for comparison of growth of bacterial isolates on NA medium containing different concentration of heavy metals. Observations on growth of fungal isolates were made after 96 h of incubation.

Bacterial bioleaching

Three different types of media, including yeast peptone glucose (YPG), potato dextrose broth (PDB) and Luria bertani (Merabishvili et al.) were used to bioleach polluted soil samples. Then the bioleaching of the heavy metals was done in three types of media (Dong et al., 2023) by the following way.

Optimization with YPG medium

The 1 mL of suspension of each bacterial isolate was inoculated into 100 mL of YPG medium (1%Yeast Extract, 2% Peptone and 2% Glucose), Flasks were placed in 250 mL of sterile conical flasks and incubated at 32°C for 72 h. After 3 days of incubation, in to these conical flasks 1 g of sterilized contaminated soil sample was added. These flasks were then sited in a shaking incubator for a further 72 h at a constant shake rate of 150 rpm and a temperature of 32°C.

Optimization with LB medium

The 1 mL suspension of each bacterial strain were inoculated into the 100 mL of autoclaved LB medium in a sterile 250 mL flasks and kept the flasks in a shaking incubator and incubated at 32°C for 72 h at 150 rpm. After 72 h of incubation, then the autoclaved 1 g of contaminated soil were added into each flask and again placed the flasks in a shaking incubator at 32°C for further 72 h at 150 rpm.

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Optimization with PDB medium

The 1 mL suspension of each bacterial isolate were inoculated into the 100 mL of autoclaved PDB medium (Potato Infusion 0.4% and Dextrose 2%) in a sterile 250 mL flasks and kept the flasks in a shaking incubator and incubated at 32°C for 72 h at 150 rpm. After 72 h of incubation, the autoclaved 1 g of contaminated soil were added into each flask and again placed the flasks in a shaking incubator at 32°C for further 72 h at 150 rpm.

Medium optimization for best growth of metallotolerant bacterial isolates

Each isolated strain of bacteria was put into a test tube comprising 10 ml of nutrient broth (NB), YPG and LB medium along with 100 mg/L of each metal in a separate flask and allowed to grow for 24 h at 30 °C. During the process, blank nutrient broth containing the similar metal concentration was used. To determine metal tolerance OD was measured in a spectrophotometer at 60 nm wavelength and results were compared with the control. (Pandit et al., 2013). Indicating the effective remediator or heavy metal that was utilized, the medium demonstrating the highest growth for bacterial isolates.

Evaluation of the potentials of metallotolerant bacterial isolates for bioremediation from heavy metals contaminated soil

Each 250 mL flask containing 100 mL of YPG (yeast peptone glycerol) media were autoclaved at 121°C for 15 min. A 2 gram of pre disinfected contaminated soil were added along with 10% (v/v) (3 × 109 Cfu/mL) of each microbial inoculum into 250 mL flask containing 100 mL of YPG medium and then followed by incubation at 30°C in shaking incubator for a week at 120 rpm. (IS-RDS3C) at 30°C for 7 days at 120 rpm (Nwaehiri et al., 2020).

To create a synergistic effect, a mixed culture consortium (MCC) of all the bacteria identified for each heavy metal was put into a 250 mL flask. After 4-5 days of experimentation, the heavy metals concentration was measured by using an atomic absorption spectrophotometer (AAS). Control flasks were cultured concurrently with the experimental flasks and contained the heavy metal salts but no test organisms. Duplicate runs of each experimental setup were performed (Oaikhena et al., 2016).

Uptake efficiency of metallotolerant bacterial isolates

For estimating the metal uptake efficiency or metal tolerance ability of the isolated bacteria, the previously adopted protocols were used and removal efficiency of the isolates for Pb, Cu and Cr was calculated by using the following equation:

$$\text{Removal efficiency\%} = \frac{\text{Initial metal conc.} - \text{Final metal conc.}}{\text{Initial metal conc.}} \times 100$$

Statistical analysis

In order to compare the means of the studied treatments, a randomized entire design was employed on the data, which were prepared using mean and standard deviation (n = 3). The cutoff for statistical importance was P 0.05. The average results from three replicates, which are listed in each column with the similar letters, are not statistically changed (P > 0.05) (Test Duncan Multiple Range) (Littell, 1989).

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Results

Isolation and identification

To isolate heavy metal resistant bacteria, 10 g of soil from the heavy metal contaminated area of Korangi Industrial Estate Karachi was collected. Then 1 g of the sample was serially diluted followed by inoculation on LB media.

Morphological characterization

The bacterial isolates were then evaluated for morphological characteristics, by using different medium which showed that *Kingella sp.* (1LB) fails to grow on MacConkey, *Listeria sp.* (2LB) produced Listeria is a gram-positive, motile, fixed, short, facultative anaerobic, non-spore-forming rod that grows in blue gray colonies on nutrient agar, *Bacillus sp.* (3LB) produced Medium-sized colony, gray-white, spherical, opaque, flat, and drying on LB medium, while *Salmonella sp.* (4LB) were colorless and transparent and typically do not alter appearance of the MacConkey medium and *Staphylococcus sp.* (5LB) generate enterococci and light pink to red isolates (*Figs. A1* and *A2*).

Gram staining

Interaction between bacteria and dye, result in either purple or transform to pink or red bacteria. Purple color indicates the presence of Gram-positive. Gram negative bacteria indicated with the appearance of pink or red, they are Gram-negative. As stated in the findings section, three isolates—2LB, 3LB, and 5LB—were determined to be gram-positive cocci whereas the other two—1LB and 4LB—were gram-negative rods bacteria (*Table 2; Fig. A3*).

Table 2. *Gram's staining and microscopy of bacterial isolates*

	1LB	**2LB**	**3LB**	**4LB**	**5LB**
Gram test	-	+	+	-	+
Shapes	RODS	COCCI	COCCI	RODS	COCCI

Biochemical characterization

The following common biochemical assays were used to identify bacterial isolates.

Catalase test

After treating the test bacterium with some drops of 3% H_2O_2 on a glass slide, it formed gas bubbles, indicating a positive catalase test. The catalase test showed 1LB negative, 2LB positive, 3LB positive, 4LB positive and 5LB positive results for bacterial isolates (*Fig. A4*).

Citrate agar test

Salmonella typhimurium inoculation turn Citrate agar to royal blue color. The citrate test came back with a positive result. Citrate agar stays green after being inoculated with Escherichia coli. The citrate test came back with a negative result. The Citrate Agar test showed 1LB negative, 2LB positive, 3LB negative, 4LB positive and 5LB negative, results for bacterial isolates (*Fig. A5*).

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Indole test

Positive result of indole test was marked by the production of a reddish color ring on surface of glass tubes quickly after the Kovac's reagent was added. No color or yellow color indicate indole negative. Results from the Indole test for bacterial isolates were 1LB negative, 2LB negative, 3LB positive, 4LB negative, and 5LB negative (*Fig. A6*).

Urease test

The production of urea is confirmed by the appearance of bright pink (fuchsia) color on the slant. Formation of pink color indicates positive result. The hydrolysis of the media proteins during prolonged incubation could result in a false-positive test. The findings of the urease test for bacterial isolates were 1LB negative, 2LB negative, 3LB negative, 4LB negative, and 5LB negative (*Fig. A7*).

Coagulase test

The bacterial cells adhering to one another following the adding of plasma indicates positive result. The nonappearance of agglutination shows negative test. The current study shows 1LB coagulase negative, 2LB negative, 3LB negative, 4LB negative and 5LB negative (*Fig. A8*).

Oxidase test

The crux of Kovac's oxidase test is the presence of cytochrome oxide, which is a saprophytic bacterium characteristic. The bacterium was positive to test whether the purple color formed between 30-60 s. Our study isolates were one oxidase positive and four oxidase negative. Showed 1LB positive, 2LB negative, 3LB negative, 4LB negative and 5LB positive, results for bacterial isolates (*Fig. A9*).

Classification of isolates on the basis of morphological and biochemical characterization

In accordance with manual of bacterial classification on the basis of above-mentioned characterization of the following were the proposed species of bacterial isolated (*Table 3*).

Table 3. *Classification of isolates on the basis of Morphological and Biochemical characterization*

Tests	1LB	2LB	3LB	4LB	5LB
Gram staining	-	+	+	-	+
Catalase	-	+	+	+	+
Citrate	-	+	-	+	-
Indole	-	-	+	-	-
Urease	-	-	-	-	-
Coagulase	-	-	-	-	-
Oxidase	+	-	-	-	+
Proposed species according to Bergey's manual	*Kingella sp*	*Listeria sp*	*Bacillus sp*	*Pseudomonas putida*	*Cupriavidus necator*

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Molecular identification and phylogenetic analysis

All of the bacterial isolates, total genomic DNA was extracted, and utilizing gDNA as a template, a PCR product with a 541 bp band was created. All of the bacterial isolates, ITS sequences were aligned, and a phylogenetic tree was generated. Many phylogenetically linked species of bacteria were similar to the bacterial isolates acquired in this study, according to an analysis of the ITS of the isolates as well as those in the NCBI database (*Fig. 2*). The phylograms topology demonstrated that the bacterial isolates used in this investigation belonged to respective bacterial strains i.e., 1LB as *Kingella sp.*, 2Lb as *Listeria sp.*, 3LB as *Bacillus sp.*, 4LB as *Pseudomonas putida and* 5LB as *Cupriavidus necator*. Furthermore, *Aspergillus niger* CBS 554.65 was used as out group.

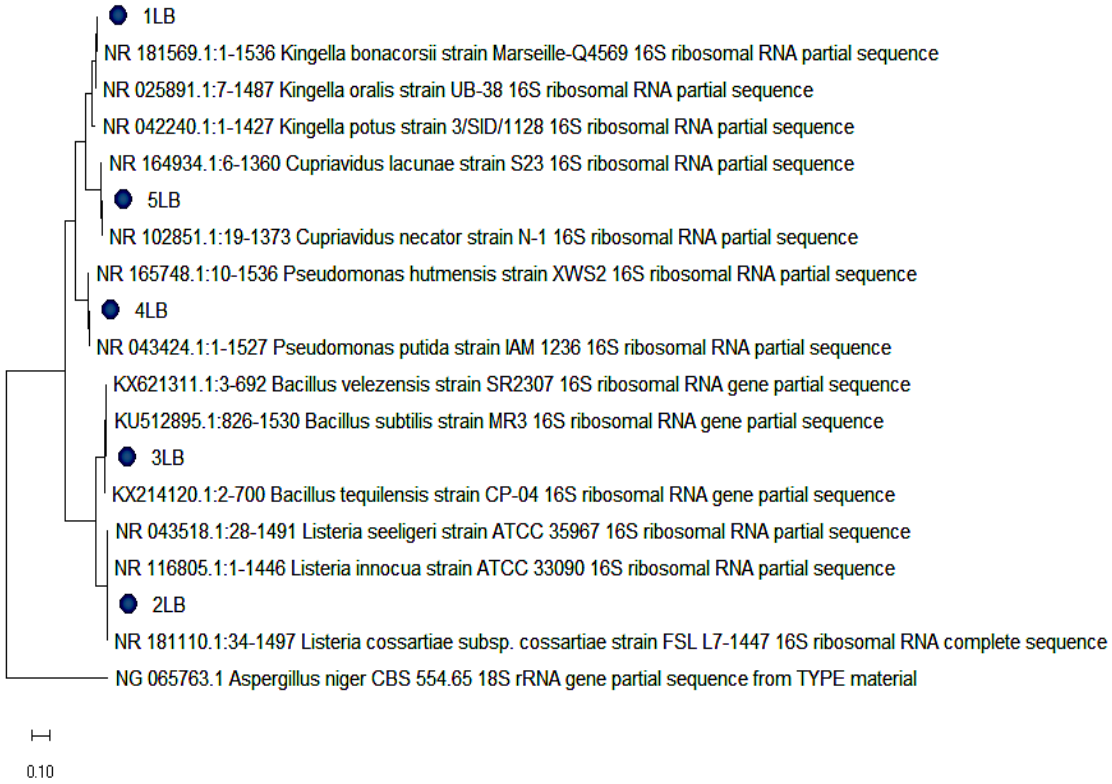

Figure 2. *Phylogenetic tree of all bacterial isolates and other relative species based on a maximum likelihood Analysis of ITS sequences. Aspergillus niger CBS 554.65 was used as out group*

Total metal content

The heavy metals contaminated soil of industrial estate were gathered and examined for heavy metals contents (Cu, Pb, and Cr), by using atomic absorption spectrophotometry (*Table 4*).

Table 4. *Total contents of heavy metals in soil sample S3*

Code	Cu (mgL^{-1})	Pb (mgL^{-1})	Cr (mgL^{-1})
S3	0.631	0.247	1.82

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Screening for heavy metal tolerant bacterial isolates

The bacterial isolates were checked for the heavy metals' tolerance using the agar well diffusion method as reported by Collins et al. (1985) in sterile NAM plates. Different concentration of heavy metals was used to check antibacterial activity. Following a 24-h incubation, the zones of inhibition for each bacterial strain were examined. According to results 1LB showed resistance against lead (Pb) and no zone formation was observed. The zone of inhibition formed by Copper and Chromium were 0.4 cm and 0.6 cm respectively. 2lb showed resistance against lead while Cu formed, inhibition zone of 0.5 cm and Cr formed zone of inhibition of 0.7 cm. 3lb showed the resistance against Cu, Pb formed zone of inhibition of 1.2 cm and Cr formed zone of inhibition of 2.5 cm. 4lb was resistant to lead, while both Cu and Chromium formed inhibition zone of 1 cm. 5lb showed resistance against lead, Cr formed zone of inhibition of 0.5 cm and Cr showed zone of inhibition of 1 cm (*Table 5, Fig. 3*).

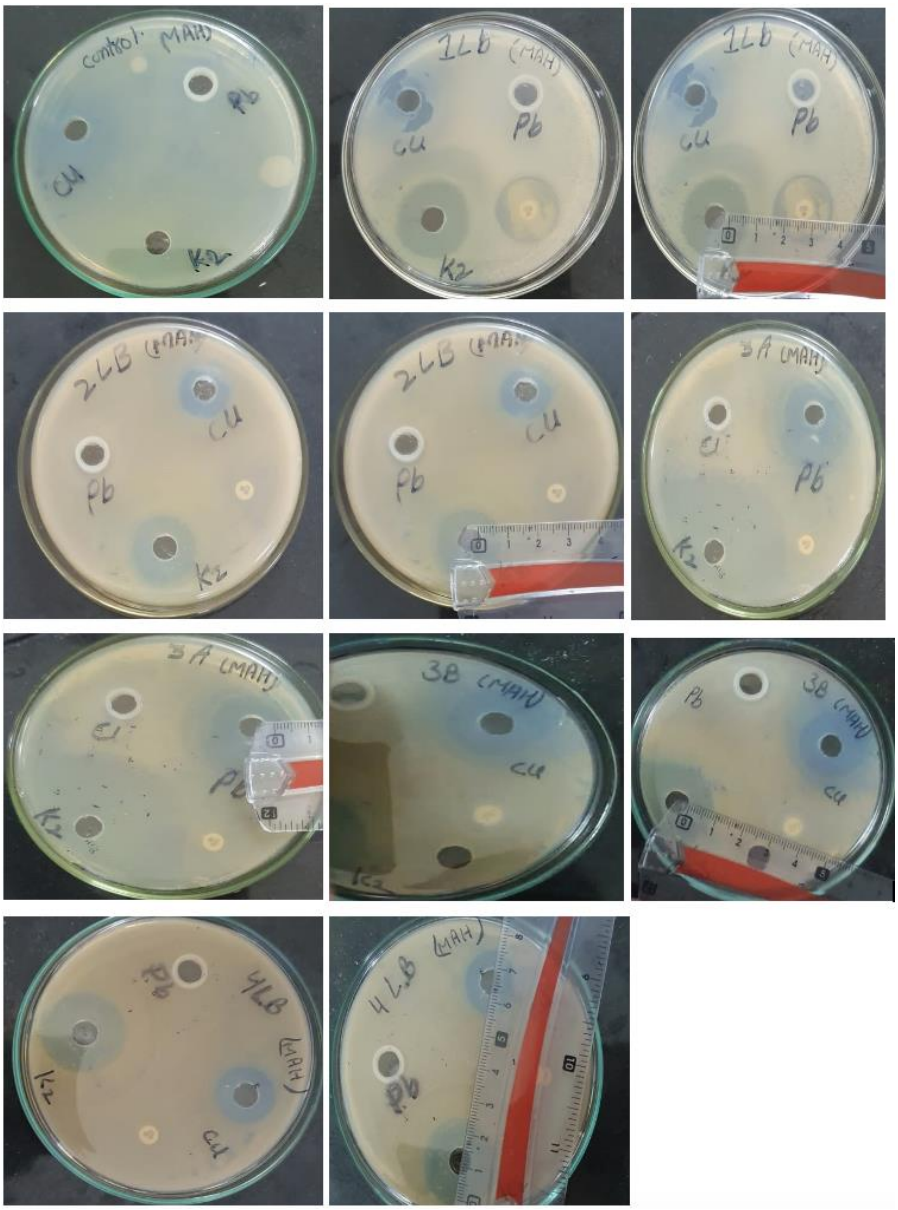

Figure 3. *Screening for heavy metal tolerant bacterial isolates 1LB-4LB*

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Table 5. *Screening for heavy metal tolerant bacterial isolates*

Sample	Pb (lead)	Cu (copper)	Cr (chromium)
1LB (*Kingella sp.*)	Resistant	0.4 mm	0.6 mm
2LB (*Listeria sp.*)	Resistant	0.5 mm	0.7 mm
3LB (*Bacillus sp.*)	1.2 mm	Resistant	2.5 mm
4LB (*Pseudomonas putida*)	Resistant	1 mm	1 mm
5LB (*Cupriavidus necator*)	Resistant	0.5 mm	1 mm

Bioremediation potential analysis

Test for tolerance trend of bacteria isolates to various concentration of heavy metal

Degree of tolerance showed by bacteria isolated to different concentration of heavy metal were analyzed. As per results, in the 1LB bacterial isolate showed highest tolerance rate against Copper (Cu). Furthermore, the tolerance trend of 2LB bacterial isolate showed maximum rate against Chromium (Cr) and minimum tolerance against Copper (Cu). Moreover, the 3LB bacterial isolates showed highest tolerance rate against Lead (Pb) and minimum against Chromium (Cr) as shown in *Figure 4A-C*.

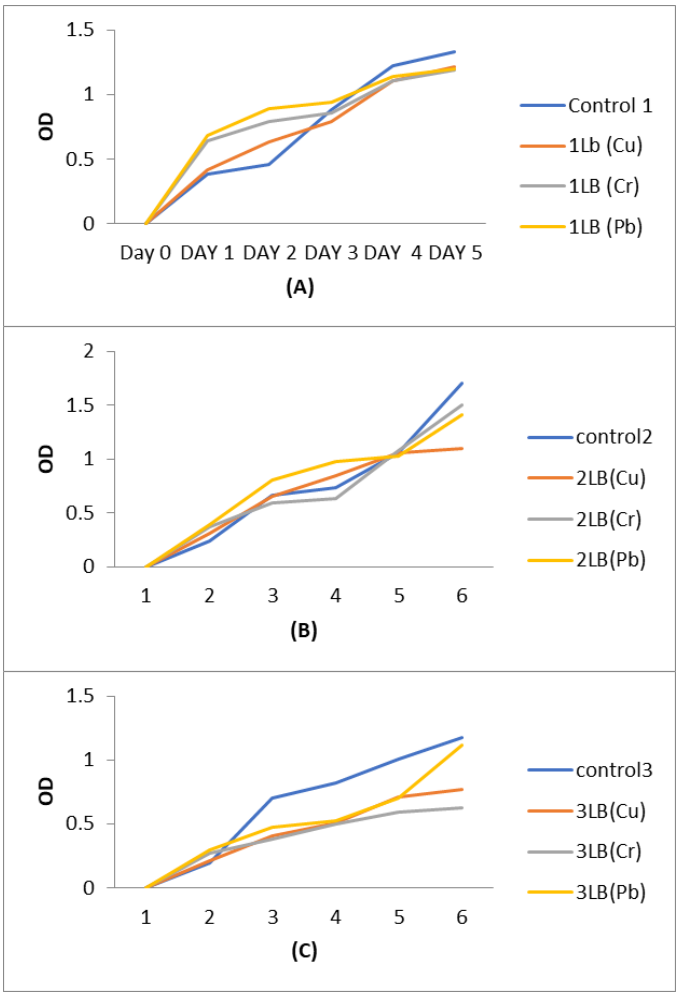

Figure 4. *Growth of different bacterial isolates (1LB, 2LB and 3LB) in presence of heavy metals at different interval of incubation*

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While, the tolerance trend of 4LB bacterial isolate showed minimum rate against Chromium (Cr) and maximum tolerance against Copper (Cu) and the 5LB bacterial isolates showed highest tolerance rate against Chromium (Cr) and minimum against Copper (Cu) as shown in *Figure 5* (Losada-Barreiro et al., 2022).

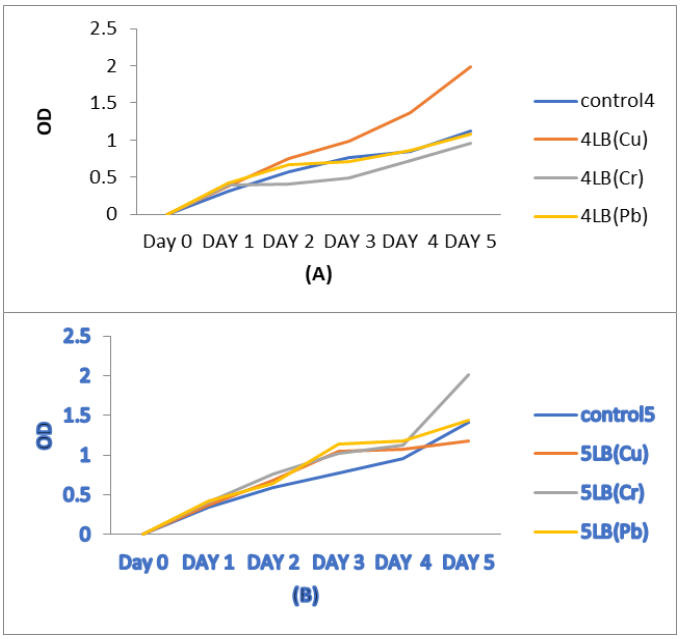

Figure 5. *Growth of different bacterial isolates (4LB and 5LB) in presence of heavy metals at different interval of incubation*

Medium optimization for best growth of metallotolerant bacterial isolates

According to the result, while using LB medium, all the bacteria isolated showed the highest tolerance level against different heavy metal concentrations. Thus, the LB medium was used for the optimization of bioremediation purposes. The medium showing the best growth for bacterial isolates indicates the efficient remediator is heavy metals (YPG) (*Fig. 6*).

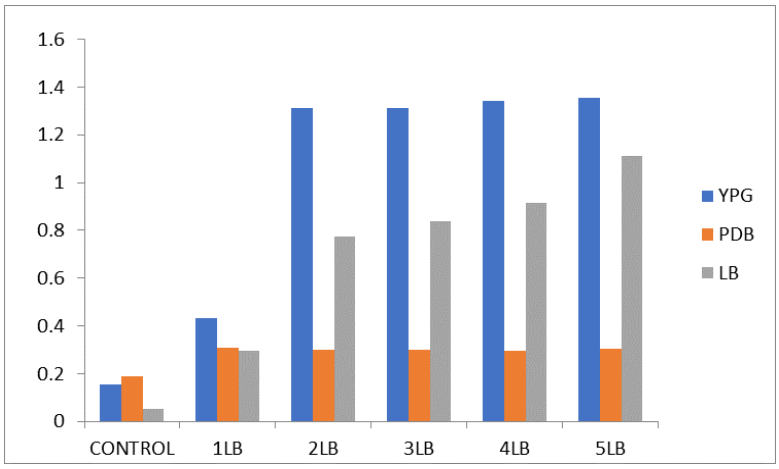

Figure 6. *Medium optimization for best growth of metallotolerant bacterial isolates by using optical density (OD)*

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Evaluation of the potentials of metallotolerant bacterial isolates for bioremediation from heavy metals contaminated soil

Bacterial heavy metal removal efficiency

Heavy metals pollution tends to lessen the number of microbial communities as they are susceptible to the heavy metals and they cannot survive into the heavy metals contaminated environment. Bacteria had the ability to survive into the heavy metals contaminated environment. All the bacterial isolates were analyzed for resistant to several concentration of heavy metals especially Cu, Cr and Pb. The 5LB bacterial strain showed removal efficacy 90.49% for Cu removal in the YPG medium.

Removal efficiency of heavy metal (lead) by bacterial isolates

The heavy metal (Lead) removal efficiency of all the indigenously isolated bacterial isolates were analysis, as shown below that indicates the potency of bacterial isolates that may be used in bioremediation of heavy metals contaminated soil. The maximum removal efficiency of 4LB bacterial strain was recorded in the YPG medium which was about 83.80% as compared to other bacterial isolates (*Table 6*).

Table 6. *The indigenous metallotolerant bacterial isolates to remove lead (Pb) [(values are presented as mean ± SEM (n = 3)]*

Code of sample	Heavy metal	Initial concentration of heavy metal Pb (mgL^{-1})	Bacterial isolate	Media	Final concentration of Pb (mgL^{-1})	Removal efficiency
S3	Pb	0.247 ± 0.051	1LB	YPG	0.07 ± 0.012	71.60%
			2LB	YPG	0.06 ± 0.022	75.71%
			3LB	YPG	0.09 ± 0.037	63.56%
			4LB	YPG	0.04 ± 0.061	83.81%
			5LB	YPG	0.09 ± 0.035	63.56%
			6MIX	YPG	0.03 ± 0.051	87.85%

Removal efficiency of heavy metal (copper) by bacterial isolates

The strength of removing heavy metal of the isolated bacterial strains were analyzed as shown below. The potency of bacterial isolates that maybe utilized for bioremediation with heavy metal of soil contaminated.

The highest removal efficiency (90.49%) of 5LB bacterial strain for copper from the soil sample S3 was recorded in the YPG medium as compared to other bacterial isolates (*Table 7*).

Table 7. *The indigenous metallotolerant bacterial isolates to remove copper (Cu) [(values are presented as mean ± SEM (n = 3)]*

Code of sample	Heavy metal	Initial concentration of heavy metal Cu (mgL^{-1})	Bacterial isolate	Media	Final concentration of Cu (mgL^{-1})	Removal efficiency
S3	Cu	0.631 ± 0.321	1LB	YPG	0.11 ± 0.011	82.57%
			2LB	YPG	0.07 ± 0.032	88.91%
			3LB	YPG	0.19 ± 0.051	69.89%
			4LB	YPG	0.08 ± 0.026	87.32%
			5LB	YPG	0.06 ± 0.019	90.49%
			6MIX	YPG	0.09 ± 0.051	85.73%

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Removal efficiency of heavy metal (chromium) by bacterial isolates

The heavy metal (chromium) removal strength of all the indigenously isolated bacterial isolates were analysis, as shown below that indicates the potency of bacterial isolates that may be used in bioremediation of heavy metals contaminated soil.

The highest removal efficiency (81.87%) of 1LB and 2LB bacterial strains for chromium from the soil sample S3 was recorded in the YPG medium (*Table 8*).

Table 8. *The indigenous metallotolerant bacterial isolates to remove chromium (Cr) [(values are presented as mean ± SEM (n = 3)]*

Code of sample	Heavy metal	Initial concentration of heavy metal Cr (mgL^{-1})	Bacterial isolate	Media	Final concentration of Cr (mgL^{-1})	Removal efficiency
S3	Cr	1.82 ± 0.451	1LB	YPG	0.33 ± 0.054	81.87%
			2LB	YPG	0.33 ± 0.021	81.87%
			3LB	YPG	1.71 ± 0.064	6.04%
			4LB	YPG	0.35 ± 0.052	80.77%
			5LB	YPG	0.34 ± 0.013	81.32%
			6MIX	YPG	1.16 ± 0.034	36.26%

Discussion

The largest city in Pakistan is Karachi, which has two planned industrial areas called Sindh Industrial Estate and Korangi Industrial Estate. The Korangi industrial area (KIA), has total area of 34.4 km which is in the district east, and about 2000 different small and medium-sized industrial units that produce goods like leather, textiles, chemicals, detergents, iron, steel, vegetable oils, beverages, and food items. Heavy metal contamination of the soil occurs as a result of production process leaks and stacking practices during industrial production (Khan et al., 2011). Heavy metal exposure at extremely high concentrations can have severe negative effects on animals, plants, and people. These metals have long-term consequences on the flora and animals. This is a significant global health issue. Majority of the heavy metals at higher concentration can cause cell death and mutations leading to serious health issues like neoplasia and oncogenic in addition to being cytotoxic (Zolfaghari, 2018). In current study indigenous bacteria from the heavy metals contaminated soil sample S3 were screened for the remediation of heavy metals Cu, Pb and Cr. In this study heavy metals resistant bacterial isolates from 1Lb-5lb were identified and characterized from contaminated soil sample S3 of Korangi industrial estate Karachi.

Initially, 150 bacterial colonies were screened on nutrient agar medium. As a result of secondary screening 25 bacterial isolates were selected. Finally, five bacterial strains were selected because they were found highly tolerant against the heavy metals. All the bacterial isolates were grown up by using heavy metals in their culture media, according to a preliminary examination of the obtained samples for their capacity to resist heavy metals. Based on their appearance, five (5) different strains of the heavy metal resistant bacterial community are obtained through serial dilutions of all the samples.

The bacterial sample were then evaluated for morphological characteristics, by using different medium which showed that *Kingella sp.* (1LB) fails to grow on MacConkey, *Listeria sp.* (2LB) produced nutritional agar made by Bacillus sp. (3LB) Listeria is a typical, short, facultative anaerobic, motile, gram-positive rod that produces blue gray colonies. On LB medium, there was a medium-sized colony that was gray-white,

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rounded, opaque and flat, drying, and Salmonella sp. (4LB) were colorless and translucent, and they normally do not affect the MacConkey medium's look and Staphylococcus sp. (5LB) generates enterococci and light pink to red colonies. form compact, small red colonies on or below the medium's surface. Similar study was also conducted by numerous scientists in according to Bergey's Manual of Determinative Bacteriology (Aneja, 2007; Bergey, 2001). Gram staining indicates, 3 isolates 2LB, 3LB and 5LB were recognized as gram-positive cocci and others i.e., 1LB and 4LB were gram negative rods bacteria as shown in results section Marzan et al. (2017) conducted a similar study. by identifying peptidoglycan, which is found in bacteria in a thick layer and distinguishing it from the other one (S4), which is gram negative. A number of researchers have described many bacterial strains that are resistant to arensic (Trevors et al., 1985; Abbas et al., 2014) in a different investigation, the strains of Bacillus cereus (MR2) and Bacillus discolorations (MR3) were Gram-positive while Proteus vulgaris (MR1), Pseudomonas fluorescence (SS4), and Pseudomonas fluorescence (SS5) were Gram-negative, rod-shaped motile bacteria. (Ahirwar et al., 2016). During the biochemical characterization the isolates 1LB strain confirmed Catalase negative, Citrate negative, Indole negative, Urease negative, Coagulase negative and Oxidase positive, while 2LB strain showed Catalase positive, Citrate positive, Indole negative, Urease negative, Coagulase negative and Oxidase positive results, 3LB strain showed Catalase positive, Citrate negative, Indole positive, Urease negative, Coagulase negative and Oxidase negative results, 4LB strain showed Catalase positive, Citrate positive, Indole negative, Urease negative, Coagulase negative and Oxidase positive results and 5LB strain showed Catalase positive, Citrate negative, Indole negative, Urease negative, Coagulase negative and Oxidase positive results. Similar study was conducted by Baker (1984), When compared to most Staphylococcus spp., which are typically oxidase-negative gram-positive bacteria, Micrococcus spp. are oxidase-positive (Jurtshuk Jr. and McQuitty, 1976). Since S4 (Micrococcus sp.) generated yellow to brown colonies on growth media as opposed to the red colony of Micrococcus roseus, their analysis revealed that it was oxidase positive and may be Micrococcus luteus. Gram staining, oxidase testing, and investigations on how S3 uses carbs revealed similarities with Gemella sp., a gram-positive, oxidase-negative organism that uses all carbohydrates. (Naveed et al., 2024; Solomon and Viswalingam, 2013).

In the current study, the 5 bacterial isolates which were characterized morphologically, were further analyzed on a molecular basis. These strains showed maximum likelihood during the NCBI blast and the topology of phylograms confirmed the linkage as 1LB (*Kingella* species), 2LB (*Listeria* species), 3LB (*Bacillus* species), 4LB (*Pseudomonas putida)* and 5LB (*Cupriavidus neactor*). A similar study conducted by Gumel et al. 99% analogy was observed between the isolate and *Pseudomonas putida* strains (Gumel, 2012). The morphological characteristics of the isolate 5LB genus Cupriavidus were in accordance to the previous observation. Similar results were also observed by Yootoum et al. when they carried molecular phylogenetic analysis of an isolate and found that this isolate is closely related to *Cupriavidus gillardii* and *Cupriavidus neactor.* The current study was conducted on the soil samples taken from heavy metals contaminated area of the Korangi industrial estate, Karachi, which is a main source of heavy metal pollution. Due to the lack of adequate waste treatment facilities, Korangi Industrial Estate contributes to pollution. The total metal content of the soil sample (S3) was recorded to be 1.044 mg/l, 0.631 mg/l, 0.24 mg/l and

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0.82 mg/l. The current study corresponds with another study in which, topsoil in the Shenyang Tiexi Industrial District had average copper and lead contents of 92.45 mg/kg and 116.76 mg/kg, respectively (Li et al., 2010). The study reported the presence of seven different heavy metals in the soil of agricultural land of the Shenyang suburb. As was found to be the least concentrated heavy metal at 11.96 mg/kg, followed by Cr at 96.2 mg/kg, Cu at 43.7 mg/kg, Pb at 102 mg/kg, and Zn at 52.7 mg/kg in the results (Xi-hui et al., 2001). Total five indigenous bacterial strains were isolated from contaminated soil in the current study and processed for their capability to remediate the heavy metals from the soil. The ability of bacteria to remove heavy metals from contaminated soil is highly effective, and they have a high tolerance rate for heavy metals (Khan, 2001). Bacteria, among other microorganisms, are important because they can align to and grow in a variety of extreme conditions, including pH, temperature, the availability of nutrients, and high metal concentrations (Anand et al., 2006). Due to multiple metabolic physiological and behavioral adaptations caused by metal stress, indigenous bacteria could grow efficiently in the heavy metal contaminated areas (Gadd, 2000). According to our study 1LB shared resistance against lead while for Cu (0.4 cm) zone of inhibition was formed. Similarly, 2LB shows resistance against lead, 4LB and 5LB also shows resistance against lead while for 2LB Cu (0.5 cm) zone of inhibition was formed. Similarly, for 3LB, 4LB and 5LB 1cm and 0.5 cm zone of inhibition was formed, respectively. Same study was conducted by (Kepler et al.,) which shows that heavy metal contaminated strains show resistance against lead and forms zone of 0.6 cm, respectively.

In current study, the bacterial isolates were further optimized on three media i.e., PDB, YPG, and LB for the bioremediation of heavy metals copper, chromium and lead from heavy metals contaminated industrial soil sample S3. Yeast potato glycerol (YPG) is recommended as a growth medium for bacterial isolates from various environmental source. Bacteria possess high potential for remediation of heavy metals due to their best development rate. Numerous bacterial genera have been discovered in the soil contaminated with metal (Ren et al., 2009). Bacteria-based bioremediation used for industrially contaminated soil and water (Khan, 2001). According to an observational study, some bacterial species were more resistant to the heavy metals (Cr, Cu, Pb), whereas other species could tolerate Pb but were sensitive to Cr. The isolates of bacteria were identified as Cr-tolerant strains in a related study (Baldrian and Gabriel, 2002). In current study the 1LB strain showed 82.56%,71.60% and 81.86% removal efficiency for Cu, Pb and Cr by using YPG medium, While, 2LB strain showed removal efficiency of 88.90%, 75.70% and 81.86% for Cu, Pb and Cr in YPG medium. The 3LB bacterial strain showed removal efficiency of Cu, Pb and Cr with 0.19% and 0.09% and 1.71% in YPG media, respectively. The 4LB bacterial strain on the other hand showed removal efficiency of 87.32%, 83.80% and 80.06% for Cu, Pb and Cr in YPG medium. The 5LB bacterial strain showed removal efficiency of 90.49%, 63.56% and 81.31%for Cu, Pb and Cr in YPG medium. Furthermore, the 6 MIX bacterial strain showed removal efficiency of 85.73%, 87.85% and 36.265% for Cu, Pb and Cr in YPG media, respectively. Among all the five indigenous bacterial isolates, 5LB showed highest uptake efficiency and removal efficiency for both copper and Chromium. Different bacterial strains exhibited different behaviors when exposed to heavy metals, with some being sensitive, moderately tolerant, and tolerant. Heavy metal intoxication causes variety of inhibitory effects in bacteria. One major effect is the blockage of crucial functional groups, followed by the conformational changes in the enzyme and its

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deactivation. Thus, slowing down or inhibiting the complementary processes taking place within the cell (Kostova, 2023). A similar study in response to other heavy metals exposure (Peña-Castro et al., 2004). Paraszkiewicz and Courbot have also examined that heavy metal stress causes production of different toxic compound as well. One such example is thiol compounds, which are stored inside the vacuoles formed by cell wall proturons (Damodaran et al., 2013).

Conclusion

There are many variables that might affect the bioremediation process, containing the type of soil, pH, temperature, nutrients, additives, and oxygen. In the biological processes for treating contaminated soil, microorganisms have a direct role in the breakdown of biological matter; therefore, their capacity to proliferate in the occurrence of heavy metals would be useful. Most heavy metals pollute the surroundings and the atmosphere, and some are poisonous to humans. Heavy metals can become exceedingly toxic after interacting with various environmental components like water, soil, and air, and humans and other living creatures may be exposed to them through the food chain. The production, quality, and safety of food are all impacted by heavy metal pollution of the soil. Thus, considering the toxicity of heavy metals, current research is aimed at isolating native heavy metals tolerant bacterial strains that could be used for biological remediation in order to detoxify the contaminated soil, as this method to remediate heavy metals is less expensive, ecofriendly and more effective. Bacterial remediation is an efficient, productive and advanced technique to remove heavy metals from the environment. In the metal contaminated environment microorganisms undergo multiple metabolic and behavioral changes as the adaptation for the survival in the heavy metal contaminated soil. In current study five bacterial isolates showed capability to remove the heavy metals but the strain (5LB) demonstrated the greatest capacity to remove Cu, Cr and Pb from the environment, making it a promising candidate for making the economical and environment eco-friendlier. The limitation, however, is that these bacterial strains could be further analyzed for any type to toxin production that may affect the human health as well as other living things as a whole. Therefore, they must be treated in a way that limits their capacity to spread disease and increases their ability to tolerate metals, which will help keep the environment free of metal pollution.

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Conflicts of interest. The authors declare no conflict of interest.

Data availability statement. The data generated during this study has been included in the manuscript.

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APPENDIX

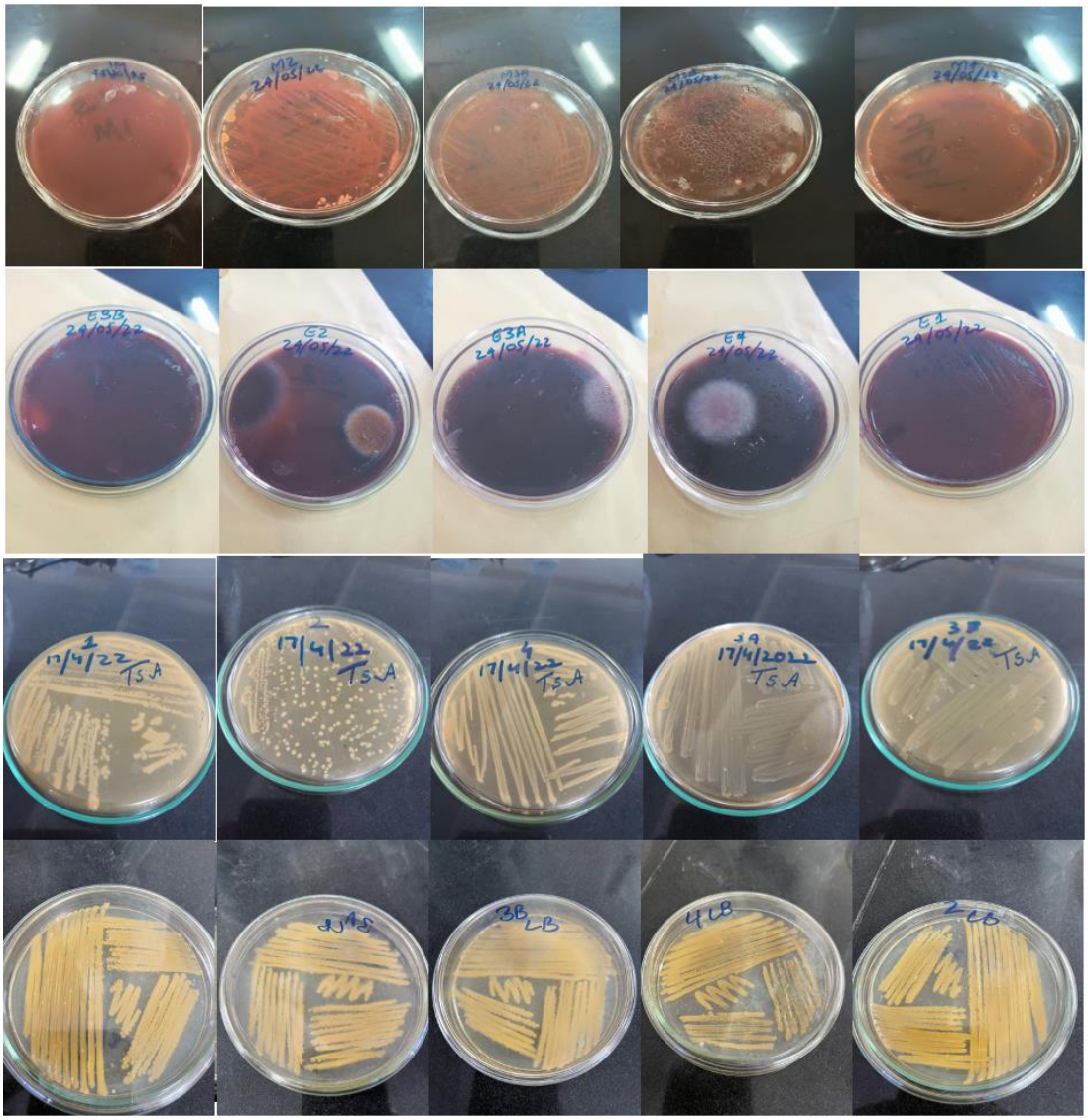

Figure A1. *Morphological characterization of bacterial isolates*

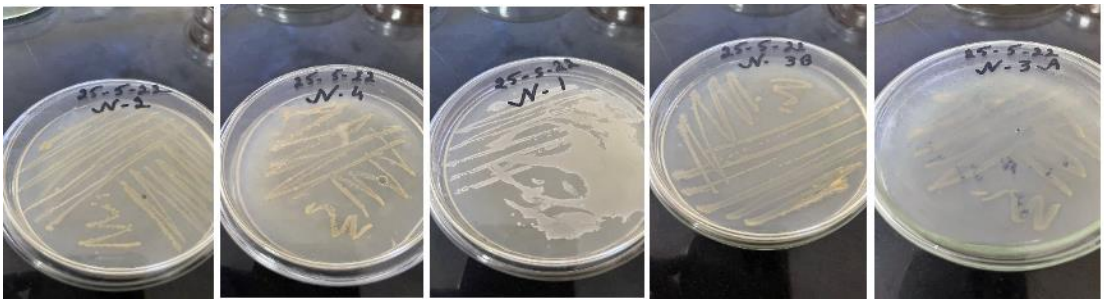

Figure A2. *Morphological characteristics of bacterial isolates*

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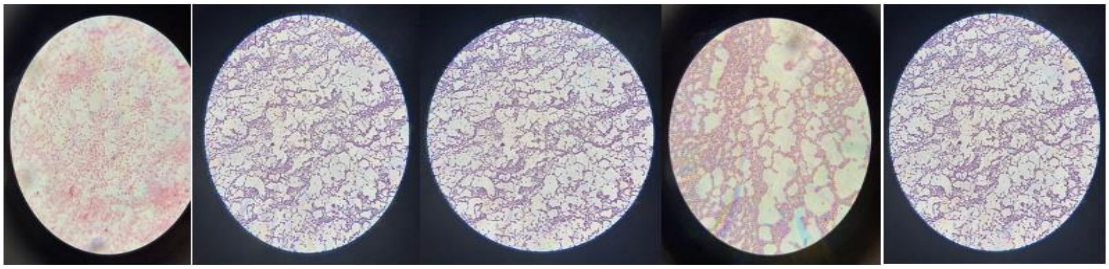

Figure A3. *Microscopy of bacterial isolates*

Figure A4. *Catalase test results for 1Lb – 5LB bacterial isolates*

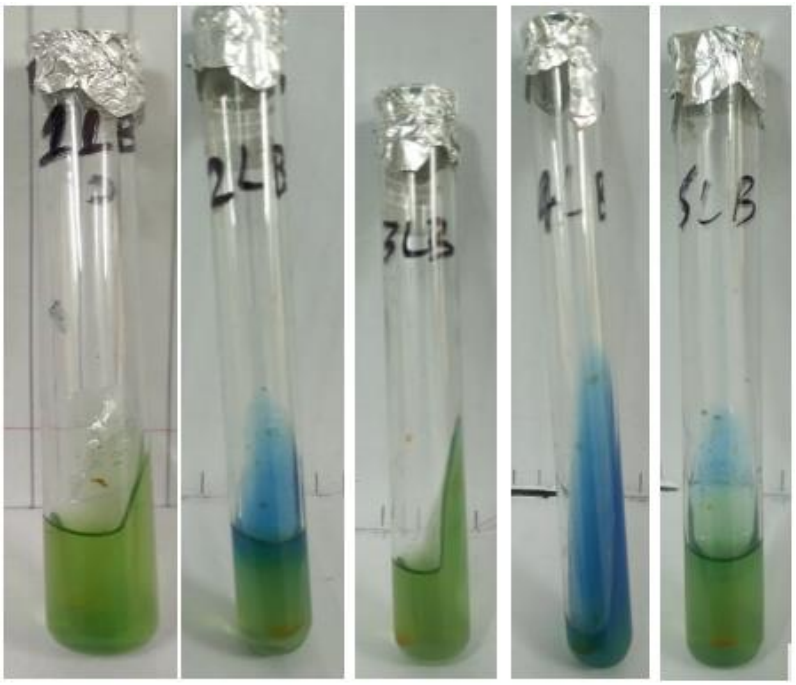

Figure A5. *Citrate Agar Test for all the bacterial isolates*

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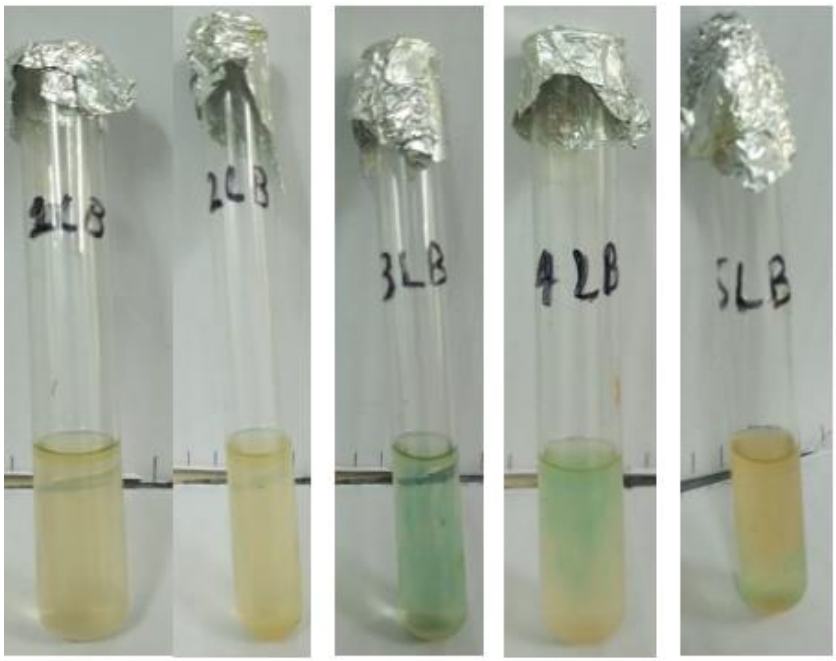

Figure A6. *Indole Test for all the bacterial isolates*

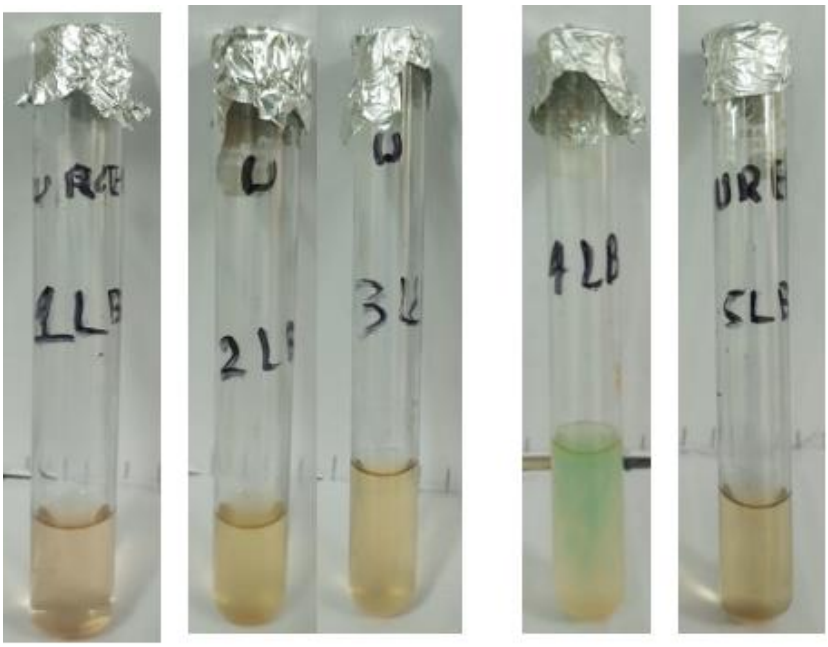

Figure A7. *Urease Test for all the bacterial isolates*

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Figure A8. *Coagulase Test for all the bacterial isolates*

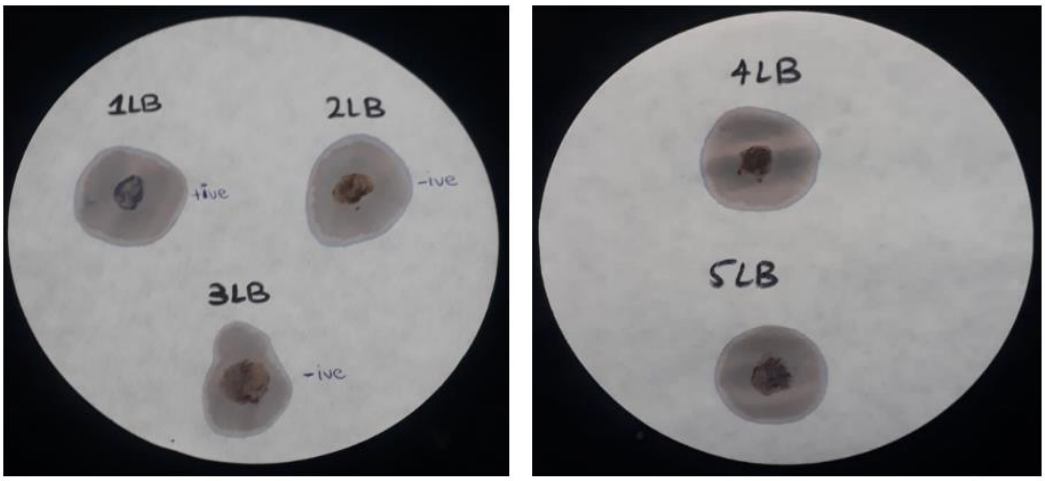

Figure A9. *Oxidase Test for all the bacterial isolates*

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