**Assessment of bioaccumulation of cadmium, nickel and chromium with acidophilic *Klebsiella* sp. isolated from biofilms and the prevalence of heavy metal resistance determinants**

**Abstract**

Expanding industrialization is one of the major causes of heavy metals contamination. These are discharged directly and indirectly into the environment including soil and water bodies, thus polluting soil and water reservoirs especially in developing countries. Hence, they are becoming a threat to humans, plants and other life forms as well as a challenge for the ecological safety. Microbial systems offer the efficient and economically feasible approaches for removal of these contaminants. This study focuses on assessment and evaluation of bacterial isolates obtained from biofilm samples of different contaminated areas for their metal bioaccumulation and removal potentials. These isolates were identified as *Klebsiella pneumoniae* MB375, *Klebsiella oxytoca* MB381, *Klebsiella pneumoniae* MB394 and *Klebsiella pneumoniae* MB398 through 16S rRNA sequencing. *Klebsiella pneumoniae* MB398 exhibited highest percentage accumulation of cadmium (91.44%), chromium (92.63%) and nickel (71.70%) with initial concentration of 200 mg L-1 for 24 hr, while 90.99% of cadmium, 92.06% chromium and 70.46% of nickel wasaccumulatedby *Klebsiella oxytoca* MB381. Significance of different environmental parameters was also highlighted in bioaccumulation process. 91.01%, 90.13% and 75.59% of chromium, cadmium and nickel respectively were accumulated by *Klebsiella pneumoniae* MB398 followed by *Klebsiella oxytoca* MB381 (chromium 88.61%, cadmium 88.21% and nickel 65.77%) with maximum inoculum volume (1%). Both these strains displayed maximum uptake of cadmium, chromium and nickel under acidic conditions (pH 5.0). The results of present research demonstrated that *Klebsiella* species can efficiently be deployed in bioremediation for removal of potentially toxic metals from contaminated environmental reservoirs.

**Introduction**

Multiple industrial units and anthropogenic activities (including leather tanning, chemicals processing, steel manufacturing and production, electroplating, batteries manufacturing, wood preservation, paints, dyes production, pulp processing, fertilizers abuse, application of sewage sludge and mining) are continuously discharging cadmium, nickel and chromium loaded effluents into the environment via diverse range of processing activities. The Concentrations of these potentially toxic metals within the environmental compartments (land and water bodies) considerably vary.

Toxic heavy metals in terrestrial and aquatic ecosystems is an environmental and public health concern. Heavy metals have persistent nature and accumulate in the environment and consequently contaminate the food chains. The inorganic pollutants are being discarded in soil, water and atmosphere due to the rapidly growing sectors of industries and agriculture, lack of proper waste management, fertilizers and pesticides. The fate of these pollutants is different. Some metals affect the biological functions and growth, while the other accumulate in different organs which cause serious diseases such as cancer (Ali, H., Khan, E., & Ilahi, I. (2019).

These metals are extremely harmful at lower concentrations as they get accumulated in the food webs and pose serious health issues. Hence, these metals are highly concerning because of their extensive applications in developing countries (especially irrigation of agricultural lands with industrial wastewater is of foremost concern), their persistence and nondegradable features along with long term consequences on all the life forms and ecological stability (Congeevaram et al. 2007; Takahashi et al. 2012; Olaniran et al. 2013; Reis et al. 2014; Banerjee et al. 2015; Tarekegn et al. 2020). Cadmium is known to be wide spread ecological contaminant and has been identified as a potent toxin (being non-essential metal), which adversely affects the living organisms even at low concentrations via entering the environmental compartments and transmission in to the food chain. Cadmium interferes in symbiosis between plants and microorganisms, disrupts the enzymatic activities, inhibits DNA mediated transformations in microbes and enhances the plants’ liability towards fungal invasions, thus, lowers the crop yields/productivity (Chellaiah, 2018; Lata et al. 2019; Xu et al. 2020).

Cadmium is selectively accumulated in some organs such the liver, kidney, gills, and exoskeleton and is not evenly distributed throughout the body. In muscle tissues, the concentrations are several orders of magnitude lower. The way that Cd is disposed of in the organisms used in the laboratory research generally resembles how it is done in nature. Numerous biotic variables, such as body size, age, sex, etc., affect bioaccumulation, but in-depth research is still missing in this area. The environmental Cd's chemical form has a significant impact on how much Cd marine species may bioaccumulate (Ray, S. (1984).

Chromium exists in nature as chromium III form, which is an essential metal and facilitates the glucose metabolism in living organisms. But, its hexavalent form (chromium VI) is highly toxic and considered as mutagenic and carcinogenic, and is known to exert deleterious effects on the microorganisms and plants (Ahemad, 2014; Thatheyus and Ramya, 2016; Quiton et al. 2018).

However, nickel in excessive concentrations exerts serious health threats to humans (by impairing respiratory system, leads to kidney, lung, nasal, larynx, liver and stomach cancers), and also affects the plants, animals and microbial communities (Chaudhary et al. 2017; Mardiyono et al. 2019). Presence of higher levels of nickel in agricultural soils has been reported to affect the crop quality, productivity and yields, which ultimately contaminates the food chain (Chen et al. 2020). Being non-biodegradable and toxic in nature all these metals need to be removed from the industrial effluents prior to their disposal into the water bodies and/or nearby land areas (Thatheyus and Ramya, 2016).

Numerous chemical and physical technologies have widely been applied for remediation of metals from contaminated sites. However, these treatment technologies are time consuming, expensive, require reagents in large quantities, ineffective at lower metal concentrations and generate complex secondary wastes, which also require special handling (Upadhyay et al. 2017; Quiton et al. 2018), thus limiting the applications of these approaches for metals treatment. Biological remediation approaches have attracted the scientific community towards designing effective pollutants’ treatment technologies because of their low operational costs, steady effects and easy recovery of valuable metals.

Through the all inclusive active action of microorganisms, bioremediation is heavily involved in the degradation, eradication, immobilization, or detoxification of various chemical wastes and hazardous materials from the environment. Degrading and transforming pollutants into less hazardous forms is the fundamental idea. Depending on a number of variables, including but not limited to cost, site conditions, type, and concentration of pollutants, bioremediation can be done in-situ or ex-situ. An alternative remediation method that provides a green technology solution to the issue of environmental degradation is biological remediation, in which microorganisms or plants are used to detoxify or remove organic and inorganic xenobiotic compounds from the environment (Abatenh, E., Gizaw, B., Tsegaye, Z., & Wassie, M. (2017)

Microorganisms including bacteria, fungi, yeast, algae and protozoa found in areas receiving industrial effluents have developed several survival mechanisms for protecting themselves from toxic effects of metals contamination like bioaccumulation, biosorption, biotransformation and biomineralization (Venil et al. 2011; Aransiola et al. 2017).

In order to avoid heavy metal mobilization or leaching into the ecosystem and to facilitate the extraction of heavy metals, microbial remediation is essential. In a metal-polluted environment, microorganisms use processes like biosorption, bioaccumulation, biotransformation, and bioleaching to survive. Due to their astounding metabolic activity, microorganisms may dwell in a wide variety of environmental conditions and can survive anywhere on the biosphere. Only when microorganisms have access to a variety of materials molecules that can assist them produce energy and nutrients to grow more cells can they begin to act against pollution Kapahi, M., & Sachdeva, S. (2019). The physicochemical features of the environment, the chemical composition and quantity of contaminants, and their accessibility to microorganisms are only a few of the variables that affect the efficiency bioremediation. Since bacteria and contaminants don't interact, the rate of deterioration is slowed down. In addition, the distribution of contaminants and bacteria in the environment varies. A complex system of elements contributes to the regulation and optimization of bioremediation processes. The presence of a microbial population capable of degrading the pollutants, the accessibility of contaminants to the microbial population, and environmental conditions are all considered here (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients). pH, temperature, moisture, soil structure, solubility in water, nutrients, site features, redox potential, oxygen content, lack of qualified human resources in this field, and physio-chemical bioavailability of contaminants are all factors that affect microorganism growth and activity (contaminant concentration, type, solubility, chemical structure and toxicity). Microorganisms have a wide range of dietary requirements, which makes them useful for bioremediation of environmental contaminants (Kensa, V. M. (2011)

Hence, wide range of microorganisms possess inbuilt competencies to uptake and tolerate higher concentrations of metal contaminants. Bacteria among microorganisms offer best possible removal or accumulation of metals because they are abundant in nature, easy to culture and handle, and require simple nutrition along with modest or no side effects (Venil et al. 2011; Upadhyay et al. 2017). Though several microbial species including various genera of fungi (*Aspergillus*, *Penicillium*, *Rhizopus*) and bacteria (*E. coli*, *Bacillus* sp. *Staphylococcus* sp., *Pseudomonas* sp.) have been studied extensively for bioaccumulation and bioremediation of metals (Venil et al. 2011; Aransiola et al. 2017; Upadhyay et al. 2017; Quiton et al. 2018), yet there exists a possibility of existence of new microbial species possessing significant tolerance for higher concentrations of metals. With these considerations, the present research was carried out to isolate and select the metal resistant indigenous bacterial isolates from biofilms grown over different contaminated sites. Additionally, bacterial growth at variable pH and temperature ranges was also examined. The conditions for bioaccumulation of metals were optimized and finally, the bioaccumulation experiments were conducted for selected metals. Furthermore, the prevalence of heavy metal resistance determinants was also assessed in current study.

**Materials and methods**

**Chemicals and medium**

NiCl2, HgCl2, CdSO4, CuSO4, CoCl2, Pb(NO3)2 and K2CrO4 (Merck) were used. For stock solution 1 gram of each metal salt was dissolved in 10 ml of autoclaved distilled water, except for mercury (0.5 g per 10 ml). M9 minimal medium (3.0g KH2PO4, 6.0g Na2HPO4, 0.5g NaCl, 1.0g NH4Cl and 12.0g agar was prepared in 1 liter of distilled water, adjusted at pH 5.0 and autoclaved, following addition of 1ml of 0.1M CaCl2, 1ml of 1M MgSO4 and 5.0g casein hydrolysate to autoclaved medium under sterile conditions. All chemicals were of analytical grade.

**Isolation of bacteria and screening for multiple metal resistance potential**

Bacteria used in present study were isolated from biofilms grown over the outlet of flour mill industrial unit, Wah; food industry, Hattar; over the nullah at back of EPA, Rawalpindi and lake situated at Ayub Park, Rawalpindi). 50 µl of each biofilm samples (serially diluted 10-1 to 10-5 in autoclaved distilled water) were plated on nutrient agar medium following incubation at 37 ˚C over 24 hours. Apparently distinct colonies were selected, purified through single colony streaking (3 to 4 rounds) and screened for maximum tolerance against various metal salts including NiCl2, HgCl2, CdSO4, CuSO4, Pb(NO3)2 and K2CrO4 using minimal medium (M9) supplemented with metal concentrations ranging from 100-1000 mg L-1. The bacteria were grown on metal supplemented plates using standard streak plate method. Majority of bacteria exhibited good potential to grow under metal stress up to 300-1000 mg L-1, while mercury was lethal and highly toxic to all the tested bacteria.

**Determination of lethal concentration (LD50) for selected metals**

The most promising bacteria with higher resistance to metals were assessed for their relative growth against Cd, Ni and Cr at different concentrations ranging between 100-1000 mg L-1 in M9 broth. Briefly, 10 ml of freshly prepared M9 broth was poured in clean test tubes. The test tubes were cotton plugged and autoclaved at 121 ̊C for 15 min. Afterwards CaCl2, MgSO4, caseine and metal salts (Cd, Ni and Cr) to be tested were added to the test tubes. The broth was then inoculated with 50 µl of bacterial suspension (24 hours grown cultures) and incubated at 37 ̊C in rotary shaker (150 rev min-1) for 24 hr. The optical density was recorded at 600 nm through UV-Vis spectrophotometer for growth of bacterial cultures. The growth of each bacterial isolate declined with the increase in metals concentration. However, four isolates MB375, MB381, MB394 and MB398 exhibited maximum relative growth up to 200 mg L-1 of respective metals. These isolates were selected for further characterization and metal accumulation experiments.

**Biochemical and molecular characterization of bacteria**

Bacterial isolates were characterized through Gram’s staining, on basis of other biochemical tests (oxidation fermentation, nitrate reduction and citrate utilization) and capability to produce various enzymes including catalase, oxidase, decarboxylases, deaminase and dihydrolase. The strains were also assessed for utilization of different carbon sources like glucose, arabinose, maltose and sucrose. All the biochemical assays were performed using standard methods described in Bergey’s manual of descriptive bacteriology. The growth patterns of bacterial isolates at different pH (5 to 11) and temperature (30, 37 and 45 ˚C) ranges were also investigated. The selected isolates were further identified using 16S rRNA gene sequences following DNA extraction as described below.

**Extraction of DNA and identification of the strains**

These bacteria were identified using 16S rRNA sequencing. Bacteria were cultured in sterilized nutrient broth and incubated at 30 ˚C for 24 hours under shaking conditions and used for genomic DNA extraction via DNeasy (QiaGen) kit system. Genomic DNA extraction was followed by amplification of 16S rRNA genes via application of forward (27F) 5́-AGAGTTTGATCCTGGCTCAG-3́ and reverse (1492R) 5́-TACGGCTACCTTGTTACGACTT-3́ primers. PCR amplified genes were purified through Invetrogen PureLinkTM kit and submitted for sequencing to Macrogen (Korea). 16S rRNA sequences compilation in FASTA format was obtained and compared with other known sequences via BLAST analysis using NCBI data base. Phylogenetic analysis of the strains was performed using MEGA 6 Package (Tamura et al., 2013).

**Optimization of different factors for bioaccumulation of metals by acidophilic bacteria**

On the basis of metal tolerance profile four bacterial isolates MB375, MB381, MB394 and MB398 were selected for optimization studies of bioaccumulation of selected metals. Metal bioaccumulation potential of bacteria MB375, MB381, MB394 and MB398 was assessed using different metal concentrations (100, 200, 300, 400, 500 and 600 mg L-1). Influence of different inoculum sizes (0.2, 0.4, 0.6, 0.8 and 1 %) on bioaccumulation capability of bacteria was also studied. M9 broth was prepared and autoclaved following supplementation of Cd, Ni and Cr (200 mg L-1), inoculated with 1% of bacterial culture and incubated at 37 ˚C, under shaking conditions (150 rpm), 24 hr. The effects of three different temperatures (30, 37 and 45 ˚C) and pH ranges 5, 6, 7, 8 and 9 on bioaccumulation efficacy of bacteria were also assessed. Final concentration of selected heavy metals was determined via flame atomic absorption spectrophotometer (SpectraA220, Australia) with wavelengths adjusted at 228.8 nm (Cd), 232 nm (Ni) and 357.9 nm (Cr) for each metal. Percentage bioaccumulation (%) was then calculated using following formula:

Percentage bioaccumulation (%) = (Im - Fm) ÷ Im X 100 eq 1 Where, Im = Initial metal concentration in the control at 0 hr and Fm = Final metal concentration in the treated medium after 24 hr

**Bioaccumulation of selected metals by acidophilic bacteria**

After optimization of experimental conditions final metal bioaccumulation experiment was conducted with 200 mg L-1 of each metal (Cd, Ni and Cr) following inoculation of 500 µl bacterial cultures with pH of the medium adjusted at 5 in shaking incubator (150 rpm) for 24 hr at 37 ˚C. M9 minimal medium was prepared in distilled water. After complete dissolution, pH of the medium was adjusted to 5.0 and poured (30 ml) in 100 ml conical flasks. The flasks were cotton plugged and autoclaved at 121 ̊C for 15 min. After autoclaving, the medium was supplemented with CaCl2, MgSO4, casien and selected metals (with initial concentration of 200 mg L-1). The metals supplemented medium, except for control, was inoculated with 1% of bacterial suspension prepared in 1 ml of autoclaved distilled water. The flasks were swirled gently to ensure homogenous mixing and incubated at 37 ̊C for 24 hr on rotary shaker (150 rpm). Afterwards, centrifugation was performed to harvest the bacterial cells (suspended in 1 ml of distilled water) for metal content analysis. Acid dissolution of bacterial cells was performed by following Ganje and Page (1974). The analysis for metal ions bioaccumulation proficiency of bacteria from the medium under these parameters was performed via atomic absorption spectrophotometer. Finally the maximum bioaccumulation yield for each metal was calculated using eq 1.

**Maximum bioaccumulation yield of metals by acidophilic bacteria**

Metal accumulation property of bacteria was given as bioaccumulated metal concentration at the end of growth (Cacc,m in mg L-1) obtained by subtracting from initial concentration of metal supplemented (C˳ in mg L-1) and bioaccumulation yield was determined by following formula:

Bioaccumulation yield = Cacc,m ÷ C˳ eq 2

Where Cacc,m = Accumulated concentration of metal (mg L-1) and C˳ = Initial dye concentration (mg L-1) (Kilic et al., 2007)

**Statistical analysis**

All the experiments were performed in a set of three (triplicates) and results were articulated as standard errors of mean (±SEM).

**Plasmid isolation**

It has been reported that a large number of bacteria possess the capability of quickly adapting to changing ecological conditions via genetic distributions occurring through plasmid transfer including resistance to antimicrobial agents and heavy metals, and degradation of toxic substances. Hence plasmids are known to carry the determinants of resistance to heavy metals (Coral et al., 2005; Li et al., 2015). Presence of plasmid DNA in bacterial isolates was determined through alkaline lysis method (Kotchoni et al. 2003) with some modifications. Pure bacterial colonies were inoculated in sterile nutrient broth (5 ml) and incubated at 37 ˚C and 150 rpm for 24 hrs. Bacterial cultures (1.5 ml) were taken and centrifuged at 5000 g for 5 min at room temperature. The resulting supernatants were aspirated and cell pellets were resuspended in 200 µl of solution I (Glucose 50.0, Tris-HCl 25.0, EDTA 10.0 mM per liter of autoclave distilled water and pH 8.0). The pellets were mixed very gently by pipetting up and down, followed by addition of 400 µl of freshly prepared solution II (0.4M NaOH and 2% SDS 20.0 ml each), mixed well by inverting gently four to six times to avoid plasmid breakage and kept on ice for 3 min. Then, 200 µl of chilled solution III (5M Sodium acetate 60.0, Glacial acetic acid 11.6 and autoclaved distilled water 28.5 ml, pH 4.5) was immediately added. The tubes were gently inverted four to six times, incubated on ice for 5 min and centrifuged (10000 g, 5 min, room temperature). The supernatants (~800 µl each) were carefully transferred into new eppendorf tubes following addition of isopropanol (600 µl), mixed gently by inverting four to six times and kept at room temperature for 10 min. The mixtures were centrifuged at 10000 g for 5 min, plasmid DNA got precipitated as pellets, and supernatants were discarded. The pellets were washed with 70% ethanol (400 µl) and centrifuged again (10000 g, 3 min, room temperature). The supernatants were removed and pellets were air-dried (ten to twenty min) to get rid of residual ethanol. Pellets were resuspended in 50 µl of TE buffer (Tris-HCl 10.0 and EDTA 1.0 mM per liter of autoclaved distilled water, pH 8.0) and run on 1% agarose gel for profiling of plasmid DNA. The plasmids were visualized under UV-illuminator and photographed using software in Dolphin gel documentation system (Wealtec, USA).

**Biofilm formation**

Biofilm formation is one of the properties of microorganisms to protect themselves against toxins, antimicrobial agents and stressed environmental conditions. Moreover biofilm formation is associated with resistance of microbes against metals and their subsequent accumulation within the biofilms. Therefore bacteria used in present study were also assessed to their potential to form biofilms. Microtiter plate assay (Cerca et al., 2007) with some modification was performed for evaluating biofilm forming potential of the bacterial strains. Bacterial strains were cultured in nutrient broth and standardized to obtain 106-108 CFUs ml-1. 20 µl of culture was inoculated to 180 µl of nutrient broth contained in 96 well microtiter plate. After 48 hours of incubation culture contents were decanted and plates were washed twice with 0.85% saline solution. Attached cells were fixed with 200 µl of methanol for 20 min, followed by discarding of fixative and staining with 0.1% crystal violet solution for 10 min. Excessive stain washed with saline solution and plates were read with microtiter plate reader (BioRad680XR) after pouring 200 µl of glacial acetic acid (33%). Furthermore, the effects of different metals (Cd, Ni and Cr ranging between 100-600 mg L-1) on biofilm formation potentials of the bacteria were also assessed. Experiments were performed in triplicates.

**Production and characterization of extracellular polymeric matrix (EPS)**

Since resistance to heavy metals and their bioaccumulation is related to secretion of extracellular polymeric substances by bacteria. Therefore, MB375, MB381, MB394 and MB398 were assessed for their potential to produce EPS. EPS from these bacteria were extracted and analyzed for pyruvic acids, uronic acids, carbohydrates and protein contents via methods described by Tahir et al. (2018).

**Results and discussions**

**Isolation, characterization and screening of acidophilic bacteria for metal resistance**

The bacterial isolates were obtained from biofilm samples using spread plate method and after purification these bacteria were further tested for tolerance against different metals. MB375, MB381, MB394 and MB398 conferring tolerance to Cd, Ni and Cr up to 1000 mg L-1 were further selected for bioaccumulation studies. These strains were assessed for determining the LC50 for each metal using different concentrations of metals (100, 200, 400, 600, 800 and 1000 mg L-1). The bacterial growth was inversely proportional to the increase in metals concentration. The LC50 of Cd and Cr calculated for MB375 was 600 mg L-1 and 400 mg L-1 for Ni. In case of MB381, 200 mg L-1 of Cd, Ni and Cr was observed to be the lethal concentration. LC50 for MB394 was recorded at 200 mg L-1 for Ni, 400 mg L-1for Cr and 600 mg L-1 for Cd. While for MB398 600 mg L-1 of Cd and Cr, and 400 mg L-1 of Ni exerted the lethal effects on growth. Ameen and coworkers (2020) screened 12 marine bacterial species for metal resistance efficiencies against chromium, nickel, cadmium and lead (10 to 600 mg L-1) and reported high chromium resistance potential of *Lactobacillus plantarum* MF042018 against chromium. All the bacterial strains used in present research were gram negative and rod shaped. Strains MB375, MB381 and MB394 exhibited optimal growth at pH 5, while MB398 displayed maximum growth at pH 7, which was indicative of their acidophilic nature. All the four strains grew maximally at 37 ˚C (mesophiles). Thomas and Rice (2014) documented higher metal uptake and accumulation capacities of gram negative bacteria compared to gram positive bacteria due to presence of active metal binding moieties (amino, carboxyl, phosphorus, sulfuryl groups) in bacterial cell walls as reported by Churchill and colleagues (1995) in case of *E. coli* and *P. aeruginosa* (gram negative), these strains accumulated nickel and chromium more efficiently in contrast to *Micrococcus luteus* (gram positive strain). Based on the biochemical characteristics each of the bacterial strain was distinct from one another as represented in Table 1.

**Phylogenetic analysis of acidophilic bacteria**

Phylogenetic analysis of partially sequenced 16S rRNA genes of bacterial isolates revealed that the strains were closely related to *Klebsiella* species (Fig 1). The individual strains were identified as *Klebsiella pneumonia* (MB375, MB394 and MB398) and *Klebsiella oxytoca* (MB381). The sequences obtained were submitted to Genbank under accession numbers KP886824, KP886826, KP886827 and KP886828, respectively. *Klebsiella pneumonia* MB375 displayed 99% similarity with *Klebsiella pneumonia* R-70 strain available in NCBI database under accession number NR037084.1.

**Influence of initial concentration on metal bioaccumulation**

Bioaccumulation potential of the bacterial isolates against cadmium, nickel and chromium was assessed at six different concentrations (100, 200, 300, 400, 500 and 600 mg L-1). The result of these experiments revealed that percentage bioaccumulation by selected bacterial strains was concentration-dependent, which declined with the increase in concentrations of the respective metals as depicted in figure 2(a). *Klebsiella pneumoniae* MB398 efficiently accumulated 93.49% of cadmium provided at the rate of 100 mg L-1 in the medium. However, *Klebsiella oxytoca* MB381could accumulate 91.88% of cadmium (100 mg L‑1) followed by *Klebsiella pneumoniae* MB375 (88.67%) and *K*. *pneumoniae* MB394 (87.49%). Percentage bioaccumulation declined following regular pattern with increase in concentration of cadmium up to 600 mg L-1. *Klebsiella pneumoniae* MB398 accumulated 95.64% of chromium (at 100 mg l-1), with little less than that by *Klebsiella pneumoniae* MB375 (92.73%) following *K*. *pneumoniae* MB394 (92.64%) and *K*. *oxytoca* MB381 (92.21%). Almost similar trend for nickel was observed as in case of cadmium and chromium. More than 72-76% of 100 mg L-1 of nickel was bioaccumulated by the four strains, which reduced to 12-20% at 600 mg L-1. Ameen et al. (2020) observed higher bioaccumulation efficacies of cadmium and lead by *Lactobacillus plantarum* at 50 and 100 mg L-1, which was negligible at higher metal concentrations. Decline in bioaccumulation potential of the bacterial strains at higher metals concentrations might be due to the saturation and/or unavailability of sufficient number of free binding sites on bacterial peptidoglycan layer. Since all the metal ions present in lower concentrations in the medium have an equal chance to interact with the available sites for binding, thus the percentage bioaccumulation is likely to become higher compared to that in presence of higher metal concentrations. At higher concentrations the saturation of binding sites results in lower bioaccumulation yields as reported by Pandiyan and Mahendradas (2011); Oves and colleagues (2013); Ergul-Ulger and companions (2014), and Haung and coworkers (2014).

**Influence of inoculum volume on metal bioaccumulation**

The bacterial strains MB375, MB381, MB394 and MB398 displayed lowest cadmium bioaccumulation (45-32%) at 0.2% inoculum volume, while, highest cadmium accumulation (84-90%) was noticed at 1% of the inoculum volume (Figure 2b). Nickel sorption by *Klebsiella pneumoniae* MB394 increased up to six times (from 10.42% to 60.03%) upon increasing the inoculum volume (0.2% to 1%). Likewise, the uptake of nickel increased to 65.77% by Klebsiella oxytoca MB381 and 75.59% by *Klebsiella pneumoniae* MB398 at 1% inoculum. Approximately, 91.01% of chromium was removed by *K*. *pneumoniae* MB398; however, 89.91%, 88.61% and 88.14% of chromium was bioaccumulated by *K*. *pneumoniae* MB375, *K*. *oxytoca* MB381 and *K*. *pneumoniae* MB394, with 1% inoculum volumes. Issazadeh et al. (2014) have documented that microorganisms possess higher surface area to volume ratios due to their small size; therefore, they provide larger contact area for interaction with metals provided in the medium and/or surrounding environment. Additionally, Devika and coworkers (2014) observed an increase in metal sorption potential of bacteria with augmented biomass due to larger surface area which ultimately increases the number of metal-binding sites onto the bacterial surfaces. Therefore, the results of present study ascertained that increase in inoculum volume is directly proportional to the metal uptake by the bacterial strains. Hence, the bioaccumulation yield of metals enhanced significantly upon increasing the bacterial biomass.

**Influence of temperature on metal bioaccumulation**

Temperature can significantly influence the microbial binding of metals (Ameen et al. 2020) as the optimal growth of the strains was also recorded at 37 ˚C, therefore, the effect of temperature on metal bioaccumulation efficiencies of bacterial strains was monitored at 30, 37 and 45 ˚C. The outcomes of experiments conducted using different incubation temperatures revealed that metals accumulation was temperature dependent. All the four bacterial strains maximally accumulated cadmium at 37 ˚C (Figure 2c). *Klebsiella pneumoniae* MB398 most efficiently accumulated 91.29% of cadmium at 37 ˚C, with second highest bioaccumulation percentage exhibited by *Klebsiella oxytoca* MB381 (89.93%). All the four bacterial strains exhibited optimum uptake of nickel at 37 ˚C, which declined with the increase in temperature to 45 ˚C. *Klebsiella pneumoniae* MB398 displayed maximum accumulation of nickel (73.42%) following *Klebsiella oxytoca* MB381 (67.84%) at 37 ˚C. Similar trend of percentage bioaccumulation in case of chromium with optimal uptake of 91.17% by *Klebsiella pneumoniae* MB398, 91.01% by *Klebsiella oxytoca* MB381, 90.10% by *Klebsiella pneumoniae* MB375 and 88.62% by *Klebsiella pneumoniae* MB394 was observed at 45 ˚C. Temperature of the medium also influences the uptake of metals by the bacterial strains because it could affect the configuration and stability of the bacterial cell wall, and may lead to the ionization of the chemical groups/moieties, which in turn may affect the binding sites, thus, leading to decline in metal accumulation, explicitly documented by Rajeshkumar and colleagues (2012). In a study, *Salmonella enterica* obtained from industrial effluents was reported to bioaccumulate cadmium optimally (15 mM) at 30-37 ˚C, which declined (13 mM) upon increase in temperature to 42 ˚C (Khan et al. 2016), thus supporting the results of present study. Al-Dhabi and coworkers (2019) reported maximum removal of cadmium by *Pseudomonas* sp. Al-Dhabi 126 at 50 ˚C and pH 6.0. Since, the bacterial strains in present study displayed optimum growth under mesophylic conditions, therefore, all these strains exhibited maximum uptake of metals at 37 ˚C.

**Influence of pH on metal bioaccumulation**

pH of the medium is one of the major factors that determine the uptake of metals because it influences ionization of the functional groups present on microbial surfaces, their (bioadsorbent’s) surface charge ratios as well as metal ions solubility (Rajeshkumar et al. 2012; García et al., 2016). Therefore, optimum pH of bacterial strains for maximum metal accumulation was assessed and evaluated in order to figure out the role of pH in metals uptake. Results of experiments performed at varying pH depicted a decline in percentage bioaccumulation with increasing pH (Figure 2d). All the four bacterial strains exhibited maximum sorption of metals under acidic conditions. *Klebsiella oxytoca* MB381 displayed highest sorption of cadmium (94.99%) at pH 5.0, followed by *Klebsiella pneumoniae* MB398 (92.57%). Almost similar findings were recorded for *Klebsiella pneumoniae* MB398 (91.35%) and *Klebsiella oxytoca* MB381 (91.10%) in case of chromium accumulation at pH 5.0, which declined under alkaline conditions. Likewise, both these strains displayed maximum uptake of cadmium (91.57 and 89.99 %) and nickel (72.65 and 70.07 %), respectively at acidic pH conditions (i.e. pH 5.0). These variabilities in bioaccumulation patterns by bacterial strains were indicative of the fact that metal accumulation varies with the type of metal (adsorbate) and biomass (adsorbent) provided as reported by Dexilin et al. (2007). Mostly the microbial surfaces possess **-**ve charges due to functional groups ionization; thus, contribute towards metal binding via provision of active binding sites. Microbial surfaces become **-**vely charged between pH ranges of 2.0 to 6.0, as the concentration of protons is higher at lower pHs due to deprotonation of metal binding sites, therefore, metal ions compete more effectively for the accessible/available binding sites which results in increased bioaccumulation/biosorption rates. While, the reduction in bioaccumulation at alkaline pHs is attributed to the formation of soluble complexes (hydroxylated complexes) of metal ions and their competition for occupation of active sites with hydroxyl ions (OH-), and beyond pH 8.0 precipitation of ions as hydroxides occurs (Rajeshkumar et al. 2012). Hence, variability in metals accumulation patterns of bacterial strains at varying pHs might be attributed to the differences in sensitivity responses of bacterial cell wall molecules (physiological properties) towards the provided pHs as documented by Oves and coworkers (2013). Rajeshkumar et al. (2012) reported 90% removal of cadmium with increase in pH up to 6.0, which declined under alkaline pH conditions (pH 10.0). Likewise, Oves and coworkers (2013) reported maximum accumulation of cadmium and copper under acidic pH conditions (pH 6.0) by *Bacillus thuringiensis*. In another research study Batool et al. (2017) reported 100% bioaccumulation of cadmium and chromium by *E*. *luteus, S. aureus* and *E*. *coli* at acidic pH over 24-72 hr of incubation, which declined with increase in pH. Thus, the findings of all these reported studies are supporting the results observed in the present study.

**Bioaccumulation experiments and Maximum bioaccumulation yield of metals by acidophilic bacteria**

After optimization of experimental conditions, final metal bioaccumulation experiment was performed with 200 mg L-1 of each metal (Cd, Ni and Cr) at pH 5.0. According to the results obtained *Klebsiella pneumoniae* MB398 efficiently removed cadmium up to 91.44% followed by *Klebsiella oxytoca* MB381 (90.99%) and *Klebsiella pneumoniae* MB375 (90.01%). *Klebsiella pneumoniae* MB398 displayed 71.70% uptake of nickel from the medium provided. Maximum uptake of nickel (70.46%) and chromium (92.06%) was observed for *Klebsiella oxytoca* MB381 (Figure 3). Bioaccumulation yields for metals uptake by the bacterial strains revealed highest bioaccumulation yield of *Klebsiella oxytoca* MB381 for chromium (45.56 mg L-1), cadmium (40.16 mg L-1) and nickel (35.61 mg L-1) followed by *Klebsiella pneumoniae* MB398 for cadmium (41.88 mg L-1), chromium (41.52 mg L-1) and nickel (37.13 mg L-1). The data is provided in Table VI (supplementary data). All these results indicated that these strains might have possessed the metal accumulator genes, thus, exhibited higher resistance and metal accumulation properties as reported by Saluja and Sharma (2014). Afzal et al. (2017) reported 49% removal of nickel by *Klebsiella variicola* isolated from industrial effluents within 24 hr at 37 ˚C and pH 7.0. In another study Ameen et al. (2020) reported 33.8% removal of nickel and 30.2% of chromium by *Lactobacillus plantarum* MF042018 within 24 hr. Wang and companions (2000) documented involvement of two mechanisms in resistance of bacteria to chromium. First is the biotransformation of chromium VI into III which is less harmful/toxic, while second refers to biosorption where pollutants are accumulated/trapped within the bacterial biomass (Volesky, 1986). In view of Volesky (1986) the attraction or affiliation for metal removal is dependent upon the chemical composition or structure of the organisms. Likewise, Batool and colleagues (2017) also reported that chemical composition of microbial species play paly an essential role in uptake and removal of heavy metals, for instance, overexpression of metallothioneins by microbial species under stressed conditions leads to increase in metals uptake from the medium, which will be an effective strategy for applications of microorganisms in remediation of metals as biosorbents. Similarly, expression of proteins on the bacterial cell surfaces as adsorbents provides an effective, efficient and inexpensive way to remediate heavy metals from surrounding environment.

**Plasmid DNA**

Plasmids (extra-chromosomal genetic elements) may range in size from several hundred base pairs (bps) to thousand kilobase pairs. Numerous bacterial plasmids with heavy metal resistance features have been reported to be mobilizable and self-transferable. Since the presence and characterization of plasmids serves as a knowledge source for ecology of plasmids and their contribution in genetic adaptations and metal resistance (Zolgharnein et al., 2007), we also conducted to experiments to assess the presence and prevalence of metal resistant plasmids in *Klebsiella* sp. According to gel electrophoretic results all the four *Klebsiella* sp. MB375, MB381, MB394 and MB398 possessed three plasmids (Figure 4). The molecular weights of plasmids isolated from these bacteria were approximately 200, 750 and 1000 bps. Mounaouer et al. (2014) also reported the presence of metals resistant plasmids in heavy metals resistant bacteria isolated from different polluted sources. In another research study, Zolgharnein and colleagues (2007) also reported the presence of small (>2 to 4 kbs) and large-sized (38-62 kbs) plasmids in bacteria isolated from industrial wastewaters. Chudobova and coworkers (2015) stated that different microorganisms have successfully adapted to uptake and accumulate the contaminants by limiting their harmful/toxic effects via different detoxification mechanisms including chromosomes and plasmid encoded, and transposons mediated metal resistance mechanisms. Most of these resistance mechanisms are plasmid-mediated, and these plasmid mediated resistance mechanisms are highly specific for particular anions and cations. The metals exert variable effects inside the bacterial cells depending upon their chemical nature and concentrations. In elevated concentrations of metals bacteria respond by expressing specific resistance mechanisms such as metallothioneins, RND efflux pumps, P-type ATPases and CDF transporters (Nies, 2003; Chudobova et al., 2017).

**Biofilm formation**

Biofilm formation potential of the bacteria was assessed over different incubation periods. All the strains depicted an inclination in biofilm formation over the passage of time (Fig 5a). *Klebsiella oxytoca* MB381 and *Klebsiella pneumoniae MB398* showed highest biofilm formation (2.281 and 2.168) after 72 hr of incubation. Furthermore, the influence of metals (Cd, Ni and Cr) with different concentrations on biofilm forming ability of the strains was also determined through 96-well microtiter plate method. The bacterial strains showed a decline in biofilm forming potential with the increase in metals concentration from 100 to 600 mg L-1 (Figure 5b). MB398 displayed highest biofilm formation (2.806) in the presence of 100 mg L-1 of chromium followed by MB381 (2.354), MB394 (1.221) and MB375 (1.064). As far as effects of nickel are concerned, MB381 displayed maximum biofilm formation (1.089) at 100 mg L-1 of nickel with second highest biofilm forming potential by MB398 (1.087) followed by MB375 (0.974) and MB394 (0.943). While an increased biofilm formation was observed for MB381 (2.424) following MB398 (2.376), MB394 (1.453) and MB375 (1.045) at 100 mg L-1 of cadmium. The bacterial cells within ecological settings prefer to live as single or multiple species aggregates referred as biofilms where these cells are surrounded with self-produced extracellular polymeric substances (EPS). These cells in biofilm mode respond to metals exposure in variable ways as they possess the capability of synthesizing EPS, metabolites, nucleic acids and proteins surrounding the cell surfaces which is their unique feature. Biofilm formation (comprising of layers of microbial cells) enhances resistance towards metals, antibiotics up to 1000 times (Prabhakaran et al., 2016). Swiecilo and Zych-Wezyk (2013) stated that the resistance of the cells towards different environmental stresses is due to the activation of various stress response mechanisms during biofilm formation and in mature biofilms. Pal and Paul (2008) reported that proteins and carbohydrates within the biofilms are the major players in the elimination process of metals. Presence of enzymes (phosphatases, peptidases) and polysaccharides within the biofilms have also been reported to increase metal uptake by the microbial cells (Karunakaran et al. 2011). According to Harrison et al. (2007) the dead cells within the biofilms may defend the living cells from the toxic effects of metals via sequestration and precipitation because these cells (dead) are chemically reactive and contain biosorption sites, which might lead to the formation of metal chelates and precipitates. It also has been reported that the metabolic end products produced by the microbial species react with metals and lead to precipitation of bio-inorganic metal complexes like co-precipitation of cadmium, nickel, copper, lead and zinc with sulfides (S2-) in biofilms of sulfur reducing bacteria, and heavy metals co-precipitation with carbonates (CO32- and HCO3-) produced during microbial respiration led to the elimination of metals from aqueous phase (Harrison et al. 2007).

**EPS production and composition**

Since extracellular substances (EPS) are the metabolic products of biofilm forming bacteria and also contribute towards bacterial resistance against metals stress due to presence of high molecular weight polysaccharides with homo- or hetro-polymeirc compositions, therefore, EPS extracted from bacteria were subjected to various tests for determining their chemical composition. The biopolymeric substances were rich in uronic acids, pyruvic acids, proteins and carbohydrate contents (Figure 6). EPS from *Klebsiella pneumoniae* MB398 possessed highest amount of uronic acids (15.48± 0.18 µg mg-1), followed by pyruvic acids (12.83± 0.21 µg mg-1), carbohydrates (10.13± 0.30 µg mg-1) and protein (1.85± 0.14 µg mg-1) contents. *Klebsiella pneumoniae* MB394 possessed EPS rich in uronic acids (11.52±0.24 µg mg-1), carbohydrates, pyruvic acid and proteins, while, carbohydrates content was highest (9.79±0.14 µg mg-1) in EPS from *Klebsiella oxytoca* MB381 compared to rest of the chemical constituents. According to Pal and Paul (2008) EPS serves as protective layer against metals stress through sequestering/binding of metal ions or delaying their diffusion within the biofilm due to the existence of ionizable function groups (amine, phosphoric, carboxyl and hydroxyl). EPS are anionic due to presence of uronic and pyruvic acids, and along with the ionizable functional groups these might be communicating with other molecules, metals and minerals. Since uronic acids are known to influence the anionic nature of the EPS, therefore, EPS can potentially be applied in biodetoxification of metals and wastewater depending upon the metal binding features of these polymers (Bramhachari and Dubey 2006; Bramhachari et al. 2007).

**Conclusion**

Four bacterial strains identified as *Klebsiella* sp. obtained from biofilm samples revealed resistance to Cd, Ni and Cr up to 1000 mg L-1 and displayed promising results for metals accumulation. Further research is in progress for identification and annotation of genes involved in metals tolerance and to comprehend genetic expressions and revealing their possible tolerance/resistance mechanisms. This would be helpful in designing of more efficient techniques for bioremediation of metal contaminants from ecological systems. The bacterial growth was inversely proportional to the increase in metals concentration. Negative Strains MB375, MB381 and MB394 exhibited optimal growth at pH 5, while MB398 displayed maximum growth at pH 7, which was indicative of their acidophilic nature. The phylogenetic analysis of acidohiplic bacteria showed that *Klebsiella pneumonia* MB375 displayed 99% similarity with *Klebsiella pneumonia* R-70 strain available in NCBI database under accession number NR037084.1. The result of the experiments for checking the Influence of initial concentration on metal bioaccumulation revealed that percentage bioaccumulation by selected bacterial strains was concentration-dependent, which declined with the increase in concentrations of the respective metals. *Klebsiella oxytoca* MB381showed highest accumulation 91.88% of cadmium. Influence of inoculum volume on metal bioaccumulation showed increase in inoculum volume is directly proportional to the metal uptake by the bacterial strains. Hence, the bioaccumulation yield of metals enhanced significantly upon increasing the bacterial biomass. Temperature can significantly influence the microbial binding of metal. The outcomes of experiments conducted using different incubation temperatures revealed that metals accumulation was temperature dependent. Since, the bacterial strains in present study displayed optimum growth under mesophylic conditions, therefore, all these strains exhibited maximum uptake of metals at 37 ˚C. At varying pH depicted a decline in percentage bioaccumulation with increasing pH. All the four bacterial strains exhibited maximum sorption of metals under acidic conditions. Bioaccumulation experiments and Maximum bioaccumulation yield of metals by acidophilic bacteria showed that all the strains exhibited higher resistance and metal accumulation properties, due to their metal accumulator genes. Biofilm formation potential of the bacteria was assessed over different incubation periods. All the strains depicted an inclination in biofilm formation over the passage of time. EPS extracted from bacteria were subjected to various tests for determining their chemical composition. EPS serves as protective layer against metals stress through sequestering/binding of metal ions or delaying their diffusion within the biofilm due to the existence of ionizable function groups. The results of present research demonstrated that Klebsiella species can efficiently be deployed in bioremediation for removal of potentially toxic metals from contaminated environmental reservoirs.

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