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Isolation and Characterization of Heavy Metal-Tolerant Bacterial Isolates from Industrial Effluents in Uttar Pradesh, India

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Authors' contributions

This work was carried out in collaboration among all authors. Author SG designed the study, performed the experiments and analyzed the data, and wrote the manuscript. Authors SG, OPV, and VT conceived the idea and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Industrial activities over the past century have significantly increased human exposure to pollutants such as heavy metals. Industrial emissions of heavy metals, which are carcinogenic, mutagenic and toxic, contaminate natural water supplies and the agricultural environment. Due to the high concentration of heavy metals in industrial effluents, the bacteria present there naturally develop resistance to heavy metals. The aim of this study is to isolate and characterize bacteria resistant to heavy metal, lead (Pb), copper (Cu), chromium (Cr), cadmium (Cd), and nickel (Ni) from sites contaminated by industrial effluents in Uttar Pradesh, India. A total of 58 bacterial isolates were isolated from 9 samples according to their various morphological parameters and 32 isolates were

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found positive for all heavy metals, which revealed that samples contained metal-resistant bacterial diversity. Two isolates were identified up to species level based on their physiological, biochemical and molecular characterization as *Comamonas testosteroni* (S4C1) and *Bacillus cereus* (S5C3). Both isolates are highly resistant to lead (Pb), copper (Cu), chromium (Cr), cadmium (Cd), and nickel (Ni) and they show different MICs against the above heavy metals at different levels. A growth experiment showed that the presence of heavy metals concentration had no discernible impact on the growth rate among the isolates. Gel analysis showed interesting patterns of protein expression were observed in the presence of various heavy metals. MALDI-TOF analysis found that specific proteins (S layer protein, F0F1ATP synthase subunit b, Flagellin, 50S ribosomal protein L4, Molecular chaperone) were overexpressed in the presence of heavy metals. As a result, identifying the heavy metal resistance bacteria and their proteins study could be useful as a preliminary investigation for the development of prospective bioremediation agents of potentially hazardous waste treatment technology.

Keywords: Resistance; Comamonas testosterone; Bacillus cereus; heavy metals; industrial effluents; environment; wastewater.

1. INTRODUCTION

One of the main causes of environmental pollution is heavy metals. The amounts of these harmful chemical compounds in the environment vary greatly because they are released as a result of the effluent that is discharged into the environment by a huge number of industrial activities, including metal processing, mining, electroplating, leather tanning, and pigment manufacture [1,2]. Excessive heavy metal concentrations in wastewater are not broken down by the traditional wastewater treatment process, which negatively affects aquatic life [3,4]. While many heavy metals, including copper, iron, and zinc, are necessary trace elements for cells at low concentrations, they can have hazardous effects at higher concentrations found in contaminated settings [5,6]. According to Chovanová et al., [7] cadmium is not only harmful to humans, animals, plants, and microorganisms, but it is also non-essential. Lead is a highly poisonous and dangerous pollutant that can be found in soil, water, and the air [8-11]. The most common forms of chromium in the environment are hexavalent (Cr(VI)) and trivalent (Cr(III)), both of which are harmful at high concentrations [12]. Beside exceedingly toxic and harmful, heavy metals are present in soluble form in the aquatic environment and disrupt ecological activities [13-15]. In addition, they can create complicated unspecific chemicals inside of cells, which has harmful effects. Many microorganisms, including bacteria, can evolve resistance and become heavy metal resistant in response to harmful quantities of metal ions [16]. Bioaccumulation [17], bio-sorption [18], bio-mineralization and precipitation [19], enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metal systems are some of the various intra- and extracellular mechanisms found in bacteria [20]. Heavy metal stress is known to change a native bacterial community's makeup so that resistant and tolerant bacteria become widely predominate [21]. The removal of metal ions by microbes has attracted a lot of attention due to its potential use in protecting the environment and recovering harmful or important heavy metals, [22]. The present research aims to isolate and characterize heavy metal resistant bacteria from industrial effluents.

2. MATERIALS AND METHODS

2.1 Materials

To prepare metal stocks solutions lead, copper, chromium, cadmium, and nickel salts were used to prepare stock solution. Applicable solutions were prepared daily by diluting the stock solution. Stocks were used to adjust the pH of the solutions. All chemical materials and culture media were procured from Sigma-Aldrich.

2.2 Study Area and Sample Collection

In this study, effluent samples were collected in sterilized screw cap bottles from the industrial discharge area, Fertilizer production unit, Pryagraj and Iffco fertilizer Bareilly, U.P., India. The samples were transported with ice boxes to the laboratory for further analysis.

2.3 Isolation of Heavy Metal Resistant Bacteria

Isolation of the bacteria from the collected samples were carried out using the serial dilution

and spread plate method, where the samples were serially diluted in 0.85% NaCl solution and then spread in sterilized nutrient agar media and incubatied at 37 ℃, for 24 hours. The isolates were further screened for the heavy metals in Luria-Bertani (LB) agar media supplemented with 100 ppm (~100 mg/L) of PbNO3, CuSO4, K2Cr2O7, CdCl2.H2O and NiCl2.6H2O. The inhibition in growth of bacteria indicates positive results [23,24].

2.4 Minimum Inhibitory Concentrations (MICs) Study

Heavy metal resistance were assessed through MIC protocol described previously by Filali et al. (2000). Isolated bacterial strains were grown on nutrient broth (NB) medium containing the different concentrations of heavy metals. The stock solution of concentration 1000 ppm (1 mg/ml) of different heavy metal was prepared and sterilized by autoclaving at 121 ºC for 15 min. The cultures were allowed to grow at a given concentration and incubated at 37 ºC for 48 hours in shaker incubator. The absorbance of bacterial growth was studied at 600 nm. The MIC was defined as the concentration at which a particular bacterial strain could not grow [25,26].

2.5 Studies of Optimum pH and Temperature on Growth Conditions

The growth of bacterial isolates in nutrient broth medium at various pH values (ranging from 3 to 12), temperatures (ranging from 10 ℃ to 65 ℃) was studied. In order to quantify the growth, the optical density of the log phase growing conditions was measured at 600 nm [27-29].

2.6 Heavy Metal Biodegradability Assay

Sterilized different culture medium was prepared and supplemented with different heavy metals. The bacterial cultures were inoculated and then media was incubated at 37 ℃ for 48 hours. The samples were collected from the control and then after 48 hours of treatment and then processed for Atomic Absorption Spectroscopy (AAS) analysis [30,31].

2.7 Morphological and Biochemical Characterization of Heavy Metal Tolerant Bacterial Isolates

The identification of the bacterial culture which showed the best results in the presence of PbNO3, CuSO4, K2Cr2O7, CdCl2H2O and NiCl26H2O was carried out through morphological, and biochemical characterization. Gram staining, catalase test, glucose fermentation test, Methyl Red and Voges-Proskauer (MRVP) test were carried out for biochemical characterization [32,33].

2.8 Molecular Characterization of Heavy Metal Tolerant Bacterial Isolates

The molecular identification of the culture was carried out through 16S rDNA sequencing. Primer of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score, the first ten sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated, and the phylogenetic tree was constructed using MEGA7 [34-36].

2.9 Identification of Stress Proteins in the Presence of Heavy Metals

The protein profile was studied after extraction of the protein from bacterial culture using PBS buffer and then quantification was carried out using Bradford assay. Further the characterization and identification of proteins was carried out using SDS page and 2D Gel electrophoresis followed by MALDI-TOF-MS [37-39].

2.10 Statistical Analysis

Three replicates of each experiment were carried out. The means ± standard deviation are used to present the data. Tukey tests were used after one-way Analysis of Variance (ANOVA) for statistical analysis, and a difference of $p < 0.05$ considered significant.

3. RESULTS AND DISCUSSION

3.1 Isolation and Preliminary Screening of Heavy Metal Tolerant Bacterial Isolates

Total 58 bacterial isolates were isolated from the selected 9 samples according to their different morphological parameters. Based on primary screening conducted on LB agar medium with a heavy metal concentration of 100 ppm (~100

mg/L). Total 32 isolates were found positive in different all heavy metals after 24-72 hours of incubation at 37℃ which showed that the collected effluent sample have metal resistant diversity of bacteria. The colonies count was performed of bacteria that were able to grow well on medium supplemented with Lead (8), Copper (4), Chromium (9), Nickel (6) and cadmium (6) similar study published by Joshi & Modi [40]; Zahoor & Rehman [41] found various heavy metal resistant microbes from industrial effluents. Among all these bacterial isolates, S4C1 and S5C3 were selected for further study as it showed maximum results with all heavy metal in screening as shown in Fig. 1.

3.2 Minimum Inhibitory Concentrations

The MIC of heavy metal resistance in bacterial isolates S4C1 and S5C3 was found to be between 300 µg/ml and 420 µg/ml (Fig. 2). S4C1 and S5C3 isolates showed a strong tendency to withstand and grow in environments with heavy metal stress, Bhardwaj et al. 2018 [42]; Hassen et al.1998 [43] found similar outcome in their findings. Therefore, Potential bacterial isolates S4C1 and S5C3 were selected for further detailed study.

3.3 Effect of pH and Temperature on Growth of Heavy Metal Tolerant Bacterial Isolates

The pH and temperature parameters in the medium were optimized for bacterial isolates S4C1 and S5C3 growth conditions. Nutrient broth (NB) medium was used for the optimization study. In the flasks with 200 ml of sterilized NB medium, a bacterial strain was cultivated at various pH (3-12) and temperature (10 ℃ - 65 ℃) conditions. According to the results of the optimization study, the bacterial isolates S4C1 & S5C3 grew best at a pH of 7 similarly Kalaimurugan et al., [44] founds the optimum growth at pH 7-9 and a temperature of 35 ℃, similarly Moghannem [45] and Sahin & Ozturk [46] found best result at 35℃ as shown in Fig. 3 and Fig. 4.

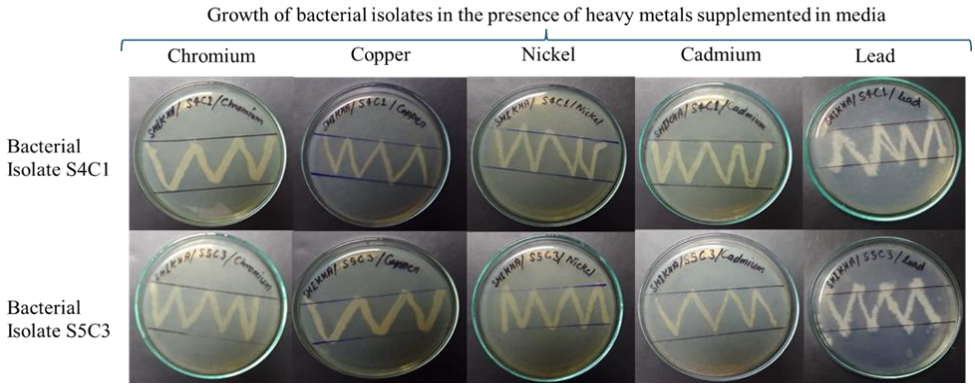

Fig. 1. Growth of bacterial isolates with heavy metal (Chromium, Copper, Nickel, cadmium and Lead) tolerance properties in the presence of 100 ppm (~100 mg/L) concentrations on LB agar media

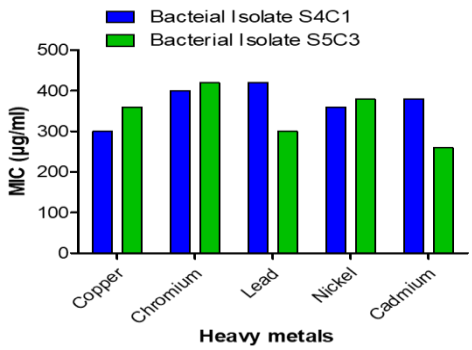

Fig. 2. Graphical representation of the minimum inhibitory concentrations values (µg/ml) of heavy metal-resistant bacterial isolates

Table 1. Minimal inhibitory concentrations (µg/ml) of bacterial isolates S4C1 and S5C3

Metal compound	MIC (µg/ml)	
	Bacterial Isolate S4C1	Bacterial Isolate S5C3
Copper	300	360
Chromium	400	420
Lead	420	300
Nickel	360	380
Cadmium	380	260

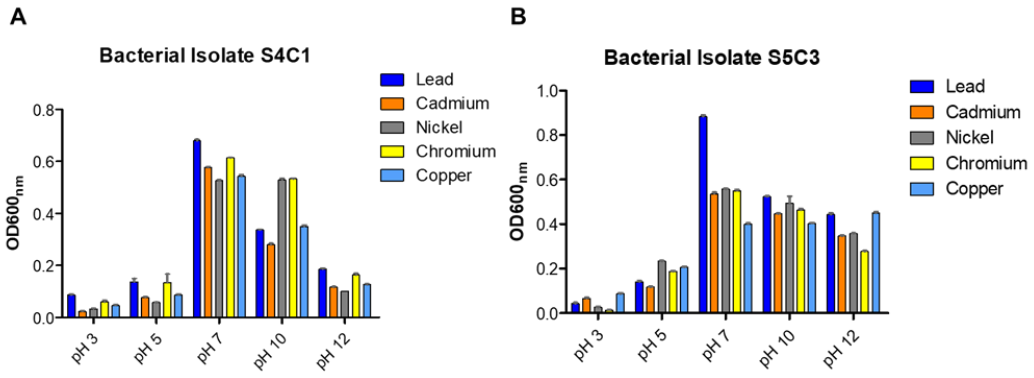

Fig. 3. Graphical representation of heavy metal resistance Bacterial isolates (A) S4C1 and (B) S5C3 growth at different pH

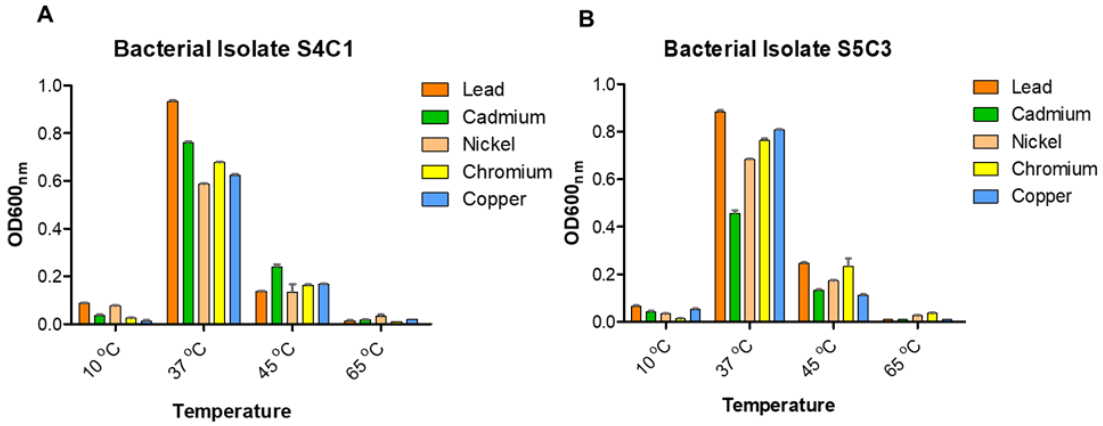

Fig. 4. Graphical representation of heavy metal resistance Bacterial isolates (A) S4C1 and (B) S5C3 growth at different temperatures

3.4 Heavy Metal Biodegradability Study

Following the optimization of the growth settings, the biodegradability of the relevant heavy metal was examined by Atomic Absorption Spectroscopy (AAS) for the selected bacterial isolates [47-49]. In order to determine the concentration of the heavy metal, a sample of the microbial growth in Nutrient Broth (NB) medium supplemented with heavy metals at pH 7.0 and 35 °C for 48 hours was taken. The results were then reviewed, and the results shown in Fig. 5.

3.5 Morphological and Biochemical Characterization of Heavy Metal Tolerant Bacterial Isolates

The morphological characteristics of bacterial isolates S4C1 and S5C3 were studied under the microscope using Gram staining technique. Gram staining is a method used to differentiate two types of bacteria (gram-positive and gram-negative) based on their different cell wall components. Table 2 displays the results of morphological and biochemical characteristics.

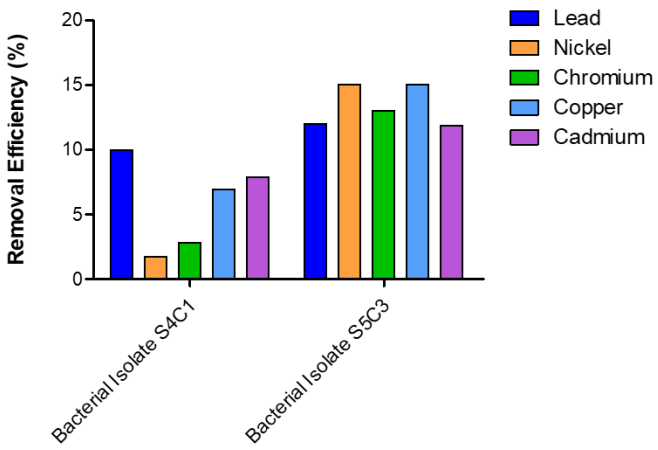

Fig. 5. Graphical representation of percentage removal efficiency of heavy metals through Atomic Absorption Spectroscopy (AAS)

Table 2. Morphological and biochemical analysis of bacterial isolates

Tests	Bacterial Isolate S4C1 (*Comamonas testosteroni*)	Bacterial Isolate S5C3 (*Bacillus cereus*)
Gram staining	Gram negative	Gram positive
Endospore staining	Negative	Positive
Catalase test	Positive	Positive
Glucose fermentation test	Negative	Positive
Mannitol test	Positive	Positive
Indole test	Negative	Negative
MR test	Positive	Positive
VP test	Negative	Negative

3.6 Molecular Characterization of Heavy Metal Tolerant Bacterial Isolates

Bacterial isolates were identified by 16 S rRNA gene sequencing to validate morphological and biochemical characterization. Isolated genomic DNA was amplified by 16S primers (Fig. 6) and 16 S rRNA gene sequencing was performed. Bacterial isolates S4C1 and S5C3 showed a high level (99–100%) similarity with known sequences in the NCBI database when compared using the Basic Local Alignment Search Tool (BLAST). This similarity was based on multiple sequence alignment by CLUSTALW along the branch length of the 16S rRNA sequence to create a phylogenic tree (Fig. 7). The MEGA 7.0 programme was used to create a Maximum Likelihood (ML) phylogenetic tree to determine the relationship between our isolates and other reference strains. Based on the first five identities, with nucleotide and amino acid similarities, respectively, the multiple heavy metal resistant bacteria isolates were identified as S4C1 is *Comamonas testosteroni* and S5C3 is *Bacillus cereus*. *Comamonas testosteroni* is aerobic gram-negative environmental bacteria that is able to break down estrogens and other sterols like ergosterol. *Bacillus cereus* is a Gram-positive bacterium commonly found in soil, food, and sea sponges. These also have the nature of forming spores, which can cause food borne diseases.

3.7 Identification of Stress Proteins in the Presence of Heavy Metals

Bacteria were grown in NB media in the presence of heavy metals lead (Pb), copper (Cu), chromium (Cr), cadmium (Cd), and nickel (Ni). The results revealed the presence of heavy metal specific stress proteins in the treated groups (Table 3). The following results were obtained when analyzing the protein profiles via SDS-PAGE (Fig. 8) and Identification by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) as shown in Fig. 9.

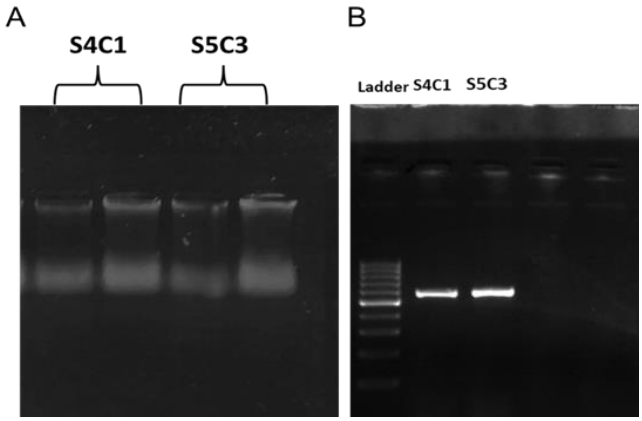

Fig. 6. A. Isolated genomic DNA Bands on 0.7% Agarose gel, B. Amplified PCR product of 16sRNA

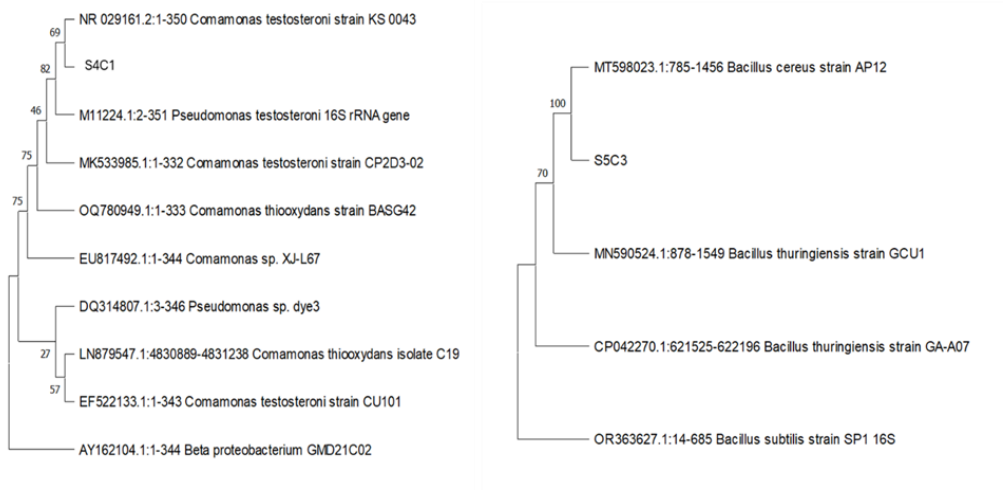

Fig. 7. Neighbor joining tree showing the phylogenetic relationship, based on 16S rRNA gene sequences, between the reference bacteria and the identified heavy metal-resistant bacterial isolates S4C1 and S5C3

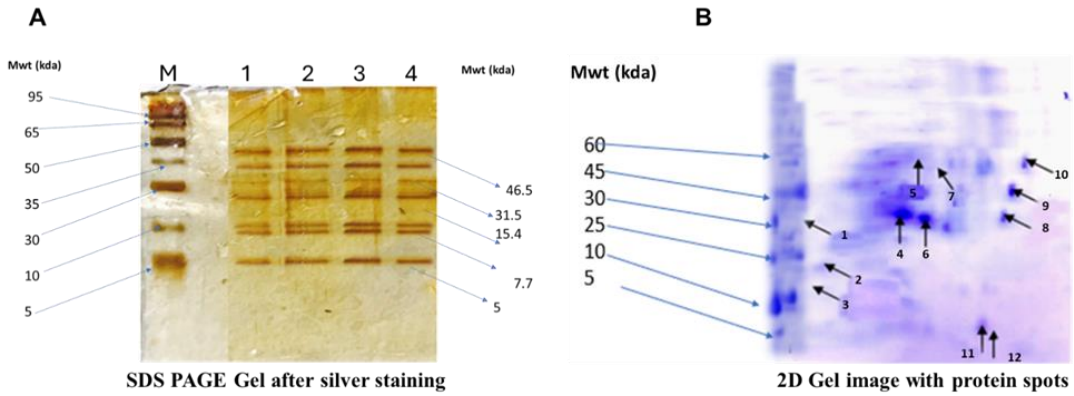

Fig. 8. Protein profiles of bacterial isolatesS4C1 (1, 2) and S5C3 (3, 4) via (A) SDS page electrophoresis and (B) 2D Gel electrophoresis

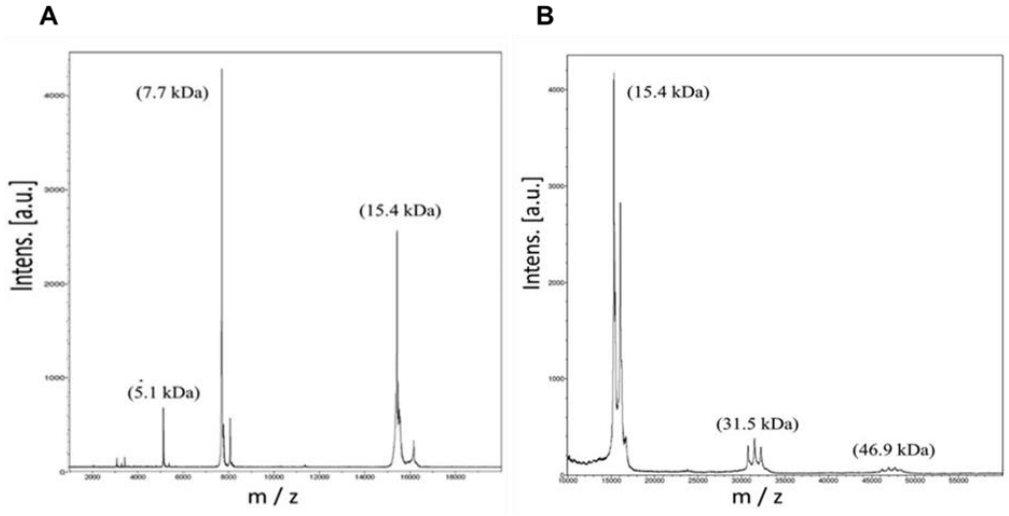

Fig. 9. Representative MALDI-TOF MS chromatogram of (A) Bacteria isolate S4C1, (B) Bacterial isolate S5C3

Table 3. Characteristics of the differentially expressed proteins

Spot no.	Mol. Wt. (kDa)	pI	Probable protein name
1	5.1	5.44	S layer protein
2	7.7	4.93	F0F1ATP synthase subunit b
4	15.4	5.64	Flagellin
5	31.5	9.95	50S ribosomal protein L4
6	46.9	6	Molecular chaperone

The ion trapping function of the S-layer proteins allows the cells to withstand alterations in a dynamic environment. It was discovered that the S-layer proteins had uranium binding properties. thereby shielding the organism from the metal's harmful effects. It is well known that the S-layer proteins include several amino acids with carboxylic side chains, which likely facilitate metal binding. When the S-layer proteins were removed from *Bacillus thuringenesis*, the organism's ability to biosorb copper decreased. According to research by Gerbino et al. [50], the S-layer proteins in *Lactobacillus kefiri* operate as a protective layer when exposed to lead. The isolated organism's increased production of S-layer proteins in response to metal exposure likely aids it in sequestering metals and inhibiting their entrance [51].

Five different types of subunits make up the F1 ATPase, with the b subunit being one of them and being present in three copies. Three catalytic sites are present in the F1 ATPase's b subunit, which is essential for the production of ATP. Four species that were able to withstand radiation exposure showed increased expression of the gene encoding the b subunit. The necessity for greater energy metabolism, which could lead to the creation of extra proteins to combat stress, is most likely implied by the increased expression of the b subunit under stressful conditions [52].

The 50S ribosomal L4 protein was overexpressed in this study when compared to the control group after exposure to metals. The 50S ribosomal L4 protein is a rRNA-binding protein from the L4 family that interacts with the 23S rRNA of the ribosome's 50S subunit several times. The processes of transcription and translation are likely aided by these proteins. The speed of bacterial growth may slow down under stressful circumstances. Starved microorganisms have slower growth rates and produce less rRNA, tRNA, and proteins. When under stress, the organism likely produces the ribosomal protein to speed up protein synthesis and provide a more hospitable environment for bacteria to proliferate. In times of duress, the L4 protein's protective properties may have served specialized functional purposes in the isolated organism to reduce metal stress [53].

Chromium and cadmium stress do not cause the expression of molecular chaperones (GroEL), but

copper stress does. Certain metals can particularly trigger certain proteins. According to research, molecular chaperones are known to assist in the folding of freshly synthesized proteins and the refolding of proteins that have been changed under stress, protecting cells from harm. Chaperonins enclose polypeptide chains and aid folding under environmental stress circumstances until the hydrophobic portions are buried. Under conditions of copper stress, the isolated organism expressed the stress protein GroEL, likely to facilitate protein folding. The molecular chaperones are also produced extracellularly in some bacterial secretion systems to help fold the unfolded proteins that are transported across the membrane [54,55].

Chromium and copper resistance appear to be influenced by flagellin expression. Flagellin is secreted to assist the organism in moving to the best colonisation sites, avoiding toxins, and boosting nutrition uptake. Flagellin overexpression in *B. cereus* CMG2K4 has been linked to nickel and cadmium tolerance in *Pseudomonas* species. According to the current study, metals are likely to affect flagellin expression in a way that promotes motility away from harmful environments. The flhA protein, a part of the flagellar type 3 secretion system, transports flagellin proteins, suggesting that flagellin may also have a role outside of the bacterial cell [38].

4. CONCLUSION

Given the abundance of heavy metals in industrial effluents, the bacteria living there would likely be resistant to heavy metals. The present investigation was initiated with the objective to isolate and identify heavy metal, Pb, Cu, Cr, Cd and Ni resistant bacteria from industrial waste contaminated sites in Uttar Pradesh, India. *Comamonas testosteronei* and *Bacillus cereus* were isolated and identified as heavy metal resistance isolates. Both isolates display high levels of resistance to Pb, Cu, Cr, Cd and Ni. The pattern of protein expression when various heavy metals were present was intriguing. It was found that certain proteins were overexpressed in the case of heavy metals. To fully comprehend the protein underlying the production of these stress proteins and their contribution to heavy metal tolerance, more studies are needed. The overall study demonstrates that bacterial isolates have high levels of metal tolerance; as a result, these isolates can be employed in bioremediation of effluents from heavy metal management enterprises.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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