Research Article

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Highly Cr(vi)-tolerant Staphylococcus simulans assisting chromate evacuation from tannery effluent

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Abstract: Chromium(vi) contaminated sites have been targeted for studying highly chromate-resistant bacteria. From a total of 23 Cr(vi)-resistant bacteria isolated on Luria-Bertani agar medium supplemented with K₂CrO₄ (1,500 μg/mL), only one strain UT8 (Staphylococcus simulans) was able to tolerate high concentration of chromate, i.e., up to 200 mg/mL in agar medium from tannery effluent. In acetate minimal medium (AMM), it showed maximum tolerance of up to 2,500 μg/mL. Cr(vi) removal potential was 94.6% after 24 h (K₂CrO₄ 1,500 μg/mL). Parametric conditions were optimized in AMM to attain maximum chromate removal. Exopolysaccharides extracted from bacterial cells exposed to chromate exhibited major absorption shifts from 2,500 to 500 cm⁻¹ revealed by Fourier transform infrared spectroscopy. Energy-disperse X-ray spectroscopy further confirmed the adsorption of oxyanions to the bacterial cells. Surface topography of the Cr(vi) treated cells showed transformation into concave shape by scanning electron microscopy. The presence of resistance genes, i.e., chromate reductase (chrR) and class I integrase (intI1), further confirmed tolerance toward chromate. Microarray data analysis of transcriptional gene expression suggested upregulation of cys gene cluster under chromate exposure. Concisely, the present investigations revealed the potential of S. simulans to be an effective candidate for chromate reclamation of wastewater.

Keywords: chromium resistance, tannery effluent, scanning electron microscopy, heavy metals, environmental contamination

1 Introduction

In the recent years, a boom in industrial activities and population boom has been witnessed, which led to environmental pollution. The inappropriate methods of waste disposal have caused the accumulation of transition metals, such as chromium (Cr), lead, cadmium, mercury, selenium, etc., in water bodies [1]. These activities are responsible for various health problems. One of the most important metal ion contaminants is Cr which is an inorganic compound present in two stable oxidation states, i.e., Cr(Ⅲ) and Cr(vi) [2]. Effluents from cement, stainless steel, paint pigments, wood preservation, and leather tanning industries increased the Cr(vi) concentrations in the environment, which is an alarming situation from the environmental safety perspective [3]. Cr(vi) is a recognized carcinogen with other toxic properties such as high water solubility, strong oxidizer, causing protein interference, and mutagenic properties. Along with these properties, Cr(vi) compounds persistent in the environment are nondegradable [4]. Cr(Ⅲ) is nontoxic and present in insoluble compounds such as oxides and hydroxides at pH >5 [3]. The reduction of Cr(vi) into Cr(Ⅲ) can be achieved by bioelectrochemical, biological, and chemical approaches [5]. Conventional methods, such as ion exchange, precipitation, solvent extraction, adsorption, membrane separation, etc., were usually applied for Cr(vi) removal; however, these procedures are costly in terms of energy usage, treatment, and sludge disposal [6].

Bioremediation is the removal of toxic pollutants by microorganisms. Microbes are particularly useful in remediating Cr(vi) polluted sites as they reduce Cr(vi) to Cr(Ⅲ). Hence, the removal of chromate by biological treatment is economical, environmentally safe, innocuous, and free from the formation of harmful secondary compounds [7]. Microorganisms develop various mechanisms to deal with environmental stress conditions such as direct and indirect removal of oxyanions, uptake through adsorption, DNA methylation, and efflux of metal ions. In the direct
approach, microbes reduce Cr(VI) into Cr(III) enzymatically, whereas in indirect method it forms complex compounds with metabolites [8]. Bioremediation also comprises an additional mechanism, i.e., chromate resistance which converts Cr(VI) to Cr(III) [9]. Chromate-tolerant bacteria also consists of various chromate reductases such as chrR, nfsA, YieF, and NemA, which catalyze the reduction of Cr(VI) to Cr(III) facilitating the electron transfer from electron donor NAD(P)H to the former and instantaneously generating a reactive oxygen species by two pathways such as class I (“tight”) and class II (“semi-tight”). These reductases are found in the cytoplasm as soluble fractions or attached to the bacterial cell membrane [10]. Along with that, the presence of integrons (mobile genetic determinants) in bacteria is also important for the spread of antibiotic and heavy metal resistance such as class I integrase (intI1) gene. These integrases have a major role in the dissemination of antibiotic and other resistance genes in gram-positive and gram-negative bacteria [11]. Hence, this study purposes to estimate the chromate removal potential of Cr(VI)-resistant strain isolated from tannery waste, detect chromate genetic determinants and integron gene, and also examine the ultrastructural alterations in surface texture and morphology of bacterium through scanning electron microscopy-energy-disperse X-ray spectroscopy (SEM-EDS) in order to understand the new findings of adaptive cellular mechanisms under Cr(VI) stress.

1.1 Novelty statement
Tannery effluent is considered a source for the isolation of Cr(VI)-tolerant bacterial strains. So these can be employed at chromate polluted sites for remediation purposes. This approach is environmentally safe and inexpensive as compared to the conventional physiochemical remediation methods. Despite high chromate tolerance, Cr(VI)-resistant bacteria also carry resistance to other heavy metals, which are also present in the effluent. Various reports have illustrated enhancing chromate removal ability by optimizing the cultural conditions.

In the present study, a high chromate tolerant bacterium UT8 was isolated from tannery effluent. This strain was able to tolerate higher chromate concentration in the agar medium. Maximum chromate removal activity (94.69%) was observed after 24 h of incubation. This study carried out detailed optimization studies to attain maximum chromate removal potential along with exopolymer production.

Results confirmed higher chromate removal along with excellent exopolysaccharide (EPS) production by strain UT8 as compared to other bacterial strains.

2 Materials and methods

2.1 Chemicals and reagents
All reagents and chemicals used in the present study were of American Chemical Society analytical grade. Following metal compounds such as K2CrO4, NiCl2, CuCl2, ZnCl2, PbCl2, CoCl2, HgCl2, and 1,5-diphenylcarbazide (DPC) were procured from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of metals mentioned above were prepared in ultrapure Milli-Q water and stored in amber glass bottles in a dark environment.

2.2 Collection of tannery liquid waste
Tannery liquid waste was obtained in sterilized glass bottles from the leather tanning industry in Kasur (Pakistan). The physiochemical characteristics of the collected sample, such as pH, temperature, color, odor, and Cr(VI) concentration (µg/mL) were measured. Under aseptic conditions, the sample was instantly brought to the laboratory in an ice container and further utilized as a source for inoculum to isolate bacteria capable of chromate removal under aerobic conditions.

2.3 Isolation and characterization of chromate-tolerant bacteria
For isolation of chromate-resistant bacteria, 50 µL of serially diluted tannery sample was spread on Luria-Bertani (LB) agar plates augmented with potassium chromate (1,500 µg/mL) and incubated for 24–48 h at 37°C temperature. The morphologically diverse bacterial colonies were picked up and restreaked. Minimum inhibitory concentration (MIC) of chromate was determined by subsequent subculturing on LB and acetate minimal medium.
(AMM) agar media supplemented with increasing K$_2$CrO$_4$ concentration (2–200 mg/mL). Of 23, only 1 bacterium UT8, was able to withstand high chromate concentration, and it was further purified by three subsequent quadrant streaks on LB agar to obtain a monotype of the isolate. Morphological and biochemical presumptive identification was performed by various tests such as catalase, oxidase, starch hydrolysis, and motility [5].

### 2.4 16S rRNA gene sequencing

The 16S rRNA sequencing of highly Cr($vi$)-resistant strain was done to confirm its taxonomic identity. For this purpose, a purified and isolated bacterial colony of strain UT8 was sent to Macrogen (Seoul, Korea). Almost complete 16S rRNA sequence was determined with primers 518F (CCAGCAGGCGGTAAATCAG) and 800F (TACCCGGGTATCTAATCC), selected for their comprehensive range of both archaea and eubacteria and also consisted of variable V5 and V6 regions of the 16S rRNA gene. The obtained sequence was analyzed in BLAST. The close neighboring sequences were downloaded and aligned using Clustal W version 1.6. Phylogenetic tree was constructed using the neighbor joining algorithm with 1,000 replicates in the bootstrap method in MEGA-X software [12].

### 2.5 Multiple metal and antibiotic tolerance

The selected chromate-tolerant bacterial strain was subjected to a multiple heavy metals and antibiotic-tolerance examination for determining the resistance potential against other toxic metals and antibiotics. Tolerance study against heavy metals such as NiCl$_2$, CuCl$_2$, ZnCl$_2$, PbCl$_2$, CoCl$_2$, and HgCl$_2$ was carried out in terms of MIC on LB agar plates. The stock solutions (stock 1 g/10 mL) of described metal salts were prepared in distilled water and autoclaved. The LB agar plates were supplemented with increasing concentrations (10–1000 μg/mL) of described metals were streaked with selected chromate-resistant bacterium UT8 and incubated under optimum growth conditions for 48 h. MIC was designated as the lowest metal concentration at which no visible bacterial growth is seen. The LB agar plates with no addition of metal solutions were taken as the control. Antibiotics such as chloramphenicol, streptomycin, and ampicillin were also used in a similar way (stock 0.1 g/10 mL) with different concentrations (10–40 μg/mL) [13].

### 2.6 Bacterial growth and chromate removal potential

The growth attributes of chromate-resistant bacterium and its removal potential was investigated by culturing the bacterium in Erlenmeyer flasks (250 mL) containing 100 mL of autoclaved LB broth in the presence and absence of Cr($vi$) (1,500 μg/mL). The flasks were kept in a shaking incubator at 120 rpm at 37°C temperature for 96 h. The control was also placed without the inoculation of the respective bacterial culture. The bacterial growth was observed by UV-Vis spectrophotometer at regular intervals in case of increase in culture absorbance at 600 nm, and Cr($vi$) removal potential was estimated by chromate-specific colorimetric DPC method at 540 nm in case of a decrease in chromate concentration [14]. The percentage of chromate removal was calculated by using the following formula:

$$\text{% chromium removal} = \frac{A_i - A_f}{A_i} \times 100$$  \hspace{1cm} (1)

where $A_i$ – initial chromate concentration (μg/mL) and $A_f$ – final chromate concentration (μg/mL).

### 2.7 Ultrastructural microscopic investigation through SEM-EDS

SEM analysis was performed to explore bacterial cell morphology for ultrastructural adaptations under Cr($vi$) exposure. For this purpose, strain UT8 was grown in LB broth under optimized conditions for 48 h under chromate stress (1,500 μg/mL). Bacterial culture with no chromate exposure was taken as a control. The cultures were centrifuged at 8,000 g for 5 min at 4°C to obtain a cell pellet. For fixing the cells, 5% glutaraldehyde was used for 12 h at 4°C and then dehydrated with increasing ethanol concentrations for 15 min (30%, 50%, 70%, 80%, 95%, and 100%) and finally freeze-dried. After that, the sample was observed by FEI Nova 450 NanoSEM using the built-in Everhart-Thornley detector (ETD) and through lens detector (TLD) detectors with an EDS [15].
2.8 Optimization of parametric conditions and infrared spectral analysis by Fourier transform infrared spectroscopy (FTIR)

It is essential to ascertain the parametric conditions to obtain a significant output of bacterial growth, EPS content, and chromate removal. Hence, the impact of temperature (28°C, 37°C, and 42°C), pH (5, 7, and 9), chromate concentration (500, 1,000, and 1,500 µg/mL), carbon (glucose, gluconate, citrate, and acetate), and nitrogen (peptone, yeast extract, ammonium sulfate, and tryptone) source was investigated both with and without Cr(vi) (1,000 µg/mL) treatment. The pH was maintained at 7.2 ± 0.1, temperature at 37°C, and agitation speed at 120 rpm. AMM and LB broth media were used and inoculated with log phase cells of strain UT8 (100 mL). Each set of experiments was set up to 96 h with duplication. At regular intervals, culture was withdrawn to monitor chromate removal, EPS production, and biomass determination [16,17]. EPS pellet was obtained by cold ethanol precipitation method, and KBr disc method was applied for FTIR analysis (Perkin Elmer spectrum BX FTIR system, Beaconsfield, Buckinghamshire). For FTIR analysis, samples were examined within the range of 500–4,000 cm⁻¹. Transmission mode got the information related to functional groups attached to EPS for chelation of chromate ions [18].

2.10 Mining of microarray data

Microarray data were obtained from Gene Expression Omnibus (GEO, http://ncbi.nlm.nih.gov/geo/) which is a publicly accessible gene expression profile database and analyzed with GEO2R. Microarray data set published by Monsieurs et al. (2011) was used to investigate differential gene expression in Cupriavidus metallidurans strain (data accessible at NCBI GEO database, accession number GSE23876) under various heavy metal exposure [20].

2.11 Statistical analysis

All experimental works were performed in triplicate. For statistical analysis of the data, standard error of the mean was calculated [21]. The graphs were plotted in GraphPad Prism 5.0 version and Origin Pro 8.5.0 software.
3 Results

3.1 Sample characteristics and isolation of chromate-tolerant bacterium

At the time of the sampling, physical characteristics such as pH, temperature, color, odor, and chromate concentration were recorded, i.e., 7.5, 38°C, gray, pungent smell, and 218 µg/mL, respectively. A total of 23 chromate-resistant bacterial strains were isolated at an initial concentration of 1,500 µg/mL K₂CrO₄. The selected strains were further purified and screened for high metal tolerance (data not given). Among the isolated strains, only one bacterium (*Staphylococcus simulans* UT8) was found to be able to resist 200 mg/mL chromate concentration on LB agar plates supplemented with the respective concentration of chromate. Whereas in AMM, it revealed resistivity up to 2,500 µg/mL K₂CrO₄. Morphological identification of K₂CrO₄-resistant strain was done. It was a gram-positive cocci with irregular bunches and nonmotile. The morphology and biochemical traits of UT8 are given in Table S2. The entire sequence of the 16S rRNA gene of bacterial strain UT8 was analyzed for similarities. The search engine of NCBI BLAST indicated 99% similarity to *S. simulans* with a bit score of 2,562 (Figure 1).

3.2 Multiple metal and antibiotic tolerance

Antibiotic- and heavy metal-resistance patterns for chromate-resistant strain were performed. The MIC for each heavy metal (NiCl₂, CuCl₂, ZnCl₂, PbCl₂, CoCl₂, and HgCl₂) was recorded (Table S3). Chromate-resistant bacterial strain UT8 revealed a wide range of MIC against various metal ions such as 250, 200, 100, 700, 1,000, and 20 µg/mL for Ni²⁺, Co²⁺, Zn²⁺, Pb²⁺, Cu²⁺, and Hg²⁺, respectively. For antibiotic resistivity, strain UT8 showed 20, 40, and 30 µg/mL MIC for ampicillin, streptomycin, and chloramphenicol, respectively.

3.3 Bacterial growth and chromate removal potential

The chromate-resistant bacterial strain UT8 was investigated for growth under chromate exposure. Under both chromate stress and nonstress conditions, maximum growth was observed after 24 h of incubation. It showed that the presence of chromate did not have damaging effect on the bacterial cells. Furthermore, the chromate removal ability of strain UT8 was 94.6% at this time period (Figure 2a). This strain was very effective in evacuating chromate ions with 32.08 µg/mL residual Cr(VI) and 97% chromate removal after 96 h of incubation (initial 1,500 µg/mL; Figure 2b). A relative study of chromate removal potential of this strain with other previously reported bacterial strains has been illustrated in Table S4.

3.4 Ultrastructural microscopic investigation through SEM-EDS

Cr(VI)-resistant bacterial strain UT8 was investigated for microscopic structural adaptation through SEM. Bacterial cells treated with Cr(VI) revealed rough, asymmetrical, bulged surface topography with depressions, and reduced

Figure 1: Phylogenetic tree of chromate-tolerant bacterium UT8 based on 16S rRNA gene sequencing and compared with other neighboring members of *Staphylococcus* genera.
cell size as compared to untreated cells that were more smooth and regular in shape (Figure 3a). The average size of cells was 883.65 nm whereas the size of bacterial cells exposed to chromate was 848.85 nm in diameter. The cells were more compact and very closely connected through EPS under chromate exposure (Figure 3b). These findings clearly suggest the effective binding of chromate ions by bacterial strain UT8 on its cell wall. The reason could be precipitation/adsorption of reduced Cr species, i.e., Cr(III) on the cell surface of the bacterium. Additionally, the EDS analysis revealed Cr peak (with 0.19 wt% analysis) for bacterial cells under chromate exposure, due to the complexation of Cr(III) with molecules present on the cell surface or presence of reduced precipitated Cr(III) species on the outer surface of the cells (Table 1).

3.5 Optimization of parametric conditions and FTIR analysis

Chromate-resistant bacterial strain UT8 was optimized for maximum Cr(vi) removal, EPS, and biomass production. The experiment was conducted at various pH, temperature, initial chromate concentration, carbon, and nitrogen sources. The results indicated maximum chromate removal by strain UT8, i.e., 90.5% at pH 7 at 1,000 μg/mL Cr(vi) concentration, whereas 95% removal was observed at
37°C after 72 h of incubation. However, at the same Cr(VI) concentration, the maximum biomass of 1,864 mg/L was achieved when glucose was used as the sole carbon source. In case of nitrogen source, yeast extract was more favorable for the growth of strain UT8 with the biomass yield of 1,145 mg/L and 89% chromate removal (initial chromate concentration 1,000 μg/mL) (Figure 4a). EPS yield increased when the bacterial cells were exposed to Cr(VI). The variable pH and temperature greatly affected the EPS yield, and the maximum EPS extracted was at pH 7, 5.1 mg/L; whereas at temperature 42°C, 8 mg/L EPS at 1,000 μg/mL initial chromate concentration (wet weight) was obtained. When the AMM was supplemented with glucose as the sole carbon source, enhanced EPS was extracted from strain UT8 under chromate stress, i.e., 14.4 mg/L while under the same medium conditions EPS was 9.2 mg/L, with no Cr(VI) stress. It clearly showed that under various physiological stress conditions, this bacterium was able to produce high EPS content for its survival. Ammonium sulfate and yeast extract were better nitrogen source substitutes for enhanced EPS production, i.e., 5.2 and 4.8 mg/L, respectively, under Cr(VI) stress (Figure 4b).

Chromate-tolerant bacterial strain UT8 showed enhanced EPS yield in the LB-medium under Cr(VI) treatment. EPS content of Cr(VI)-treated cells was 0.8 g/L as compared to the EPS produced by the untreated cells, i.e., 0.33 g/L. EPS was further investigated for the presence of functional groups through FTIR technique. FTIR analysis of EPS showed variations in the functional groups exposed to chromate, which assisted in chelation of Cr(VI) ions. EPS produced by cells unexposed to Cr(VI) demonstrated a diverse absorption peak at 3,265 cm⁻¹ due to −OH/−NH stretching vibrations; an absorption peak at 2,352 cm⁻¹ indicated weak C−H stretching band and at 1,623 cm⁻¹ a C−O stretch for amino sugars and proteins. The absorption peaks from 760 to 1,250 cm⁻¹ revealed various polysaccharides. An asymmetrical C−O−C ester linkage causes a band formation at 1,055 cm⁻¹ whereas 860 cm⁻¹ was due to β-glycosidic associations present in sugar monomers. EPS extracted from bacterial cells treated with Cr(VI) showed major variations from 2,500 to 500 cm⁻¹. The important changes in absorption band were observed in 4,000–3,500, 3,500–2,750, 2,500–2,000, 1,750–1,200, and 1,000–800 cm⁻¹ (Figure S1). These variations in Cr(VI)-treated bacterial cells were predominantly due to the specific functional groups carboxyl, amino, sulfonate, and hydroxyl, which react with metal ions to get ionized and form a permanent bond with the metal ions.

### Table 1: Elemental content in chromate-tolerant bacterium UT8 grown in LB broth supplemented with 1,500 μg/mL chromium(VI)

<table>
<thead>
<tr>
<th>Elements</th>
<th>Apparent concentration</th>
<th>wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>47.33</td>
<td>67.04</td>
</tr>
<tr>
<td>O</td>
<td>21.97</td>
<td>26.86</td>
</tr>
<tr>
<td>Na</td>
<td>2.54</td>
<td>1.62</td>
</tr>
<tr>
<td>P</td>
<td>3.71</td>
<td>1.95</td>
</tr>
<tr>
<td>Cl</td>
<td>1.65</td>
<td>1.33</td>
</tr>
<tr>
<td>K</td>
<td>1.56</td>
<td>1.01</td>
</tr>
<tr>
<td>Cr</td>
<td>1.05</td>
<td>0.19</td>
</tr>
</tbody>
</table>

3.6 Detection of chrR and intI1 genes and bioinformatics

Chromate-resistant strain UT8 possesses the property of chrR which is known to catalyze the reduction of Cr(VI) to Cr(III). The PCR amplicons of chrR gene were produced by primers chrR and chrF and intI1 gene by primers intI1F and intI1R in chromate-resistant bacterium Staphylococcus aureus. The amplicons were of 268 and 280 bp when it was analyzed in gel electrophoresis (Figure 5a). Bioinformatics tool was applied to understand the homology between chrR and nitroreductase genes (nfsA). Already published FASTA sequences of chrR gene related to Pseudomonas species were analyzed in MEGA-X software which showed 94% homology with each other, whereas nfsA gene in Staphylococcus species indicated 99% similarity (Figure S2a and b). Approximately, 57% homology was observed between both the genes (Figure S2c), suggesting that nitroreductase gene is involved in Cr(VI) reduction.

3.7 Mining of microarray data

Monsieurs et al. (2011) published a microarray data set in GEO NCBI (data accessible at NCBI GEO database, accession number GSE23876) which were further analyzed by GEO2R. In this study, transcriptional gene expression was measured in C. metallidurans CH34 strain under various heavy metal exposure. Upon chromate exposure, a cys gene cluster was excessively expressed (Rmet_2811, 2813) (Figure 5b and c). This cluster is linked to sulfur ion metabolic pathway which is structurally similar to Cr(VI) ion. The transcriptional expression of cys cluster was significantly higher in chromate exposure with p values 0.0028 and 0.0043 for Rmet_2811 and Rmet_2813, respectively. Other than that,
the expression of genes involved in carbohydrate metabolism was also overly expressed as metal removal requires a lot of energy and the cell needs ATPases for the removal of toxic ions.

4 Discussion

These days Cr(VI) pollution in the environment is an emerging issue. Rapid industrial development and
Figure 5: (a) Detection of chromate reductase and integrase gene in this study. Lane 1: 1 kb DNA Ladder-Thermo Fisher, Lane 2: Chromate reductase gene chrR 268 bp, and Lane 3: integrase gene intI1 280 bp, (b) microarray investigation of cys cluster Rmet_2813, and (c) Rmet_2815 in Cupriavidus metallidurans CH34.
deforestation accumulated toxic contaminants in the environment which are the leading cause of various health illnesses. Therefore, one of the possible solutions to these problems is the reclamation of Cr(vi)-contaminated areas by the application of chromate resistant bacteria. Hence, this study conducted the chromate removal potential and SEM-EDS analysis of Cr(vi)-resistant bacterial strain UT8. From a total of 23 chromate tolerant strains, only one bacterium was able to effectively resist high concentrations of Cr(vi) (200 mg/mL). This is one of the few reports describing the highest MIC of chromate revealed by a Cr(vi)-resistant bacterial strain. However, in AMM, it revealed tolerance up to 2,500 µg/mL K2CrO4. Previous reports described various Cr(vi)-tolerant microbes such as a gram-positive bacterial strains S. aureus and Pedicoccus pentosaceus (2,000 mg/L) and Streptomyces sp. CG52 (500 mg/L) [22]. Bacterial strain UT8 (MN92132) resists higher chromate concentrations in the LB agar medium.

SEM analysis of chromate-resistant bacterial strain UT8 clearly illustrated variations in cell surface morphology and texture under chromate exposure. The bacterial cells were of normal size; however, few cells revealed reduction in size after metal exposure. Bacterial cells tend to change their shape upon chromate exposure by forming a concave shape. This might be due to the survival mechanisms under unfavorable environmental conditions. The presence of Cr(vi) on the cell surface was confirmed by EDS analysis which indicated the chromate adsorption on the bacterial cell surface. Extracellular aggregation and EPS were also clearly observed in between the cells after Cr(vi) treatment, which might be the sites for Cr biosorption [23]. Our research findings are in accordance with earlier study [24]. El-Naggar et al. (2020) also reported similar findings of SEM micrographs before and after adsorption of chromate ions on the cell surface of Pseudomonas alcaliphila NEWG-2 and also recorded structural variations [25]. It was further confirmed by EDS, which is an excellent tool for the investigation of elemental content and characterization of chemical compounds in various sorbents [25]. Bai et al. (2018) also presented the EDS analysis of the cell surface of the thermophilic bacterium Caldicellulosiruptor saccharolyticus, which consisted of Cr in the form of precipitated particles accumulated around the cell surface [26]. The peaks of other elements such as calcium, sodium, carbon, oxygen, phosphorus, and potassium are previously reported to be involved in the localization of Cr on bacterial cell surface [27]. Elsayed et al. also reported similar findings regarding the cell shape irregularity and precipitation of Cr species on the bacterial cell surface through EDS analysis [28]. In a study, EDS analysis of raw aerogel revealed metal peak after Cr adsorption (1.1 wt%) [29].

Bacterial cell wall acts as an initial defense against environmental stress. The production of EPS is reported to increase under stress conditions and to act more as a binding site for the chelation of metallic ions on the surface of bacterial cells [14,30]. The composition of EPS biomolecules was confirmed with IR spectral data using the FTIR technique. Biomolecules such as carbohydrates and proteins signify a peculiar C–H, N–H, and O–H absorption band shifts. Previously, some researchers described the characteristic peaks for 1,3 β glucan linkage and carbonyl group at 1202.90, 1401.15, and 1554.42 cm⁻¹, respectively. A vibration range of 1026.32 and 1144.60 cm⁻¹ is representative of the C–O–C, C==O, and C–C groups found in pyranose glucose and specific carbohydrates, respectively [15]. These shifts in EPS sample unexposed to Cr(vi) were compared to EPS loaded with chromate ions and revealed the partial bonding between oxyanions and carbonyl and hydroxyl groups present in the extracted EPS. The absorption peak between 1,695 and 1,655 cm⁻¹ showed NH₂ and –COO amide I and α-helix of protein. A previous study described the association of C–O–C, C==O, and C–C groups in the uptake of metal ions by JL2810 EPS through FTIR spectroscopy [31]. The variations in vibrational shifts may be due to the oxygen bond existing in EPS molecular structure with oxyanions during the adsorption which may result in the reduction of negative charge/electron cloud density of groups [15]. These results are in accordance with Shahnaz et al. [32] describing shift in some peaks after Cr(vi) adsorption. In general, the findings of the present study suggested that various functional groups, hydroxyl, carboxyl, hydroxyl, and amines, present in the extracted EPS might help in metal ion uptake, especially proteins and carbohydrates.

The maximum chromate removal percentages achieved were 94.6% and 97% at 1,500 µg/mL chromate concentration, at 24 and 96 h, respectively. Bacterial strain UT8 was proficient in efficiently removing chromate from aqueous solution. Our experiment indicated that the rate of chromate removal and cell growth were parallel, and the growth rate was not reduced by the presence/absence of metal ions which indicated a constitutive pathway of chrR [33]. The growth curve of strain UT8 appears to be saturated after a certain period, indicating that the cellular mechanisms have been modified/ altered to develop chromate tolerance and cell survival when chromate was applied. Initially, the low rate of chromate removal by strain UT8 after 24 h might be related to the lethal and mutagenetic
effects of chromate ions on the cellular metabolism of the bacterium. Cr(Ⅵ)-tolerant strain UT8 also showed resistivity toward other heavy metals such as Pb²⁺, Zn²⁺, Cu²⁺, Hg²⁺, Ni²⁺, and Co²⁺, and antibiotics, viz. ampicillin, streptomycin, and chloramphenicol. The pattern of resistance was Cu²⁺ > Pb²⁺ > Ni²⁺ > Co²⁺ > Zn²⁺ > Hg²⁺. The tolerance for other heavy metals in bacteria perhaps reflects the level of toxic metal pollution in the environment and could be the direct association of these metal ions with bacterial cells. However, heavy metal-tolerant microorganisms can also be isolated from uncontaminated environments or they readily adapt to higher concentrations of lethal metal ions by developing resistance mechanisms [27]. Bharagava and Mishra (2018) also suggested the prevalence of higher metal tolerance in a population raised from increased environmental contamination [27]. Nevertheless, bacterial heavy metal tolerance is significant when applying bacteria into soils for remediation of metal polluted areas. The antibiotic resistance property might be generally linked with transmissible plasmids. These characters may have appeared because of the contact of bacterial cells with antibiotics that can cause a spontaneous co-selection of tolerance factors for metal ions and antibiotics [27].

The strain UT8 was further investigated for maximum chromate ion removal, EPS production, and biomass determination under optimized parametric conditions, viz. pH, temperature, initial chromate concentration, carbon, and nitrogen sources. Under neutral conditions with chromate stress of 1,000 µg/mL, maximum chromate removal was achieved, whereas Cr(Ⅵ) removal was lower in acidic and alkaline media. This may result from inadequate bacterial cell growth and lower reductase activity under these conditions. Temperature, pH, and initial concentration of Cr(Ⅵ) salt have a direct influence on chromate removal [34]. A previous study also describes similar results of Cr(Ⅵ) reduction by Bacillus sp. CRB-1 [35]. Wani et al. (2019) also described the similar effect of pH on chromate removal and suggested that this could be the result of insufficient cell biomass [36]. A correlation exists between the removal of chromate and temperature. The chromate removal was increased under increasing temperature and the optimum temperature noticed was 37°C. Under optimized temperature, the activity of chrR increases which aids in Cr(Ⅵ) removal while higher temperatures damage the enzymatic activity of reductases and cause changes in membrane structures, which could be the reason for lower chromate removal efficacy [37]. In a study, maximum chromate removal by strains MAI1 and MAI2 was examined at 25°C and 35°C; however, less chromate removal was attained at 45°C. Cell growth and Cr(Ⅵ) removal are badly affected by temperatures above 40°C due to the possibility of cell death and protein denaturation [36]. Bacterial strain UT8 showed a variation in chromate removal under different initial Cr concentrations. This strain was capable of removing 92 ± 0.35%, 90 ± 0.74%, and 84 ± 0.69% Cr(Ⅵ) with initial K₂CrO₄ concentration, i.e., 500, 1,000, and 1,500 µg/mL, respectively. McLean et al. (2000) reported that the decline in Cr(Ⅵ) removal with increasing chromate ion concentration appears to suggest the toxicity of chromate toward bacterial cells and reduce the active participants involved in Cr(Ⅵ) oxidation [38]. Shi et al. (2009) reported their findings and stated the significant chromate removal at low Cr(Ⅵ) concentrations which considerably increased with increasing chromate concentration[39]. For Cr(Ⅵ) elimination, various compounds act as electron donors which may be used by chromate-reducing microbes. Glucose and yeast extract significantly affected chromate removal in strain UT8 under optimized conditions as carbon and nitrogen sources. In a study, Pseudomonas fluorescens LB300, Agrobacterium radiobacter, Escherichia coli ATCC33456, and Bacillus cereus strains demonstrated enhanced Cr(Ⅵ) reduction when glucose was applied as a single source of carbon [40].

EPS production by strain UT8 was examined under a variable pH and temperature. Our observations indicated that EPS increased under neutral pH when exposed to chromate as compared to the unexposed cells. Our results are in accordance with a previous report [41]. Czaczek and Myszka (2007) stated that bacterial growth and EPS yield are affected by extreme pH resulting in variations in surface morphology of cells and the composition of EPS [42]. In our study, the highest EPS yield was obtained at 42°C after 96 h of incubation supplied with 1,000 µg/mL Cr(Ⅵ). In the literature, it is reported that EPS acts as an initial barrier to bacterial cells, which have a direct association with metal ions in the aqueous medium. They not only protect the bacterial cells but also aid in metal remediation in the environment [43,44]. Extracted EPS yield was increased with increasing Cr(Ⅵ) concentration; however, at 1,500 µg/mL chromate concentration, a reduction in EPS content was observed. This might be due to the toxic effect of higher Cr(Ⅵ) concentration on the bacterial cells, which causes reduced cell growth, resulting in lower biomass [45]. A similar pattern of EPS content was described by Øvåes et al. (2013) in their study on Pseudomonas aeruginosa strain under various chromate concentrations [46]. EPS production of strain UT8 was investigated under various carbon and nitrogen sources. When glucose and ammonium sulfate were supplied in AMM amended with chromate stress (1,000 µg/mL), substantial EPS yield was observed. Cerning et al. (1994)
reported similar findings of EPS production by *Lactobacillus casei* CGI11 glucose, the most efficient source of carbon for maximum EPS yield [47].

Tolerance toward chromate can be ascribed to several mechanisms such as Cr(vi) genetic determinants on plasmids, efflux channels, direct reduction by specific enzymes or indirect through secondary metabolites, Cr(vi) biotransformation, DNA methylation, uptake, or adsorption of chromate ions [48]. A partially amplified *chrR* and *intI* genes were detected in Cr(vi)-resistant bacterium UT8. Similar results were also reported in gram-positive bacteria *Arthrobacter aurescens*, *Bacillus atrophaeus*, and *Rhodococcus erythropolis* by Patra et al. (2010) in their study [49]. Baldiri et al. (2018) described *chrR* gene as a genetic determinant highly like the phylogenetically distant microbes [10]. In a previous study, a 321-bp amplicon of *chrh* gene was obtained, which confirmed the occurrence of this gene in two bacteria, reaffirming their Cr(vi) reducing property [50]. Some researchers reported the presence of *chrR* gene in *P. aeruginosa* which is responsible for chromate resistance [51]. Various other types of genes have been reported in the literature, which are involved in Cr(vi) ion resistance/transport/reduction/efflux. These genes occur either in the bacterial chromosome or in the plasmid. Phylogenetic analysis of *chrR* and *nfsA* gene revealed a 57% homology with one another, describing the potential of both the genes in chromate reduction. The presence of any single gene may support the survival of bacteria in the chromate contaminated environment. Integrons are mobile genetic elements that are commonly part of bacterial genomes, allowing the effective gene transfer and expression of exogenous genes [52]. Integron has an essential component, i.e., *intI* gene that encodes integron integrase and specifically *intII* (encoding Class1 integrons), which is commonly applied as a biomarker for horizontal gene transfer [53].

The expression profiles of certain determinants of resistance to Cr(vi) and other heavy metals in *C. metallidurans* CH34 were then compared. The GEO NCBI gene expression profile database was used to extract expression data for Cr(vi)-resistant genes within *C. metallidurans* CH34 (accession no. GSE23876). It was further analyzed by GEO2R that revealed a cys cluster (Rmet_2811-15), which is a Cr-stimulative gene cluster. This gene cluster is part of a bigger cluster cysGNDHUIVB associated with sulfite and sulfate metabolism. A structural analogy exists between sulfur and Cr(vi) ions, which can facilitate the entry of Cr(vi) ions into the cell through sulfate absorption. The expression of this gene cluster was significantly high relative to its response in other heavy metal stress conditions. The expression of cys cluster was six times upregulated in chromate stress; whereas in cadmium, lead, and nickel, its expression was downregulated [20]. Cr(vi) detoxification results in sulfur deficiency, subsequently leading to upregulation of pathways related to sulfur. Juhnke et al. (2002) previously described the reduction in chromate uptake due to the suppression of sulfate uptake system which is the main system of chromate tolerance in *C. metallidurans* CH34 [54]. The close relationship between sulfur metabolism and Cr(vi) response is also proven by the overexpression of other heavy metal response regions that are affiliated with (thio) the transport of sulfate (Rmet_1376-79-cysTWAB) and biosynthesis of cysteine and the formation of thioester bond in acetylxoenzyme A synthesis (Rmet_0607-11). The pathways related to sulfur supplies an adequate concentration of thiol groups, which form a permanent bond with metal ions, thus restraining them in a relatively nontoxic form [20]. The chromosomal *chr* cluster (Rmet_3864-66), comprising three genes that generally go undetected, still suggested contributing to Cr(vi) resistance previously. Two transcriptional regulators belong to this region, i.e., one putative activator *chrB* (Rmet_3866), one repressor *chrF* (Rmet_3864), and a chromate efflux pump *char* (Rmet_3865). The expression analysis of this region revealed a remarkable twofold upregulation of both *chrB* and *char* genes, combined with fivefold upregulation of the repressor *chrF*. The microarray literature survey provided important understanding of transcriptomic expression of various heavy metal stress responses in CH34 strain, making it a highly resistant bacterium able to survive in anthropogenic environments.

### 5 Conclusion

This study concludes that strain UT8 *S. simulans* is highly tolerant to chromate and has a maximum chromate removal potential of 94.6% after 24 h of incubation (K2CrO4 1,500 µg/mL). Ultrastructural visualization revealed variations in SEM micrographs when bacterial cells were exposed to chromate. It was additionally supported by EDS analysis. FTIR spectroscopy showed the vibrational shifts in the extracted EPS of strain UT8 exposed to Cr indicated the adsorption of Cr(vi). Due to this property, strain UT8 could be harnessed to reclaim Cr(vi) polluted areas. Hence, these investigations are highly significant to those industries discharging high concentrations of Cr(vi) for bioremediation.
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