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Biosorption-driven Chromium Removal by *Bacillus pumilus* Isolated from Natural Wastewater: Characterization and Surface Morphological Transformations

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Abstract

Release of heavy metal-containing, especially chromium (Cr), untreated industrial effluents into the water resources pose serious ecological and public health concerns. Thus, the present study is an attempt to isolate, identify and characterize a native Cr(III) resistant bacterium and explore the pathway underlying its possible bioremediation potentiality for chromium. Primary analysis of the wastewater samples collected from a heavy metal contaminated pond revealed presence of Cr (0.31 ppm) beyond the permissible limit. Following culture, a pure bacterial isolate (C₄) was recovered. Morphological and biochemical characterization identified C₄ as a Gram-positive, rod-shaped and non-lactose fermenting bacterium capable of utilizing citrate as the only source of carbon and exhibiting positive catalase, oxidase, urease, and methyl red reactions, indicating considerable metabolic versatility. Molecular characterization using partial 16S rRNA gene sequencing identified C₄ as *Bacillus pumilus* (GenBank accession number: PQ061175.1). Growth kinetic assay revealed its variable chromium tolerance and strong adaptability. Findings of the scanning electron microscopic studies demonstrated the phenotypic adjustments of C₄ under chromium stress. FTIR spectral investigation demonstrated that the hydroxyl, amino, carboxyl, carbonyl, proteinaceous, lipid, and polysaccharide functional groups collectively contribute to the biosorption and immobilization of Cr(III) by *Bacillus pumilus*. Finally, atomic absorption spectroscopy (AAS) study confirmed superior Cr bio-absorption capacity (~22% in 24 h) of *Bacillus pumilus*. Collectively, our findings for the first time elucidate the explicit cell-wall biosorption mechanisms that make *B. pumilus* an exceptional environmentally sustainable biocatalyst for the restoration of industrial wastewater and ecosystems contaminated with chromium.

Keywords: Chromium, Bacillus, Heavy metal resistant, Isolation, Bacteria, Characterization

Introduction

Rapid industrialization, urbanization, and intensification of agriculture have made heavy metal contamination a serious global environmental concern. Heavy metals are especially dangerous among other environmental contaminants due to their toxicity, persistence, non-biodegradability, and propensity to build up in living things via the food chain.



In India, agricultural lands located near municipal dumping grounds are especially vulnerable to heavy metal contamination due to continuous exposure to contaminated wastewater and landfill leachate (Patra et al., 2001; Kuanar et al., 2022). Because of its widespread use in industries like leather tanning, electroplating, textile processing, pigment manufacturing, stainless steel production, wood preservation, and metal finishing, chromium (Cr) is regarded as one of the most hazardous and environmentally significant heavy metals. In the environment, chromium is mostly found in two stable oxidation states: hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)]. Although Cr(III) is an essential micronutrient at trace concentrations, excessive levels may become toxic to living organisms. In contrast, Cr(VI) is highly soluble, mobile, and considerably more toxic owing to its strong oxidizing nature and high bioavailability (Dey et al., 2014). Chromium contamination can adversely affect soil microorganisms, reduce soil fertility, impair plant growth, and enter the food chain, ultimately posing significant health risks to humans, including oxidative stress, organ dysfunction, reproductive disorders, and carcinogenic effects (Shin et al., 2023). In order to treat the contaminated wastewater, conventional chromium removal techniques such as chemical precipitation, ion exchange, membrane filtration, adsorption, and electrochemical treatment have been widely used (Shin et al., 2023). Nevertheless, these methods are frequently linked to high running costs, insufficient metal removal, the production of secondary pollutants, and higher energy usage. Consequently, the development of economically viable and environmentally sustainable solutions for chromium treatment is gaining traction.

Bioremediation has emerged as a promising eco-friendly approach for the treatment of heavy metal-contaminated environments (Chakraborty et al., 2026). This method transforms, immobilizes, or eliminates hazardous metals from polluted soil and water by using microorganism inherent metabolic capacities. Among them, chromium-resistant bacteria have been evident to evolve diverse adaptation mechanisms enabling to survive under elevated Cr concentrations (Pande et al., 2020). However, the underlying exact mechanism of bioremediation remained unexplored. In addition to heavy metal removal, many beneficial bacteria improve soil fertility, enhance nutrient cycling, and support sustainable agricultural productivity (Bakiyaraj et al., 2014). Thus, identification and characterization of native bacterial strains that are naturally adapted to polluted environments are crucial for the effective use of microbial bioremediation due to their greater survival and remediation efficiency than introduced strains (Chen et al., 2019). Therefore, exploring chromium-resistant bacteria from contaminated sites is essential for developing effective and sustainable bioremediation technologies.

The explicit novelty of our study stems from the exploration of a uniquely adapted indigenous strain, *Bacillus pumilus* C₄, isolated directly from the severe multi-metal stress environment of the Dhapa (West Bengal, India) landfill ecosystem. While general metal tolerance in *Bacillus* species has been reported, this work provides a novel, integrated insight into the extraordinary Cr(III) resistance and rapid biosorption capacity of *B. pumilus* C₄. By systematically coupling growth kinetics, FE-SEM ultrastructural cell-surface profiling, and FTIR biomolecular functional group identification with quantitative AAS removal efficiency (4.5-22%), this study elucidates the explicit cell-wall biosorption mechanisms that make *B. pumilus* C₄ an exceptional biocatalyst for industrial wastewater restoration.

Materials and methods

Chemicals and reagents

All microbiological culture media used in this study, including Luria-Bertani (LB) medium were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Chromium(III) was used as the target heavy metal and was supplied as chromium(III) chloride hexahydrate (CrCl₃·6H₂O) by HiMedia Laboratories Pvt. Ltd., India. All other chemicals and reagents were acquired from Merck India Ltd. and were of highest analytical reagent (AR) grade.

Site selection and sampling

Dhapa, a dumping zone located in the district of North 24 Parganas, have been reported from West Bengal, India earlier as a heavy metal, particularly chromium, contaminated area (Patra et al., 2001; Das et al., 2014). Thus, water sample was taken from this specific area located between 22.548096° N and 88.406178° E (Fig. 1.). The site experiences a tropical wet-and-dry weather with mean annual temperature of approximately 26 °C. The maximum temperature (~ 39 °C) occurs during summer, whereas the minimum (~ 14 °C) is recorded in winter. The region receives substantial monsoonal rainfall from June to September, which plays a vital role in both waste leaching and agricultural practices. The accumulation of industrial and municipal solid waste in this zone contributes significantly to the enhanced levels of chromium and other heavy metals detected in the surrounding groundwater (Flores et al., 2022; Ghosh et al., 2024). Immediately after collection, the water samples were delivered in a suitable condition to the laboratory and kept until needed for additional microbiological analysis (Kalsoom et al. 2021).

Estimation of the concentrations of different heavy metals in wastewater samples

Under carefully monitored laboratory conditions, collected wastewater samples were examined. The concentrations of heavy metals, including arsenic (As), lead (Pb), chromium (Cr), and cadmium (Cd), were measured by means of Atomic Absorption Spectroscopy (AAS; Thermo Scientific; Model: iCE 3000 Series) following the

method of Roy et al. (2017). To eliminate possible metal contamination, all glassware was immersed in 15% (v/v) nitric acid (HNO_3) for adequate decontamination, followed by thorough rinsing with double-distilled water and air drying before use. Stock and working solutions of chromium (III) were freshly prepared using double-distilled water to ensure analytical accuracy, reproducibility, and consistency throughout the experimental procedures.



Fig. 1. Water sample collection site at Dhapa West Bengal, India (22.548096° N, 88.406178° E)

Isolation, media optimization and cultivation of bacterial strains

Wastewater samples collected from the study site were processed immediately for the isolation of indigenous bacterial strains. In short, two milliliters of each sample were suspended in sterile Milli-Q water and serially diluted up to 10^{-9} under aseptic conditions. Aliquots (0.1 mL) from appropriate dilutions (10^{-9}) were spread uniformly onto sterile Luria-Bertani (LB) agar plates containing tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L), and agar (20 g/L), pH 7.0 was adjusted. Before being used, the medium was autoclaved for 20 minutes at 121°C at 15 psi pressure. Inoculated plates were incubated at $30 \pm 2^\circ\text{C}$ for 48 hours in order to facilitate the development of different bacterial colonies. Colonies with unique morphological traits (designated as C1-C5) such as variations in size, shape, elevation, margin, surface texture, and pigmentation were chosen and repeatedly streaked onto new LB agar plates until pure colony with unique morphological traits was achieved. The purified isolates were maintained on LB agar slants at 4°C for routine use, while long term preservation was carried out in LB broth supplemented with 20% (v/v) sterile glycerol and stored at -80°C . The strains were inoculated in 100 mL of Luria-Bertani (LB) broth, and the mixture was continuously shaken at 120 rpm at 30°C to assess the growth characteristics of the purified isolates. Growth curves generated from these observations were used to identify the exponential growth phase, and actively growing cultures were chosen for all ensuing investigations (Roy et al., 2017).

Biochemical and morphological traits of the isolate

Following incubation, several colonies (C1-C5) appeared on the agar plates. Among them, a predominant colony type (C4) was selected based on its distinct morphology, size, and pigmentation characteristics (Seragadam et al., 2021). To create a pure culture, this colony was further sub cultured in LB broth and incubated for 96 hours at 37°C. C4 isolate was then subjected to a series of biochemical tests for preliminary identification, according to Bergey's Manual of Systematic Bacteriology's instructions (Bakiyaraj et al., 2014) which included Gram staining, different enzyme activity assays (catalase, oxidase, urease, and indole), methyl red and citrate utilization. In brief, generation of bubbles following treatment of the bacterial isolate with 3% hydrogen peroxide confirms positive results for the catalase test (Rojas et al., 2023). In urease test, appearance of pink on the slant of bacterial isolate confirms urease production and suggests positive outcome (Bertin et al., 2022).

Similarly, in case of indole test, appearance of a red ring following treatment of the bacterial isolate with Kovac's reagent in Tryptone broth confirms positive response (MacWilliams, 2012). In case of methyl red assay, generation of red coloration indicated positive response while yellowish appearance indicated negative one (McDevitt, 2009). During citrate utilization test, colour change of Simmon's citrate medium from green to blue indicates positive result (MacWilliams, 2009). In case of oxidase test, saturating a filter paper with oxidase reagent and placing a

loopful of bacterial isolate on to it changes its colour from dark blue to purple, confirming positive result for the test (MacFaddin, 2000). Cellular morphology was examined under a compound microscope at 100× magnification (LABOMED, LP Plan Acrho 9133040), providing detailed observations of cell shape, surface texture, and external appendages, supported the results of our Gram staining and biochemical tests. (Doane et al., 2020).

Growth kinetics study of the isolate under chromium exposure

Using the broth dilution method, growth kinetics of Cr(III) for the bacterial isolate C₄ was calculated with minor adjustments to previously published protocols (Zhu et al., 2022; Bukhari, 2023). In order to investigate the effect of Cr(III) on the growth kinetics, fresh overnight cultures of the C₄ isolate (1 mL) were inoculated individually in sterile Luria-Bertani (LB) broth supplemented with increasing concentrations (5, 10, 20, 50, 100, and 200 ppm) of Cr(III), prepared from chromium(III) chloride hexahydrate (CrCl₃·6H₂O). For 96 hours, all inoculation cultures were incubated at 30°C in a rotary shaker running at 120 rpm to allow growth. A UV-Visible spectrophotometer (PerkinElmer Lambda 365+) was used to measure the optical density at 600 nm (OD₆₀₀) at a regular interval of 6 h to evaluate the bacterial growth after incubation. Uninoculated LB broth containing identical concentrations of Cr(III) served as negative controls to verify that any changes in optical density resulted solely from bacterial growth (Rojas-Solis et al., 2023).

16S rRNA-based bacterial identification

In order to isolate genomic DNA from our overnight bacterial cultures, a standard CTAB and phenol-chloroform extraction method was followed. Initially, the bacterial cells were harvested and followed by resuspending the pellets in 500 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). In order to lyse the cell wall, lysozyme (20 mg mL⁻¹) was added, and incubated for 30 minutes at 37 °C. Next, cells were completely lysed and cellular proteins were degraded by adding 1% SDS and proteinase K (200 µg mL⁻¹), leaving it to incubate at 56 °C for an hour. Similarly, to remove interfering polysaccharides, 100 µL of a CTAB/NaCl solution [10% CTAB in 0.7 (M) NaCl] was mixed to it and incubated for ten minutes at 65 °C. The remaining proteins and lipids were removed by extracting the lysates twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by a final wash with straight chloroform. In order to recover the DNA, we precipitated it using 0.7 volumes of isopropanol, washed the resulting pellet with 70% ethanol, and let it air-dry. After dissolving the pure DNA pellet in 50 µL of nuclease-free water, it was treated with RNase A (10 µg mL⁻¹) at 37 °C for 15 minutes to remove RNA contamination.

Finally, the yield and purity of the isolated DNA of C₄ bacterial strain was checked using A260/280 spectrophotometry readings alongside agarose gel electrophoresis. Using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast>), partial 16S-rRNA gene sequences of the bacterial isolates were examined for taxonomic identification by contrasting them with bacterial and archaeal sequences found in the NCBI database. Sequences with over 99% similarity with the isolate were screened for further phylogenetic analysis. Following isolation of the genomic DNA from the selected bacterial isolates, expression of the 16S-rRNA gene was studied through PCR using universal primers (F: AGAGTTTGATCCTGGCTCAG; R: CGGTACCTTGTTACGACTT). The amplified sequences were compared and analyzed using the BLAST-Nucleotide algorithm to search for similarity with known sequences in GenBank. The query sequences revealed 99% sequence similarity with the reference 16S-rRNA sequences from bacterial strains in the database. In order to ascertain the evolutionary links between the isolated strains and related sequences found in NCBI, a phylogenetic tree was proposed based on the obtained similarity matrix (Doane et al., 2020; Mohammad et al., 2022).

Metabolic profiling using Fourier Transform Infrared Spectroscopy (FTIR)

In order to explore the biomolecules involved in metal binding and biosorption, FTIR was used to examine changes in the surface functional groups of bacterial biomass after exposure to Cr(III). Comparative spectral analyses were performed between untreated bacterial cells (control) and two treated groups exposed to Cr-(III) at a concentration of 5 ppm (lowest) and 200 ppm (highest), respectively, with the help of a FTIR spectrometer (Spectrum Two PerkinElmer, USA). Fresh overnight bacterial cultures were harvested by centrifugation (Thermo Scientific Sorvall ST8R) at 10,000 rpm for 15 min at room temperature to obtain sufficient cell biomass. The resulting pellets were washed thrice in sterile 50 mM phosphate buffer solution (PBS) to remove residual culture medium and loosely bound metal ions (Kasprowicz et al., 2010). A sterile mortar and pestle were used to grind the cleaned biomass into a fine powder after it had been dried for eight hours at 50°C in a hot-air oven. For FTIR analysis, approximately 1 mg of the dried bacterial biomass were scanned over the spectral range of 4000–500 cm⁻¹ with a spectral resolution of 4 cm⁻¹. All spectra were acquired and processed using Spectrum IR software (version 10.7.2). Variations in the position and intensity of characteristic absorption bands between control and Cr(III)-treated samples were interpreted to identify the functional groups and biomolecular constituents associated with chromium binding and bacterial metal sequestration (Kasprowicz et al., 2010; Faghihzadeh et al., 2016; Kepenek et al., 2019).

Scanning Electron Microscopy (SEM) analysis

Scanning Electron Microscopy (SEM) was employed to examine the surface morphology and ultrastructural changes in the isolated C₄ bacterial strain exposed to Cr-(III) at a concentration of 5 ppm (lowest) and 200 ppm

(highest), respectively. Untreated cultures grown without Cr(III) served as the control. Immediately after 96 h of incubation, C₄ cultures were centrifuged at 10,000 rpm for 15 min at room temperature to extract the bacterial cells. The resulting cell pellets were washed three times with 50 mM PBS (pH 7.2-7.4) to remove residual culture medium and loosely associated metal ions (Pandey et al., 2015). To retain cellular architecture, cleaned cells were fixed in 4% (v/v) glutaraldehyde for 24 h. Following fixation, the samples underwent a graded ethanol dehydration series. The dehydrated specimens were dried by the critical-point drying technique and mounted onto aluminum SEM stubs using conductive adhesive. A small layer of gold was then sputter-coated onto the samples to enhance electrical conductivity and image quality. Surface morphology and structural alterations of both untreated and Cr(III)-treated bacterial cells were examined using a field-emission scanning electron microscope (Zeiss Gemini FE-SEM 450) operated at an accelerating voltage of 15 kV. The acquired micrographs were compared to evaluate chromium-induced changes in cell morphology and to provide insights into the adaptive responses of the bacterial isolates toward Cr(III) exposure. (Pandey et al., 2015; Merino et al., 2025).

Evaluation of Cr(III) bioremediation potential through Atomic Absorption Spectroscopy (AAS)

The Cr(III) bioremediation efficiency of the isolated bacterial strain C₄ was evaluated under controlled batch culture conditions using AAS (Thermo Scientific; Model: ICE 3000 Series) following the method of Roy et al. (2017). In 25 mL of sterile Luria-Bertani (LB) broth containing 10⁷ CFU mL⁻¹ of C₄ bacterial isolate, chloride hexahydrate was supplemented at concentrations of 5, 10, 20, 50, 100 and 200 ppm. All flasks were incubated at 37 ± 1°C in a rotary shaker maintained at 150 rpm for 96 h to facilitate bacterial growth and metal uptake. Parallel control flasks containing Cr(III)-amended LB broth without bacterial inoculation were maintained under identical incubation conditions. Following the incubation period, the bacterial biomass was separated from the culture supernatant by removing 10 mL of culture from each flask and centrifuging it for 15 minutes at 10,000 rpm at room temperature. The resultant supernatant was collected and processed for AAS (Thermo Scientific, ICE 3000 Series) to quantify the residual Cr(III) content. The bioremediation efficiency of the C₄ bacterial isolate under different Cr concentration was determined by comparing the residual Cr(III) concentration in the treated samples with that of the corresponding uninoculated controls. The percentage of chromium (III) removal was subsequently calculated to evaluate the metal-removal performance of the bacterial cultures (Roy et al., 2017; Alves et al., 2022).

Statistical analysis

Data has been represented as mean ± SEM (standard error of the mean) of three independent replicates. One-way analysis of variance (ANOVA) and a *post-hoc* Duncan's multiple range test (DMRT) were used to assess statistical significance across the various experimental groups, with *p* < 0.05 serving as the threshold level of significance. Microsoft Excel and GraphPad Prism (version 8.0) were used to represent the data.

Results and Discussion

Concentration of different heavy metals in wastewater

In our AAS study, primary estimation of chromium in the wastewater samples through AAS revealed the presence of chromium at a concentration of 0.31 ppm (Table 1.) and other heavy metals. Such high level of total chromium substantially violates the permissible thresholds (0.05 mg/L) established by the Indian Standards (IS 3025, Part 37: 2022) urging severe ecological liabilities and urgency for the development of eco-friendly, low-cost and large scale applicable sustainable remediation methods.

Table 1. Estimated concentrations of different heavy metals in Dhapa wastewater following Atomic absorption spectroscopy (AAS).

Sl. No.	Parameter	Standard	Concentration (ppm)
1	Cadmium (Cd)	IS 3025: PART 41	< 0.02
2	Total Chromium (Cr)	IS 3025: PART 52	0.31
3	Lead (Pb)	IS 3025: PART 47	0.26
4	Total Arsenic (As)	IS 3025: PART 37	< 0.10

Biochemical characterization of the C₄ bacterial isolate

Isolation and re-culture of the pure C₄ isolate following Gram staining and microscopic analysis verified it as a Gram-positive and rod-shaped bacterium. (Fig. 2.; Table 2.). Biochemical screening revealed that the isolate C₄ possesses a versatile metabolic and enzymatic profile (Fig. 2.; Table 2.). The organism is capable of utilizing alternative carbon sources, as demonstrated by a positive citrate utilization test (indicating active citrate permease), while a negative indole test confirms the absence of tryptophanase activity. Analysis of glucose metabolism showed a distinct positivity for the Methyl Red (MR) assay, proving the strain can switch between mixed-acid and 2, 3-butanediol fermentation pathways. Culturing on Eosin Methylene Blue (EMB) agar yielded a negative result, reinforcing its classification as a non-lactose fermenting bacterium. Furthermore, the strain C₄ was found to be strongly positive for catalase (vigorous H₂O₂ effervescence), oxidase (presence of cytochrome c oxidase), and urease (ability to hydrolyze urea) (Fig. 2.; Table 2.).

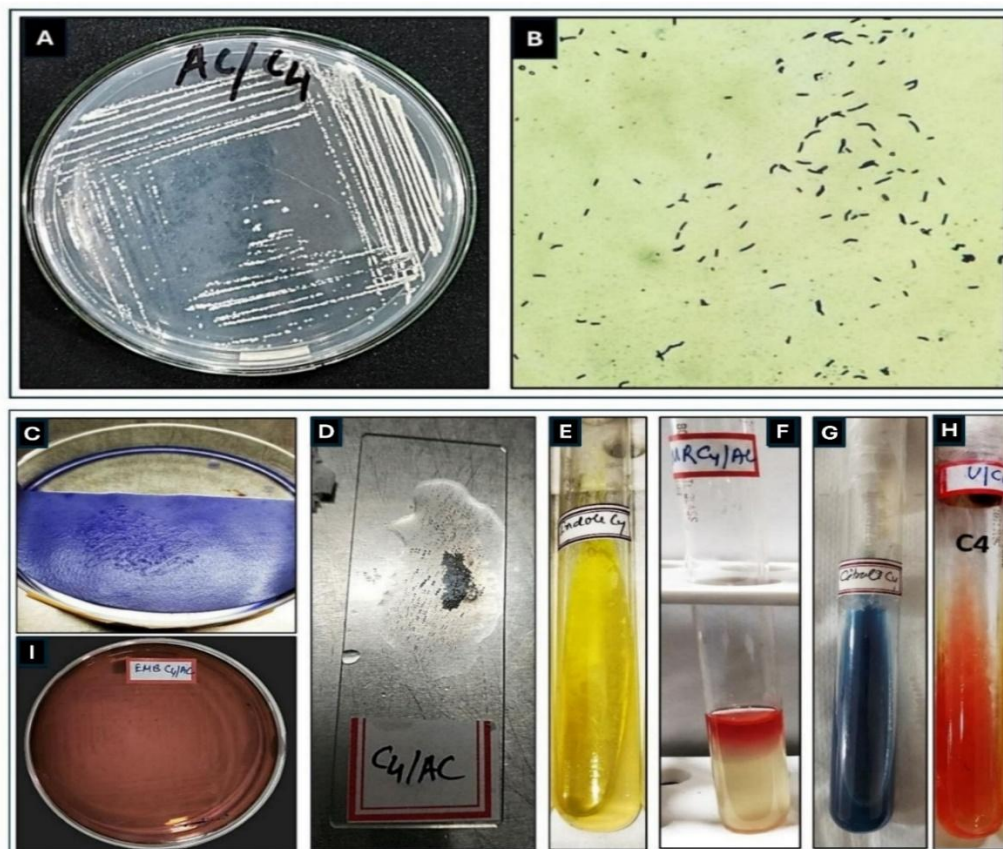


Fig. 2. Photographic representation of (A) Pure culture of isolate C₄ (*Bacillus pumilus*) on LB agar medium and (B) gram-stained microscopic image (100x), and results of the different biochemical assays such as (C) oxidase test, (D) catalase test, (E) indole test, (F) methyl red test and (G) citrate test, (H) urease test and (I) EMB test, performed for the characterization of the bacterial strain C₄ (*Bacillus pumilus*) isolated from wastewater.

Evidences gathered from biochemical analysis suggested its robust respiratory and hydrolytic machinery with strongly positive catalase, oxidase, and urease activities, equipping it to effectively manage oxidative stress and potentially immobilize metals via carbonate precipitation (Jiang et al., 2019; Chaima et al., 2024). Furthermore, its positive citrate utilization test indicates an active citrate permease system for alternative carbon sourcing, while a negative indole test confirms the absence of tryptophanase activity (Darwesh et al., 2021). Notably, its positivity for the Methyl Red (MR) assay demonstrates advanced metabolic plasticity, allowing its potentiality to dynamically switch between mixed-acid and 2,3-butanediol fermentation pathways to prevent lethal self-acidification during prolonged glucose metabolism (Darwesh et al., 2021; Maqsood et al., 2024).

Identification of the bacterial isolates

Based on the 16s rRNA gene resemblance with other closely related bacteria whose gene sequences were retrieved from the GenBank database, the isolated highly chromium-resistant bacteria C₄ was identified as *Bacillus pumilus* (accession no. PQ061175.1) (Fig. 3.). This high degree (99% similarity) of genetic similarity not only confirmed the taxonomic identity of the isolate *Bacillus pumilus* but additionally confirmed its connection to bacterial species that are known to be resistant to heavy metals (Chen et al., 2019).

Growth kinetics of *Bacillus pumilus* under chromium exposure

Observations gathered from our growth kinetics study suggested that the isolate (C₄) *Bacillus pumilus* exhibited strong resistance to chromium. Growth kinetics monitored over a 96-h incubation period demonstrated that *B. pumilus* maintained active growth across all tested Cr(III) concentrations. The growth profile was very similar to the untreated control at lower metal doses (5-20 ppm), suggesting that these concentrations had little negative effect on cellular metabolism (Fig. 4.). Exposure to higher (10 folds) Cr(III) concentrations (50-200 ppm) exerted a moderate extension of the lag phase in *B. pumilus*, suggesting a critical adaptation window for up regulating stress-response genes, activating efflux pumps, or synthesizing protective extracellular polymeric substances (Sher et al., 2023). Following this initial delay, the isolate successfully transitioned into an active exponential growth phase, ultimately achieving high, baseline-comparable optical densities that confirm its robust chromium-tolerance mechanisms and position *Bacillus pumilus* strain as an exceptional candidate for targeted bio-augmentation protocols in heavily chromium contaminated aquatic matrices (Chakraborty et al., 2025). to sustained bacterial growth in contaminated environments.

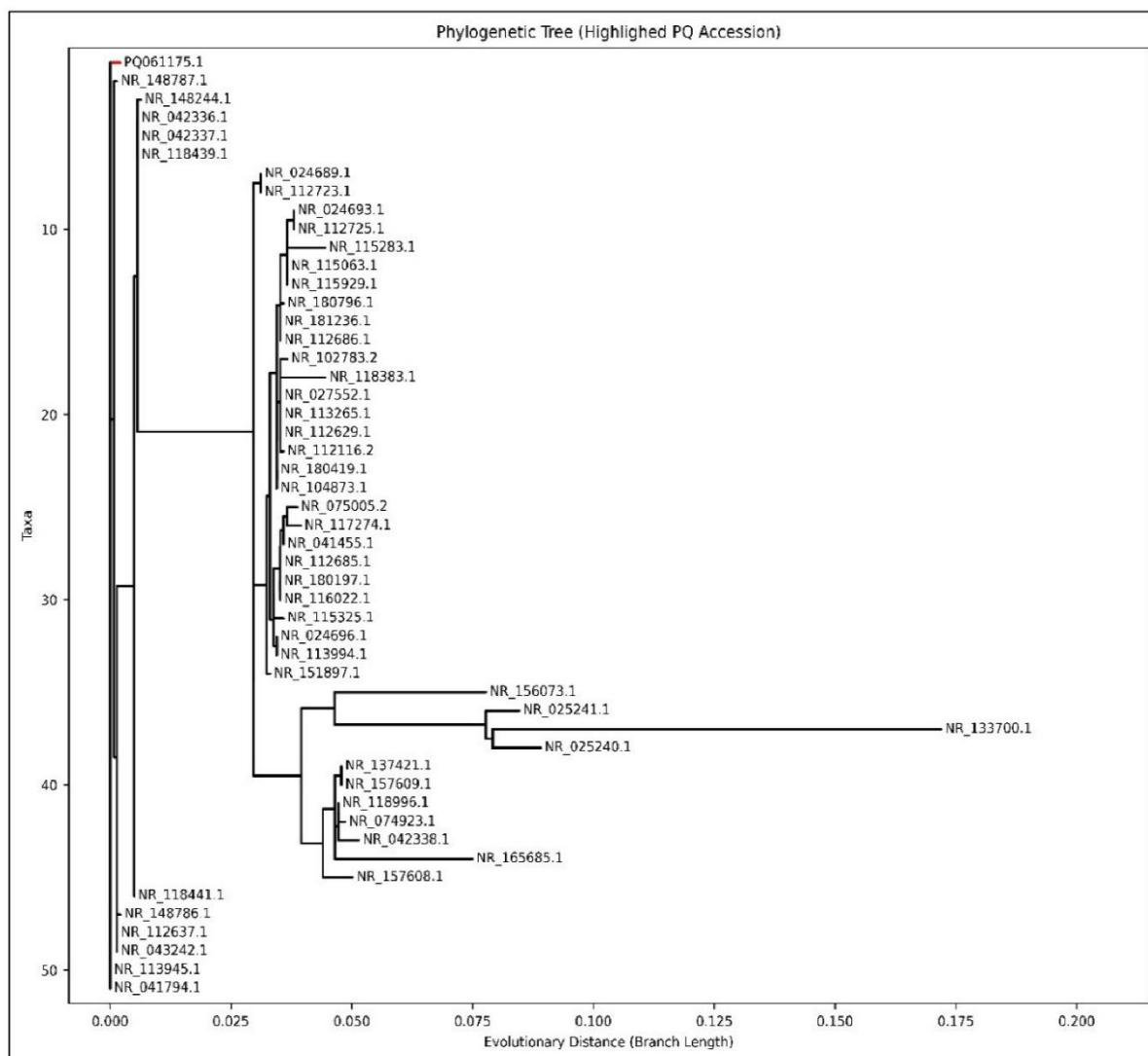


Fig. 3. Phylogenetic tree representing identity of bacterial isolates following 16srRNA sequence data where the isolate designated as C₄ is *Bacillus pumillus* (accession no PQ061175.1)

Such tolerance is likely associated with a combination of physiological and biochemical defense mechanisms that reduce intracellular metal toxicity. Such notion may be supported as earlier studies (Sher et al., 2023; Chakraborty et al., 2025) have demonstrated that bacteria resistant to chromium use a variety of tactics to endure metal stress, including adsorption of metal ions onto the cell surface, biosorption by extracellular polymeric substances, intracellular sequestration, active metal efflux systems, enzymatic detoxification, and the production of metal-binding biomolecules. These mechanisms collectively reduce the toxic effects of chromium and contribute

Morphological response of *Bacillus pumilus* to Cr(III) following SEM analysis

Bacillus pumilus (C₄) surface morphology was examined using SEM both before and after exposure to Cr(III). Untreated cells exhibited a typical rod-shaped morphology with smooth, intact cell surfaces and well-preserved cellular architecture, indicating healthy physiological conditions (Fig. 4.). Cultures subjected to lower Cr(III) concentrations (5 ppm) revealed similar morphological traits, indicating that mild Cr stress did not result in appreciable changes to cell morphology or membrane integrity. In contrast, exposure to higher Cr(III) concentration (200 ppm) resulted in distinct ultrastructural modifications such as irregular cell surfaces, slight distortion of cell shape, increased aggregation of bacterial cells, and deposition of electron-dense material on the cell surface, indicating interactions between Cr(III) ions and bacterial biomass (Fig. 4.). Moreover, such observations also suggested the physiological adaptation to elevated metal concentrations rather than total loss of cellular integrity (Shin et al., 2023). The significant chromium tolerance of *B. pumilus* is demonstrated by the retention of cellular shape at lower concentrations of Cr(III) and the capacity of the bacteria to maintain structural integrity despite mild surface changes at higher concentrations. Biosorption and extracellular metal-binding activities, which are known to be significant mechanisms supporting microbial chromium sequestration and detoxification, are probably linked to the observed aggregation and surface-associated deposits (Shin et al., 2023). These findings support the suitability of *B. pumilus* as promising candidate for chromium (III) bioremediation in contaminated aquatic environments.

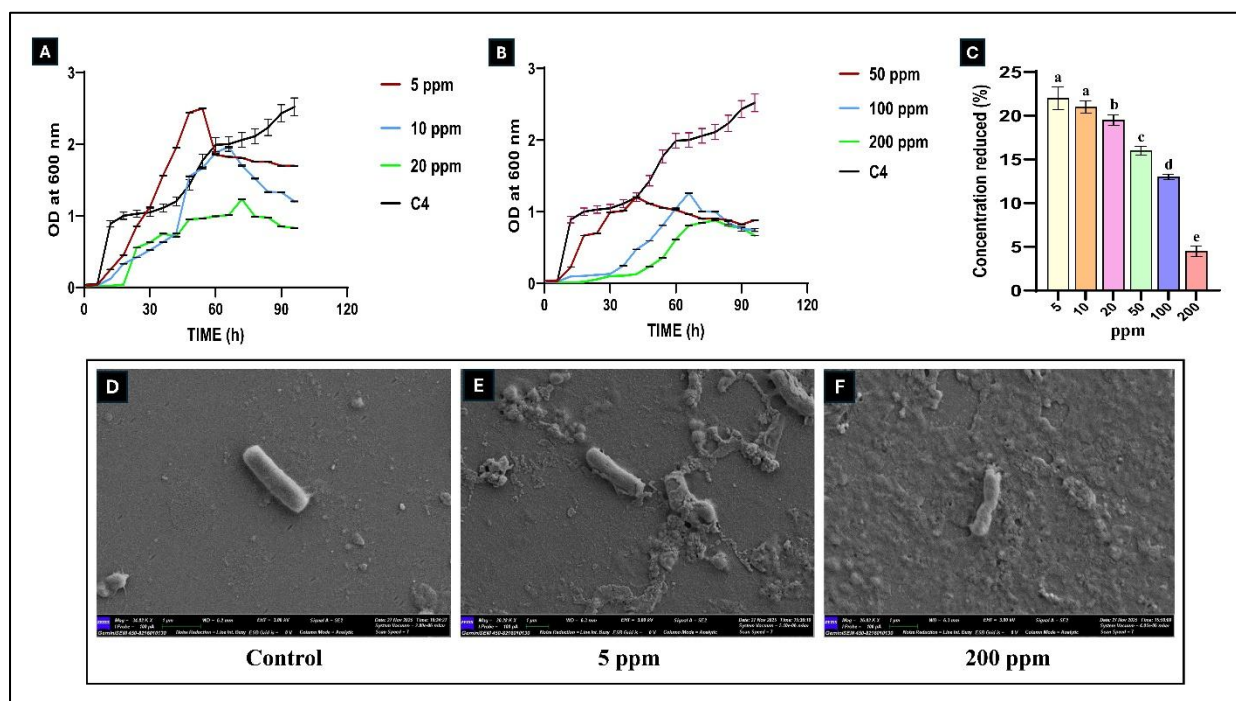


Fig. 4. Graphical representation of the data (Mean $\pm$ SEM) of growth kinetics study where the effects of (A) low concentrations (5-20 ppm) and (B) their 10-fold high concentrations (50-200 ppm) of chromium were investigated on the growth pattern of the bacterial strain C4 (*Bacillus pumilus*) isolated from wastewater. (C) Diagrammatic representation of the bioremediation potential (% reduced) of chromium (in ppm) in 24 h by *Bacillus pumilus* following Atomic Absorption Spectroscopy (AAS). Different letters on the error bar indicated significant change in between the different experimental groups following one-way ANOVA and a *post-hoc* Duncans Multiple Range Test (DMRT) considering ($p < 0.05$) as threshold of significance; while same alphabet indicated no significant change. (D-F) Representative images of Scanning Electron Microscopy (SEM) of *B. pumilus* subjected to different concentrations (5 ppm and 200 ppm) of chromium exposure.

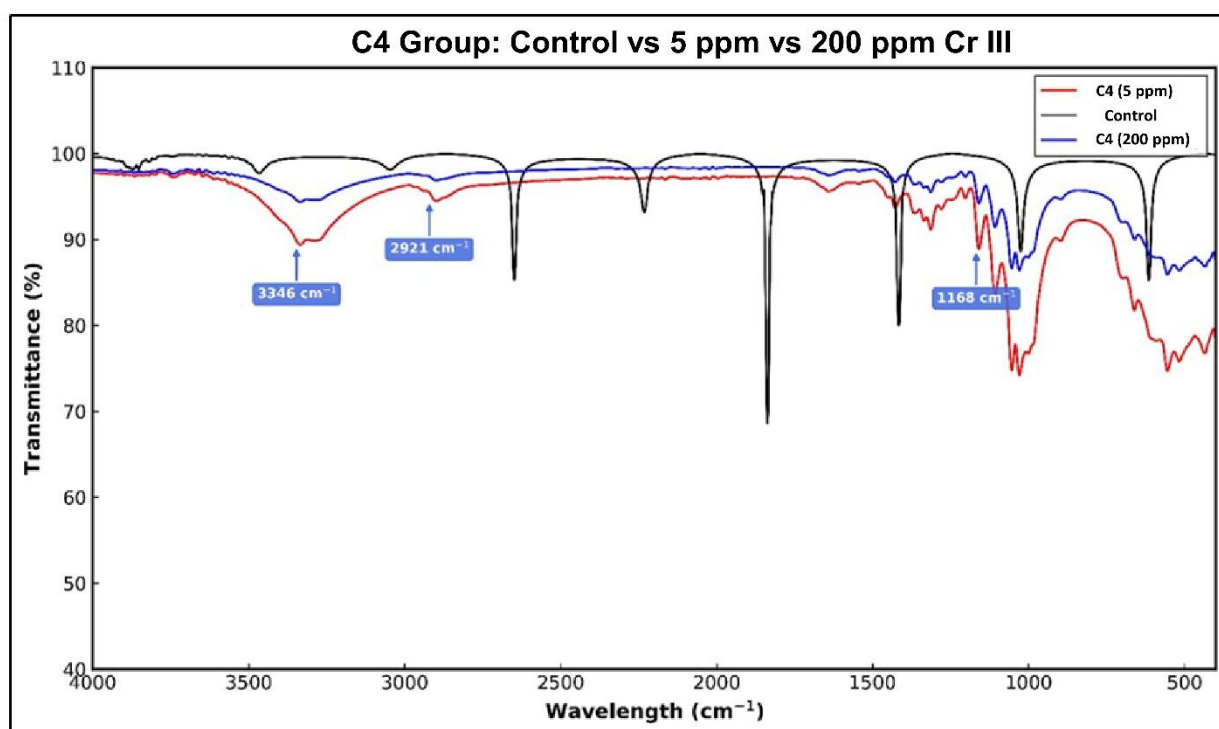


Fig. 5. FTIR-based evidences of minimal alteration in the cell wall composition of the C4 isolate (*Bacillus pumilus*) upon exposure to low and high concentrations of chromium(III).

FTIR analysis of Cr(III) exposed *Bacillus pumilus* revealed its underlying bio-absorption mechanism

The FTIR spectral analysis of untreated *B. pumilus* biomass and Cr(III)-treated samples (5 ppm and 200 ppm) revealed noticeable alterations in the intensity and shape of several characteristic absorption bands (Fig. 5.; Table 3.), indicating the participation of multiple cell surface functional groups in chromium binding and adaptation. A broad absorption band observed in the region of 3645-3200 cm⁻¹ (centered around 3346 cm⁻¹), corresponding to hydroxyl (-OH) and amino (N-H) stretching vibrations, was evident in the control sample. Following exposure to Cr(III), this band became progressively broader and more intense, particularly at 200 ppm, suggesting the involvement of hydroxyl- and amine-containing biomolecules in chromium complexation through hydrogen bonding and coordination interactions. The absorption band between 2960 and 2878 cm⁻¹ (approximately 2921 cm⁻¹), attributed to the asymmetric stretching vibrations of aliphatic -CH₂ and -CH₃ groups associated with membrane lipids, exhibited slight intensity changes at 5 ppm Cr(III) and more pronounced variations at 200 ppm, indicating structural modifications of membrane components under elevated chromium stress. A weak broad band within the 2500-2490 cm⁻¹ region, assigned to the O-H stretching vibration of carboxylic acid groups, showed a slight decrease in intensity and increased broadening at higher chromium concentration, suggesting the participation of carboxyl groups in metal coordination.

Table 2. Phenotypic profile of *Bacillus pumilus* (C₄).

Type	Parameter	Characteristic
Morphological	Gram stain	Positive
	Cell shape	Rod shape
	Colony colour	Creamy white, off-white
	Colony shape	Circular to slightly irregular
	Colony size (mm)	Large, typically 2.0 mm to 8.0 mm (0.2 to 0.8 cm) after 24-48 hours
	Margin	Entire
	Elevation	Flat
	Texture	Smooth
	Opacity	Opaque
	Cell size	Small
	Motility	Motile
	Growth temperature range	35°C - 55°C
	Optimum pH range	37°C
Biochemical	Citrate	Positive
	Methyl red	Positive
	Oxidase	Positive
	Catalase	Positive
	Urease	Positive
	Indole	Negative
	EMB test	Negative

Table 3. Characterization of the functional groups on the surface *Bacillus pumilus* (C₄) through FTIR spectral peak analysis, showing shift positions (cm⁻¹) and structural modulations up on exposure to low and high concentrations of chromium(III).

Peak Region / Position (cm ⁻¹)	Functional Group	Vibration Mode	Possible Assignment	Control C ₄	C ₄ + Cr(III) (5 ppm)	C ₄ + Cr(III) (200 ppm)
3645-3200 (≈3346)	Hydroxyl (-OH) / N-H	O-H / N-H stretching	Hydrogen-bonded alcohols, phenols, amines	Broad O-H/N-H band present	Increased O-H band intensity indicating interaction with Cr(III)	Greater band broadening and intensity changes suggesting stronger metal binding
2960-2878 (≈2921)	Aliphatic -CH ₂ / -CH ₃	C-H asymmetric stretching	Long-chain aliphatic hydrocarbons, membrane lipids	Present	Slight intensity variation	More pronounced intensity changes due to chromium stress
2500-2490	Carboxylic acid (-COOH)	O-H stretching	Carboxylic acid groups	Weak broad band	No significant change	Slight decrease/broadening due to metal coordination

1775-1700	Carbonyl (C=O)	C=O stretching	Esters, ketones, carboxylic acids, anhydrides	Present	Slight shift/ intensity variation	Moderate band broadening indicating possible interaction with Cr(III)
1655-1600	Aromatic C=C / Amide I	C=C or C=O stretching	Aromatic compounds and proteins	Present	Minor shift in band position	Slight broadening with increased intensity, Indicating protein involvement
1550-1500	Aromatic ring / NO ₂	Ring stretching	Aromatic biomolecules	Present	No major change	Slight intensity variation
1460-1450	Methylene (–CH ₂)	CH ₂ bending	Aliphatic hydrocarbons	Present	Minor intensity change	Moderate intensity variation
1380-1346	Methyl (–CH ₃)	CH ₃ bending	Aliphatic compounds	Present	Slight increase in intensity	Small spectral change under higher chromium concentration
1168-1087	Alcohol / Ether (C–O)	C–O stretching	Alcohols, ethers, polysaccharides	Present	Increased C–O band intensity indicating Cr(III) interaction	More pronounced intensity changes suggesting stronger adsorption
1100-1000	Ether / Carbohydrates	C–O–C stretching	Glycosidic bonds, extracellular polysaccharides	Present	Slight increase in intensity	Broadening and intensity enhancement due to metal binding
964	=C–H	Out-of-plane bending	Unsaturated hydrocarbons	Present	No significant change	Slight reduction in intensity
700-600	Aromatic C–H/ C–Cl	Out-of-plane bending / C–Cl stretching	Aromatic or halogen-containing compounds	Present	Minor variation	Slight broadening observed
669-585	C–Cl	Stretching vibration	Halogen-containing compounds	Present	No appreciable change	Slight intensity variation
514-405	C–Br / C–I / Metal–O	Heavy atom stretching	Heavy atom vibrations / possible metal–oxygen interaction	Present	Weak band maintained	Slight enhancement suggesting possible Cr–O interaction

The carbonyl stretching region (1775-1700 cm⁻¹), characteristic of esters, ketones, and carboxylic acid derivatives, displayed moderate band broadening and minor spectral shifts after chromium treatment, implying interactions between carbonyl-containing functional groups and Cr(III) ions. Similarly, the absorption band at 1655-1600 cm⁻¹, assigned to aromatic C=C vibrations and the amide I region of proteins, underwent slight broadening and an increase in intensity, particularly in cells exposed to 200 ppm Cr(III). These spectrum alterations suggest that aromatic biomolecules and proteinaceous components may support cellular adaption processes or chromium sequestration. The aromatic ring vibration region (1550-1500 cm⁻¹) remained relatively stable under low chromium concentration but exhibited slight intensity variations at higher Cr(III) levels, reflecting subtle modifications in aromatic cellular constituents. Likewise, the methylene bending vibration at 1460-1450 cm⁻¹ and the methyl bending vibration between 1380 and 1346 cm⁻¹ showed moderate intensity variations, suggesting minor alterations in lipid and aliphatic biomolecular structures as a consequence of chromium exposure.

Significant spectral changes were also observed in the polysaccharide-associated regions. The C-O stretching bands located between 1168 and 1087 cm⁻¹, attributed to alcohols, ethers, and polysaccharides, displayed increased intensity following Cr(III) treatment, with the most pronounced enhancement occurring at 200 ppm. This observation suggests that oxygen-containing functional groups play a major role in chromium adsorption.

Similarly, the C-O-C stretching region between 1100 and 1000 cm^{-1} , associated with glycosidic linkages and extracellular polysaccharides, exhibited clear band broadening and intensity enhancement, demonstrating the active participation of extracellular polymeric substances (EPS) in metal binding. In contrast, the absorption band at 964 cm^{-1} , corresponding to out-of-plane bending vibrations of unsaturated =C-H groups, showed only a slight reduction in intensity, suggesting minimal participation of these functional groups in chromium interaction. Additionally, a minor broadening was noted in the aromatic C-H/C-Cl vibration region (700-600 cm^{-1}), while the C-Cl stretching band between 669 and 585 cm^{-1} exhibited only slight intensity variation. The low-frequency region between 514 and 405 cm^{-1} , assigned to heavy atom vibrations such as C-Br, C-I, and possible metal-oxygen (Cr-O) interactions, showed weak but noticeable enhancement after chromium exposure, particularly at the higher concentration. Collectively, these FTIR spectral modifications demonstrate that hydroxyl, amino, carboxyl, carbonyl, proteinaceous, lipid, and polysaccharide functional groups collectively contribute to the **biosorption** and immobilization of Cr(III) by *Bacillus* sp. (C4), with more substantial spectral alterations occurring under higher chromium concentrations, reflecting enhanced metal-biomolecule interactions and adaptive cellular responses (Fig. 5.; Table 3.).

Atomic Absorption Spectroscopy (AAS)-based quantification confirmed efficient chromium bioremediation by *Bacillus pumilus*

The capacity for Cr bioremediation of the isolated bacterial strain (*Bacillus pumilus*) was further validated by quantitative measurement using AAS. According to our findings, *Bacillus pumilus* showed significant ($p < 0.05$) chromium removal efficiency in all the Cr-treated groups where highest efficiency of 22% was noted in 5 ppm treated group and lowest efficiency of 4.5% in 200 ppm treated group when compared to the control values (Fig. 4.). Thus, the quantitative AAS analysis confirmed that the isolated *Bacillus pumilus* strain has measurable potential for chromium bioremediation. This is consistent with the well-documented capacity of *Bacillus* species to tolerate heavy metals and detoxify them through mechanisms such as biosorption onto the cell wall, intracellular bioaccumulation, enzymatic reduction of toxic Cr(VI) to the less mobile and less toxic Cr(III), and extracellular polymeric substance (EPS) binding (Tang et al., 2021; Wróbel et al., 2023). Such a notion may be supported as similar findings were observed in our FTIR analysis also. Noteworthy, removal efficiency was inversely related to the initial Cr concentration. The highest efficiency (22%) occurred at 5 ppm, whereas the lowest (4.5%) occurred at 200 ppm. This trend is a common feature of microbial metal remediation and can be explained in several ways. At low concentrations, metal ions are less toxic to the cells, so metabolic activity, growth, and reductase enzyme function remain largely intact, and the available binding sites on the cell surface are sufficient relative to the number of metal ions (Tang et al., 2021; Wróbel et al., 2023). At high concentrations, these binding sites become saturated, and the oxidative stress and membrane damage caused by Cr can inhibit growth and enzyme activity, which limits the proportion of metal the cells can remove. The lower percentage removal at 200 ppm therefore likely reflects the toxicity threshold and limited biosorption capacity of the biomass rather than a lack of remediation ability.

Taken together, these findings suggest that *B. pumilus* is most effective in low to moderately contaminated environments, while its performance under heavy contamination may require optimization. Strategies such as increasing the inoculum density, optimizing pH, temperature, and contact time, using immobilized cells, or employing the strain in a consortium with other metal-tolerant bacteria could improve removal at higher concentrations. Nevertheless, the consistent and statistically significant removal across all treatments supports *B. pumilus* as a promising, eco-friendly candidate for the bioremediation of chromium-contaminated sites.

Conclusion

In conclusion, this study highlights the novel discovery and functional evaluation of *Bacillus pumilus* C4, a native bacterial strain isolated from the heavily polluted Dhapa dumping ground wastewater, exhibiting superior tolerance ($>200 \text{ mg/L}$) and bioremediation capability (22% removal in 24 h) toward trivalent chromium. The core novelty of our findings lies in demonstrating how natural environmental selection in municipal landfill sites drives cell-surface phenotypic adaptation: spectroscopic (FTIR) and microscopic (SEM) analyses proved that *B. pumilus* C4 neutralizes metal toxicity through extracellular polymeric substance (EPS) entrapment, cell aggregation, and functional complexation via hydroxyl, amino, carboxyl, and polysaccharide groups. These distinctive traits position *Bacillus pumilus* C4 as a novel, highly promising bio-augmentative candidate for green, cost-effective industrial effluent treatment. Future research will focus on elucidating specific genetic operons governing metal transport, evaluating performance in continuous-flow bioreactors, and assessing multi-metal field performance. We need to evaluate how these strains perform within microbial consortia and conduct field-scale trials to ensure their stability, efficacy, and practical scalability under unpredictable real environmental conditions.

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Author Contributions

AC: Writing - original draft, Preparation of figures; SP: Writing - review; Preparation of figures; PB: Writing - review & editing; NCS: Writing - review & editing; PKDM: review & editing; PKP: Writing - review & editing, Conceptualization, Supervision. AC, SP, PB, NCS, PKDM, PKP conceived the concept, wrote and approved the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare no competing interests.

Ethics approval

The study was approved (file no. IEC/01/07/2026) by the Institutional Ethical Committee, Swami Vivekananda University, West Bengal, India.



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