



Mitigating Lead and Chromium Toxicity in Agriculture: The Role of *Bacillus tequilensis* RCDB-1 as a Dual Bioremediation and Biofertilizer Agent

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(Received: 29 January 2026

Revised: 25 March 2026

Accepted: 10 April 2026)

KEYWORDS

Heavy metal tolerant, Tomato plant, Enzymatic activity, Antioxidant, Bioremediation, Biofertilizer, Sustainable agriculture, Plant growth promotion

ABSTRACT:

This study investigates the heavy metal tolerance and plant growth-promoting (PGP) potential of the bacterial strain of *Bacterial strain CDB-1* (PP762244) under Lead (Pb) and Chromium (Cr) stress. The growth kinetics of CDB-1 were evaluated across varying metal concentrations, pH levels, and temperatures to determine its physiological limits. Results demonstrated that *Bacterial strain CDB-1* possesses robust tolerance to increasing concentrations of Pb and Cr, maintaining high metal-removal efficiency. In vivo experiments using tomato (*Solanum lycopersicum*) plants revealed that bacterial inoculation significantly alleviated metal-induced phytotoxicity, promoting enhanced shoot height, root elongation, and nodulation compared to non-inoculated metal-treated controls. Furthermore, Physiological analysis demonstrated a distinct dose-dependent enzymatic response to metal stress; as Pb and Cr concentrations increased to 350 mg/L, superoxide dismutase (SOD) activity was significantly upregulated by 47.5% to counteract oxidative damage. Conversely, dehydrogenase (DHA) activity exhibited a 49.1% inhibition, indicating a metabolic trade-off between antioxidant defense and energy production. Greenhouse experiments further confirmed that the inoculation of CDB-1 enhanced the growth parameters of *S. lycopersicum*, including plant height and root length, under heavy metal stress compared to non-inoculated controls. To the best of our knowledge, this study provides critical insights into the dual role of *Bacterial strain CDB-1* as both a growth promoter and a biosorbent. This study suggests the potential of using EPS-producing microbes as a sustainable biotechnological tool to improve crop productivity and detoxify heavy metal-polluted agricultural ecosystems.



1. Introduction

The global environment is currently facing an unprecedented crisis as the overexploited of natural resources leads to severe contamination of soil and water ecosystems. Among the most persistent pollutants are heavy metals and metalloids, which, unlike organic pollutants, are non-biodegradable and tend to bioaccumulate within the food chain [1]. While some elements are essential for biological functions in trace amounts, non-essential heavy metals such as lead (Pb) and chromium (Cr) pose a lethal threat to living organism. Chronic exposure to these toxic elements is linked to devastating health disorders, including anaemia, renal failure, reproduction toxicity, and various forms of cancer [2]. Traditional remediation technologies, including electrochemical degradation, photocatalysis, and chemical coagulation, have been widely employed. However, these methods are frequently criticized for being prohibitively expensive, time consuming, and prone to generating secondary hazardous waste [1]. Consequently, there is an urgent need for sustainable, “green alternatives” [3]. Microbial assisted bioremediation, particularly microbial produced extracellular materials like exopolysaccharide or enzymes and proteins specially membrane proteins has been emerged as a transformative biotechnological solution. These biological products offer several advantages, including biocompatibility, biodegradability [4]. Microbial produced products possess a broad range of unique biological

properties and bioactivities for future biotechnological and bioremediation applications, which are rarely found [5-7]. In the present study, a heavy metal-tolerant bacterial strain was isolated from cow dung and subjected to comprehensive growth kinetic analyses under heavy metal stress. The tolerance profile was evaluated across a range of pH conditions to determine the optimal levels for maximum growth and bioremediation efficiency. Furthermore, we investigated the accumulation of heavy metals within tomato seedlings (*Solanum lycopersicum*) to assess the strain’s protective effect on plant growth and metal translocation.

To confirm the bioremediation potential, Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was performed to quantify metal uptake. Additionally, the physiological response of the strain was evaluated through enzymatic assays, specifically measuring the activities of Superoxide Dismutase (SOD) and Dehydrogenase to elucidate the mechanisms of oxidative stress management and metabolic adaptation under heavy metal exposure.

2. Method

2.1. Material

Luria-Bertani Agar Media (HIMEDIA, Nashik), Luria-Bertani broth (HIMEDIA, Nashik), Chromium, 1,2, Tri, Lead acetate (Loba Chemical Mumbai), Ciprofloxacin Antibiotics (HIMEDIA, Nashik), Barium Chloride (HIMEDIA, Nashik), Sulfuric Acid, Agar Powder (HIMEDIA, Nashik),



Zinc oxide (Loba Chemical Mumbai), Soil, Tomato seeds, and other fine chemicals were purchased from HIMEDIA (INDIA).

2.1. Impact of heavy metals stress (Pb and Cr) on bacterial growth profile

To study the growth profile of bacterial strain, heavy metal stock solutions were prepared by dissolving analytical-grade Lead (Pb) $\text{Pb}(\text{NO}_3)_2$ and Chromium (Cr) $\text{K}_2\text{Cr}_2\text{O}_7$ salts in double distilled water (ddH_2O). The stock concentration was standardized at 2000 mg/L for Pb and 500 mg/L for Cr. To assess the concentration dependent impact on bacterial growth and adsorption, working solution were prepared by serial dilution of the primary stocks. Final concentration for the experiment trial ranged from (0, 250, 500, 1000, 1500, 2000 mg/L) for Pb and (0, 50, 100, 150, 200, 250, 500 mg/L) for Cr [8]. For the microbial growth kinetics and metal adsorption assay; 100 mL of LB broth was used for each individual test. The seed culture was prepared by inoculating one loopful of bacterial culture in 3 mL of freshly prepared LB broth and grown for 12 hr at 37 °C. Each flask was inoculated with 1% (v/v) standardized seed culture of After that, the LB broth was prepared in a 100ml Erlenmeyer flask and inoculated with the seed culture of the bacterial strain, ensuring the initial $\text{OD}_{600\text{nm}}$ of approximately (0.1) [9]. The culture was incubated in an orbital shaker at 30°C for 150 rpm for 24-72 hr. Growth kinetic were recorded by measuring the absorbance at 600nm at regular intervals for 0 to 4 days using UV-Vis

spectrophotometer. Following incubation, the culture was centrifuge at 8000xg for 10 min at room temperature to separate the biomass and to calculate the EPS yield.

2.2 Optimization of pH for heavy metal adsorption

The biosorption of heavy metal by EPS is highly dependent on the solution pH, as it dictates both the specification of metals ions in solution and the ionization state of the functional groups on the EPS surface [10]. In this study, the adsorption efficiency of lead (Pb) and Chromium (Cr) was evaluated across a range of pH value to obtain the optimal conditions for maximum and efficient metal sequestrations. The adsorption of metals (Pb and Cr) by EPS under various pH values. To conduct this study, bacterial culture was grown with optimized concentration of heavy metal with various pH range from 6, 7, and 8. To study this, the cell free culture supernatants were oven dried and 10 mg of dried powder of each sample were placed in a microwave digestion vessel. 9 mL of Nitric acid (65%-68%, GR) and 3 mL of concentrated HCl (35% GR) were added to the digestion vessel as the digestion solution. The following mixture solution were digested for 10 min at 180°C using a microwave digester (Multiwave 5001, Anton Parr). After digestion the following solution was transferred into a 50 mL volumetric flask and made up to 100 mL with ultrapure water. [8]. Time depended on adsorption efficiency was checked using the



Inductively Couple Plasma Optical Emission Spectrometer (ICP-OES, Thermo Scientific ICAP Pro) with argon gas (99.999%). The remaining residual concentration were calculated from triplicate run.

2.3. Bacterial mitigation of Lead (Pb) and Chromium (Cr) accumulation in *Solanum Lycopersicon*

To evaluate this mitigation of heavy metal via bacterial isolates; the tomato (*Solanum Lycopersicon*) seeds were inoculated with the exopolysaccharide-producing bacterial strain CDB-1 and sown in post containing a mixture of soil, vermicompost, and cocopeat (2:1:1 ratio by weight). To stimulate heavy metal stress, lead (Pb) and chromium (Cr) were applied at concentrations of 1500 mg/L and 500 mg/L, respectively. The experiment comprised six treatment group: (1) Control (No metal or bacteria) (2) CDB-1 alone, (3) Pb only, (4) Pb + CDB-1, (5) Cr only, and (6) Cr + CDB-1. The plant was maintained for 60 days under controlled laboratory conditions, ensuring consistent natural light and temperature. Irrigation was performed using distilled water for control and metal-only groups, while the inoculated groups were treated with bacterial culture. Specially 20mL of the respective treatment was applied initially, followed by a 100 mL supplementation on the fifth day. After 60 days, growth parameters including plants high, leaf length, root length, and root nodule formation were evaluated to assess the impact of

bacterial inoculation on heavy metal stress mitigation [11-12].

2.4. Intracellular and extracellular heavy metal accumulation and enzymatic response

2.4.1. Superoxide Dismutase (SOD) activity.

The accumulation of heavy metal and the subsequent enzymatic response of bacterial strain were determined following the methods of [13] with modifications. To assess the SOD activity, bacterial cultures (CDB-1 alone and CDB-1 treated with Pb and Cr) were harvested by centrifugation at 10,000 rpm for 30 minutes. The resulting pellets were washed three times with phosphate buffer saline (PBS) pH 7.0. Bacterial cells were lysed using ultrasonication (30 second on and off cycle, amplitude 20 %) under iced conditions. The crude lysate was collected after centrifugation for 10,000xg, 10 min at 4°C. The crude lysate was collected in fresh tube and place on chilled ice. The SOD assay is based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by superoxide anions. Enzyme extracts were prepared by mixing of 50 mM phosphate buffer (PBS), 130 mmol L⁻¹ methionine, 750 μmol L⁻¹ nitroblue tetrazolium (NBT), and 100 μmol L⁻¹ EDTA-Na₂. To this mixture, 100 μL of the prepared enzyme extract was added, followed by the addition of 120 μL of 10 μmol L⁻¹ riboflavin. To initiate the reaction, the mixture was incubated under a



4000 Lux light at a distance of 30 cm for 45 min. The absorbance of the reaction mixture was measured at 560 nm using spectrophotometer [14] to quantify the antioxidant response under heavy metal stress representing the inhibition of NBT photochemical reduction. All the experiment were performed in triplicates.

2.4.2. Dehydrogenase activity:

To assess the dehydrogenase activity was measured as an indicator of microbial metabolic activity. Harvested cells were resuspended in PBS and treated with a 1% (w/v) solution of 2,3,5-triphenyltetrazolium chloride (TTC). The crude lysate mixture was incubated in the dark at 37°C for 24 hr allowing the colourless TTC to be reduced to red-coloured triphenylformazan (TPF) by active microbial dehydrogenase. Following incubation, the samples were centrifugation at 10,000 rpm, for 10 minutes. The absorbance of the supernatant (containing the extracted TPF) was measured at 485 nm using a spectrophotometer. All the experiment were performed in triplicates.

2.4.3. Calculation of enzymatic activities.

The activities of SOD and DHA were quantified to evaluate the physiological response to heavy metal stress. The SOD was determined based on the percentage inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The inhibition (%) was calculated via following formula; In which the SOD activity was

determined based on the inhibition of NBT reduction.

$$\text{SOD Inhibition (\%)} = \frac{S_i - S_f}{S_i} \times 100\%$$

Where S_i is the absorbance of the control group (without Pb^{2+} treatment and Cr treatment), and S_f is the absorbance of the Pb and Cr treated sample. The resulting value reflects the enhancement of antioxidant activity in response to oxidative stress induced by Pb and Cr exposure.

In addition, the extent of metabolic suppression caused by Pb and Cr were quantified by the reduction in dehydrogenase activity inhibition, calculated as following formula:

$$\text{DHA Inhibition (\%)} = \frac{D_i - D_f}{D_i} \times 100\%$$

Where D_i represents the absorbance of the untreated control group, and D_f corresponds to the absorbance of the Pb and Cr treated sample. This percentage quantifies the degree of enzymatic inhibition and overall metabolic decline due to Pb and Cr toxicity

3. Result and Discussion

3.1. Impact of heavy metals stress (Pb and Cr) on bacterial growth profile

In this study, the growth effect of the EPS-1 producing bacterial strain with various heavy metal ions, Lead [Pb] and Chromium [Cr], in different concentrations was shown in (Figure 1.a and b) under the heavy metal amended



condition the growth of bacterial strain was

limited bacterial growth, and this might be due

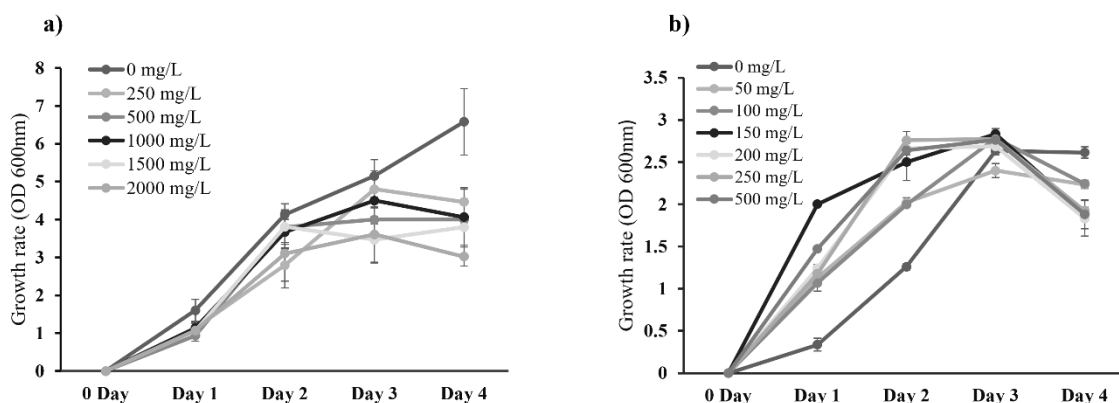


Figure 1: Growth of the Isolated bacteria CDB-1 strain under heavy metal stress. Bacterial growth was monitored via Optical density (OD_{600nm}) over 96 hr period across a concentration gradient of (a) lead, Pb (0-2000 mg/L), and (b) chromium, Cr (0-500 mg/L).

taken OD 600nm and as comparison also taken control condition was the only EPS-1. After 24 hr the bacterial strain growth was observed under Pb and Cr amended conditions with different concentrations. Among the heavy metal ions tested at different concentration Cr strongly limited the growth of the bacterial strain more than Pb metal ions [8]. The result showed a slight decrease in the growth under different concentration are 250 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L in Lead (Pb), and 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L, 500mg/L in Chromium (Cr) of heavy metals. The bacterial growth limitation observed that the concentration of 1500 mg/L in Pb growth rate of EPS-1 enhanced bacterial growth as compared to the control. The concentration of 500 mg/L in the Cr growth rate of EPS-1 enhances growth as compared to the control. These results showed that the tested metal ions

to metal ions toxicity [23].

3.2. Effect of different pH on bacterial growth under heavy metals stress

The effect of pH on the growth rate of selected bacteria were studied under the heavy metals Pb and Cr stress. The growth rate of the strain exhibited a pH depended on profile, with varying optimal points observed upon the addition of lead (Pb) and Chromium (Cr) [24]. As shown in Figure 2. (a and b), the bacterial growth profile in the presence of chromium and lead were monitored over 24 hr at varying pH range (6,7, and 8) to evaluate its metabolic resilience. Figure 2 a; the bacterial strain demonstrated a robust growth phase, reaching maximum optimal density (OD 600nm) between 12 and 15 hr. Notably, pH 8.0 treatment group exhibited the highest peak absorbance approx. 4.8, suggesting that alkaline condition optimize metabolic efficiency and Cr tolerance



for this specific strain. In case, of lead a similar trend was observed under Pb stress, with the exponential growth phase occurring between 6 and 15 hr. For lead treated culture, pH 7.0 appeared to be the most favourable, maintaining a steady peak OD 600 nm of nearly 4.3 [25]. This result indicates in (Figure 2. a and b) that the growth rate of EPS-1 observed at pH-7 observed a maximum growth rate of EPS-1 with Pb, and pH-8 observed a maximum growth rate of EPS-1 with Cr [26]. After 18 hrs, all treatment groups transitioned into the decline phase, likely due to nutrient depletion and the accumulation of secondary metabolites in the broth. The ability of bacterial strain to maintain high biomass in the presence of these toxic heavy metals confirms it

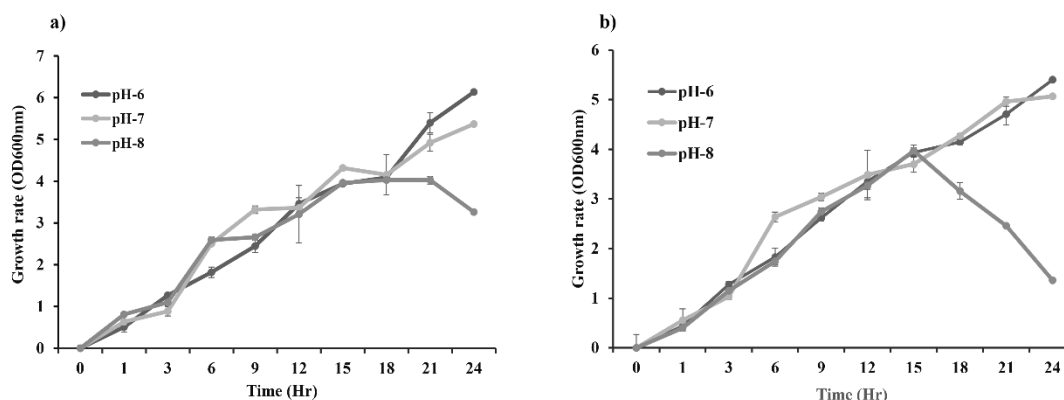


Figure 2: Effect of different pH on bacterial growth under heavy metal stress. Bacterial growth was monitored via Optical density (OD_{600nm}) over 24 hours. (a) Lead (Pb) and (b) Chromium (Cr). All the experiments were maintained at a constant temperature of 37 C. (a) Under Pb conditions, maximum growth was observed at an optimal pH of 7.0. (b) Under Cr treated, after 15 Hr the growth was shifted toward the decline phase, and pH 6.0 and 7.0 showed maximum growth under heavy metal stress.

potential as a robust bio-remediation agent. The metal sequestration capacity of bacterial culture cell

Table1: ICP-OES quantification of residual Pb and Cr (mg/L) in aqueous solution across a pH gradient (Initial concentration C₀=100 mg/L)

pH Level	Residual Lead (Pb) (mg/L)	Residual Chromium (Cr) (mg/L)
C ₀	100	100
6	58.2	73.1
7	44.3	37.4
8	33.2	30

free supernatant was quantified using ICP analysis across a pH gradient (Figure 3) and (Table 1). In this experimental setup, the data point at C₀ represent the initial concentration (C₀) of 100 mg/L for both lead (Pb) and Chromium (Cr). Based on our observations as

the pH increased, a significant decline in the residual concentration (C_e) was observed which also have been observed and reported in previous study



supporting that microbial can be the selection of organism for heavy contamination removal. In this study, lead Pb concentration decreased from the initial 100 mg/L to approximately 58 mg/L at pH 6.0. Further optimization to pH 8.0 resulted in a final residual concentration of 33 mg/L, achieving a total removal efficiency of 67%. In case of Chromium (Cr); While Cr showed a more gradual initial decline compared to Pb at pH 6.0 (73 mg/L remaining), it exhibited

Where the C_0 is the Initial concentration, C_e is final concentration.

3.3. Impact of Heavy metal stress and bacterial inoculation on Tomato growth

The growth promoting and protective efficacy of the bacterial isolates was evaluated in tomato (*Solanum lycopersicum*) plants under both normal and heavy metal stress conditions. Following a 60-day growth period, all the treatment groups were successfully reached the

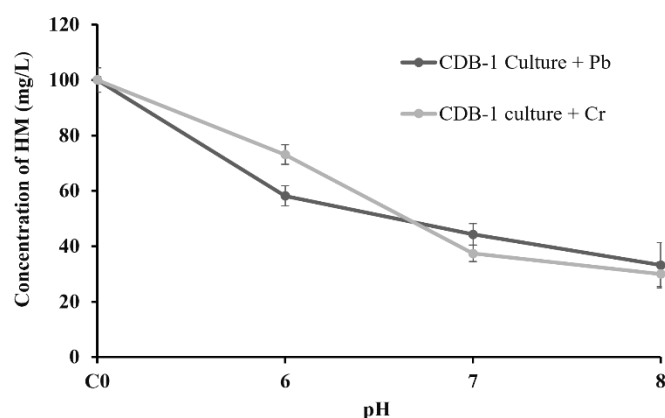


Figure 3: pH-dependent adsorption profile of Pb and Cr by bacterial culture. The y-axis represents the residual metal concentration (mg/L) measured via ICP analysis.

superior sequestration at alkaline conditions. At pH 8.0, the residual Cr concentration reached its lowest point of 30 mg/L, representing a 70 % removal efficiency. This data suggested and strongly supported the previously reported data on heavy metal sequestration by bacteria. C_0 ; Initial concentration of Metals.

The remaining residual of heavy metals in the cell-free supernatant was calculated using the following formula.

$$\text{Removal efficiency \%} = \frac{C_0 - C_e}{C_0} \times 100$$

following stage; however, distinct morphological disparities were evident across the group Figure 3a. The control group and the bacterial-only treatment group exhibited robust vegetative growth, characterized by healthy foliage and standard height, consistent with established growth patterns [27]. In contrast, plants exposed to lead (Pb) and chromium (Cr) without bacterial inoculation experienced from severe growth inhibition, manifested as startled stems and significance reduced leaf surface area. Specifically, Pb and Cr toxicity resulted in

a marked suppression of root elongation (Table 1). However, the application of the bacterial

the bacterial inoculated metal groups (CBD-1+Pb and CBD-1+Cr) highlight the promising

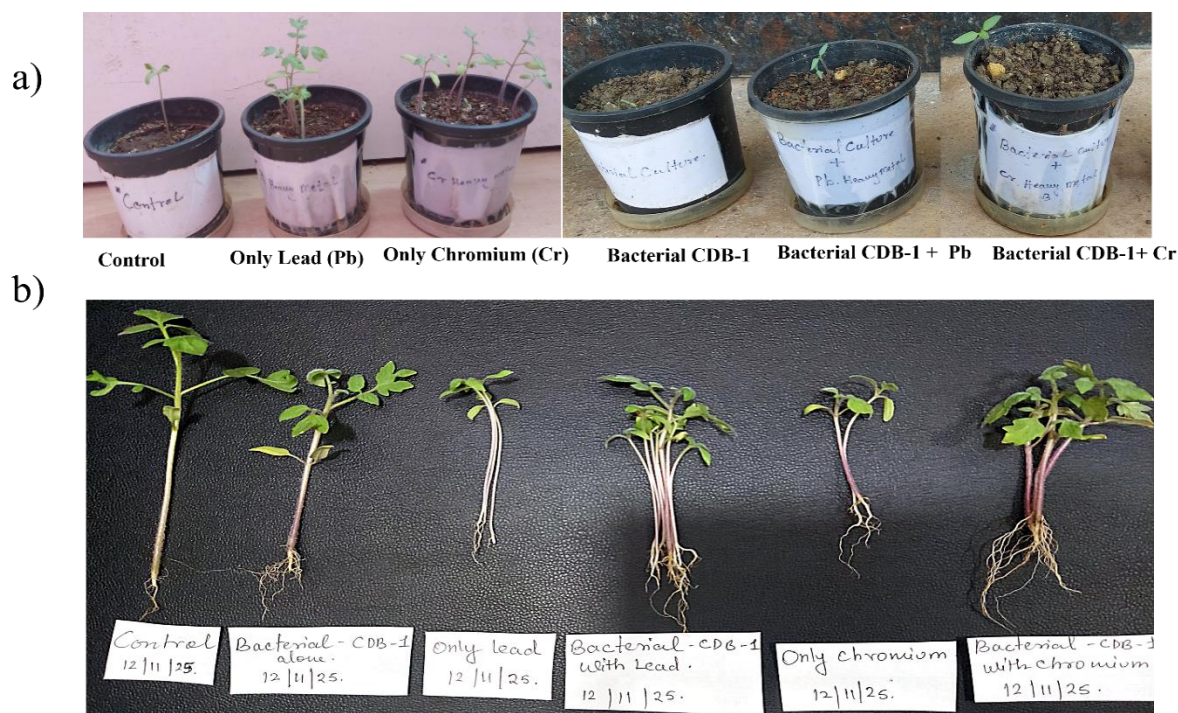


Figure 4. Morphological response of tomato (*Solanum lycopersicum*) plants to heavy metal stress and Bacterial CDB-1 inoculation after 60 days. (a) Comparative development of above-ground biomass across treatments. From left to right: control (untreated), Bacterial CDB-1 alone, Lead only (Pb), Bacterial CDB-1+ Pb, Chromium only (Cr), CDB-1+Cr. (b) representative root architecture and biomass demonstrating the inhibitory effects of heavy metals and the subsequent alleviation by bacterial CDB-1.

strain to metal-stressed plants partially alleviated these phytotoxic effects, promoting noticeably better growth compared to the metal alone treatment (Figure 3b). These results suggested that the bacterial isolate enhances plant tolerance by reducing the bioavailability of Pb and Cr. This mitigation is likely achieved through extracellular polymeric substance (EPS) mediated sequestration and biosorption, which effectively restricts the uptake of toxic metal ions by the plant roots [28,19]. The significant recovery of root and shoot system in

efficacy of the strain in enhancing plant growth and facilitating phytoremediation in metal-contaminated soils.

3.4. Intracellular and extracellular heavy metal accumulation and enzymatic response



Table 2. Effect of Bacterial CDB-1 and Heavy Metals (Pb, Cr) on Plant Growth Parameters

Treatment Groups	No. of Plants (n)	Mean No. of Leaves	Mean Plant Height (cm)	Mean Root Length (cm)	Total Root Nodules	Leaf colour Observation
Control	1	4	17.5	2.5	4	Green
Bacterial CDB-1	1	5	14	4	7	Light to Dark Green
Only Lead (Pb)	3	3.0 ± 0.0	9.17 ± 1.04	2.33 ± 0.76	3	Greenish
CDB-1 + Lead	5	3.2 ± 1.1	9.56 ± 1.79	2.70 ± 0.84	6	Greenish to Green
CDB-1 + Lead	2	3.5 ± 0.7	9.05 ± 0.07	3.15 ± 0.07	4	Greenish
CDB-1 + Chromium	4	3.5 ± 0.6	11.50 ± 1.87	4.48 ± 1.34	8	Greenish to Light Green

Heavy exposure of heavy metals toxicity often triggers oxidative stress in bacteria by generating reactive oxygen species (ROS), which leads to cellular dysfunction and metabolic inhibition. To evaluate the physiological adaptation of the bacterial strain to heavy metal stresses using Pb and Cr, the activity of dismutase (SOD) and dehydrogenase (DHA) were quantified. SOD shows as the primary antioxidant defense by neutralizing superoxide radicals, while DHA activity serves

as a key indicator of overall bacterial metabolic vigor. Our result shows that the enzymatic response of strain CDB-1 to Pb and Cr treatment revealed a different physiological trade-off (Figure 4). A dose-dependent increase in the inhibition of NBT reduction was observed, indicating an upregulation of SOD activity to counterpart metal induced oxidative stress (Figure 4 (a)). In contrast, dehydrogenase (DHA) activity showed significant inhibition, approximately 54-57% at 350 mg/L for both

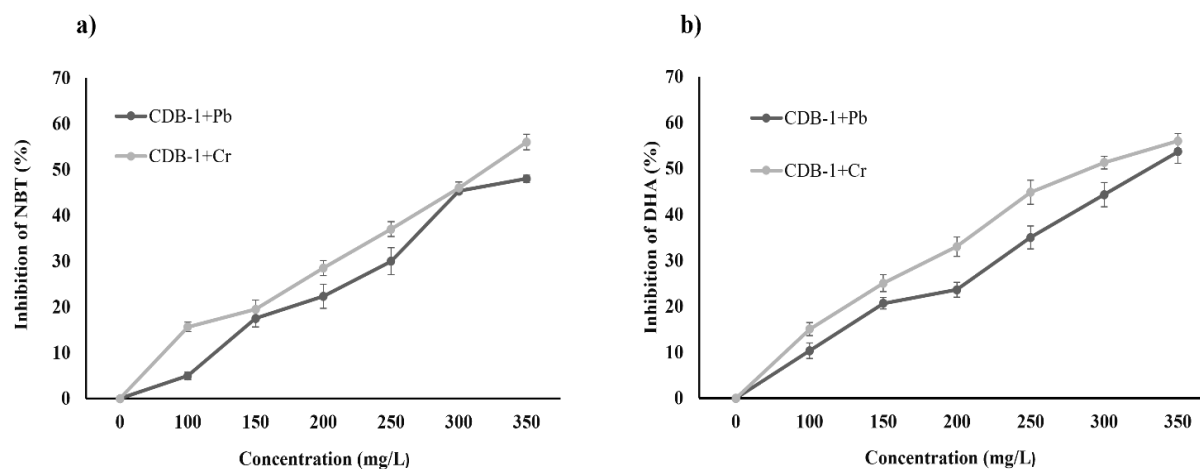


Figure 4. Effect of Pb and Cr on the enzymatic activity of Bacterial strain CDB-1. (a) Superoxide and (b) Dehydrogenase activity were evaluated in bacterial strain CDB-1 culture treated with Pb and Cr concentrations ranging from 0 to 350 mg/L⁻¹.



metal pb and Cr (Figure 4(b)). This concurrent rise in antioxidant defense and decline in metabolic activity suggested that while CDB-1 successfully activates protective mechanism, the heavy metal stress imposes a significant energetic and metabolic burden on the cell.

5. Conclusion

This study demonstrated the bioremediation potential of bacterial isolates CDB-1 against Pb and Cr toxicity. Our finding indicates that CDB-1 showed significant tolerance, maintaining potential growth even at high metal concentration of 2000 mg/L Pb and 500 mg/L Cr. Optimization revealed that strain's metabolic efficiency is highly dependent on environmental parameters, at pH 7.0 for Pb and a slightly alkaline pH 8.0 for Cr. The physiological analysis revealed a critical survival mechanism of antioxidant and metabolic vigor. as heavy metal concentration increased, the strain significantly upregulated SOD activity up to 47.5 % to mitigate oxidative damage, while simultaneously experienced a dose dependent suppression of dehydrogenase (DHA) activity. Also, the ability of CDB-1 to thrive under extreme metal stress and its successful integration with tomato plant (*Solanum lycopersicum*) highlight its dual role as both a biofertilizer and a bioremediation agent. This research provides a mechanistic foundation for utilizing bacterial strains to enhance crop resilience and detoxify heavy metal contaminated agriculture soils, offering a

sustainable strategy for environmental restoration.

Competing interests

The authors report no conflict of interest

Availability of data and materials

In texts, all the accessible websites and organizations have been provided. Authors declare that all data were generated in-house and no paper mill was used.

Acknowledgment

The authors acknowledge the help and facilities provided by Amity University Chhattisgarh.

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