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Simultaneous removal of ternary heavy metal ions by a newly isolated *Microbacterium paraoxydans* strain VSVM IIT(BHU) from coal washery effluent

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Abstract In the present work, the removal of Cr (VI), Cd (II) and Pb (II) at 50 mg/L of each metal ion concentration was investigated by *Microbacterium paraoxydans* strain VSVM IIT(BHU). The heavy metal binding on the bacterial cell surface was confirmed through X-ray photoelectron spectroscopy and energy dispersive X-ray. X-ray photoelectron spectroscopy analysis also confirmed the reduction of Cr (VI) to Cr (III). Heavy metal removal dynamics was investigated by evaluating dimensionless, and the value of N_k (9.49×10^{-3}, 9.92×10^{-3} and 1.23×10^{-2} for Cr (VI), Cd (II) and Pb (II) ions) indicated that the removal of heavy metals by bacterial isolate was mixed diffusion and transfer controlled. It was found that both the experimental and predicted values for isolated bacterial strain coincided with each other with a good R^2 value in the L-M Algorithm range of 0.94–0.98 for the ternary metal ion system. The bacterial isolate presented a maximum heavy metal ion removal efficiency of 91.62% Cr (VI), 89.29% Pb (II), and 83.29% Cd (II) at 50 mg/L.

Keywords Ternary heavy metal ions complex · Bacterial isolate · Cr (VI) reduction · Artificial Neural Network · Dimensionless number · Antioxidants

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Introduction

Heavy metals are used in pigment, paint, battery, steel, coal, chrome plating and tannery industries (Sarangi et al. 2008). The effluents from these industries cause heavy metal contamination in the aquatic ecosystem (Singh et al. 2021a, 2020). Human beings are exposed to heavy metals by the intake of contaminated water, food and contact with skin, which can lead to a variety of cancers, nephrological and neurological disorders (Balali-Mood et al. 2021). Therefore, it is imperative to remove toxic heavy metals, such as Cr (VI), Cd (II) and Pb (II) ions from the water (Garg et al. 2012). The

traditional heavy metal removal techniques, including membrane filtration, oxidation, precipitation, adsorption and ion exchange (Singh et al. 2021b) have been extensively practised in the past. These methods have limitations like insufficient removal of heavy metals, high operating cost and generation of secondary chemical sludge after wastewater treatment (Goksungur et al. 2005). Thus, it is demanding to find a suitable heavy metal removal methods for wastewater treatment (Singh et al. 2020). Biomass has been widely used as a bio-remediating agent to remove heavy metals (Elahi et al. 2020). Using living biomass results in the generation of minimum or no secondary chemical sludge and lower operation costs (Hedayatkhah et al. 2018). Additionally, they are widely and easily available across the globe (Bar-On et al. 2018). Among the living biomass, bacteria are highly resistant to toxic metals and accumulate heavy metal ions in their intracellular space (Jacob et al. 2018). Bacterial strains such as *Pseudomonas* (El-Naggar et al. 2020), *Klebsiella* (Tekerlekopoulou et al. 2013), *Microbacterium* (Humphries et al. 2005) and *Bacillus* (Li et al. 2020) have been used for heavy metal removal. Additionally, bacterial mediated heavy metal removal is considered a cost-effective and eco-friendly option (Ibrahim et al. 2012).

Due to their tolerance to heavy metals, bacteria may flourish in environments with high concentrations of heavy metal ions (Liu et al. 2012). Liu et al (2012) reported that *Microbacterium* spp. can tolerant up to 4.08 mM of chromate ions. The antioxidant system in the bacteria reduces the toxicity of heavy metals (Garg et al. 2013; Kubrak et al. 2010). Antioxidants are expressed in the cell when stress is created due to metal toxicity. These antioxidants capture reactive oxygen species (ROS) and minimize toxicity (Kumar et al. 2013). The expression of antioxidants participates in the detoxification of heavy metals with their subsequent intracellular bioaccumulation (Joutey et al. 2015).

The present work aims at the removal of the ternary metal ion complex of Cr (VI), Pb (II) and Cd (II) by a bacterial isolate. The reduction of Cr (VI) into Cr (III) by bacterial isolate was also investigated. The effects of heavy metal concentrations on bacterial cell development and morphological alterations were studied. Artificial Neural Network (ANN) study was conducted to predict optimum conditions for removing heavy metal ions. The contrivance of bacterium-mediated bioremediation was investigated in terms of antioxidant production, and dimensionless numbers were calculated to describe the mechanism of heavy metal removal.

Materials and methods

Characterization of bacterial isolate

Heavy metal resistant bacterium specie was isolated from the drain (Baliya Nala, Singrauli, Madhya Pradesh, India) situated in the vicinity of coal washery units (Singh and Mishra, 2021a). The bacterial isolate was isolated by serial dilution followed by spreading on agar plate. 1 mL of wastewater was mixed in 9 mL of normal saline water and was serially diluted up to 10^{-10} dilution. 0.2 mL of each dilution was poured on Luria–Bertani (LB) agar plates containing 100 mg/L heavy metal ions. The plates were incubated for 24 h at 37 °C. The most tolerant heavy metal resistant pure bacterial culture was identified by DNA isolation followed by 16S rRNA gene sequencing and Basic Local Alignment Search Tool (*BLAST*) analysis. Obtained 16S rRNA gene sequence was submitted to NCBI GenBank and accession number was obtained (Singh and Mishra, 2021a).

Surface morphology of control and heavy metal exposed bacterial cells were analysed through a scanning electron microscope (SEM) (ZEISS EVO, Carl Zeiss Microscopy make, Germany). Samples for SEM–EDX were prepared by the procedure mentioned in Singh and Mishra (2021a). The elemental composition, including Cr (VI), Cd (II) and Pb (II) in control and in heavy metal exposed bacterial cells was investigated by energy dispersive X-ray (EDX) (EDAX Inc. make, USA) and X-ray photoelectric spectroscopy (XPS) (Thermo Fisher Scientific make, USA). The surface functional groups on the bacterial cell surface were identified by the Attenuated Total Reflectance-Fourier Transform Infrared spectrophotometer (ATR-FTIR) (NICOLET spectrophotometer (iS5) make, USA). Samples for ATR-FTIR and XPS were prepared by modifying the method of Shao et al (2019).

Effect of heavy metal concentration on the growth of isolated bacterial strain

The fresh bacterial pre-inoculum was prepared in 20 mL of LB broth in 100 mL conical flask. The

stock solution of Cr (VI), Cd (II) and Pb (II) were prepared by adding predetermined amount of Potassium Dichromate ($K_2Cr_2O_7$), Cadmium Nitrate Tetrahydrate ($Cd(NO_3)_2.4H_2O$) and Lead (II) Nitrate ($Pb(NO_3)_2$) in 1 L of distilled water, respectively. The effect of Cr (VI), Pb (II) and Cd (II) concentration was observed on the bacterial growth between 50 and 200 mg/L of each metal in the ternary metal ion complex system. The LB broth medium containing heavy metals and control was inoculated with 1% of fresh bacterial inoculum and flask was placed in the incubator for 24 h at 37 °C and 180 rpm. The bacterial cells were grown in 20 mL LB broth in 100 mL flask in control and in heavy metal containing growth medium. The sample was collected at every 2 h up to 24 h. The all experiments were performed in the triplicate and average of the readings were recorded. Bacterial growth in the liquid broth was determined in the form of cell density by measuring absorbance at 600 nm.

Estimation of heavy metals

Cr (VI), Cd (II) and Pb (II) removal was investigated at 50 and 100 mg/L of each. Bacterial cells were grown in LB broth medium using 1% of bacterial inoculum (0.5–0.6 optical density) at 180 rpm, pH 7 and 37 °C for 1 to 5 days. After incubation, the sample was withdrawn, centrifuged and cell pellet was discarded. The remaining heavy metal ion concentration in the supernatant was estimated by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES), Perkin Elmer, Optima 7000 DV make, USA. The heavy metal removal from the aqueous phase was calculated by Eq. 1.

$$\text{Removal}\ (\%) = \frac{C_i - C_e}{C_i} \times 100 \quad (1)$$

where, C_i and C_e are the initial and equilibrium concentrations of heavy metals (mg/L).

Antioxidants activity

The activity of antioxidants activity was observed in the bacterial isolate exposed to 100 mg/L of ternary heavy metal complex of Cr (VI), Pb (II) and Cd (II). The culture was incubated for 24 h at 37 °C and 180 rpm. The samples were collected at stationary phase (population density was 1.63 ± 0.08 OD at 600 nm). A homogenized solution of bacterial cell pellet in PBS buffer was centrifuged at 10,000 rpm for 12 min and the supernatant was collected. The activities of antioxidants, including glutathione S-transferase (GST), superoxide dismutase (SOD), peroxidase (POX) and catalase were analyzed in the supernatant by methods described in Habig et al. (1974); Beers Jr and Sizer (1952); Ewing and Janero (1995); Reuveni et al. (1992); and Singh and Mishra (2021a).

Derivation of dimensionless numbers

Heavy metal bio-sorption on the bacterial surface is controlled by rearrangement, film, bulk diffusion, and intra-particle diffusion (Imaga and Abia 2015). Dimensionless numbers have been deduced and explained in Section S1.1 of the supplementary information.

ANN

An output experimental data pattern can be predicted when relevant input experimental data is given (Yildiz 2018). Regarding data analysis, the Levenberg–Marquardt (LM) algorithm is commonly used. In most of the attempts, it has been picked due to its ease of use and high level of training capability (Singh and Mishra 2020a, 2020b). In the present work, contact time, pH and temperature were used as input constraints.

Statistical analysis

All experiments were carried out in triplicate (n = 3), and the average values were used to generate the graphs. The experimental errors (± standard deviation) were determined and displayed in graphs as error bars.

Result and discussion

Characterization

The metal-resistant bacterial strain was isolated from coal mining effluent and identified as *Microbacterium paraoxydans* strain VSVM IIT(BHU). The bacterial

strain was submitted to NCBI GenBank under accession no. MN650647.

Identification of bacterial isolate by 16S rRNA gene sequencing

Identification and phylogenetic analysis of bacterial isolate has been done in our previous study (Singh and Mishra, 2021a). Genomic DNA of bacterial isolate was extracted and 16S rRNA gene was amplified by polymerase chain reaction (PCR). Amplification of 16S rRNA gene was confirmed through gel electrophoresis which showed DNA band between 1200 and 1600 bp (Singh and Mishra, 2021a). The bacterial isolate 16S rRNA sequence showed maximum similarity with *Microbacterium paraoxydans.* The 16S rRNA gene sequence of bacterial isolate was submitted to NCBI GenBank under the bacterial name *Microbacterium paraoxydans* strain VSVM IIT(BHU) with accession number (MN650647) (Singh and Mishra, 2021a). The phylogenetic analysis of bacterial isolate is shown in Fig. 1.

Microbacterium paraoxydans strain VSVM IIT(BHU) (accession no. MN650647) showed monophyletic grouping with *Microbacterium paraoxydans* strain D2O3 which indicated handy evolution relationship between both the strains. *Microbacterium paraoxydans* strain VSVM IIT (BHU) (accession no. MN650647) showed grouping with other strains. An external strain *Microbacterial barkeri* strain DSM 20145 confirmed this grouping, located outside of the entire tree.

Fan et al. 2018 isolated heavy metal tolerant bacterial strain from coal mining area and identified it by 16S rRNA sequencing followed by phylogenetic analysis. Marzan, et al. 2017 isolated heavy metal resistant bacterial isolates from tannery effluent. Authors identified bacterial isolates as *Gemella* sp., *Micrococcus* sp. and *Hafnia* sp. by 16S rRNA gene sequence analysis. Jiang et al. 2017 isolated heavy metal resistant bacterial strains from ramie rhizosphere soil present around mine refinery and identified it by 16S rRNA sequencing followed by phylogenetic analysis.

SEM and EDX

The surface morphology and elemental analysis of the bacterial isolated are presented in Fig. 2. It became evident from Fig. 2a that the bacterial cells were rod-shaped with a smooth surface. Bacterial cells secrete a sticky extracellular polymeric substance (EPS) that later accumulated on the exterior surface of the cells giving bacteria their rough texture (Das et al. 2013). The EPS mainly consist of polysaccharides and proteins (Zeng et al. 2020). Sodhi et al. (2020) studied the heavy metal reduction by *Alcaligenes* sp. MMA and analysed surface morphology using SEM. Shao et al. (2019) investigated the morphological changes in *Bacillus* sp. exposed to Pb (II) and Cr (VI), and observed that the surface of the cells was rough. Reis-Mansur

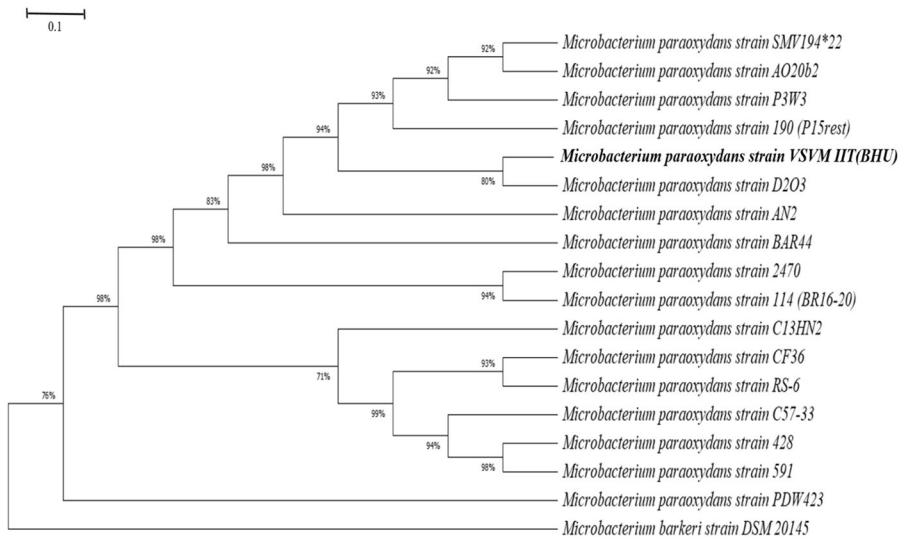

Fig. 1 Evolutionary phylogenetic tree of *Microbacterium paraoxydans* strain VSVM IIT (BHU) (accession number MN650647) and other similar bacterial strain (Singh and Mishra, 2021a)

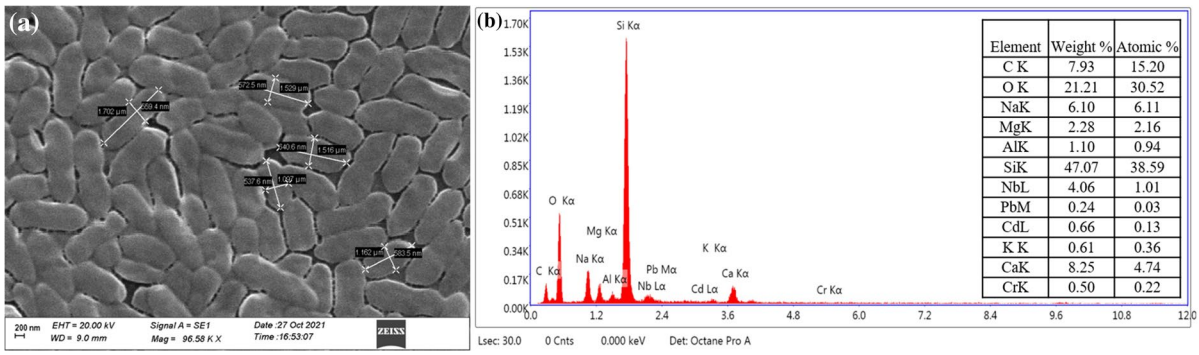

Fig. 2 SEM (**a**) and EDX (**b**) of bacterial

et al. (2019) isolated *Microbacterium* sp. LEMM J01 from Antarctic soil and reported elongated and rod-shaped bacterial structures. Gao et al. (2013) carried out the isolation of *M. neimengense sp.* from the rhizosphere of the maize and observed a similar kind of bacterial morphology.

EDX analysis confirmed the presence of those elements which are component of the bacterial cell wall. It also indicated towards the binding heavy metal ions on the surface of bacterial cells. C, O, Na, Mg, Al, Si and Ca were reported in the EDX of bacterial cells (Fig. 2b). Si, C and O available in the majority were responsible for the biosorption of heavy metals on the bacterial surface (Singh et al. 2020). The presence of C on the bacterial cells makes them suitable for the biosorption of heavy metals (Labied et al. 2018). Si has a robust heavy metal binding affinity, forming a complex structure with heavy metals (Oh et al. 2007). Both O and Si combine to form a mesoporous amorphous structure on the bacterial cell surface, which creates a suitable environment for the biosorption of heavy metals (Bois et al. 2003). Cr (VI), Pb (II) and Cd (II) also appeared on the surface of bacterial cells grown in a medium spiked with the ternary metal heavy-ion system (Fig. 2b), confirming their biosorption. Syed and Chinthala (2015) investigated Cu (II), Pb (II) and Cd (II) bioremediation by *Bacillus* sp. isolated from Solar Salterns. Authors performed EDX analysis for the heavy metal exposed bacterial species and reported successful biosorption of Cd (II), Pb (II) and Cu (II) on the surface of bacterial cells. Goswami et al (2017) confirmed the biosorption of heavy metal ions on the *Rhodococcus opacus* bacterial cells in SEM–EDX results. Shamim et al. (2013) investigated Pb (II) removal by *Aeromonas caviae* strain KS-1. In the EDX analysis, the authors observed Pb (II) binding on the bacterial cells.

XPS

The control and cells grown in 100 mg/L concentration of each metal ion were incubated at 37 °C for 24 h. The results of XPS of control and heavy metal exposed bacterial isolate are shown in Fig. 3.

The C and O elements were present on the bacterial cell in majority (Fig. 3a). The N was also observed in the XPS analysis in the bacterial cells. The XPS indicated that Pb (II), Cr (VI) and Cd (II) were bound to the bacterial cells (Fig. 3b). Heavy metals interact with surface functional groups (i.e., amino, hydroxyl, and carboxyl groups) present on the bacterial cell surface (Hossan et al. 2020). Both surface adsorption and bioaccumulation get involved when heavy metal interact with living bacterial cell. Heavy metal adsorbs on the surface of the bacterium through surface functional groups and enters into the bacterial cell through cell surface receptor (Singh and Mishra 2021a). The presence of Cr (VI), Cd (II) and Pb (II) was detected in the binding energy range of 579 to 575 eV, 417 to 410 eV and 145 to 141 eV for Cr2p3, Cd3d3 and Pb4f5, respectively (Fig. 3b). Zhao et al (2021) investigated Cd (II) removal by sulphate-reducing bacteria. Authors reported Cd (II) binding on the bacterial surface as a Zn–S–Cd complex. Kim et al (2021) performed XPS analysis of *Sporosarcina pasteurii* exposed to Zn, Pb, Cu, Cd and observed the presence of divalent cations in XPS.

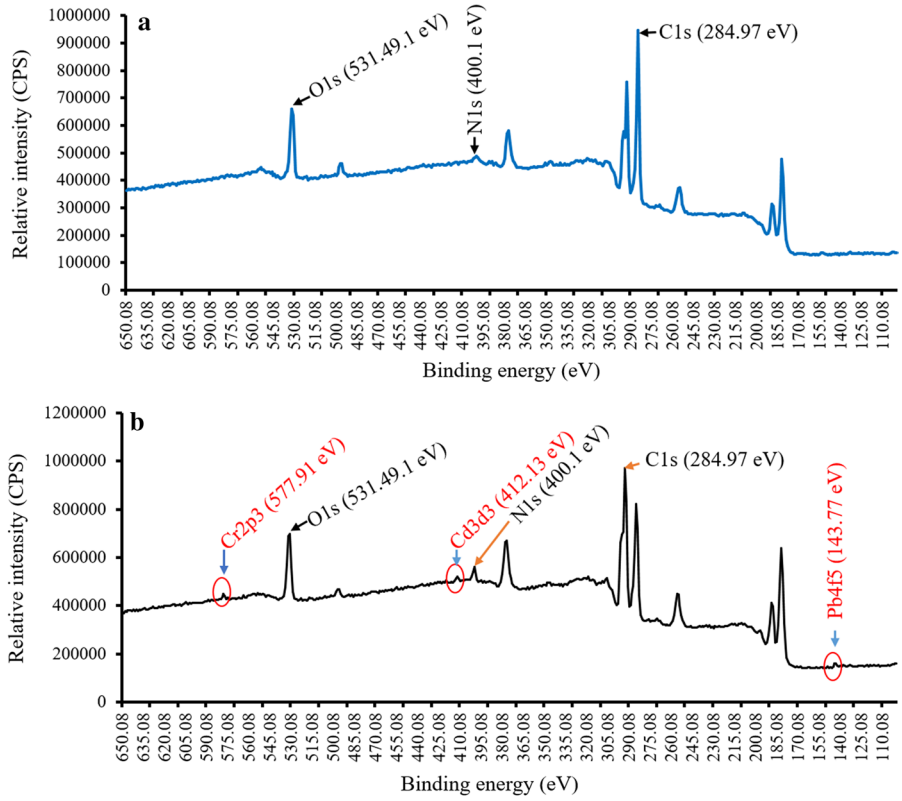

Fig. 3 XPS results of control (**a**) and heavy metal exposed bacterial isolate (**b**)

ATR-FTIR

The surface functional groups on bacterial cells were investigated through ATR-FTIR. Results indicated a shifting in the functional groups in the cells exposed to heavy metals. The ATR-FTIR of bacterial cells grown in control and heavy metals is shown in Fig. 4.

Transmittance peaks at 1300–1400 and 1,600–1,700 cm^{-1} indicated C=C and C–H stretching, respectively (Singh et al. 2020). ATR-FTIR peaks at 1,050 – 1,210 cm^{-1} confirmed C–O and C–N groups of aliphatic compounds (Saha et al. 2013; Kibami et al. 2017). Shifting in FTIR spectra in the range of 1000–1400 cm^{-1} region revealed that bacterial cells were exposed to heavy metals. Changes in the peaks between 1300 and 1400 cm^{-1} reflected the involvement of C=C groups. Shifting the ATR-FTIR spectra at 1050 cm^{-1}, 1210 cm^{-1} reflected C–O and C–N groups. Bacterial cells secrete extracellular polymeric substance (EPS) consisting of polysaccharides and proteins. These extracellular polymeric substances are involved in the binding of heavy metal ions on bacterial cell surface (Zeng et al. 2020). A

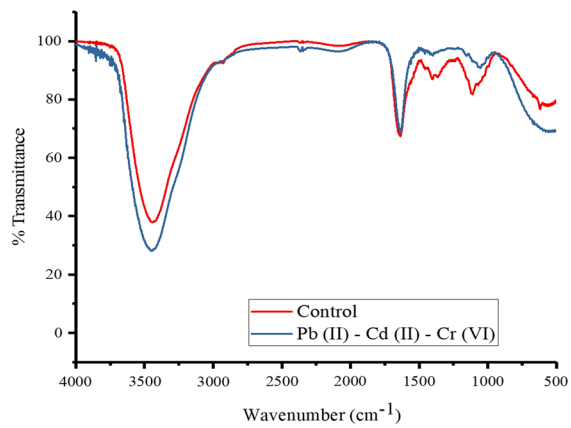

Fig. 4 ATR-FTIR spectra of ternary metal exposed and control bacterial cells

comparison of both types of spectra represented Cd (II), Pb (II) and Cr (VI) binding on the bacterial cell through both non-covalent and covalent interactions (Singh et al. 2020). Wang et al (2018) investigated Pb (II) biosorption in the *Arthrobactor* strain GQ-9 and substantiated the participation of functional groups

present on the bacterial surface, such as –OH, –NH, C=O and C–N in the Pb (II) binding.

Bacterial growth at various heavy metal concentrations

The effect of the ternary metal complex on bacterial growth was observed at 50, 100, 150 and 200 mg/L of each metal. The microbial growth in control and heavy metal exposed growth medium are shown in Fig. 5.

It became apparent from Fig. 5 that maximum and minimum growth was observed in control and at 200 mg/L concentration of ternary metal ions complex. Bacterial cells were grown in 50 mg/L Cr (VI)-Pb (II)-Cd (II) and showed a better growth rate after the control. The bacterial growth parameters such as growth rate (μ) and generation time (g) are listed in Table 1.

The microbial growth rate gradually decreased with an increase in concentration of heavy metals due to heavy metal toxicity. Heavy metal ions generate ROS in the bacteria, inhibiting the expression of several bacterial intracellular proteins, including growth factors (Briffa et al. 2020). ROS production significantly increases in the bacterial cells with the rise in heavy metal concentration in the growth medium. ROS-mediated stress in bacterial cells damages the cell or inhibits bacterial growth (Lazarova et al. 2014).

Table 1 Effect of heavy metal ion concentration on bacterial growth parameters

Heavy metal concentration (mg/L)	Growth rate (μ) (h^{-1})	Generation time (g) (h)
0 (control)	0.13	5.33
50	0.10	6.93
100	0.09	7.7
150	0.08	8.66
200	0.05	13.86

Nath et al (2019) isolated *Bacillus megaterium* strain GCCSO1 from the contaminated soil sample that showed resistance against Cd (II), Pb (II), Cu (II) and Fe (II). It showed a maximum growth in the presence of copper and lead and minimum growth in the presence of cadmium. Dabir et al (2019) isolated *M. oxydans* CM3 and *Rhodococcus* sp. AM1 from coal and aluminium mines and observed bacterial growth inhibition in the presence of Cd (II) and Pb (II) at 400 mg/L.

Removal of heavy metals

Microorganisms utilize trace amounts of heavy metals for their metabolic activities (Tian et al. 2019). They acquire a resistance against heavy metal ions, which helps in their bioaccumulation (Voica et al. 2016). Metal resistance property is based on the

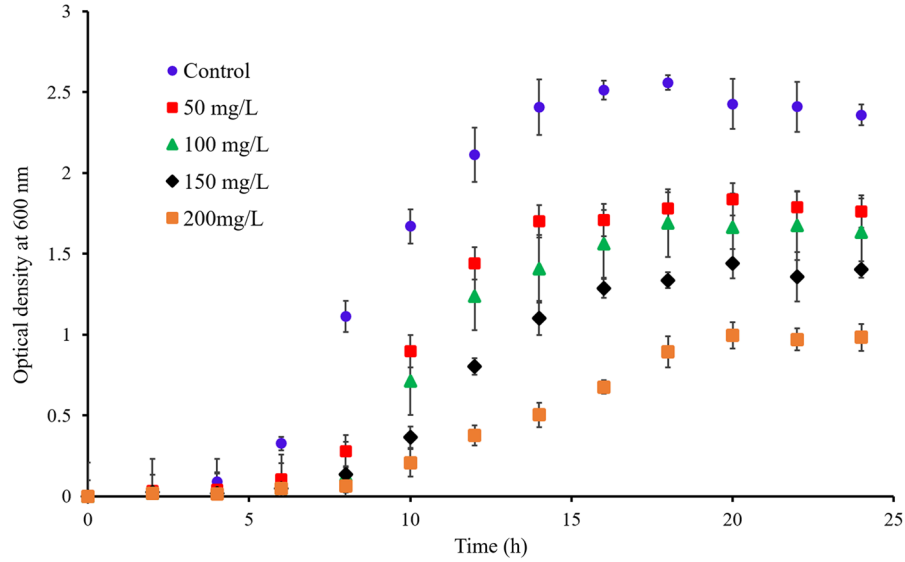

Fig. 5 Effect of heavy metal ions on bacterial growth

antioxidant expression and expression of metal-binding protein within the bacterial cells (Ianeva 2009; Voica et al. 2016). Heavy metal enters into the cytoplasm of bacterial cell through several cell surface receptors. Heavy metal induces the production of oxidative stress in the cell which causes damage in cell proteins and DNA. Metal binding protein minimizes the heavy metal toxicity and enhances the storage of heavy metals into the bacterial cell (Diep et al. 2018). Metallothioneins are the well-known metal binding protein which play an important role in the supply of essential metals ions into the cell and bioaccumulation of toxic heavy metal ions into the cell. Metallothioneins, small protein of about 300 amino acids, are located in the cytoplasm. Metallothioneins mainly consist aromatic (10%) and high proportion of cysteine residues (15–35%) (Balzano et al. 2020).

Moreover, protein folding, intracellular enzymatic activity and ionization rate are the key players in heavy metal bioremediation (Zhang and Li 2011). Bioremediation of Heavy metal also depends on the initial concentration of heavy metals in the growth medium, reaction time, pH, temperature, agitation rate, inoculum dose, types and composition of growth medium, bacterial strain and bacterial resistance (Tang et al. 2021). When heavy metal ions enter into the bacterial cell, heavy metal mediated oxidative stress is generated inside the cell. This oxidative stress is detrimental to the cell. The antioxidants present in the bacterial cell neutralizes this mediated stress and enhance the uptake or bioremediation capacity of bacterial cell (Shao et al. 2019). Optimized pH and temperature for *Microbacterium paraoxydans* strain VSVM IIT(BHU) were 7 and 37 °C (Singh and Mishra 2021a). In the present study, the removal of ternary metal complex of Cd (II), Cr (VI) and Pb (II) was investigated in the range of 50 to 100 mg/L of metals at pH 7 and 37 °C. The removal efficiency of a bacterial isolate is shown in Fig. 6.

The Cr (VI), Pb (II) and Cd (II) removal were recorded as 91.62%, 89.29% and 83.29% at 50 mg/L, respectively (Fig. 6a). It was observed that the removal efficiency decreased when the bacterial isolate was grown in 100 mg/L of metal ions. 75.51% (Cr), 73.84% (Pb) and 68.51% (Cd) removal was reported at 100 mg/L (Fig. 6b). Bacterial strain followed the heavy metal removal pattern as Cr (VI) > Pb (II) > Cd (II). The least removal of Cd (II) was reported as compared other two heavy metals owing to its higher toxicity.

Membrane proteins mediate the bioaccumulation of toxic heavy metals in the bacterial cells (channels and receptors). Intracellular heavy metal ions bind with intracellular proteins like metallothionein. The Cr (VI) is reduced into Cr (III) in the intracellular cell compartment, a process that depends on a number of enzymatic reactions. The Cr (III) accumulates in the bacterial cells (Chojnacka 2010). Huang et al (2001) investigated the Cd (II), Pb (II) and Cr (VI) removal by *E. coli* and *B. subtilis* at 50 mg/L initial metal ions concentration. *E. coli* and *B. subtilis* were able to remove 63.39% and 69.90% Cd (II), 68.51% and 67.36% Pb (II) and 60.26% and 54.56% Cr (VI). Sharma et al (2022) isolated Pb (II) and Ni (II) tolerant bacterial strain from the metal contaminated site of Haryana, Chandigarh and Mohali, India. Authors reported that 39 bacterial isolate were tolerant to 500 mg/L Pb (II) at pH 7. Rajivgandhi et al (2022) investigated Pb (II) removal by *Bacillus cereus* RMN 1 (MK521259) isolated from metal contaminated sites (Thoothukudi city, the southern Part of India). Authors investigated Pb (II) and Cu (II) removal at 300 mg/L, pH 7 for 60 min. Authors reported 85.2% and 60.2% removal of Pb (II) and Cu (II), respectively. Khadim et al (2019) investigated Cd (II) and Ni (II) bioremediation by ureolytic bacteria isolated from barn horse soil. Authors claimed that bacterial isolate was able to removal 96% Cd (II) and 89% Ni (II) at 500 mg/L, pH 7 and incubation for 48 h. Dabir et al (2019) isolated *M. oxydans* CM3 and *Rhodococcus* sp. AM1 from coal and aluminium mines and recorded Pb (II) removal efficiency of *M. oxydans* CM3 and *Rhodococcus* sp. AM1 as 58 and 39% at 400 mg/L.

Intracellular heavy metal ions generate ROS which damage the cell. Antioxidant enzymes can neutralise this heavy metal-mediated stress (Banerjee et al. 2015). These antioxidants actively participate in the heavy metal bioremediation by bacteria. The Cr (VI) is reduced into Cr (III) in the bacterial cell. The surface functional groups on the bacterial cell and intracellular protein such as chromate reductase participate in the reduction of Cr (VI).

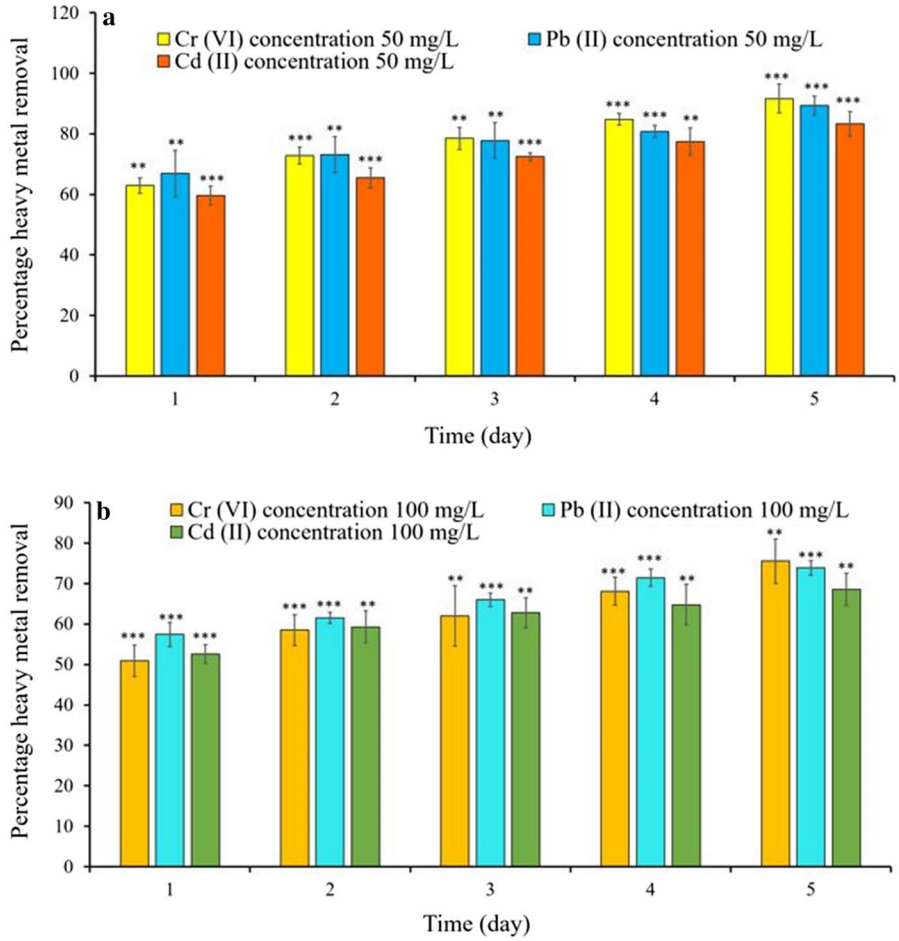

Fig. 6 Heavy metal removal in ternary metal ion system at 50 mg/L (**a**) and 100 mg/L (**b**). The experimental errors (± standard deviation) were in the range of ± 1.27–7.65 and are shown as error bars. The statistical analysis were performed using Paired T-test using (Graphpad Prism 9.00). (*p≤0.05; **p≤0.01; ***p≤0.005)

Expression of antioxidants

The antioxidants protect cells from oxidative damage (Ge et al. 2011). The expression of antioxidants has been analyzed in the control and heavy metal exposed bacterial cells (Fig. 7).

The antioxidant activity increased in the bacterial cells when grown in the medium containing 100 mg/L each metal ion (Fig. 7). The samples of heavy metal exposed bacterial culture was collected after 24 h at stationary phase and population density was 1.63 ± 0.08 OD at 600 nm. The bacterial isolate exposed to heavy metals showed higher antioxidants expression than control. The antioxidants expression in the metal exposed bacterial cells enhanced up to 36.02% (GST), 54.49% (catalase), 45.13% (SOD) and 96.32% (POX). The enhanced expression of antioxidants in the heavy metal exposed

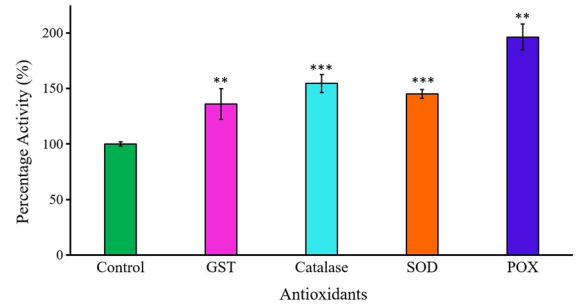

Fig. 7 Expression of antioxidants in the bacterial cells grown in control and ternary metal complex of Pb (II), Cd (II) and Cr (VI). The experimental errors (± standard deviation) were in the range of ± 2.00–13.76 and are shown as error bars. The statistical analyses are with reference to control. *p≤0.05; **p≤0.01; ***p≤0.005 (Paired T-test using Graphpad Prism 9.00)

bacterial cells showed bioaccumulation and toxicity of heavy metals in the intracellular space.

Elahi et al (2019) conveyed that the activity of antioxidants in the Cr (VI) exposed *M. testaceum* B-HS2 increased considerably. Liao et al (2020) re-counted Cr (VI) reduction by *Pannonibacter phragmitetus* and observed that the expression of antioxidants plays an essential role in Cr (VI) reduction. Banerjee et al (2015) evaluated the heavy metal removal efficiency of *Enterobacter cloacae* B1 and testified that SOD activity enhanced in the Cd (II) and Pb (II) exposed bacterial cells. The catalase activity also increased in the Cd (II) exposed bacterial cells. Steunou et al (2020) explored the Cd (II) metal ion mediated stress in bacterial cells. The authors observed that SOD activity in the bacterial cells increased when cells were exposed to Cd (II).

Metal bioremediation mechanism

Heavy metal uptake dynamics

Passive diffusion is responsible for most of the uptake of Cd (II), Pb (II) and Cr (VI) by bacteria. Passive diffusion is the simplest and unregulated technique of transporting a chemical molecule/ ion across a membrane (Arnot et al. 2010). When molecules are transported from one area to another, the driving force is concentration gradient. The present work explored the dimensionless numbers in the ternary metal ion complex. The dimensionless numbers are tabulated in Table 2.

The mixed diffusion and transfer control was observed as rate-controlling step (N_k between 10^{-3} and 10^{1} in all cases) in the ternary metal ion biosorption together with thorough surface coverage and minimized surface tension (φ and λ between 10^{-2} to 10^{4} and 10^{-12} to 10^{8}) (Joos and Serrien 1989; Ferri and Stebe 2000). Heavy metal ions in the aqueous medium cross the plasma membrane and enter the bacterial cell's intracellular space. Passive diffusion is a simple mechanism in which small molecules cross the plasma membrane. The small molecules dissolve in the phospholipid bilayer, cross the bacterial plasma membrane, and enter the intracellular of the bacterial cell (Ma et al. 2009a, b). Singh and Mishra (2020a) conducted a similar investigation on nickel removal by composite material and observed that nickel ion adsorption on composites was mostly regulated by diffusion. Additionally, Singh and Mishra (2020b) identified an adsorption mechanism based on dimensionless numbers for removing zinc, nickel and copper ions from residual neem twig ash and stated that the process was diffusion controlled. According to Singh and Mishra (2021b) Zn, Ni, and Cu adsorption on composite material was diffusion-controlled during their simultaneous removal. Gupta and Diwan (2016) reported heavy metal uptake mechanism in the bacteria. Authors reported that heavy metal enter into the bacterial through passive diffusion (metabolic independent) and active transport (metabolic dependent). Ma et al (2009a, b) reported that heavy metal ions enter into bacterial cells through passive diffusion. The transport of heavy metal into the cytoplasm also depends on concentration gradient of heavy metal ions in the cell across membrane (Ma et al. 2009a, b).

Table 2 Value of dimensionless numbers for metal ions in ternary metal ion system

Dimensionless numbers	φ	λ	N_k
Cr (VI)	1300.00	8.03×10^{-4}	9.49×10^{-3}
Cd (II)	1243.15	1.54×10^{-3}	9.92×10^{-3}
Pb (II)	1001.90	3.86×10^{-3}	1.23×10^{-2}

Reduction of Cr (VI) into Cr (III)

Cr (VI) is several times more toxic than Cr (III). *M. paraoxydans* strain VSVM IIT(BHU) showed the ability to reduce Cr (VI) into Cr (III). The reduction of Cr (VI) into Cr (III) is shown in Fig. 8.

XPS spectra of heavy metal exposed bacterial cells were collected from the Cr2p domain. Cr (III) presence was observed at binding energy 577.0—579.0 and 586.5—588.0 eV, corresponding to Cr2p3/2 and Cr2p1/2 orbital, respectively (Park et al., 2007). The minor Cr (VI) peak was observed at 579.0—581.0 and 588.5—590.0 eV (Park et al., 2007). These results indicated that chromium bound to the *M. paraoxydans* strain VSVM IIT(BHU) was in a trivalent oxidation state mostly, which revealed reduction of Cr (VI) into Cr (III).

Aranda-Garcia and Cristiani-Urbina (2020) investigated Cr (VI) biosorption by *Quercus crassipes* shell in a continuous up-flow fixed-bed column, and

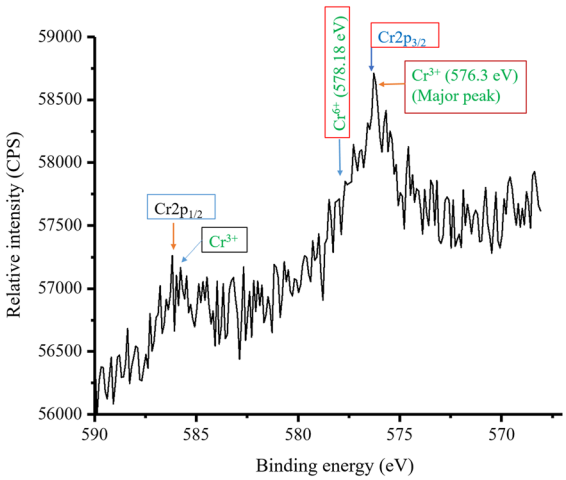

Fig. 8 Reduction of Cr (VI) into Cr (III)

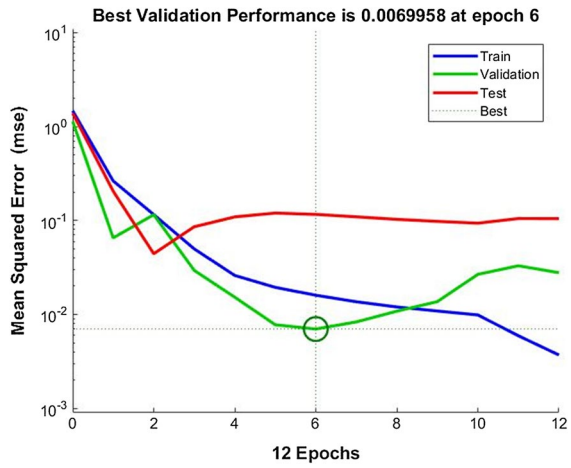

Fig. 9 Performance between MSE and number of epochs for heavy metal removal

demonstrated that most of the chromium adsorbed on the biosorbent was in the trivalent state. Dave and Bhatt (2018) investigated Cr (VI) reduction by a novel bacterial consortium and observed that Cr (VI) was reduced into Cr (III) by bacterial consortium. Nancharaiah et al (2010) examined Cr (VI) immobilization and its reduction into Cr (III) by a mixture of microbes. Gautam et al. (2021) also observed Cr (VI) reduction by *Alkalihalobacillus clausii* CRA1 in the aqueous phase.

ANN

Contact time, pH and temperature were provided as network inputs, with the optical density of the sample being used as the target. The feed-forward back-propagation network type was applied in conjunction with the L-M algorithm to predict the output function. The network was trained until the smallest number of epochs were recorded. Thereafter, the experimental data was merged with the network simulation. The experimental findings were compared with the predicted output function. The mean square error (MSE) of the ANN model for the Cd (II), Cr (VI), and Pb (II) ions in the ternary metal-ion system is depicted in Fig. 9.

The L-M algorithm produced the lowest MSE through data training, testing, and validation (encircled point). The biosorption of the ternary metal complex has been shown as the regression between experimental and model values (Fig. 10).

The coloured lines in the plot represent predicted values produced from ANN whereas the circles represent experimental data. The experimental and theoretical results appeared to coincide indicating a strong regression coefficient ($R^2 = 0.94–0.99$). It was confirmed that the L-M algorithm was effective in accurately expecting the output function with the minimum MSE at epoch 6 and maximum validation performance in 10 neurons at 0.0069958 for Pb (II), Cr (VI) and Cd (II) ions in the ternary metal ion system.

The correlation plot between experimental and model values revealed the highest regression of 0.96 with a minimal deviation of 0.003% between the experimental and model values (Fig. 11).

Figure 11 demonstrates the applicability of the L-M algorithm. Ghosh and Sinha (2015) employed ANN modelling to optimize the reduction of copper by *Stenotrophomonas maltophilia* PD2 biomass and found R^2 of 0.958 from the trained network. Similarly, Talib et al (2019) used ANN to investigate the removal of Cr (VI) by *Acinetobacter radioresistens* strain NS-MIE and came up with an R^2 of 0.9991. Additionally, Ahmad et al. (2014) observed R^2 of 0.997 between the experimental data and model output, when was used ANN to predict the biosorption efficiency of immobilised *Bacillus subtilis* for removing Cd (II) ions. After executing ANN for modelling biosorption of Pb (II) ions, Khan et al (2017) obtained R^2 in the range of 0.95–0.99.

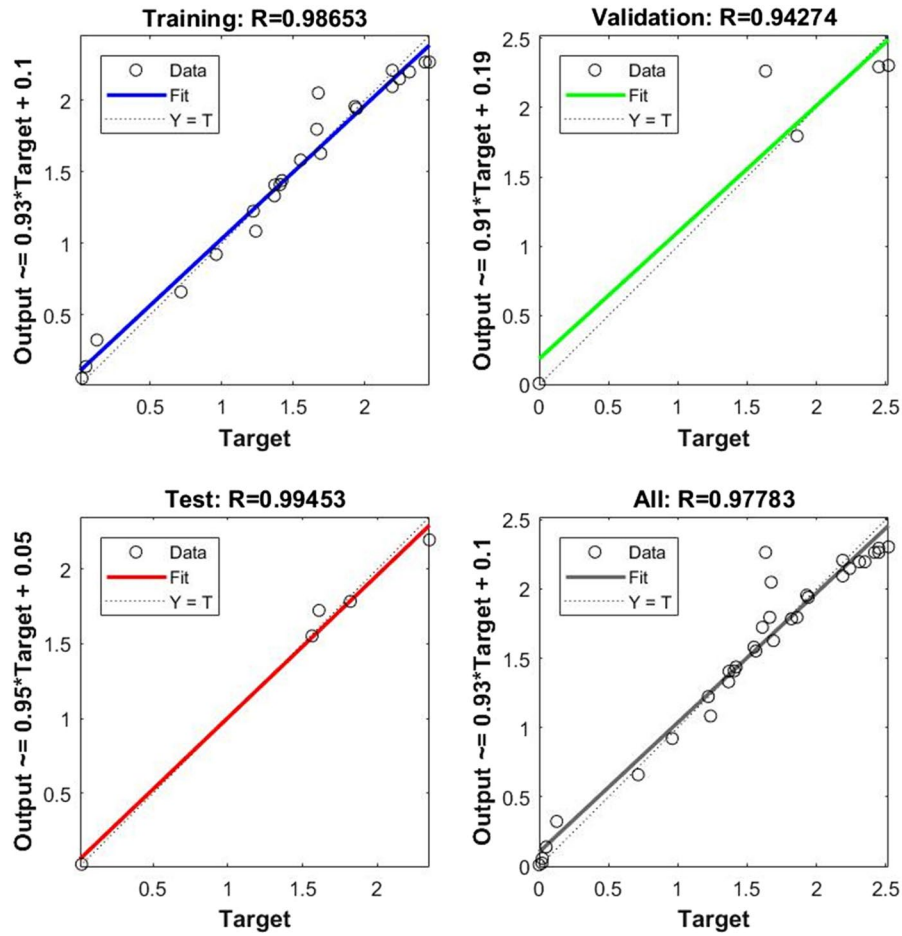

Fig. 10 Regression plot for ternary metal ion system

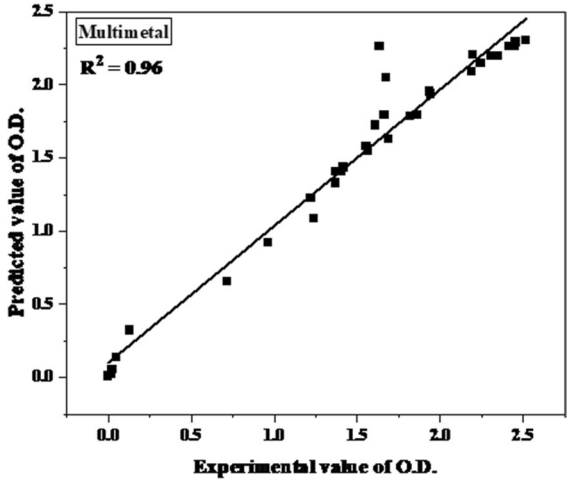

Fig. 11 Correlation plot for ANN predicted and experimental values for ternary metal ion complex

Comparative study of heavy metal removal

The comparison of heavy metal removal efficiency of bacterial isolate with other microorganisms is shown in Table 3. It is evident from Table 3 that the heavy metal removal efficiency of *M. paraoxydans* strain VSVM IIT(BHU) (accession no. MN650647) is substantially higher (Cr (VI) (91.62%), Cd (II) (83.29%) and Pb (II) (89.29%)) as compared to other bacterial strains. Though these readings have been derived in different environmental conditions, yet they show the importance and possible application of bacterial isolate on a larger scale.

Conclusion

Microbacterium paraoxydans strain VSVM IIT(BHU) accession no. MN650647 showed maximum removal

Table 3 Comparison of heavy metal removal efficiency

Bacterial species	Heavy metal removal efficiency (%)	Initial metal concentration (mg/L)	Incubation time (hours)	References
Cr (VI) removal efficiency				
E. coli	60.26	50	60	Huang et al. (2001)
Enterobacter cloacae B2-DHA	81	1	120	Rahman et al. (2015)
B. subtilis	54.56	50	60	Huang et al. (2001)
P. aeruginosa CA207Ni	45.0	450	24	Oyetibo et al. (2013)
Rhodococcus sp. AL03Ni	90.52	450	24	Oyetibo et al. (2013)
B. cepacia AL96Co	89.25	450	24	Oyetibo et al. (2013)
Bacillus cereus	96.85	50	48	Zhao et al. (2012)
M. paraoxydans strain VSVM IIT(BHU) (accession no. MN650647)	91.62	50	120	Present study
Pb (II) removal efficiency				
Urease-producing Strain Pb01	90	200	168	Zhang et al. (2020)
Phosphate-solubilizing bacteria Pb02	90	200	168	Zhang et al. (2020)
Achromobacter Sp. TL-3	80	1500	36	Batta et al. (2013)
E. faecium Pb12	81.36	0.91	2	Bhakta et al. (2012)
M. paraoxydans strain VSVM IIT(BHU) (accession no. MN650647)	89.29	50	120	Present study
Cd (II) Removal efficiency				
M. oxydans CM3	58	400	72	Dabir et al. (2019)
Rhodococcus sp. AM1	39	400	72	Dabir et al. (2019)
E. faecium Pb12	62.64	0.97	2	Bhakta et al. (2012)
Pseudomonas sp. M3	70	100	24	Abbas et al. (2014)
Burkholderia sp. strains ha-1	81.78	5 mM	24	Yu et al. (2021)
Burkholderia sp. strains hj-2	79.37	5 mM	24	Yu et al. (2021)
Burkholderia sp. strains ho-3	63.05	6 mM	24	Yu et al. (2021)
M. paraoxydans strain VSVM IIT(BHU) (accession no. MN650647)	83.29	50	120	Present study

of Pb (II), Cr (VI) and Cd (II) at 50 mg/L. The bacterial isolate showed high heavy metal tolerant capacity and it can grow at 200 mg/L heavy metal concentration. The Cr (VI) was also reduced into Cr (III) by *M. paraoxydans* strain VSVM IIT(BHU). The growth of bacterial cells decreased with the increase in heavy metal concentration from 50 to 200 mg/L. Antioxidants activities increased in the heavy metal exposed bacterial isolate, which showed heavy metal toxicity and its bioaccumulation into intracellular space. The heavy metal removal dynamics results revealed that metal removal was regulated by the mix diffusion and transfer process. The smallest MSE (0.0069958) and the largest R^2 values (0.98) were obtained with the L-M Algorithm to predict the optical density of heavy metal ions. The bacterial isolate identified in the present study has significant potential in removing toxic metal ions from the liquid phase.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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