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Microbially induced carbonate precipitation-based stabilization of a mixture of highly toxic heavy metals by Citrobacter sp. strain PO2

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ABSTRACT

This study evaluated the effectiveness of microbial-induced carbonate precipitation (MICP) in immobilizing heavy metals, using the most resistant bacterial strain isolated from petroleum-contaminated soil. Among the 16 strains isolated from soil near oil wells in Khuzestan, Iran, Citrobacter sp. strain PO2 was identified as the most effective in stabilizing a mixture of highly toxic heavy metals. The strain has been deposited in the NCBI database under accession number PP864728. The analysis was performed using inductively coupled plasma optical emission spectrometry (ICP-OES), scanning electron microscopy (SEM), and energy-dispersive spectroscopy (EDS) techniques. The results showed that the isolated bacterium removed 96.08% of copper, 97.47% of zinc, 99.89% of lead, 72.60% of cadmium, and 60.59% of nickel from a medium containing a mixture of heavy metals at a concentration of 800 mg/L, after 24 hours of incubation at 30 °C. At a concentration of 1500 mg/L, the bacterial strain removed 93.56%, 94.64%, and 97.59% of copper, zinc, and lead, respectively, from the mixed heavy metal solution, while no significant removal of cadmium and nickel was observed at this concentration. At a concentration of 1200 mg/L, bacterial removal efficiencies were 22.07% for cadmium and 6.29% for nickel, respectively. The toxicity of the heavy metals was ranked as follows: Pb > Cu = Zn > Cd > Ni. The MICP process thus represents a promising biological approach for stabilizing heavy metals, offering significant potential for applications in ecological restoration.

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INTRODUCTION

Heavy metals are highly toxic pollutants that persist in the environment (Mitra et al., 2022). Various techniques are employed to remove heavy metal contaminants, including adsorption, filtration, evaporation, chemical precipitation, redox reactions, and electrodialysis (Fei et al., 2023). However, these methods can be costly, sometimes inefficient, and may not always comply with environmental regulations, often leading to the generation of hazardous waste (Khatun et al., 2024). It is worth noting that several biological methods, including bioaccumulation, bioextraction, biosorption, bioflocculation, and phytoremediation, have been applied to remove these pollutants (Abbas et al., 2014; Khatun et al., 2024). However, these approaches are often ineffective due to their high costs and limited efficiency. These biological methods are often time-consuming and lead to the accumulation of heavy metals within biomass or adsorbents, which must subsequently be removed from the environment to prevent the potential re-release of contaminants (Choudhary et al., 2011, Kondakindi et

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*Corresponding Author Email: *gh.r.ghezelbash@gmail.com* al., 2024). The biological mineralization method, particularly microbial-induced carbonate precipitation (MICP) technology, has gained significant attention in recent years as an effective, low-cost, and environmentally friendly approach for removing heavy metals by forming more stable precipitates in the natural environment (Zhang et al., 2023; Lin et al., 2023). The primary mechanism underlying MICP involves the microbial urease-driven production of carbonate ions, resulting in the precipitation of metal carbonates in the presence of dissolved metal ions. However, urease-producing microorganisms can also induce the formation of heavy metal carbonates instead of calcium carbonate, effectively immobilizing and stabilizing toxic heavy metals in the environment (Dejong et al., 2014; Kalkan 2020).

Over the past decade, numerous studies have highlighted the potential of MICP as a promising strategy for the immobilization of heavy metals in contaminated environments. One of the earliest contributions in this field was made by Gomaa et al. (2018), who investigated the efficiency of ureolytic, heavy metal–resistant bacteria in promoting biomineralization. Among 22 bacterial strains isolated from calcareous soils in Egypt, *Micrococcus luteus* was identified as the most potent, exhibiting both high urease activity and strong metal tolerance. Their findings showed that this strain was capable of removing 60.66% of cadmium (Cd) and 97.20% of lead (Pb) from contaminated media. Following this, Qiao et al. (2021) further explored the use of MICP to remove various heavy metals through bacterially mediated carbonate precipitation. Their study demonstrated that metal removal primarily occurred via the formation of metal carbonates. Utilizing two isolates of *Sporosarcina* (KP-4 and KP-22), they achieved removal efficiencies of 75.10% for copper (Cu) in a system containing 160 mg/L of mixed metals. Additionally, the strains were able to remove 98.03% of zinc (Zn), 59.46% of nickel (Ni), and 96.18% of cadmium (Cd), underscoring the versatility of these microorganisms in multimetal remediation contexts. More recently, Mao et al. (2023) examined the combined effect of *Leptolyngbya* sp. XZMQ and *Bacillus* sp. XZM on arsenic immobilization in soils collected from Shanxi, China. Following a 90-day incubation under controlled greenhouse conditions, they observed stabilization of up to 26% of arsenite [As (III)] and arsenate [As (V)], although no specific crystalline mineral phases were identified in association with the immobilized arsenic.

Previous studies have primarily focused on the removal of one or a few heavy metals, with most bacterial isolates lacking resistance to a broad spectrum of metals simultaneously. Therefore, the primary objective of this study is to isolate a multi-resistant bacterial strain capable of concurrently removing multiple heavy metals. To accomplish this, a diverse range of heavy metals was selected for both screening resistant isolates and inducing carbonate precipitation under multi-metal conditions. The ultimate goal is to achieve simultaneous precipitation of various metals through a single bioremediation process.

MATERIALS AND METHODS

Isolation and screening of urease-producing heavy metal-resistant bacteria

Soil samples were collected from areas surrounding oil wells and a metal industries company in Ahvaz (Khuzestan, Iran). A total of eight soil samples were aseptically collected in sterile containers and promptly transported to the laboratory for microbiological culturing. For sample preparation, 10 g of each specimen was transferred into an Erlenmeyer flask containing 90 mL of sterile physiological saline (0.9 sodium chloride [NaCl]). The mixtures were then placed on a shaker at 150 rpm for 30 minutes at 30 ºC to facilitate the release of bacteria into the aqueous phase (Baudoin et al., 2003). A 100 µL aliquot of the resulting suspension was subsequently plated on nutrient agar culture supplemented with various concentrations of heavy metals. The concentrations of all metals used in the screening medium were set at 100, 300, and 500 ppm. All inoculated plates were incubated at 30°C for 3 to 7 days, after which the resulting colonies were harvested for subsequent studies. Isolates that were able to grow at all three concentrations in the presence of the metals were considered multi-metal-resistant strains and selected for further analysis. Purified bacterial isolates were then cultured on a urea-specific agar medium. Urease-producing bacteria were identified based on a distinct colorimetric response: the hydrolysis of urea by urease enzymes led to ammonium release, raising the pH of the medium and resulting in the development of a pink color. This reaction enabled clear differentiation between urease-positive and urease-negative strains (Wang et al., 2017). All chemicals and laboratory consumables used in this study, such as heavy metal salts, urea, microbial media and syringe filters, were purchased from Merck (Germany), Samchun (South Korea) and Biolife (Italy).

Screening of metal carbonate precipitate producing isolate

Purified bacterial isolates were cultured on a precipitation medium at 30 °C for two weeks. Screening of colonies was performed by light microscopy to detect the formation of metal crystal precipitates. The composition of the precipitation medium included 20 g/L urea, 2.12 g/L $NaHCO_3$, 10 g/L NH_4Cl, 3 g/L nutrient broth, 25 g/L $CaCl_2 \cdot 2H_2O$, and 15 g/L agar. The pH was adjusted to 8.0 using 1 N NaOH (Chahal et al., 2011). The only modification to the above precipitation medium was the substitution of $CaCl_2 \cdot 2H_2O$ with 800 mg/L of the studied heavy metals, added either individually or in combination. The metal salts used as heavy metal sources included $CuSO_4 \cdot 5H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4$, $Pb(NO_3)_2$ and $Cd(NO_3)_2 \cdot 4H_2O$.

Minimum inhibitory concentration and minimum bactericidal concentration determination

For the purpose of determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), a colony of the isolates was cultured on prepared tryptic soy agar (TSA) medium (containing casein peptone 15 g/L, soy peptone 5 g/L, sodium chloride 5 g/L, agar 15 g/L; pH 7.3 ± 0.2). Then, a bacterial suspension was prepared in sterile distilled water and adjusted to approximately 0.5 McFarland standard. Subsequently, the suspension was diluted until the bacterial concentration reached approximately 5×10^5 CFU/mL. Serial dilutions of each metal (lead, copper, nickel, cadmium, and zinc) were prepared separately. For each concentration, 1.8 mL of tryptic soy broth (TSB) medium containing the respective metal was mixed with 200 µL of the diluted bacterial suspension in sterile tubes, resulting in a final volume of 2 mL. The tubes were then incubated for 48 hours. The lowest concentration of metal at which no visible turbidity was observed was recorded as the MIC. After 24 hours of incubation, the MBC was defined as the lowest concentration at which no bacterial growth was observed on the solid medium. (Pfaller et al., 2010).

Bacterial growth profile and microbial growth rate coefficient

Initially, isolates were separately cultured overnight on TSA culture medium supplemented with urea and varying concentrations of Cu, Ni, Zn, Pb, and Cd, both as single metals and in mixed metal solutions. Concentrations of 100, 200, 300, 400, 600, 800, and 1500 mg/L of the aforementioned metal ions were added to all liquid and solid media, both individually and in combination. Subsequently, a bacterial suspension with an optical density at 600 nm (OD_{600}) of 0.9–1.0 was prepared in sterile water from the activated strain. This suspension was inoculated into the culture medium containing either individual or mixed target heavy metals and 2% urea, at a 1% (v/v) inoculation ratio. The cultures were then incubated at 30°C for 40 hours under controlled conditions with shaking at 120 rpm. The control culture contained no metal ions. Turbidity measurements at 600 nm were recorded for all samples every 2 hours throughout the incubation period, and growth curves were subsequently generated accordingly (Cheng et al., 2019). After incubation, the culture media containing either individual metals or metal mixtures were centrifuged at 10,000 rpm for 5 minutes to collect the precipitates. These precipitates were thoroughly washed to remove any remaining bacterial cells, dried at room temperature, and subsequently analyzed. The concentration of residual metals in the supernatant was measured at each stage of metal precipitation to assess the extent of metal removal.

Mathematical equations can be applied during the growth phase to model bacterial growth:

$$\frac{dN}{dt} = \mu N$$

$$N = N_0\, e^{\mu(t-t_0)}$$

After simplifying these equations, we will have:

$$Ln\, N - Ln\, N_0 = \mu\,(t - t_0)$$

In this equation, N represents the bacterial turbidity at the initial time point (t_0), t donates time, and μ represents the growth rate coefficient. By plotting the natural logarithm N against time during the logarithmic growth phase, the microbial growth rate coefficient (μ) was determined from the slope of the resulting line (Perni et al., 2005).

Quantitative determination of urease activity based on ammonium release

Similar to the previous step, a bacterial suspension of the activated isolates was prepared in sterile water, adjusted to a turbidity corresponding to an OD_{600} of 0.9–1 Subsequently, 1% (v/v) of this suspension was inoculated into TSB supplemented with 2% urea and either individual or mixed heavy metals (100, 200, 300, 400, 600, 800, and 1500 mg/L). A parallel culture without any metal addition served as the control. Urease activity, based on ammonium production, were monitored at 6-hour intervals over a 36-hour incubation period. Two sterile reaction tubes were prepared for each urease activity measurement. In each tube, 900 µL of Tris buffer containing 2% urea was mixed with 100 µL of washed bacterial cells. Immediately, 100 µL of 1.5 M trichloroacetic acid (TCA) was added to one tube to stop the enzymatic reaction. This tube served as a control to account for the ammonium produced during the culturing process prior to the enzymatic activity assay, thereby ensuring an accurate measurement of ammonium released during the 10-minute enzymatic reaction. The other tube, containing metals at various concentrations, either individually or in combination, was incubated at 37 °C for 10 minutes, after which 100 µL of TCA was added to terminate the enzymatic reaction. The samples were then centrifuged at 10,000 rpm for 5 minutes. Subsequently, 500 µL of the supernatant was transferred to a new tube containing 1,750 µL of distilled water and 250 µL of Nessler's reagent (a solution of potassium tetraiodomercurate (II), typically prepared by mixing mercury (II) iodide and potassium iodide in an alkaline medium). The absorbance of the resulting solution was measured at 480 nm immediately (time zero) and at 6-hour intervals for a total duration of 36 hours (Mortensen et al., 2011). The enzymatic activity of the isolated bacteria was measured in media containing heavy metals at seven different concentrations. Enzyme activity was defined as the amount of enzyme that releases one micromole of ammonia per minute per milliliter of the final reaction volume.

Analysis of sediments resulting from bacterial activity

Various methods were used to investigate metal removal and metal carbonate precipitation. Metal precipitates formed in bacterial cultures with heavy metals were collected, dried, and analyzed by scanning electron microscopy (SEM) (MIRA3 TESCAN, Czech Republic). The composition of the precipitates was determined using energy-dispersive spectroscopy (EDS). Dissolved metal concentrations in the culture supernatants were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) (PerkinElmer Optima 8300, US). Residual metals in the supernatant after incubation were measured by ICP-OES to evaluate metal removal efficiency (De Muynck et al., 2010; Dhami et al., 2013).

Identification of the selected isolate

The urease-producing isolate was identified through morphological, biochemical (Garrity et al., 2005), and molecular analyses (Tanca et al., 2017). Genomic DNA extraction was performed using the manual boiling method (Dashti et al., 2009). PCR amplification of the universal 16S rRNA gene was performed using universal primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (3'-TACGGYTACCTTGTTACGACTT-5'), synthesized by Cinagen Company, yielding a fragment of approximately 1344 bp. The PCR reaction mixture (25 µL) consisted of 9.5 µL sterile distilled water, 1 µL genomic DNA, 1 µL of each primer, and 12.5 µL master mix. Thermal cycling conditions included initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. PCR products were confirmed via electrophoresis on 1% agarose gel and subsequently sequenced by Pishgam Biotechnology Company (Tehran, Iran). Bidirectional sequencing was conducted using Macrogen's automated sequencing services (Huelsenbeck et al., 2001). Sequence alignment and identification were performed by comparing the obtained sequences with those in the NCBI GenBank database using BLASTN (Hoffman et al., 2010). Sequence assembly was done using FinchTV v1.4.0 and BioEdit v7.1.3.0. Phylogenetic analysis was carried out through Bayesian inference using MrBayes v3.2.6. The Markov chain Monte Carlo (MCMC) was run for 6 million generations with a sampling frequency of 1000, four chains, and a burn-in of 25%. The analysis was terminated when the standard deviation of split frequencies dropped below 0.01 (Ronquist et al., 2003). Phylogenetic trees were visualized using TreeGraph2 software.

RESULTS AND DISCUSSION

Screening results of urease-producing, heavy metal-resistant bacteria

Among the 16 bacterial strains isolated from petroleum-contaminated soil, strain OP2 was selected as the most promising candidate owing to its rapid urea hydrolysis and robust growth in media supplemented with varying concentrations of heavy metals. The bacterium was isolated from soil sampled at a depth of 0–15 cm in the vicinity of an oil well at the Marun Oil Field, Khuzestan Province, Iran (31.1217° N, 49.3097° E). Strain PO2 was identified as a multi-resistant bacterium, exhibiting resistance to five heavy metals: copper (Cu), nickel (Ni), zinc (Zn), lead (Pb), and cadmium (Cd). Multimetal-resistant bacteria exhibit enhanced survival in heavy metal–contaminated environments and maintain metabolic activity under toxic stress, making them effective agents for bioremediation (Ali et al., 2020; Alabssawy & Hashem, 2024). The initial screening of urease-producing bacteria was based on a color change in a urea-containing medium with a pH indicator, where urease activity increases the pH and results in the development of a pink color (Achal et al., 2011). Studies have demonstrated that microbial carbonate precipitation increases proportionally with bacterial urease activity, highlighting the direct relationship between urease activity and carbonate formation (Hammes et al., 2002).

Results of screening isolates producing metal carbonate precipitation

Figure 1 illustrates the ability of strain PO2 to precipitate metal carbonates at a concentration of 800 mg/L. Distinct carbonate crystals of $CuCO_3$, $NiCO_3$, $ZnCO_3$, $PbCO_3$, and $CdCO_3$, along with mixed-metal carbonate formations, were observed under 20× magnification. These results highlight the strain's potential for multi-metal bioprecipitation and environmental remediation. Several microbial pathways can lead to carbonate precipitation, including urea hydrolysis,

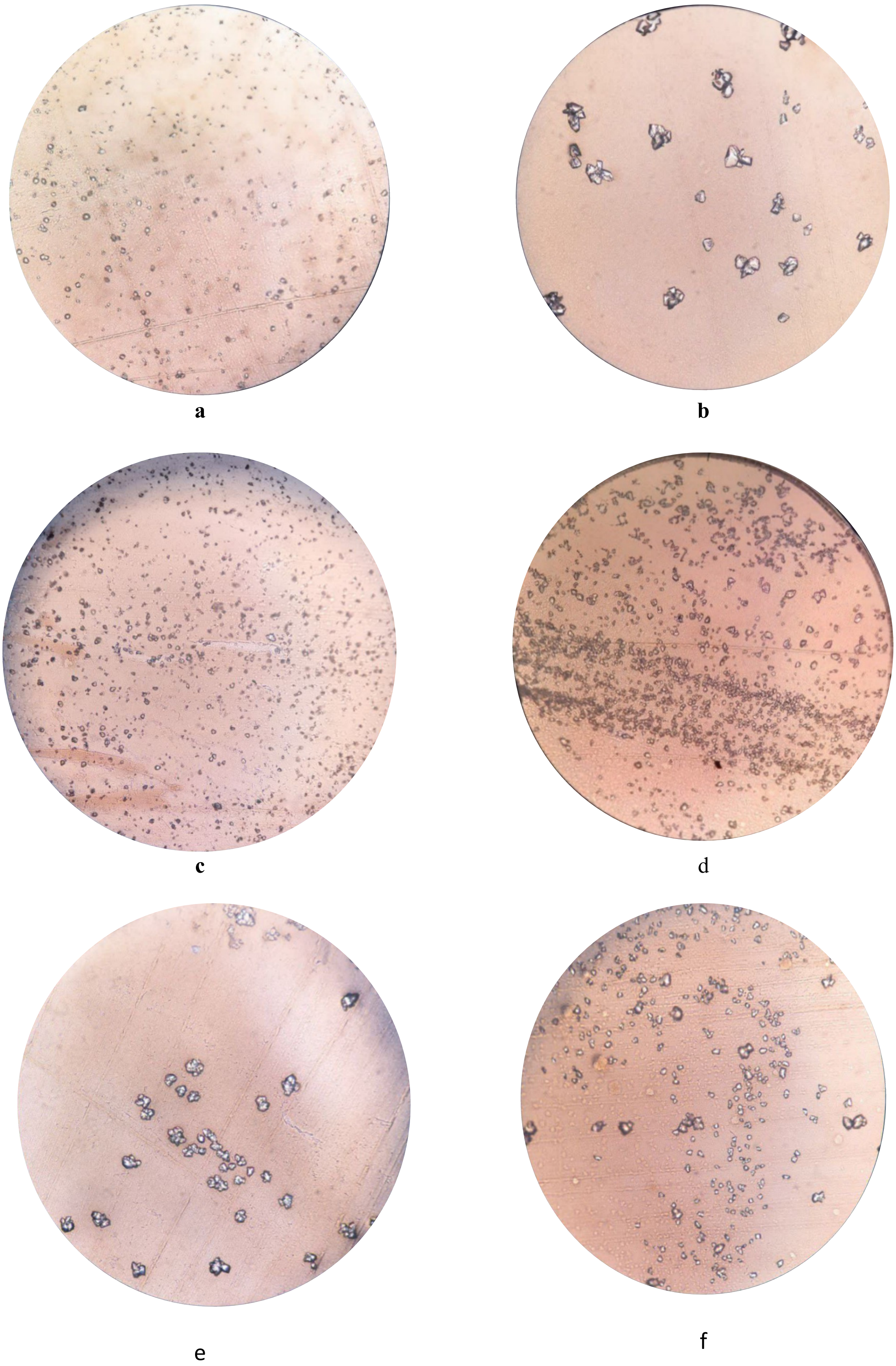


Fig. 1. Carbonate of metals (a), $CuCO_3$ (b), $NiCO_3$ (c), $ZnCO_3$ (d), $PbCO_3$ (e), $CuCO_3$ (f), Mixed metals formed by strain PO2 at the concentration of 800 mg/L with a magnification of 20x.

denitrification, and sulfate reduction. In metal-rich environments, these processes may facilitate the formation of metal carbonates such as those of calcium, copper, cadmium, or lead. Among them, urea hydrolysis by urease-producing bacteria is the most extensively studied and efficient mechanism, commonly referred to as MICP (Torgal et al., 2015). In ureolysis-based MICP, microbial hydrolysis of urea leads to carbonate generation and pH elevation, creating favorable conditions for the precipitation of divalent metal ions as solid carbonates (MCO_3) (Lin et al., 2022). It is important to note that not all metal carbonates readily precipitate under the same conditions. The precipitation efficiency depends heavily on the solubility product (Ksp) of each metal carbonate, as well as the stability of intermediate species in solution. Among the tested metals, $PbCO_3$ (Ksp $\approx 7.4 \times 10^{-14}$) and $CdCO_3$ (Ksp $\approx 5.2 \times 10^{-12}$) exhibit very low solubility, indicating a strong tendency to precipitate. $ZnCO_3$ (Ksp $\approx 1.4 \times 10^{-11}$) and $CuCO_3$ (Ksp $\approx 2.5 \times 10^{-10}$) are moderately more soluble but still favor precipitation under ureolytic conditions. In contrast, $NiCO_3$ (Ksp $\approx 6.6 \times 10^{-9}$) is significantly more soluble, and may require higher carbonate concentrations or elevated pH to achieve effective precipitation (Haynes, 2016).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

The study found that strain PO2 exhibited the highest resistance to the toxicity of heavy metals such as lead, copper, and zinc at concentrations up to 1500 mg/L, while showing the lowest resistance to cadmium and nickel at concentrations around 1200 mg/L. The resistance hierarchy to heavy metal toxicity was established as Pb > Cu = Zn > Cd > Ni, with lead demonstrating the greatest resistance and nickel the lowest. Detailed results are presented in Table 1. Heavy metals can be toxic to living organisms, particularly when they interfere with the activity of microorganisms involved in bioremediation processes (Kang et al., 2015; Mugwar & Harbottle, 2016). This hierarchy is consistent with previous reports on bacterial metal tolerance: lead and copper are generally better tolerated due to their tendency to form less bioavailable complexes and their frequent presence in contaminated environments, whereas cadmium and nickel are more bioavailable and cytotoxic, disrupting cellular processes even at lower concentrations (Majzlik et al., 2011; Mugwar & Harbottle, 2016). Such resistance is frequently observed among bacteria isolated from petroleum-contaminated soils, where co-selection pressure results in indigenous strains capable of both hydrocarbon degradation and heavy metal tolerance (Velusamy et al., 2011; Palaniyandi et al.).

Growth profiles and growth rate coefficients of bacterial isolates

Strain PO2 exhibited high tolerance to lead, copper, and zinc, but was strongly inhibited by cadmium and nickel, particularly at higher concentrations. Table 2 shows the effect of individual heavy metals on the bacterial growth rate coefficient. At lower concentrations (100 and 200 mg/L), strain PO2 exhibited consistently high growth rate coefficients across all tested heavy metals. The highest growth rates were recorded with lead (0.92 at 100 mg/L) and copper (0.85 at 100 mg/L), indicating minimal inhibitory effects at these concentrations. Although cadmium and nickel caused significant inhibition at higher concentrations, substantial growth was still observed at the lower doses. These findings demonstrate that strain PO2 has a strong tolerance to low levels of heavy metals. At 800 mg/L, lead (0.74), copper (0.59), and zinc (0.50) supported relatively higher growth compared to cadmium (0.38), nickel (0.30), and the metal mixture (0.28). At 1500 mg/L, the growth rate decreased for all conditions, with no detectable growth in the presence of

Tabel 1. MBC test results of strain PO2

Isolate	Cu (mg/L)	Ni (mg/L)	Cd (mg/L)	Zn (mg/L)	Pb (mg/L)
Strain PO2	1750	1250	1300	1800	2000

Table 2. Growth rate coefficients of strain PO2 exposed to various heavy metals at different individual concentrations.

Concentration mg/L	Metals	Specific growth rate
100	Lead	0.92
	Copper	0.85
	Zinc	0.84
	Cadmium	0.80
	Nickel	0.73
	Mixed metals	0.50
200	Lead	0.85
	Copper	0.82
	Zinc	0.80
	Cadmium	0.71
	Nickel	0.65
	Mixed metals	0.43
300	Lead	0.82
	Copper	0.80
	Zinc	0.71
	Cadmium	0.63
	Nickel	0.61
	Mixed metals	0.39
400	Lead	0.80
	Copper	0.71
	Zinc	0.61
	Cadmium	0.51
	Nickel	0.50
	Mixed metals	0.36
600	Lead	0.80
	Copper	0.65
	Zinc	0.57
	Cadmium	0.44
	Nickel	0.36
	Mixed metals	0.34
800	Lead	0.74
	Copper	0.59
	Zinc	0.50
	Cadmium	0.38
	Nickel	0.30
	Mixed metals	0.28
1500	Lead	0.61
	Copper	0.52
	Zinc	0.43
	Cadmium	0
	Nickel	0
	Mixed metals	0.22

cadmium and nickel, and a further reduction in the mixed-metal treatment (0.22). It is noteworthy that at a concentration of 1500 mg/L, nickel and cadmium ions were removed from the culture medium. Figure 2 illustrates the growth curves of strain PO2 in the presence of the tested heavy metals, both individually and in combination. During the removal of heavy metals using the MICP method, it is essential to assess the growth ability of microorganisms under varying concentrations of heavy metals. Therefore, the resistance of microbial strains used in the MICP process is very important. Moreover, efforts are currently underway to develop metal-resistant strains through various genetic and biochemical mechanisms (Hao et al., 2021). There are at least six biochemical mechanisms that can confer resistance, and different mechanisms may act alone or in combination. Furthermore, multiple resistance mechanisms can coexist within the same species for a particular metal. The largest group of metal resistance systems functions through the energy-dependent efflux of toxic ions (Seifan et al., 2019; Gillieatt and Coleman, 2024). Understanding these diverse resistance mechanisms is crucial for developing effective microbial strains for bioremediation of

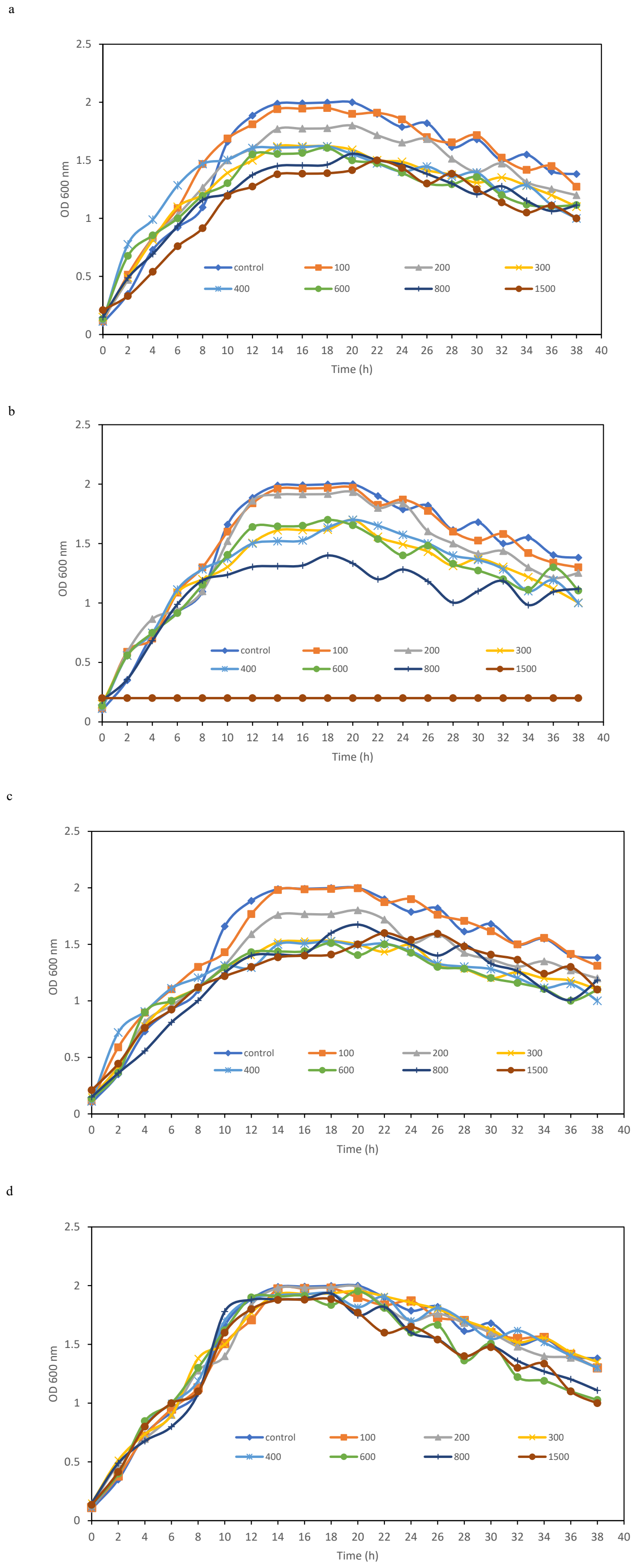
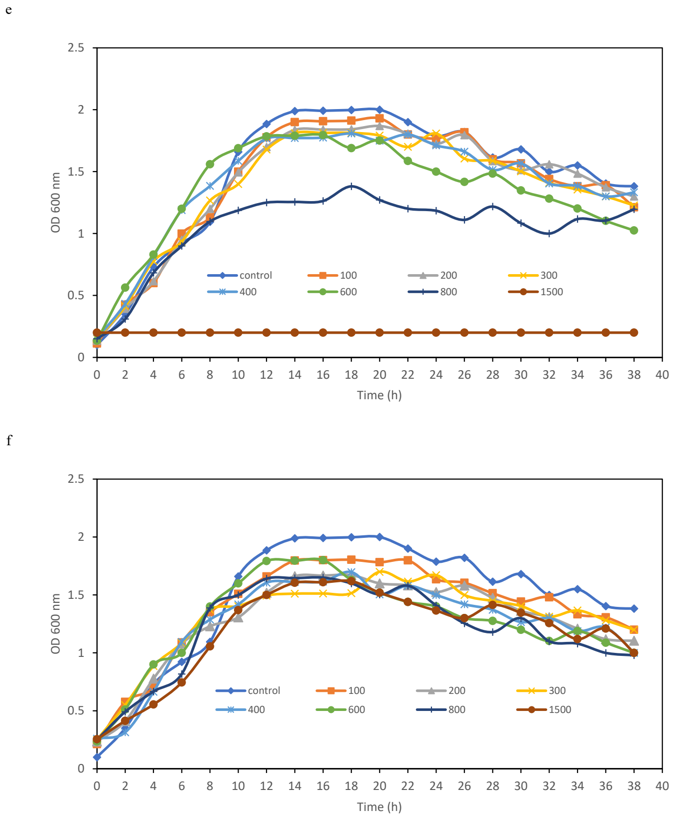


Fig. 2. Growth curves of strain PO2 in the presence of (a) Cu, (b) Ni, (c) Zn, (d) Pb, (e) Cd, and (f) a mixture of all metals. Metal concentrations, whether tested individually or in combination, ranged from 100 mg L^{-1} to 800 mg L^{-1}. At 1500 mg L^{-1}, cadmium and nickel were excluded because the strain did not grow at this concentration.



Continued Fig. 2. Growth curves of strain PO2 in the presence of (a) Cu, (b) Ni, (c) Zn, (d) Pb, (e) Cd, and (f) a mixture of all metals. Metal concentrations, whether tested individually or in combination, ranged from 100 mg L^{-1} to 800 mg L^{-1}. At 1500 mg L^{-1}, cadmium and nickel were excluded because the strain did not grow at this concentration.

heavy metals (Saha et al., 2021).

Urease activity measurement results

Figure 3 illustrates the enzymatic activity of strain PO2 in the presence of the heavy metals examined in this study, both individually and in combination. The enzymatic activity in the absence of metals was also assessed and is included in the figure as a control. The urease activity of strain PO2 in the absence of metal was 0.939 U. When exposed to 100 and 1500 mg/L of copper, the activity changed to 0.701 and 0.860 U, respectively. In the presence of cadmium at the same concentrations, the activity was recorded at 0.830 and 0.619 U. Similarly, exposure to the metal mixture resulted in activities of 0.871 and 0.710 U, respectively. At a concentration of 1500 mg/L, urease activity was completely inhibited (0 U) in the presence of nickel and cadmium. Accordingly, these two metals were omitted from the enzyme buffer in the metal mixture at this concentration to ensure measurable enzymatic activity. Heavy metals can inhibit enzyme activity by interacting with the enzyme's active site, particularly with thiol (-SH) groups. The inhibitor binds to the enzyme, preventing substrate binding at the active site, leading to inhibition of enzymatic activity. The extent of inhibition depends on the concentrations of both substrate and inhibitor. Additionally, inhibitors may bind to other regions of the enzyme, causing conformational changes that hinder substrate-enzyme complex formation, thereby reducing urease activity (Fopase et al., 2019).

Results of inductively coupled plasma (ICP) analysis

Following the removal of bacterial debris, cell remnants, and metal carbonate precipitates

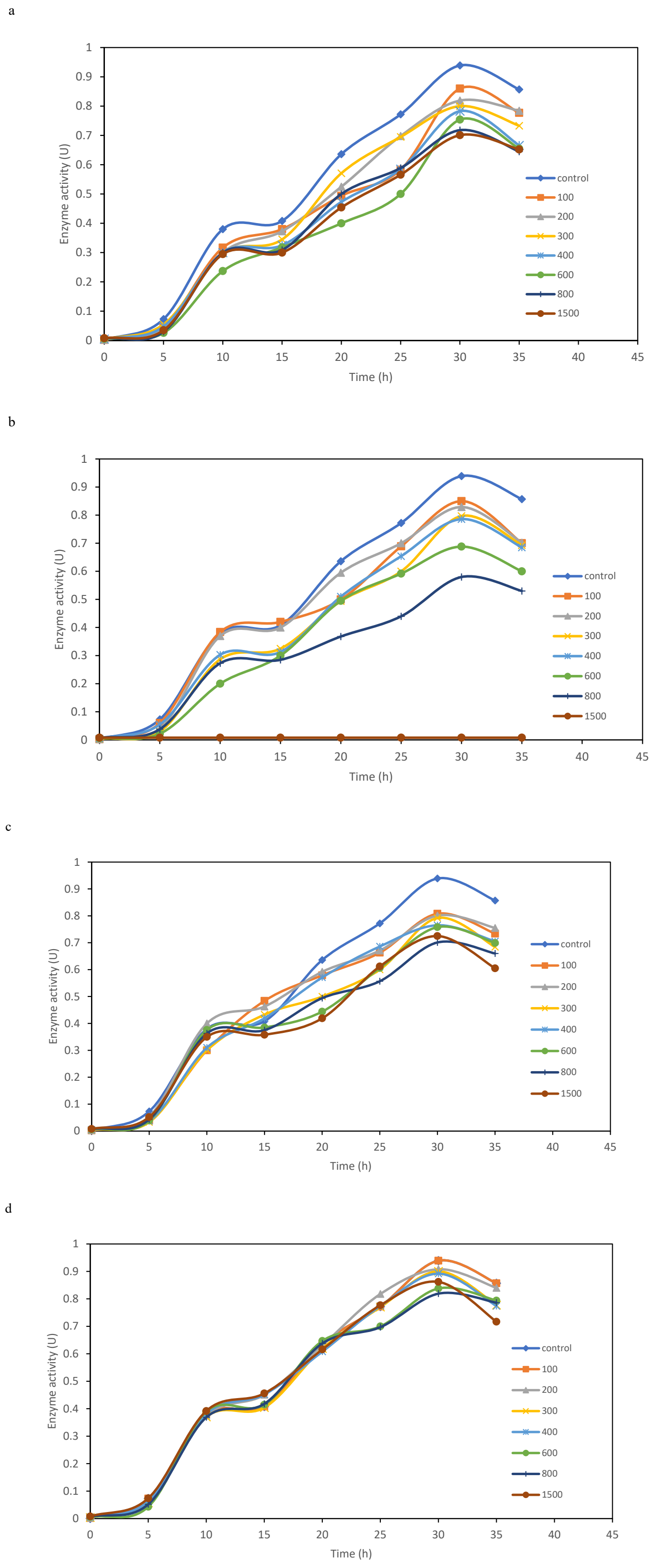
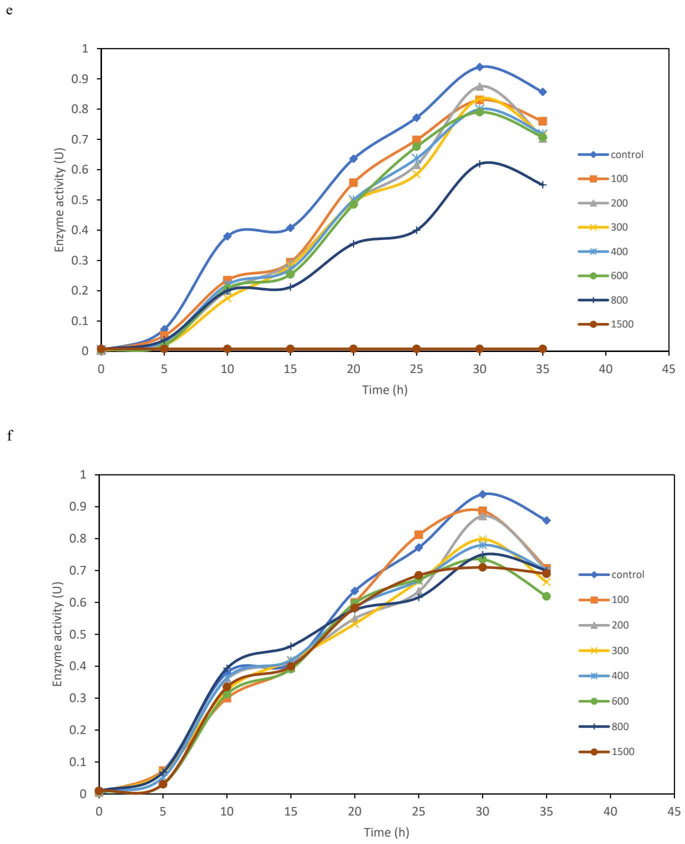


Fig. 3. Enzyme activity curve of strain PO2 in the presence of a) Cu, b) Ni, c) Zn, d) Pb, e) Cd, and (f) a mixture of all metals. Metal concentrations, whether tested individually or in combination, ranged from 100 mg L⁻¹ to 800 mg L⁻¹. At 1500 mg L⁻¹, cadmium and nickel were excluded because the strain did not grow at this concentration.



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from the culture medium, ICP analysis was carried out on the supernatant. The concentrations of residual metals in the supernatant are summarized in Tables [3, 4]. The ability of the multi-heavy-metal-resistant strain PO2 to precipitate various metals contributed to a substantial reduction in metal levels remaining in the culture medium. Table 3 summarizes the initial and final concentrations of individual metals following cultivation, along with their respective percentage reductions. Table 4 provides the corresponding data for cultures exposed to multiple metals. Previous researchers have employed ureolytic bacterial strains with heavy metal resistance to remove or immobilize specific metals (Li et al., 2013; Kumari et al., 2014; Kang et al., 2015; Zhao et al., 2017). In the present study, strain PO2 demonstrated the ability to remove heavy metals through urease activity.

Scanning Electron Microscopy (SEM) and Energy-Dispersive Spectroscopy (EDS) Analysis

To confirm the presence of heavy metals in the precipitated sediments and to quantify their atomic ratios, SEM and EDS were employed. Figures [4–5] illustrate the distribution of heavy metals within the sediment samples. The atomic ratios of metals in the precipitates formed by strain PO2 were determined at metal concentrations of 800 and 1500 mg/L, both in single-metal and mixed-metal conditions. At a concentration of 800 mg/L, the atomic ratios for Cu, Ni, Cd, Zn, and Pb in single-metal versus mixed-metal treatments were 0.72 vs. 0.48 (Cu), 0.03 vs. 0.46 (Ni), 0.23 vs. 2.24 (Cd), 0.95 vs. 0.11 (Zn), and 18.70 vs. 2.28 (Pb), respectively. At 1500 mg/L, the ratios were 0.29 vs. 0.48 (Cu), 0.31 vs. 0.08 (Zn), and 14.54 vs. 0.03 (Pb). Interestingly, nitrogen (N) was detected in some samples, although it is not typically associated with carbonate

Table. 3. Effectiveness of MICP in the removal of single heavy elements using ICP analysis

Metals	Concentration before treatment (mg/L)	Concentration after treatment (mg/L)	Metal removal percentage
Copper	100	0	100
	200	0	100
	300	0.42	99.86
	400	0.65	99.83
	600	12.35	97.94
	800	34.012	95.74
	1500	100.18	93.32
Zinc	100	0.11	99.89
	200	0.25	99.87
	300	0.37	99.87
	400	2.019	99.49
	600	6.58	98.90
	800	24.52	96.93
	1500	79.012	94.73
Lead	100	0	100
	200	0	100
	300	0	100
	400	0	100
	600	0.12	99.98
	800	0.64	99
	1500	74.26	95.02
Cadmium	100	0.4	99.6
	200	0.74	99.6
	300	4	98.66
	400	10.33	97.41
	600	83.59	86.06
	800	200.12	74.98
	1200	935.16	22.07
	1500	1500	0
Nickel	100	0.12	99.88
	200	0.32	99.84
	300	0.62	99.79
	400	23.75	94.06
	600	64.47	89.25
	800	219.14	72.60
	1200	1124	6.29
	1500	1500	0

crystallization or heavy metal hydroxide precipitation. This anomaly may explain why certain precipitates did not match the standard diffraction patterns listed in the International Center for Diffraction Data (ICDD) database. In a recent study, Jalilvand et al. (2020) isolated urease-producing, heavy-metal-resistant bacteria from contaminated calcareous soils near the Angoran mine in Zanjan Province, Iran. They identified *Variovorax boronicumulans* as a ureolytic bacterium capable of biomineralizing heavy metals. SEM and EDS analyses in their study confirmed the bacterium's ability to precipitate carbonates of Zn, Cu, and Cd. In this study, distinct EDS peaks corresponding to metals, carbon, and oxygen confirmed that the observed metal carbonate precipitation was induced by bacterial ureolytic activity. In our study, as well as in previous reports (Li et al., 2013; Gomaa et al., 2018), the size and morphology of the resulting crystals varied depending on the type of heavy metal present during the biomineralization process. These findings highlight the influence of metal type on the biomineralization pathway and the resulting crystalline structures.

Identification results and phylogenetic relationships

Strain PO2 was selected from 16 isolates due to its exceptional performance in metal

Table. 4. The effectiveness of MICP in the removal of heavy element mixtures using ICP analysis.

Concentration before treatment (mg/L)	Mixed metal solution	Concentration after treatment (mg/L)	Metal removal percentage
100	Copper	0.20	99.86
	Zinc	0.10	99.9
	Lead	0	100
	Cadmium	0.12	99.88
	Nickel	0.4	99.06
200	Copper	0.35	99.82
	Zinc	0.15	99
	Lead	0	100
	Cadmium	0.32	99.84
	Nickel	0.96	99.02
300	Copper	0.55	99.81
	Zinc	0.22	99
	Lead	0.1	99.96
	Cadmium	0.62	99.79
	Nickel	11.24	96.25
400	Copper	12	97
	Zinc	50.01	98.74
	Lead	0.42	99.89
	Cadmium	23.75	94.06
	Nickel	30.64	92.34
600	Copper	20	96.66
	Zinc	8.075	98.65
	Lead	0.61	99.89
	Cadmium	54.21	90.96
	Nickel	64.47	89.25
800	Copper	31.3	96.06
	Zinc	20.2	97.47
	Lead	0.87	99.89
	Cadmium	219.14	72.60
	Nickel	315.23	60.59
1500	Copper	90.56	93.96
	Zinc	80.26	94.64
	Lead	36.02	97.59

Table 5. Results of biochemical tests of *Citrobacter* sp. strain PO2**.**

Biochemical tests		Result
Urease		+
Catalase		+
Oxidase		–
Carbohydrate Fermentation	Mannitol	+
	Sorbitol	+
	Mannose	+
	Trehalose	+
	Glucose	+
	Maltose	+
	Sucrose	+
	Inositol	–
	Melibiose	–
Indole		–
Citrate		+
MR		+
Indole production		–
Gelatinase		–
Lipase		–
Phosphatase		–

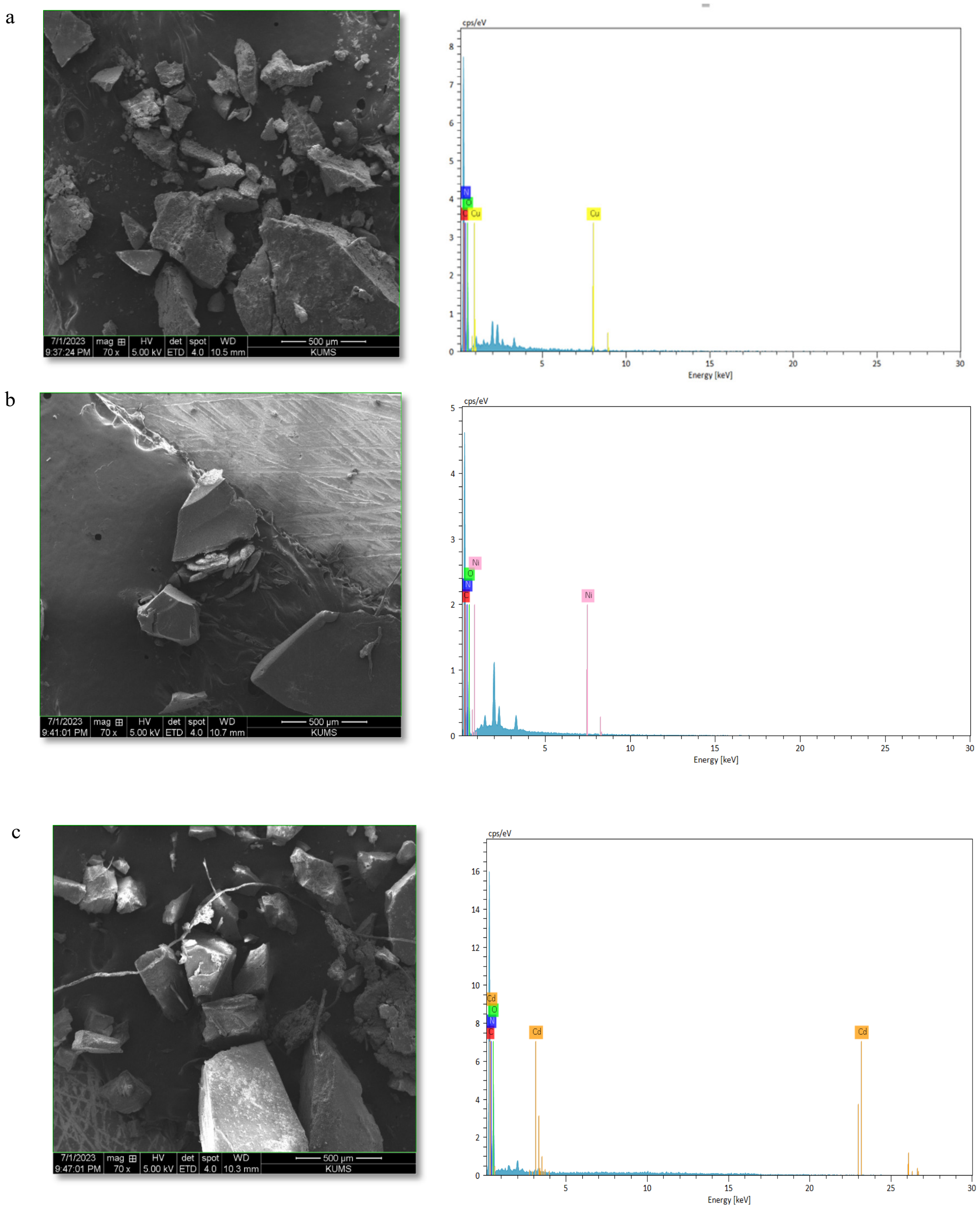

Fig. 4. SEM and EDS analysis results of metal carbonate precipitates formed by strain PO2 at a concentration of 800 mg/L in the presence of (a) Cu, (b) Ni, (c) Cd, (d) Zn, (e) Pb, and (f) a mixture of these metals.

carbonate production and high urease activity. It showed 100% sequence similarity to *Citrobacter cronae* strain TUE 2-1 and 99.78% similarity to *Citrobacter freundii* strain ATCC 8090 based on universal 16S rRNA gene sequencing. The results of the biochemical tests are presented in Table [5], and the phylogenetic tree of the isolated bacterium is shown in Figure [6]. Phylogenetic analysis based on universal 16S rRNA gene sequencing positioned strain PO2 within the *Citrobacter* genus. While it exhibited the highest similarity to *Citrobacter cronae* strain Tue2-1, PO2 clustered separately from some other well-characterized *Citrobacter*

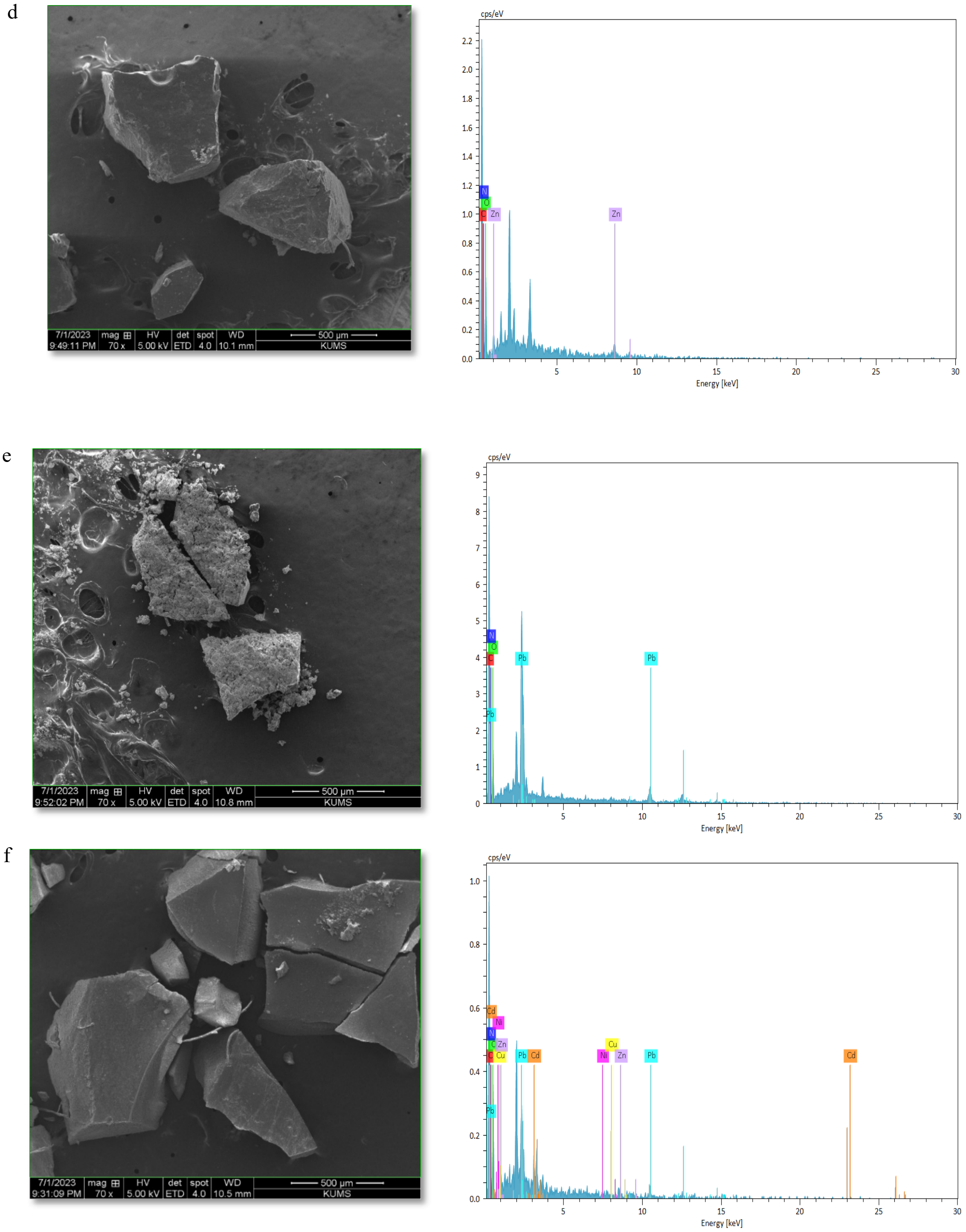

Fig. 4. SEM and EDS analysis results of metal carbonate precipitates formed by strain PO2 at a concentration of 800 mg/L in the presence of (a) Cu, (b) Ni, (c) Cd, (d) Zn, (e) Pb, and (f) a mixture of these metals.

species. Most *Citrobacter* species formed a coherent clade, with closely related genera such as *Klebsiella* and *Kluyvera* appearing on adjacent branches, reflecting genus-level divergence. These findings support the classification of strain PO2 as a member of the *Citrobacter* genus. Therefore, the sequence was submitted to the NCBI database under accession number PP864728 and designated as *Citrobacter* sp. strain PO2.

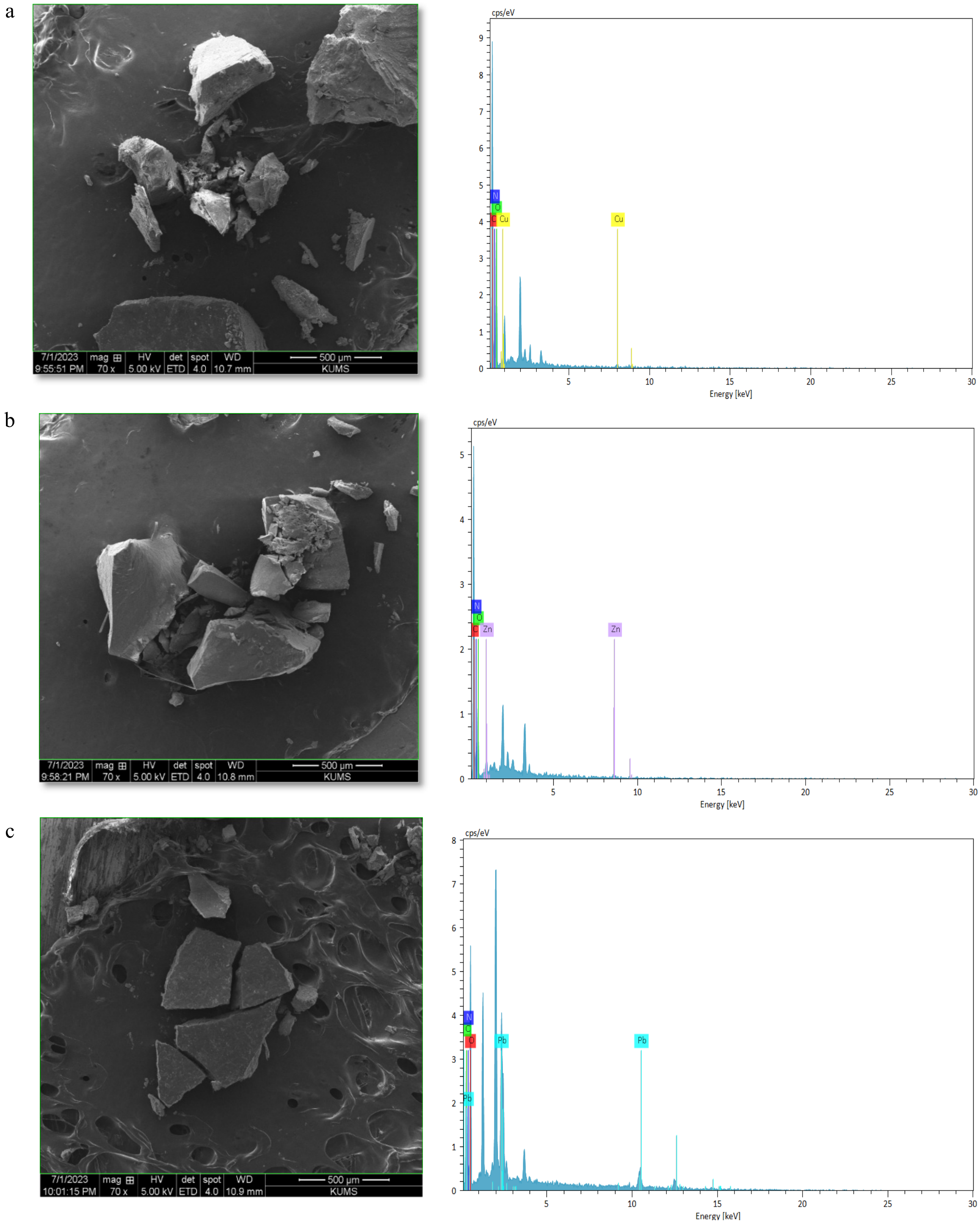

Fig. 5. SEM and EDS analysis results of metal carbonate precipitates formed by strain PO2 at a concentration of 1500 mg/L in the presence of (a) Cu, (b) Zn, (c) Pb, and (d) a mixture of these metals.

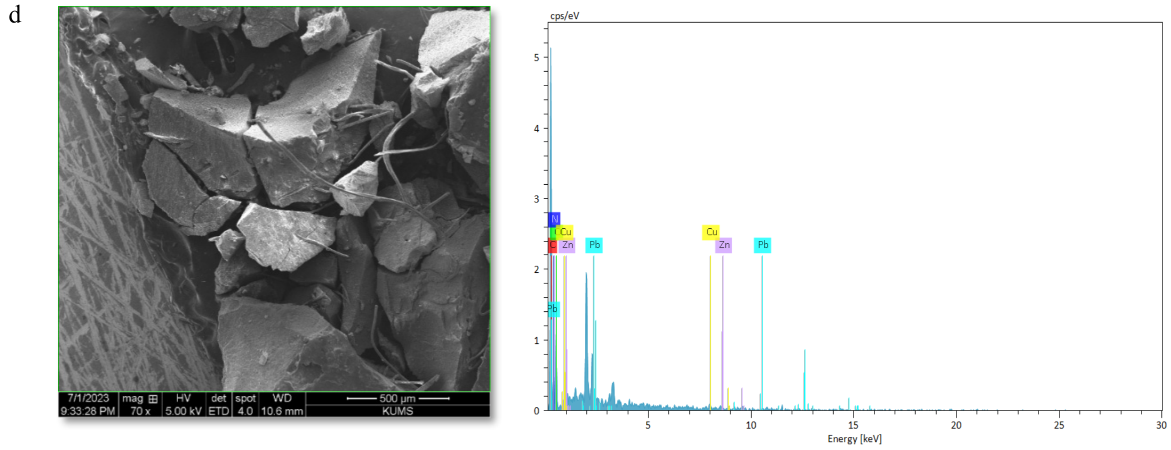

Fig. 5. SEM and EDS analysis results of metal carbonate precipitates formed by strain PO2 at a concentration of 1500 mg/L in the presence of (a) Cu, (b) Zn, (c) Pb, and (d) a mixture of these metals.

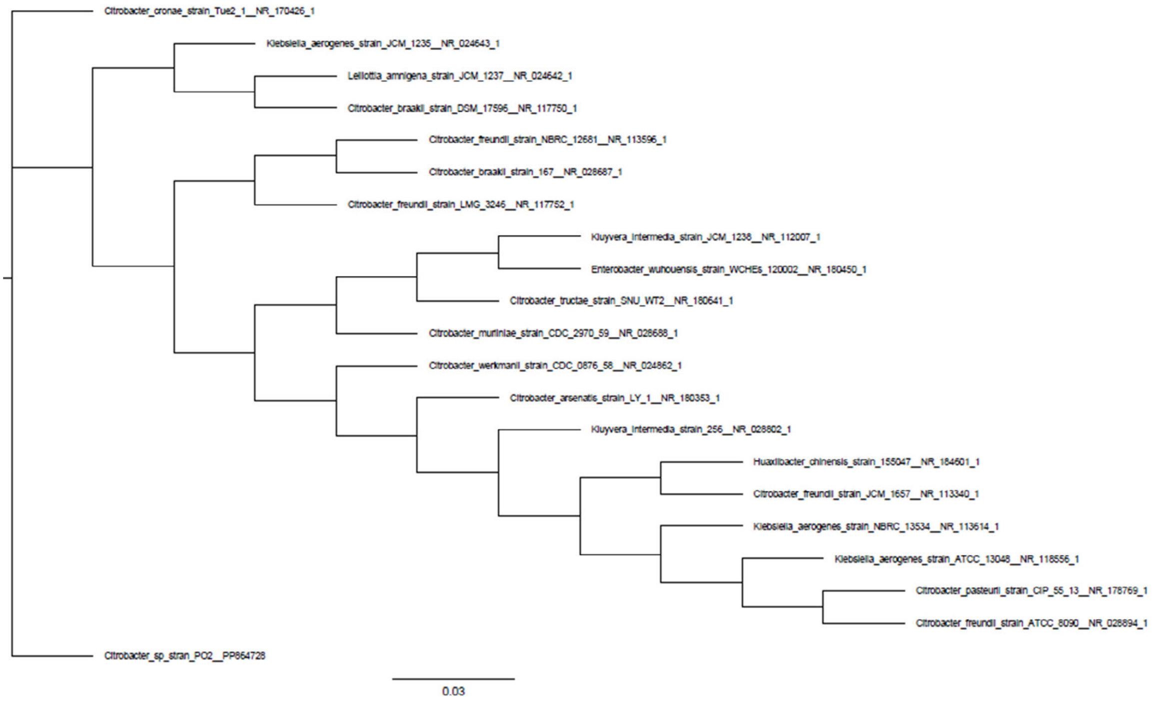

Fig. 6. The phylogenetic tree of the isolated bacteria *Citrobacter* sp. strain PO2

CONCLUSION

This study aimed to evaluate the efficacy of a MICP-capable strain, isolated from petroleum-contaminated soil, in removing a mixture of highly toxic heavy metals (Ni, Cu, Pb, Zn, and Cd). The findings confirmed that effective simultaneous removal of multiple metals requires a urease-producing bacterium with multi-metal resistance. The strain isolated in this study exhibited excellent performance in stabilizing the heavy metal mixture, achieving up to 98% removal within a few hours, even at the highest tested concentration of 1500 mg/L. However, the efficiency of the MICP process may be affected by various factors, including metal toxicity, urease activity, type of precipitate formed, temperature, and pH. The microbial mineralization mechanism involves carbonate precipitation driven by a pH increase resulting from urea hydrolysis catalyzed by bacterial urease. This rise in pH creates supersaturated conditions favorable for the co-precipitation of multiple metals. Overall, the results indicate that MICP offers strong potential for heavy metal stabilization and may serve as an effective approach for remediating metal-contaminated soils and wastewater.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

REFRENCES

Abbas, S. H., Ismail, I. M., Mostafa, T. M., & Sulaymon, A. H. (2014). Biosorption of heavy metals: a review. *J Chem Sci Technol*, *3*(4), 74-102.

Achal, V., Pan, X., & Zhang, D. (2011). Remediation of copper-contaminated soil by *Kocuria flava* CR1, based on microbially induced calcite precipitation. *Ecological Engineering*, 37(10), 1601–1605.

Alabssawy, A. N., & Hashem, A. H. (2024). Bioremediation of hazardous heavy metals by marine microorganisms: a recent review. *Archives of microbiology*, *206*(3), 103.

Ali, N., Dashti, N., Al-Mailem, D., Eliyas, M., & Radwan, S. (2012). Indigenous soil bacteria with the combined potential for hydrocarbon consumption and heavy metal resistance. *Environmental Science and Pollution Research*, *19*(3), 812-820.

Baudoin, E., Benizri, E., & Guckert, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil biology and biochemistry*, *35*(9), 1183-1192.

Bolouri Bazaz, J., Bolouri Bazaz, M. T., & Karrabi, S. M. (2021). Analyzing the use of molasses as an alternate and optimal culture medium in MICP process of sandy soils. *Amirkabir Journal of Civil Engineering*, 53(7), 18–18.

Choudhary, S., & Sar, P. (2011). Uranium biomineralization by a metal-resistant *Pseudomonas aeruginosa* strain isolated from contaminated mine waste. *Journal of Hazardous Materials*, 186(1), 336–343.

Cheng, L., Shahin, M. A., & Chu, J. (2019). Soil bio-cementation using a new one-phase low-pH injection method. *Acta Geotechnica*, 14(3), 615–626.

Castanier, S., Le Métayer-Levrel, G., & Perthuisot, J. P. (1999). Ca-carbonates precipitation and limestone genesis—the microbiogeologist point of view. *Sedimentary Geology*, 126(1-4), 9–23.

Dashti, A. A., Jadaon, M. M., Abdulsamad, A. M., & Dashti, H. M. (2009). Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. *Kuwait Med J*, *41*(2), 117-122.

De Muynck, W., De Belie, N., & Verstraete, W. (2010). Microbial carbonate precipitation in construction materials: a review. Ecological engineering, 36(2), 118-136.

Dhami, N. K., Reddy, M. S., & Mukherjee, A. (2013). Biomineralization of calcium carbonates and their engineered applications: a review. Frontiers in microbiology, 4, 314.

Dejong, J. T., Soga, K., Kavazanjian, E., Burns, S., Van Paassen, L. A., Al Qabany, A., Aydilek, A., Bang, S. S., Burbank, M., Caslake, L. F., & Chen, C. Y. (2014). Biogeochemical processes and geotechnical applications: progress, opportunities and challenges. In *(pp. 143–157)*.

Fakhar, A., Gul, B., Gurmani, A. R., Khan, S. M., Ali, S., Sultan, T., ... & Rizwan, M. (2022). Heavy metal remediation and resistance mechanism of Aeromonas, Bacillus, and Pseudomonas: A review. *Critical Reviews in Environmental Science and Technology*, *52*(11), 1868-1914.

Fei, Y., & Hu, Y. H. (2023). Recent progress in removal of heavy metals from wastewater: A comprehensive review. *Chemosphere*, *335*, 139077.

Fopase, R., Nayak, S., Mohanta, M., Kale, P., & Paramasivan, B. (2019). Inhibition assays of free and immobilized urease for detecting hexavalent chromium in water samples. *3 Biotech*, 9, 1–2.

Gadd, G. M., & White, C. (1993). Microbial treatment of metal pollution—a working biotechnology? *Trends in Biotechnology*, 11(8), 353–359.

Garrity, G. M. (2005). *Bergey's Manual of Systematic Bacteriology: Volume Two: The Proteobacteria (Part C)*. Brenner, D. J., Krieg, N. R., & Staley, J. T. (Eds.). Springer US.

Gillieatt, B. F., & Coleman, N. V. (2024). Unravelling the mechanisms of antibiotic and heavy metal resistance co-selection in environmental bacteria. *FEMS Microbiology Reviews*, *48*(4), fuae017.

Gomaa, E. Z. (2018). Biosequestration of heavy metals by microbially induced calcite precipitation of ureolytic bacteria. *Romanian Biotechnological Letters*, 24(1), 147–153.

Haynes, W. M. (2016). CRC Handbook of Chemistry and Physics (97th ed., Internet version). CRC Press.

Hammes, F., & Verstraete, W. (2002). Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Reviews in Environmental Science and Biotechnology*, 1, 3–7.

Hao, X., Zhu, J., Rensing, C., Liu, Y., Gao, S., Chen, W., ... & Liu, Y. R. (2021). Recent advances in exploring the heavy metal (loid) resistant microbiome. *Computational and structural biotechnology journal*, *19*, 94-109.

Hoffman, M. T., & Arnold, A. E. (2010). Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. *Applied and Environmental Microbiology*, 76(12), 4063–4075.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17(8), 754–755.

Jalilvand, N., Akhgar, A., Alikhani, H. A., Rahmani, H. A., & Rejali, F. (2020). Removal of heavy metals zinc, lead, and cadmium by biomineralization of urease-producing bacteria isolated from Iranian mine calcareous soils. *Journal of Soil Science and Plant Nutrition*, 20, 206–219.

Kang, C. H., Oh, S. J., Shin, Y., Han, S. H., Nam, I. H., & So, J. S. (2015). Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea. *Ecological Engineering*, 74, 402–407.

Kang, C. H., Shin, Y., Anbu, P., Nam, I. H., & So, J. S. (2016). Biosequestration of copper by bacteria isolated from an abandoned mine by using microbially induced calcite precipitation. *The Journal of General and Applied Microbiology*, 62(4), 206–212.

Khatun, M., Kobir, M. M., Miah, M. A. R., Sarkar, A. K., & Alam, M. A. (2024). Technologies for remediation of heavy metals in environment and ecosystem: A critical overview of comparison study. *Asian Journal of Environment & Ecology*, 23(4), 61-80.

Kondakindi, V. R., Pabbati, R., Erukulla, P., Maddela, N. R., & Prasad, R. (2024). Bioremediation of heavy metals-contaminated sites by microbial extracellular polymeric substances–A critical view. *Environmental Chemistry and Ecotoxicology*, *6*, 408-421.

Kumari, D., Pan, X., Lee, D. J., & Achal, V. (2014). Immobilization of cadmium in soil by microbially induced carbonate precipitation with *Exiguobacterium undae* at low temperature. *International Biodeterioration & Biodegradation*, 94, 98–102.

Kurmaç, Y. (2009). The impact of toxicity of metals on the activity of ureolytic mixed culture during the precipitation of calcium. *Journal of Hazardous Materials*, 163(2-3), 1063–1067.

Li, M., Cheng, X., & Guo, H. (2013). Heavy metal removal by biomineralization of urease producing bacteria isolated from soil. *International Biodeterioration & Biodegradation*, 76, 81–85.

Lin, H., Zhou, M., Li, B., & Dong, Y. (2023). Mechanisms, application advances and future perspectives of microbial-induced heavy metal precipitation: A review. *International Biodeterioration & Biodegradation*, *178*, 105544.

Lin, J. Y., Garcia, E. A., Ballesteros, F. C., Garcia-Segura, S., & Lu, M. C. (2022). A review on chemical precipitation in carbon capture, utilization and storage. *Sustainable Environment Research*, *32*(1), 1-20.

Majzlik, P., Strasky, A., Adam, V., Nemec, M., Trnkova, L., Zehnalek, J., Hubalek, J., Provaznik, I., & Kizek, R. (2011). Influence of zinc (II) and copper (II) ions on *Streptomyces* bacteria revealed by electrochemistry. *International Journal of Electrochemical Science*, 6(6), 2171–2191.

Mao, Q., Xie, X., Pinzon-Nuñez, D. A., Xie, Z., Liu, T., & Irshad, S. (2023). Native microalgae and Bacillus XZM remediate arsenic-contaminated soil by forming biological soil crusts. *Journal of Environmental Management*, *345*, 118858.

Mitra, S., Chakraborty, A. J., Tareq, A. M., Emran, T. B., Nainu, F., Khusro, A., Idris, A. M., Khandaker, M. U., Osman, H., Alhumaydhi, F. A., & Simal-Gandara, J. (2022). Impact of heavy metals on the environment and human health: Novel therapeutic insights to counter the toxicity. *Journal of King Saud University-Science*, 34(3), 101865.

Mortensen, B. M., Haber, M. J., DeJong, J. T., Caslake, L. F., & Nelson, D. C. (2011). Effects of environmental factors on microbial induced calcium carbonate precipitation. *Journal of Applied Microbiology*, 111(2), 338–349.

Mugwar, A. J., & Harbottle, M. J. (2016). Toxicity effects on metal sequestration by microbially-induced carbonate precipitation. *Journal of Hazardous Materials*, 314, 237–248.

Nazar, B. L., & Schoolwerth, A. C. (1979). An improved microfluorometric enzymatic assay for the determination of ammonia. *Analytical Biochemistry*, 95(2), 507–511.

Perni, S., Andrew, P. W., & Shama, G. (2005). Estimating the maximum growth rate from microbial growth curves: definition is everything. *Food Microbiology*, 22(6), 491–495.

Pfaller, M. A., Andes, D., Diekema, D. J., Espinel-Ingroff, A., Sheehan, D., CLSI Subcommittee for Antifungal Susceptibility Testing. (2010). Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: time for harmonization of CLSI and EUCAST broth microdilution methods. *Drug Resist. Updat.*, 13(6), 180–195.

Qiao, S., Zeng, G., Wang, X., Dai, C., Sheng, M., Chen, Q., Xu, F., & Xu, H. (2021). Multiple heavy metals immobilization based on microbially induced carbonate precipitation by ureolytic bacteria and the precipitation patterns exploration. *Chemosphere*, 274, 129661.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19(12), 1572–1574.

Saha, L., Tiwari, J., Bauddh, K., & Ma, Y. (2021). Recent developments in microbe–plant-based bioremediation for tackling heavy metal-polluted soils. *Frontiers in Microbiology*, *12*, 731723.

Seifan, M., & Berenjian, A. (2019). Microbially induced calcium carbonate precipitation: a widespread phenomenon in the biological world. *Applied Microbiology and Biotechnology*, 103, 4693–4708.

Tanca, A., Manghina, V., Fraumene, C., Palomba, A., Abbondio, M., Deligios, M.,Silverman, M. & Uzzau, S. (2017). Metaproteogenomics reveals taxonomic and functional changes between cecal and fecal microbiota in mouse. *Frontiers in Microbiology*, *8*, 391.

Thairu, Y., Nasir, I. A., & Usman, Y. (2014). Laboratory perspective of gram staining and its significance in investigations of infectious diseases. *Sub-Saharan African Journal of Medicine*, 1(4), 168.

Torgal, F. P., Labrincha, J. A., Diamanti, M. V., Yu, C. P., & Lee, H. K. (2015). Biotechnologist and biomimetics for civil engineering. Springer International, pp. 21–56.

Velusamy, P., Awad, Y. M., Abd El-Azeem, S. A. M., & Ok, Y. S. (2011). Screening of heavy metal resistant bacteria isolated from hydrocarbon contaminated soil in Korea. *J Agric Life Environ Sci*, *23*(1), 40-43.

Wang, Z., Zhang, N., Cai, G., Jin, Y., Ding, N., & Shen, D. (2017). Review of ground improvement using microbial induced carbonate precipitation (MICP). *Marine Georesources & Geotechnology*, 35(8), 1135–1146.

Zhang, Y., Hu, X., Wang, Y., & Jiang, N. (2023). A critical review of biomineralization in environmental geotechnics: Applications, trends, and perspectives. *Biogeotechnics*, 100003.

Zhao, Y., Yao, J., Yuan, Z., Wang, T., Zhang, Y., & Wang, F. (2017). Bioremediation of Cd by strain GZ-22 isolated from mine soil based on biosorption and microbially induced carbonate precipitation. *Environmental Science and Pollution Research*, 24, 372–380.