

Study On The Performance Of Carbonate-Mineralized Bacteria Combined With Eggshell For Immobilizing Pb and Cd in Water And Soil

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Abstract

Microbially induced carbonate precipitation (MICP) is an advanced bioremediation approach to remediate heavy metals (HMs) contaminated water and soil. In this study, metal tolerant urease-producing bacterial isolates, namely UR1, UR16, UR20 and UR21, were selected based on their urease activity. The efficiency of these isolates in water for Pb and Cd immobilization was explored. Our results revealed that UR21 had the highest removal rates of Pb (81.9%) and Cd (65.0%) in solution within 72 h through MICP. The scanning electron microscopy-energy dispersive x-ray and x-ray diffraction analysis confirmed the structure and the existence of PbCO_3 and CdCO_3 crystals in the precipitates. In addition, the strain UR21, in combination with urea/eggshell waste (EGS) or both, was further employed to investigate the effect of MICP on soil enzymatic activity, chemical fractions and bioavailability of Pb and Cd. The outcomes indicated that the applied treatments reduced the proportion of soluble-exchangeable Pb and Cd, resulted an increment in carbonated bound Pb and Cd in the soil. The DTPA extractable Pb and Cd was reduced by 29.2% and 25.2% with the treatment of UR21 + urea + EGS as compared to the control. Besides, the application of UR21 and EGS significantly increased the soil pH, cation exchange capacity, and enzyme activities. Our findings may provide a novel perceptible for an eco-friendly and sustainable approach to remediate heavy metal contaminated environment through a combination of metal-resistant ureolytic bacterial strain and EGS.

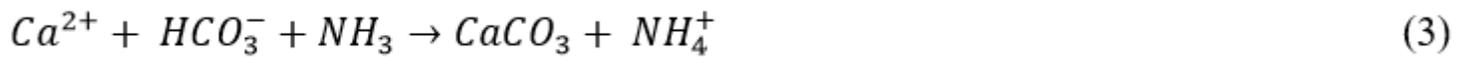
Introduction

Contamination of natural surroundings by heavy metals (HMs) through industrialization, smelting, mining activities, excessive use of fertilizers and pesticides in farming areas and combustion of hazardous waste materials has become a widespread environmental problem (Guo et al. 2020; Hua et al. 2021; Wei et al. 2021). Cadmium (Cd) and lead (Pb) has gained special attention due to their high perseverance and toxicity (Hamid et al. 2019). Once introduced into the surroundings, these HMs could permanently exist and accumulate in the environment, which could endanger human and other organisms health by way of the food chain (Azeez et al. 2019; Wei et al. 2021). Therefore, there is an immediate need for an appropriate way to remediate HMs polluted water and soil.

The adsorption, chemical precipitation, membrane separation, and biological methods have been purposed for the remediation of HMs contaminated water and soil (Muhammad et al. 2021; Zhai et al. 2018). Among these suggested approaches, biological methods that incorporate the use of biological microorganisms called microbial remediation is well-accepted due to their low economical costs, environment-friendly and the reduced amount of remaining contaminants (Fang et al. 2018; Hamid et al. 2019).

Microbially induced carbonate precipitation (MICP), one of the innovative bioremediation strategies mediated by living organisms has gained wide attention (Bhattacharya et al. 2019; Mwandira et al. 2017; Zhu et al. 2017). Many MICP methods have been emerged, involving cyanobacteria exopolysaccharides (Kawaguchi and Decho 2002), urea hydrolysis (Stocks-fischer et al. 1999) and induction of carbonic

anhydrase (Zhuang et al. 2018). The most effective microbial mechanism for achieving MICP is the hydrolysis of urea catalyzed by the enzyme urease found in urease-producing bacteria (Qian et al. 2017; Wong 2015). The basic principle associated with MICP based on the hydrolysis of urea by urease enzyme into ammonia and carbamate (Eq. (1)), which further results the formation of CaCO_3 in the presence of Ca^{2+} ions (Eq. (2)-(3)). Heavy metal ions similar to Ca^{2+} , including Pb^{2+} , Cd^{2+} , and Cu^{2+} , can be introduced into calcite crystals by replacing Ca^{2+} in the lattice or accessing the interstice or crystal defect during calcite precipitation (Achal et al. 2011). This contributes in the mineralization of metal ions into stable mineral forms (Eq. (4)), thereby reducing the mobility and toxicity of metals (Bhattacharya et al. 2018; Li et al. 2013). The wide application of MICP and the ability of its product to trap heavy metals could contribute to a new in situ remediation approach for contaminated soil.



Recently, using MICP strategy for remediation of heavy metals has drawn increasing attention, a number of urease-producing bacteria have been reported to remove HMs, such as the strains of *Bacillus cupriavidus*, *pararhodobacter*, and *Pseudomonas* (Kang et al. 2014; Kang et al. 2016). Annu et al. (2016) reported that 65.0–82.0% of Cd is removed by *Saccharomyces cerevisiae*. Kang et al. (2014) showed that excellent removal efficiency of lead (68.1%) was recorded by the strain *Enterobacter cloacae*. In addition, the MICP process may also strengthen the biochemical standards of sandy soils (Gao et al. 2019; Sun et al. 2019). Environmental factors such as temperature and pH, affects the urease activity and heavy metal restriction capacity of ureolytic bacteria (Bhattacharya et al. 2018; Zhao et al. 2017). For example, earlier reports have shown that various bacterial or fungal strains secreting urease can only respond to acidic or alkaline conditions, but few can hardly resist the acidic or alkaline conditions, thus creating several barriers for the application. Therefore it is important to find the microorganism that can survive and maintain their urease activity under a wide range of environmental conditions.

Urea is the main component utilize by ureolytic bacteria to produce carbonate precipitate through the MICP process to immobilize HMs. However, several findings have shown that the application of excessive urea to HMs-polluted soil can cause soil acidification, salinization, and microbial biomass reduction (Geisseler and Scow 2014; Tian and Niu 2015). This restricts the use of MICP to remediate HMs polluted soil. Therefore, it is essential to find a material comparable to urea and extend the implementation value of the MICP. The eggshell wastes (EGS), rich in calcium sources including calcium carbonate (94%), magnesium carbonate (1%), calcium phosphate (1%) and organic matter (4%) (Tsai et al. 2006). Some

reports have suggested that EGS can immobilize HMs in soil (Ashrafi et al. 2015; Lee et al. 2013). The possible underlying mechanisms involved in the absorption of metal ions by EGS are as follows: (1) the porous composition of EGS enables it an effective natural bio-adsorbent and (2) the electrostatic interaction between the species of negative charge (e.g., carbonate ion) and the positive metal ion charge (Sankaran et al. 2020; Shaheen et al. 2013). Moreover, the addition of EGS could effectively improves the soil fertility (Luo et al. 2018). Concerning the requirement of calcium for the MICP process and the eggshell waste is rich in other nutrients. So, we hypothesized that the mixture of urease-producing bacteria and EGS might have better immobilization rate of Pb and Cd in the soil as compared to a single treatment, which is relatable with the combined effect of urease-producing bacteria and urea. In addition, no related research has been recorded so far to remediate Pb and Cd polluted soil by employing the combination of urease-producing bacteria and EGS. Therefore, the aims of the current study were to: (1) isolate and identify Pb and Cd tolerant bacteria that secret urease; (2) estimate the capability of bacteria for Pb and Cd removal in water through MICP and to confirm the mechanism involved through scanning electron microscopy-energy dispersive x-ray spectrometry (SEM-EDS) and x-Ray diffraction; (3) compare the Pb and Cd immobilization effect of urease-producing bacteria supplemented with urea and EGS in soil; (4) assess the influence of urease-producing bacteria supplemented with urea and EGS on soil physicochemical properties.

Materials And Methods

Soil sampling and eggshell waste preparation

The soil used in this experiment was collected from uncontaminated farmland in suburbs of Xi'an, Shaanxi province. The basic properties of the soil were illustrated in Table 1. The soil samples were air-dried and sieved to eliminate the impurities such as stones and organic residual. Finally, the soil was spiked with $\text{Pb}(\text{NO}_3)_2$ and $\text{Cd}(\text{NO}_3)_2$ solutions, the final Pb and Cd content in the soil was about 106 mg kg^{-1} and 10 mg kg^{-1} , respectively. The eggshells were collected from a bakery, washed to eliminate impurities using deionized water, oven-dried for 4h at 70°C and then crushed into powder form with the help of a blender.

Table 1 Physico-chemical characteristics of soil before experiment

Soil Characteristics	Values
pH	7.8
Cation exchange capacity (CEC)	17.2 cmol kg ⁻¹
Electrical conductivity (EC)	375 μ s cm ⁻¹
Total nitrogen (N)	0.07%
Total available phosphorous (AP)	16.1 ppm
Total available potassium (AK)	172 ppm

Isolation of the ureolytic bacteria

For the isolation of urease-producing metal resistant bacterial strains, the soil was collected from a Pb mine tailing located in Huayin, Shaanxi province, China (N34°34'1.46"110°05'11.81"). 1 g of soil was taken from the sample, serially diluted and the soil suspensions were spread on the BPU agar plate (4 g L⁻¹ beef extract powder, 4 g L⁻¹ peptone, 20 g L⁻¹ urea, 18 g L⁻¹ agar powder, pH 7) for primary isolation. The plates were placed in the incubator under 30°C for 18 h. For further screening, the isolates with different morphology were transferred on YA plates (yeast extract powder 18 g L⁻¹, ammonium sulfate 12 g L⁻¹, agar powder 18 g L⁻¹, pH 7) in the presence of PbCl₂ and CdCl₂ with increasing concentrations from 100–500 mg L⁻¹ and were incubated at 28°C for 24 h. The isolates were inoculated repeatedly on the plate in order to get pure culture. Subsequently, single colonies were picked and stored in a liquid containing the mixture of 25% (v/v) glycerol and YA broth at -80°C.

Determination of biological characteristics of the isolated strains

The minimum inhibitory concentration (MIC) of Pb and Cd, which inhibits bacterial growth was accomplished by following the protocol of Nokman et al. (2019). The urease activity of the isolated strains were measured as described by Zhao et al. (2019). Furthermore, the ability of the isolates to form calcite was determined by following the procedure of Kang et al. (2015). The molecular identification of these bacterial strains were performed with the procedure of Zhao et al. (2019).

Influence of environmental factors on the urease activity of bacterial strains

To evaluate the influence of environmental conditions such as (temperature and initially provided pH) on the urease activity, the strains were analyzed by taking one factor under consideration at a time. Firstly, the 12 h grown culture was transferred (2% v/v) into separate autoclaved 250 mL flasks (carrying 100 mL YA broth) and then placed in an incubator under the series of selected physical conditions with the continues aeration at 150 rpm. The ureolytic bacteria were incubated for 36 h at 150 rpm under the temperature between 24 to 36°C with a difference of 4°C to evaluate the impact of varying temperature on the urease activity of the isolates under the neutral pH. Similarly, the isolates were further tested under the pH within the range of 6.5 to 8 with the difference of 0.5 to determine the influence of different pH on

the urease activity of isolates. At the end of the experiment, the urease activity of the bacterial strains were determined.

Removal efficiency of the bacterial strains for Pb and Cd in solution

5 mL of fresh bacterial culture was transferred to 45 mL of YA media supplemented with 0.7 M urea and 2 mM of Pb from PbCl_2 , 2 mM of Cd from CdCl_2 separately. The samples were then shaken in an incubator (180 rpm) at 30°C for 72 h. The control (without bacteria) was also set for comparison purpose and placed in the incubator. The cultured samples were placed in a centrifugation chamber at 6000 rpm for 12 min. The amount of Pb and Cd in the supernatant was measured by using an atomic absorption spectrometer (ZEEnit-700P-analytikjena). The removal efficiency of Pb and Cd was determined by analyzing the variation between the control supernatant and bacteria inoculated supernatant. The precipitates obtained at the end of the experiment were gathered and further characterized by scanning electron microscope (SEM, JSM-7500F), energy dispersive spectrometer (EDS, EMPYREAN) and X-ray diffraction (XRD).

SEM-EDS and XRD analyses of precipitates

The resulting precipitates were dried for 12 h in a lyophilizer and after completely drying, gold plating is applied to the samples. The structural morphology of the precipitate was examined by scanning electron microscopy (SEM) at a stable voltage of 20 kV and an EDS spectrometer was performed for a detailed elemental composition of the precipitated crystal components. The analysis of X-Ray Diffraction (XRD) was used to further investigate the structure of calcite as well as metal precipitates.

Soil incubation experiment

Based on the high removal efficiency for Pb and Cd, the strain UR21 was further employed for the soil incubation experiment. Five treatment were established including CK (control group), S1 (UR21), S2 (UR21 + urea), S3 (UR21 + EGS) and S4 (UR21 + urea + EGS). The 300 g of Pb and Cd contaminated soil were taken in 500 mL glass beakers, thoroughly mixed with 25 mL of ($\text{OD}_{600} = 1.0$) bacterial suspension, 2% of EGS and 1% of urea in above-mentioned treated groups by accounting the earlier reports and demand for microbial induced carbonate precipitation (Ashrafi et al. 2015; Chen and Achal 2019). A room temperature ($30 \pm 5^\circ\text{C}$) was maintained during the 40 days experiment. All treatments and control were performed in triplicate and soil samples were collected after 10, 20, 30, 40 days of incubation.

In all samples, the available Cd was collected by shaking soil samples (5 g) in a shaker for 2 hours with 20 mL of DTPA-TEA solution (Lu et al. 2017). The solution was filtered using a 0.45- μm membrane filter, and the filtrate was further evaluated by employing the flame atomic absorption spectrophotometer (ZEEnit-700P-analytikjena). A pH meter (PB-10 Sartorius, Germany) was used to calculate the pH and CEC was measured as described by Jien and Wang (2013).

Soil Pb and Cd fractions

After the soil incubation experiment, the contaminated soil in the beakers was extracted, mixed, air-dried, and pushed through 100-mesh sieves. To assess the fraction of Pb and Cd in contaminated soil, the Tessier sequential extraction process was utilized (Tessier et al. 1979) and briefly mentioned elsewhere (Hamid et al. 2019). Five different fractions were defined by this method included: soluble-exchangeable (EXC), carbonate-bound (CAR), Fe-Mn oxides bound (OX), organic-matter bound (ORG), and residual fractions (RES).

Soil microecology

After 40 days of the incubation, the soil samples were obtained and the impact of MICP on soil enzymatic activity was determined. Soil enzymatic activity including urease and catalase was measured as described by Yu et al. (2019). The acquired enzymatic activities (urease and catalase) of the collected sample were presented as $\text{NH}_4^+\text{-N g}^{-1} 24 \text{ h}^{-1}$ and as $\text{mL (0.1 mol L}^{-1} \text{ KMnO}_4) \text{ h}^{-1} \text{ g}^{-1}$, respectively.

Statistical analysis

All experiments were conducted in triplicates, the difference between treatments were calculated using one way ANOVA compared using post-hoc test ($P < 0.05$) by SPSS 21.0. The standard errors was estimated by employing MS-Excel, version 2013.

Results And Discussion

Isolation and identification of the bacterial isolates

A total of 27 unique colony types were isolated from the soil. Four strains (UR1, UR16, UR20 and UR21) were selected based on their urease activity. The 16S rRNA gene was sequenced and compared with the known sequence in the NCBI database. The results revealed that the isolates UR1, UR16, and UR21 are closely related to one another and correspond to the genus of *Bacillus sp.* and the isolate UR20 belongs to the genus *Citrobacter sp.* (Fig. S1). The obtained sequences of UR1, UR16, UR20 and UR21 were submitted to GenBank under the accession No. MT022031, MT022032, MT022034 and MT022035, respectively.

Biological characteristics of the bacterial strains

The toxicity of heavy metals is a serious aspect taken into account for the remediation process because the performance and existence of the bacteria in a contaminated environment predominantly depend on it (Nokman et al. 2019). Among the selected bacterial isolates, UR20 exhibited a high degree of resistance to Pb and the MIC value was 1400 mg L^{-1} , followed by the isolate UR21 showed the MIC value of 1300 mg L^{-1} . Whereas, the MIC value for the Cd was 500 mg L^{-1} and 400 mg L^{-1} for UR20 and UR21, respectively (Table 2). The increasing amount of heavy metal has a toxic effect on the growth of microorganisms. The ability of isolates to resist the different heavy metals provides an opportunity to use them in multiple metal-contaminated sites for bioremediation (Satyapal et al. 2018).

Table 2 Minimum inhibitory concentrations of heavy metals

Isolates	MIC results	
	Lead conc. (mg L ⁻¹)	Cadmium conc. (mg L ⁻¹)
UR 1	1100	500
UR 16	1200	400
UR 20	1400	500
UR 21	1300	400

MIC is defined as the minimum concentration of heavy metal that inhibits the bacterial growth.

The calcite production capability of urease-producing microorganism leads to the precipitation of soluble metal ions and finally convert them to carbonates (Gomaa et al. 2018). The highest calcite production was noticed in UR21 (20.2 mg mL⁻¹) followed by UR1 (20.2 mg mL⁻¹), UR20 (17.1 mg mL⁻¹) and UR16 (19.6 mg mL⁻¹) as shown in Fig. 1. The metabolic product (CO₃²⁻) was released by the microorganisms that react with (Ca²⁺) ion present in the medium and contribute to the precipitation of minerals (Dhami et al. 2013). The carbonate concentration was increased when calcium (Ca²⁺) and carbonate (CO₃²⁻) ions are prominent (Qian et al. 2010). Urease influences the chemical process associated with the formation of biominerals through four distinct parameters, including calcium concentration, cell surface, pH, and accessibility of nucleation sites (Anbu et al. 2016). The first two factors are responsible for the concentration of CO₃²⁻ and the nucleation site helps in the production of calcium carbonate (Taylor et al. 2013). The cell surface of the bacteria is covered with negatively charged groups that function as a binding site for the divalent cations (e.g. Ca²⁺, Mg²⁺) and these nucleation sites lead to the deposition of calcite (Anbu et al. 2016). This research showed that the isolates were able to produce a high amount of calcium carbonates under suitable conditions due to their urease activity.

Urease plays a vital role in the formation of carbonate crystals in a media supplemented with calcium and urea (De Muynck et al. 2010). The urease activity of the four isolates was shown in (Fig. 1). The highest urease activity was observed by the isolate UR20 of 58.1 U mL⁻¹ followed by the isolates UR21, UR16 and UR1, with the urease activity of 55.2 U mL⁻¹, 39.9 U mL⁻¹ and 27.2 U mL⁻¹, respectively. It is commonly believed that the formation of calcium carbonate depends on enzyme activity, a high rate of urease activity accelerates the production of calcium carbonate (Al-salloum et al. 2016). The ability of bacterial strains to hydrolyze urea is affected by many factors including temperature, pH, incubation period and concentration of urea, and by providing a suitable condition to a specific strain the enzymatic activity of that strain can be enhanced (Sheng et al. 2020).

Urease activity of the bacterial isolates under different environmental conditions

The isolates grown under different temperatures have shown different urease activity. The maximum ability to hydrolyzed urea was observed when the bacterial isolates was incubated at temperatures 28°C and 32°C (Fig. 2(a)). The ability of the bacterial strains to hydrolyzed urea was comparatively low when incubated at the temperature of 24°C. These results indicated that the enzymatic activity of the bacterial strains could be influenced by the temperature. The appropriate temperature to get maximum enzyme activity varies from 24 to 37°C (Soon et al. 2014). Imran et al. (2019) demonstrated that the enzyme activity of the isolate rises 5.0 to 10 times when the temperature raised from 10 to 20°C. Consequently, based on these experimental results, our study indicated that temperature was one of the crucial factors that influence the urease activity because the amount of urea hydrolyzed varies with temperature.

The results obtained after growing isolates under different pH revealed that the bacterial strains exhibit the highest ability to hydrolyzed urea at the pH of 7.5 and 8.0. The urease activity of the bacterial isolate is relatively low at the pH of 6.5 as shown in Fig. 2(b). The heterotrophic facultative and aerobic bacteria which are widely used in MICP known to grow well under weak alkaline conditions (Sheng et al. 2020). The investigation shows that the strain *Bacillus megaterium* and *B.cereus* can grow well at the pH range from 6.5 to 11.5. whereas, some strains like *B. subtilis* and *B. thuringiensis* can grow at the pH range from 6.0 to 10 (Kaur et al. 2013). The pH has not only affects the urease activity but also affects the metabolism of bacteria. Our investigations showed that the initial pH of the medium plays an important role to attain maximum ureases activity.

Removal of Pb and Cd in solution

The addition of bacterial isolates in YA broth containing Pb and Cd supplemented with urea showed high removal efficiency of heavy metals after 72 h of incubation. The highest removal rate for Pb reached to 81.9% and 77.1% by the isolate UR21 and UR20, respectively (Fig. 3(a)). Similarly, in the case of Cd, UR21 had the highest removal rate of 65.0% (Fig. 3(b)). It was observed that the removal rate of Pb and Cd become stable from 60 to 72 h. It is likely that the metal ion occupied all the available binding sites and the functional group provided by the bacteria, thus, there are no more available sites for the metal ion to bind (Zhao et al. 2019). Our studies and the previous research revealed that the deposition rate of Pb is higher as compared to Cd (Rahman et al. 2019). Heavy metals have different sizes of ionic radius and the variation in the ionic radius of these elements could be responsible for the diversity in the precipitation. The ionic radius of Pb is higher than Cd so it might be one of the reasons that the removal rate of Pb in the solution is higher (He et al. 2019). The remediation rate of heavy metals mainly depends on the number of bacterial cells. The increase in growth of the bacteria promoted the production of urease enzyme, which enhanced the removal rates of the heavy metals (Kang et al. 2015; Li et al. 2008). The Pb and Cd ions present in the solution attached with the available binding sites (such as carboxyl, amino, and phosphate) of the bacterial cell and then utilized the carbonates produced by the hydrolysis of urea to generate lead and cadmium carbonates, which convert these soluble metals into insoluble form and reduced their toxic level by lowering bioavailability (Zhao et al. 2017). Zhao et al. (2019) reported that free Cd ion present in soil incorporates with the carbonate ion produced by the ureolytic isolate to form CdCO_3

precipitates. The present study demonstrated that the strain UR21 has great potential in immobilization of Pb and Cd.

SEM-EDS and XRD analysis of the precipitates

The precipitates obtained after the remediation process was observed under SEM to understand the different morphological characteristics. The surface of Pb and Cd carbonate crystals had several bacterial imprints, which demonstrated that the bacteria surface act as a nucleation site for the precipitation of carbonates. As illustrated in the Fig. 4, the PbCO_3 crystals produced during bacterial ureolysis were needle-shaped which are also consistent with the findings of Kang et al. (2015). Whereas, the CdCO_3 crystals produced were roughly rhombohedral in shape (Fig. 4). The variation in the morphology of the crystals formed is based on the urease activity of the bacteria and the bacterial species (Park et al. 2010). The EDS analysis of the precipitates indicated the existence of lead (Pb), carbon (C) and oxygen (O) in lead carbonate crystals. Similarly, cadmium (Cd), carbon (C) and oxygen (O) were detected in cadmium carbonate crystals. The analysis by SEM and EDS evidently revealed that the isolates could efficiently transform the soluble Pb and Cd into insoluble PbCO_3 and CdCO_3 crystals and thus promoted the remediation of Pb and Cd.

XRD analysis also indicated that the carbonate precipitation was accelerated by the MICP process (Fig. 4). The XRD spectra showed an increment of calcium carbonate peaks in the form of vaterite and calcite. More importantly, XRD spectra verified that the strain UR 21 immobilized Pb and Cd specifically in the form of lead carbonate and cadmium carbonate, which has low toxicity as compared to Cd ions. In contrast to PbCO_3 , the peaks of CdCO_3 were slightly low. Similar results were also reported by other scholars (Chen and Achal 2019; Kang et al. 2016). These findings revealed that Pb^{2+} and Cd^{2+} with an ionic radius similar to Ca^{2+} was introduced into the CaCO_3 crystal by replacing Ca^{2+} in the lattice or accessing the interstices (Achal et al. 2011). Furthermore, Pb^{2+} and Cd^{2+} ions may be adsorbed to the surface of calcite through its lattice (Zhu et al. 2016).

Fractionation of Pb and Cd in soil

The mobility and toxicity of the Pb and Cd in the environment was estimated by their fractions. The results revealed that different treatments had a significant effect on the Pb and Cd fractions in soil. The prevalent fraction of Pb in the control was exchangeable-Pb (64.64%), followed by the carbonated-Pb fraction (16.7%), while the Fe-Mn oxide-Pb, organic matter-Pb and the residual-Pb was accounted for 10.6%, 4.12% and 3.91%, respectively. The exchangeable-Pb fraction in soil decreased to varying degrees after 40 days of incubation, revealing that the exchangeable fraction of Pb was transform into other fractions as illustrated in Fig. 5(a). As compared to control, the proportion of soluble-exchangeable Pb was declined to 19.5%-43.8% in all treated groups and the proportion of carbonated-Pb was enhanced to 18.5%-35.9%. Meanwhile, the proportion of Fe-Mn oxide-Pb was increased to 1.56%-6.69% in all treated groups except S4 group whereas, the proportion of organic-Pb and residual-Pb in soil was less than 6%. A similar pattern was found in the case of Cd, the dominant fraction in the control group was

exchangeable-Cd (70.68%), followed by carbonated-Cd (11.01%), while the Fe-Mn oxide-Cd, organic matter-Cd and the residual-Cd was comprises for 8.37%, 4.17% and 5.78%, respectively (Fig. 5(b)). The exchangeable-Cd was reduced to 20.22%-37.78% and the carbonated-Cd was increased to 17.61%-36.71% in other treatments. Whereas, as compared to control group, no significant difference in Fe-Mn oxide-Cd, organic matter-Cd and the residual-Cd were observed, which are in accordance with the findings of Ren et al. (2020).

It's worth mentioning that both the exchangeable Pb and Cd content dramatically decreased with the addition of UR 21, especially with the treatment of UR21 + urea + EGS in the S4 group, revealing the pivotal role of UR21 in the MICP pathway. Based on the fractional variations in Pb and Cd exchangeable and carbonated forms, it could be concluded that during the MICP process, converting the soluble-exchangeable to carbonated bound Pb/Cd was the key strategy for reducing HMs toxicity and mobility. This trend of fraction variation was also observed in earlier reports (Chen and Achal 2019; Zhu et al. 2016). The minimal association between UR21 and Fe-Mn oxide proportion of Pb and Cd could be due to the reduced bioavailability (Krishnamoorthy et al. 2006). Moreover, the possible reason behind the unchanged fraction of residual-bound Pb and Cd is that it is tightly bound and very stable under natural conditions (Zhao et al. 2019).

DTPA available Pb and Cd

The content of DTPA available Pb and Cd in the treated soil was declined as the incubation time increased. According to Figure (6a,b), compared with the control, the S4 treatment reduced the DTPA-extractable Pb and Cd contents in the soil by 29.2% and 25.2% after 40 days of incubation. At the same time, the S3 group also exhibited a prominent reduction in DTPA-extractable Pb (by 27.1%) and Cd (by 20.6%). The results suggested that the immobilization ability of available Pb is slightly higher than the available Cd. Meanwhile, it can be seen in Fig. 6a,b that the immobilizing efficiency of strain UR21 combine with EGS was relatively high as compared to strain UR21 combined with Urea. The following reason may be contributed to achieving those outcomes. Firstly, the EGS contain many substances involving amino acid that had functional group similar to urea, which could be used by urease (Nagamalli et al. 2017). Secondly, the enrichment of calcium carbonate source provided for the ureolytic bacteria by the addition of EGS to the soil, triggered the MICP process (Peng et al. 2020). Therefore the outcome revealed that the EGS can be combined with urease-producing bacteria as an alternate of urea, to achieve high immobilization efficiency in the soil.

Effect of MICP on soil pH and CEC

The pH and CEC values of the soil were assessed to analyze the effect of MICP on the physicochemical properties of soil. The soil pH gradually increased during the period of 40 days incubation (Fig. 7(a)), The soil pH in S4 group increased to 0.41 units followed by S3, S2 and S1 group (0.36, 0.07 and 0.06 units) respectively. However, as compared to control group, the CEC value of the soil was 35.41 cmol kg⁻¹ in S3 group followed by S4 group which was 33.91 cmol kg⁻¹ after 40 days of incubation (Fig. 7(b)). Our results indicated that as compared to the UR21 alone treatment, the combination of UR21 with urea and

EGS significantly enhanced the soil pH. The rise in pH is attributed largely to the presence of eggshells, most of which is composed of CaCO_3 . The possible reason could be the neutralization reaction between carbonate and H^+ in an acidic environment in the presence of eggshells (Luo et al. 2018). In addition, the elevation of pH could be due to the generation of NH_4^+ , formed by the decomposition of urea during the MICP process (Zhu et al. 2016). CEC is an indicator of nutrient abundance in the soil. The result suggested that the application of EGS improves the soil CEC value, which is consistent with the findings of Yong et al. (2010). The increase in the CEC in the presence of EGS could be due to the enrichment of Ca^{2+} which is consistent with the findings of Peng et al. (2020). The elevation of CEC in soil plays a vital role in the stabilization of heavy metals, the ions of the heavy metal are exchanged with Mg^{2+} , Na^+ , and other cations thus get precipitated in the soil (Bolanle-ojo et al. 2014).

Effect of MICP on soil enzymatic activity

Heavy metal pollution may have a detrimental effect on the biological activities of soil microbes (Pan and Yu 2011). The urease and catalase activity of soil are sensitive biomarker of heavy metals. Hence, they are employed to determine the impact of soil amendments on biological functions of soil (He et al. 2019; Shi and Ma 2017). The effects of MICP on enzymatic activity in the contaminated soil are diverse, and possibly correlated with the biological activities of the bacteria and soil environmental conditions (Peng et al. 2020). The change in soil urease and catalase activity after 40 days of incubation was illustrated in Fig. 8. The results indicated that the soil urease activity was promoted most in S2 group, increased by 120% followed by S4 treatment with an increment of 82%, respectively as compared to control. The possible reason could be the addition of urea may contribute to enhance the urease activity in the S2 and S4 group. Besides, the S4 treatment has the maximum carbonated bound Pb and Cd along with the urease activity, but the least exchangeable Pb and Cd fraction (Fig. 5). Which also indicated that Cd passivation was strongly influenced by soil UR21, which generate urease to break down urea. As a result of this, Cd precipitates as carbonate. Earlier report also showed that the urease, a main enzyme involved in the MICP pathway (Chen and Achal 2019), not only regulates the heavy metal immobilization in soil, but it is also closely linked to the geochemical mechanism of the N-cycle (Wang et al. 2017).

In contrast to urease activity, the results suggested that the soil catalase activity was significantly high in S4 group, increased by 71% followed by S2 treatment with an increment of 50%, respectively as compared to control. Although catalase does not play an important role in the MICP process, but it serves as an essential indicator to evaluate the activation of soil microbes (Lemanowicz 2019).

Conclusion

The current study revealed that the UR21 exhibited high Pb and Cd removal rates in solution. The SEM-EDS and XRD analysis confirmed that the strain UR21 can efficiently reduce the heavy metal concentration by converting the soluble Pb and Cd into insoluble carbonate minerals. Moreover, the application of eggshell waste and UR21 effectively reduced the soluble-exchangeable fraction and bioavailability of Pb and Cd in the soil after 40 days of incubation. The soil pH, cation exchange capacity,

and enzyme activities were also improved especially with the combination of UR21, urea and eggshell waste. This study emphasized that the combined application of urease-producing bacteria and eggshell could be a successful approach for green and sustainable remediation of Pb and Cd contaminated water and soil in the near future.

Declarations

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Author contributions WT planned the experiment, carried it out, and analyzed the results. YN participated in each experiment and completed the writing of the manuscript. AF and ISA participated in the analysis of data. LX and LH provided technical assistance.

Data availability The datasets used in the current study are available from the corresponding author on reasonable request.

Ethical approval No ethical approval was necessary for this study.

Consent to participate All participants in this study consent to participation.

Consent to publish All authors consent to this publication.

Competing interests The authors declare no conflict of interest

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Figures

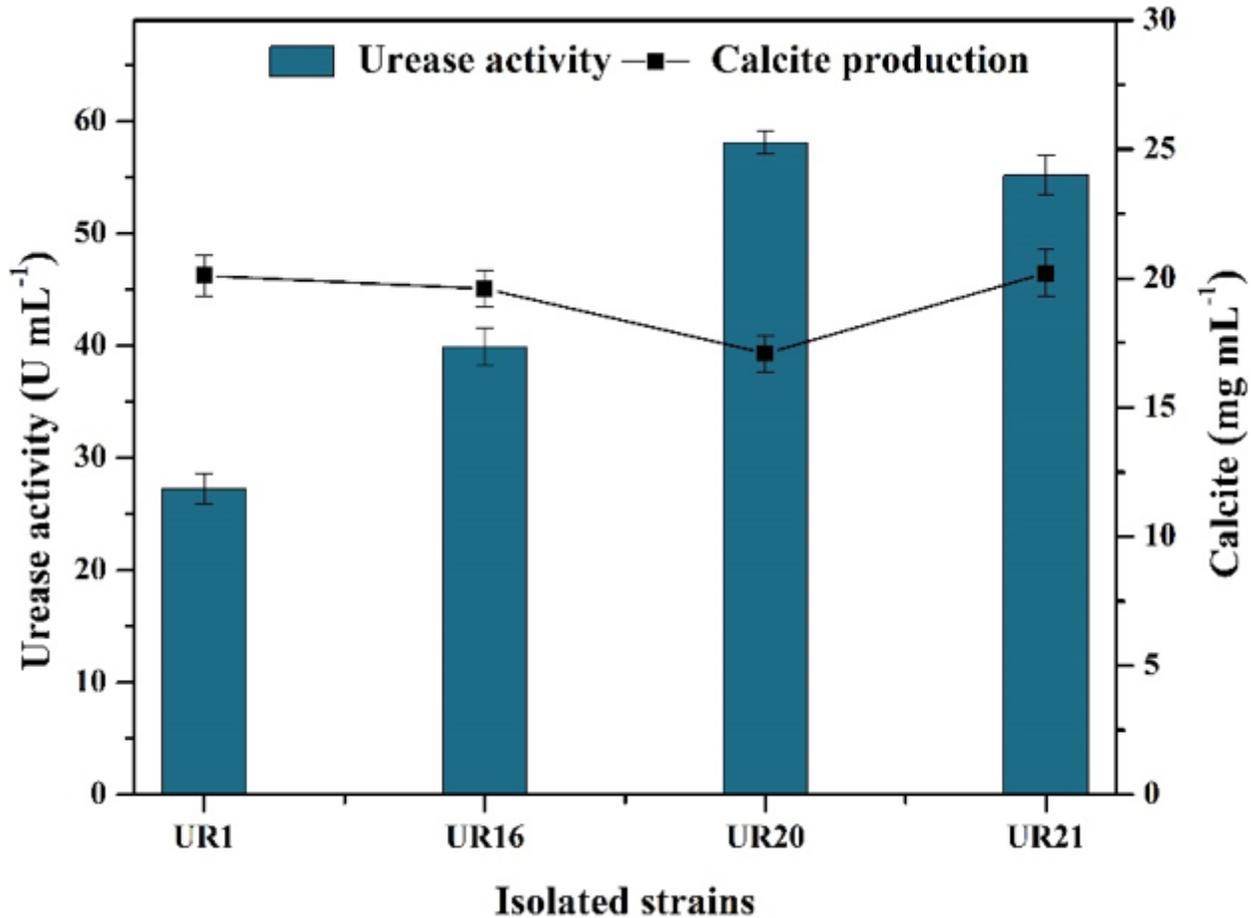


Figure 1

Urease activity and calcite production of four bacterial strains (values are means \pm SD).

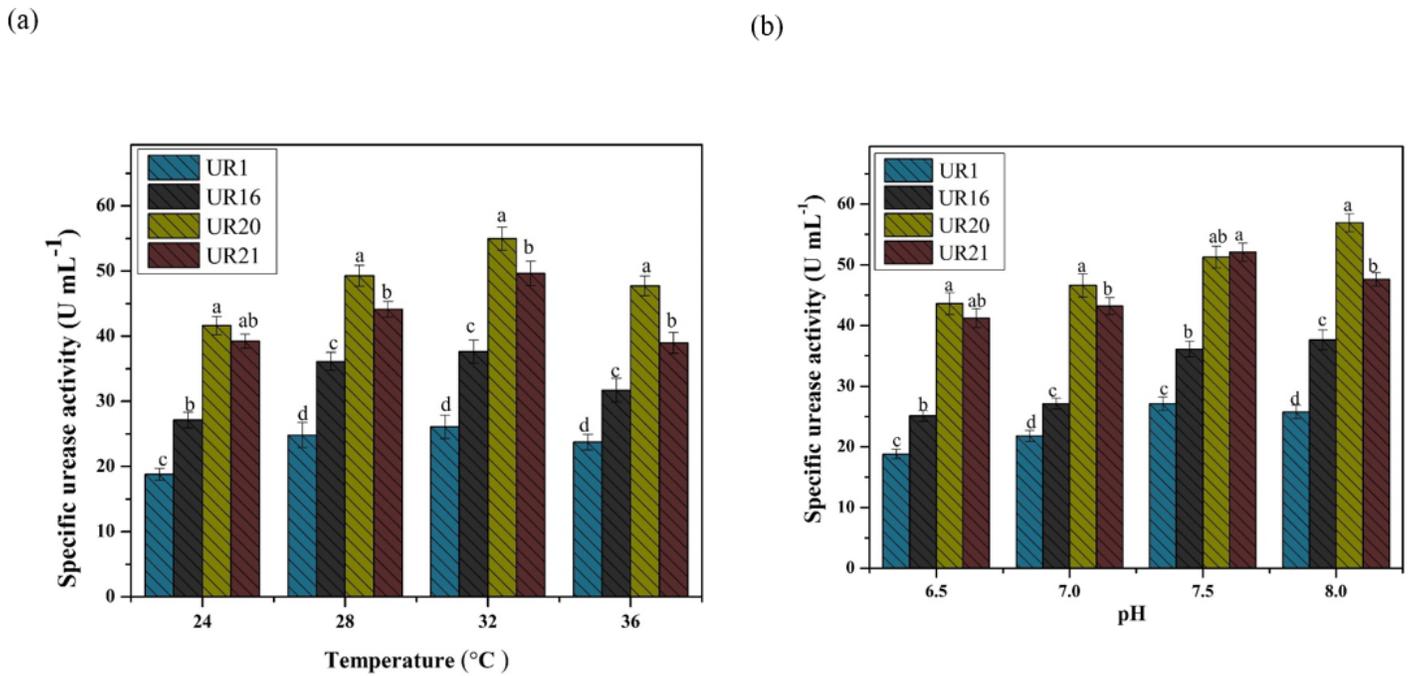


Figure 2

Effect of the different environmental conditions on the urease activity of the bacterial strains. Effect of incubation temperature (a) and initial pH (b) on urease activity. The experiment was conducted in triplicates and lowercase letters above the error bars indicate significant differences among different treatments ($p < 0.05$).

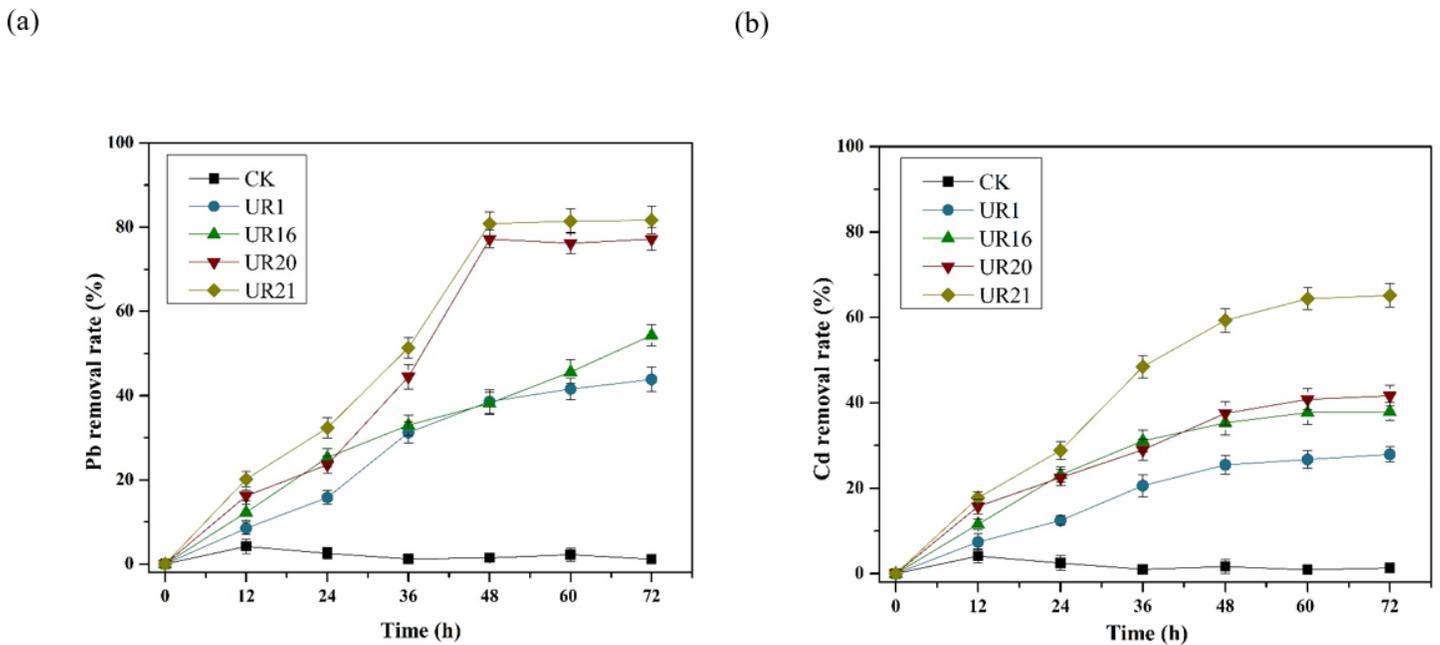


Figure 3

Removal rate of Pb and Cd in solution by different isolates after 72 h of incubation (values are means \pm SD). Lead (a) and cadmium (b).

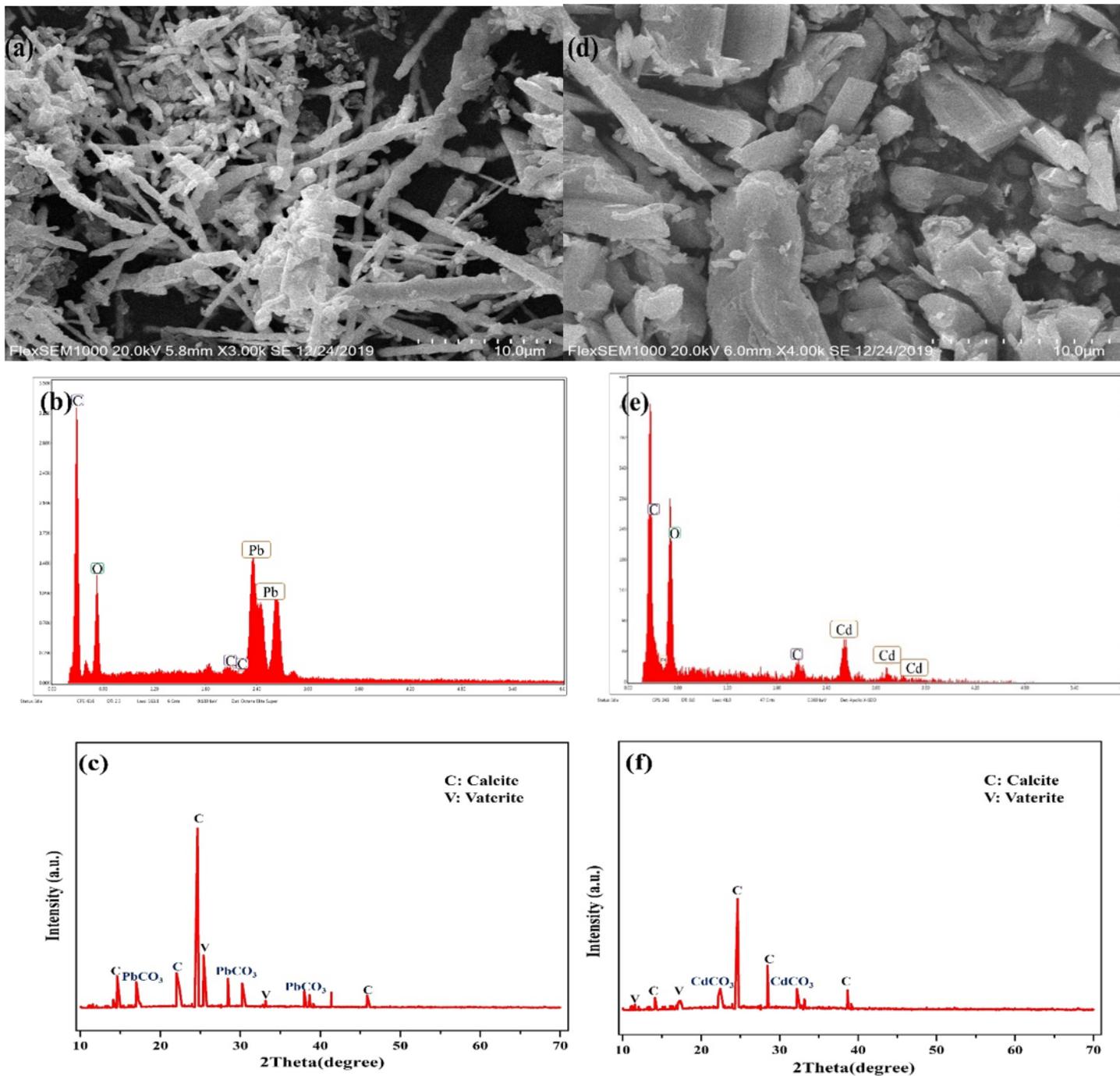


Figure 4

SEM images, EDS spectrum and XRD analysis of metal precipitates. Lead carbonate (a, b, c) and cadmium carbonate (d, e, f).

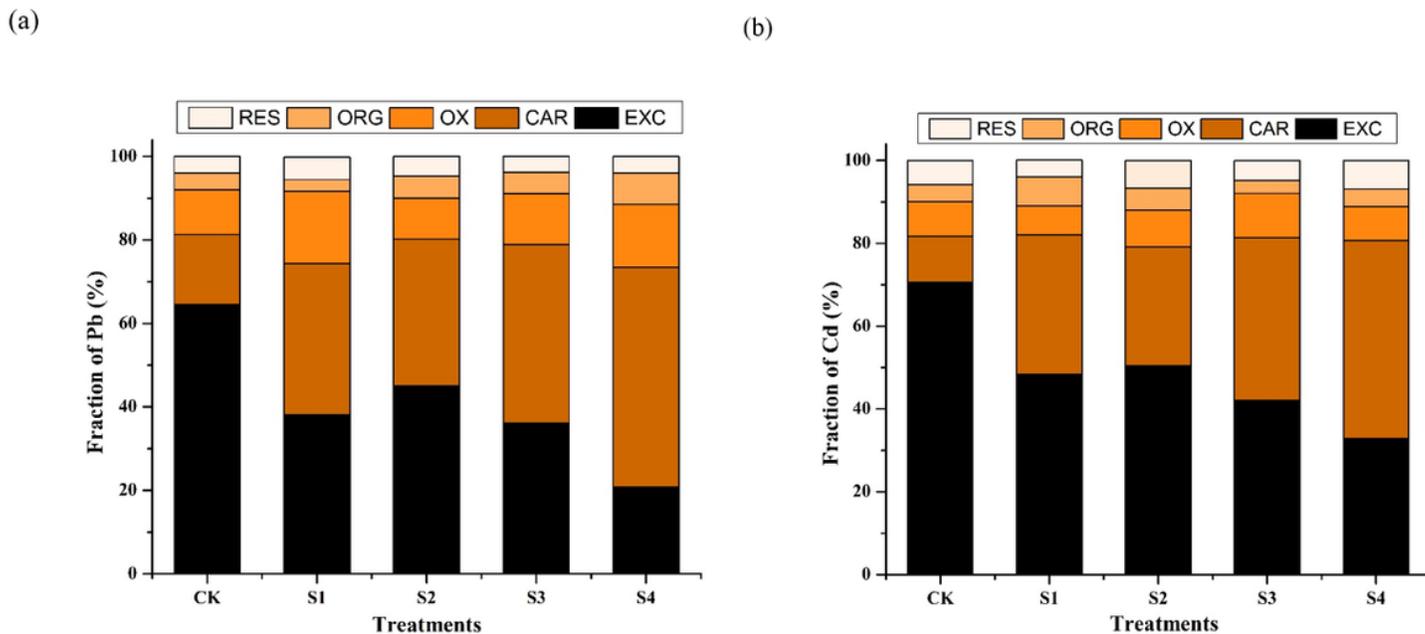


Figure 5

The effect of different treatments on the speciation of (a) Pb and (b) Cd in the contaminated soil (EXC, exchangeable; CAR, carbonate bound; OX, Fe-Mn oxide bound; ORG, organic matter bound; RES, residual).

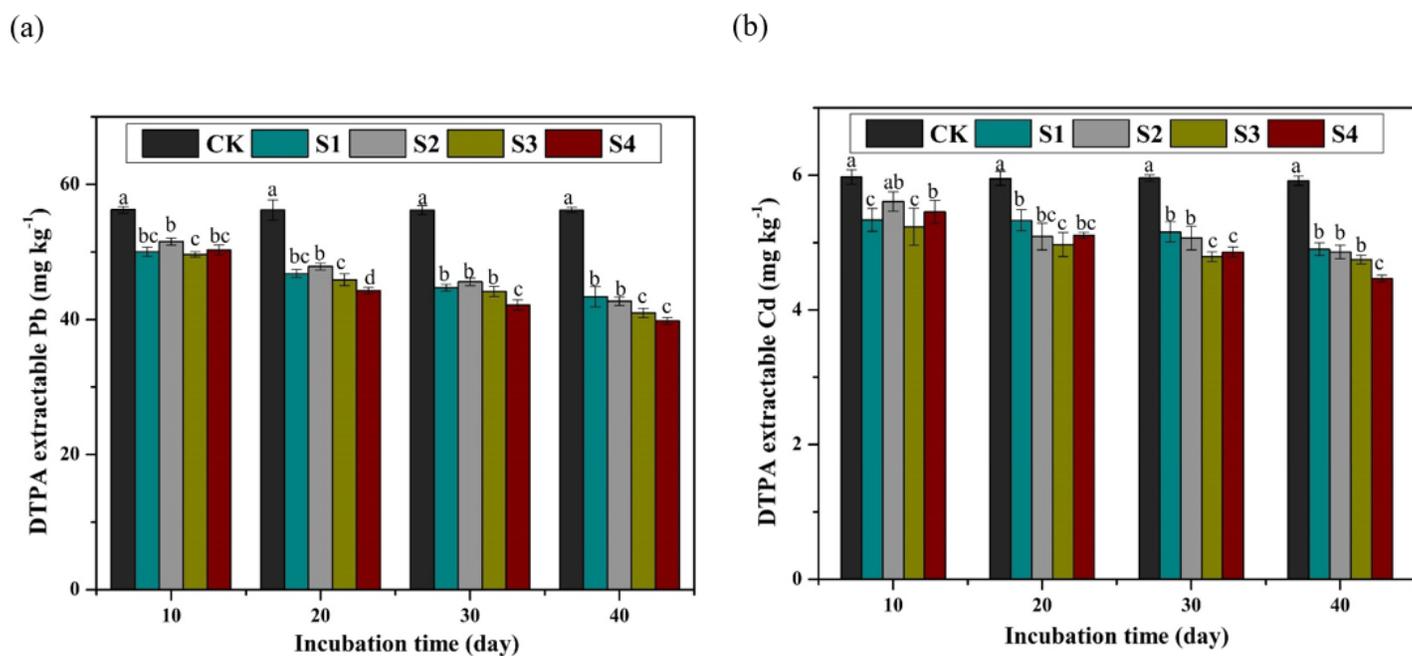
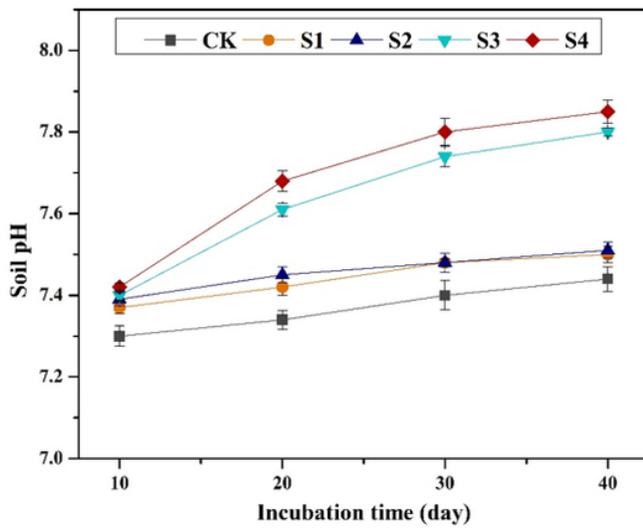


Figure 6

Dynamics of DTPA-Pb (a) and DTPA-Cd (b) in the soil during 40 days of incubation. Lowercase letters above the error bars indicate significant differences among different treatments (p < 0.05).

(a)



(b)

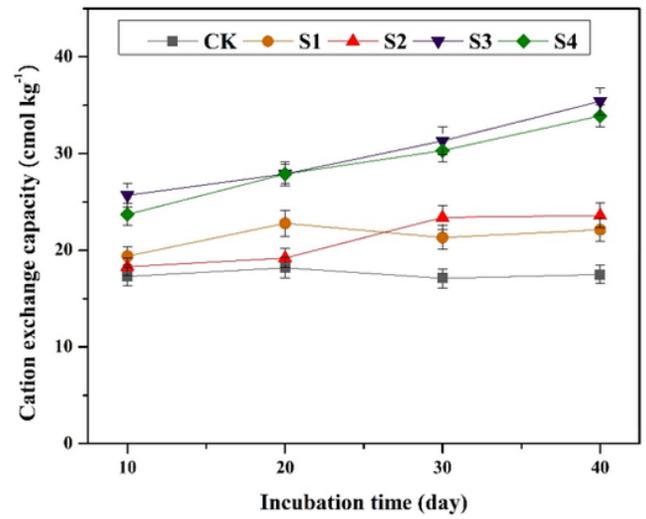


Figure 7

Dynamics of soil pH (a) and soil CEC (b) in the presence of different treatments during 40 days of incubation. Error bars represent the standard deviation of three sampled pots.

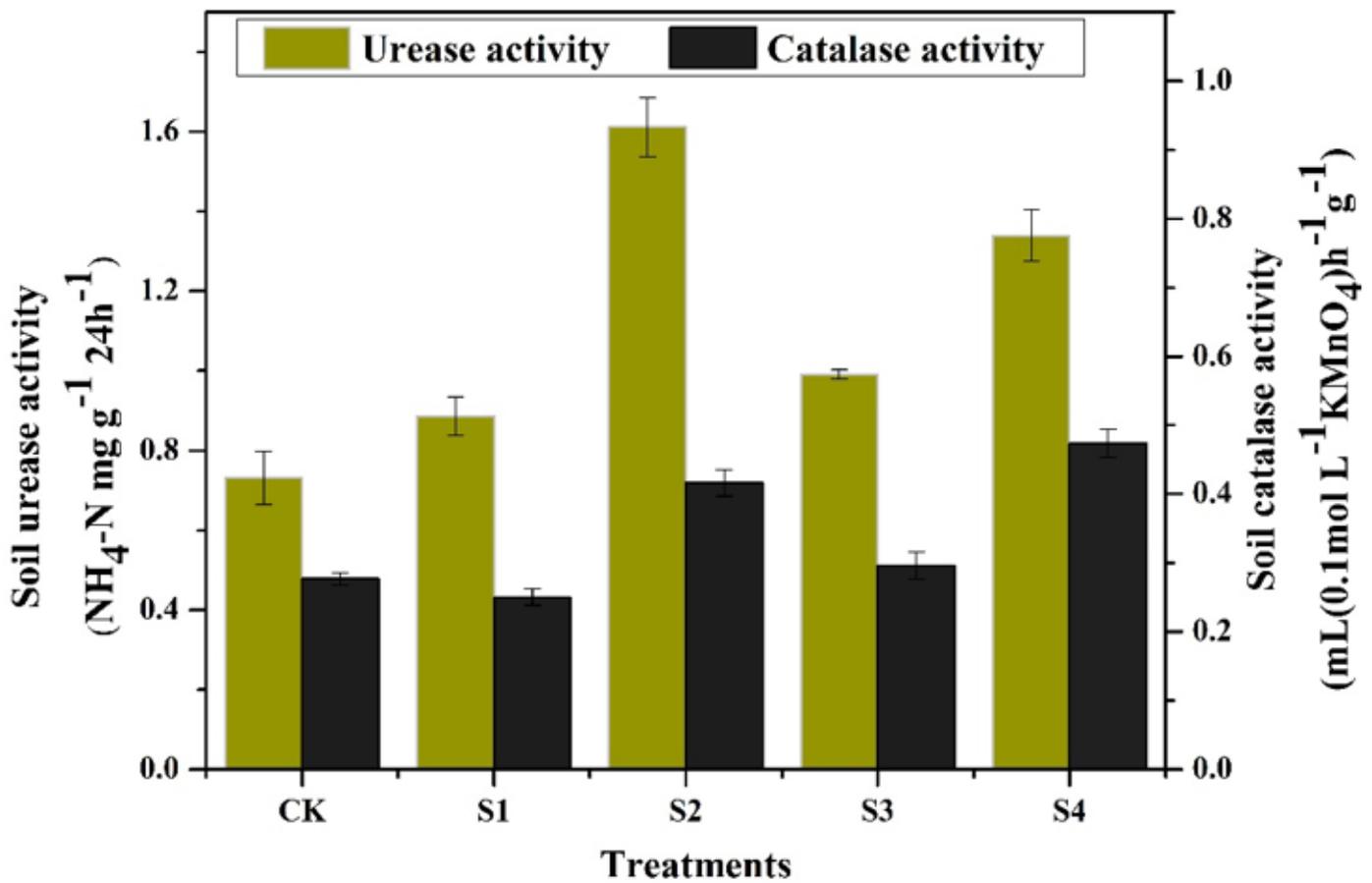


Figure 8

Dynamics of soil enzymatic activities (urease and catalase) in the soil after 40 days of incubation. Lowercase letters above the error bars indicate significant differences among different treatments ($p < 0.05$).

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