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ORIGINAL PAPER

Performance of heavy metal-immobilizing bacteria combined with biochar on remediation of cadmium and lead co-contaminated soil

Ting Wei · Han Gao · Fengqiu An · Xiulian Ma · Li Hua · Junkang Guo

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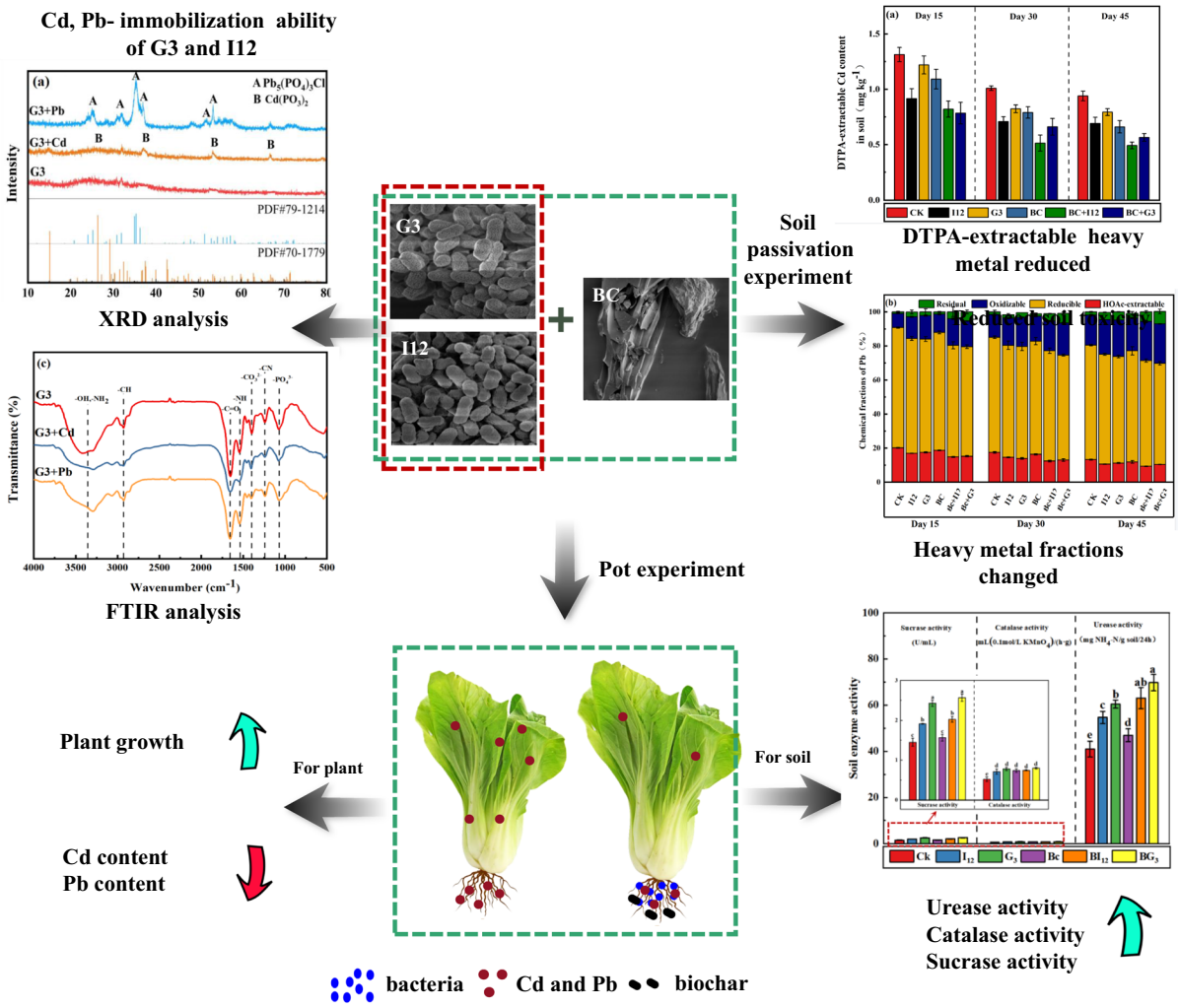
Abstract Heavy metal pollution of soil has become a public concern worldwide since it threats food safety and human health. Sustainable and environmental-friendly remediation technology is urgently needed. Therefore, we investigated the properties and heavy metal removal ability of *Enterobacter asburiae* G3 (G3), *Enterobacter tabaci* I12 (I12), and explored the feasibility of remediation Cd, Pb co-contaminated soil by the combination of G3/I12 and biochar. Our results indicated that both strains are highly resistant to Cd, Pb and maintain plant growth-promoting properties. The removal efficiency of G3 for Cd and Pb were 76.79–99.43%, respectively, while the removal efficiency of I12 for Cd and Pb were 62.57–99.55%, respectively. SEM–EDS and XRD analysis revealed that the morphological and structural changes occurred upon heavy metal exposure, metal precipitates were also detected on cell surface. FTIR analysis indicated that functional groups (–OH, –N–H, –C = O, –C-N, $–PO_4$) were involved in Cd/Pb immobilization. Application of the bacteria, biochar, or their combination decreased the acid-extractable Cd, Pb in soil while increased the residual fractions, meanwhile, the bioavailability of both metal elements declined. Besides, these treatments increased soil enzyme (sucrase, catalase and urease) activity and accelerated pakchoi growth, heavy metal accumulation in pakchoi was depressed upon bacteria and/or biochar application, and a synergistic effect was detected when applying bacteria and biochar together. In BC + G3 and BC + I12 treated plants, the Cd and Pb accumulation decreased by 24.42% and 52.19%, 17.55% and 47.36%, respectively. Overall, our study provides an eco-friendly and promising in situ technology that could be applied in heavy metal remediation.

T. Wei · H. Gao · X. Ma · L. Hua · J. Guo (✉)
School of Environmental Science and Engineering, Shaanxi University of Science & Technology, Xi'an 710021, People's Republic of China
e-mail: guojunkang029@163.com

F. An
College of Environmental and Chemical Engineering, Xi'an Polytechnic University, Xi'an 710048, Shaanxi, China

Graphical abstract



Keywords Heavy metal · Biochar · Heavy metal-immobilizing bacteria · Remediation · Heavy metal accumulation

Introduction

Soil is the basis of human survival. With the continuous industrialization and urbanization, heavy metal pollution of soil is becoming more and more serious, which impairs the yield and safety of various food íiícrops (Wei et al., 2022a; Yao et al., 2012; Zhang et al., 2023; Hua et al., 2021). Heavy metals enter human body via the food chain transfer, directly or indirectly threats people's health by inducing a wide variety of acute and chronic diseases (Munir et al., 2021). Therefore, soil heavy metal remediation technologies are urgently needed. The current technologies are classified into physical remediation, chemical remediation, bioremediation, and combined remediation. The main technologies are guest soil method, heat treatment method, electric remediation method, stabilization method, leaching method etc. (Wu et al., 2019). Traditional physical and chemical remediation methods need a large number of chemicals and energy sources, and also cause problems such as the

irreversibility of soil properties and secondary pollution (Yang et al., 2020). Microbial remediation, a newly developed bioremediation technology, has attracted widespread attention since it has the advantages of simple operation, unlikely to cause secondary pollution, and can be applied to in situ remediation on site (Li et al., 2013). Microorganisms participate in various biochemical processes in soil and respond rapidly to the changes of soil environment (Lao et al., 2020). Especially that the microorganisms living in soils contaminated with heavy metals had evolved various resistant mechanisms. They adsorb metal ions through anion action or induce the transformation of heavy metals via biological reduction, oxidation, complex coordination of organic matter and dissolution, thus reducing the biotoxicity and alleviating the stress of heavy metals (Ojuederie et al., 2017). Some heavy metal-tolerant bacteria also have plant growth-promoting properties. Renu et al. (2021) found that bacterial strains screened from contaminated sites not only reduced heavy metal stress of As, Cr, Ni, and Pb, but also promoted the growth of purslane plants and reduced the phytotoxicity of heavy metals. Others have observed similar phenomena (El-Meihy et al., 2019; Pal et al., 2019). However, microbial remediation technology has certain limitations, the toxicity of heavy metals and changes of the surrounding environment could affect the growth and remediation ability of microorganisms (Wu et al., 2021).

Biochar is a well-known carbon-rich, heavy metal immobilization substance. It is derived from pyrolysis of biomass under oxygen-limited condition (Hua et al., 2019; Kumi et al., 2020; Ren et al., 2022). Due to the advantages of well-developed pore structure, large specific surface area, and rich in active metal-binding groups, biochar could efficiently immobilize heavy metal ions in soil via diverse mechanisms, including adsorption, reduction, electrostatic interaction and surface complexation (Taraqqi-A-Kamal et al., 2021; Wei et al., 2022b). This leads to the reduced heavy metal toxicity to various cultivated plants as well as indigenous and inoculated soil microorganisms. Besides, biochar could release nutrient elements, such as C, N, K, P etc. to the surrounding environment (Ma et al., 2020), thus promotes the plant growth and improves the survival and reproduction of microbes. Furthermore, biochar provides a protective environment for microorganisms in contaminated soils, mitigating the harmful effects caused by toxic metal ions (Zhang et al., 2013). Moreover, biochar has a wide source of raw materials and relatively low cost, thus it is well-recognized as an excellent heavy metal remediation material.

With the increasing complexity and severity of heavy metal pollution, it is difficult for a single remediation measure to achieve the ideal remediation effect. Therefore, the combined application of different remediation strategies has gradually become a research hotspot. Based on the advantages of microorganisms and biochar in heavy metal remediation, we hypothesized that the combined application of these two substances has a synergistic effect and can achieve better remediation effects. Therefore, in this study, the Pb, Cd removal efficiency of heavy metal-tolerant bacteria was determined and the underlying mechanisms were revealed. Furthermore, the effects of individual or combined application of biochar and the bacterial strains on Cd, Pb bioavailability and chemical fraction change, soil enzyme activity, plant growth and heavy metal uptake of pakchoi (*Brassica chinensis* L.) plants were explored. The current study may provide a cost-effective and eco-friendly in situ strategy for the remediation of Pb and Cd contaminated soil.

Materials and methods

Physiochemical properties of the soil

Soils used in the current study were collected from suburb of Hanzhong, Shaanxi Province, China. It belongs to clay loam according to the USDA Soil Taxonomy. The $CdCl_2$ and $Pb(NO_3)_2$ solutions were added into soil and mixed thoroughly to get Cd and Pb supplemented soil. The soil was then incubated for 75 days at room temperature. Subsequently, the co-contaminated soil was air-dried and sieved through a 2-mm mesh before applied in the pot experiment. Soil was suspended in deionized water with a ratio of 1: 2.5, then the mixture was left to stand for 30 min and the pH was determined using a pH meter. The content of organic matter in soil was tested as the methodology described by Zeng et al. (2011). The available phosphorus in the soil was extracted with sodium bicarbonate solution and then analyzed by molybdenum antimony anti-colorimetric method (Durak et al., 2010). Available nitrogen content in soil

was assessed using the alkaline hydrolysis diffusion method as previously described (Kowalenko et al., 2009). Cation exchange capacity (CEC) of soil was evaluated via hexaamminecobalt trichloride method (Yu et al., 2020). The soil samples were acid digested with HNO_3, and then subjected for Cd and Pb analysis using ICP-AAS (ShiMazda, Japan) (Monica et al., 2015).

Bacterial strains and their properties

The bacterial strains used in this study were previously isolated from Cd and Pb co-contaminated soil, 16 s rRNA analysis indicated that the strains were *Enterobacter tabaci* I12 (I12) and *Enterobacter asburiae* G3 (G3), the Genbank accession No. were No. OM698360.1 and OM698359.1, respectively. Both strains are non-pathogenic. The indole-3-acetic acid (IAA) production ability of the bacterial strains was assessed by Salkoeski's colorimetric method (Babu et al., 2013). The siderophore production ability of the bacteria was determined using chrome azurol sulfonate (CAS) method (Naik et al., 2011), and the phosphorus-solubilization ability was determined using the methodology described by Shen et al. (2016). The nitrogen fixation capacity of G3 and I12 was estimated qualitatively by inoculating the purified strains on sterile Ashby medium several times and subsequently observing its growth condition (Oves et al., 2019). The minimum inhibition concentration (MIC) of G3 and I12 against Cd and Pb was assessed as previously described by Wei et al. (2021)

Characteristics of biochar

The maize straw biochar was purchased from Henan Lize Environmental Protection Technology Co., Ltd. (China). The biochar is alkaline, with the pH of 10.3, the BET surface area, oganic matter content and CEC value of the biochar were 25.61 $m^2\ g^{-1}$, 259. 20 g kg^{-1} and 86.03 $cmol^+\ kg^{-1}$, respectively. The biochar were sieved through a 1.5 mm-mesh before applied in the pot experiment.

$Cd^{2+}$and Pb^{2+} removal ability of the bacterial strains

The 5% bacterial cells ($OD_{600}=1.0$) were inoculated into sterilized LB liquid medium that contained 50 mg L^{-1} Cd^{2+} (in the form of $CdCl_2$) or 600 mg L^{-1} Pb^{2+} (in the form of $PbNO_3$), cells were then cultured at 37℃ for 72 h. After that, the medium was centrifuged, filtered and the Cd, Pb in the supernatant was determined using ICP-AAS. The heavy metal removal ability of the bacterial strains was determined using the following formula:

$$Q = \left[\left(C_0 - C/C_0\right)\right] * 100\%$$

C_0: initial concentration of Cd^{2+}, Pb^{2+} in solution (mg L^{-1}).

C: equilibrium concentration of Cd^{2+}, Pb^{2+} in solution (mg L^{-1}).

FTIR、SEM–EDS and XRD analysis

The bacterial cells collected before and after Cd^{2+} and Pb^{2+} adsorption were freeze-dried and subsequently subjected for fourier transform infrared spectroscopy (FTIR) to reveal the changes of surface functional groups upon metal ions adsorption. The harvested bacterial cells were suspended in 2.5% glutaraldehyde, stored at 4℃ for 2 h, then centrifuged and washed several times with phosphate buffer (0.1 M, pH 7.0), subsequently dehydrated with different concentrations of ethanol (50%, 75%, 85%, 90%, 95% and 100%). Finally, the cells were freeze-dried and the morphological changes of cells before and after Cd^{2+} and Pb^{2+} adsorption were observed by scanning electron microscopy (SEM), and the elemental analysis was determined by X-ray energy dispersive spectroscopy (EDS). X-ray diffraction (XRD) was employed to investigate the newly formed metal-containing substances.

Soil passivation experiment

Soil passivation experiment was performed to evaluate the effects of bacteria and biochar on heavy metal availability and fractions in soil. Briefly, 50 g soil was added into a 100 ml beaker, biochar and/or bacterial strains were mixed well with the soil. Biochar was added at the ratio of 2%, and 2.5 ml bacterial suspension ($OD_{600}=1.0$) was added into the beaker. The contaminated soil itself was used as control. All beakers were kept at room temperature and the soil moisture content was maintained at about 60%. Samples were collected at 15 day

intervals. The bioavailability of Cd and Pb in soil were extracted by DTPA and analyzed by ICP-AAS (Wei et al., 2022b). Chemical fractions Cd and Pb in the soil were assessed by Community Bureau of Reference (BCR) method (Hua et al., 2019).

Pot experiment

The pot experiment was conducted in the growth chamber with the temperature of 26 ℃, relative humidity of 65%, 16 h day/8 h night. Six treatments were established: (1) CK: plants were cultivated in Cd and Pb co-contaminated soil; (2) G3: plants were cultivated in contaminated soil and inoculated with the bacterial strain G3; (3) I1: plants were cultivated in contaminated soil and inoculated with the bacterial strain I12; (4) BC: plants were cultivated in contaminated soil with 2% biochar supplementation; (5) BC + G3: plants were cultivated in contaminated soil that supplemented with 2% biochar and inoculated with the bacterial strain G3; (6) BC + I12: plants were cultivated in contaminated soil that supplemented with 2% biochar and inoculated with the bacterial strain I12. Specifically, the test soil was added to each pot (500 g soil per pot). The pakchoi seeds (*Brassica chinensis* L.) were sterilized with 3% NaOCl and evenly sowed. After germination, the plants were thinned from 8 to 3 and the plants of uniform size were kept for the following experiment. After cultivated for 3 weeks, plants were subjected for 25 ml bacterial suspension ($OD_{600} = 1.0$) inoculation. The bacterial suspension was prepared as previously described (Wei et al., 2022b). The biochar was added to the soil by mixing the soil and biochar thoroughly before the pot experiment started. 20 pots were set up for each treatment and the whole experiment was performed 3 times. Plant samples were collected 3 weeks after bacterial inoculation.

Growth parameters of the plants

Plant height and root length of pakchoi plants were recorded with a meter rod. Fresh weight was recorded with a scale. Pakchoi samples were rinsed with distilled water, dried at 80 ℃ overnight to obtain constant weight and then the dry mass was recorded.

Cd and Pb accumulation of the plants

Root and shoot samples were acid (HNO_3) digested on a digestion furnace, the digestion procedure was performed as previously by Guo et al. (2018). Subsequently, heavy metal content in plants was assessed by ICP-AAS (ShiMazda, Japan). The total metal accumulation in plants was calculated as follow:

$$\text{Total heavy metal accumulation} = C_{root} \times DW_{root} + C_{shoot} \times DW_{shoot}$$

Table 1 Physicochemical properties of the tested soil

pH	Organic matter (g kg^{-1})	Available P (mg kg^{-1})	Available N (mg kg^{-1})	Total Cd (mg kg^{-1})	Total Pb (mg kg^{-1})	CEC ($cmol^{+}$ kg^{-1})
7.91	23.11 ± 1.26	1.59 ± 0.05	26.87 ± 0.67	3.15 ± 0.01	539.50 ± 0.33	8.45 ± 0.53

Values are expressed as mean ± standard error (n = 4)

Table 2 Properties of the bacterial strains

Bacterial strains	IAA (mg L^{-1})	Siderophore (%)	P-solubilization (mg L^{-1})	N_2 fixation	Minimum inhibitory concentration (mg L^{-1})	
					Cd^{2+}	Pb^{2+}
G3	4.640 ± 0.488	55.191 ± 0.613	60.222 ± 1.530	+	1050	1500
I12	9.087 ± 0.312	38.448 ± 1.067	33.294 ± 1.056	+	1000	1500

Values are expressed as mean ± standard error (n = 4)

C_{root}, $C_{shoot,}$ DW_{root}, DW_{shoot} indicate heavy metal concentration in root, heavy metal concentration in shoot, dry weight of root and dry weight of shoot, respectively.

The bioconcentration factors and translocation factor are determined as follows (McKone et al., 2007; Thakur et al., 2016):

Bioconcentration factors (BCF): BCF = heavy metal content in plants/heavy metal content in soil.

Translocation factor (TF) = heavy metal content in shoot/heavy metal content in root.

Soil enzyme activity

The catalase activity of soil was evaluated by $KMnO_4$ titration. $KMnO_4$ was used to titrate the residual H_2O_2 after H_2O_2 decomposition reaction, expressed as the volume of 0.1 mol/L $KMnO_4$ consumed in 1 h per g of dry soil (Jin et al., 2009). Soil urease activity was tested by mixing 5 g soil sample together with citrate buffer (20 mL, pH 6.7) and 10% urea solution (10 mL) for 24 h. Subsequently, the obtained ammonium was spectrophotometrically determined at the wavelength of 578 nm (Ma et al., 2020). The sucrase activity was determined according to the protocol described by Jiang et al. (2022).

Data analysis

Datas are shown as mean ± standard error. Data analysis was performed by the SPSS 19.0 for Windows. Student's t test and One-way ANOVA were employed to evaluate the significant differences among treatments. Figures were produced using Origin 9.0.

Results and discussion

Properties of the soil

The physicochemical properties are the main characteristic of the soil. The soil used in this study is alkaline, with the pH of 7.9, the organic matter content was 23.11 g kg^{-1}, the available P and available N content was 1.59 ± 0.05 mg kg^{-1}, 26.87 ± 0.67 mg kg^{-1}, respectively. The total Cd and Pb content was 3.15 ± 0.01 mg kg^{-1} and 539.50 ± 0.33 mg kg^{-1}, respectively. The CEC of the soil was 8.45 ± 0.53 $cmol^{+}$ kg^{-1} (Table 1).

Properties of bacterial strains

Soil microorganisms are indigenous residents of the soil. In heavy metal-polluted soil, the microbes have evolved diverse mechanisms to adapt the polluted environment (Coelho et al., 2015). Thus, various heavy metal-tolerant bacteria and fungi have been isolated from the contaminated soil and used for the heavy metal remediation (Zhuang et al., 2007; Kumar Mishra et al., 2017). Both G3 and I12 that isolated from heavy metal contaminated soil exerted high Cd and Pb resistance. The MIC of G3 for Cd and Pb were 1050 mg kg^{-1} and 1500 mg kg^{-1}, respectively. While the MIC of I12 for Cd and Pb were 1000 mg kg^{-1} and 1500 mg kg^{-1}, respectively. Besides, G3 and I12 maintained plant growth-promoting traits. They could produce phytohormone IAA (Table 2), a well-known plant growth regulator, which could stimulate cell division, differentiation and formation of new organs, enrich root surface area, accelerate the uptake of nutrients by plants, and ultimately promote the growth and development of plants (Yu et al., 2017). Moreover, both G3 and I12 have the ability of phosphorus solubilization, nitrogen fixation, and siderophore production (Table 2). Bacterial strains with the ability of phosphorus solubilization and nitrogen fixation could improve the survival probability of strains in severe environments, accelerate the colonization of plants and improve the soil quality and fertility (Chen et al., 2022; Mao et al., 2017; Singh et al., 2021). Siderophores, an extracellular organic compound with small molecular weight, is usually secreted by microbes under iron-limited conditions. It acts as a ferric iron chelator, but could also bind other divalent metal ions, and reduce their harmful effects to plants and microbes (Nair et al., 2007). Overall, G3 and I12 not only have high metal resistance, but also have plant growth-promoting traits. The existence of beneficial bacteria in the rhizosphere brings various benefits to plants, such as promote their growth, stimulate nutrient uptake, protect plants forms various abiotic and biotic stresses (Mishra et al., 2021; Souza et al., 2015). Thus, G3 and I12 could potentially be

used for heavy metal remediation and plant growth stimulation.

Cd and Pb removal ability of the bacterial strains

Both G3 and I12 had ability to remove metal ions. The Cd^{2+} removal rate of G3 was 76.79%, and the Pb^{2+} removal rate was 99.43%. The removal rate of Cd^{2+} by I12 was 62.57%, and the removal rate of Pb^{2+} by I12 was 99.55% (Fig. 1). Previous studies indicated that bacteria could remove metal ions via precipitation, biosorption by metal-binding functional groups, and convert metals to less toxic forms by intracellular and extracellular enzymes (Ojuederie et al., 2017). Some bacteria could produce extracellular polymers and form biofilms, which enable the cells to bind metal ions (Fang et al., 2002). Besides, bacteria could uptake metal ions via active transportation and thus reduce the metal ions in the supernatant (Nies et al., 2000). To investigate the metal removal mechanism of G3 and I12, SEM–EDS, XRD and FTIR were used to investigate the precipitates in the presence and absence of Pb and Cd.

SEM–EDS, FTIR and XRD analysis of G3 and I12 in the absence and presence of Cd, Pb

Before Cd and Pb exposure, G3 and I12 cells were short rod-shaped and the cell surfaces were smooth

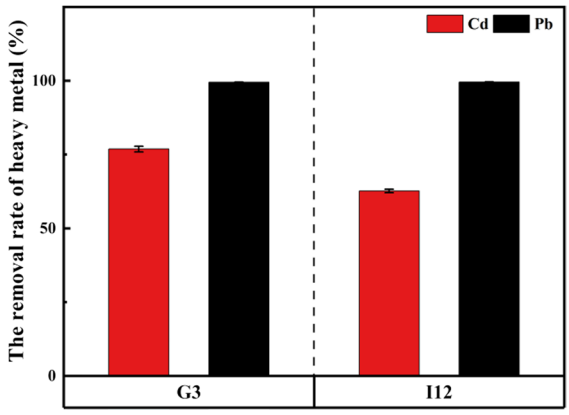


Fig. 1 Removal efficiency of heavy metals by bacteria G3 and I12. Cd removal rate **a**; Pb removal rate **b** Values are the mean ± SD

(Fig. 2a, g). After Cd and Pb exposure, the morphology of G3 did not change significantly, but a large number of deposits appeared on cell surface (Fig. 2b, 2c). It is speculated that Cd and Pb bind to the active sites of the cell wall, forming precipitates that adhere to the cell surface. In addition, irregular aggregation of bacterial cells was observed, which may due to the denaturation of proteins and polysaccharides on cell surface under heavy metal stress and thus lead to cell aggregation. Similarly, a large number of adherents and deposits were also observed on I12 upon Cd and Pb exposure (Fig. 2h, i). Meanwhile, the cells exhibited rough surface and irregular shape after incubation with Cd, which is likely due to the Cd induced cell damage. These findings are consistent with the previous studies that large amounts of deposits detected on the cell surface upon metal ion exposure (Teemu et al., 2008; Yang et al., 2020). EDS analysis indicated that peak of Cd^{2+} /Pb^{2+} were detected, indicating that G3 and I12 had Cd and Pb immobilizing ability (Fig. 2d, e, f, j, k, l). It is reported that extracellular polymers can precipitate heavy metals. Johnson et al. (2009) found that sulfate-reducing bacteria CL4 precipitate zinc by releasing glycerol. Fayaz et al. (2011) pointed out that after exopolysaccharide is secreted into solution by bacteria, it can bind heavy metal ions.

The XRD and FTIR analyses were performed to reveal the heavy metal immobilization mechanisms. Comparative analysis of XRD patterns with the known crystalline substances confirmed that the immobilization of Cd and Pb by the two bacterial strains through the formation of Cd/Pb-containing salt precipitates (Fig. 3a, b). For G3, the main precipitates were $Pb_5(PO_4)_2Cl$ and $Cd_3(PO_4)_2$, and for I12, the main precipitates were $Pb_5(PO_4)_2Cl$ and $CdCO_3$. FTIR spectrum exhibited significant differences in the absorption peaks before and after metal ion exposure (Fig. 3c, d). For G3, the absorption peaks of hydroxyl group (–OH) shifted from 3301 cm^{-1} to 3279 cm^{-1} upon Cd exposure, and the peak of carboxyl group (–C = O) shifted from 1395 cm^{-1} to 1406 cm^{-1}, implying the formation of hydroxyl and carboxyl complexation (Fig. 3c). Meanwhile, an asymmetric stretching vibration was observed at about 3000 cm^{-1}, indicating that -CH_2 was involved in Cd binding. Moreover, the absorption peak of amide C-N (at 1243 cm^{-1}) group shifted and the intensity decreased, indicating protein chelation

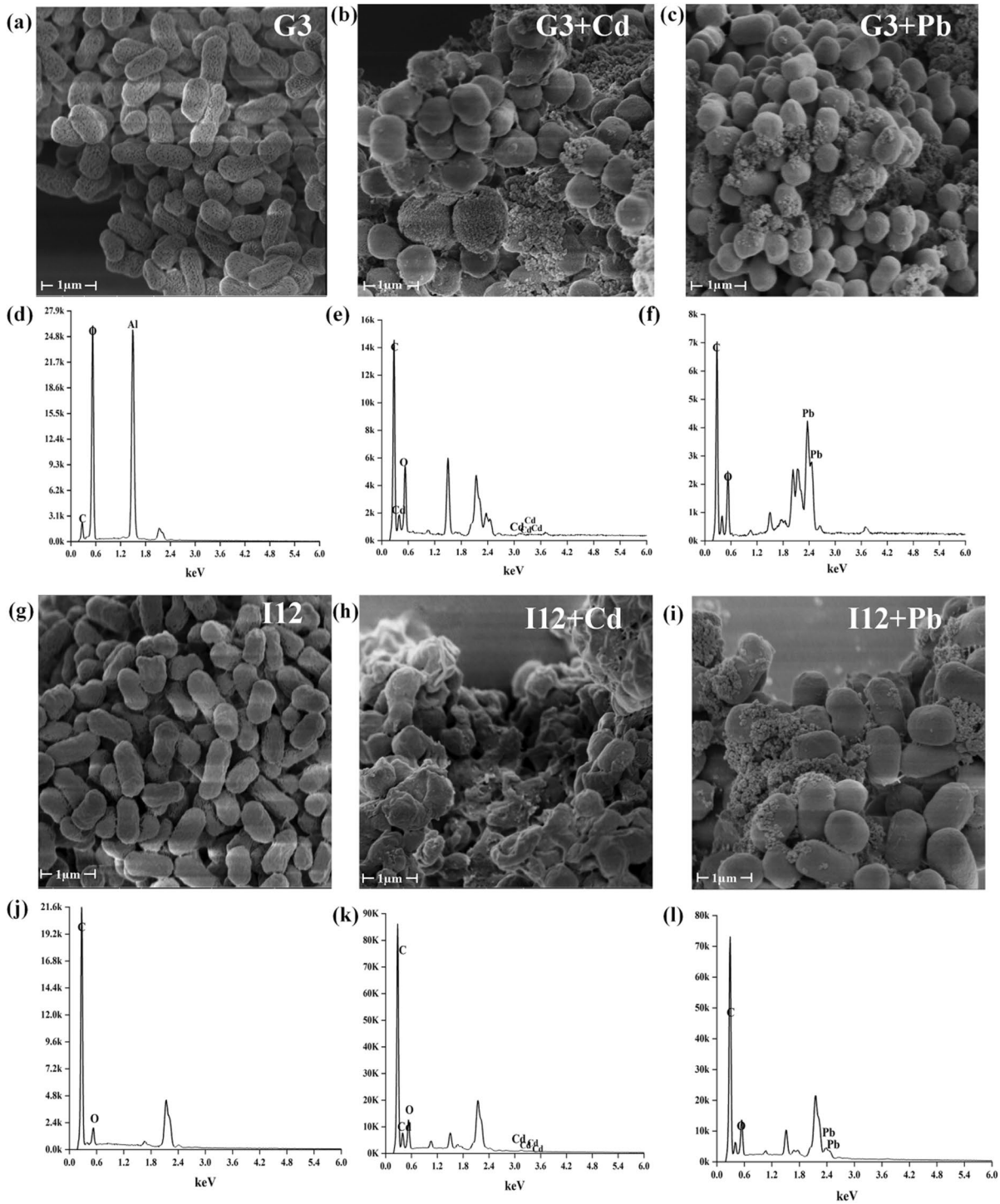


Fig. 2 SEM–EDS of the bacterial strains before and after Cd^{2+}、Pb^{2+} adsorption. SEM of G3 **a**; G3 + Cd **b**; G3 + Pb **c**; I12 **g**; I12 + Cd **h**; I12 + Pb **I**. EDS of G3 **d**; G3 + Cd **e**; G3 + Pb **f**; I12 **j**; I12 + Cd **k**; I12 + Pb **l**

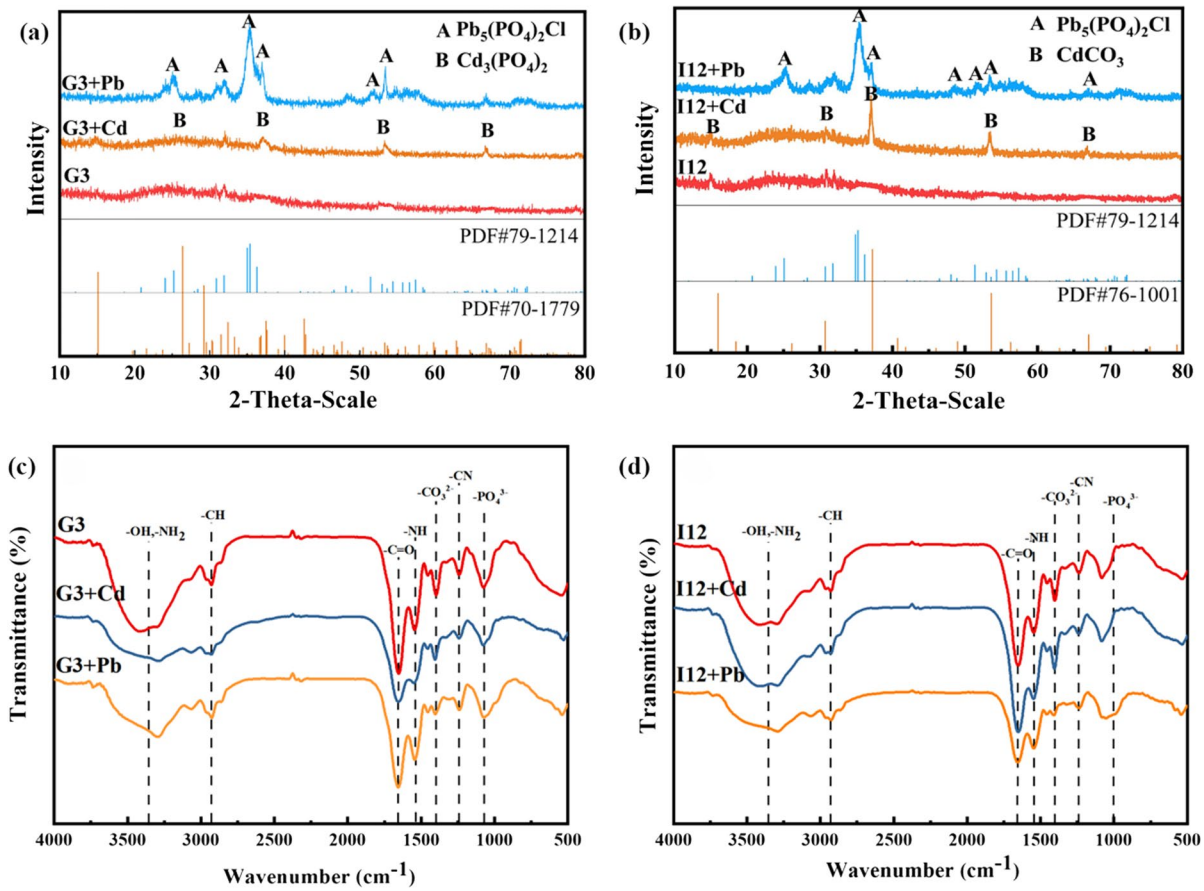


Fig. 3 XRD and FTIR analysis of strains before and after adsorption of Cd^{2+} and Pb^{2+}. XRD of G3 **a** and I12 **b**, FTIR of G3 **c** and I12 **d**

was involved in Cd immobilization. Upon Pb exposure, the shift of absorption peak at 3290 cm^{-1} (–OH) was observed. New absorption peak appeared at 3063 cm^{-1} -3071 cm^{-1}, indicating that –NH-containing substances in bacterial cells interact with Pb^{2+}. Besides, the absorption peak at 1075 cm^{-1} widened, indicating the involvement of $–PO_4$ in Pb immobilization. Similarly, for I12, FTIR indicated that the functional groups -OH were involved in Cd binding and –OH, –C = O, $–PO_4–$ were involved in Pb binding (Fig. 3d). It is reported that bacterial cell walls could provide binding sites for the adsorption of heavy metals (Jin et al., 2017; Nithya et al., 2011; Pan et al., 2011), various charged functional groups (including hydroxyl, carboxyl, and phosphate groups etc.) present on polysaccharides, peptidoglycan, protein, (lipo) teichoic acids of bacterial cells are involved in the removal of heavy metal ions (Yang et al., 2020; Yi et al., 2017). Neutralization of some special groups on cell surface significantly impaired their ability of heavy metal removal (Beveridge et al., 1980; Chakravarty et al., 2012), which confirmed that functional groups on cell surface played a key role in the heavy metal binding.

Effects of bacteria and biochar on DTPA-extractable heavy metal in soil

The DTPA-extraction is often used to evaluate the bioavailability, mobility and toxicity of heavy metals in soil (Qi et al., 2021). In the control soil, the DTPA-Cd and DTPA-Pb were 1.31 mg kg^{-1} and 143.51 mg kg^{-1}, respectively. However, the content of DTPA-Cd and DTPA-Pb in soil decreased at 15d, 30d and 45d upon bacteria and biochar treatment (Fig. 4).

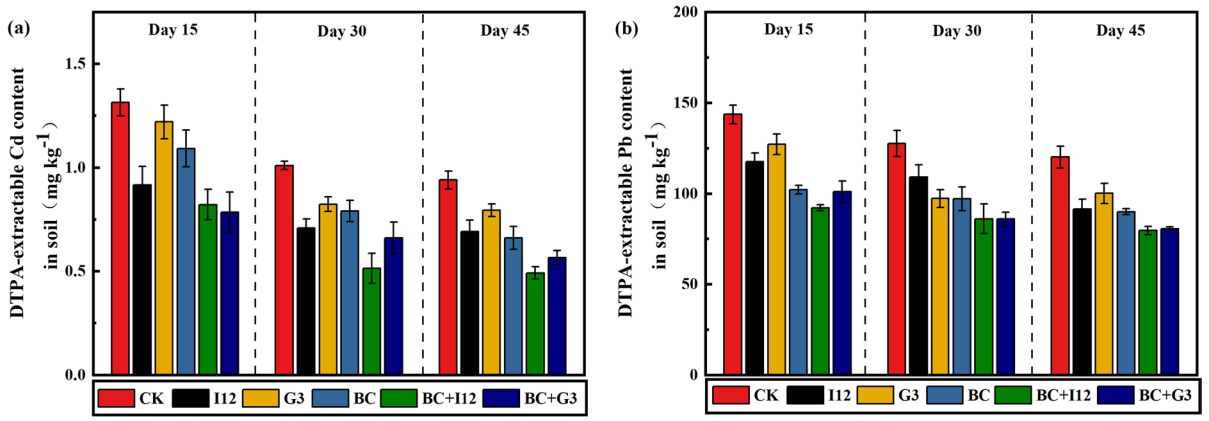


Fig. 4 Effect of different treatment on DTPA-extractable Cd and Pb content in soil. **a** DTPA-Cd; **b** DTPA-Pb.Values are the mean ± SD

The decreasing rate ranged from 7.14–62.59% for Cd and 11.41% -44.64% for Pb. These results indicated that I12 and G3 could immobilize metal ion via various functional groups on cell surface, which may have contributed to the decrease of DTPA-extractable heavy metals. Besides, biochar has high specific surface area and porous structure, and it is reported to interact with heavy metals via electrostatic interaction, adsorption, surface complexation and reduction, make itself a good absorbent for metal ions, and thus reduced the bioavailability and toxicity of heavy metal in soil (Andrey et al., 2019; Awitdrus et al., 2021). Decreasing of DTPA-extractable metal ion by biochar and microbe addition was also reported by others (Duan et al., 2021; Han et al., 2020).

Effects of bacteria and biochar on the chemical form of heavy metals in soil

Upon entering the soil, heavy metals form various chemical fractions via diverse reactions, including dissolution, adsorption, complexation and precipitation, resulting in different environmental behaviors and biological toxicity (Violante et al., 2010). The mobility and biotoxicity of heavy metals from high to low were acid-soluble fraction > reducible fraction > oxidizable fraction > residual fraction (Liu et al., 2018). The HOAc -extractable heavy metal could be readily uptake by various crops and it is regarded as the most harmful form. The residue fraction is the most stable form with the least activity and hardly be absorbed and induce harmful effects to crops (Wei et al., 2022b). For the control soil, the main chemical form of Cd was the HOAc-extractable fraction (63.56%), followed by the reducible state (32.27%), while the oxidizable and residual forms accounted for a smaller percentage (4.07% and 0.098%, respectively) (Fig. 5a). After the application of I12, G3, BC, BC + I12 and BC + G3, the HOAc-extractable and reducible heavy metals decreased, while the oxidizable and residual fractions increased. After 45 days of treatment, the acid soluble fraction of Cd decreased by 17.23%, 18.96%, 16.56% 25.56%, and 27%, respectively. Pb in the untreated soil is mainly present in the reducible state (70.63%), followed by the HOAc-extractable state (20.17%) (Fig. 5b). Similarly, the application of I12, G3, BC, BC + I12 and BC + G3 resulted in a decline of reducible and HOAc-extractable Pb and increase of the oxidizable and residual fractions. After 45 days of treatment, the acid soluble fraction of Pb was reduced by 47.60%, 44,30%, 41.20%, 53.89% and 48.40% upon I12, G3, BC, BC + I12 and BC + G3 treatment, respectively. Changes of chemical forms indicated that Cd and Pb could be converted from HOAc-extractable form to others upon bacteria and biochar treatment. Similar observations have been found by others (Seneviratne et al., 2017; Tu et al., 2020). Biochar maintains various functional metal-binding groups (hydroxy, carboxylic and phenolic groups. etc.), which contributed to the heavy metal fixation and changes of Cd and Pb fractions (Chen et al., 2020; Namgay et al., 2010; Ren et al., 2018). The

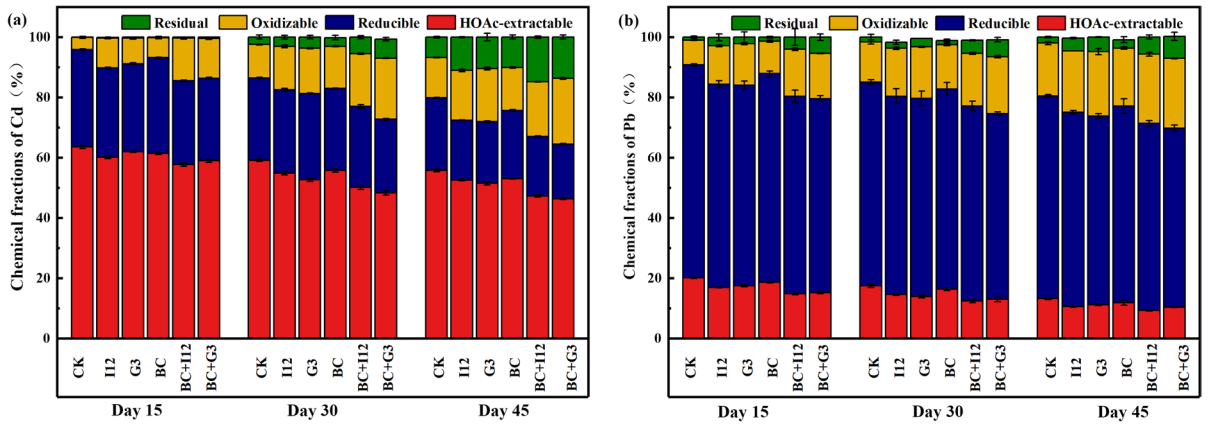


Fig. 5 Chemical fractions distribution of Cd and Pb in soil under different treatments. **a** Chemical fractions of Cd; **b** Chemical fractions of Pb. Values are the mean ± SD, significant differences were calculated with Student's t-test ($p < 0.05$)

SEM–EDS, XRD and FTIR analysis verified that G3 and I12 could bind metal ions and form precipitates on cell surface, therefore, they are also involved in the chemical speciation transformation of Pb and Cd.

Effects of of bacteria and biochar on soil enzyme activity

Soil enzymes, including urease, sucrase and catalase, play an essential role in the decomposition of soil organic matter, nutrient cycling and redox reactions (Burns et al., 2013). Besides, soil enzymes are highly sensitive to various stress conditions, and could be used as an integrative biological indicator for assessing the quality and fertility of soil (Bhattacharjya et al., 2021). Urease is an important component of the soil nitrogen cycle, which can promote the hydrolysis of urea to form NH^{4+}, thus promoting the absorption and utilization of nitrogen by plants (Gao et al., 2010; Wang et al., 2014). Catalase is involved in catalyzing the decomposition of harmful hydrogen peroxide, thus alleviating the toxic effect of heavy metals on various organisms and improving the physical and chemical properties of soil (Huang et al., 2013; Zhang et al., 2020). Sucrase catalyzes the decomposition of sucrose, thus providing energy and nutrients for the growth of microorganisms in soil. Our findings indicated that bacteria, biochar or their combination significantly increase the activity of the three tested enzymes (except that biochar did not increase sucrase activity), especially in soils treated with BC + G3, the urease, sucrase and catalase activities were increased by 70.35%、77.19% and 53.95%, respectively (Fig. 6). It is reported that treatment with beneficial bacteria could stimulate the secretions of various organic substances (amino acids, sugars, organic acids, etc.) of plant root, which in turn promoted the proliferation of microorganisms, and ultimately lead to the increased soil enzyme activity (Wei et al., 2022b). Biochar could immobilize heavy metal ions, reducing their toxicity to microorganisms. Their addition could adjust the soil carbon–nitrogen ratio, regulate the coordination of

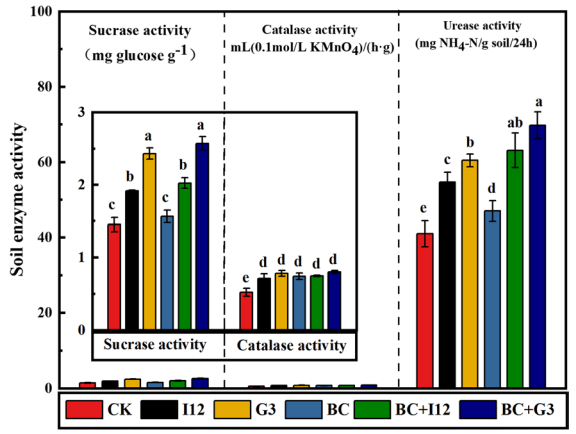


Fig. 6 Effects of different treatment on soil enzyme activities. **a** sucrase activity; **b** catalase activity; (c) urease activity. Values are the mean ± SD, significant differences were calculated with Student's t-test ($p < 0.05$)

rhizosphere microecological environment and nutrients (Feng et al., 2021), and provide the nutrients for the growth and development of microorganisms. Moreover, the porous structure of biochar can provide habitat for microorganisms, thus improving the survival rate of microorganisms, and thus increase the number of enzymes secreted by microorganisms (Glaser et al., 2002; Zhu et al., 2017). The increased soil enzyme activity implied that the soil health and quality were elevated after bacteria and biochar addition.

Effects of bacteria and biochar on the growth of pakchoi plants

Heavy metal pollution induces growth inhibition in various plants, since it causes malnutrition, metabolic disorders, hormone imbalance, photosynthesis and respiration disorders (Guo et al., 2018; Wei et al., 2018). In this study, the growth parameters of pakchoi plants were determined (Fig. 7). The bacteria, biochar and their combination impacted the shoot and root length, fresh and dry weight of pakchoi plants

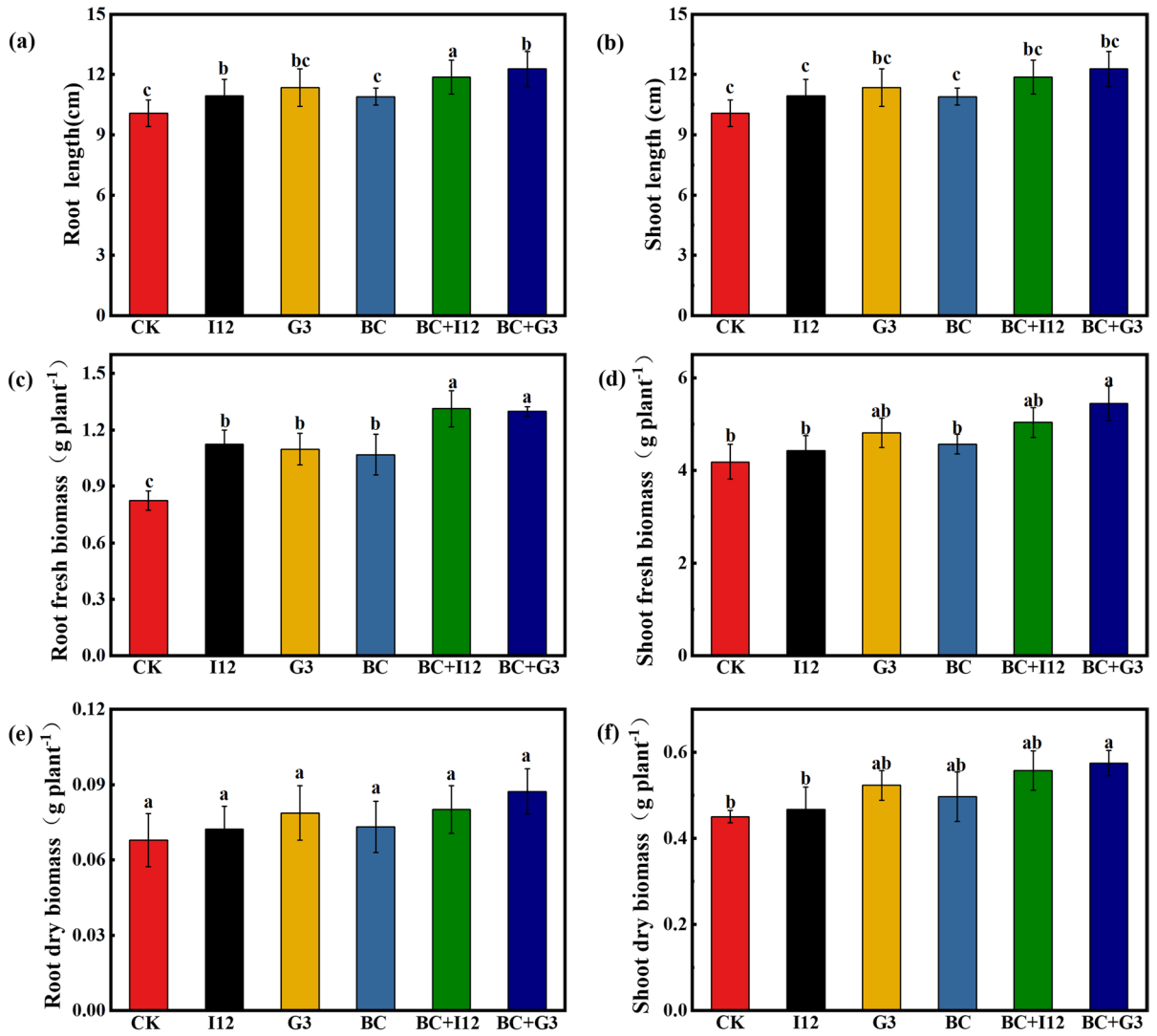


Fig. 7 Effects of different treatment on growth of pakchoi plants. **a** root length; **b** shoot length; **c** root dry biomass; **d** shoot dry biomass; **e** root fresh weight; **f** shoot fresh weight. Values are the mean ± SD, significant differences were calculated with Student's t-test ($p < 0.05$)

as compared to control plants ($p < 0.05$). Especially in BC + G3 treated plants, the fresh weight of underground and aboveground parts increased by 30.28% and 57.78%, respectively. While the dry mass of root and shoot rise by 28.57% and 27.6%, respectively. These outcomes indicate that both bacteria and biochar show beneficial effects on growth of pakchoi plants under heavy metal stress, and a synergistic effect was observed upon the addition of both bacteria and biochar in soil. The improved growth of pakchoi plants upon bacteria treatment could be attributed to the biosynthesis of phytohormone IAA (Zahir et al., 2010), increased availability of nutrients (N, P) (Ahmad et al., 2015), and production of sidesphore (Kong et al., 2017). In addition, beneficial microbes could enhance the antioxidant response in plants by enriching enzymatic and nonenzymatic antioxidants (Huo et al., 2021; Ullah et al., 2019), thus mitigating the heavy metal induced growth inhibition. Biochar could enhance the cation exchange capacity and organic carbon content of the soil, elevate the availability of various nutrients (such as N, P, and K) for plant uptake (Nigussie et al., 2012), improve soil structure to retain soil water content and enhanced soil fertility and microbial activity (Yamato et al., 2006), thus providing a favorable environment for the plant growth. Furthermore, both bacteria and biochar maintain the heavy metal-binding capability, which induced the transformation of highly toxic metal speciation into less toxic speciation, which decreased the heavy metal mobility and phytotoxicity to plants (Figs. 4, 5) and alleviated the growth inhibition caused by Cd and Pb exposure.

Effects of bacteria and biochar on heavy metal accumulation of pakchoi plants

Heavy metal elements in soil enter plant roots via the apoplasmic and symplastic pathways, and then they are transported upwards and accumulated in various plant tissues (Li et al., 2018; Tao et al., 2017). Pakchoi plant is reported to have a strong enrichment capacity for Cd and Pb (Weng et al., 2018). However, the application of bacteria and biochar alone or in combination significantly reduced the Cd, Pb concentration in shoot and root of pakchoi plants (Fig. 8a, b), when applied together, bacteria and biochar showed a synergistic effect. BC + G3 decreased Cd content in root and shoot by 33.83% and 39.88%, it also decreased Pb content in root and shoot by 50.50% and 55.66% as compared with the control. BC + I12 decreased Cd content by 33.01% in root and 43.93% in shoot, it decreased Pb content by 43.20% in root and 51.29% in shoot. The total Cd and Pb accumulation in plants also decreased upon combined bacteria and biochar treatment (Fig. 8c, d). Bochar is known to be an promising adsorbent that immobilizes heavy metal ions efficiently via various functional groups (Tu et al., 2020; Venegas et al., 2016), anions released from biochar could form phosphate and carbonate complex with metal ions (Liu et al., 2018; Wei et al., 2022b), thus prevented their entry into plant roots. Besides, the bacterial strain G3 and I12 used in this study are capable of binding and precipitating Cd and Pb, reducing heavy metal mobility and availability in soil (Figs. 4, 5), and eventually resulted in lower Cd and Pb level in plants. Furthermore, biochar could provide nutrients and habitat for microorganisms (Wu et al., 2021), which may enhanced the reproduction of heavy metal-immobilizing bacteria, and thus indirectly restricts the transportation of metal ions from soil to plants.

Conclusion

The bacterial strains G3 and I12 are highly resistant to Cd and Pb, and they have plant growth-promoting traits, including IAA and siderophores production, nitrogen fixation, and phosphorous solubilization. Both strains have high Cd and Pb removal efficiency, SEM–EDS, XRD and FTIR analyses indicated that they could bind metal ions via various functional groups and form precipitates on cell surface. When applied bacteria alone or in combination with biochar in Cd and Pb co-contaminated soil, they stimulated the transformation of Cd and Pb from HOAc-extractable fraction to residual fraction, thus decreasing the metal bioavailability. Besides, bacteria and biochar application enhanced the soil sucrase, urease and catalase activities, and their application accelerated pakchoi growth while decreased Cd and Pb accumulation in plants. Generally, the combined application of bacteria and biochar showed better heavy metal remediation effects than apply them individually, indicating there is a

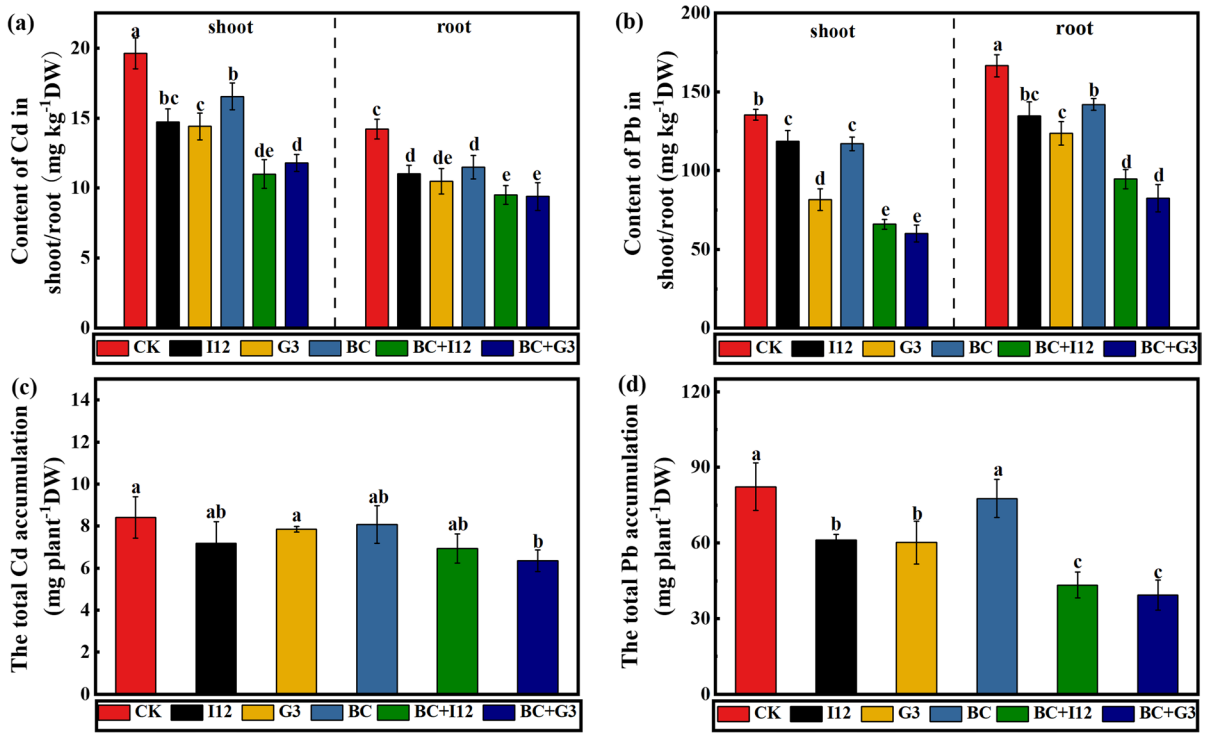


Fig. 8 Effects of different treatment on **a** Cd concentration, **b** Pb concentration, **c** the total Cd accumulation, **d** the total Pb accumulation of pakchoi plants. Values are the mean ± SD, significant differences were calculated with Student's t-test ($p < 0.05$)

synergistic effect between them. This study reveals that the combination of heavy metal-immobilizing bacteria and biochar is a promising in situ technology for remediation of heavy metal polluted soil.

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

Declarations

Conflict of interest The authors declare no competing interests.

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