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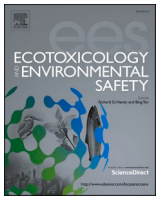

Application of mixotrophic acidophiles for the bioremediation of cadmium-contaminated soils elevates cadmium removal, soil nutrient availability, and rice growth

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ABSTRACT

A major challenge in radically alleviating the threats posed by Cd-contaminated paddy fields to human health is to reduce the Cd levels in both soils and rice grains. In this study, the microbial extraction (ME) treatment using a mixotrophic acidophilic consortium was used for the bioremediation of Cd-contaminated soils. The results showed that the ME treatment enhanced the total Cd (40%) and diethylenetriamine pentaacetic acid-soluble Cd (DTPA-Cd, 64%) removal efficiencies in contaminated soils. In addition, ME treatment decreased the levels of Cd acid-soluble and reducible fractions and thereby reduced Cd uptake in rice tissues. Microbial community analysis indicated that the indigenous soil microbial diversity and composition were not changed after the ME treatment, but the relative abundance of functional microbes associated with Cd removal was improved. Notably, soil available nutrient levels were elevated upon inoculation with mixotrophic acidophiles, resulting in an increase in rice growth and grain weight. This study provides a scientific basis for the potential application and evaluation of ME treatment in the field for remediating Cd-contaminated paddy soils.

1. Introduction

Cadmium (Cd) is one of the most toxic and carcinogenic heavy metals in paddy field ecosystems (Goswami et al., 2017). The atmospheric deposition, sewage irrigation, fertilizer and sewage sludge application are the main sources of Cd input in agricultural soils (Bind et al., 2019; Kumar et al., 2021; Yadav et al., 2021). Rice (Oryza sativa), as the major crop for the global population, easily assimilates Cd compared to other crops (Shi et al., 2020). Cd is nonessential for and nonmetabolizable by living organisms but accumulates at high levels in humans through the soil-rice-food chain, posing tremendous environmental risks and health threats (Dutta et al., 2019). Cd contamination has become an ecological problem worldwide, and is in urgent need of remediation to decrease the adverse hazards of Cd exposure and insure the food safety (Huang et al., 2019).

Soil treatment techniques can be divided into immobilization methods and mobilization methods (Liu et al., 2018). An immobilization strategy can decrease Cd mobility through precipitation, sorption, and chelation between passivators and soil Cd (Jia et al., 2021). However, the immobilization cannot fundamentally eliminate Cd from soils, and the environmental risks resulting from Cd precipitate dissolution are inestimable (Kushwaha et al., 2018). Phytoremediation is a well-studied in situ remediation technology that uses hyperaccumulators to extract Cd from soils in an eco-friendly, low-cost, and labor-saving manner (Wang et al., 2021). However, this method is restricted by its high time cost due to low plant biomass, slow plant growth, and low Cd availability in soils (Xu et al., 2020). Microbial extraction is a mobilization technique that utilizes microorganisms to promote the extraction of

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metals and metalloids from solid materials (Nguyen et al., 2021). This provides a cost-effective approach to minimize soil Cd content by microbial transformation of the insoluble fraction of Cd to a water-soluble fraction of Cd. Cd^{2+} dissolved in the leaching solution can be separated from the solid matter and fundamentally removed from soils (Jacob et al., 2018). In addition, some natural microbes can produce growth-promoting agents, facilitating the acquisition of sufficient nutrition by crops and stimulating the optimal growth (Ahemad, 2019; Liu et al., 2021). Microbial leaching technology has been thoroughly investigated for the extraction of heavy metals from secondary sources (Srichandan et al., 2019), metallurgical slags (Potysz et al., 2018), sewage sludges (Gu et al., 2018), and electronic wastes (Baniasadi et al., 2019). Therefore, using microorganisms in combination with other techniques for heavy metal-contaminated soil remediation has gradually become a research focus in the safe use of agricultural land.

The biological reagents used in soil treatments involve autotrophic and heterotrophic microbes based on the difference in the metabolic substrates of organisms (Xu et al., 2020). Autotrophic bacteria and archaea can oxidize inorganic sulfur compounds and/or ferrous ions, accelerating the dissolution of sulfide fractions of heavy metals (Hao et al., 2021). Heterotrophic microorganisms are able to produce organic acids or complexing agents that can mobilize metals from nonsulfidic fractions (Ghosh and Paul, 2015). Recently, mixotrophic acidophilic consortia with high phylogenetic diversity and functional diversity have been used in the bioleaching of toxic metal-contaminated soils (Hao et al., 2019). The cooperative leaching system is competitive and preferable for the mobilization of multiple chemical fractions of Cd compared to systems that use only autotrophic or heterotrophic bioleaching (Beolchini et al., 2009). However, most studies on the bioremediation of Cd-contaminated soils using mixed cultures are laboratory investigations. Suitable experimental conditions in laboratory-scale remediation system, such as a low solid-to-liquid ratio (< 10%, wt/vol), long experimental period, and adequate stirring time between soils and leaching solution, can guarantee high Cd removal efficiency, but these conditions are difficult to achieve in pilot-scale tests (Gan et al., 2015; Li et al., 2016; Yang et al., 2016). In addition, previous studies have focused mainly on using microbial treatment to improve Cd extraction yields. Comprehensive evaluations of the effects of bioremediation operations on soil nutrient changes, indigenous microbial ecology, and crop yield after microbial treatment remain lacking (Li et al., 2017). These results will help identify an effective and sustainable remediation method to reduce the environmental pressure caused by Cd pollution.

In this study, the microbial extraction performance using a mixotrophic acidophilic consortium was investigated to remediate Cd-contaminated paddy soils with a high solid-to-liquid ratio and short-term stirring. We hypothesized that the mixotrophic acidophiles can effectively remove Cd from soils and have little effects on soil nutrients and microbial community. The objectives of this study were to: (i) measure the effects of mixotrophic acidophiles on total Cd and available Cd removal efficiencies; (ii) investigate the effects of mixotrophic acidophiles on indigenous microbial community changes and soil properties; (iii) evaluate the effects of mixotrophic acidophiles on rice growth and grain weight.

2. Materials and methods

2.1. Sampling of Cd-contaminated paddy soils

The Cd-contaminated soils used in this study were collected from an agricultural paddy field (top 20 cm) located in Xiangtan County, Hunan Province, Southeast China (27°77′ N, 112°88′ E). The rice fields were once adjacent to a steel smelter factory and had been contaminated mainly by Cd-bearing irrigation water for a decade. After removing stones and plant roots from the soils, the obtained soil samples were quickly packed on dry ice and brought back to the laboratory. One part of the soils was stored at 4 °C for soil physiochemical measurements. The other part was used for the subsequent soil remediation experiments.

2.2. Enrichment and cultivation of mixotrophic acidophiles

For enrichment of mixotrophic acidophiles, acid mine drainage (AMD) samples were obtained from a copper mine in Jiangxi Province, China (29°04′ N, 117°71′ E). The AMD (100 mL) and fresh Cd-contaminated soils used in this study (10 g) were added to a 2 L Erlenmeyer flask containing 900 mL of basal salt medium. The medium contained 3 g/L $(NH_4)_2SO_4$, 0.1 g/L KCl, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, and 0.01 g/L $Ca(NO_3)_2$. In addition, 1 g/L elemental sulfur, 0.7 g/L glucose, and 0.3 g/L yeast extract were added as the energy sources. The cultures were shaken at 175 rpm and 32 °C. After the pH value of the bacterial solution decreased to 2.5 and the bacterial density was approximately 10^7 cells/mL, the subculture operation was performed by transferring the bacterial solution as inoculum (100 mL) into fresh basal salt medium (900 mL), and culturing under the aforementioned culture conditions. The subcultivation of mixotrophic acidophiles was performed 10 times, and the culture was then used for the subsequent bioremediation experiment. The bacterial solution properties of mixotrophic acidophiles are listed in Table 1.

2.3. Soil remediation experiments

Soil remediation experiments were carried out in polyethylene pots (30 cm tall and 40 cm diameter). Fresh soil samples (10 kg) were weighed into each pot to a height of 15 cm. In this study, three experimental treatments were prepared: (i) control with deionized water (CK); (ii) chemical extraction with pH 2.5 basal salt medium (CE); and (iii) microbial extraction with a pH 2.5 bacterial consortium solution (ME). The three different solutions (2 L) were added to each pot and stirred for 20 laps (0.5 lap per second) manually to blend the soils and the solution. To ensure sufficient bacterial amounts for inoculation, the supernatants in three remediated groups were separately collected after one day of standing, and replaced by the corresponding fresh leaching solution (2 L) and handled as mentioned above. The soil remediation experiments lasted for seven days at ambient temperature. Each treatment was performed in triplicate. The soil samples in the three remediation experiments were collected on day 7 to analyze the variations in soil physiochemical properties, Cd removal, and the microbial community.

The supernatants collected (approximately 1.9 L per day) in the three remediated groups were separately stored in 15 L buckets (a total of seven times). Functional unimolecular nanomaterials (150 g) developed by the humic acid coated with Fe_3O_4 nanoparticles were added into the buckets to adsorb the extracted Cd^{2+} in the supernatant at a mass-to-volume ratio of 1% (Liu et al., 2008). The supernatant was stirred at 300 rpm for 5 min using a digital display electric mixer (JJ-1, Liangyi Instrument Equipment Co., Ltd., Shenzhen, China). The Cd^{2+} concentrations before and after adsorption treatment were determined by ICP–OES (Optima 5300DV, PerkinElmer, Shelton, USA).

Table 1
Properties of bacterial solution of mixotrophic acidophiles (mean ± SD, n = 3).

Property	Bacterial solution
pH	2.5 ± 0.1
ORP, mV	494.7 ± 1.4
Total K, mg/L	242.8 ± 0.8
Total P, mg/L	62.6 ± 0.6
SO_4^{2-}, g/L	4.9 ± 0.2
Gluconic acid, mg/L	2755.1 ± 26.5
Oxalic acid, mg/L	25.3 ± 1.2
Citric acid, mg/L	19.4 ± 1.1
Malic acid, mg/L	0.3 ± 0.2

2.4. Pot experiments

Seeds of the indica rice cultivar Jinyou 13 were obtained from the Hunan Academy of Agricultural Sciences to evaluate Cd uptake in rice tissues after the three different soil treatments. The seeds were sterilized with 3% NaOCl for 10 min and then rinsed with deionized water. The seeds were then germinated in gauze soaked with distilled water under dark conditions at 30 °C for four days. The germinated seeds were selected and sown in polyethylene pots filled with the treated soils. After three weeks, the uniform seedlings were thinned to five plants per pot. During the rice growth period (110 days), the pots were placed in a glasshouse with a day/night regimen of 14 h/10 h at 30/25 °C and a relative humidity of 60%. After harvest, the rice roots, stems, leaves, and grains in each group were separated, dried, and acid digested with HNO_3/$HClO_4$ (5:2, vol/vol), and the Cd levels in the rice tissues were measured by ICP–OES (Hao et al., 2019).

2.5. Chemical analysis

Soil samples were air-dried and digested by an acid mixture of HNO_3, HF, and $HClO_4$ (10:5:1, vol/vol), and total element concentrations (total Cd, P, and K) were measured by ICP–OES. The soil pH and oxidation reduction potential (ORP) were measured in a 1:2.5 (wt/vol) soil-to-deionized water suspension using a pH meter (BPH-220, Bell Instrument Equipment Co. Ltd., Dalian, China). The alkali-hydrolyzed N, total N and exchangeable Ca^{2+} levels were measured by the acid titration method. The DTPA-Cd content in the soils was evaluated by the diethylenetriamine pentaacetic acid (DTPA) extraction method. Briefly, air-dried soils (5 g) and extraction agent (25 mL) containing 0.005 M DTPA, 0.1 M triethanolamine, and 0.01 M $CaCl_2$ were added to 100 mL shake flasks and shaken at 25 °C for 2 h (180 rpm). The suspension was centrifuged for 5 min at 3000 rpm and then measured by ICP–OES. The distribution of Cd fractions (acid-soluble fraction, reducible fraction, oxidizable fraction, and residual fraction) in soils was carried out using the improved BCR sequential extraction procedure (Hao et al., 2021).

The pH and ORP values in bacterial solution were directly measured by the pH meter. The concentrations of total P and total K were determined by ICP-OES. The SO_4^{2-} contents in bacterial solution were evaluated by the barium sulfate precipitation method (Zhang et al., 2015). The organic acid (citric acid, oxalic acid, malic acid, and gluconic acid) concentrations in the bacterial solution of mixotrophic acidophiles were determined by Agilent 1200 HPLC (Agilent Technologies Co. Ltd., Santa Clara, USA).

The total Cd and DTPA-Cd removal efficiencies in soils were calculated as follows: removal efficiency (%) = $(M_1 - M_2) / M_1 \times 100$, where M_1 is the Cd quantity in soils before remediation treatment, and M_2 is the Cd quantity in soils after three remediation treatments. For the Cd removal efficiencies in different rice plant tissues, M_1 represented the Cd quantity in plant tissues of the CK group, and M_2 represented the Cd quantity in the CE- and ME-treated groups. For the adsorption efficiency of nanomaterials to Cd^{2+} in the supernatant, M_1 represented the Cd concentration before the adsorption treatment, and M_2 represented the Cd concentration after the adsorption treatment. Besides, we calculated bioconcentration factor (BCF) as the following formula: BCF = Cd contents in rice tissues / Cd contents in soils.

2.6. Microbial community analysis

2.6.1. DNA extraction and amplicon sequencing

Genomic DNA of mixotrophic acidophiles used for the ME treatment was extracted by the E.Z.N.A. Water DNA Kit (Omega BioTek Inc., USA). Genomic DNA from the soil samples in the CK, CE, and ME treatments on day 7 was extracted using an E.Z.N.A. Soil DNA Kit (Omega Bio-Tek Inc., USA). The primers 515F and 806R with a sample-specific barcode sequence were used to amplify the V4 region of bacterial 16 S rRNA genes. PCR amplification was performed in a total volume of 25 μL containing 10 μM each primer (1 μL), template DNA (1 μL), 2 × Taq PCR Master Mix (Vazyme, Piscataway, USA) (12.5 μL), and DNase-Free deionized water (9.5 μL). The cycling procedure was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 30 s and a final extension step at 72 °C for 10 min. The PCR products of each sample were amplified in triplicate, mixed and purified by the E.Z.N.A.TM Gel Extraction Kit (Omega Bio-Tek Inc., USA). For each sample, recovered PCR products were pooled in approximately equimolar amounts to form a composite sample. The MiSeq 500 cycle kit was used for 2 × 250 bp paired-end sequencing using the Illumina MiSeq platform.

2.6.2. Data preprocessing

The raw Illumina data were processed on the Galaxy pipeline developed by Prof. Zhou's laboratory (http://zhoulab5.rccc.ou.edu/) at the University of Oklahoma. The detailed procedure and threshold values were described by previous studies (Kong, 2011; Magoč and Salzberg, 2011). Sequences with a clustering threshold of 97% were clustered into the same operational taxonomic units (OTUs) (Edgar, 2013). The taxonomic annotation for each OTU was obtained through the Ribosomal Database Project (RDP) classifier with a minimal 50% confidence score (Wang, 2007).

The alpha diversity (Shannon index, Pielou evenness, and OTUs) of the soil microbial community was determined on the website of the Institute for Environmental Genomics (IEG), University of Oklahoma (http://ieg.ou.edu/). A Venn diagram was drawn at the OTU level using 'Venny 2.1′ (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Detrended correspondence analysis (DCA) for comparing the heterogeneity of soil physicochemical properties and microbial community structure among the three treatments was conducted on the R statistical platform with the vegan package. Pearson correlation tests were performed to assess the contributions of environmental variables to the bacterial community (DCA 1). Partial least square path modeling (PLSPM) was constructed using the 'amap', 'shape', 'diagram', and 'plspm' packages to show the association of Cd removal with soil physicochemical properties, microbial community, and grain weight.

2.7. Statistical analysis

Statistical analyses were conducted with IBM SPSS Statistics version 21.0 software. Differences in soil properties, Cd removal efficiency, soil microbial diversity, and Cd content in rice tissues among the three treatments were analyzed using one-way analysis of variance (ANOVA). The least significant difference test (LSD) was used to identify the significance ($P < 0.05$) of differences between means.

3. Results

3.1. Cd removal efficiencies and changes in soil physicochemical properties

The soil physicochemical properties are shown in Table 2. The total Cd content in this soil (14.9 mg/kg) exceeded the Environmental Quality Standard for Soils of China (0.4 mg/kg) and was approximately 37 times higher than the standard threshold (EPM, 2018). The ME treatment using a mixotrophic acidophilic consortium reduced the Cd levels in soils. After the ME treatment on day 7, the total Cd (14.9 mg/kg) and DTPA-Cd (6.2 mg/kg) levels in the original soils decreased to 9.0 mg/kg and 2.2 mg/kg, respectively, and the corresponding removal efficiencies reached 40% and 64% (Fig. 1a). There were no statistically significant ($P > 0.05$) differences in the total Cd levels between the CK and CE groups. However, the total Cd and DTPA-Cd levels in ME-treated soils were significantly ($P < 0.05$) lower than those in the CK and CE treatments. For each Cd fraction, in ME-treated soil, the removal efficiencies of Cd acid-soluble and reducible fractions were significantly ($P < 0.05$) higher than those in the CK and CE groups (Fig. 1b). No significant ($P <$

Table 2
Soil physicochemical properties (mean ± SD, n = 3) before and after deionized water extraction (CK), chemical extraction (CE), and microbial extraction (ME).

Property	Before treatment	After treatment		
	Original soil	CK	CE	ME
Total Cd, mg/kg	14.9 ± 0.3 a	14.0 ± 0.1 ab	12.2 ± 1.8 b	9.0 ± 1.5c
DTPA-Cd, mg/kg	6.2 ± 0.6 a	5.7 ± 0.1 a	3.8 ± 0.4 b	2.2 ± 0.5c
pH	6.5 ± 0.1 a	6.3 ± 0.1 a	5.7 ± 0.1 b	4.4 ± 0.1c
ORP, mV	199.4 ± 4.5c	216.4 ± 3.6 b	210.6 ± 1.7 b	277.5 ± 1.9 a
Alkali-hydrolyzed N, mg/kg	218.0 ± 15.6 b	227.7 ± 23.4 b	294.7 ± 20.0 a	280.7 ± 4.2 a
Available P, mg/kg	1.9 ± 0.2 b	0.8 ± 0.1 d	1.4 ± 0.1c	2.2 ± 0.1 a
Total N, g/kg	2.3 ± 0.1 ab	2.2 ± 0.2 b	2.3 ± 0.1 ab	2.4 ± 0.1 a
Total P, g/kg	0.7 ± 0.1 a	0.6 ± 0.1 b	0.7 ± 0.1 a	0.7 ± 0.1 a
Total K, g/kg	14.4 ± 0.2 a	14.7 ± 0.6 a	14.1 ± 0.5 a	14.8 ± 0.3 a
Organic matter, g/kg	41.2 ± 1.0 a	37.8 ± 2.6 b	41.1 ± 0.7 a	40.8 ± 0.5 a
Exchangeable Ca^{2+}, cmol/kg	10.6 ± 0.3 b	9.5 ± 0.3c	10.9 ± 0.2 b	12.2 ± 0.8 a
Available Mn, mg/kg	72.2 ± 9.6c	45.7 ± 4.5 d	84.6 ± 1.8 b	128.0 ± 4.4 a

Different lowercase letters in the same row indicate significant differences ($P < 0.05$, LSD) among the three treatments.

0.05) differences were observed in Cd removal from oxidizable and residual fractions among the three treatments. Compared with those in the CK and CE groups, the percentages of activated Cd fractions (acid-soluble and reducible fractions) declined after ME treatment, and the stabilized Cd fraction (residual and oxidizable fractions) percentages increased in the soils (Fig. 1c). These results indicated that the ME treatment reduced the amounts of acid-soluble and reducible fractions of Cd and decreased Cd availability in contaminated soils. In the adsorption experiment, the adsorption efficiency of Cd^{2+} in the supernatant extracted from the soils reached 99.6%, demonstrating that the nanomaterials efficiently adsorbed and removed Cd^{2+} from the supernatant.

Variations in soil physicochemical properties were evaluated before and after the three treatments. DCA showed that the replicates in each treated group on day 7 were clustered together and separated from the other two treated groups, indicating that the soil physicochemical properties were changed after the three treatments (Fig. 1d). The ME treatment significantly ($P < 0.05$) lowered the soil pH while increasing the ORP values compared to the CK and CE groups (Table 2). The levels of alkali-hydrolyzed N, total N, total P, and organic matter in the CE and ME treatments significantly ($P < 0.05$) increased compared to those in the CK group. Moreover, the available P, exchangeable Ca^{2+}, and available Mn levels in the ME group were significantly ($P < 0.05$) higher than those in the CK- and CE-treated soils, indicating that the ME treatment increased the soil nutrient levels and improved the availability in soils.

3.2. Soil microbial community

The soil microbial communities after the CK, CE, and ME treatments on day 7 were analyzed by 16 S rRNA amplicon sequencing to explore the effects of the different treatments on indigenous soil microbes. The alpha diversity of the soil microbial community was not changed among the three groups (Fig. 2a). The Shannon index, Pielou evenness, and observed richness (OTU numbers) showed no significant ($P > 0.05$) differences among the three treatments. The Venn diagram showed that there were 1261 shared OTUs (36.5%) among the three soil samples. The soils in CK, CE, and ME treatments had only 384, 470, and 534 unique OTUs, respectively (Fig. 2b). DCA showed that the microbial community structures of the CK and CE soils were clustered together but were different from those of the ME soil samples (Fig. 2c). The dominant

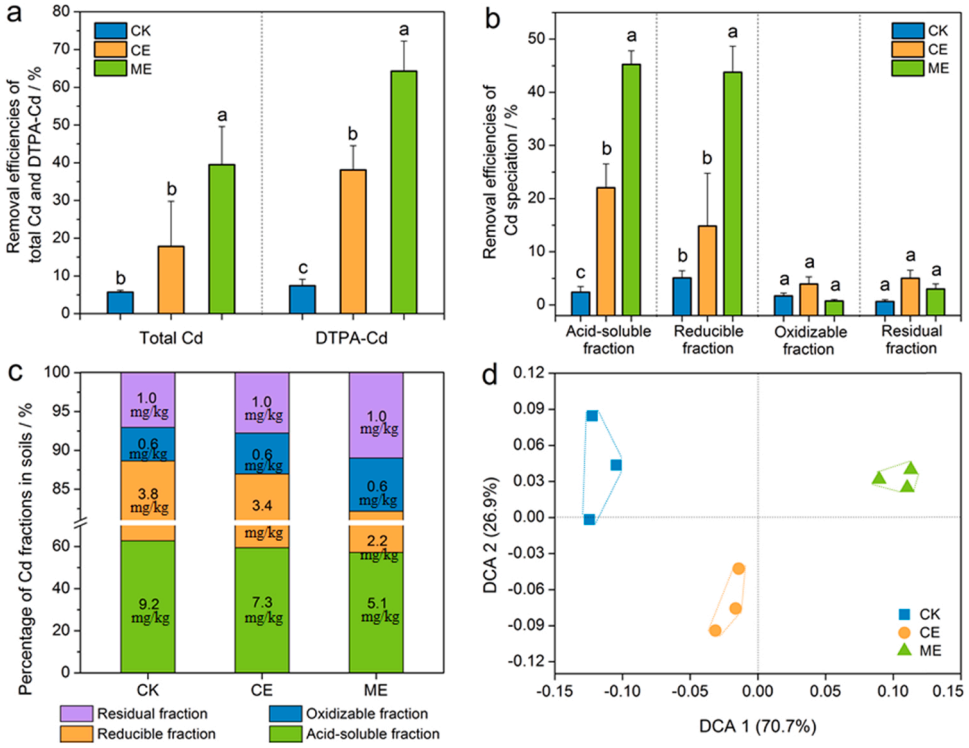

Fig. 1. Effects of the deionized water extraction (CK), chemical extraction (CE), and microbial extraction (ME) treatments on the total Cd and DTPA-Cd removal (a), each Cd fraction removal (b), Cd fractional distribution (c), and soil physicochemical properties (d). Different lowercase letters above the error bars indicate significant differences ($P < 0.05$, LSD) among the three treatments. Quantitative values represent the levels of each Cd fraction in soils after the three treatments.

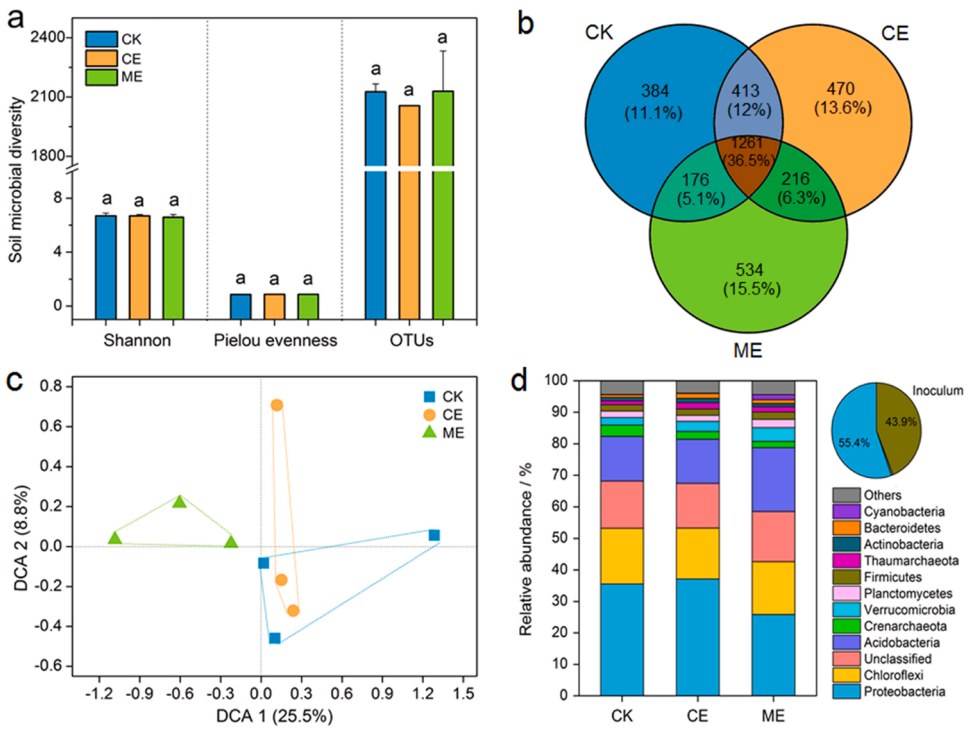

Fig. 2. Variations in alpha diversity indexes (a), OTU distribution (b), microbial community structure (c), and microbial community composition (d) after the deionized water extraction (CK), chemical extraction (CE), and microbial extraction (ME) treatments. Different lowercase letters above the error bars indicate significant differences ($P < 0.05$, LSD) among the three treatments.

microbial phyla in the mixotrophic acidophilic consortium were *Firmicutes* (55.4%) and *Proteobacteria* (43.9%) (Fig. 2d). The phyla *Proteobacteria*, *Chloroflexi*, and *Acidobacteria* were dominant and accounted for more than 65% of those communities in the three treated soils. ME treatment increased the relative abundance of the phylum *Acidobacteria* compared to that in the CK and CE groups, while the relative abundance of *Proteobacteria* was decreased. The results demonstrated that the ME treatment did not alter the soil microbial community composition at the phylum level among the three treatments but changed the relative abundance of dominant microbes.

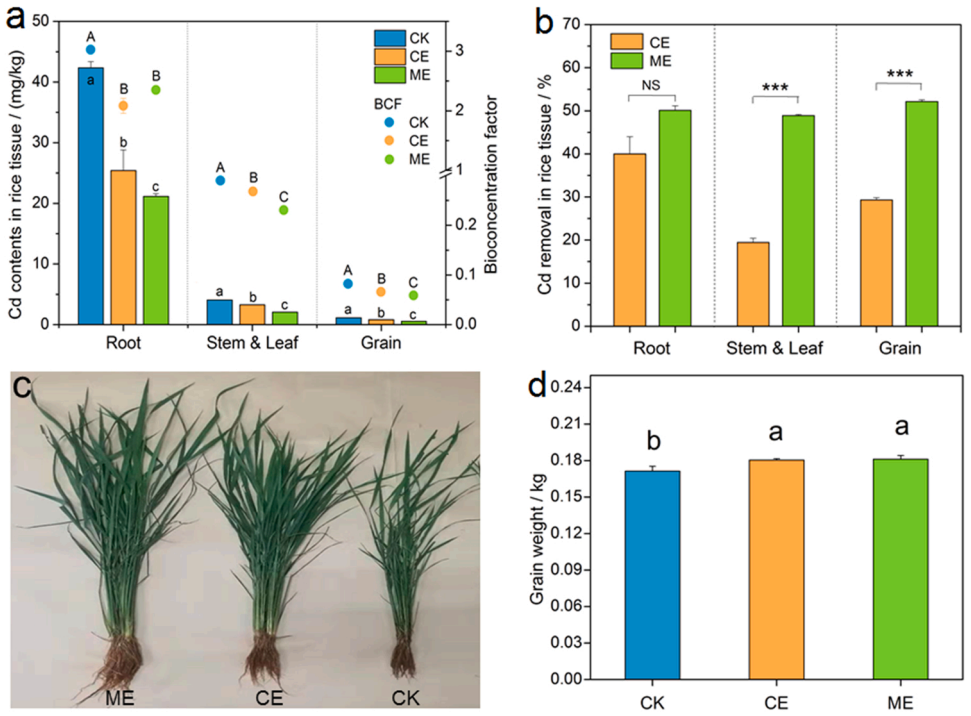

Fig. 3. Effects of the deionized water extraction (CK), chemical extraction (CE), and microbial extraction (ME) treatments on Cd uptake (a), Cd removal in rice tissues (b), rice growth (c), and grain weight (d). Different lowercase, capital letters, and asterisks above the error bars indicate significant differences ($P < 0.05$) among the three treatments.

3.3. Cd uptake and Cd removal in rice tissues

The effects of different treatments on Cd uptake were investigated by planting rice in CK-, CE-, and ME-treated soils. Significant differences ($P < 0.05$) in Cd accumulation levels in each rice tissue were observed between the plots for the three treatments (Fig. 3a). The ME treatment significantly ($P < 0.05$) reduced Cd uptake in different rice tissues. Besides, compared with the BCFs in rice root (3.03), stem and leaf (0.29), and grain (0.08) of the CK group, the BCFs were significantly ($P < 0.05$) decreased by 2.35, 0.23, and 0.06 after the ME treatment, respectively. The Cd removal efficiencies of the ME treatment in rice stems, leaves, and grains calculated from the Cd levels compared to the CK treatment were significantly higher than those of the CE group (Fig. 3b). After the ME treatment, the treated soils had a positive effect on rice plant growth. The rice plants grown in ME-treated soils were taller and thrived better than those grown in CK- and CE-treated soils (Fig. 3c). The grain weights after harvesting in the CK-, CE-, and ME-treated soils were also estimated (Fig. 3d). No significant differences ($P > 0.05$) in grain weights were observed between the ME and CE treatments, but the grain weights in these treatments were significantly ($P < 0.05$) higher than those in the CK treatment.

3.4. Associations among soil bacteria, soil physicochemical properties, and Cd removal

Pearson correlation analysis showed that the soil total Cd and DTPA-Cd levels were significantly ($P < 0.01$) positively correlated with the soil pH (Fig. 4a), indicating that Cd removal was improved by the decrease in soil pH. Soil pH also had a significantly ($P < 0.05$) negative correlation with the levels of total N, exchangeable Ca^{2+}, available P, and available Mn. In addition, soil microbial community differentiation (DCA 1) was positively correlated with the soil pH but negatively associated with the soil ORP, available P, and available Mn levels. Correlations among the soil bacterial genera, soil Cd removal, and grain weight are shown in Fig. 4b. The results clearly showed that three genera, namely, *Aquabacterium*, *Terrimonas*, and *Mycobacterium*, were positively correlated with total Cd and DTPA-Cd removal. Bacterial genera such as *Acidobacteria*_Gp1, Gp13, Gp25, *Rhizomicrobium*, and *Subdivision3_genera_incertae_sedis* had a significant ($P < 0.01$) positive correlation with F2 (reducible fraction) removal. The grain weight was positively correlated with the genera *Aciditerrimonas*, *Basilea*, and *Geothrix*. The relative abundance of some genera associated with soil Cd removal and grain weight were altered after the three treatments (Fig. 4c). Compared to the CK and CE treatments, the ME treatment significantly ($P < 0.05$) increased the relative abundances of *Acidobacteria*_Gp1, *Acidobacteria*_Gp13, *Subdivision3_genera_incertae_sedis*, and *Candidatus Koribacter*, which were beneficial to soil Cd removal. Additionally, the relative abundance of *Aquabacterium* was significantly ($P < 0.05$) higher in the CE and ME treatments than in the CK treatment.

PLSPM was conducted to disentangle the influences of ME treatment on soil environments, Cd removal, and grain weight (Fig. 5). The results showed that the ME treatment had a significantly positive effect on soil nutrient levels (path coefficient = 0.942, $P < 0.001$), and soil nutrients

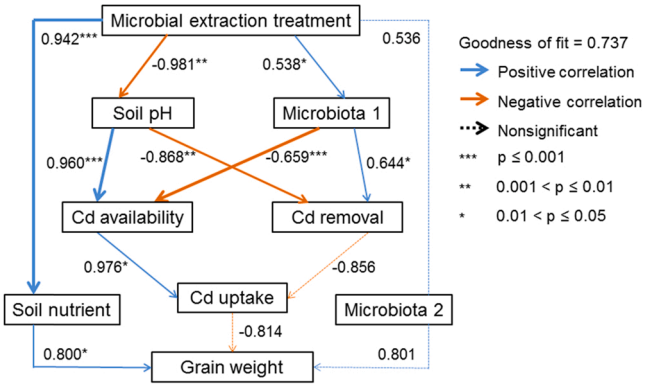

Fig. 5. Partial least square path modeling shows the effects of microbial extraction treatment on soil physicochemical properties, soil microbial community, Cd removal, and grain weight. Microbiota 1 included the genus of *Terrimonas*, *Mycobacterium*, and *Aquabacterium*. Microbiota 2 included the genus of *Aciditerrimonas*, *Geothrix*, and *Aquabacterium*.

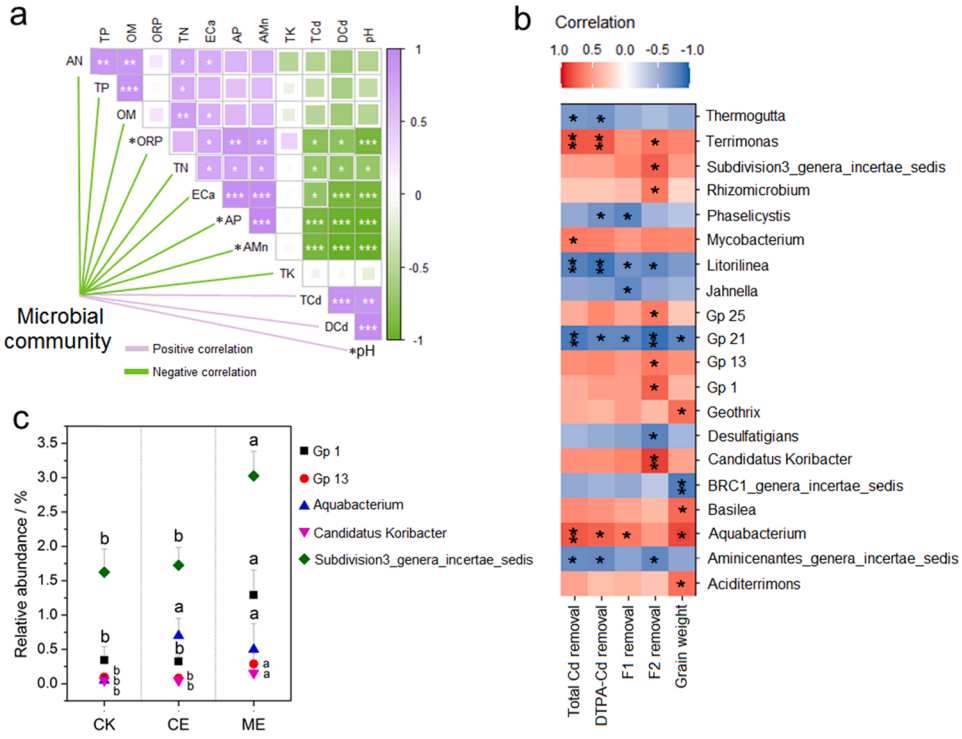

Fig. 4. Association between the soil physicochemical properties and microbial community (DCA 1) (a), linkage between Cd removal, grain weight, and soil bacteria at the genus level (b), and the relative abundance of microbial genera associated with Cd removal in deionized water extraction (CK)-, chemical extraction (CE)-, and microbial extraction (ME)-treated soils (c). Different lowercase letters and asterisks above the error bars indicate significant differences ($P < 0.05$) among the three treatments.

exerted a significantly direct effect on grain weight (path coefficient = 0.800, $P < 0.05$). ME treatment exerted a significantly negative effect on soil pH values (path coefficient = −0.981, $P < 0.01$) and showed significantly indirect effects on Cd availability (path coefficient = 0.960, $P < 0.001$) and Cd removal (path coefficient = −0.868, $P < 0.01$). In addition, the decrease in Cd availability under ME treatment decreased Cd uptake in rice tissues (path coefficient = 0.976, $P < 0.05$). The ME treatment also had a direct impact on soil microbial abundance. Microbial consortium 1, including *Terrimonas*, *Mycobacterium*, and *Aquabacterium*, had significantly direct effects on Cd availability (path coefficient = −0.659, $P < 0.001$) and Cd removal (path coefficient = 0.644, $P < 0.05$), whereas microbial consortium 2, including *Aciditerrimonas*, *Geothrix*, and *Aquabacterium*, had no significant effect on grain weight. In general, PLSPM demonstrated that the ME treatment could regulate soil pH values and microbial abundance through a direct pathway to increase soil Cd removal and decrease soil Cd availability, thereby reducing Cd uptake in rice tissues. Moreover, ME treatment could directly increase the soil nutrient levels and indirectly improve the grain weight.

4. Discussion

In this study, we systematically investigated the effects of ME treatment using mixotrophic acidophiles on the bioremediation of Cd-contaminated paddy soils under conditions of a high solid-to-liquid ratio and short-term stirring. The results demonstrated that ME treatment enhanced the soil Cd removal efficiency and decreased Cd accumulation in rice tissues. Moreover, the soil available nutrient levels were elevated after ME treatment and promoted rice growth and grain weight.

ME treatment significantly ($P < 0.05$) reduced the total Cd and DTPA-Cd levels in contaminated soils (Table 2) and increased the removal efficiencies of acid-soluble and reducible Cd fractions. Cd biotoxicity and Cd accumulation in rice crops are not only determined by the total Cd levels, but are also more dependent on the percentages of each Cd fraction in soils (Deng et al., 2019). The acid-soluble fraction of Cd mostly coprecipitated with carbonate minerals, which was sensitive to the soil pH change (Tessier et al., 1979). Cd release in the acid-soluble fraction was achieved through the dissolution of solid materials with a pH 5 reagent (Gleyzes et al., 2002). In this study, the soil pH after the ME treatment decreased to 4.4 with the bacterial solution added (Table 2), resulting in a 45.2% removal efficiency of acid-soluble Cd (Fig. 1b). Compared with CK-treated soils, the soil pH significantly declined by 1.9 units in ME-treated soils, attributing to a large number of H^+ would be generated with the addition of bacterial solution containing sulfuric acid and organic acids (Table 1). This result was similar to the previous studies. Cd-contaminated soils washing using the citric acid solution (pH 3) decreased the soil pH from 5.7 to 3.7 within seven days (Yang et al., 2022). Liu and Lin (2013) also reported that the soil pH was decreased from 6.80 to 2.80 after citric acid washing. The decrease in soil pH could increase the Cd mobility in soils, but was needed for Cd release from the soils to be stable in solution phase. The reducible fraction of Cd was bound to hydrous oxides of Fe, Mn, and Al in soils. The reducing reagent ($E^0 = -1.87$ V and pH 2.0) of hydroxylamine hydrochloride was used to extract this fraction (Huang et al., 2021). After the ME treatment, the soil pH and ORP values were 4.4 and 277.5 mV, respectively, removing only 43.8% of the reducible fraction of Cd. The oxidizable fraction of Cd incorporated into insoluble polymeric materials and organic matter should be dissolved under hyperoxidation conditions, such as in the presence of the oxidizing reagent H_2O_2 or NaClO. The residual fraction of Cd was coated on the crystalline lattice of silicate and primary and secondary minerals. The Cd released in the residual fraction should be digested by a strong acid mixture. For these four Cd fractions, acid-soluble and reducible fractions with higher bioavailability can be easily activated and accumulated by plants; conversely, oxidizable and residual fractions are relatively stable and nonextractable. After ME treatment, lower acid-soluble and reducible fraction percentages and levels of Cd were observed compared to those under the CK and CE treatments (Fig. 1c), which facilitated alleviation of the environmental stress and health risk of Cd contamination.

High-throughput sequencing technology expeditiously enabled us to obtain more information on the soil microbial community. Notably, the dominant microbial taxa identified among mixotrophic acidophiles were dissimilar to those recorded in ME-treated soils. Further analysis indicated that there were no significant differences between the CK, CE, and ME treatments in terms of both alpha diversity and the relative abundance of dominant microbes (Fig. 2), which indicated that the introduced bacterial consortium had minor noticeable effects on the indigenous soil microbial community. There could be several reasons for this nonsignificant change in bacterial composition after the three soil treatments. One of the possible reasons is that a total of 14 L of bacterial solution (10^7 cells/mL) was added to 10 kg of contaminated soil (10^9 cells/g) in batches over a 7-day experimental period. The bacterial amounts in the enriched bacterial consortium were less than the amounts of indigenous microbes, which hardly altered the soil microbial community structures. Another mechanism explaining our results is the buffering capacity of soils, such as neutralizing acidity and resistance to invasion by exogenous microbes, allowing the soil to persistently recover from the disturbances associated with the introduced bacterial consortium (Deng et al., 2022). Enriched bacterial acidophiles were not colonized in ME-treated soils, and therefore, the potential environmental risks such as continuous soil acidification could be avoided. The increased Cd removal observed in the ME treatment was mainly attributed to a beneficial indirect effect of the bacterial consortium on Cd mobilization in contaminated soils. Metabolites such as organic acids and sulfuric acid produced by the enriched mixotrophic acidophiles might play a key role in Cd removal performance (Camargo et al., 2018). Therefore, it is worth noting that the metabolic activity and function of the bacterial consortium observed in this study deserve further exploration.

The mixotrophic acidophile consortium used in the ME treatment was enriched from the AMD and Cd-contaminated soils. Indeed, our previous study based on a 15-day column bioleaching experiment showed that the addition of mixotrophic acidophiles to contaminated soils removed 34% of total Cd and 87% of DTPA-Cd (Hao et al., 2019). In this study, compared to the CK and CE treatments, the ME treatment did not alter the soil microbial community structure but caused a proportional change in the abundance of some indigenous microbes (Fig. 4c), which showed a significantly ($P < 0.05$) positive association with Cd removal (Fig. 4b). The genera *Gp1* and *Gp 13* belonging to the phylum *Acidobacteria* were sensitive to pH shifts and preferred relatively acidic soil conditions (Mendes et al., 2015). This phylum could create acidic microsites in soils and increase Cd availability in Cd-contaminated soils with near-neutral pH (Conradie and Jacobs, 2021). The genera *Aquabacterium* and *Candidatus Koribacter*, which have nitrate-dependent Fe (II) oxidation and manganese oxidation capacities, respectively, could accelerate microbial iron/manganese redox cycling and efficiently dissolve the intermetallic compounds (Allward et al., 2018; Li et al., 2015). These results were reasonable given that the enriched bacterial genera in the ME-treated soils were thought to be positively associated with metal-bearing compound oxidation and in turn enhanced Cd removal from Cd-contaminated soils.

The ME treatment significantly ($P < 0.05$) decreased Cd accumulation in rice tissues (Fig. 3a). The introduced bacterial consortium reduced Cd availability in contaminated soils, alleviating the Cd migration from soils to grains (Fig. 3b). A previous study reported that Cd shared many physical characteristics, such as charge and ionic radius, with Ca (Perfus-Barbeoch et al., 2002). Cd could permeate into Ca channels through guard cells and root cells and was accumulated by the plant tissues. In this study, the increased exchangeable Ca^{2+} content after the ME treatment might have suppressed Cd translocation in the soil–plant system through competitive assimilation with Ca (Table 2). In

addition, the gene expression of *OsNRAMP5* and *OsNRAMP1* in rice roots, regulating Cd tolerance and transport in rice plants, could be inhibited by the inoculation of exogenous microbes, thereby inhibiting Cd transport and reducing shoot Cd accumulation (Sasaki et al., 2012; Takahashi et al., 2011). More importantly, the grain weight of plants grown in ME-treated soils was significantly ($P < 0.05$) higher than that of plants in the CK group (Fig. 4d). These results were reasonable and could be explained by the following scenarios: first, the bacterial consortium enhanced Cd removal and reduced the Cd available fractions in soils, thereby alleviating the Cd toxicity in rice plants at the cellular level. Second, the increase in available nutrient levels in soils (e.g., alkali-hydrolyzed N, available P, total N, total P, exchangeable Ca^{2+}, and available Mn) after ME treatment was beneficial to the rice growth (Jankong et al., 2007). Soil fertility parameters could be used as indicators to evaluate the effects of ME treatment on soils. Compared with CK-treated soils, the average contents of alkali-hydrolyzed N, available P, and total P in ME-treated soils were increased by 23.3%, 175.0%, and 16.7%, respectively. The decreases of soil pH values after ME treatment contributed to the dissolution and transformation of unavailable nitrogen and phosphorus into the available fractions (Guo et al., 2018; Ren et al., 2015), thus in turn increasing their contents. Clearly, the soil exchangeable Ca^{2+} was significantly elevated from 9.5 cmol/kg to 12.2 cmol/kg owing to extreme release of Ca during the ME treatment, which can be attributed to the strong replacement by protons (Liu and Lin, 2013).

From a practical perspective, the actual Cd removal efficiency of the ME treatment used in this study was higher than the Cd phytoextraction efficiency reported in previous studies, although laboratory results cannot fully reflect the field conditions (Phieler et al., 2015). In this study, the ME treatment using the mixotrophic acidophiles was able to remove an estimated 40% of the total Cd within 7 days from the Cd-contaminated soils. This figure (40%) was higher than not only that (4.63%) of the *A. carambola* inoculated with a bacterial consortium enriched from the AMD at a low soil Cd level (4.4 mg/kg) but also that (1.15%) recorded for the *E. splendens* inoculated with AMF and *Penicillium* at a high soil Cd level (7 mg/kg) within 1 year (Li et al., 2017; Wang et al., 2007). In addition, previous study showed that soil washing with EDTA removed 71% of total Cd, but the soils after EDTA washing could not support the growth of buckwheat (*F. esculentum*). Soil washing with the biodegradable chelating agents such as EDDS and IDS was less efficient and removed only 20% and 31% of Cd (Gluhar et al., 2020).

We believe that the ME performance can be applied to actual field applications. The scale-up cultivation of functional strains or microflora has been achieved the industrial level. The amount of mixotrophic acidophiles can satisfy the demands in field ME treatment. A microbial cultivation workshop can be placed near contaminated rice paddies, and the bacterial solution can be transported into paddy fields through irrigation canals or carrier vehicles. Irrigation water can be used for the cultivation of mixotrophic acidophiles. It is worth noting that the stability of microbial community structure and function in the scale-up cultivation process of mixotrophic acidophiles is the important scientific issue that needs further study. This stability may be guaranteed by increasing the inoculum dose and by removing the microbial biomass of irrigation water, which used for the microbial cultivation. The manual mixing operation used in this study can be replaced by conventional agricultural tillage methods to blend the bacterial solution and contaminated soils. After plowing and natural sedimentation, the supernatants can be drained out and separated from the soils. Deep soil layer should be protected from the stir disturbance in mixing operation. The automated machinery with the capability of plowing at a constant depth needs to be designed and applied to blend the solid liquid mixtures. Finally, the supernatant liquid can flow out through the drainage ditches and be collected in a reservoir under gravity. Impermeable films should be laid at the bottom and sides of the drainage ditches and reservoir. The absorbents of heavy metals should be added into the reservoir to adsorb Cd^{2+} in the supernatant liquid. The purified liquid can be reused for the cultivation of a bacterial consortium. It is therefore expected that ME treatment using mixotrophic acidophiles can elevate Cd removal from contaminated soils from the laboratory scale to the field scale.

It was thought that the ME treatment barely damaged the surrounding soils and groundwater systems. First, the mixotrophic acidophiles were enriched from the natural environments (AMD and Cd-contaminated soils). After the ME treatment, the introduced microbe did not colonize the treated soils and only affected the changes in the proportions of indigenous bacteria. Continuous soil acidification could thus be avoided. However, the indigenous microbial community structure and function after the ME treatment need to be continuously examined over a long period of time. Second, the soil physicochemical properties were not changed dramatically by the ME treatment. In contrast, the ME performance improved the soil nutrient levels or availability and further promoted rice growth and grain weight. Third, paddy fields had a dense plow pan layer, which can prevent the supernatant liquid from seeping down and contaminating the groundwater system. Fourth, the ME treatment procedure is as follows: (i) the cultured bacterial solution is transported into paddy fields and then mixed with contaminated soils; (ii) the supernatant liquid outflows into the collecting reservoir through the drainage ditches; and (iii) after adsorption, the purified solution is reused for the cultivation of mixotrophic acidophiles. Therefore, the ME treatment performance is a relatively closed loop operation and has little chance of polluting the surrounding environment and therefore can be considered an environmentally friendly bioremediation technology for paddy field ecosystems.

5. Conclusions

In the present study, the bioremediation of cadmium-contaminated soils was conducted by the ME treatment using the mixotrophic acidophiles. We identified the impact of ME treatment on soil Cd removal, nutrient turnover, rice growth, as well as microbial communities. Specifically, the ME treatment significantly increased the removal efficiencies of total Cd and available Cd from Cd-contaminated soils, and triggered a significant reduction in Cd accumulation of rice tissues. The ME treatment elevated the soil nutrients levels such as alkali-hydrolyzed N, available P, total K, and exchangeable Ca^{2+}, which enhanced the rice growth and grain weight. More significantly, the addition of mixotrophic acidophiles had little effect on soil microbial community composition but just improved the proportions of indigenous functional bacteria positively associated with the soil Cd removal. Future study will be carried out to explore the feasibility of ME treatment applied in field scale and to verify the potential ecological impacts on paddy field environments.

CRediT authorship contribution statement

Baoxing Yuan: Investigation; Data curation; Visualization; Writing–original draft. **Lihua Huang**: Methodology; Software. **Xueduan Liu**: Conceptualization; Supervision; Funding acquisition. **Lianyang Bai**: Conceptualization; Supervision; Project administration. **Hongwei Liu**: Visualization. **Huidan Jiang**: Methodology. **Ping Zhu**: Investigation. **Yunhua Xiao**: Software; Visualization. **Jiaobiao Geng**: Software; Visualization. **Qianjin Liu**: Supervision; Funding acquisition. **Xiaodong Hao**: Conceptualization; Investigation; Data curation; Methodology; Visualization; Writing–review & editing; Supervision; Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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