Immobilization of lead in tailings by Bacillus subtilis through microbially induced phosphate precipitation (MIPP)

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Abstract: The accumulation of heavy metal tailings has raised serious environmental concerns, especially in China. Therefore, remediation and utilization of heavy metal tailings have become urgent and vital. In this paper, an emerging technology MIPP (microbially induced phosphate precipitation) was applied and the commercial Bacillus subtilis were employed to mitigate the Pb contamination risks in tailings. Through aqueous Pb-removal experiments and tailings remediation experiments, the process and mechanism of MIPP treatment for heavy metal tailings were systematically explored. In addition, the optimal Pb-removal conditions were discussed. The results shows that the commercial Bacillus subtilis can be served as a preferable MIPP-stabilization commercial strain with lower application cost, better heavy metal tolerance, and excellent Pb stabilization compared to traditional strains isolated from contaminated sites. The mobile Pb was almost converted into Pb₅(PO₄)₆ through MIPP treatment contributing to the Pb immobilization. According to SPLP (the synthetic precipitation leaching procedure), the leaching concentration of Pb from tailings dropped from 0.86 mg/L before treatment to less than 0.02 mg/L after MIPP remediation, which is far below China's groundwater environmental requirements (0.1 mg/L). This technology has the advantages of on-site construction and environmental friendliness. The treated tailings are expected to be safely used as construction sand for road base and mine backfilling.
Keywords: Bacillus subtilis, microbially induced phosphate precipitation (MIPP), heavy metal tailings, remediation, leaching.

1. Introduction
Tailings stack has reached 6 billion tons in China, which has occupied a large amount of land and can contaminate surrounding soils and water [1, 2]. The heavy metals (HMs) in tailings cannot be degraded by microorganisms or chemicals, but may persist for a long time and threaten humans [3]. Therefore, the safe treatment and resource utilization of these wastes becomes urgent and significant.

In this paper, lead (Pb) - zinc (Zn) tailings obtained from Sichuan, China with prominent Pb pollution are remediataed. Pb is a toxin, accumulating in the human body mainly through the food chain, tailing dust, contaminated water, which can harm the liver, kidney, nervous system, and blood vessels, especially for children [4]. Many chemical methods have been used to reduce the bioavailability and mobility of Pb. However, most of them have the disadvantages of high operating costs, secondary pollution, incomplete precipitation, and inhibition of soil fertility [5]. Recently, several studies on the removal of Pb through bio-mineralization via plants, macro-algae, fungi, and bacteria have been reported. Bio-mineralization is a universal natural phenomenon inducing (heavy) metal ions to precipitate into insoluble minerals, which are critical in controlling the metal cycling and mineral formation on the surface of the earth [6]. Among them, MIPP (microbially induced phosphate precipitation) is attracting attentions among researchers for Pb stabilization, since the phosphate is able to effectively immobilize the unstable Pb. In the MIPP biomineralization process, metabolic activity like organic acid, phosphatase, or phytase produced from microbes can increase the availability phosphate incorporated and then mineralize toxic ions as a result of precipitating phosphorus-containing minerals [7, 8].

The phosphorus microorganism currently applied for heavy metal pollution mitigation is mostly isolated from the contaminated site in order to assure the local microbial ecological balance and a strong tolerance of bacteria to pollutants. However, the intricate isolation process of optimal bacteria undoubtedly increases the economic and time cost. Therefore, it is urgent for exploiting a commercial, ecological, and effective MIPP strain. Bacillus subtilis (BS), which is widely existing in the natural soil ecosystem and reported to have high phosphorus solubilization capability [9], is selected and used in this paper. The the tolerance of BS to Pb and the remediation mechanism of MIPP for Pb containing tailings are elucidated in the study. MIPP is expected to have the advantages of in-situ construction, flexible process, and environmental friendliness. After treatment, the tailings are expected to be used as construction sand.

2. Materials and methods

2.1. Materials
The tailings were taken from a Pb-Zn tailings pond in Miyi County, Sichuan Province. The content of major heavy metals in tailings is shown in table 1. It can be seen that the content of Pb in tailings is as high as 2279.47 mg/kg, and that of Zn reaches 30451.60 mg/kg. According to US EPA Method 1312 (2003) (SPLP: synthetic precipitation leaching procedure), the leaching concentrations of Pb under
acid rain environments are 0.86mg/L, which is much higher than the regulation limit of 0.1mg/L according to GB/T 14848 (2017) in China while other heavy metals meet the requirements. Therefore, this paper focuses on the stabilization of Pb in tailings.

Table 1. Content of major heavy metals in tailings (mg/kg).

|     | Cr   | Cd      | Pb       | Cu       | Zn     | Ni       | Mn     | As     |
|-----|------|---------|----------|----------|--------|----------|--------|--------|
|     | 63.39| 140.39  | 2279.47  | 127.07   | 30451.60| 36.42    | 720.00 | 61.88  |

Industrially mass-produced and non-toxic sodium glycerolphosphate (SGP) was used as the organophosphorus, which was purchased from Suzhou Tianke Trading Co., Ltd.. The stock solution of Pb was prepared by dissolving Pb(NO$_3$)$_2$ (AR) in deionized water. Pb solutions used in this work were prepared by diluting the stock solution to desired concentrations.

2.2. Bacterial strains, growth conditions

Bacillus subtilis (CCTCC AB 98002) was employed in this paper due to its reported dephosphorylation ability [10]. In addition, BS is metabolically efficient, non-pathogenic, and widely found in natural soil. Luria-Bertani medium (LB: 10 g NaCl, 10 g tryptone, and 5g yeast extract in 1 000 mL H$_2$O) was selected as a liquid nutrient for fermentation, preservation, and reaction of strains. The bacteria were cultured in a shaker at the speed of 120 rpm at 30 °C for 20h, then the bacterial suspension was used for MIPP.

2.3. Pb toxicity assays

The tolerance of bacteria to heavy metal toxicity is exhibited through MIC (minimal inhibitory concentration), which refers to the lowest concentration of heavy metals that cause total inhibition of growth [11]. For this, BS was grown in Pb-containing (0, 100, 300, 500, 700, 1000, 1100, 1200, 1300mg/L) LB medium for 20h under 180r/min and 30C. Then the CFU (colony forming unit) of such surviving Pb-exposed cells was determined by dilution coating plate method on corresponding LB agar plates after incubation of 48 h. The minimum Pb concentration of culture solutions without bacterial life signs is served as MIC.

2.4. Pb removal efficiency

The removal effect of MIPP on different concentrations of Pb (100, 300, 500, 700, 1000mg/L) in contaminated water was studied. The appropriate amount of Pb stock solution and SGP were added to 80 mL of bacterial suspension followed by pH adjust with NaOH and HCl to make up 100 mL of neutral reactant solution containing target concentration of Pb and 0.05M of SGP. Two blank control reaction solutions, one containing bacterial suspension and Pb (500 mg/L) but no SGP, and the other containing SGP and Pb (500 mg/L) but no bacterial suspension, were set up to determine the contribution of bacterial suspension and SGP alone on Pb removal. The experimental conditions for all samples were kept the same.

It has been confirmed that moderate amounts of Mg could promote cellular life activities and stabilize the structure of phosphatases [12], thus Mg was additionally added and expected to improve the treatment effect in this paper. Based on the aforementioned reaction solution preparation, preset amount of MgCl$_2$ was additionally added to 80 mL of bacterial suspension, resulting in the final 100
mL of neutral reactant solution containing 500mg/L Pb, 0.05M SGP and target concentration of Mg\(^{2+}\) (0.01, 0.02, 0.03M).

All the above reaction solutions were set at room temperature for 3 days and then centrifuged. The concentration of Pb in the supernatant was determined by ICP-OES (Agilent 720ES) and the representative centrifugation precipitation was analyzed by X-ray diffraction analysis (XRD) and transmission electron microscopy (TEM).

2.5. Remediation of tailings
In the customized PVC two-part mold, the dried tailings sand was compacted in 5 layers to the target relative density (Dr) of 90\%, and the diameter of the prepared sample was 39.1mm with a height of 80mm. Neutral reactant solutions containing 80\% bacterial suspension, 0.05M SGP, and 0.03M MgCl\(_2\) were injected from the bottom of the sample at a rate of 0.5mL/min through a peristaltic pump (Langer Pump BT100-1L) until they overflowed from the top of the sample. The samples were cured at 20±3°C for 3 days. After that, the samples were subjected to SPLP test. All trials were conducted in triple to obtain data with significance at p≤0.05.

2.6. Characterization of Pb-loaded precipitation

**XRD:** The sample for XRD analysis was dried frozenly and finely grounded to pass the 200 mesh sieve. The crystalline phases of minerals were analyzed by XRD (Shimadzu XRD-6000) with Cu Ka radiation (k¼0.15418nm) at 40kV and 30mA. The angle was set to 10–70°, with 2°/min (2θ).

**TEM:** The sample preparation process is based on previous literature [13]. Thin sections of samples were prepared with an ultramicrotome (Leica, Germany), placed on 200-mesh Formvar-coated copper grids, and viewed with the Libra 120 Plus TEM (Carl Zeiss).

3. Results and discussion

3.1. Resistance of bacteria to Pb toxicity
Heavy metals can damage and inhibit bacterial life activities mainly through binding the functional groups related to cell metabolism and change the enzyme structure. As shown in figure 1a, along with the increase of Pb concentration, fewer bacteria survived, especially when Pb concentration is greater than 750 mg/L. When the Pb concentration reached 1200 mg / L, no BS survived. It can be concluded the Pb concentration of 1200 mg / L is the MICs of BS. The tolerance of bacteria applied here to lead exceeds the average resistance capacity of bacteria isolated from the lead-contaminated site (figure 1b), thus is suitable to the vast majority extent of Pb pollution in contaminated sites or water.
3.2. **Pb removal efficiency**

As can be seen from figure 2a, with the treatment of MIPP system, more Pb was precipitated by phosphate as the Pb content increased, but at the same time more Pb remained in the supernatant, and the removal rate of Pb gradually decreased, mostly due to the bacteria and phosphatase were inhibited severely. In general, the removal of Pb by MIPP was efficient in the range of Pb concentration of 0-1000 mg/L, with the removal rate exceeding 98%. The results of the blank control groups showed that the bacterial suspension alone could only remove about 25% of Pb in the polluted water. The Pb concentration drops from 500 mg/L to 373.3 mg/L mainly due to the adsorption and complexation effect. In addition, the SGP alone was able to reduce the Pb concentration from 500 mg/L to 154.5 mg/L, with a removal rate of about 70%, because the phosphoric groups in SGP can chelate Pb well. The removal performance of Pb through adsorption and complexation perhaps further promotes the remediation effectiveness, in addition to the contribution of biomineralization.

**Figure 1.** Tolerance of bacteria to Pb: CFU of BS surviving in Pb-contaminant medium (a); the MIC of bacteria to Pb used herein and reported in the literature [11, 13-18] (b).

**Figure 2.** Removal of Pb in the concentration range of 100-1000 mg/L (a) and in presence of small amounts of Mg (0.01, 0.02, 0.03M) (b).
According to figure 2b, the removal of Pb was significantly improved in the presence of Mg, and was better with the increase of Mg concentrations. Importantly, Pb concentrations after treatment with Mg can meet environmental standards for groundwater and surface water compared to remediation without Mg.

3.3. Remediation of tailings
After MIPP treatment by Bacillus subtilis, the concentrations of Pb in the leaching solution of the stabilized tailings were lower than the detection limit of 0.02mg/L by ICP-OES, which meets the requirements of the groundwater environment in China (≤ 0.1mg/L), indicating that there is no environmental risk for the tailings after remediation. The stabilized tailings are expected to be an alternative of construction sand with significant economic and ecological benefits.

3.4. Pb removal mechanism and process
According to the XRD pattern in figures 3(a) and (b), after treatment with MIPP technique, the Pb ions in the contaminated water were converted to Pb_6(PO_4)_3, attributed to the mineralization effect of BS. In this process, BS induced the decomposition of sodium glycerophosphate to release inorganic phosphorus (a small amount of H_2PO_4^- and PO_4^{3-}, and a large amount of HPO_4^{2-} following thermodynamic principle). Then Pb combined preferably with PO_4^{3-} to form Pb_6(PO_4)_3 because the solubility of phosphate is lowest. It is noteworthy that in the case of added Mg, there is absence of Mg in the mineralization product despite it is more abundant than Pb (figure 3(b)). This could be caused by the facts that some Mg ions were taken up by bacteria as essential elements, as well as being embedded in phosphatases to stabilize the protein structure, resulting in a small amount of free Mg^{2+} remaining, and the electronegativity of Pb (2.33) is greater than that of Mg (1.31), which makes it easier for Pb to bind and precipitate with P, while inhibiting the precipitation of the remaining Mg^{2+}. Furthermore, there is also no peak of Pb-GP (Pb-Glycerophosphate) in the precipitation after MIPP treatment (figure 3(a) and (b)) although the SGP has the potential to complex and precipitate 70% of Pb (figure 3(c)), which is mostly because Pb-GP is extremely unstable in the face of BS and is easily hydrolyzed by the phosphatase secreted from BS.

![Figure 3. XRD patterns of Pb-stabilized bio-precipitates in cases of MIPP treatment (a), MIPP](image-url)
treatment in present of Mg (b), and chelation of SGP (c).

As shown in figure 4a, during the microbial mineralization reaction, some heavy metals enter into bacterial cells and precipitation with the acid ions hydrated in internal bubbles; some are absorbed by functional groups on the bacterial surface and by extracellular polymeric substances surrounding the bacteria, which then promptly bind to the released acid ions; the others in the bacterial suspension precipitates with the diffused hydrolyze acid ions from bacteria [19]. The TEM observations (figure 4b) also indicate this process. A small amount of mineralized product was formed in the internal and cell surface of the cells, and most of them were mineralized in the bacterial suspension. The mineralization products in the bacterial suspension were flocculent, while that inside the bacteria were tightly agglomerated which may be related to the organized microbial activity.

![Conceptual diagram (a) and TEM images (b) of bio-mineralization.](image)

**Figure 4.** Conceptual diagram (a) and TEM images (b) of bio-mineralization.

In summary, mineralization is the main mechanism of heavy metals removal by bacterial in the MIPP system, which converted Pb in the contaminated water and tailings into environmentally stable $\text{Pb}(\text{PO}_4)_6$. Besides, the adsorption and complexation of Pb by bacteria and SGP either directly contributed to the removal of Pb, or acted as intermediates and nucleation sites for mineralization, playing an important role in the MIPP system. The principle of Pb fixation in MIPP can be demonstrated by the following equation (1-7):

1. $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} (\text{SGP}) + \text{H}_2\text{O} \xrightarrow{\text{Bacillus subtilis}} \text{Na}_2\text{HPO}_4 + \text{C}_3\text{H}_6\text{O}_3$
2. $\text{HPO}_4^{2-} \xrightarrow{} \text{H}^+ + \text{PO}_4^{3-}$
3. $6\text{PO}_4^{3-} + 9\text{Pb}^{2+} \rightarrow \text{Pb}_6(\text{PO}_4)_6$
4. $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} (\text{SGP}) + \text{Pb}^{2+} \rightarrow 2\text{Na}^+ + \text{C}_3\text{H}_7\text{PbO}_6\text{P} (\text{GP-Pb})$
5. $6\text{C}_3\text{H}_7\text{PbO}_6\text{P} (\text{GP-Pb}) + 3\text{Pb}^{2+} + 6\text{H}_2\text{O} \xrightarrow{\text{Bacillus subtilis}} \text{Pb}_6(\text{PO}_4)_6 + 6\text{C}_3\text{H}_6\text{O}_3 + 6\text{H}^+$
6. $\text{Pb}^{2+} + \text{BS} \rightarrow \text{BS-Pb}^{2+}$
7. $\text{BS-9Pb}^{2+} + 6\text{PO}_4^{3-} \rightarrow \text{BS-Pb}_6(\text{PO}_4)_6$

**4. Conclusions**

In this paper, industrial bacteria $\text{BS}$ were used to stabilize Pb in tailings by MIPP technology. The toxicity resistance of bacteria, Pb removal effect, mechanism and process of MIPP, as well as leaching
potential of the MIPP-treated tailings were examined.

The MIPP technology can effectively stabilize Pb in contaminated wastewater and tailings. The removal rate exceeds 98% for wastewater with Pb concentrations of 0-1000 mg/L, and up to 99.99% in the presence of small amounts of Mg²⁺. The treated wastewater meets the environmental standards of groundwater (0.1mg/L) in China. Through the remediation of MIPP, the leaching concentration of Pb declines from 0.86mg/L of the untreated sample to less than 0.02mg/L, which also complied with the environmental standards. Immobilizing Pb by phytase-induced phosphate to form Pb₄(PO₄)₆ precipitates are the linchpins of stabilization. Compared with strains isolated from contaminated sites in previous literatures, the commercial BS used in this study has excellent heavy metal tolerance and lower application cost, and is therefore a more promising stabilizer for MIPP technology.

EIPP technology provides a new perspective for disposal and utilization of heavy metal tailings with excellent ecological benefits. The treated tailings are expected to be safely used as building materials, such as road base and mine backfilling. Further researches on the durability and field-scale application of the treated tailings are necessary. In addition, organic phosphorus in wastewater or solid wastes is also desired to be extracted and applied in the following researches to reduce the cost of this technology.

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