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Synergistic Effect of Pb Resistant Bacteria and *L. macroides* US3 Biostimulant in Ecorestoration of Pb-Treated Soil

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ABSTRACT
Lead pollution poses a formidable threat to agriculture, bioaccumulating in crops, and ultimately harming human health. Even low-level exposure can significantly reduce crop yields, diminish nutritional value, and precipitate economic losses and food insecurity. To combat this threat, we investigated the efficacy of a novel biostimulant, *Lysinibacillus macroides* US3, previously isolated from the rhizosphere of a plant with enhanced plant growth attributes to promote ecorestoration of a Pb-remediated soil. Four Pb-resistant bacterial (LRB) strains: *Bacillus infantis* K66, *Halopseudomonas xiamenensis* B13, *Lysinibacillus fusiformis* KAF67, and *Pseudomonas* spp. A27 harbouring the gene cluster PbrABCT were employed in the treatment of Pb contaminated soil. The treatment efficacy was remarkable, with final Pb removal percentages of 85%, 82%, 83%, and 83%, respectively while control had a 41% removal. To achieve ecorestoration and facilitate agricultural reuse of soil, maize seeds were planted in the treated soil, and 10% w/v of US3 biostimulant was introduced as liquid culture into the pots except the control pot. Post-cultivation analysis revealed enhanced plant growth and biomass yield in US3 inoculated pots, 48% Pb uptake by maize in control pots while undetected in inoculated pots, 53% residual Pb in control soil while undetected in inoculated soil. The synergistic application of Pb-resistant bacteria and US3 biostimulant effectively ecorestored Pb-stressed soil, demonstrating a promising approach for sustainable Pb mitigation. This study highlights the potential of microbial-based solutions for environmental remediation and agricultural sustainability.

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INTRODUCTION

Lead (Pb) pollution poses a formidable threat to agriculture, as it bioaccumulates in crops, and ultimately harming human health (Chiwetalu *et al*., 2022). Even low-level exposure can significantly reduce crop yields, diminish nutritional value, and precipitate economic losses and food insecurity. Among available removal techniques, microbial remediation has attracted much attention due to its lower cost, higher efficiency, and less impact on the environment; hence, it is an effective alternative to conventional, physical or chemical Pb-remediation technologies (Ehis-Eriakha and Akemu, 2022). Microorganisms have various remediation mechanisms to cope with Pb pollution, which are basically categorized into biosorption, bioprecipitation, biomineralization, and bioaccumulations (Shan *et al*., 2023). Studies have demonstrated that bacteria such as *Azotobacter chroococcum*, *Paenibacillus jamilae*, and fungus like *Aspergillus niger* are efficient in eliminating Pb from the environment (Shan *et al*., 2023; Gul *et al*., 2024). Progress in microbial technology has resulted in the creation of vigorous bacteria and the identification of associated genes, which may improve the effectiveness of removal procedures.Various kinds of molecular defenses are available to bacteria in order to combat Pb toxicity. Some microbes have a group of genes that become active when Pb is present. These genes called pbrTRABCD aid in developing bacteria Pb resistance by turning on different Pb detoxification systems (Li *et al* 2020). Microbial-based remediation of Pb pollution offers a promising approach to environmental management and sustainability. This eco-friendly technique leverages microorganisms to remove or immobilize Pb contaminants, mitigating their harmful effects on ecosystems. However, while microbial remediation can effectively reduce Pb concentrations, it may not necessarily eliminate the ecotoxicity posed by Pb pollutants.

To address this limitation, regenerating the soil system using Plant Growth-Promoting Rhizobacteria (PGPR) is essential. PGPR can enhance soil fertility, promote plant growth, and reduce Pb toxicity, thereby restoring ecosystem balance (Ehis-Eriakha and Adetunji, 2022). This integrated approach combines microbial remediation with PGPR-mediated soil regeneration, providing a comprehensive strategy for managing Pb pollution and promoting environmental sustainability. This study highlights the efficacy of PGPR Lysinibacillus macroides US3 as biostimulant to restore Pb polluted soil treated individually with four resistant bacteria.

MATERIALS AND METHODS

The experimental design for maize crop cultivation was complete randomized design (CRD). Post bioremediation of Pb treated soil with four Pb resistant bacteria, the soil was cultivated with *Lysinibacillus macroides* US3. Planting of seeds was carried out using the technique of Ju *et al*. (2020). Seedlings were transferred into each planting pot (diameter 12cm and depth 10 cm, with 4 kg of soil inoculated with strain US3 per pot and the control pot which was uninnoculated. The pots were irrigated with 250 ml of water weekly under greenhouse condition. This experiment lasted for 28 days and plat growth attributes such as root length and shoot length were monitored at weekly interval. Table 1 shows the experimental design for maize crop cultivation in duplicates

Table 1: Experimental Design for Maize Crop Cultivation

Control Pot 1	Pot 2	Pot 3	Pot 4	Pot 5
4 kg of lead spiked soil + 3 maize seedlings	4 kg of *Pseudomonas* spp lead treated soil + 3 maize seedlings *Lysinibacillus macrolides* strain US3	4 kg of *Bacillus infantis* lead treated soul + 3 maize seedlings + *Lysinibacillus macrolides* strain US3	4 kg of *Lysinibacillus fusiformis* lead treated soil + 3 maize seedlings + *Lysinibacillus macrolides* strain US3	4 kg of *Halopseudomonas xiamenensis* lead treated soil + 3 maize seedlings + *Lysinibacillus macrolides* strain US3

Seed inoculation

Thirty five (35) maize seeds were subjected to a one-minute surface sterilization with 5% sodium hypochlorite, followed by three thorough washes with sterile distilled water. After being air-dried, the seeds were soaked in a bacterial solution, and the mixture was stirred repeatedly for five minutes. Bacterized seeds were spread out gently on a Petri plate and allowed to air dry for a whole night at room temperature. Serial dilutions were used to count the bacterial cells, and the result was estimated to be 10^6 CFU/seed (O.D. = 0.8) (Li *et al*., 2020).

Examination of plant development characteristics

To assess plant growth, shoot and root lengths, fresh weights, and dry weights were measured. The lengths of shoots and roots were recorded bi-weekly for 28 days. The fresh weights of shoots and roots were measured immediately after harvesting. The dry weights of shoots and roots were determined after oven-drying the samples until all internal cell water was removed. The percentage of dry weight was calculated using the formula: Dry Weight Percentage = (Dry Weight / Fresh Weight) × 100 (Cowdhury *et al*., 2020).

Determination of Pb accumulation in soil and the roots of maize crops:

The maize seedlings were gathered, they were divided into distinct roots and shoots, dried separately for 48 hours in an oven set to 650 ºC. Following the Allen, *et al*. (2020) procedure, the oven-dried materials were powdered and dissolved. To do this, 200 mg of the sample's dust was obtained and dissolved using beakers and a hot induction plate in aquaregia (H2SO4: HNO3: HClO4, v/v) in the ratio 1:3:1. After allowing the dissolved samples to cool, they were filtered using nylon syringe filters with a 0.22 m pore size. Double distilled water was used to dilute the samples, and the total volume was 50 ml. Following that, these digested samples were kept at room temperature while an atomic absorption spectrometer was used to calculate the concentration of heavy metals in the plant's roots and shoots (Jiang, 2020).

RESULTS AND DISCUSSION

The 16S rRNA gene sequencing analysis revealed a diverse bacterial community, with four dominant species identified as *Halopseudomonas xiamenensis* strain B13, *Bacillus infantis* strain K66, *Lysinibacillus fusiformis* KAF67 and *Pseudomonas* spp. These bacterial groups belong to the phylum Firmicutes and Proteobacteria. The sequences aligned gave 95 - 100% similarity with those deposited in GenBank and as such considered close relatives.

Table 2: Molecular Identification of Pb Resistant Bacteria

Isolate Code	Closest Relative Ascension No. (bases compared)	Similarity (%)	Phylum	Bacteria Identification
Pbr A	MH734834	100	Proteobacteria	*Halopseudomonas xiamenensis* strain B13
Pbr B	KU922247	98	Firmicutes	*Bacillus infantis* strain K66
Pbr C	MN759656	100	Firmicutes	*Lysinibacillus fusiformis* KAF67
Pbr D	MT250857	95	Proteobacteria	*Pseudomonas* spp

All isolates significantly reduced Pb concentration compared to control soil. *Bacillus infantis* strain K66 demonstrated the highest percentage reduction in Pb levels, closely followed by *Lysinibacillus Fusiformis* strain KAF67, *Pseudomonas* spp. strain A27, and *Halopseudomonas Xiamenensis* strain B13 while control pot exhibited the lowest percentage of Pb reduction (Fig. 1). This observation underscores the effectiveness of the selected microbial strains in mitigating Pb contamination, with *Bacillus infantis* strain K66 emerging as particularly promising in Pb remediation.

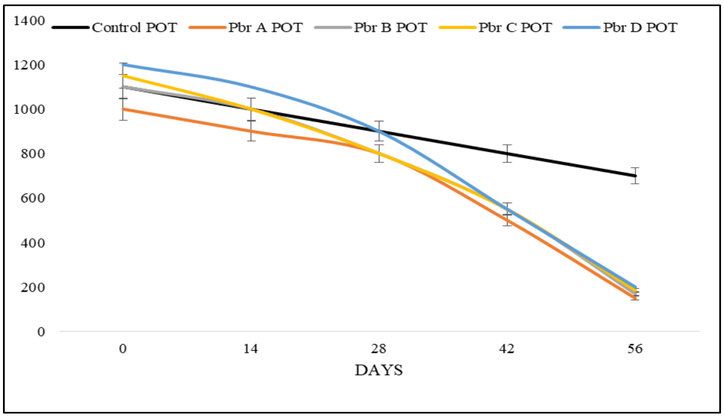

Figure 1: Dynamics of Pb reduction for treatments and control groups (Day 0 - 56)

Inoculating maize seeds with *Lysinibacillus macroides* strain US3 in Pb-treated soil significantly enhanced maize growth under Pb stress. The treated plants showed increased root length, shoot length, chlorophyll content, and dry and wet weights compared to the control. Notably, the absence of US3 in the control pot resulted in minimal growth enhancement, highlighting the bacterium's crucial role in mitigating Pb toxicity in maize cultivation.

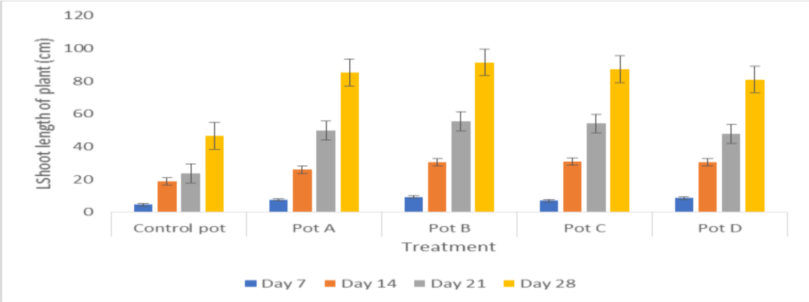

Figure 2: Shoot length of maize plant at day 7, 14, 21 and 28 (cm).

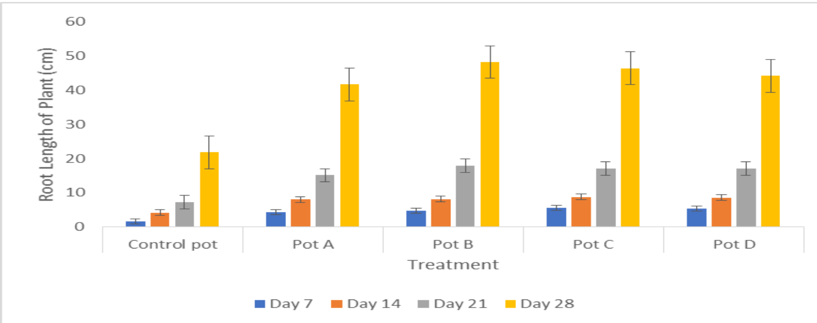

Figure 3: Root length of maize plant at day 7, 14, 21 and 28 (cm)

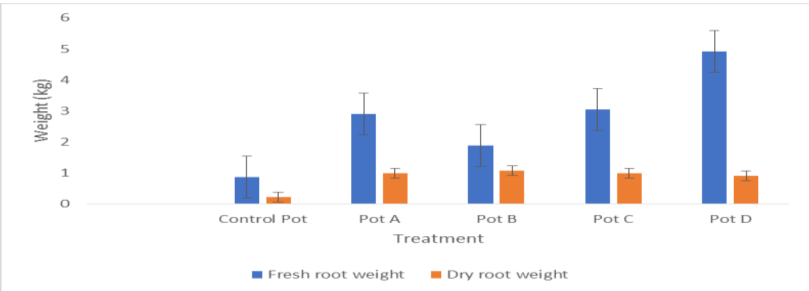

Figure 4: Fresh and dry root weight of the maize plant after cultivation (cm)

Post cultivation, the analysis on Pb uptake in the maize root revealed a notable difference in treated and control pots, it was observed that the control pot exhibited a significant Pb accumulation of 48%, whereas Pb uptake was remarkably absent in the treated pots after 28 days (Table 3). In contrast, the analysis of residual Pb content in the soil revealed that 53% of the initial Pb content remained in the control soil, indicating incomplete immobilization, while detectable levels of Pb were also found in the treated pots, suggesting some degree of Pb retention.

Table 3: Pb Uptake in Maize Root (%) Post Cultivation for 28 days

Treatment	Percentage (%)
Control	53
Pbr1	not detected
Pbr2	not detected
Pbr3	not detected
Pbr4	not detected

Table 4: Residual Pb Content Present In Soil after Cultivation of Maize (%)

Treatment	Percentage (%)
Control	53
Pbr1	not detected
Pbr2	not detected
Pbr3	not detected
Pbr4	not detected

Discussion

Lead (Pb) pollution poses a significant threat to both biotic and abiotic components of the environment. Fortunately, bioremediation has emerged as a cost-effective and eco-friendly approach to detoxify polluted environments. This study explored the synergistic use of four Pb-resistant bacteria (LRB) - *Halopseudomonas xiamenensis* strain B13, *Bacillus infantis* strain K66, *Lysinibacillus fusiformis* KAF67, and *Pseudomonas* spp. - previously isolated from a quarrying site, along with a plant growth-promoting rhizobacterium (PGPR) *Lysinibacillus macroides* US3 to achieve bioremediation and ecorestoration of Pb polluted soil. Lead-resistant bacteria have been implicated in various environmental processes, particularly bioremediation (Sevak *et al*., 2021). The efficacy of the four LRB in Pb removal was evident in the significant decline in Pb concentration across all treatments compared to the control (Fig. 1). This finding is consistent with previous studies, which have documented several bacterial strains capable of removing Pb through diverse mechanisms, including uptake, immobilization, precipitation, biosorption, and other processes. The results of this study reinforce the potential of certain bacterial groups in removing Pb from polluted environments.

The four strains exhibited high Pb removal efficiencies, with strain K66 emerging as the most effective and efficient. Research suggests that certain bacterial genera, possess a greater affinity and sensitivity to Pb, which may explain the variations in Pb reduction gradients across treatments (Mitra *et al*., 2021; Shan *et al*., 2023). Notably, *Bacillus* strains are known for their inherent Pb-resistant properties, which enable them tolerate a wide range of Pb derivatives and Pb-based compounds. This was evident in the exceptional performance of strain K66. This finding is consistent with previous studies, which have demonstrated the efficacy of *Bacillus subtilis* X3 in bioprecipitating Pb in various forms, including $Pb_5(PO_4)_3OH$, $Pb_{10}(PO_4)_6(OH)_2$, and $Pb_5(PO_4)_3Cl$ (Qiao *et al*., 2019). The remarkable Pb removal efficiency of strain K66 further supports its potential as a valuable tool in bioremediation studies.

Beyond bioremediation, ecorestoration of treated environments is crucial for restoring biodiversity and biological activities (Ehis-Eriakha *et al*., 2024). One promising approach to ecorestoration of stressed soil is the use of microbe-based nutrient supplements to enrich nutrient-deficient soil. Plant growth on treated soil is a key strategy for achieving ecorestoration. However, residual toxic effects from pollutants can hinder plant growth by disrupting physiological processes. To address this challenge, this study investigated the use of a plant growth-promoting bioinnoculant, *L. macroides* US3, to support maize growth in treated soils. The results showed that soils amended with US3 supported maize growth, as indicated by improved growth parameters, compared to maize grown on unamended control soil. The beneficial traits of strain US3, including nitrogen fixation, phosphate solubilization, and indole acetic acid production, contributed to its plant growth-promoting effects. These findings are consistent with reports that Plant Growth Promoting (PGP) bacteria enhance plant performance and alleviate stress caused by xenobiotic contamination *(Zhan et al., 2022; Nawaz et al., 2024).*

The addition of strain US3 to Pb-treated soil had a multifaceted impact, enhancing plant growth while promoting the removal of residual Pb from the soil, preventing Pb uptake by the plant, and creating a favorable environment for maize growth. In stark contrast, the control (PGPR-unamended) pot exhibited detectable Pb uptake in maize roots and residual Pb in the soil, which hindered maize growth and development due to Pb toxicity. This highlights the benefits of US3 amendment in promoting plant growth under Pb stress and facilitating ecorestoration. Consistent with our findings, previous studies have shown that heavy metal-resistant and plant growth-promoting bacteria (PGPB) can increase metal uptake efficiency and translocation, enhance heavy metal tolerance in plants, promote growth, and ultimately contribute to accelerated ecorestoration of contaminated soils (Wang *et al*., 2022).

Leveraging on the potential of microbe-based solutions for ecorestoration, various studies have demonstrated the effectiveness of heavy metal-resistant bacteria in promoting plant growth and mitigating metal toxicity. Das *et al*. (2014) reported that As-resistant bacteria capable of oxidizing as and exhibiting PGP traits could support plant growth on As-stressed soils while reducing as toxicity and uptake. Similarly, seed inoculation with bacteria has been shown to improve barley plant growth and nutrient uptake from Pb- and Cd-contaminated soil, while preventing Pb and Cd accumulation in plants (Belimov *et al*., 2002; Cruz-Hernández *et al*., 2022). Abdelkrim *et al*. (2018) further demonstrated the effectiveness of PGPR inoculation in promoting plant growth and tolerance under Pb stress, with significant increases in plant biomass and Pb removal observed in inoculated plants. These findings collectively highlight the potential of microbe-based solutions to enhance plant growth and promote ecorestoration of treated environments.

CONCLUSION

This study underscores the effectiveness of Pb-resistant bacteria, particularly *Bacillus infantis* strain K66, in removing Pb from polluted environments, thereby showcasing its potential as a potent bioremediation agent. Furthermore, the results highlight the synergistic benefits of combining bioremediation with bioinoculation, enhancing plant growth and promoting ecorestoration of treated environments. These findings have far-reaching implications for the development of sustainable, eco-friendly bioremediation strategies to mitigate Pb pollution and restore ecosystem health.

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