RESEARCH ARTICLE

Ex-situ Bioremediation of Heavy Metal-Contaminated Soil Using Bacillus cereus and Micrococcus luteus Consortium: A Phytotoxicity Assessment

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Abstract

Pollution by arsenic, lead, and mercury poses an environmental and health risk. Microbial bioremediation is an environmentally benign process for remediating contaminated soils. The purpose of this study was to assess the influence of two bacterial strains isolated and identified from the soil at Gevra coal mine, Korba, and Mand coalfield, Raigarh: *Bacillus cereus* and *Micrococcus luteus* on the rate of depollution of arsenic, lead, and mercury-contaminated soils. To test this effect, sterile soil was bioaugmented with B. cereus and M. luteus strains individually and in combination for 25 days at 30˚C. The bioaugmentation of the sterile soil with a mixture of B. cereus and M. luteus strains resulted in the highest rate of reduction of Pb^{2+} (80.33%), Hg^{2+} (79.42%), and As^{3+} (74.77%) compared to the rate of bioaugmentation by each bacterial strain individually. These findings are supported by the study of sterile soil, which revealed an increase in the mobility and bioavailability of Pb^{2+}, As^{3+}, and Hg^{2+}. Ecotoxicological responses indicated lower heavy metal concentrations were not associated with lower soil toxicity. These promising results provide another perspective for a soil bioremediation bioprocess that employs bacterial bioremediation.

Keywords: Bioremediation, Bioaugmentation, Phytotoxicity, LC-MS, FTIR.

Highlights:

- Bacterial consortium has been developed for bioremediation of heavy metals using Bacillus cereus and Micrococcus luteus.
- Bacterial consortium indicated great remediation ability to a combination of heavy metals (Pb^{2+}, As^{3+}, and Hg^{2+}) via bioaugmentation method for soil.
- LC-MS analysis revealed information about bioremediation products after bioremediation.
- Phytotoxicity analysis indicated that bioremediation soil is becoming more viable and vital for germination seedlings.

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Introduction

Heavy metal contamination in soils, surface water, groundwater, sediments, and air is a global issue (Mishra, 2017; Xie *et al.,* 2016). Soil pollution by heavy metals is a serious environmental and social problem because of the dangers that these elements can produce, not only for human health but also for biodiversity and the structure of soil organisms and microbial communities (Tran and Popova, 2013). Some heavy metals are essential and required by organisms as micronutrients, while others serve no biological purpose and are toxic even in low quantities (Bruins *et al.,* 2000). Heavy metals are persistent contaminants of great concern since they are non-biodegradable; their risk is exacerbated by their buildup in the environment via the food chain, resulting in serious irreversible damage (Pushkar *et al.,* 2015). This damage can result in anemia, reproductive problems, kidney failure, brain impairment, and cardiovascular disease (Kang *et al.*, 2015). As a result, the necessity to adopt specialized treatment procedures becomes vital. Chemical leaching (Zhang *et al.,* 2020; Abo-Alkasem *et al.,* 2023), reverse osmosis, membrane filtration, stabilization/solidification (Liu X *et al.,* 2021), evaporation, ion exchange, electrochemical treatment, sorption, and precipitation have recently been used to remediate contaminated sites and remove contaminants (Kang *et al.,* 2015; Liu X *et al.,* 2021). However, they have several limitations, including the production of harmful chemical sludge, which is bad for the environment (Yin K *et al.,* 2019; Zhou B *et al.,* 2023). Recently, scientists have been interested in various bioremediation systems due to their amazing benefits and great efficiency in cleaning heavy metal-contaminated soils (Yin K. *et al.,* 2019). Microbial bioremediation is used as a biological treatment for polluted soil (Abbes C *et al.,* 2018; Ayangbenro A.S. *et al.,* 2017). When compared to other de-pollution methods, it is efficient, promising, and environmentally beneficial (Chen M *et al.,* 2021). A diverse range of microorganisms is used as biological tools in the bioremediation process to rehabilitate heavy metal-polluted environments by developing resistance mechanisms

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such as bioaccumulation, biosorption, biotransformation, and biomineralization that adapt to the various toxic metals found in ecosystems. Several bacteria are employed in remediation approaches, including *Pseudomonas stutzeri* LBR and *Cupriavidus metallidurans* LBJ, which were isolated and identified in the lab from Tunisian sediment. (Mansouri A. *et al.,* 2019).

The concept of mixing multiple species with complementary features, known as a consortium, has grown during the previous decade (Santos *et al.,* 2019). The use of consortia is expected to bring numerous advantages over individual species. First, within a consortium, certain members can compensate for qualities that others lack, resulting in bigger overall effects (Kumar *et al.,* 2016; Shilev *et al.,* 2020). Other combinations include plant-growth-promoting bacteria with *Arbuscular mycorrhizal* fungus (Yadav *et al.,* 2022) and drought-tolerant strains with nitrogen fixer isolates (Badde *et al*., 2022). Second, collaborative efforts among consortium members can aid in the development and operation of target strains (Sun *et al.,* 2022). This interaction was also discovered between strains, where plants may obtain stronger salt-stress tolerance (Hashem *et al.,* 2016) and increased organic phosphorous mineralization (Jiang *et al.,* 2021) than inoculated with either bacterium alone. Third, the connection between inoculants and indigenous species is most likely established through antibiotic release and quorum sensing within bacterial consortia (Santoyo *et al.,* 2021). These characteristics allow the employment of microbial consortiums to get more consistent and successful results (Aguilar P.A. *et al.,* 2020; Santoyo *et al.,* 2021; Khan, 2022). Despite the advantages presented above, it is still necessary to understand the use of microbial consortia (Kaminsky *et al.,* 2019; Ramakrishna *et al.,* 2019; Jack *et al.,* 2021).

While several studies have found heavy metal bioremediation via microorganisms (Adams *et al.,* 2014; G.U. Chibuike and S.C. Obiora, 2014; Dirisu, 2015), there is a lack of knowledge on the use of a combination of heavy metal tolerant bacteria in synergy for heavy metal bioremediation (Gupta M.K. *et al.,* 2014). As a result, the purpose of this work was to assess the individual and synergistic capacity of heavy metal-tolerant *B.cereus* and *M.luteus* for bioremediation of Pb^{2+}, As^{3+}, and Hg^{2+} contaminated soils.

Methods

Source of Isolates and Chemicals

The used bacterial isolates were previously isolated from Gevra coal mine Korba (22.336312, 82.545748) and Mand coalfield Raigarh (22°16′6″N 83°20′38″), Chhattisgarh, and were identified as *Bacillus cereus* (Genbank no.-OQ691646) and *Micrococcus luteus* (Genbank no.-OQ691646). The chemicals were used in analytical grade and purchased from the Kasliwal brothers chemical shop in Raipur, Chhattisgarh.

Development of the Bacterial Consortium

One loop full culture of both bacteria was taken to be grown into a nutrient broth medium (Himedia, India) and then incubated at 37°C for 24 hours separately. After incubation to obtain (OD_{600} 0.3–0.6), biomass cultures were centrifuged (R-4C, Remi). This was then suspended in a minimal medium (Singh *et al.,* 2021). The absorbance was measured by UV-vis spectrophotometer (Type 119, Systronics) at 600nm. After that, both cell suspensions were mixed with an equal amount to prepare a bacterial consortium.

Bioremediation Experiment of Heavy Metal via Consortium

The experimental setup was conducted as described by Njoku *et al.,* (2020) with a slightly modified method. The nutrient broths containing an initial concentration of $Pb(NO_3)_2$, $HgCl_2$, and $NaAsO_2$ at 3000 mg/L separately and 1000 mg/L combination of these three metals in the conical flask were incubated with 1 ml of 24 hours cultures of *Bacillus cereus, Micrococcus luteus*, and developed consortium respectively, for 96 h in an orbital shaking incubator at 120 rpm at 37°C. Without microbes, control flasks also were incubated containing the heavy metal simultaneously with the test flasks. The heavy metals accumulated were determined using an atomic absorption spectrophotometer in the microbial biomass and were extracted by acid digesting with nitric acid. The acid-digested biomass was filtered and made up of a volume of 50 mL with distilled water. The given formula calculated the bioaccumulation factor and percentage of remediation.

$$\%\ remediation\ =\ \frac{Initial\ heavy\ metal\ level\ -\ Final\ heavy\ metal\ level}{Initial\ heavy\ metal\ level}\times 100$$

$$Bioaaccumulation\ Factor = \frac{heavy\ metal\ content\ in\ the\ microorganism}{heavy\ metal\ in\ the\ medium\ after\ 96\ h}$$

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was employed to identify functional groups in bacterial strains that could be involved in metal uptake during the biosorption process. This method has been useful in providing structural information on metal cation binding in microorganisms (Gupta *et al.,* 2020). FTIR analysis was performed on cells before and after metal uptake in an aqueous solution containing three metals (As^{3+}, Pb^{2+}, and Hg^{2+}) at concentrations of 10 mg/L. The FTIR (IRTracer-100, Shimadzu) investigation resulted in the appearance of spectra of the control before and after metal uptake (Pagnucco G. *et al.*, 2023).

Liquid Chromatography-Mass Spectrometry (LC-MS)

The bioremediation of heavy metals (As^{3+}, Pb^{2+}, and Hg^{2+}) by *Bacillus cereus* and *Micrococcus luteus* was further confirmed by LC-MS to establish structural information and molecular weights of remediate products created after bacterial treatment and can help to propose the appropriate microbial pathways (Sreedevi *et al.,* 2022). LC-MS (LCMS-8060NX, Shimadzu) analysis was performed utilizing a water, Micromass Q-TOF micro, and a Waters Alliance 2795 separation module with a Unisol YVR C18 4.6*250mm 5um column. Instrumentation control of data collections was carried out using data analysis MRM and unit resolution.

Experimental design with contaminated soil sample

For the bioremediation of heavy metal-contaminated soil bioaugmentation test was performed. In a conical flask containing 500mL M9 medium mixed with 10 g of soil

(Containing 500 mg/kg As^{3+}, Pb^{2+}, and Hg^{2+} separately) and then bacterial ($70x10^7$) cells were inoculated. The experiment setup was carried out in 12 flasks of soil. The experimental design composed with 12 conditions with soil was: (i) (Pb^{2+}, BC1): soil + M9 media + *B. cereus* (ii) (Pb^{2+}, ML1): soil + M9 media + *M. luteus* (iii) (Pb^{2+}, BM 1): soil + M9 media + *B. cereus* + *M. luteus* (iv) (As^{3+}, BC1): soil + M9 media + *B. cereus* (v) (As^{3+}, ML1): soil + M9 media + *M. luteus* (vi) (As^{3+}, BM 1): soil + M9 media + *B. cereus* + *M. luteus* (vii) (Hg^{2+}, BC1): soil + M9 media + *B. cereus* (viii) (Hg^{2+}, ML1): soil + M9 media + *M. luteus* (ix) (Hg^{2+}, BM 1): soil + M9 media + *B. cereus* + *M. luteus* (x) (Pb^{2+}, As^{3+}, Hg^{2+} BC1): soil + M9 media + *B. cereus* (xi) (Pb^{2+}, As^{3+}, Hg^{2+}, ML1): soil + M9 media + *M. luteus* (xii) (Pb^{2+}, As^{3+}, Hg^{2+}, BC ML) soil + M9 media + *B. cereus*+ *M. luteus*. All experimental flasks were incubated in a shaking incubator (150rpm) at 30˚C for 25 days. All tests were performed in triplicate. The bacterial count and heavy metal level were assessed every five days by atomic absorption spectrophotometer.

Phytotoxicity Analysis

To assess the toxicity of heavy metals contaminated soil and its effect on plant growth, a phytotoxicity study was carried out. The experiment was conducted at room temperature on the *C. arietinum* L. crop. Initially, the seeds were washed with distilled water. Then 200 gms of test soil was kept in a pot and 10 seeds of *C. arietinum* L. were equally spread on the soil surface. Distilled water (4ml) was evenly added to the soil and kept in the dark for germination at 25°C for 4 days. After 4 days, seeds were germinated with visible roots, or lengths were measured of the root. The control test was studied in uncontaminated soil collected from nearby sites. *C. arietinum* L. seedlings meristem cells were also analyzed under a fluorescence microscope (Olympus BX63). The toxicity was evaluated in terms of percentage germination and lengths of plumule and radicle after 7 days. The following formula calculated seed germination (%).

$$Germination\% = \frac{Number\ of\ seeds\ germinated}{Total\ numbers\ of\ seeds} \times 100$$

Statistical analysis

Comparisons of the test and control samples were done using Microsoft excel and analysis of variance (ANOVA).

Results

Bioremediation of heavy metals by Bacterial Consortium

The heavy metals residues present after 96 hours of remediation in the broths are given in Table 1. In the control test, arsenic (0.21%) decreased from 1325.89 to 1323.92 mg/L, lead (0.14 %) 2135.68 to 2132.54 mg/L, and mercury (0.42%) from 1869.14 to 1861.68 mg/L. Afterward, heavy metal-contaminated broths incubated with *B. cereus* obtained 54.43% As^{3+}, 16.97% Pb^{2+}, and 38.46% Hg^{2+} remediation percentages obtained. Whereas *M. luteus* inoculated contaminated broths were obtained 49.42, 25.22, and 32.38% remediation of As^{3+}, Pb^{2+}, and Hg^{2+}, respectively. A developed consortium of *B. cereus* and *M. luteus* were remediated As^{3+} 77.73%, Pb^{2+} 55.22%, and Hg^{2+} 70.57% percentage loss after incubation of 96 hrs (Table 1). There was significant variation in the residual arsenic and lead content between the control and treated medium after 96 hours at $p < 0.05$.

The remediation approach of the three metals combinations with consortium was also carried out (Table 2). In the control test with a combination of arsenic, lead, and mercury percentages were reduced, As^{3+}(1.18%) from 253.89 to 250.23 mg/L, Pb^{2+}(0.91 %) 439.56 to 435.25 mg/L and Hg^{2+}(2.86%) content reduced from 123.89 to 120.36 mg/L obtained after 96 hrs of incubation. The mixture of the metals in broth inoculated with *B. cereus*, the As^{3+} intensity (58.46%) decreased from 260.69 to 108.98 mg/L, Pb^{2+}(52.83%) 458.78 to 216.54 mg/L and Hg^{2+}(63.23%) 136.25 to 50.25 mg/L obtained after 96 hours of bioremediation. On the other hand, in broth with a combination of the metals inoculated with *M. luteus*, the As^{3+} content (60 %) decreased from 245.69 to 98.36 mg/L, the Pb^{2+}(72.58%) 456.95 to 125.87 mg/L and the Hg^{2+}(72.93%) 133.78 to 36.89 mg/L. In the case of microbial

Table 1: Heavy metal concentration in the broths (mg/L) and % loss of the metals after 96 h.

Heavy Metal	*Inoculated Microorganism*	*Heavy Metals Level mg/L*		*Percentage loss after 96 hours*	*Impact of Organism percentage Loss*
		Initial (0 hrs)	*Final (96 hours)*		
Arsenic	Without microbes	1325.89 ± 0.49	1323.92 ± 0.31	0.21	-
	Bacillus cereus	1286.23 ± 0.24	556.78 ± 0.24	54.43	54.25
	Micrococcus luteus	1315.84 ± 0.37	665.29 ± 0.47	49.42	48.89
	B. cereus + M. luteus	1298.78 ± 0.53	289.18 ± 0.26	77.73	77.12
Lead	Without microbes	2135.68 ± 0.23	2132.54 ± 0.33	0.14	-
	Bacillus cereus	2115.57 ± 0.35	1756.29 ± 0.22	16.97	16.11
	Micrococcus luteus	2125.78 ± 0.64	1589.72 ± 0.33	25.22	24.35
	B. cereus + M. luteus	2068.84 ± 0.25	926.62 ± 0.61	55.22	54.32
Mercury	Without microbes	1869.14 ± 0.62	1861.68 ± 0.17	0.42	-
	Bacillus cereus	1825.28 ± 0.71	1123.55 ± 0.28	38.46	37.45
	Micrococcus luteus	1856.65 ± 0.85	1255.29 ± 0.19	32.38	31.25
	B. cereus + M. luteus	1852.59 ± 0.26	845.86 ± 0.27	70.57	69.51

Table 2: Heavy metal concentration in broths with combined heavy metals (mg/L) and % loss of the metals after 96 hours

Heavy metal	*Inoculated microorganism*	*Heavy metals level mg/L*			*Percentage loss after 96 hours*	*Impact of organism percentage Loss*
		HMs	*0 Hour*	*96 hours*		
$As^{3+}+Pb^{2+}+Hg^{2+}$	Without microbes	As	253.89 ± 0.14	250.23 ± 0.11	1.18	-
		Pb	439.56 ± 0.25	435.25 ± 0.19	0.91	-
		Hg	123.89 ± 0.54	120.36 ± 0.28	2.86	-
$As^{3+}+Pb^{2+}+Hg^{2+}$	*Bacillus cereus*	As	260.69 ± 0.16	108.98 ± 0.24	58.46	57.68
		Pb	458.78 ± 0.20	216.54 ± 0.39	52.83	52.21
		Hg	136.25 ± 0.22	50.25 ± 0.15	63.23	62.34
$As^{3+}+Pb^{2+}+Hg^{2+}$	*Micrococcus luteus*	As	245.69 ± 0.31	98.36 ± 0.22	60	59.28
		Pb	456.95 ± 0.44	125.87 ± 0.46	72.58	71.36
		Hg	133.78 ± 0.46	36.89 ± 0.52	72.93	72.12
$As^{3+}+Pb^{2+}+Hg^{2+}$	*B.cereus +M. luteus*	As	246.88 ± 0.39	46.36 ± 0.42	81.30	79.62
		Pb	423.65 ± 0.15	68.80 ± 0.39	83.92	83.10
		Hg	128.12 ± 0.16	21.52 ± 0.36	83.59	82.86

consortium including *B.cereus* and *M. luteus* inoculated with a mixture of the metals in the broth, the As^{3+} concentration (81.30%) decreased from 246.88 to 98.36 mg/L, Pb^{2+}(83.92%) 423.65 to 68.80 mg/L and Hg^{2+}(83.59%) 128.12 to 21.52 mg/L remediated after 96 hrs incubation. There was a significant difference among the treatment and control samples after 96 hours at $p < 0.05$.

The level of the accumulated heavy metals through isolates incubated in broths was presented in Table 3. Initially, 0 mg/L concentration of the heavy metals in the microorganisms was shown in broth with no heavy metals after 96 hours. However, broths supplemented with heavy metals incubated with the microorganisms accumulated the heavy metals. In the test for As^{3+} containing broth *B. cereus* accumulated 729.45 mg/L, *M. luteus* accumulated 650.55 mg/L and the consortium of *B. cereus* and *M. luteus* accumulated 1009.60 mg/L. On the other hand, the test with Pb^{2+} in broth with *B. cereus* accumulated 359.28 mg/L, *M. luteus* accumulated 536.06 mg/L while the consortium includes *B. cereus* and *M. luteus* accumulated 1142.22 mg/L of Pb^{2+} with significant difference observed between treatment and control samples after 96 hrs incubation. In the case of Hg^{2+}, *B. cereus* accumulated 701.73 mg/L, *M. luteus* accumulated 601.36 mg/L and the consortium accumulated 1006.73 mg/L. The *B. cereus* + *M. luteus* with As^{3+} have bioaccumulation factor 3.49, where the consortium is shown against Pb^{2+} and Hg^{2+} accumulation factor 1. *B. cereus* broth containing As^{3+} also observed 1 bioaccumulation factor and the rest of obtained less than 1 accumulation factor.

FTIR

FTIR spectra were obtained for bacterial strains before and after treatment with a mixture of three metals, ranging from 4,000 to 400 cm^{-1} (Figure 1 a,b). The FTIR profiles of metal-free bacterial strains showed a variety of peaks, indicating the complexity of the bacterial cell surface. Common bands were visible in the strains before metal uptake across both bacterial types, albeit fewer IR peaks were identified in the pre-treated strains than in the heavy metal-treated strains. The IR bands corresponded to functional groups, including amino (N-H, NH2), alkyne (C ≡ C), carbonyl (C=O), carboxylic (C-O), hydroxyl (-OH), and groups (Figure 1a) (Mamera M *et al.,* 2020; Rahman N *et al.,* 2014). Table 4 shows the band allocations and specific functional groups for both strains. Metal exposure resulted in changes in band intensity, shifts in absorption bands, and the appearance of

Table 3: Heavy metals accumulate in the microorganisms.

Description	*Metal con. in isolate (0 h)*	*Metal con. in isolate (96 h)*	*Metal con. in broth (96 h)*	*Bioaccumulation factor*
B.cereus + As^{3+}	0	729.45 ± 0.18	556.78 ± 0.24	1.31
M.luteus + As^{3+}	0	650.55 ± 0.22	665.29 ± 0.47	0.97
B.cereus + *M.luteus* + As^{3+}	0	1009.60 ± 0.36	289.18 ± 0.26	3.49
B.cereus + Pb^{2+}	0	359.28 ± 0.13	1756.29 ± 0.22	0.20
M.luteus + Pb^{2+}	0	536.06 ± 0.42	1589.72 ± 0.33	0.33
B.cereus + *M.luteus* + Pb^{2+}	0	1142.22 ± 0.24	926.62 ± 0.61	1.23
B.cereus + Hg^{2+}	0	701.73 ± 0.32	1123.55 ± 0.28	0.62
M.luteus + Hg^{2+}	0	601.36 ± 0.37	1255.29 ± 0.19	0.47
B.cereus + *M.luteus* + Hg^{2+}	0	1006.73 ± 0.42	845.86 ± 0.27	1.19

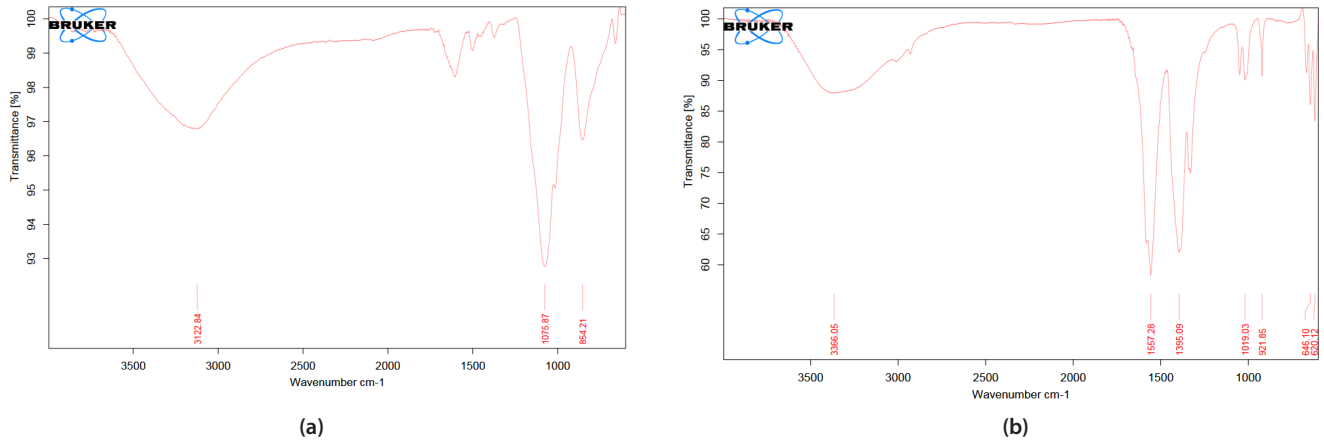


Fig. 1: (a) Metal-free consortium **(b)** Consortium with metals. Before and after absorption of mixed metals.

new peaks. The number of IR bands increased as the strains interacted with the multi-metal environment. Notably, IR shifts and new peaks were more common in consortiums treated with heavy metals. Metal-loaded *B. cereus* and *M. luteus* showed alterations in IR spectra, indicating the presence of functional groups associated with aromatic organics, alkynes (C ≡ C), and alkanes (C-H). In contrast, the *B. cereus* and *M. luteus* strains showed changes in aromatic organics, alkanes (C-H), hydroxyl, amine, and aldehyde functional groups. Furthermore, additional peaks appeared in the spectra of the *B. cereus* and *M. luteus* metal-loaded strains, showing the presence of aromatic compounds, alkanes (C-H), carboxyl (C-C), alcohol (R-CHO), amine (P-NH, NH2), and hydroxyl (O-H) functional groups (Fig 1 b). These findings highlighted the subtle changes in bacterial cell surfaces caused by the uptake of several metals, with unique variations depending on bacterial strain.

Liquid chromatography-mass spectrometry analysis

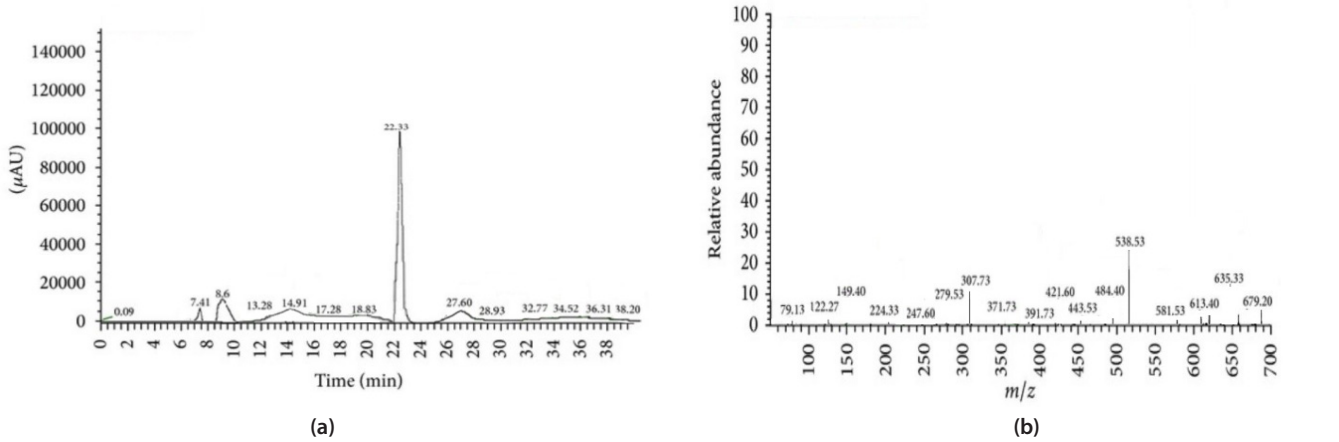
Metal homeostasis necessitates intracellular metal complexation in the presence of a cellular surplus, followed by metal release to metal-requiring apoproteins. Excess metal ions are kept in cellular storage locations such as vacuoles (Hall, 2002). The proteinous and non-proteinous metal ion trafficking components of the consortium are identified using LC-MS (Mahmoud M et al., 2024). Fig. 2a and 2b show the LC-MS chromatograms for Pb^{2+}, Hg^{2+}, and As^{3+}. The data were collected from the Luna PFP (2) analytical column, which used ammonium formate and methanol as eluting buffers. Fig. 2(a) depicts the retention time in minutes, while Fig. 2(b) depicts the m/z ratio of each component in the consortium treated with heavy metals. The peaks in the chromatograms were analyzed using the database to determine the components. Compared to the literature, Fig. 2(a) shows two peaks at 6 to 10 minutes retention, 7.4 and 8.6, suggesting the presence of cysteine (Cys) and glutamine (Glu) residues, which are the subunits of phytochelatins (γ-glutamylcysteine). Fig. 2(a) also indicated two significant peaks at 14 to 25 minutes of retention time, that is, 14.9 and 22.3 suggested the existence of two forms of phytochelatins (PC2 and PC3, respectively). In contrast, Fig. 2(b) indicated m/z peaks of glutathione, PC2, and PC3 at 307, 538, and 679, respectively (Odoemelam *et al.,* 2011).

Bioremediation of soil by microbial consortium

The bioremediation result of sterile heavy metal contaminated soil via bioaugmentation by the bacterial isolates *B. cereus* and *M. luteus* are given below. To examine the Pb^{2+}, Hg^{2+}, and As^{3+}

Table 4: IR absorption band changes and possible assignment for the metal-free and metal-loaded consortium.

FTIR peak	*Consortium metal free*	*Consortium with metals*	*Functional group*	*Bond*	*Assignment*
1		620	$C_2H_2R_2$	C-H out-of-plane-bend	Alkene
2		646	$C_2H_2R_2$	C-H out-of-plane-bend	Alkene
3	854	921	1,3-Disubstituted (Aromatic compounds)	C-H out-of-plane-bend	Aromatic
4		1019	R-OH	C-O stretches	Alcohol
5	1075		RCOOR	C-O stretch	Carbonyl
6		1395	C-C	C-C bend	Alkane
7		1557	P-NH_2	NH_2	Amine
8	3122		C=C-H	C-H stretch	Alkene
9		3366	RO-H hydrogen bond	O-H stretch	Hydroxyl



(a) (b)

Fig. 2: (a) Chromatogram produced by LC-MS analysis for Pb^{2+}, Hg^{2+}, and As^{3+} at various retention times (min). **(b)** Chromatogram produced by LC-MS analysis for Pb^{2+}, Hg^{2+}, and As^{3+} at various *m/z* ratios.

reduction rate, the growth kinetic of isolates were tracked at 30˚C over 25 days. Initially, within 15 days of the experiment, bacterial growth rates were increased and microbial consortium led to an essential lessening of Pb^{2+}, Hg^{2+}, and As^{3+} levels in sterile soil. The microbial consortium reduced the As^{3+} concentration from 500.00 to 126.12 mg/kg (74.77% loss), the Pb^{2+} level from 500.00 to 98.32 mg/kg (80.33% loss), and Hg^{2+} fell from 500.00 to 102.89 mg/kg (79.42% loss). In the case of a combination of heavy metals (Pb^{2+}, Hg^{2+}, and As^{3+}) consortium reduced the level of heavy metals from 1500.00 to 642.75 mg/kg (298.89 mg/kg As^{3+}, 256.89 mg/kg Pb^{2+} and 109.65 mg/kg Hg^{2+}) over 25 days at 30°C (57.15% loss). Therefore, the microbial consortium showed significant results on soil bio-augmented decrease in heavy metal concentration compared to the individual stain (Fig. 3-6).

Phytotoxicity Analysis

Reducing heavy metal concentration through remediation treatment does not reduce soil toxicity (Jiang Y *et al.,* 2021). To assess soil toxicity, the germination index (GI%) of *C. arietinum* L. was employed, and the toxicological effects of heavy metals were removed using microbial consortia bioremediation techniques.

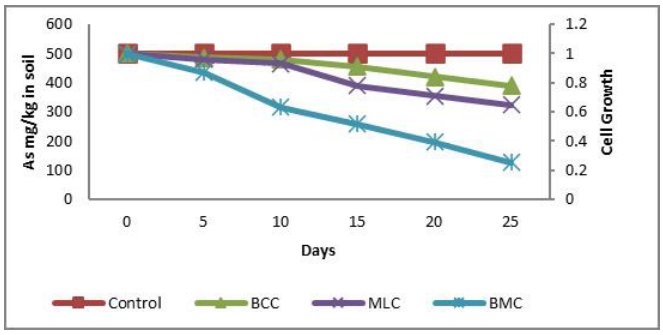


Fig. 3: Bioaugmentation of polluted soil (As^{3+}) by *B. cereus* and *M. luteus*. (BCC) Polluted soil bioaugmented by the *B. cereus*. (MLC) Polluted soil bioaugmented by *M. luteus*. (BMC) Polluted soil bioaugmented by a consortium.

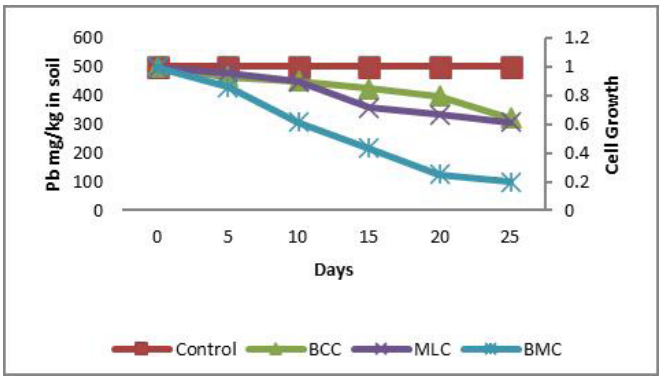


Fig. 4: Bioaugmentation of polluted soil (Pb^{2+}) by *B. cereus* and *M. luteus***.** (BCC) Polluted soil bioaugmented by the *B. cereus*. (MLC) Polluted soil bioaugmented by *M. luteus*. (BMC) Polluted soil bioaugmented by a consortium.

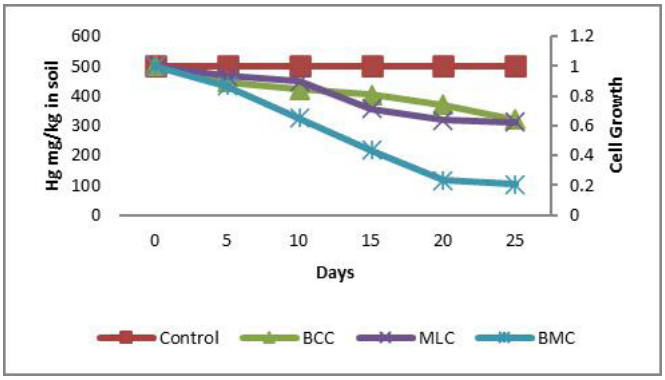


Fig. 5: Bioaugmentation of polluted soil (Hg^{2+}) by *B. cereus* and *M. luteus***.** (BCC) Polluted soil bioaugmented by the *B. cereus*. (MLC) Polluted soil bioaugmented by *M. luteus*. (BMC) Polluted soil bioaugmented by a consortium.

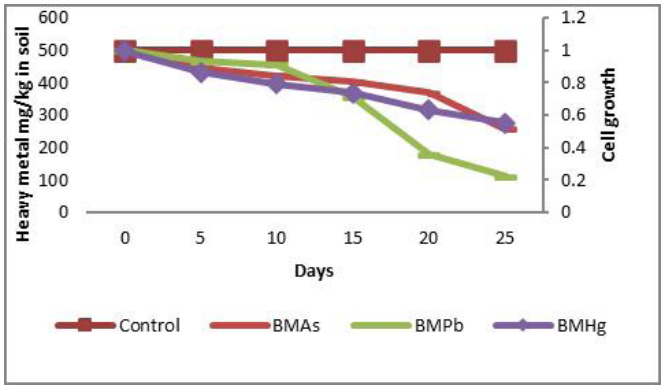


Fig. 6: Bioaugmentation of polluted soil (Pb^{2+}, Hg^{2+}, and As^{3+}) by *B. cereus* and *M. luteus*. (BCAs, BCPb & BCHg) Polluted soil bioaugmented by the *B. cereus*. (MLAs, MLPb & MLHg) Polluted soil bioaugmented by *M. luteus*. (BMAs, BMPb & BMHg) Polluted soil bioaugmented by a consortium.

The uncontaminated soil (control) demonstrated normal germination and root length, indicating GI (86%) of the initial composite material compared to the contaminated soil (Table 5). The ecotoxicity estimation revealed the 59% germination index of treated soil after 25 days (Fig. 7). The obtained index was significantly lower than the initial soil state. These changes were observed in the decrease of final heavy metal concentrations. Albeit, it could be understood the drop in GI% of *C. arietinum* L. seeds in treated soil, was because of the accumulation of heavy metal in the microbial consortium and increased soil quality (Fig. 8-11). To further analyze the effect of microbial consortium treatment on heavy metal contaminated soils, the

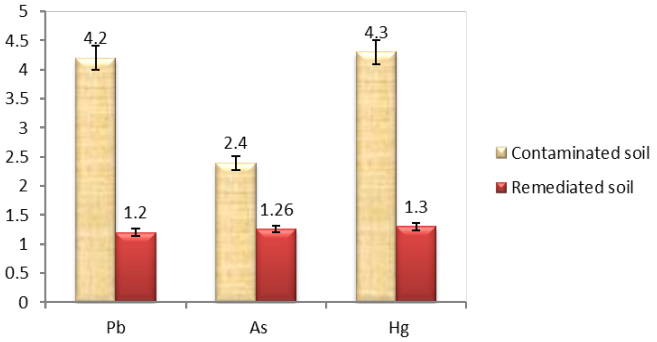


Fig. 7: Heavy metal transfer factor in *C. arietinum L* plant in normal and remediated soil

Fig. 8: Plant growth in uncontaminated soil

Fig. 9: Plant growth in contaminated soil

Fig. 10: Plant growth in remediated soil

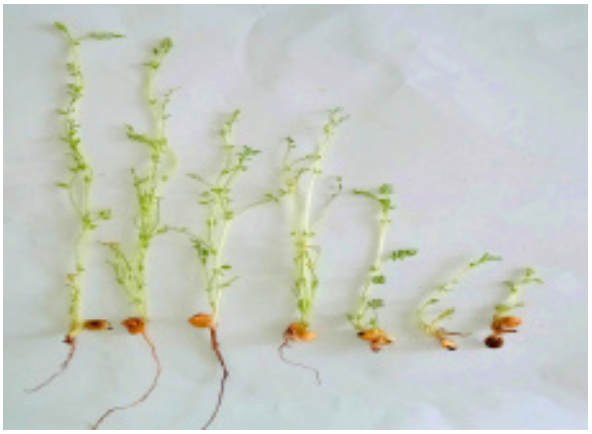


Fig. 11: Week-wise plant growth in remediated soil

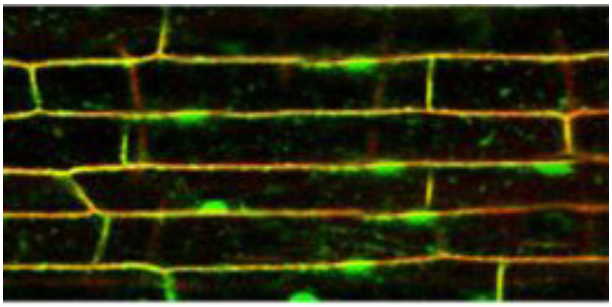


Fig. 12: Single plane confocal images of root apical meristem of *C. arietinum L.* stained with both FDA (green) and PI (red).

Table 5: Effects of *B. cereus* and *M. luteus* on *C. arietinum L* under normal and remediated soil

Parameters	*Treatments*			
	Plant in Control (Metal free soil)	*Plant in metal-ontaminated soil*	*Plants in bio-remediated soil*	*CD at 5% significance level*
Shoot length (cm)	21.1 ± 1.28	0.1 ± 0.001	19.8 ± 1.32	1.3
Root length (cm)	16.3 ± 0.2	0.001 ± 0.002	15.6 ± 0.81	0.7
Chlorophyll content (mg/g)	2.12 ± 0.23	0.13 ± 0.01	1.86 ± 0.016	0.26
Dry weight of plant (g)	0.210 ± 0.11	0.002 ± 0.001	0.198 ± 0.012	0.012
Pb^{2+}, Hg^{2+}, and As^{3+}in shoot (µg/g)	Not detected	Pb^{2+} 38.15 ± 2.52 As^{3+} 31.24 ± 3.87 Hg^{2+} 28.11 ± 3.51	Pb^{2+}11.56 ± 3.62 As^{3+} 10.13 ± 2.32 Hg^{2+} 08.11 ± 3.14	Pb^{2+} 3.30 As^{3+} 3.08 Hg^{2+} 3.46
Pb^{2+}, Hg^{2+}, and As^{3+} in root (µg/g)	Not detected	Pb^{2+} 42.08 ± 4.31 As^{3+} 24.17 ± 2.91 Hg^{2+} 43.56 ± 3.25	Pb^{2+} 12.52 ± 3.12 As^{3+} 12.65 ± 2.87 Hg^{2+} 13.16 ± 1.68	Pb^{2+} 3.36 As^{3+} 1.91 Hg^{2+} 3.31

C. arietinum L. seedlings vitality' root apical meristem was also performed. Fluorescent staining evaluated that the vitality of the meristematic zone increased (increase of FDA fluorescence) and dead cells decreased (decrease of PI fluorescence) after soil remediation compared with uncontaminated soil. Soil phytotoxicity revealed that the germination rate of *C. arietinum* L. was repressed due to the accumulation of heavy metals and bioremediation soil, becoming more viable and vital for germination seedlings (Fig. 12).

Conclusion

The findings of this study indicate that microbial bioremediation using the strains *B. cereus* and *M. luteus* is an effective approach for reducing the concentrations of As^{3+}, Pb^{2+}, and Hg^{2+} in sterile polluted soil, thereby contributing to environmental protection. A significant reduction in the concentrations of As^{3+} (77.74%), Pb^{2+} (80.33%), and Hg^{2+} (79.42%) was notably observed in the bioaugmented sterile soil (57.15%) in the presence of the consortium of both bacterial strains. Soil phytotoxicity assessments revealed that a decrease in heavy metal levels did not always correspond to a reduction in soil toxicity. In conclusion, microbial bioremediation of soil contaminated with As^{3+}, Pb^{2+}, and Hg^{2+} by the strains *B. cereus* and *M. luteus* yielded highly promising results, demonstrating the effectiveness of an environment friendly biological approach for remediating heavy metal-contaminated soil.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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