Asia-Pacific Journal of Science and Technology: Volume: 30 Issue: 04 Article ID: APST-30-04-01 Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

Evaluating the bioremediation potential of *Bacillus* cereus and micrococcus iuteus consortium in soil polluted by heavy metal

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Received 1 June 2024
Revised 13 June 2024
Accepted 9 October 2024

Abstract

Pollution by heavy metal is an environment health threat due to their toxicity. Bioremediation through microbes is an ecofriendly method used to treat contaminated soils. Microbial consortiums are used as a natural strategy to remediate contaminated soil giving enhanced results compared with individual species. Bioremediation of heavy metals via *Bacillus cereus* and *Micrococcus luteus* were investigated. *B. cereus and M. luteus* were exposed to 3000 mg/L each of Lead nitrate, Mercuric Chloride and Sodium arsenate. Further, the organisms were exposed to 1000 mg/L of all three metals at 30°C for 96 hrs. Phytotoxicity of treated soil was also investigated. The microbial consortium combination of *B. cereus and M. luteus* has the maximum accumulation of As (1009.60 mg). Individually *B. cereus and M. luteus* demonstrated the highest uptake of As (729.45mg) and Hg (601.36 mg) respectively. The highest accumulation of the combined three heavy metals was observed by *M. luteus*. Soil phytotoxicity responses exposed that reduced heavy metal concentration correlate with decreased soil toxicity. The results indicated the efficiency of using the target organisms for the bioremediation of heavy metal and their probable agent for bioremediation.

Keywords: Heavy Metal, Microbial consortium, Bioremediation, Phytotoxicity, Contamination

1. Introduction

Chhattisgarh's mining industry is increasing rapidly. It hold the 2nd place in coal production in India and thereby contributing to the country's economic growth [1]. However, the rising mining activities lead to soil, water and air pollution. Different heavy metals contain arsenic, chromium, lead, mercury and cadmium rise in concentration presents in microbial natural environment [2]. Certain bacteria develop resistant against high levels of heavy metal stress which is quite necessary for their survival [3].

Heavy metals pollutants cannot directly have an effect on the biogeochemical cycles and soil fertility. Heavy metals present in soil negatively affects microbial activity and carbon cycle thus leading to impaired uptake of nutrients in plants. Heavy metals can get transferred to living organism through food chain posing high risk of causing human health problems. Most of heavy metals at very low amount are carcinogenic, mutagenic and non-biodegradable in nature [4]. Several conventional remediation techniques have been developed to remediate heavy metal contaminated soils but these approaches could have a damaging effect on soil properties and are expensive. Instead, biological treatment methods are cost efficient, effective and environmentally sustainable [5]. Mainly commercially available biological treatment products included single species to have achieved specific goals [6] because of single species has term limitation on their function to decontamination of heavy metal containing soil. Albeit shortcomings of the microbes can be moderately compensated through combining of pesticides and fertilizers, therefore more sustainable approaches are desirable [7].

The current year, a new thought expanded to combining multiple species with corresponding traits, recognized as a consortium [8]. The microbial consortium is used to obtain expected result with some advantages compares

over individual species. In consortium combination of different species which could have alternative properties to enhance effect on treatment of contaminants. The consortiums combined with those microbes which can produce indole 3 acetic acid as well as solubilize phosphates were much capable to promote high growth than single strain [9].

The main objectives of this study was the effectiveness of bacteria *B. cereus* and *M. luteus* in remediating heavy metal contaminated soil through the bioaugmented process through bacterial strains individually and microbial consortium.

2. Materials and methods

2.1 Source of Microbes and Chemicals

The used bacterial isolates were previously isolated from Gevra coal mine Korba and Mand coalfield Raigarh, Chhattisgarh and were identified as *B. cereus* and *M. luteus* [10]. The chemicals and reagents were used analytical grade (Lead nitrate, Mercuric Chloride and Sodium arsenate) and purchased from Kasliwal Brothers chemical shop in Raipur, Chhattisgarh.

2.2 Development of the Bacterial Consortium

One inoculating loop full culture of both bacteria was taken to be grown into Nutrient broth medium (Himedia, India) and then incubated at 37**°**C for 24 hours separately. After incubation to obtain biomass cultures were centrifuged, this was then suspended in double distilled water. The absorbance was measured by a Spectrophotometer at 600 nm. After that, both suspensions of the cells were blended with an equal amount to prepare bacterial consortium.

2.3 Degradation Experiment of Heavy Metal via Consortium

The experimental setup was conducted as previously described by K. L Njoku et al [4] with a slightly modification. The Nutrient broths containing initial concentration of Lead nitrate, Mercuric Chloride and Sodium arsenate at 3000 mg/L separately and 1000 mg/L combination of these three salts in conical flask were incubated with 1 ml of 24 hours old cultures of *B. cereus, M. 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 experiments were done in triplicates.

2.4 Assessment of Heavy Metals

Initially zero hour incubated culture and after 96 hrs incubated cultures were taken to determine initial as well as the final remaining heavy metal in the broth and microorganisms. The cultured flasks were centrifuged and filtered using Whatman filter paper to obtain biomass. The obtained microbial biomass was washed out numerous times with double distilled water and dried in the hot air oven at 70 °C for 24 hrs. and accumulated heavy metals was determined via Atomic Absorption Spectrophotometer (Agilent, 240FS AA) in the biomass of microbial were extracted by acid digesting with nitric acid. The acid digested biomass was filtered and made up volume 50 mL with de-ionized water. The bioaccumulation factor and percentage of degradation were calculated by given formula [11].

$$\%\ degradation = \frac{Initial\ HM\ level\ -\ Final\ HM\ level}{Initial\ HM\ level} X\ 100$$

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

2.5 Bioremediation tests of contaminated soil sample

For bioremediation of heavy metal contaminated soil bioaugmentation test was performed. In a conical flask containing 500 mL M9 medium mixed with 10 gms of soil (Containing 500 mg/kg As, Pb and Hg separately) and then bacterial ($70x10^7$) cells were inoculated. The experiments setup was carried out in 12 flasks for soil. The experimental design composed with 12 conditions with soil was given in Table 1. All experimental flasks were incubated in shaking incubator (150 rpm) at 30˚C for 25 days. All tests were performed in triplicate. The bacterial count and heavy metal level was assessed in every five days by Atomic Absorption Spectrophotometer.

Table 1 Experimental design of Heavy Metal Contaminated Soil for Bioaugmentation.

SN.	Name	Pb (II) (500mg/Kg) Contaminated Soil	As (III) (500mg/Kg) Contaminated Soil	Hg (II) (500mg/Kg) Contaminated Soil	Media	Micro-organism
1	PbBC1	10gms	-	-	M9 media	*B. cereus*
2	PbML1	10gms	-	-	M9 media	*M. luteus*
3	PbBM1	10gms	-	-	M9 media	*B. cereus* + *M. luteus*
4	AsBC1	-	10gms	-	M9 media	*B. cereus*
5	AsML1	-	10gms	-	M9 media	*M. luteus*
6	AsBM1	-	10gms	-	M9 media	*B. cereus* + *M. luteus*
7	HgBC1	-	-	10gms	M9 media	*B. cereus*
8	HgML1	-	-	10gms	M9 media	*M. luteus*
9	HgBM1	-	-	10gms	M9 media	*B. cereus* + *M. luteus*
10	PbAsHgBC1	10gms	10gms	10gms	M9 media	*B. cereus*
11	PbAsHgML1	10gms	10gms	10gms	M9 media	*M. luteus*
12	Pb As Hg BC ML	10gms	10gms	10gms	M9 media	*B. cereus* + *M. luteus*

2.6 Phytotoxicity tests

Phytotoxicity study was conducted following the methodology of Attila Bodor et al [12] with slight Modification. The tests were conducted at the initial and 25 days long bioremediation processed soil to assess changes in soil properties after the treatment. For this test, 20 gms of test soil was kept in a sterilized 90 mm Petri dish (Borossil), and 10 seeds of chickpea were equally spread on the soil surface. Distilled water (4ml) was evenly added to the soil. Afterwards, black gram seeds were closed in Petri dish and kept in the dark for germination at 25 ℃ for 4 days. After 4 days seeds were germinated with visible roots or lengths were measured of root. The control test was studied in uncontaminated soil collected from a nearby polluted site. Germination seed index (%) was calculated between the percentage of seed germination subtracted from the percentage root length and divided by hundred.

2.7 Root apical meristem cells analysis

Chickpea seedling's meristem cells were analyzed in accordance with the methods of Attila Bodor et al. [12]. After the root was washed with distilled water, approximately 10 mm long root tips were cut and dipped into buffer solutions. As a counter stain 10 μM fluorescein diacetate solutions were applied for 25 min for viable cells. and 3 μg mL^{-1} propidium iodide solution (1 min. applied) for dead cells was used. Stained meristems were placed onto glass slides and observed under a fluorescence microscope (Olympus BX63). The fluorescence intensity of apical meristem was observed through cellSens image analysis software.

2.8 Statistical analysis

Means and standard deviations as well as comparative analysis between the test and control samples were done with Microsoft excel and analysis of variance (ANOVA) with Origin pro 9.0 software.

3. Results and discussion

3.1 Heavy Metal Percentage Loss in Broth

The residual individual heavy metals and in combination were analyzed in the broths after 96 h of incubation. In control test (without the microorganisms), Arsenic (0.21% loss) decreased from 1325.89 mg/L to 1323.92 mg/L, lead (0.14 % loss) decreased from 2135.68 mg/L to 2132.54 mg/L, while mercury (0.42% loss) decreased from 1869.14 mg/L to 1861.68 mg/L after 96 hrs of remediation. Afterward, heavy metal contaminated broths incubated with *B. cereus* obtained 54.43% loss of As, 16.97% loss of Pb as well as 38.46 % loss of Hg whereas *M.luteus* inoculated in contaminated broths resulted in 49.42% loss of As, 25.22% loss of Pb and 32.38% loss of Hg after incubation of 96 hrs. Microbial consortium of *B. cereus* and *M. luteus* bioremediated percentage of heavy

metals, As 77.73%, Pb 55.22% and Hg 70.57% loss after incubation of 96 hrs (Table 2). There was significant variation in the residual arsenic and lead content between the control and treated medium after 96 hrs at $P < 0.05$.

In the control test, without microbes and combination of arsenic, mercury and lead reduced after 96 hrs of incubation from 253.89 mg/L to 250.23 mg/L (As -1.18% loss), 439.56 to 435.25 mg/L (Pb- 0.91 % loss). While Hg (2.86% loss) reduced from 123.89 mg/L to 120.36 mg/L. The combination of the metal in broth inoculated with *B. cereus*, the As concentration (58.46 % loss) decreased from 260.69 mg/L to 108.98 mg/L, Pb (52.83 % loss) decreased from 458.78 mg/L to 216.54 mg/L at the same time as Hg decreased from 136.25 mg/L to 50.25 mg/L (63.23% loss) obtained after 96 hrs of bioremediation. On other hand, broth with combination of the metals inoculated with *M. luteus*, the As content (60 % loss) decreased from 245.69 mg/L to 98.36 mg/L, the Pb level reduced from 456.95 mg/L to 125.87 mg/L (72.58% loss) and the Hg level (72.93% loss) decreased from 133.78 mg/L to 36.89 mg/L. In the case of microbial consortium including *B.cereus and M. luteus* inoculated with a mixture of the metals in broth, the As concentration (81.30 % loss) decreased from 246.88 mg/L to 98.36 mg/L, Pb decreased from 423.65 mg/L to 68.80 mg/L (83.92% loss) and Hg level reduced from 128.12 mg/L to 21.52 mg/L (83.59 % loss) remediated after 96 hrs incubation (Table 3). There was a significant difference among the treatment and control samples after 96 h at P <0.05.

Table 2 Heavy Metal concentration in the Broths (mg/L) and loss percentages after 96 h.

Heavy Metal	Inoculated Microorganism	HM Level mg/L		Percentage loss after 96 hours	Impact of Organism percentage Loss
		Initial (0 hrs)	Final (96 hrs)		
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 3 Heavy Metal concentration in Broths with combined HMs (mg/L) and loss percentages after 96 h.

Heavy Metal	Inoculated Microorganism	HMs Level mg/L			Percentage loss after 96 hours	Impact of Organism percentage Loss
		HMs	0 Hour	96 hours		
As+Pb+Hg	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+Pb+Hg	*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+Pb+Hg	*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+Pb+Hg	*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

3.2 Heavy metals accumulated by the consortium

The level of the accumulated heavy metals through isolates incubated in broths is presented in Table 4. The initial concentration of 0 mg/l in the microbes was shown in broth-free heavy metals after 96 hrs. However broths supplemented with heavy metal and incubated with the microorganisms accumulated the heavy metals. In the test for As containing broth with *B. cereus* accumulated 729.45 mg/L, *M.luteus* accumulated 650.55 mg and the consortium of *B. cereus* and *M. luteus* accumulated 1009.60 mg. On the other hand, test against Pb in broth with *B. cereus* accumulated 359.28 mg, *M. luteus* accumulated 536.06 mg. While the consortium accumulated 1142.22 mg of Pb. A significant difference between the treatment and control samples was observed after 96 h at $p < 0.05$. In the case of Hg, *B. cereus* accumulated 701.73 mg, *M. luteus* accumulated 601.36 mg, and the consortium accumulated 1006.73 mg/L. The consortium against As, has a bioaccumulation factor of 3.49 whereas it demonstrated a bioaccumulation factor of 1 against Pb and Hg. *B. cereus* broth containing As also demonstrated a bioaccumulation factor of 1 while it was less than 1 in other cases.

Table 4 HMs Accumulated 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	Nil	729.45±0.18	556.78±0.24	1.31
M.luteus + As	Nil	650.55±0.22	665.29±0.47	0.97
B.cereus + *M.luteus* + As	Nil	1009.60±0.36	289.18±0.26	3.49
B.cereus + Pb	Nil	359.28±0.13	1756.29±0.22	0.20
M.luteus + Pb	Nil	536.06±0.42	1589.72±0.33	0.33
B.cereus + *M.luteus* + Pb	Nil	1142.22±0.24	926.62±0.61	1.23
B.cereus + Hg	Nil	701.73±0.32	1123.55±0.28	0.62
M.luteus + Hg	Nil	601.36±0.37	1255.29±0.19	0.47
B.cereus + *M.luteus* + Hg	Nil	1006.73±0.42	845.86±0.27	1.19

3.3 Bioremediation of soil by microbial consortium

The bioremediation results of sterile soil contaminated with heavy metal via bioaugmentation by the bacterial isolates *B. cereus* and *M. luteus* are given below. To examine As, Pb, and Hg reduction rate, the growth kinetic of isolates were studied at 30˚C over 25 days. Initially, for the first 15 days of the experiment bacterial growth rates were increased and microbial consortium led to an essential lessening of As, Pb and Hg level in sterile soil. The microbial consortium reduced the As concentration from 500.00 mg/kg to 126.12 mg/kg (74.77% loss) (Figure 1), Pb level from 500.00 mg/kg to 98.32 mg/kg (80.33% loss) (Figure 2) and Hg fell from 500.00 mg/kg to 102.89 mg/kg (79.42% loss) (Figure 3). In the case of a combination of heavy metals (As, Pb & Hg), the consortium reduced the level of heavy metals from 1500.00 mg/kg to 642.75 mg/kg (298.89 mg/kg As, 256.89 mg/kg Pb and 109.65 mg/kg Hg) over 25 days at 30ºC (57.15% loss) (Figure 4). Therefore, the microbial consortium showed significant decrease in the heavy metal concentrations compared to the individual stain in the bioaugmented soil. Therefore, there is a significant difference ($p<0.05$) between sterile soil bioaugmented with the bacterial consortium and individually bioaugmented soil compared to the control.

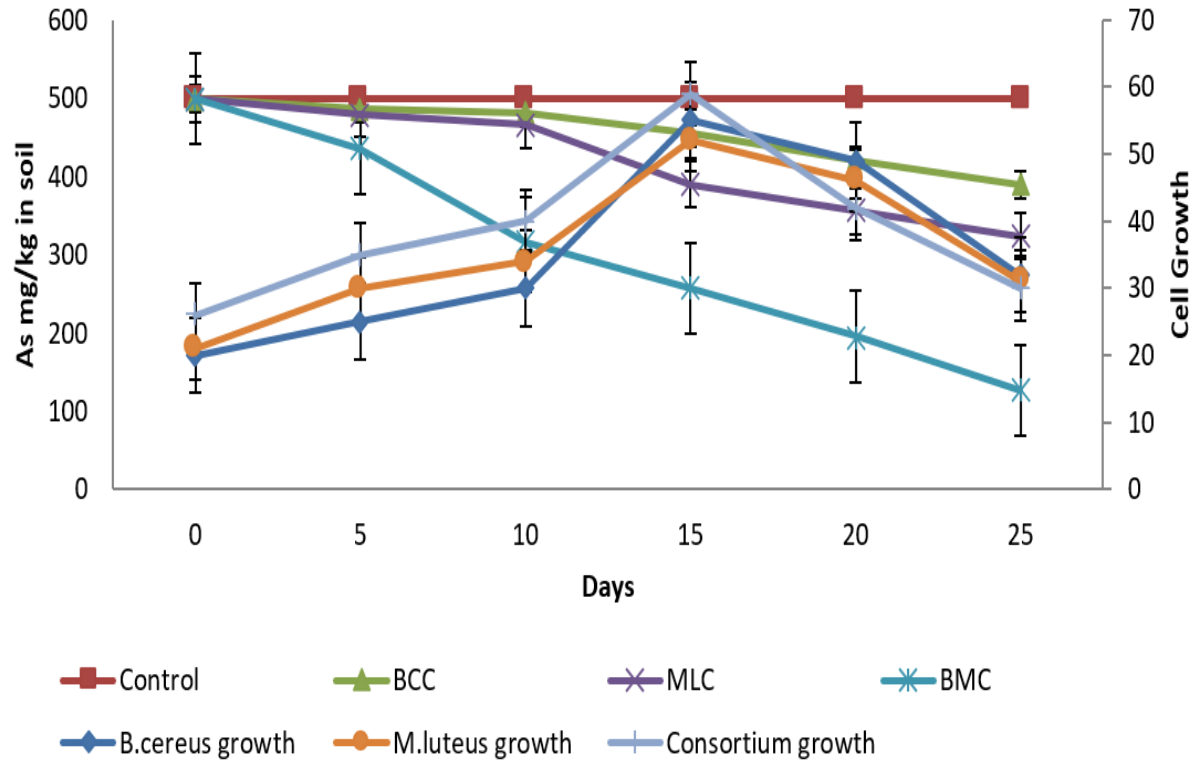

Figure 1 Bioaugmentation of arsenic polluted soil by *B. cereus* and *M. luteus*.
*(BCC) Polluted soil bioaugmented by the *B. cereus*.
*(MLC) Polluted soil bioaugmented by *M. luteus*.
*(BMC) Polluted soil bioaugmented by Consortium.

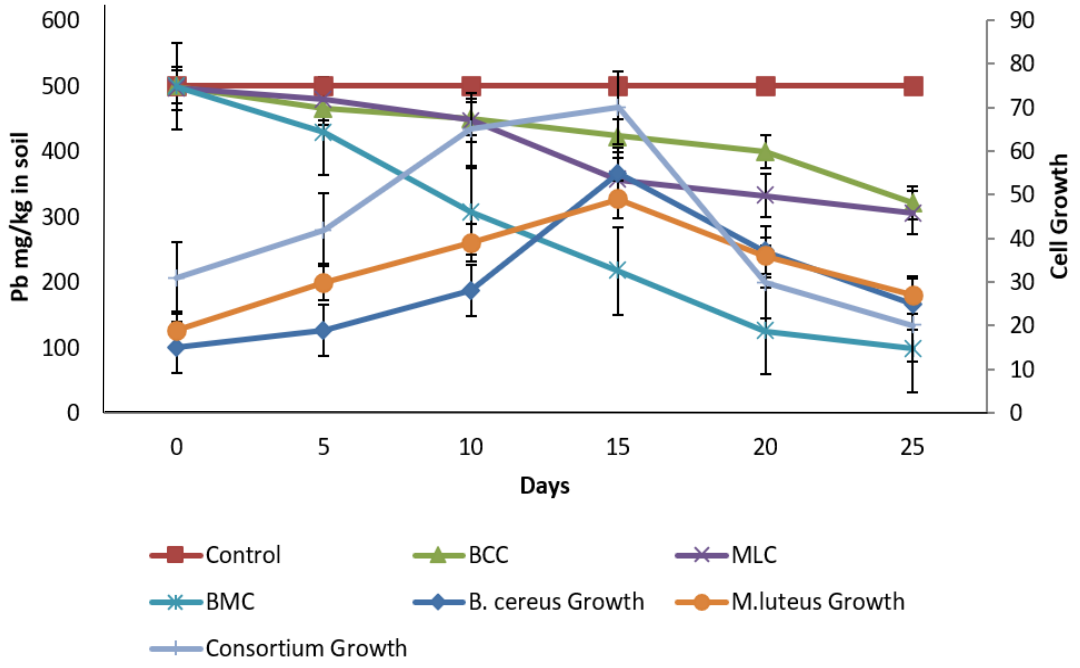

Figure 2 Bioaugmentation of lead polluted soil by *B. cereus* and *M. luteus*.
*(BCC) Polluted soil bioaugmented by the *B. cereus*.
*(MLC) Polluted soil bioaugmented by *M. luteus*.
*(BMC) Polluted soil bioaugmented by Consortium.

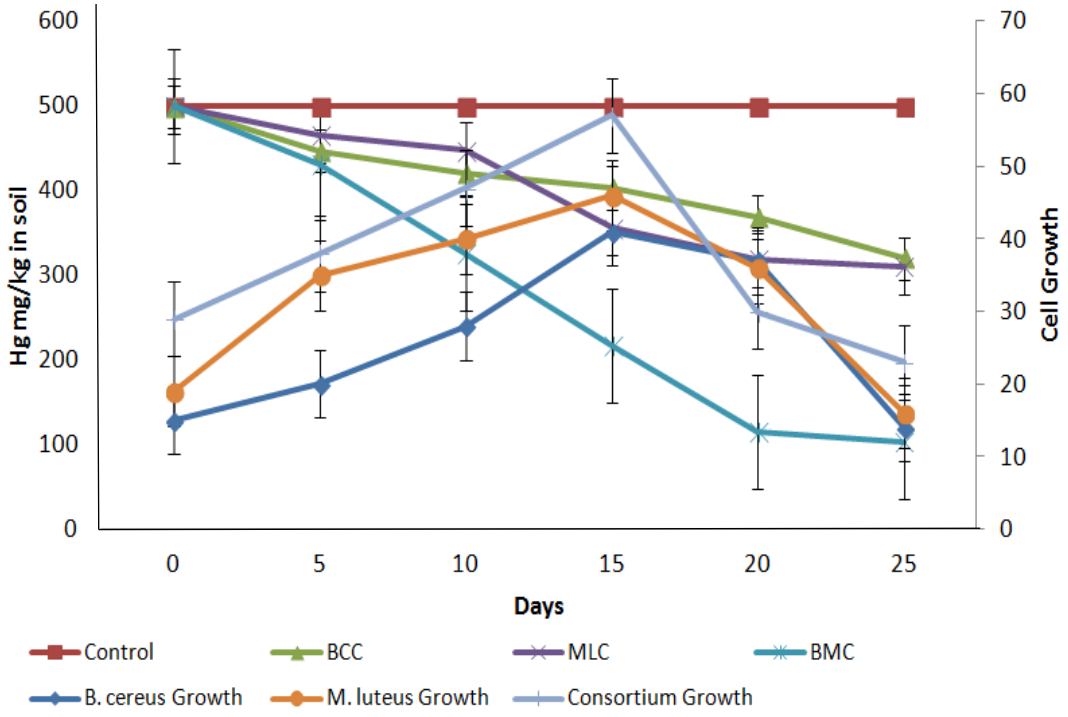

Figure 3 Bioaugmentation of mercury polluted soil by *B. cereus* and *M. luteus*.
*(BCC) Polluted soil bioaugmented by the *B. cereus*.
*(MLC) Polluted soil bioaugmented by *M. luteus*.
*(BMC) Polluted soil bioaugmented by Consortium.

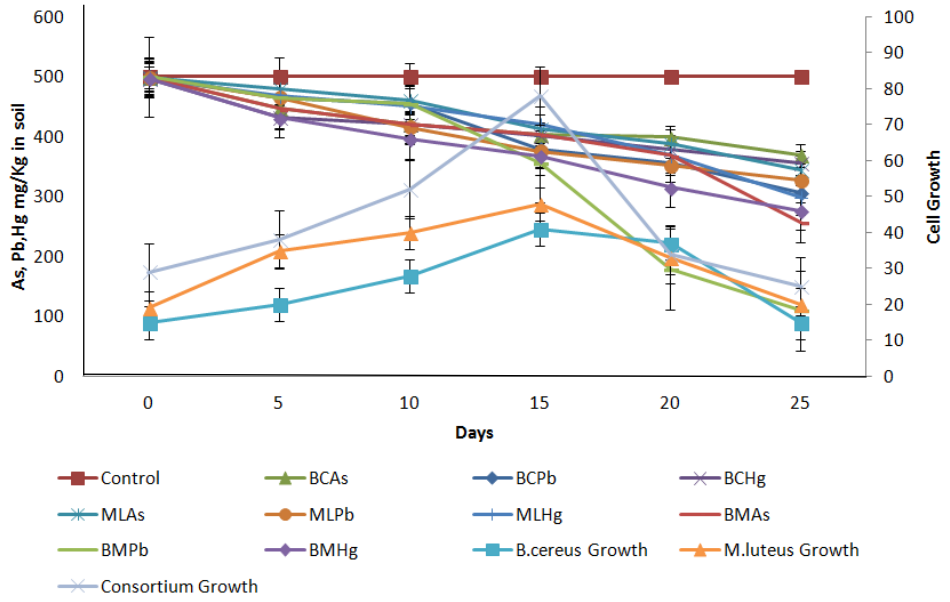

Figure 4 Bioaugmentation of As+Pb+Hg polluted soil by *B. cereus* and *M. luteus*.
*(BCAs, BCPb & BCHg) Polluted soil bioaugmented by the *B. cereus*.
*(MLAs, MLPb & MLHg) Polluted soil bioaugmented by *M. luteus*.
*(BMAs, BMPb & BMHg) Polluted soil bioaugmented by Consortium.

3.4 Soil Phytotoxicity

The heavy metal concentrations were reduced in remediated soil but not supported to reduce soil toxicity [13]. To evaluate the soil toxicity, the germination index (GI%) of chickpea was used. The uncontaminated soil (control) presented normal germination and root length, which indicated GI (86%) of the initial composite soil compared with contaminated soil. The ecotoxicity estimation revealed a 59% germination index of treated soil after 25 days (Figure 5). The obtained index was significantly lower than the initial soil state. These changes were observed till the decrease of final heavy metal concentrations. However, it could be understood that the drop in GI% of chickpea seeds in treated soil, was because of the accumulation of heavy metal in microbial consortium and increased soil quality. To further analysis the effect of microbial consortium treatment on heavy metal contaminated soils, the chickpea seedlings vitality' root apical meristem was also performed using fluorescent staining that evaluated, 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 black gram was repressed due to the accumulation of heavy metals and biodegraded soil, become more viable and vital for germination seedling.

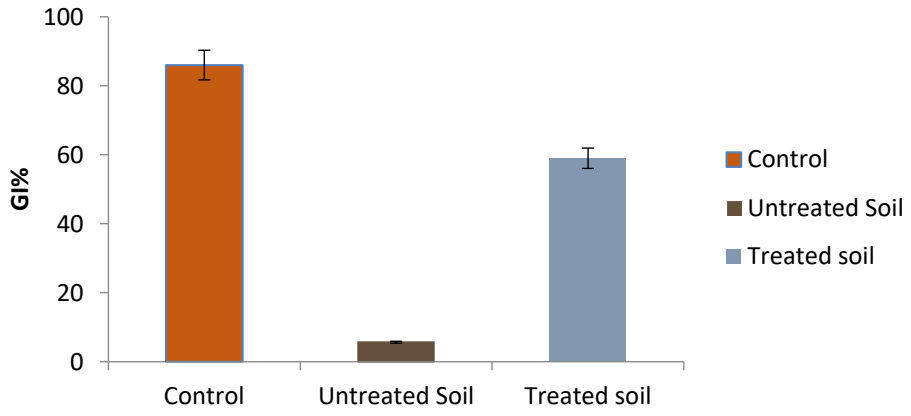

Figure 5 Germination index of chickpea seedlings.
*(Control) GI % of chickpea in uncontaminated soil.
*Untreated soil GI% of chickpea and
*(Treated) GI % of chickpea in treated soil (298.89 mg/kg As, 256.89 mg/kg Pb and 109.65 mg/kg Hg)

4. Discussions

This work was based on the microbial bioremediation of heavy metal-contaminated soil (Hg, Pb, and As), and a phytotoxicity study based on the germination index was evaluated on chickpeas. For soil bioremediation, two bacterial strains *B. cereus* and *M. luteus* were used [14]. Bioremediation competence is influenced by the resistance capacity of *B. cereus* and *M. luteus* bacteria to Hg, Pb, and As through their accumulation and adsorption ability on the membrane [15]. The heavy metal levels in the broth after 96 hrs indicate that though their levels can be reduced with time, the activities of the two microorganisms enhance their loss, hence, can assist in media contaminated with such metals [16].

This work started with a physicochemical characterization of the soil sample. The pH of the polluted soil was analyzed as an acidic pH, which supports the accumulation ability of metal ions on the bacterial cell surface with a maximum uptake capacity at pH 5 [17]. A bioaugmentation experiment was carried out over 25 days in sterile soil. These tests helped us to evaluate the rate of reduction in Hg, Pb, and, As concentration according to the bacterial growth kinetics [18]. The initial 15-day experiment results showed that the increase in the bacterial growth rate led to a dropping in the concentration of Hg, Pb, and As in the soil. After 15 to 25 days, the decrease in the number of bacterial cells in the sterile soil may be due to the reduction of the carbon source. The Hg, Pb, and As concentration reduction rate was observed in sterile soil augmented with the consortium of *B. cereus* and *M. luteus* strains, with a value of the combination of Hg, Pb, and As (57.12%) [19]. The result obtained that the bacterial consortium of the strains *B. cereus* and *M. luteus* are characterized by a high efficiency of mercury, lead, and arsenic biosorption [20]. Further, *Bacillus cereus* NWUAB01 with 69% removal efficiency of lead was isolated in mining soil [21].

Soil phytotoxicity established that, while the germination rate of black gram was inhibited most probably due to the accumulation of metal ions [22]. The seedlings of chickpea became more viable and vital when grown in the remediated soil [23]. In this work, vitality and membrane integrity of the chickpea seedlings' root apical meristem were integrated in the soil phytotoxicity analysis to obtain the effectiveness of bioremediation. However, this work did not intend to reveal the reasons behind the changes in the responses of the root apical meristems our results involve the ecotoxicological responses induced in plants inhibition of germination as well as root development. Our conclusion of this work further supports that a dropping in metal ions and reduced soil toxicity [24].

5. Conclusions

The current study showed that natural reduction capacity is very low to decrease of As, Pb, and Hg from the contaminated environment. Whereas *B. cereus* and *M. luteus* activities can be enhanced by reducing heavy metals from the environment. Different influence method was investigated of the *B. cereus and M. luteus* as single and combined in the treatment of heavy metals from the environment. The combination of isolates indicated a higher reduction rate against As, Pb, and Hg as compared with individual isolates. This revealed that *B. cereus* and *M. luteus* have the potential capacity to get rid of heavy metals from the environment. Overall, this study provides valuable insights into the diverse and dynamic responses of chickpeas to Pb(II), As(III) and Hg(II) metal ions on their adaptability and resilience in metal-contaminated environments. It emphasizes the complexity of metal–plant interactions and their ecological implications. These findings can be valuable for understanding how plants adapt to heavy metal contamination in various environmental settings. Understanding these impacts can help in developing strategies to mitigate the adverse effects of heavy metal contamination and the need for research, monitoring, and mitigation strategies to address these issues and to safeguard both environmental and human well-being.

Further research is needed to uncover the underlying mechanisms driving these responses and to develop effective remediation techniques. These findings have important implications for understanding plant–metal interactions in contaminated ecosystems.

6. Conflict of Interest

The authors have declared that no conflict of interest exists.

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