Fungal-bacterial biofilm mediated heavy metal rhizo-remediation

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Abstract

Heavy metal pollution due to excessive use of chemical fertilizers (CF) causes a major damage to the environment. Microbial consortia, closely associated with the rhizosphere are able to remediate heavy metal-contaminated soil by reducing plant toxicity. Thus, this study was undertaken to examine the remedial effects of microbial biofilms against contaminated heavy metals. Fungi and bacteria isolated from soil were screened for their tolerance against Cd^{2+}, Pb^{2+} and Zn^{2+}. Fungal-bacterial biofilms (FBBs) were developed with the highest tolerant isolates and were further screened for their bioremediation capabilities against heavy metals. The best biofilm was evaluated for its rhizoremediation capability with different CF combinations using a pot experiment conducted under greenhouse conditions with potato. Three bacterial and two fungal isolates were selected to develop FBBs upon the tolerance index (TI) percentage. Significantly ($P < 0.05$) the highest metal removal percentage was observed in *Trichoderma harzianum* and *Bacillus subtilis* biofilm under *in situ* condition. The biofilm with 50% of recommended CF (50CB) significantly ($P < 0.05$) reduced the soil available Pb^{2+} by 77%, Cd^{2+} by 78% and Zn^{2+} by 62% compared to 100% recommended CF (100C). In comparison to initial soil, it was 73%, 76% and 57% lower of Pb^{2+}, Cd^{2+} and Zn^{2+}, respectively. In addition, 50CB treatment significantly ($P < 0.05$) reduced the metal penetration into the tuber tissues in comparison with 100C. Thus, it is concluded that *T. harzianum–B. subtilis* biofilm is an ideal combination to remediate soil contaminated with Cd^{2+}, Pb^{2+} and Zn^{2+}.

1. Introduction

Recently, the rates of chemical fertilizers (CF) and agrochemical applications have increased tremendously. While CF has been shown to increase crop yield, incorrect agrotechnical applications and fertilization practices can cause entire agroecosystems to be seriously disrupted and contribute to the development of toxic pollutants in the soil. In most agrochemicals, heavy metals are the main inorganic pollutants, posing long-term risks to living organisms and the environment due to the fact that they are exceedingly hazardous at trace concentrations, bioaccumulation and their non-biodegradability (Meliani and Bensoltane, 2016). In agricultural soils, heavy metals can become mobile, with a tiny part of the overall mass seeping into aquifers or migrating to human diets via crop uptake (Hookoom and Puchooa 2013). For instance, elevated concentrations of arsenic (As), cadmium (Cd), lead (Pb), and zinc (Zn) have been discovered in potato samples from overused phosphate-fertilized soils, which increases the daily consumption of metals in food (Cheraghi et al., 2013). It has been well documented that synthetic fertilizers and pesticides contain variable levels of heavy metals as impurities or active ingredients (McLaughlin et al., 2000). For example, chemical phosphatic fertilizers such as triple super phosphate (TSP) can contain high amounts of Cd (Alkhader, 2015) and Cd contamination in soils of many countries is due to the use of Phosphate (P) fertilizers (McLaughlin et al., 1996). The constant application of phosphate fertilizers introduces other contaminants into agricultural soils, such as Hg, As, and Pb (Premarathana et al., 2005), which eventually accumulate in substantial concentrations in plants, resulting in phytotoxic symptoms (Alkhader, 2015).

The removal of heavy metals is performed by different chemical and physical methods such as precipitation, oxidation, reduction, membrane filtration, reverse osmosis and evaporation. However, in terms of eliminating heavy metal pollutants from contaminated soil, most of these procedures are both expensive and ineffective (Baz et al., 2015). In this context, rhizoremediation through rhizosphere soil beneficial microorganisms has been considered as one of the promising methods to reduce soil toxicities (Burdet al., 2000; Afzal et al., 2017). Rhizoremediation is the most environmentally friendly method of removing and degrading contaminants in the rhizosphere region through the interaction of bacteria and plant roots (Abtenh 2017). The rhizosphere region with heavy metal-contaminated soil provides environment for microbes (Idris et al., 2004) which are highly resistant to most of the heavy metals (Verma and Rawat, 2021). Plants feed microorganisms with necessary nutrients, and microorganisms convert hazardous chemicals into harmless minerals that plants can consume (Abtenh 2017). However, it is evident that a consortium of microbes living in the rhizosphere can detoxify contaminants more effectively than a single strain or species (Verma and Rawat, 2021). Rhizoremediation may also be accomplished by introducing viable microbial communities to infected areas artificially or by stimulating viable native populations (Tanu and Hoque, 2012).

Biofilms are microbial cell clusters, including bacteria and fungi, that adhere to a specific surface including soil (Meliani and Bensoltane, 2016). Biofilm formation is a method adopted by soil microorganisms to cope with extreme environmental conditions, such as high heavy metal concentrations (Harrison et al., 2007). Therefore, this strategy has the potential to be used as a rhizo-remediation tool to remediate heavy metal contamination from the rhizosphere. Researchers are interested in biofilm-based rhizo-remediation due to their high microbial biomass and capacity to immobilize contaminants (Quintelas et al., 2009). Biofilms directly absorb dissolved organic molecules and nutrients via a concentration gradient, whereas dissolved heavy metals are often deposited on the biofilm surface due to the interactions between metal ions and microbe surfaces. The extracellular polymeric substances (EPS), the metal-sorbing biomass in the biofilm, are often immobilized as a biofilm matrix. Biofilm EPS has been proven to play a substantial role in metal removal depending on pH, solubility and concentration of metals and organic matter, and biomass scale (Jung *et al.*, 2001). Multispecies biofilms formed by fungal–bacterial interactions have been employed more efficiently in bioremediation (Frey-Klettet al., 2011). Fungal–bacterial biofilms (FBBs) are bacteria in a fungal surface-attached biofilm form that can be generated in vitro from microbial monocultures (Seneviratne et al., 2008). Thus, it is worthwhile studying FBB-based rhizo-remediation methods for heavy metal bio-removal. This study therefore focused to isolate heavy metal tolerant bacterial and fungal species to develop FBBs, and then to evaluate them for heavy metal tolerability and rhizo-remediation ability.

2. Materials And Methods

2.1 Isolation and screening of fungi and bacteria

The soil organic humus horizon (A00/A01) was taken from a location where industrial effluents were stagnating in Biyagama industrial zone, Sri Lanka, and it was sieved (< 2 mm) and stored overnight at

4°C. The soil samples were serially diluted (10-fold) before plating on sterile Nutrient Agar (NA, 20 g/L medium; Himedia™, India) and Potato Dextrose Agar (PDA, 49.01 g/L medium; Himedia™) plates. Fungal and bacterial isolates were screened for the tolerance to Cd^{2+}, Pb^{+2} and Zn^{2+} in the form of $Cd(NO_3)_2$, $Pb(NO_3)_2$ and $ZnCl_2$. Briefly, the minimum inhibitory concentration (MIC) was determined by inoculating all the fungal and bacterial isolates on PDA and NA media incorporated with filter sterilized (0.22 µm pore size) heavy metals separately at concentrations ranging from 100 to 600 mg/L with interval of 100 mg/L. The fungal and bacterial isolates without adding the heavy metals served as the controls. Plates inoculated with fungi and bacterial were incubated at 29 ± 1 ^{o}C for 5 days and at 25 ± 1 ^{o}C for 3 days, respectively, during which mycelial radial growth and bacterial growth were monitored. Heavy metal tolerance capability of the microbial isolates was determined using the growth percentage of the microbial isolates in comparison to the control. The Metal Tolerance Index (Ti) was computed as the ratio of the treated colony's extended radius to the untreated colony's extended radius. The highest tolerant fungal and bacterial isolates were combined in all possible ways to develop FBBs, according to the method described in Seneviratne and Jayasinghearachchi (2003). Briefly, both fungi and bacteria were cultured separately in yeast mannitol broth (YMB) without agar for 7 days and they were combined in to one culture at day 14. The adhesion of bacterial cells to fungal filaments was observed continuously under an optical microscope model BX43F by staining with lactophenol cotton blue.

2.2 Bio removal of heavy metals in in-situ liquid media

The biofilms with the best attachments and monocultures were further screened for their bioremediation capabilities against heavy metals Cd^{2+}, Pb^{2+} and Zn^{2+}. Briefly, 10 mL of five-day old biofilms and monocultures were inoculated into a series of 250 mL Erlenmeyer’s flasks containing 50 mL of PDB and NB added with 400 mg/L of Cd^{2+}, Pb^{2+} and Zn^{2+}. The inoculated media were incubated on a rotary shaker at 150 rpm and 29 ± 1 ^{o}C for 5 days together with controls containing only the medium having heavy metal, but without biofilms and monocultures. Microbial mass was separated after 5 days through filtration and the filtrates were centrifuged at 12,000 rpm for 20 min. Afterwards, the supernatant of the filtrates was acidified with concentrated HNO_3. The contents of the filtrate were analyzed after proper digestion and dilution, using atomic absorption spectrophotometer for the availability of the heavy metals. Based on the following equation, metal bioaccumulation in biomass was represented as the quantity expelled from a solution containing the metal.

Metal removal (%) = [(C0 − Ct)/C0] × 100

where, C0 is initial metal concentration in the solution (mg/L), Ct is metal concentration after incubation in the solution (mg/L). Three replicates were maintained for each treatment.

Heavy metal loaded and unloaded biofilm biomass were analyzed using Fourier Transform Infrared Spectroscopy (FT-IR, Bruker alpha, German). Spectra were collected at a resolution of 4 cm^{-1} over a range of 500–4000 cm^{-1}. Spectra were analyzed using OPUS 7.5 software. The best biofilm combination or the monoculture which showed the highest metal removal percentage was subjected to a pot experiment with

potato to evaluate its rhizo-remediation capability against the three heavy metal ions under greenhouse condition.

2.3 Rhizo-remediation experiment

The experiment was conducted in a top-vent type film plastic greenhouse at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. The mean day temperature was 31.7 ± 1.2 ^{o}C during the growing period while minimum and maximum temperatures were 21.1 ± 0.8 ^{o}C and 32.3 ± 1.5 ^{o}C, respectively inside the protected house. The light intensity was 2.5 ± 0.73 klux in the morning, 5.25 ± 1.5 klux at midday and 3.62 ± 1.1 klux in the evening. The daily mean relative humidity was 79.4 ± 12.7% inside the protected house during the daytime. To provide a generally homogeneous medium across all treatments, the fine earth portion of Red Yellow Podsolic soil was recovered using a 2-mm stainless-steel sieve, and the sifted soils were permitted to air dry for over a week. The medium was spiked with solutions having 6 mg Cd^{2+}/L, 25 mg Pb^{2+}/L and 25 mg Zn^{2+}/L in the form of $Cd(NO_3)_2$, $Pb(NO_3)_2$ and $ZnCl_2$. The available heavy metal concentration of the soil was determined (Kisku et al., 2011) through acid digestion method (1 perchloric acid:4 nitric acid) using Atomic Absorption Spectrophotometry (AAS). A blend of urea (2.0 g/kg), triple super phosphate (TSP) (3.33 g/kg), and muriate of potash (MOP) (1.33 g/kg) was mixed with the soil as a basal fertilizer mixture based on the soil dry weight. Disease free seed tubers (‘Granola’ variety) were obtained from the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka (government certified) and sprouted for one week before being planted in each pot (3 tubers per pot) with the amended soil mixture. Four days following the seed tuber planting, 100 ml of diluted (250 times with clean water) FBB mixture was sprayed directly in to each pot medium using a spray tank.

For the first two weeks, all pots were irrigated twice a week with 250 mL of water, then every other day as the plants grew taller. Any leachate accumulated in the plastic container beneath the pots was poured back into their respective pots. All the pots were arranged according to CRD inside the greenhouse. The treatment combinations were 100% CF (100C), 50% CF (50C), 50% CF + FBB (50CB), FBB alone (B), and no amendments (0CB) with five replicates for each treatment. After 90 days from planting, plants were harvested without damaging the tubers and were washed carefully with de-ionized water to remove unwanted materials. The tubers were then placed in black polythene bags and transported to the laboratory for further analysis. Soil samples were also collected into black polythene bags separately to analyze available heavy metal contents.

2.4 Determination of bioavailability of heavy metals

Rhizo-remediation efficiency of CF and FBB treatment combinations on heavy metals were evaluated by analyzing the availability of heavy metals in soil samples and tuber biomass. Briefly, the peripheral peel of the tubers (1 mm thickness: TM0) was removed carefully using a sharp sterilized blade after washing the surface thoroughly with de-ionized water. Tuber mass layers with different thicknesses (tuber mass < 1 cm, TM1; 1 cm < tuber mass < 2 cm, TM2; tuber mass > 2 cm, TM3) were obtained after peeling off the TM0 layer. Approximately, 10 g of chopped tuber samples from each tuber layers were measured and air-

dried for a day separately, to reduce the water content, followed by oven-drying at 70°C for 48 hours to constant weight.

The dried samples (both tuber and soil samples separately) were ground manually with ceramic mortar and pestle followed by passing through a 2mm non-metal sieve to ensure uniform particle size. Samples were digested (approximately 1 g) with 10 mL of 16 N concentrated nitric acid diluted to 50 mL with deionized water, and the extract was used to determine Pb^{2+}, Cd^{2+} and Zn^{2+} concentrations using the Atomic absorption spectrophotometer (AAS Model No. GBC 933AA) at 217.0 nm, 228.8 nm and 213.9 nm wavelengths, respectively (standards were $Pb(NO_3)_2$, $Cd(NO_3)_2$ and $ZnCl_2$). Samples were analyzed in triplicates. Based on the absorbance data, heavy metal concentrations in the different layers of tuber mass and soil samples were determined. Mean concentrations of the treated soils were compared with the concentration of initial soil (Kisku et al., 2011).

2.5 Statistical Analysis

The effects of different CF and FBB amendments on heavy metal bioavailability, was analyzed using the analysis of variance (ANOVA) in Minitab® 16.2.1, 2010.Mini Table 2017 software. A Tukey's Simultaneous mean separation test (α = 0.05) was used to test for significant differences in the treatment means.

2.6 Molecular identification of heavy metal tolerant microbial components

Using a modified thermolysis approach, genomic DNA of heavy metal resistant fungal isolates was extracted from 5-day old fungal cultures grown on plates (Zhang et al., 2010). Universal primers for fungal DNA ITS1 (5'–TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC-3') were used to amplify fungus DNA (White et al., 1990). In a total volume of 20 µL, each amplification sample contained 2 µL of 10× PCR buffer (Fermentas), 1.2 µL of dNTP mixture (2.5 mmol l-1 each), 0.8 µL of deioned formamide, 0.4 µL of MgCl2 (25 mmol l-1), 0.8 µL of each primer (10 µmol l-1), 0.2 µL of Taq DNA polymerase (5 U µL-1) and 1 µL of genomic DNA (20 ng/mL). Polymerase Chain Reaction (PCR) products were purified (Bao et al., 2012) and subjected for sequencing. Identification of the heavy metal tolerant bacterial isolates was done through 16S rDNA sequence analysis. The genomic DNA of each isolate cultured in YMB for 24 to 48 hours was extracted according to the manufacturer's procedure using the ZR Bacterial DNA Kit™ (Zymo Research California USA). Forward primer 27F (5'-AGA GTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3') were used for the PCR procedure. In 25 µL PCR mixture, 0.5 ng of genomic DNA, 2X Master Mix (One PCR) of 80 mM Tris-HCl (pH 9.2), 0.1% TritonTMX-100, 150 mM of dNTP, 1.0 mM of $MgCl_2$, 0.005 U of Taq DNA Polymerase and 0.2 µM of forward and reverse primer were included with volume adjustment with nuclease-free water. The 16S rDNA amplification of the isolates was detected using agarose gel electrophoresis. The amplified products were sequenced at the Macrogen Sequencing facility in Korea, and the sequences were compared using pairwise alignment with BLAST algorithm to those stored in the

Genbank databases of the National Center for Biotechnology Information (NCBI) (Landeweert et al., 2003; Javadi et al., 2012).

3. Results

3.1 Analysis of fungal and bacterial isolates for tolerance to heavy metals

Initially six fungi and ten bacteria were isolated from soil samples and the MICs of the Pb^{2+}, Cd^{2+} and Zn^{2+} for the fungal and bacterial isolates were studied. The fungal and bacterial isolates were highly resistant to metal ions and grew rapidly at lower metal ions concentrations. Higher metal ion concentrations reduced the growth compared to the control. All fungal isolates showed higher MIC for all metal ions than that of the bacterial isolates. Only four fungal isolates (F1-F4) showed a visible growth on PDA at the heavy metal concentration of 400 mg/L whereas five bacterial isolates (B1-B5) showed visible growth at the heavy metals' concentration of 300 mg/L. None of the bacteria and fungi was grown at the metal concentration of 500 mg/L. Therefore, TI percentage was calculated for fungal and bacterial isolates at the metal concentration of 400 mg/L and 300 mg/L, respectively. Fungal isolates F1 and F2 showed higher TI percentage than that of all other fungal isolates (Fig. 1a). The significantly ($P < 0.05$) highest TI percentage for Pb^{2+} and the numerically highest TI percentage for Cd^{2+} and Zn^{2+} were recorded by fungal isolate F2. Bacterial isolates B2, B3 and B5 showed higher TI percentages than that of all other bacterial isolates (Fig. 1b). Out of all bacterial isolates, the highest TI percentages for Pb^{2+} and Cd^{2+} were recorded by B3 whereas B5 showed the highest TI percentage for Zn^{2+}. Therefore, three bacterial isolates (B2, B3 and B5) and two fungal isolates (F1 and F2) were selected to develop FBBs based on the TI percentage values.

3.2 Molecular identification of heavy metal tolerant microbial components

Nucleotide sequence analysis of the responsive microbial components through GenBank search revealed that the isolates had high sequence similarity to the species B2- *Bacillus firmus* (MN643058.1), B3- *Bacillus subtilis* (NC_000964.3), B5- *Pseudomonas fluorescens* (NZ_JRXU00000000.1), F1-*Rhizopus oryzae* (DQ080073.1) and F2-*Trichoderma harzianum* (KR868300.1) (Table 1) among the nucleotide sequences available in the NCBI database.

Table 1
Molecular identification of heavy metal tolerant fungal and bacterial isolates

Sample identity	Length of the fragment (bp)	Closest Relative	Similarity (%)	Accession Number
B2	731	*Bacillus firmus*	99	MN643058
B5	842	*Pseudomonas fluorescens*	98	NZ_JRXU00000000
B3	621	*Bacillus subtilis*	99	NC_000964
F2	725	*Trichoderma harzianum*	100	KR868300
F1	810	*Rhizopus oryzae* (DQ080073.1)	98	DQ080073

3.3 Biofilm formation and bio-removal of heavy metals in in-situ liquid media

The fungal filaments in FBBs served as a surface for bacterial cells to colonize (Fig. 2a-f). Out of all FBB combinations, the highest attachment strength between bacterial cells and fungal filament was observed in the combination of *T. harzianum* (F2) and *B. subtilis* (B3). *R. oryzae* (F1) did not contribute to develop FBBs with any bacterial isolates. Based on the attachment strength, FBBs with two bipartite associations (*T. harzianum* and *B. subtilis*, *T. harzianum* and *B. firmus*) and FBB with one tri partite association (*T. harzianum, B. subtilis* and *B. firmus*) were evaluated for heavy metal bio-absorption capacity with their individual fungal and bacterial isolates.

In comparison with single cultures, all selected FBBs showed high heavy metal removal percentage after day 5. It was noted that Cd^{2+} removal percentage was higher than Pb^{2+} removal percentage in all FBBs whereas Pb^{2+} removal percentage was higher than Cd^{2+} removal in all single cultures (Fig. 3). Out of all the FBBs, the significantly ($P < 0.05$) highest heavy metal removal percentage was observed in *T. harzianum* and *B. subtilis* combination for all the heavy metals. Therefore, the FBB combination *T. harzianum* and *B. subtilis* was selected to evaluate its rhizoremediation ability of Cd^{2+}, Pb^{2+} and Zn^{2+} metal ions in a greenhouse pot experiment with potato.

The sorption of metal ions on biofilms was investigated using FT-IR spectroscopy. A number of absorption peaks were observed in the FT-IR spectra of the FBBs (Fig. 4). Around 3,600–3,100 cm^{-1}, a large absorption peak was observed with maximum absorption at 3,329 cm^{-1}. The spectrum also displayed an absorption peaks at 2,920 cm^{-1} and at 2851 cm^{-1}. Clear peaks were observed at 1,640 cm^{-1}, 1550 cm^{-1} and at 1080 cm^{-1}. In comparison to heavy metal untreated biomass, the FT-IR spectra of all heavy metal treated biofilm biomasses revealed a slight shift in the area of 1720–1150 cm^{-1}. Further, the intensities of the peaks of that region were reduced in the Cd^{2+} and Pb^{2+} treated biomass whereas the intensities were higher in Zn^{2+} treated biomass in comparison with metal untreated biomass. Moreover, a

small peak which appeared in the heavy metal untreated biomass around 1720 cm^{-1} (C = O stretching) disappeared in the Cd^{2+} and Pb^{2+} treatments, whereas it appeared in Zn^{2+} treated biomass. When compared to the spectra obtained for heavy metal untreated biomass, the spectrum after interaction with heavy metal ions revealed the absence of an asymmetrical stretching band at 2851 cm^{-1}. Further the peak intensities in the region 3300 – 2600 cm^{-1} were lower in all the metal treated biomasses than the metal untreated biomass. It was noted that the peak around 1550 cm^{-1}, corresponding to –NH bending shifted slightly after heavy metal biosorption.

3.4 The effect of CF and FBB treatments on rhizo-remediation of heavy metals

The initial soil available Pb^{2+}, Cd^{2+} and Zn^{2+} metal ion concentrations prior to the application of fertilizer treatments were 26.4 mg/L, 6.2 mg/L and 27.3 mg/L, respectively. It was clearly observed that the availability of all metal ions significantly ($P < 0.05$) reduced in FBB treated soil compared to non-biofilm treatments (Table 2). Out of all treatments, 50CB showed the lowest soil heavy metal availability for all metal ions. No significant differences were observed between the treatments 50CB and B for the availability of all heavy metals. Interestingly, treatment 50CB significantly ($P < 0.05$) reduced the soil available Pb^{2+} by 53%, soil Cd^{2+} by 49% and soil Zn^{2+} by 50% compared to 100C, and soil Pb^{2+} and Cd^{2+} by 44% and soil Zn^{2+} by 42% compared to initial soil metal ion concentrations. Further, FBB treated soil reduced the pH in comparison with FBB non-treated soil and the lowest was recorded in 50CB treatment. It was noted that the treatment 100C increased the availability of Pb^{2+}, Cd^{2+} and Zn^{2+} metal ions in soil at harvest by approximately 16%, 8% and 10% respectively compared to the metal ion availability before the application of CF.

Table 2
The effect of different CF and FBB treatments on soil heavy metal availability and pH

Treatment	Soil available heavy metal concentrations (mg/L)			Soil pH
	Pb^{2+}	Cd^{2+}	Zn^{2+}	
Initial soil	$26.40^{b} \pm 2.65$	$6.20^{b} \pm 0.82$	$27.3^{b} \pm 3.45$	$5.30^{a} \pm 0.75$
100C	$31.52^{a} \pm 3.32$	$6.73^{a} \pm 0.94$	$30.41^{a} \pm 4.2$	$4.81^{c} \pm 0.82$
50C	$27.90^{b} \pm 3.14$	$6.05^{b} \pm 0.76$	$25.7^{b} \pm 3.12$	$5.05^{b} \pm 0.45$
50CB	$14.75^{d} \pm 1.12$	$3.45^{d} \pm 0.07$	$15.52^{e} \pm 1.67$	$4.60^{d} \pm 0.55$
B	$15.86^{d} \pm 1.25$	$3.57^{d} \pm 0.11$	$15.89^{e} \pm 2.11$	$4.79^{c} \pm 0.32$
0CB	$23.42^{c} \pm 2.14$	$5.02^{c} \pm 1.57$	$22.26^{c} \pm 3.05$	$5.22^{a} \pm 0.63$

Mean ± SD. Treatments 100C, 50C, 50CB, B and 0CB are 100% CF, 50% CF, 50% CF + FBB, FBB alone and No amendments, respectively. Means in the same column followed by the same letter are not significantly different at 5% probability level.

3.5 Determination of bioavailability of heavy metals in potato tubers

It was observed that all treatments reduced the penetration of all heavy metals into the inner tuber mass with the increment of the distance from the peel of the tuber (Fig. 5). Interestingly, the biofilm treatments significantly ($P < 0.05$) reduced the bioavailability of all tested heavy metal in all tuber tissue layers compared to non-biofilm treatments. The lowest bioavailability was recorded by the treatment 50CB for all heavy metals in all tuber tissue layers. Bioavailability of all metal ions was not recorded in both TM2 and TM3 tuber biomass layers in FBB treatments whereas all the metal ions were detected in all tuber layers of non-FBB treatments. Out of all treatments, the highest metal ion bioavailability in the tuber layers was recorded in the treatment 100C. The 50CB reduced the bioavailability of Pb^{2+}, Cd^{2+} and Zn^{2+} by 76%, 62% and 81%, respectively in TM0 layer, and the corresponding values of 100C were 9%, 13% and 28%, respectively.

4. Discussion

Despite the fact that the sampling site was chosen with the aim of isolating metal-tolerant microorganisms, only a limited number of microorganisms were able to isolate from it. Pollution of soil and water by toxic compounds such as heavy metal ions, in general, may result in a decrease in microbial population and diversity. This is due to the stress exerted causing the extinction of sensitive inhabitant microbial species, as well as the enhanced growth of other resistant species. (Iram et al., 2009). This might be the primary reason of reducing the number of microorganisms during the initial isolation in the current study. However, microorganisms isolated from heavy metal-contaminated natural habitats,

frequently display resistance to heavy metal contaminants (Yazdani et al., 2010). Therefore, the natural tolerance shown by the microorganisms in such environments was considered to select the location to isolate microorganisms.

In our study, the fungal isolates had higher tolerance against heavy metal contamination compared to bacterial isolates (Fig. 1a, b). A prior research found that heavy metals impact bacteria and fungus differently in soil, with fungus being more resistant to heavy metals as a group than bacteria (Rajapaksha et al., 2004). The existence of fungal species has been widely reported in many contaminated/polluted areas with higher heavy metal concentrations, such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and As^{3+} (Zafar et al., 2007; Fazli et al., 2015; Oluwatosin et al., 2018). In the current study, only four out of six fungal isolates showed high tolerance against the treated metal ions. This might be due to the fungal genera, species, and strains have different morphological and physiological properties, and hence their responses to heavy metal ion concentrations differ (Saba *et al.*, 2017). The results showed that *T. harzianum* and *R. oryzae* had the highest metal tolerance against all metal ions. Remarkable tolerance of heavy metal such as Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and As^{3+} has shown by *Trichoderma* and *Rhizopus* species (Zafar et al., 2007; Zeng et al., 2010; Oluwatosin et al., 2018).

Toxic metal tolerance in bacteria has been well-studied. However, overall efforts appear to be limited considering the variety of toxic metal ions and bacteria in the soil (Tanu and Hoque, 2012). In the present study, it was evident that all the *Bacillus* sp. showed high tolerance and the highest was recorded by *B. subtilis* against the treated metal ions. High degree of tolerance has been reported by *Bacillus* sp. to heavy metals especially Cr^{3+} and Cd^{2+} (Tanu and Hoque, 2012). Further, *B. subtilis* has been reported as the most tolerant species to Pb^{2+} (Tharannum et al., 2012; Alzahrani and Ahamed, 2015) and Cd^{2+} (Sizentcov *et al.*, 2020). Heavy metals are impossible to be degraded biologically, therefore they persist in the environment for extended periods (Khan et al., 2009). However, it is evident that soil beneficial microorganisms are capable of detoxifying and bioremediating heavy metals such as Pb^{2+} and Cd^{2+} (Harrison, 1997; Casova et al., 2009). The genus *Trichoderma* has been reported to have effective soil colonization and a high biodegradation potential (Lorito et al., 2010). The inoculation of plant growth promoting rhizo-bacteria *Methylobacterium oryzae* and *Burkholderia* sp. to potato has significantly reduced the toxicity of Ni^{2+} and Cd^{2+} under pot culture conditions (Madhaiyan et al., 2007; Khan et al., 2009).

In the current study, FBBs had higher metal removal and tolerance capacity than their single cultures in the liquid medium (Fig. 3). There have been instances of biofilms being used to remove heavy metals (Meliani and Bensoltane, 2016; Ogbuagu et al., 2017). Biofilm communities of Gram positive and Gram negative bacteria, including *Streptococcus aureus*, *B. subtilis*, *B. licheniformis*, *Pseudomonas aeruginosa* and *Serratia marscecens* have been reported to have Cd^{2+} and Zn^{2+} bioremediated (Khan et al., 2009). Further, the reduced rates of CF, when coupled with FBB has allowed detoxification of allelochemicals and heavy metals (Doering and Uehlinger, 2006; Ogbuagu et al., 2011). Extracellular polymeric substances (EPS) formed by biofilms have been shown to protect the microbial population against external toxic

contaminants. This is mainly by creating a metabolic gradient within the structure, which results in an unequal distribution of heavy metal ions, allowing relatively limited amounts of pollutants to enter the biofilm's microbial cells, allowing for improved tolerance and resistance (Herath et al., 2014). Furthermore, bioinorganic processes and their products in biofilms aid in the transformation of toxic oxidation states of heavy metal ions into non-toxic states (Herath et al., 2014). The production of EPS by fungal mycelium has the potential to increase EPS production in FBBs (Seneviratne and Indrasena, 2006). The EPS is largely composed of a complex combination of polysaccharides, proteins, nucleic acid, and several other organic compounds, which can include functional groups such as hydroxyl, carboxyl, amino, and phosphate and may also engage in metal ion binding (Flemming and Wingender, 2010).

The involvement of different functional groups of the biofilm in metal sorption was further validated by FT-IR spectroscopic analysis (Fig. 4). The presence of many absorption peaks in the FBBs demonstrates the complexity of the FBBs biomass and EPS. The presence of O–H and N–H stretching, which represent the hydroxyl and amine groups, was shown by the broad absorption peak around 3600–3100 cm^{-1}. Absorption peaks at 2920 cm^{-1} and 2851 cm^{-1} revealed asymmetrical and symmetrical C-H stretching, confirming the existence of an aliphatic methylene group. The carbonyl group stretching from aldehydes and ketones is shown by the peak at 1,640 cm^{-1}. These groups could be conjugated or not to aromatic rings (Kellner et al., 1998). Due to the existence of a protein peptide link, the peak at 1550 cm^{-1} can be attributed to N–H stretching of secondary amide bonds. The strong band at 1080 cm^{-1} represents –CN stretching of the protein fractions on the EPS (Kang et al., 2007). Slight shifting of the FT-IR spectrum, disappearing of a peak around 1720 cm^{-1} (C = O stretching) and a reduction of peak intensities in the region of 1720– 1150 cm^{-1}, may signify the involvement of carbonyl group stretching in the binding of Cd^{2+} and Pb^{2+} metal ions.

Further, a disappearance of a band at 2851 cm^{-1} and a reduction of peak intensities in the region of 3300 – 2600 cm^{-1} for the heavy metal treated biomass indicated that the biosorption of metal ions occurs at hydroxyl, CH2 groups present on the surface of the biomass. A reduction of peak intensity at 1080 cm^{-1} after metal biosorption indicates the involvement of protein fractions available on EPS for the metal binding (Kang et al., 2007). It was noted that the peak around 1550 cm^{-1}, corresponding to –NH bending shifted slightly after heavy metal biosorption. This might be due to the involvement of amino groups in metal biosorption (Park et al., 2005). Therefore, the peak shifts in the spectrum observed with the presence of metal ions, as well as alterations in those peak areas, showed the interaction of those functional groups on the surface of the biofilm biomass via the heavy metal biosorption process.

The current study clearly showed that the FBB combination reduced the degree of heavy metal availability in soil, reducing the possibility of such soil toxicities reaching tuber tissues. Microbial communities are known to alter heavy metal mobility and availability to plants through the release of chelating agents, acidification, phosphate solubilization, and redox shifts (Abou-Shanab et al., 2003a; Smith and Read, 1997). Plants and bacteria can form nonspecific relationships in which typical plant functions and biochemical mechanisms stimulate the microbial population, which degrades

contaminants in the soil. These biochemical mechanisms boost the microbial community associated with plant roots to enhance remediation activity. It has been found that the presence of ectomycorrhizal or vesicular-arbuscular fungus on plant roots reduces metal absorption by the plants (Tam, 1995; Yan de *et al*., 2007). The reason might be that some plants may employ rhizosphere-dwelling plant growth-promoting bacteria or mycorrhizal fungus to minimize the negative effects of heavy metals and thus influence heavy metal uptake by plants. It has been reported that a strain of *Pseudomonas maltophilia* has converted mobile and toxic Cr^{6+} to nontoxic and immobile Cr^{3+}, which also reduced the mobility of other hazardous ions such as Hg^{2+}, Pb^{2+}, and Cd^{2+} in the context (Blake et al., 1993; Park et al., 1999). It is noteworthy that the pot culture experiment in the current study showed a reduction in metal ion availability by the application of FBBs in the form of FBB while reducing the pH in soil in comparison with FBB untreated soil (Table 2). It has been reported that the medium pH has a considerable impact on metal ion adsorption; the higher the acidity, the higher the adsorption (Lopez *et al*., 2000). The uptake of Zn^{2+}, Cd^{2+}, and Pb^{2+} by *Penicillium chrysogenum* mycelium was pH-dependent, with the optimum uptake of Pb^{2+} occurring in the pH range of 4 to 5 (Usman et al., 2020). Organic acids including gluconic acid produced by microbial biofilms is the main reason for the pH reduction in the medium (Seneviratne and Indrasena, 2006; Teitzel *et al*., 2003). Further, pH increases the negative charge at the surface of the microbial cells and EPS, which stimulate the immobilization by the electrochemical attraction and adsorption of cations (Lopez *et al*., 2000; Flemming and Wingender, 2010).

In the treatment 100C, all measured metal ions in the soil increased at harvest compared to initial soil metal ion availability. This may be due to the impact of external CF applications like urea and phosphate fertilizers like TSP. It has been reported that intensification of agricultural practices such as excessive use of synthetic agrochemicals, CF, organic manures result in accumulation of heavy metals like Cd^{2+} and Pb^{2+} in cultivated lands (Lambert and Indraratne, 2014). Phosphate fertilizers are considered as the key source of Cd^{2+} accumulation in agricultural soils among mineral fertilizers. Phosphorites (phosphate rocks) are used to make these fertilizers, which can include a high concentration of Cd^{2+} (Casova et al., 2009). For instance, TSP has been recorded the highest Cd^{2+} concentration (23.5 mg/kg) among the phosphate fertilizers used in potato cultivation in Sri Lanka (Premarathne *et al*., 2011). Further, urea added soils showed higher acid phosphatase activity, thereby decreasing the soil pH (Shetty et al., 2019). The current study showed a reduction of soil pH by the treatment 100C. Metal ions are more readily available in soil due to the solubilization and mobilization of metal ions in soil by short-chain organic acid anions, amino acids, and other low-molecular-weight organic molecules in this acidic rhizosphere environment (Rengel, 2015).

5. Conclusions

The FBB developed from *T. harzianum* and *B. subtilis* can be used as a potential candidate for the bio-removal of Pb^{2+} Cd^{2+} and Zn^{2+} metal contaminants in the liquid medium. However, in the soil medium with potato, 50CB can be considered as the best treatment to remediate the soil contaminated with Pb^{2+} Cd^{2+} and Zn^{2+} metals while reducing the metal penetration into potato tubers.

References

1. Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, van Berkum P, Moawad H, Ghanem K, Ghozlan HA (2003a) Rhizobacterial effects on nickel extraction from soil and uptake by Alyssum murale. New Phytol 158(1):219–224
2. Abtenh E (2017) Application of microorganisms in bioremediation-review. J Environ Microbiol 1(1):2–9
3. Afzal AM, Rasool MH, Waseem M, Aslam B (2017) Assessment of heavy metal tolerance and biosorptive potential of *Klebsiella variicola* isolated from industrial effluents. AMB Express 7(1):184
4. Ahmed NA, Nawaz, Badar U (2005) Screening of copper tolerant bacterial species and their potential to remove copper from the environment. Bull Environ Contam Toxicol 74:219–226
5. AlKhader AMF (2015) The Impact of Phosphorus Fertilizers on Heavy Metals Content of Soils and Vegetables Grown on Selected Farms in Jordan. Agrotechnol. 5 (1):1–5
6. Alzahrani OM and NT (2015) Ahamed Isolation and Characterization of Heavy Metal Resistant *Bacillus subtilis* spp. Collected from Water Sources of Taif Province of Saudi Arabia. Int J Curr Microbiol Appl Sci 4(6):350–357
7. Bao Z, Ikunaga Y, Matsushita Y, Morimoto S, Takada-Hoshino Y (2012) Combined analyses of bacterial, fungal and nematode communities in Andosolic agricultural soils in Japan. Microbes Environ 27:72–79
8. Baz El, Baz M, Barakate M, Hassani L, Gharmali AE, Imziln B (2015) Resistance to and Accumulation of Heavy Metals by Actinobacteria Isolated from Abandoned Mining Areas. The Scientific World Journal
9. Beveridge TJ (1989) Role of cellular design in bacterial metal accumulation and mineralization. Annu Rev Microbiol 43:147–171
10. Blake RC, Choate DM, Bardhan S, Revis N, Barton LL, Zocco TG (1993) Chemical transformation of toxic metals by a Pseudomonas strain from a toxic waste site. Environ Toxicol Chem 12:1365–1376
11. Burd GI, Dixon DG, Glick BR (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can J Microbiol 46(3):237–245
12. Casova K, Cerny J, Szakova J, Balík J, Tlustos P (2009) Cadmium balance in soils under different fertilization managements including sewage sludge application. Plant Soil Environ 55(8):353–361
13. Cheraghi M, Lorestani B, Merrikhpour H, Rouniasi N (2013) Heavy metal risk assessment for potatoes grown in overused phosphate-fertilized soils. Environ Monit Assess 185(2):1825–1831
14. Doering M, Uehlinger U (2006) Biofilms in the Tagliamento. Eawag: Swiss Federal Institute of Aquatic Science and Technology 60: 11–13
15. Fazli MM, Soleimani N, Mehrasbi M, Darabian S, Mohammadi J, Ramazani A (2015) Highly cadmium tolerant fungi: their tolerance and removal potential. J Environ Health Sci Eng 13:19 1–9
16. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633

17. Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A (2011) Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental and food microbiologists. Microbiol Mol Biol Rev 75(4):583–609
18. Harman GE, Lorito M, Lynch JM (2004) Uses of *Trichoderma* spp. to remediate soil and water pollution. Adv Appl Microbiol 56:313–330
19. Harrison JJ, Ceri H, Turner RJ (2007) Multi-metal resistance and tolerance in microbial biofilms. Nat Rev Microbiol 5:928–938
20. Harrison MJ (1997) The arbuscular mycorrhizal symbiosis: an underground association. Trends Plant Sci 2:54–60
21. Herath HMLI, Rajapaksha AU, Vithanage M, Seneviratne G (2014) Developed fungal–bacterial biofilms as a novel tool for bioremoval of hexavelant chromium from wastewater. Chem Ecol 30(5):418–427
22. Hookoom M, Puchooa D (2013) Isolation and identification of heavy metals tolerant bacteria from industrial and agricultural areas in Mauritius. Curr Res Microbiol Biotechnol 1:119–123
23. Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004) Bacterial communities associated with flowering plants of the Ni hyperaccumulator Thlaspigoesingense. Appl Environ Microbiol 70(5):2667–2677
24. Iram S, Iftikhar A, Barira J, Saeeda Y (2009) Fungal tolerance to heavy metals. Pak J Bot 41(5):2583–2594
25. Jang A, Kim SM, Kim SY, Lee SG, Kim IS (2001) Effect of heavy metals (Cu, Pb, and Ni) on the compositions of EPS in biofilms. Water Sci Technol 43(6):41–48
26. Javadi MA, Ghanbary MAT, Tazick Z (2012) Isolation and molecular identification of soil inhabitant Penicillia. Ann Biol Res 3:5758–5761
27. Kang SY, Lee JU, Kim KW (2007) Biosorption of Cr(III) and Cr(VI) onto the cell surface of *Pseudomonas aeruginosa*. Biochem Eng J 36:54–58
28. Kellner RJ, Mermet JM, Otto M (1998) Analytical chemistry. Wiley/ VCH Verlag GmbH Press, New York
29. Khan MS, Zaidi A, Wani PA, Oves M (2009) Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ Chem Lett 7:1–19
30. Kisku GC, Pandey P, Singh MP, Misra NV (2011) Uptake and accumulation of potentially toxic metals (Zn, Cu and Pb) in soils and plants of Durgapur industrial belt. J Environ Biol 32:831–838
31. Lambert MTJ, Indraratne SP (2014) Cadmium and lead contents in paddy and uncultivated grumusols in murunkan, mannar district. *Proceedings of the Peradeniya University International Research Sessions, Sri Lanka*18: 508
32. Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003) Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl Environ Microbiol 69:327–333

33. Lo´pez FA, La´zaro N, Priego JM, Marque´s AM (2000) Effect of pH on the biosorption of nickel and other heavy metals by *Pseudomonas fluorescens*. J Ind Microbiol Biotechnol 2:146–151
34. Lorito M, Woo SL, Harman GE, Monte E (2010) Translational research on *Trichoderma*: from 'Omics' to the field. Annu Rev Phytopathol 48:395–417
35. Madhaiyan M, Poonguzhali S, Sa T (2007) Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere 69(2):220–228
36. McLaughlin MJ, Hamon RE, McLaren RG, Speir TW, Rogers SL (2000) Review: A bioavailability-based rationale for controlling metal and metalloid contamination of agricultural land in Australia and New Zealand. Aust J Soil Res 38:1037–1086
37. McLaughlin MJ, Tiller KG, Naidu R, Stevens DP (1996) Review: The behavior and environmental impacts of contaminants in fertilizers. Aust J Soil Res 34(1):1–54
38. Meliani A, Bensoltane A (2016) Biofilm-Mediated Heavy Metals Bioremediation in PGPR Pseudomonas. J Bioremediat Biodegrad 7(5):1–9
39. Ogbuagu DH, Nwachukwu KN, Balogun BA (2017) Application of biofilms in removal of heavy metals from wastewater in static condition. Int J Microbiol Immunol Res 5(2):6–13
40. Ogbuagu DH, Okoli CG, Emereibeole EI, Anyanwu IC, Onuoha O, Ubah NO, Ndugbu CO, Okoroama ON, Okafor A, Ewa E, Ossai R, Ukah F (2011) Trace metals accumulation in biofilms of the upper and middle reaches of Otamiri River in Owerri. Nigeria J Biodivers Environ Sci 1(3):19–26
41. Oluwatosin GO, Olusegun OA, Akinyemi O, Cornelius CB, Mark SMR (2018) Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. J Microbiol 49:29–37
42. Park CH, Keyhan M, Matin A (1999) Purification and characterization of chromate reductase in *Pseudomonas putida*. General Meeting of American Society for Microbiology 99: 536–548
43. Park D, Yun YS, Park JM (2005) Studies on hexavalent chromium biosorption by chemically-treated biomass of *Ecklonia* sp. Chemosphere 60:1356–1364
44. Premarathana HMPL, Hettiarachchi CM, Indraratne SP (2005) Accumulation of Cadmium in Intensive Vegetable Growing soils in the Up Country. Trop Agric Res 17:93–103
45. Premarathna HMPL, Hettiarachchi GM, Indraratne SP (2011) Trace Metal Concentration in Crops and Soils Collected from Intensively Cultivated Areas of Sri Lanka. Pedologist 54(3):230–240
46. Quintelas C, Rocha Z, Silva B, Fonseca B, Figueiredo H, Tavares T (2009) Removal of Cd(II), Cr(VI), Fe(III) and Ni(II) from aqueous solutions by an *E. coli* biofilm supported on kaolin. Chem Eng J 149:319–324
47. Rajapaksha RM, C P, Tobor-Kapłon MA, Baath E (2004) Metal Toxicity Affects Fungal and Bacterial Activities in Soil Differently. Appl Environ Microbiol 70(5):2966–2973
48. Rengel Z (2015) Availability of Mn, Zn and Fe in the rhizosphere. J Soil Sci Plant Nutr 15(2):397–409
49. Saha JK, Selladurai R, Coumar MV, Dotaniya ML, Kundu S, Patra AK (2017) Status of soil pollution in India. In: Soil pollution-an emerging threat to agriculture. Springer, Singapore, pp 271–315

50. Seneviratne G, Indrasena IK (2006) Nitrogen fixation in lichens is important for improved rock weathering. J Biosci 31:639–643
51. Seneviratne G, Jayasinghearachchi HS (2003) Mycelial colonization by *Bradyrhizobia* and *Azorhizobia.* J. Biosci. 28: 243–247
52. Seneviratne G, Zavahir JS, Bandara WMMS, Weerasekara MLMAW (2008) Fungal-bacterial biofilms: their development for novel biotechnological applications. World J Microbiol Biotechnol 24:739–743
53. Shetty P, Acharya C, Veeresh N (2019) Effect of Urea Fertilizer on the Biochemical Characteristics of Soil. Int J Appl Sci Biotechnol 7(4):414–420
54. Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Academic Press Inc., San Diego
55. Tam PCF (1995) Heavy metal tolerance by ectomycorrhyzal fungi and metal amelioration by *Pisolithustinctorium*. Mycorrhiza 5(3):181–187
56. Tanu FZ, Hoque S (2012) Bacterial Tolerance to Heavy Metal Contents Present in Contaminated and Uncontaminated Soils. Bangladesh J Microbiol 29(2):56–61
57. Teitzel GM, Parsek MR (2003) Heavy Metal Resistance of Biofilm and Planktonic *Pseudomonas aeruginosa*. Appl Environ Microbiol 69(4):2313–2320
58. Tharannum S, Krishnamurthy V, Mahmood R (2012) Characterization of chromium remediating bacterium *Bacillus subtilis* isolated from electroplating effluent. Int J Eng Res Appl 4(2):961–966
59. Usman ZU, Mukesh Y, Vandana S, Sharma JK, Sanjay P, Sidhartha D, Anil SK (2020) Microbial Bioremediation of Heavy Metals: Emerging Trends and Recent Advances. Res J Biotechnol 15(1):164–178
60. Verma P, Rawat S (2021) Rhizoremediation of Heavy Metaland Xenobiotic-Contaminated Soil: An Eco-Friendly Approach. Springer Nature Singapore 95–113
61. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York, pp 315–322
62. Yang RY, Tang JJ, Chen X, Hu SJ. (2007). Effects of coexisting plant species on soil microbes and soil enzymes in metal lead contaminated soils. Appl. Soil Ecol. 37:240–246.
63. Yazdani M, Chee KY, Faridah A, Soon GT (2010) An in vitro study on the Adsorption, Absorption and uptake Capacity of Zn by the Bioremediator *Trichoderma atroviride*. Environ Asia 3:53–59
64. Zafar S, Aqil F, Ahmad I (2007) Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technol 98:2557–2561
65. Zeng X, Su S, Jiang X, Li L, Bai L, Zhang Y (2010) Capability of pentavalent arsenic bioaccumulation and bio-volatilization of three fungal strains under laboratory conditions. Clean Soil Air Water 38:238–241
66. Zhang YJ, Zhang S, Liu XZ, Wen HA, Wang M (2010) A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. Lett Appl Microbiol 51(1):114–118

Figures

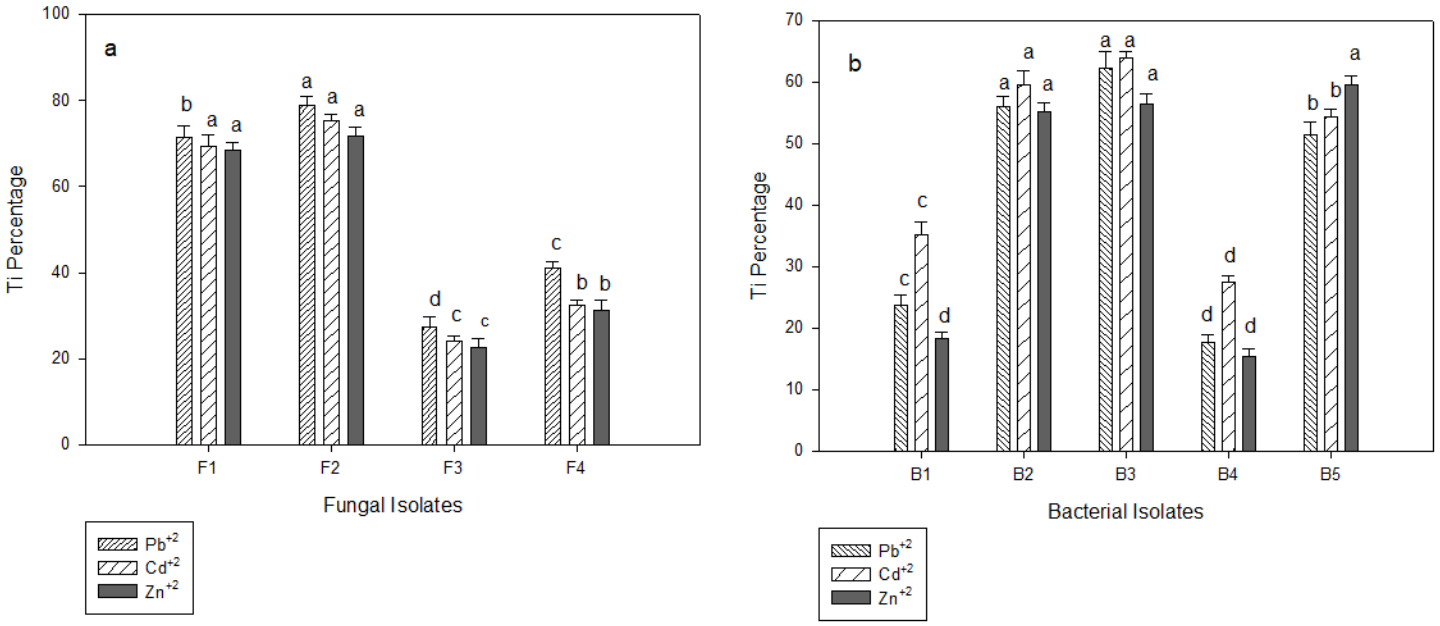

Figure 1

Tolerance Index (Ti) percentages of fungal and bacterial isolates against Cd2+, Pb2+ and Zn2+ metal ions. (a) – Ti percentages of fungal isolates against Cd2+, Pb2+ and Zn2+ metal ions. (b)- Ti percentages of bacterial isolates against Cd2+, Pb2+ and Zn2+ metal ions. Columns with the same letter are not significantly different at 5% probability level. vertical bars show standard deviations.

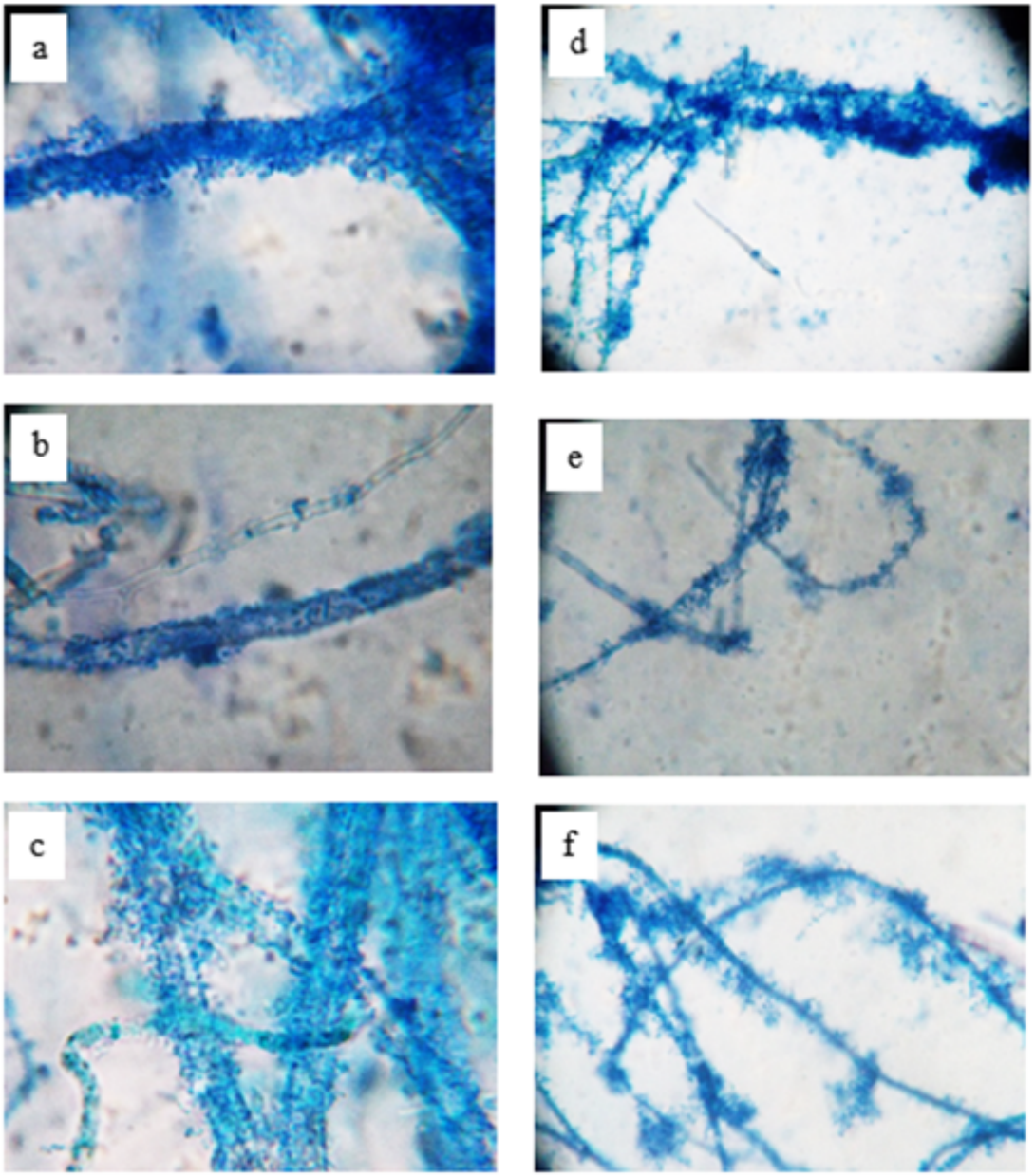

Figure 2

Bacterial colonization on Trichoderma harzianum mycelium in FBBs (a)- colonization of Bacillus subtilis, (b) Bacillus firmus, (c) B. subtilis and B. firmus, on T. harzianum mycelium in FBBs at x 1000 magnification. (d) B. subtilis (e) B. firmus (f) B. subtilis and B. firmus on T. harzianum mycelium in FBBs at x 400 magnification. Darkness is due to cotton blue stain absorbed by EPS produced by the biofilms. Stain, lactophenol cotton blue.

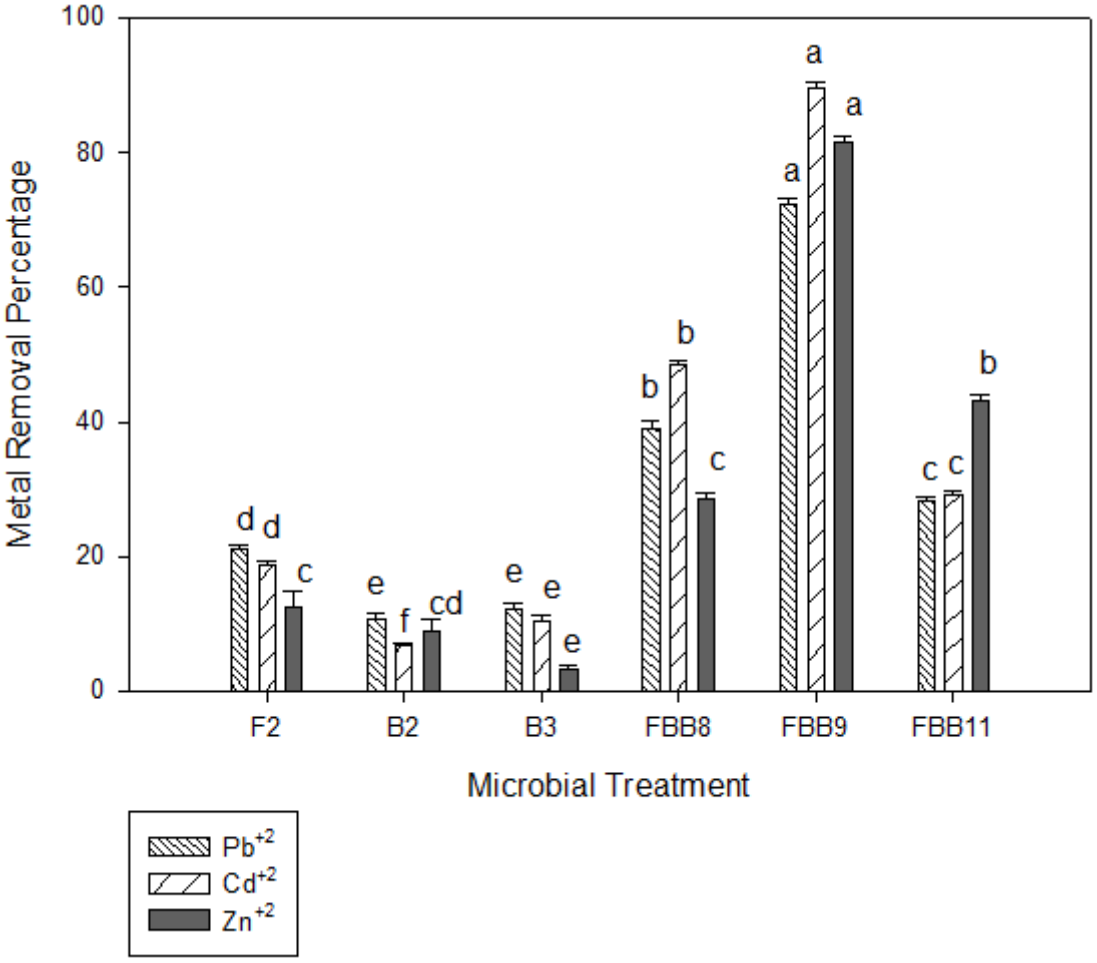

Figure 3

Metal removal percentages of fungal bacterial biofilms (FBBs) with their single cultures against Cd2+, Pb2+ and Zn2+ metal ions. Columns with the same letter are not significantly different at 5% probability level. vertical bars show standard deviations.

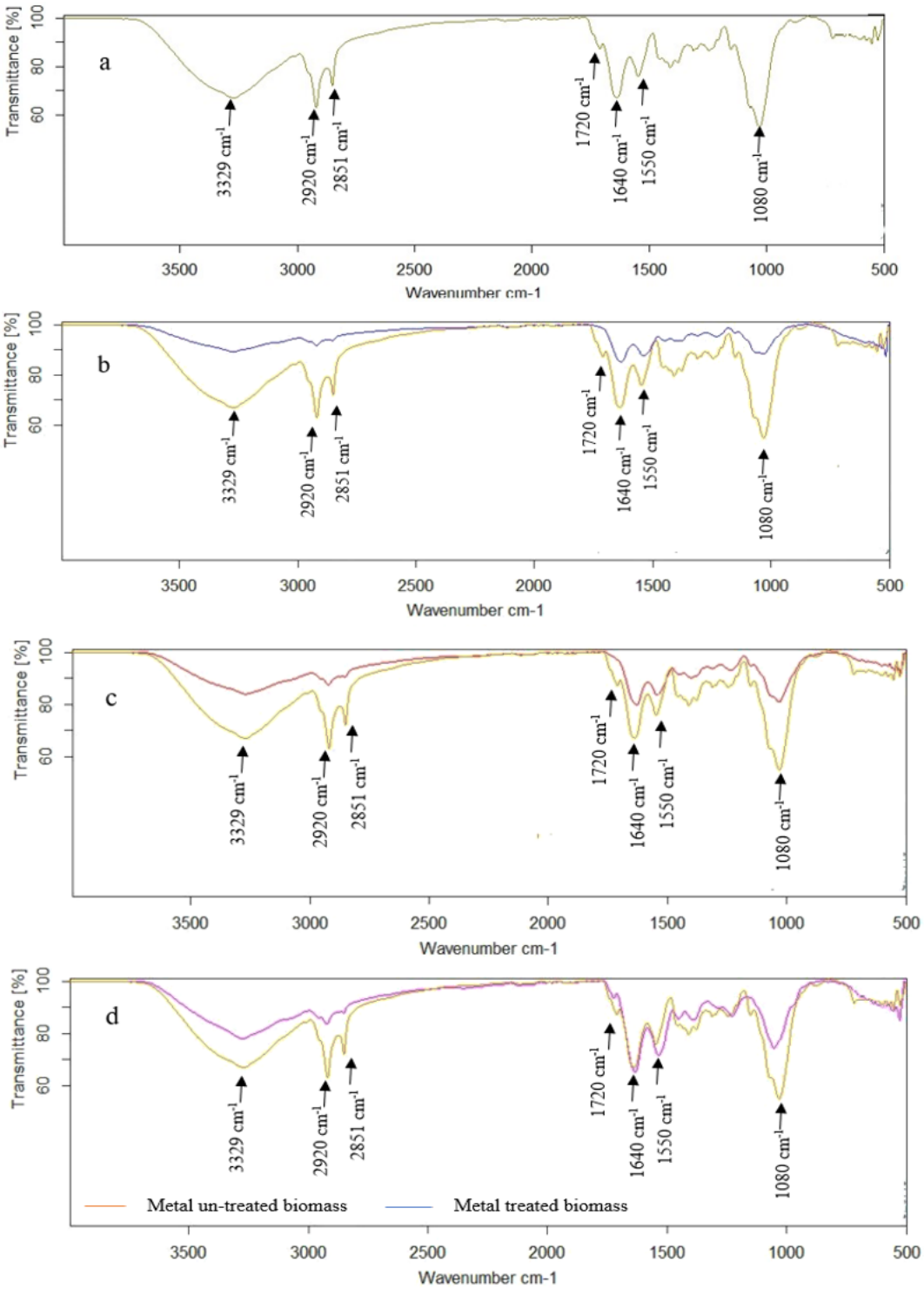

Figure 4

FT-IR spectrum of FBB (a) before heavy metal loading. (b) after Cd2+ loading, (c) after Pb2+ loading (d) after Zn2+ loading.

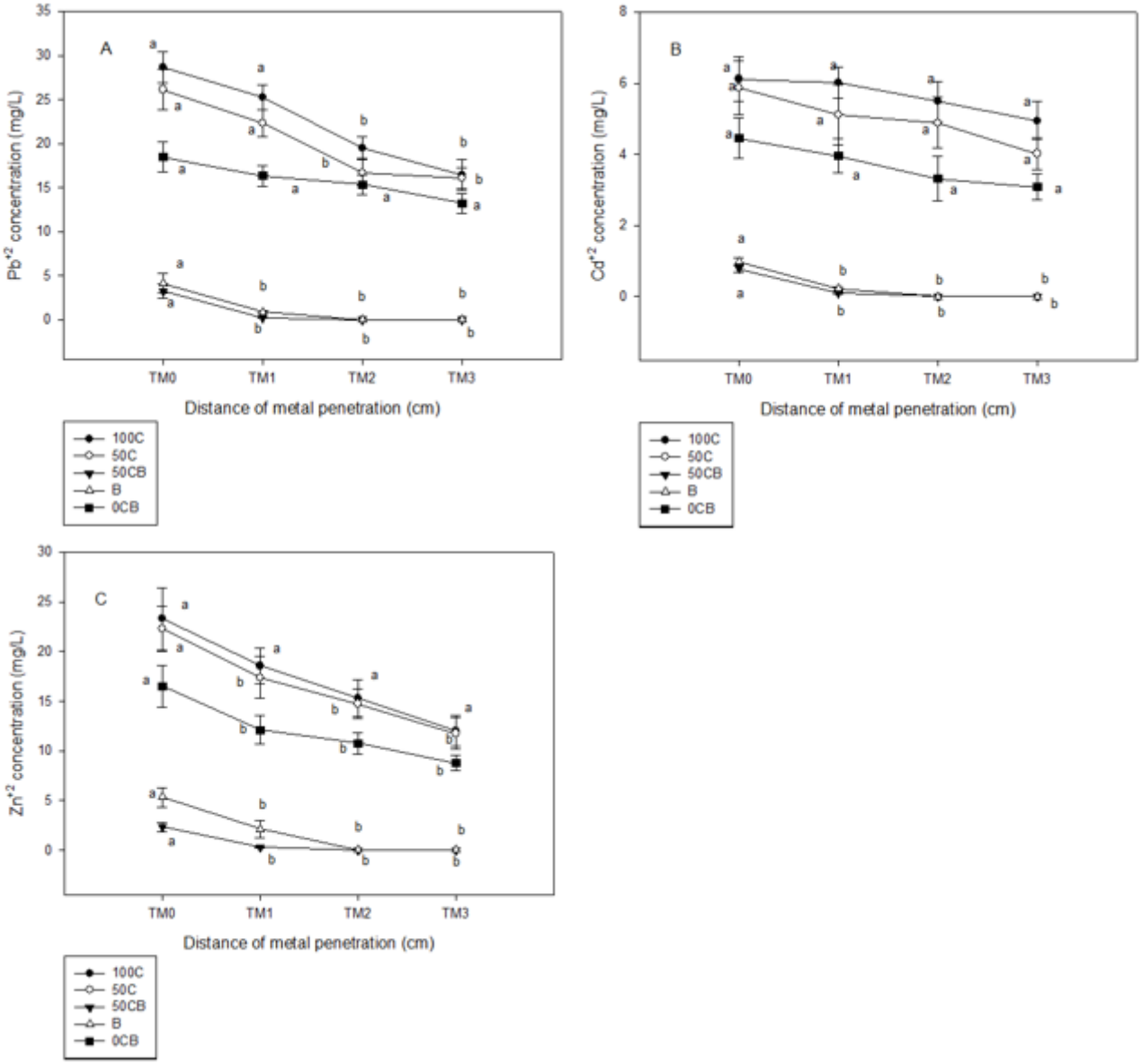

Figure 5

Changes in concentrations of heavy metals with the metal penetration distance in potato tuber mass. (A) Changes of Pb2+ metal ion, (B) Cd2+ metal ion, (C) Zn2+ metal ion concentrations with the penetration distance in potato tuber mass. Concentrations with the same letter along a line are not significantly different at 5% probability level.