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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 major damage to the environment. Microbial biofilms, closely associated with the rhizosphere can 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+}. Three bacterial and two fungal isolates were selected upon the tolerance index (TI) percentage. Fungal-bacterial biofilms (FBBs) were developed with the most 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 potatoes. Significantly ($P < 0.05$), the highest metal removal percentage was observed in *Trichoderma harzianum* and *Bacillus subtilis* biofilm under *in situ* conditions. When compared to the 100% recommended CF, the biofilm with 50% of the recommended CF (50CB) significantly ($P < 0.05$) reduced soil available Pb^{2+} by 77%, Cd^{2+} by 78% and Zn^{2+} by 62%. 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 100 C. Thus, the function of the developed FBB with *T. harzianum–B. subtilis* can be used as a potential solution to remediate soil polluted with Pb^{2+} Cd^{2+} and Zn^{2+} metal contaminants.

Keywords Metal contamination · Tolerance · Toxicity · Rhizosphere

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 the 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 because 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 (Alengebawy et al. 2021). 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 (Roberts 2014; Fan et al. 2018). The constant application of phosphate fertilizers introduces

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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). Due to the long-term use of agrochemicals, heavy metals such as Cu, Zn, and Cd have been reported to have a higher accumulation potential in agricultural soil (Wang et al. 2020). These heavy metals are included in most agrochemicals as ingredients (Alloway 2013). Excess Zn in the soil has a negative impact on soil characteristics, microbes, and enzymes, and accumulation of Zn in plant roots or shoots causes severe damage. (Lukowski and Dec 2018; Alengebawy et al. 2021).

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 (Dixit 2015; Suman et al. 2018). In this context, rhizoremediation through rhizosphere soil beneficial microorganisms has been considered as one of the promising methods to reduce soil toxicities (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 an environment for microbes (Idris et al. 2004) which are highly resistant to most 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, 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 (Hennebel et al. 2015; Sharma 2021). Multispecies biofilms formed by fungal–bacterial interactions have been employed more efficiently in bioremediation (Frey-Klett et 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 evaluating them for heavy metal tolerability and rhizo-remediation ability.

Materials and methods

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 in the first week of August in the year 2019, and it was sieved (< 2 mm) and stored overnight at 4 °C. The soil samples were serially diluted (10-fold) and 100 µl of each "dilution" was plated on nutrient agar (NA), actinomycetes isolation agar (AIA), starch casein agar, potato dextrose agar (PDA) and Czapek Dox agar. 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 an 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 °C for 5 days and at 25 ± 1 °C for 3 days, respectively, during which mycelial radial growth and bacterial growth were monitored. The 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 into 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.

The 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 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 °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. Afterward, 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.

$$Metal\,removal\,(\%) = [(C0 - Ct)/C0] \times 100$$

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 conditions.

Rhizo-remediation experiment

The experiment was conducted from September to December 2019 in a top-vent type film plastic greenhouse at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. The mean daily temperature was 31.7 ± 1.2 °C during the growing period while minimum and maximum temperatures were 21.1 ± 0.8 °C and 32.3 ± 1.5 °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 $79.4 \pm 12.7\%$ 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 the 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) were 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 into 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 (100 C), 50% CF (50 C), 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.

Determination of bioavailability of heavy metals

Rhizo-remediation efficiency of CF and FBB treatment combinations on heavy metals was 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 deionized 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 layer were measured and air-dried for a day separately, to reduce the water content, followed by oven-drying at 70 °C for 48 h 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 2 mm 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).

Statistical analysis

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

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 deionized 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 to sequencing.

Identification of the heavy metal tolerant bacterial isolates was done through 16 S rDNA sequence analysis. The genomic DNA of each isolate cultured in YMB for 24 to 48 h was extracted according to the manufacturer's procedure using the ZR Bacterial DNA Kit™ (Zymo Research California USA). Forward primer 27 F (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 16 S 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).

Results

Analysis of fungal and bacterial isolates for tolerance to heavy metals

Initially, six fungi and fourteen 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 seven bacterial isolates (B1-B7) 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 a 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
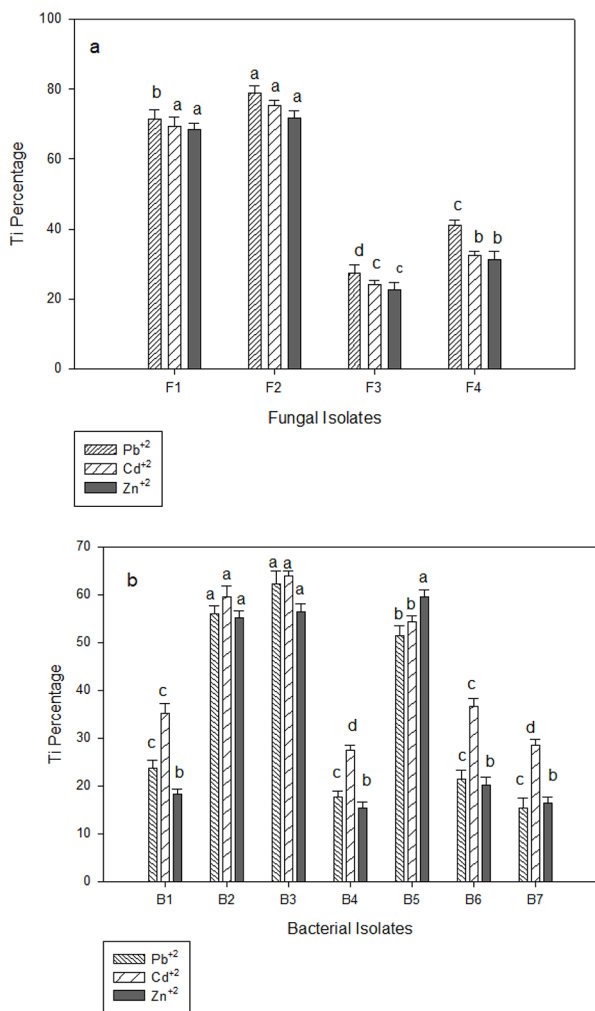
Fig. 1 Tolerance Index (Ti) percentages of fungal and bacterial isolates against Cd^{2+}, Pb^{2+} and Zn^{2+} metal ions. (a) – Ti percentages of fungal isolates against Cd^{2+}, Pb^{2+} and Zn^{2+} metal ions. (b)- Ti percentages of bacterial isolates against Cd^{2+}, Pb^{2+} and Zn^{2+} metal ions. Columns with the same letter are not significantly different at 5% probability level. vertical bars show standard deviations

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.

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 altitudinis* (LC667806.1), B3-*Bacillus subtilis* (OM061702.1), B5- *Pseudomonas fluorescens* (CP003041.1), F1-*Rhizopus oryzae* (MH877016.1), and

F2-*Trichoderma harzianum* (MK738146.1) (Table 1) among the nucleotide sequences available in the NCBI database.

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. Out of all FBB combinations, the highest attachment strength between bacterial cells and the fungal filament was observed in the combination of *T. harzianum* (F2) and *B. subtilis* (B3) (Figs. 2a and c and 3a and b). *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. altitudinis*) (Fig. 2a-d) and FBB with one tripartite association (*T. harzianum, B. subtilis*, and *B. altitudinis*) 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. 4). 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 with *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 potatoes.

The sorption of metal ions on biofilms was investigated using FT-IR spectroscopy. Several absorption peaks were observed in the FT-IR spectra of the FBBs (Fig. 5). 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 absorption peaks at $2,920\ cm^{-1}$ and $2851\ cm^{-1}$. Clear peaks were observed at $1,640\ cm^{-1}$, $1550\ cm^{-1}$, and $1080\ cm^{-1}$. In comparison to heavy metal untreated

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	634	*Bacillus altitudinis*	100	LC667806.1
B5	770	*Pseudomonas fluorescens*	100	CP003041.1
B3	621	*Bacillus subtilis*	100	OM061702.1
F2	618	*Trichoderma harzianum*	100	MK738146.1
F1	840	*Rhizopus oryzae* (DQ080073.1)	100	MH877016.1

Fig. 2 Bacterial colonization on *Trichoderma harzianum* mycelium in FBBs (a)- colonization of *Bacillus subtilis*, (b) *Bacillus altitudinis*, on *T. harzianum* mycelium in FBBs at x 400 magnification. (c) *B. subtilis* (d) *B. altitudinis* on *T. harzianum* mycelium in FBBs at x 1000 magnification. Darkness (x) is due to lactophenol cotton blue cotton blue stain absorbed by EPS produced by the biofilms
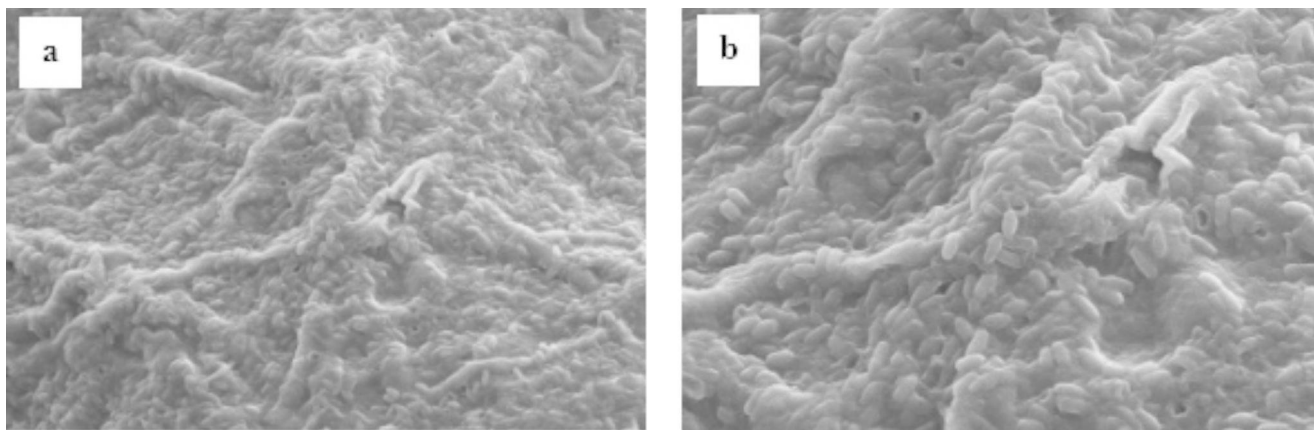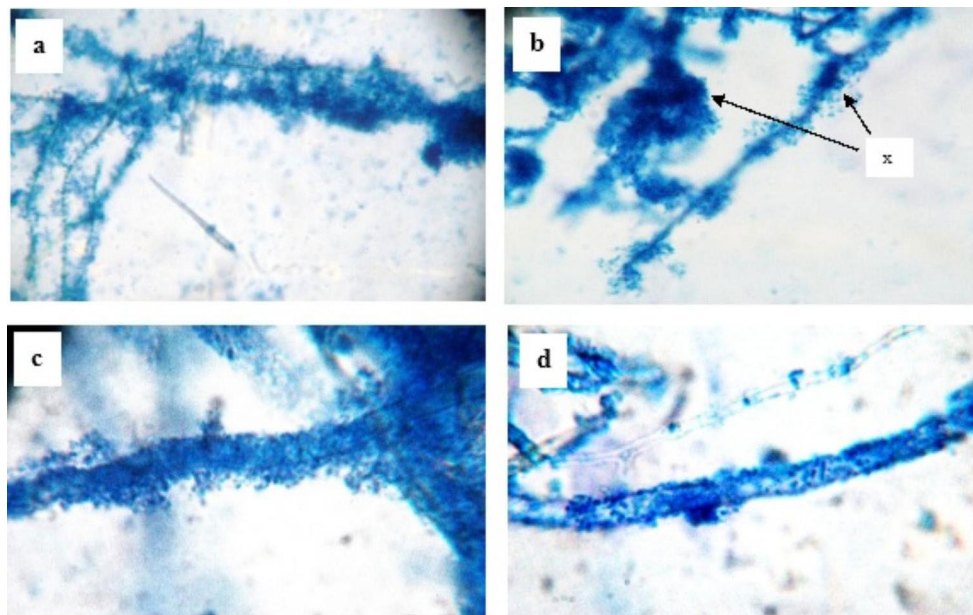
Fig. 3 SEM images of the most responsive FBB of *B. subtilis* on *T. harzianum*. The scale bar represents (a) 3 μm (b) 1 μm, respectively

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 that 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.

The effect of CF and FBB treatments on rhizoremediation of heavy metals

The initial soil available Pb^{2+}, Cd^{2+}, and Zn^{2+} metal ion concentrations before the application of fertilizer treatments were 26.4 mg/L, 6.2 mg/L, and 27.3 mg/L, respectively. It was observed that the availability of all metal ions significantly (P < 0.05) was 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

Fig. 4 Metal removal percentages of fungal bacterial biofilms (FBBs) with their single cultures against Cd^{2+}, Pb^{2+} and Zn^{2+} metal ions. Columns with the same letter are not significantly different at 5% probability level. vertical bars show standard deviations
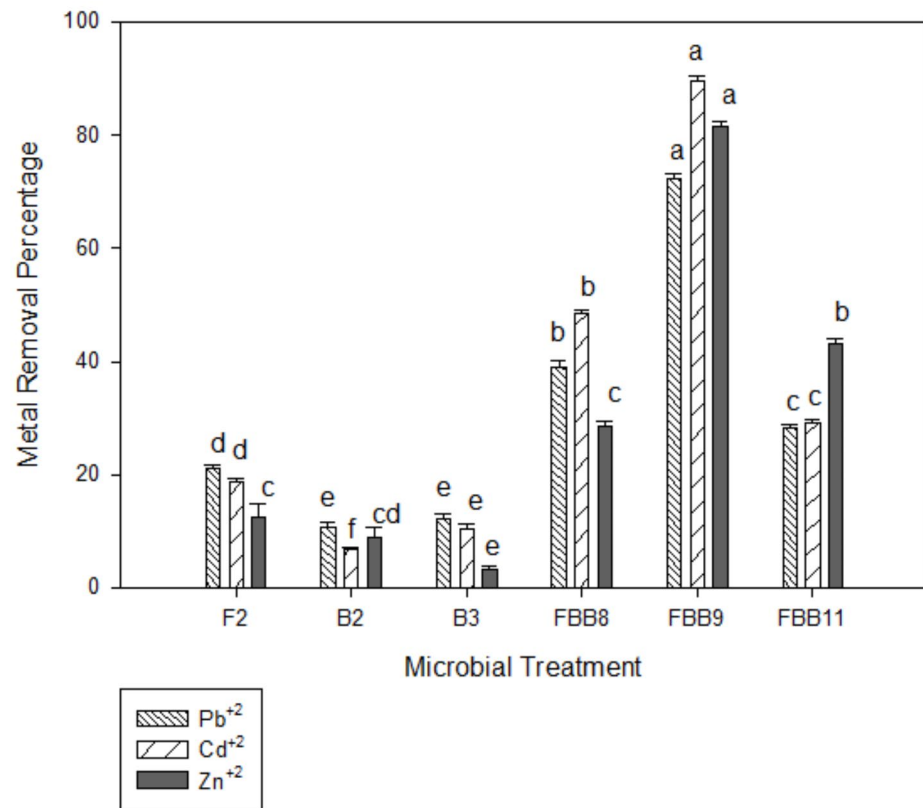
($P < 0.05$) reduced the soil available Pb^{2+} by 53%, soil Cd^{2+} by 49% and soil Zn^{2+} by 50% compared to 100 C, and soil Pb^{2+} and Cd^{2+} by 44% and soil Zn^{2+} by 42% compared to initial soil metal ion concentrations. Further, FBB treated soil reduced the pH in comparison with FBB non-treated soil, and the lowest was recorded in 50CB treatment. It was noted that the treatment 100 C increased the availability of Pb^{2+}, Cd^{2+} and Zn^{2+} metal ions in the soil at harvest by approximately 16%, 8%, and 10% respectively compared to the metal ion availability before the application of CF.

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. 6). Interestingly, the biofilm treatments significantly ($P < 0.05$) reduced the bioavailability of all tested heavy metals 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. The 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 100 C. 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 100 C were 9%, 13% and 28%, respectively.

Discussion

Even though the sampling site was chosen to isolate 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 for 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.
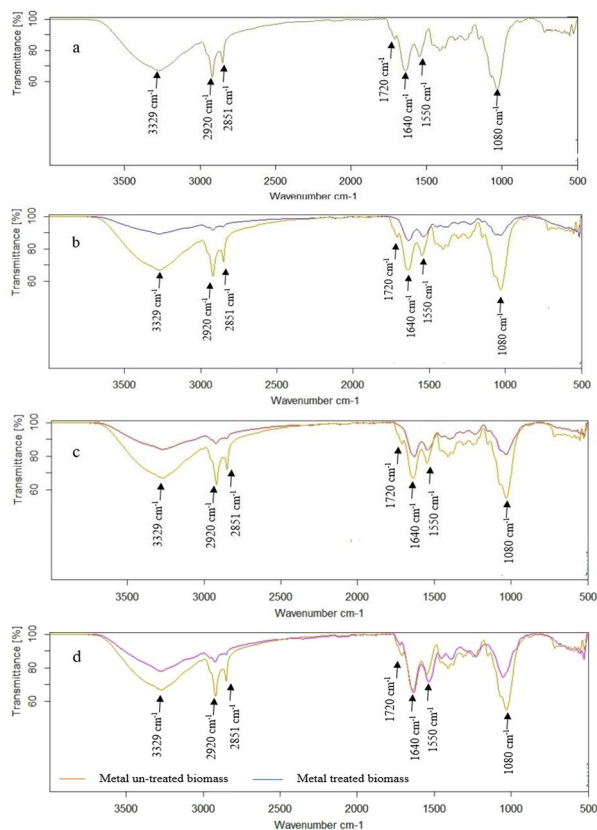
Fig. 5 FT-IR spectrum of FBB (a) before heavy metal loading. (b) after Cd^{2+} loading, (c) after Pb^{2+} loading (d) after Zn^{2+} loading

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	$26.40^b \pm 2.65$	$6.20^b \pm 0.82$	$27.3^b \pm 3.45$	$5.30^a \pm 0.75$
100 C	$31.52^a \pm 3.32$	$6.73^a \pm 0.94$	$30.41^a \pm 4.2$	$4.81^c \pm 0.82$
50 C	$27.90^b \pm 3.14$	$6.05^b \pm 0.76$	$25.7^b \pm 3.12$	$5.05^b \pm 0.45$
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 100 C, 50 C, 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

In our study, the fungal isolates had higher tolerance against heavy metal contamination compared to bacterial isolates (Fig. 1a, b). 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 having different morphological and physiological properties, and hence their responses to heavy metal ion concentrations differ (Saha et al. 2017). The results showed that *T. harzianum* and *R. oryzae* had the highest metal tolerance against all metal ions. The remarkable tolerance of heavy metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and As^{3+} has been 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. A 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+} (Syed and Chinthala 2015). Heavy metals are impossible to be degraded biologically, therefore they persist in the environment for extended periods (Khan et al. 2009). However, soil beneficial microorganisms are capable of detoxifying and bioremediating heavy metals such as Pb^{2+} and Cd^{2+} (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 rhizobacteria *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. 4). 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+} bioremediation (Khan et al. 2009). Further, the reduced rates of CF, when coupled with FBB have allowed detoxification of allelochemicals and heavy metals (Doering and Uehlinger 2006; Ogbuagu et al. 2011). Microorganisms can sequester, precipitate, biosorb, and change the oxidation states of various metals (Ibrahim et al. 2021). Metal sequestration happens by cell wall components and by intercellular metal bindings peptides and proteins (Balzano et al. 2020). The biomolecules
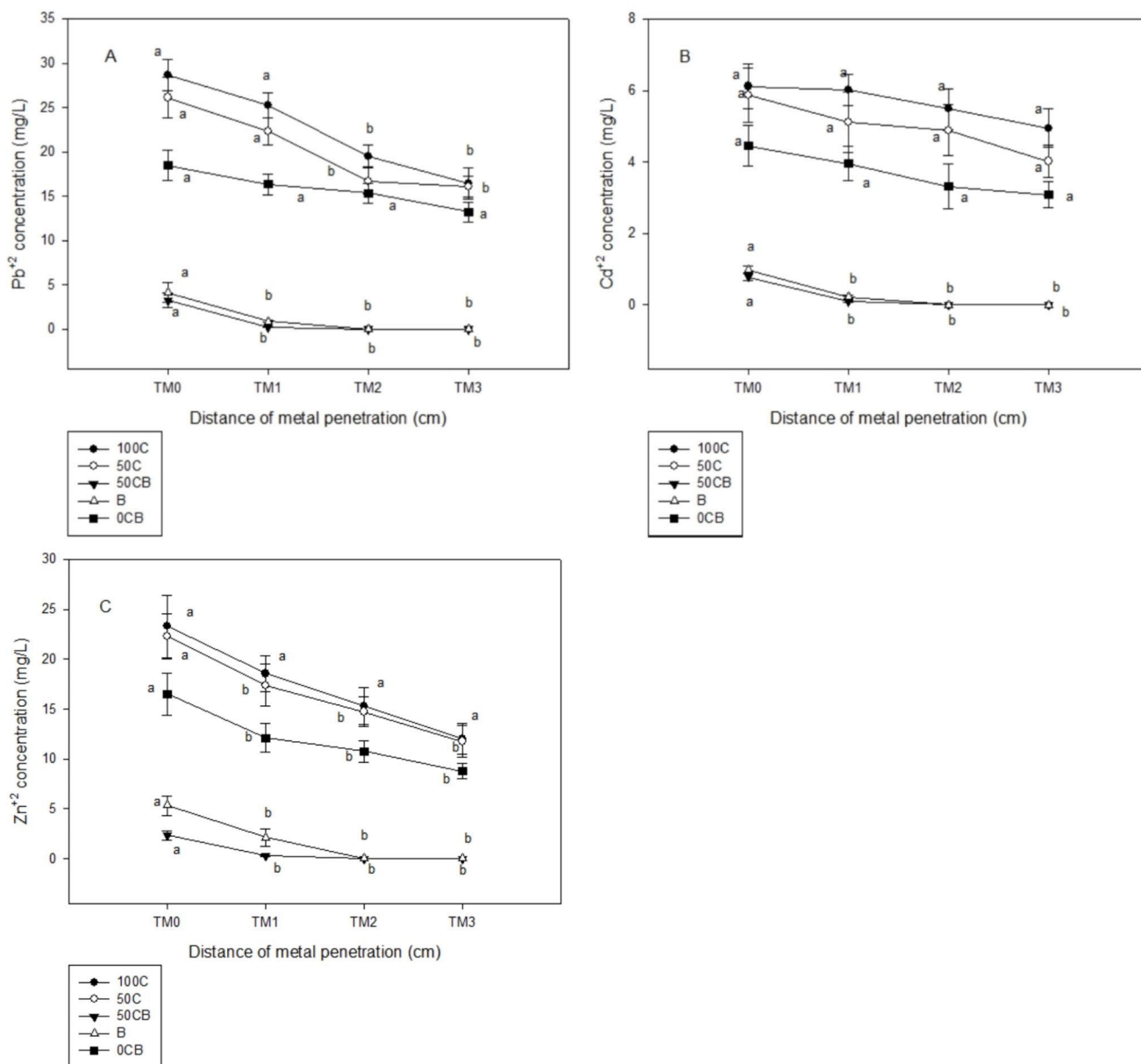
Fig. 6 Changes in concentrations of heavy metals with the metal penetration distance in potato tuber mass. (A) Changes of Pb^{2+} metal ion, (B) Cd^{2+} metal ion, (C) Zn^{2+} metal ion concentrations with the penetration distance in potato tuber mass. Concentrations with the same letter along a line are not significantly different at 5% probability level

of microbial cell walls contain negatively charged functional groups such as phosphate, hydroxyl, and carbonyl, which bind quickly with toxic metal ions and help them in bioremediation (Dixit et al. 2015). With the help of enzymes (such as dioxygenases, peroxidases, and oxidoreductases), microorganisms convert the toxic metal into a less toxic or harmless form. Further, 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 (Selatnia et al. 2004). Scanning electron microscopy also indicated high EPS secretion by FBBs (Fig. 3a,b). 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). Exopolysaccharide (EPS) is the most important component in bacterial cells that can sequester ions. Additional EPS-producing microbes i.e.; *Bacillus* sp., *Pseudomonas* sp., and *Agrobacterium* sp. (Selatnia et al. 2004) may produce a powerful polysaccharide rich in anionic groups that helps to remove hazardous metals from the environment (Hennebel et al. 2015).

The involvement of different functional groups of the biofilm in metal sorption was further validated by FT-IR spectroscopic analysis (Fig. 5). 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}. 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; Sessitsch et al. 2013). 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 (Yan de et al. 2007; Mishra et al. 2019). 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 Hassen et al. 2008; Chellaiah 2018). 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 the 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 (Sharma 2021). 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 are 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 stimulates the immobilization by the electrochemical attraction and adsorption of cations (Flemming and Wingender 2010).

In the treatment 100 C, 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 the 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 (Premarathna 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 of 100 C. 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). Although bioremediation abilities of textile dyes by *T. harzianum – B. subtilis* biofilm combination has been reported by another study (Henagamage 2019), the heavy metal bioremediation ability of the aforementioned biofilm combination has not been reported yet. Hence, this was the first study presenting the potential of *T. harzianum – B. subtilis* biofilm on heavy metal bioremediation in soil.

Conclusions

The use of biofilm based biofertilizers as a key to modern agriculture is fundamental, based on its renewable, low cost, and eco-friendly potential in ensuring sustainable agriculture. The FBB developed from *T. harzianum* and *B. subtilis* can be used as a potential candidate for the bioremoval 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. Hence, the function of developed FBB can be used as a potential solution to remediate soil polluted with Pb^{2+} Cd^{2+} and Zn^{2+} metal contaminants.

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