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Characterization of lead resistant endophytic *Bacillus* sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*

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ABSTRACT

The aim of this study was to isolate and characterize endophytic bacteria from the roots of the metal hyperaccumulator plant *Alnus firma*. A total of 14 bacterial endophytes were isolated from root samples and assayed for tolerance to heavy metals. Isolate MN3-4 exhibited maximum bioremoval of Pb and was subsequently identified as *Bacillus* sp. based on 16S rRNA sequences. The pH and initial metal concentration highly influenced the Pb bioremoval rate. The growth of isolate MN3-4 was moderately altered in the presence of metals. Scanning electron microscopy, energy dispersive spectroscopy, biological-transmission electron microscopy, and Fourier transform infrared spectroscopy studies revealed that isolate MN3-4 had extracellularly sequestered the Pb molecules with little intracellular accumulation. Isolate MN3-4 did not harbor *pbrA* and *pbrT* genes. Moreover, isolate MN3-4 had the capacity to produce siderophores and indoleacetic acid. A root elongation assay demonstrated an increase (46.25%) in the root elongation of inoculated *Brassica napus* seedlings compared to that of the control plants. Obtained results pointed out that isolate MN3-4 could potentially reduce heavy metal phytotoxicity and increase Pb accumulation in *A. firma* plants.

1. Introduction

Mining activities are a major source of heavy metal contamination in the surface environment. Elevated levels of heavy metals are found in and around metalliferous mines due to the discharge of mine waste into the ecosystem [1,2]. South Korea has a long history of metal-mining activities, with the most extensive activities reported in the early 20th century. Currently, most of the mines are abandoned because of economic conditions and a major decline in ore reserves. At present, there are approximately 1000 abandoned mines in Korea, and most of them have been left unmanaged, resulting in severe contamination of nearby agriculture soils and streams [2–5]. To address this concern, several chemical processes such as precipitation, oxidation, reduction, and a membrane process have been tried using different surface-modified substrates [2,6–8]. Previous research has shown the ineffectiveness and disadvantages of these conventional processes [8,9].

Phytoremediation is considered a clean, inexpensive, and non-environmentally disruptive technology that uses certain plants for the remediation of contaminated sites. Hyperaccumulating plants have attracted great interest for phytoremediation of heavy metals. However, several factors restrict the application of phytoremediation for heavy contamination, including the (a) growth rate of the plants, (b) phytotoxicity of metals, (c) reduced root biomass, and (d) limited metal uptake [10,11]. As a result, efforts have been devoted to enhancing the phytoremediation activity of plants using either plant growth-promoting rhizobacteria or endophytes.

Endophytic bacteria are defined as a nonpathogenic bacteria present within the interior tissues of healthy plants. These bacteria are reported in most plant species and span a wide range of bacterial phyla. Endophytes can facilitate plant growth and increase plant resistance to pathogens, drought, salinity, and even herbivores. A great deal of research suggests that endophytic bacteria equipped with a metal sequestration pathway can diminish the phytotoxicity of pollutants and increase the phytoavailability of heavy metals by producing siderophores and organic acids [11–14]. Combining increased phytoavailability and reduced internal bioavailability of metals will allow hyperaccumulators to accumulate larger amounts

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of metals without increasing phytotoxicity. Simultaneously, a plant provides a favorable niche for endophytic bacteria so that biotic and abiotic stresses against endophytic colonization are reduced. Recently, many researches have shown the effectiveness of endophytic bacteria for enhanced remediation of metals. Sheng et al. [14] reported that the endophytic bacteria *Pseudomonas fluorescens* G10 and *Mycobacterium* sp. G16 reduce Pb toxicity in *Brassica napus*. Barzanti et al. [15] reported that the plant *Alyssum bertolonii* harbors several groups of endophytic bacteria, increasing plant growth and nickel accumulation.

Alnus spp. is the abundant plant species found in most of the abandoned mines of Korea. The genus belongs to the birch family (Family: *Betulaceae*) and comprises about 30 subspecies of monoecious trees and shrubs. *Alnus* spp. has the capability to fix nitrogen and use insoluble metals through the employment of inoculated mycorrhiza [16]. Pilot scale studies in reclaimed dredging areas have confirmed the potential application of *Alnus firma* and *Alnus hirsuta* for phytoremediation of Pb and other metals [17]. However, interactions between *A. firma* and their associated endophytic bacteria have not been reported. A better understanding of the interactions is a critical prerequisite for the development of effective phytoremediation using *A. firma*. Hence, the objectives of this study were (a) to isolate and characterize Pb-resistant endophytic bacteria from the metal hyperaccumulator *A. firma* collected from mine tailings, (b) characterization of the resistance mechanism in high Pb-resistant strains, and (c) to assess the potential strain for plant growth-promoting factors.

2. Materials and methods

2.1. Isolation of Pb-resistant endophytic bacteria from A. firma root interior

Three healthy *A. firma* plants were collected randomly from the Pb-contaminated mine tailings in Korea. The properties and heavy metal contents of the soil sample were as follows: pH 4.95; moisture content, 18%; organic matter, 6 g/kg; Pb, 846 mg/kg; Cd, 6.53 mg/kg; and As, 46.6 mg/kg. Plant samples were washed with tap water, followed by five rinses with sterile deionized water, and then separated into roots and stems. Healthy root samples were surface sterilized by sequential immersion in 95% ethanol and 3% hypochlorite solution for 2 min. Surface sterilized root samples were washed several times in sterile water to remove surface sterilization agents. The disinfection process was confirmed by plating 100 μl of final rinse water onto Luria–Bertani (LB) agar plates. Root materials (0.5 g fresh weight) were ground in a mortar containing 5 ml of sterile water. The suspension was serially diluted, and 0.1 ml of the dilution was plated using the spread plate technique onto LB agar supplemented with 25 mg/l of $Pb(NO_3)_2$. Plates were incubated at 37 °C for 3 days and observed for bacterial growth. Morphologically distinct colonies were identified, purified, and stored at 4 °C for further study.

2.2. Determination of lead and other heavy metal resistance

The resistance to lead and other heavy metals, such as zinc, cadmium, nickel, and copper, was determined using the agar dilution method [18]. A log phase culture of the isolates was aseptically inoculated on LB agar plates and individually supplemented with increasing concentrations of the previously mentioned metals (50–1500 mg/l). The plates were incubated at 37 °C for 24 h and observed for bacterial growth. The lowest concentration of a metal, which completely inhibited the growth of the isolates was considered as the minimal inhibitory concentration (MIC). All metal salts were added to the medium after autoclaving and cooling to 50 °C from filter sterilized stock solutions. The metal salts used for the study included $Pb(NO_3)_2$ (Wako, Osaka, Japan), $Cd(NO_3)_2$ (Junsei, Tokyo, Japan), $ZnCl_2$ (Junsei, Tokyo, Japan), $NiSO_4{\cdot}6H_2O$ (Daejung, Shiheung-Si, Korea), and $CuCl_2{\cdot}2H_2O$ (Wako, Osaka, Japan).

2.3. Removal of metals by isolates MN1-5 and MN3-4

Batch experiments were performed to test the metal removal activity of isolates MN1-5 and MN3-4. Several reactor sets were prepared, and the experiments were repeated twice. Briefly, 100 μl of the bacterial suspensions were aseptically inoculated in 250 ml Erlenmeyer flasks containing 100 ml of LB broth individually amended with metals (Cd, 50 mg/l; Ni, 50 mg/l; Pb, 100 mg/l; Zn, 100 mg/l; and Cu, 100 mg/l). The flasks were incubated in a shaking incubator (180 rpm) at 37 °C for 24 h. After incubation, 2 ml of the sample was collected from the flasks and centrifuged at 10,000 rpm for 5 min. One milliliter of the supernatant was immediately filtered through a 0.2 μm membrane and analyzed for residual metal concentration using inductively coupled plasma mass spectrometry (ICP) (Leemans Labs, Inc., USA).

2.4. Removal of Pb at different pH and initial metal concentrations

Batch experiments were performed to assess the influences of pH and initial metal concentration on Pb removal. Briefly, 100 μl of the bacterial suspensions MN3-4 was aseptically inoculated into 500 ml Erlenmeyer flasks containing 200 ml of Pb (50 mg/l) amended LB broth with different pH levels (4, 6.5, and 9). The flasks were incubated in a shaking incubator (180 rpm) at 37 °C for 24 h. Samples were collected at the prescribed time intervals (0, 12, 24, 36, and 48 h) and centrifuged at 10,000 rpm for 5 min. One milliliter of the supernatant was immediately filtered through a 0.2 μm membrane and analyzed for the residual Pb concentration using ICP.

Similarly, 100 μl of the bacterial suspensions MN3-4 was inoculated in 200 ml of LB broth amended with different concentrations of Pb (80, 160, and 320 mg/l). The flasks were incubated in a shaking incubator (180 rpm) at 37 °C for 24 h. Samples were collected at the prescribed time intervals (0, 12, 24, 48, 72, and 100 h) and centrifuged at 10,000 rpm for 5 min. One milliliter of the supernatant was immediately filtered through a 0.2 μm membrane and analyzed for the residual Pb concentration using ICP.

2.5. Growth kinetics of isolate MN3-4

One milliliter of the mid-log phase culture (10^8 cells/ml) of the bacterial suspensions MN3-4 was used for inoculation in 50 ml LB broth supplemented with metal (Cd, 75 mg/l; Ni, 75 mg/l; Pb, 750 mg/l; Zn, 100 mg/l; and Cu, 400 mg/l). The flasks were incubated in a shaking incubator (180 rpm) at 37 °C, and the growth was measured at different time intervals according to the increase in optical density at 600 nm in a UV–vis spectrophotometer [19]. Cultures grown in the absence of metal were used as a control.

2.6. Scanning electron microscopy–energy dispersive spectroscopy, Fourier transform infrared spectroscopy, and biological transmission electron microscopy

Scanning electron microscopy–energy dispersive spectroscopy (SEM–EDS), Fourier transform infrared spectroscopy (FT-IR), and biological transmission electron microscopy (Bio-TEM) analyses were carried out to detect Pb and their compounds that were either absorbed by the cell surface or entrapped within the exopolymeric substance (EPS). Samples (100 ml) of broth cultures from the test flask with Pb (100 mg/l) were centrifuged at 6000 rpm for 5 min.

The cells were washed twice with deionized sterile water and dehydrated using a lyophilizer (llshin, Lab Co., Ltd., South Korea). Lyophilized cells were placed on a brass stub, sputter-coated with gold, and examined by SEM–EDS (JSM-6400, Japan-JEOL). *Bacillus* sp. MN3-4 was characterized with respect to its surface functional groups using FT-IR. Lyophilized cells were mixed with KBr at a ratio of 1:100 and compressed into films for FT-IR analysis using a Perkin–Elmer Spectrum. Infrared (IR) absorbance data were obtained for wave numbers 500–4000 cm^{-1}. Isolate MN3-4 was cultured in LB medium supplemented with Pb (100 mg/l) at 37 °C for 24 h. After incubation, 10 μl of the sample was loaded in grids and air dried. Electron micrographs were collected using Bio-TEM (H-7650, Japan HITACHI).

2.7. Genomic DNA extraction and identification of isolate MN3-4

Cells were harvested from 10 ml overnight cultures of LB broth, and the pellets were lysed in lysis buffer containing 25% sucrose, 25 mM EDTA, 50 mM Tris–HCl, and 5 mg/ml of lysozyme [20]. The chromosomal DNA was extracted according to the standard procedure of Maniatis et al. [21].

The 16S rDNA was amplified using polymerase chain reaction (PCR) with the universal primers 27f and 1492r [22]. The PCR products were purified using a PCR purification kit (QIAGEN Inc., Valencia, CA, USA). Purified amplicons were sequenced in both forward and reverse directions using an automated sequencer ABI PRISM (Model 3700, Foster City, CA, USA). The sequences were compared using the BLAST program (http://www.ncbi.nlm nih.gov/BLAST) for the identification of isolates.

2.8. Amplification of Pb-resistant genes

The membrane transport protein genes *pbrA* and *pbrT* were amplified using the primers *pbrA*1 (5′-ATGAGCGAATGTGGCTCGAAG-3′), *pbrA*2 (5′-TCATCGACGCAACAGC CTCAA-3′) [23], *pbrT*f (5′-ATGGTGATTGCTTTAGTT-3′), and *pbrT*r (5′-TTAGGCTTGC TTCTTTTT-3′). The reaction conditions for amplification were as follows: pre-denaturalization at 95 °C for 4 min, then 35 cycles of denaturing at 95 °C for 30 s, annealing for 1.5 min (60 °C for *pbrA* and 49 °C for *pbrT*), extension at 72 °C for 1.5 min, and a final step for extension at 72 °C for 7 min.

2.9. Plant growth-promoting factors

Siderophore secretion was measured using the "universal" method of Schwyn and Neilands [24]. In brief, 0.5 ml of blue Chrome azurol S (CAS) solution was added to 0.5 ml of filtered supernatant of isolate MN3-4. A reference solution was prepared using the uninoculated medium. Positive reactions were estimated by the change in color of the assay reagent from blue to orange. The assay was considered to be negative when no change in color was observed within 3 h.

The ability of the isolates to use ACC as a nitrogen source is a consequence of the enzymatic activity of ACC deaminase. The isolates were cultured in DF minimal medium supplemented with 3 mM ACC instead of $(NH_4)_2SO_4$ as the nitrogen source. Following inoculation, the tubes were incubated at 37 °C on a rotatory shaker at 180 rpm for 48 h. After incubation, the development of turbidity in the tubes when compared with that of the control tube was considered a positive result. The ACC was added to the DF minimal medium from a filter sterilized stock solution after autoclaving and cooling to 45–50 °C [25].

The production of IAA was determined according to the method of Gordon and Weber [26]. In brief, isolate MN3-4 was cultured for 2 days in Dworkin and Foster (DF) minimal medium supplemented with 0.5 mg/ml of tryptophan. After incubation, 1 ml of cell suspension was transferred into the tube, mixed vigorously with 2 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$), and allowed to stand at room temperature for 20 min. Development of a pink color in the tubes indicated IAA production. The phosphate-solubilizing potential of the isolate was determined according to Pikovskaya [27]. The development of a clear zone at the inoculation site on the culture plates was considered to be an index of phosphate solubilization.

Table 1
Minimal inhibitory concentration of various heavy metals to the isolates. All the isolates exhibited maximum resistance to lead.

Strains	Pb	Cd	Zn	Ni	Cu
MN1-1	700	100	100	100	300
MN1-2	500	–	100	–	200
MN1-3	700	100	100	100	200
MN1-4	500	–	100	–	200
MN1-5	1500	100	200	100	600
MN1-6	500	–	100	–	200
MN2-1	500	–	–	–	200
MN2-2	700	100	100	100	500
MN2-3	500	–	–	–	200
MN2-4	500	–	100	–	200
MN3-1	1000	100	100	100	250
MN3-2	500	–	–	–	200
MN3-3	500	–	100	–	300
MN3-4	1500	150	150	150	800

(–) >50 ppm.

2.10. Root elongation assay

The plant root elongation activity of isolate MN3-4 was determined according to the method of Belimov et al. [28], with minor modification. In brief, *Brassica juncea* seeds were surface sterilized with 1% sodium hypochlorite solution and rinsed several times with sterile water. The surface sterilized seeds were inoculated with isolate MN3-4 by soaking for 1 h in 0.1 M phosphate buffer containing 10^8 cells/ml. Seeds soaked in sterile water were used as a control. The inoculated and control seeds were aseptically transferred into Petri dishes lined with sterile filter paper. Five milliliters of $PbNO_3$ solution (50, 100, and 150 mg/l) was aseptically added to the plates and incubated in a growth chamber at 25 °C with 12 h light/dark for 6 days. The assay was repeated in triplicate with three plates (with six seeds per dish) for each treatment. Results were subjected to analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences) v 12 software (Chicago, USA). Statistical significance was assessed using Duncan's multiple range test (DMRT) at $p < 0.05$.

3. Results and discussion

This study represents an attempt to correlate the endophytic bacteria and its role in enhancing the accumulation of metals in *A. firma*. The surface sterilization protocol was effective in removing epiphytic microorganisms, and the bacterial isolates can be considered to be true endophytic bacteria. A total of 14 morphologically different lead-resistant bacterial colonies were isolated from the root samples and repeatedly screened for their lead resistance in 1/5-strength LB medium to prevent lead precipitation [29]. The results showed that two isolates, designated MN1-5 and MN3-4, were found to be highly resistant (<1500 mg/l) to Pb and other metal ions (Table 1). The Pb resistance appears to be somewhat higher than that typically reported for isolates from other hyperaccumulator plants [29]. However, a direct comparison of our results with those of other studies is difficult because of the (a) composition of the medium used, (b) diffusion rate, (c) complexation, and

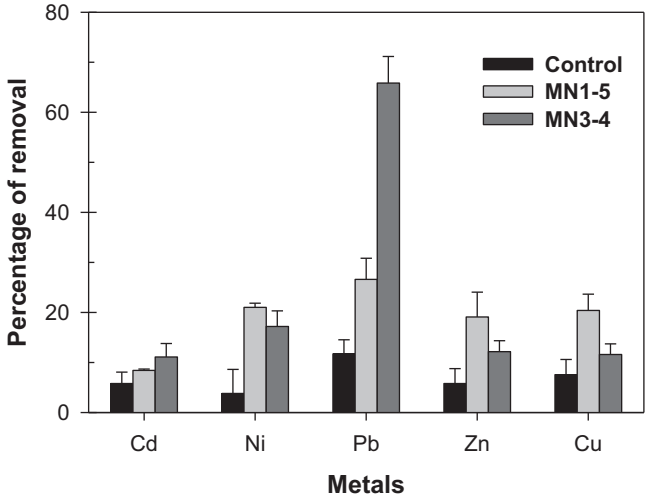

Fig. 1. Removal of heavy metals by isolates MN1-5 and MN3-4.

(d) bioavailability of metals to bacteria, resulting in a difference in the MICs of the metals [19,30].

A metal hyperaccumulator accumulates more than one metal and can therefore provide a specific niche for endophytes that could be adopted to survive in the presence of different metals. For instance, endophytic bacteria isolated from *Sedum alfredii* and *A. bertolonii* were resistant to more than one metal [15,31]. The present study also showed that endophytic bacteria isolated from *A. firma* were found to exhibit resistance to more than one metal (Table 1). All of the isolates exhibited maximum resistance to Pb and minimum resistance to either Cd or Ni. The order of the toxicity of metals in the isolates was found to be Pb > Cu > Zn > Ni > Cd. Results indicated that these endophytic bacteria populations had a marked adaptation to heavy metals under prolonged constant metal stress. The high Pb-resistant strains MN1-5 and MN3-4 were selected for further studies.

The phytotoxicity of metal is a critical factor affecting the success of phytoremediation. Recent studies suggest that some endophytic bacteria can reduce metal phytotoxicity via adsorption, intracellular accumulation, intracellular sequestration, or extracellular precipitation. Hence, the high Pb-resistant isolates MN1-5 and MN3-4 were screened for metal removal, with the results depicted in Fig. 1. Isolate MN3-4 effectively removed Pb (65.8%), followed by those of Ni (17.2%), Zn (12.1%), and Cu (11.6%). Similarly, MN1-5 also exhibited maximum removal of Pb (26.6%), followed by those of Ni (21.0%), Cu (20.4%) and Zn (19.1%). However, both of the isolates exhibited limited activity toward Cd. Since isolate MN3-4 exhibited maximum Pb removal efficiency, it was selected for further studies. PCR amplification of the targeted 16S rDNA resulted in the predicted 1.5 kbp amplicon in isolate MN3-4. The amplified products were sequenced and compared with 16S rRNA sequences in the NCBI database. Based on the partial sequence comparison by BLAST, the isolate was identified as *Bacillus* sp (99% identity with GenBank accession nos. HQ121399, AB126755, JF907013). The isolated 16S rRNA sequence of *Bacillus* sp. MN3-4 was deposited in the GenBank (accession no. JN689332). The results are consistent with previous studies reporting the presence of *Bacillus* sp. in heavy metal hyperaccumulators [29].

The Pb removal efficiency of *Bacillus* sp. MN3-4 at different pH levels was evaluated, and the results are presented in Fig. 2a. More than 95% of Pb ions were removed at pH 9, and 73% of Pb ions were removed at pH 7. However, a drastic change in the Pb removal rate was observed at pH 4. The differences in the removal rate can be attributed partly to variation in the population density. Moreover, the interaction between the Pb ions and bacteria is also strongly

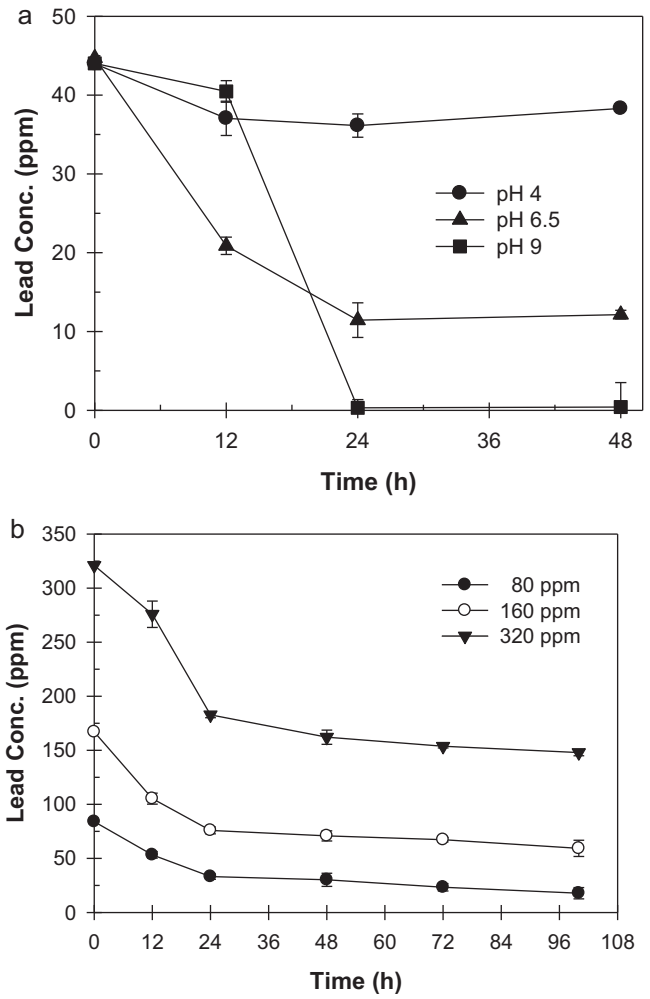

Fig. 2. Influence of pH and initial lead concentrations on lead removal by isolate MN3-4. (a) pH and (b) initial lead concentrations.

influenced by the pH of the solution. In an acidic pH, because of the protonation of the binding sites resulting from a high concentration of protons, negative charge intensity was reduced, resulting in the reduction or inhibition of the binding of metal ions [32]. Most bacterial surfaces are negatively charged because of the ionization of functional groups, thus contributing to the metal binding. At low pH, some functional groups will be positively charged and may not interact with the metal ions [33]. Conversely, metal ion adsorption is usually increased under neutral pH conditions by a proportional increase in the number of ionized acidic groups. These effects are reflected in the Pb removal rate of isolate MN3-4. Similarly, the Pb removal rate was also altered according to the initial concentration of Pb (Fig. 2b) and was decreased according to the increasing concentration of metals. The maximum removal rate (78.8%) was observed at 80 mg/l after 100 h of incubation, and the minimum removal rate (53.9%) was observed at 320 mg/l. The decreased removal rate at high concentration can be attributed to a reduced number of reactive sites for Pb ions. The results are consistent with previous studies reporting a decrease in the metal removal rate with a high concentration of metals [34].

The growth response of isolate MN3-4 in the presence of metals is presented in Fig. 3. A significant difference in the growth rate was observed according to the metal. The extended lag phase (24 h) was observed for Cd, and the minimum lag phase (4 h) was observed for Ni (Fig. 3). However, the mixed metals completely altered the growth of isolate MN3-4. The differences in the growth rate can

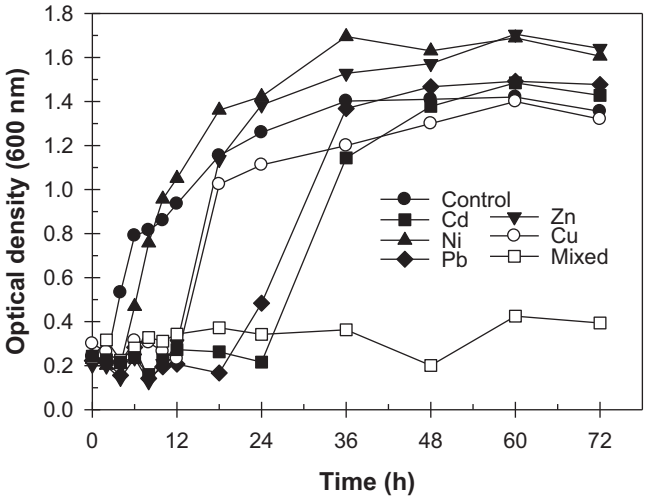

Fig. 3. Growth kinetics of isolate MN3-4 in the presence of metals. The growth rate of the isolates was moderately altered according to the metal.

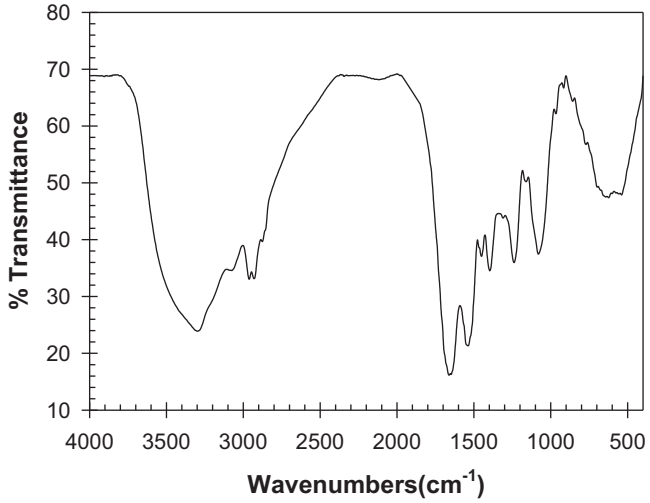

Fig. 5. Fourier-transform infrared spectra of isolate MN3-4.

be attributed to the bioavailability and toxicity of the metals. The results are consistent with previous studies reporting the difference in the growth rates of the *Bacillus* sp. according to the metal [19].

To further validate the Pb removal, isolate MN3-4 was characterized by SEM–EDS, FT-IR, and Bio-TEM. The EDS spectra of isolate MN3-4 before and after Pb removal are presented in Fig. 4a and b. The spectra showed the presence of phosphorus, chloride, and potassium in both the control and test samples. However, the Pb peak was observed only in the test sample, which

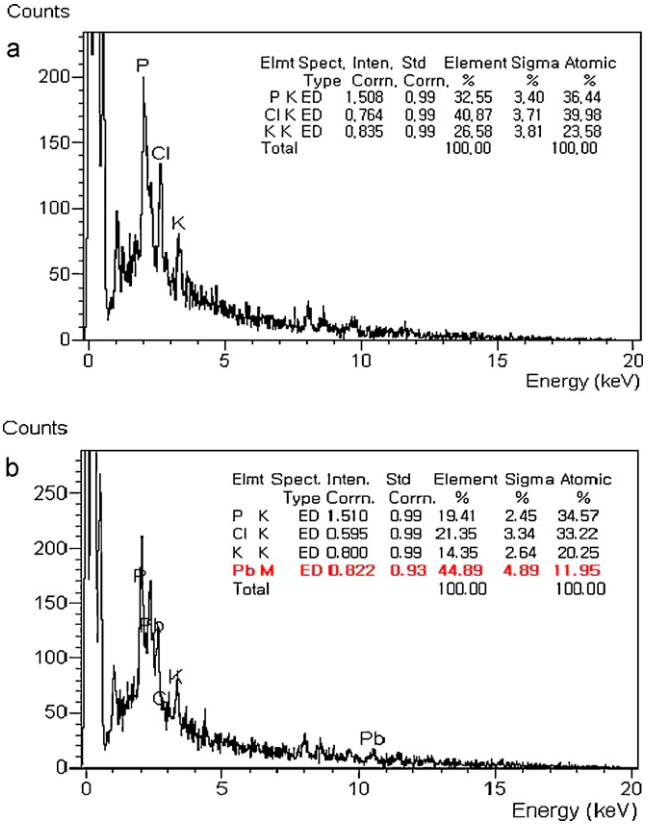

Fig. 4. Scanning electron microscopy–energy dispersive spectra of isolate MN3-4. (a) Control and (b) treated sample (after treatment with lead). Lead peaks were observed only in the treated sample, which indicates that the bacteria had either adsorbed or accumulated the lead ions.

indicates that the bacteria had either adsorbed or accumulated the metal ions. Numerous chemical groups are thought to contribute to the biosorption and bioaccumulation of metals in bacteria. These groups comprise hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, amide, and phosphonate groups [35]. In order to investigate the functional groups involved in the biosorption and bioaccumulation of Pb in isolate MN3-4, FT-IR analysis was carried out, and the results are presented in Fig. 5. The spectrum showed the presence of characteristic bacterial signatures. The broad peak (3400–3200 cm^{-1}) was assigned to the stretching of the O–H group because of inter- and intramolecular hydrogen bonding of polymeric compounds. O–H stretching indicates the "free" hydroxyl groups and bonded O–H bands of carboxylic acids. The second broad peak (2920–2850 cm^{-1}) indicated alkyl C–H stretching vibration of aliphatic acids. Peaks observed at 1658–1664 cm^{-1} and 1554–1532 cm^{-1} were due to the stretching vibrations of C=O bonds [36]. Furthermore, the spectrum showed the presence of prominent carboxyl (1398 and 916 cm^{-1}) and amide groups (1234, 1548 and 1648 cm^{-1}), which are preferentially expected for bacterial cultures [37].

A TEM microgram of the isolates is presented in Fig. 6. TEM revealed that isolate MN3-4 extracellularly sequestered lead with little adsorption and accumulation within the bacterial cells. Lead accumulation was visible as dark granules either outside or inside the bacterial cells and was confirmed by elemental analysis (Fig. 5). The extracellular sequestration could be due to the exopolymers produced by isolate MN3-4. *Pseudomonas marginalis* isolated from the lead-contaminated soil had sequestered lead ions in exopolymeric layers [38]. In addition to lead sequestration, the exopolymers also adsorb the lead ions and thereby limit the entry of metal ions into the cells. The reaction between the exopolymers and metal ions is thought to involve specific ionic interactions. Another mechanism of lead sequestration is the intracellular accumulation of lead as dense granules within the cytoplasm of isolate MN3-4. Intracellular accumulation is not a commonly observed mechanism of metal resistance because the bacterial cells have toxic effects as the metal crosses the cellular membranes [39]. Despite the metal toxicity, intracellular accumulation was sufficient to protect isolate MN3-4 from lead toxicity. The polymeric sequestration of lead and intracellular lead partitioning requires further study.

Numerous studies have reported that the microorganisms improved heavy metal resistance through horizontal gene transfer [40]. Proteins encoded by metal-resistant genes can reduce or eliminate metal toxicity. A better-characterized lead-resistant mechanism in bacteria includes active efflux pumps, such as P-type

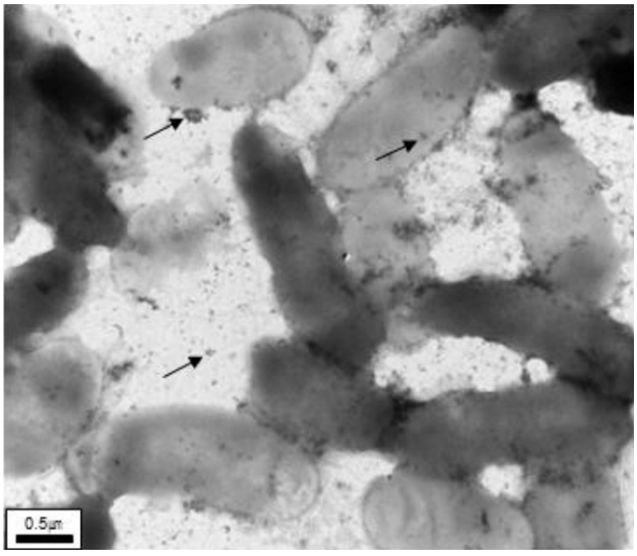

Fig. 6. Transmission micrographs of lead-resistant bacteria MN3-4 showing extracellular lead exclusion by an exopolymer surrounding the cell and intracellular accumulation. *Arrows* indicate the dark granules.

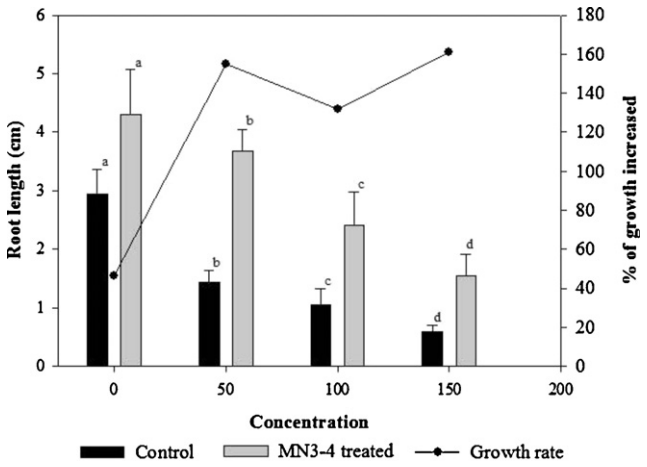

Fig. 7. Root length of *Brassica juncea* seedlings inoculated with the *Bacillus* spp. MN3-4 strains and grown in the absence or presence of lead in filter paper culture.

ATPases, which transport lead ions against the concentration gradient using energy provided by ATP hydrolysis. Hynninen et al. [41] found that the efflux transporter *pbrA* and a phosphatase *pbrB* cooperate in a lead-resistant mechanism in *Cupriavidus metallidurans* CH34. Hence, isolate MN3-4 was screened for the efflux transporter *pbrA* and lead uptake protein *pbrT*. Gel analysis of the PCR products showed no visible amplicon for either *pbrA* or *pbrT*. The results suggest that isolate MN3-4 may harbor another efflux transporter gene or that the primers were inappropriate for the amplification of the *pbrA* and *pbrT* genes. This phenomenon requires further study.

Endophytic bacterial associations are important in natural and managed ecosystems because of their nutritional and non-nutritional benefits to host plants. The beneficial effects of endophytic bacteria on their hyperaccumulators are similar to that of plant growth-promoting rhizobacteria, including IAA production, phosphate solubilization, siderophore production, ACC deaminase activity, and nitrogen fixation. IAA is a plant hormone that is widespread among bacteria–plant-associated systems. Zaidi et al. [42] reported that IAA indirectly increased the phytoaccumulation of metals by increasing plant biomass. In this study, estimation of IAA in culture filtrate showed that isolate MN3-4 had the ability to produce IAA (0.65 ± 0.04 M) when the culture medium was supplemented with L-tryptophan. Siderophores are low molecular weight organic molecules that show high affinity for Fe^{3+} ions, but they can also form complexes with metals that can be assimilated by the plants. Braud et al. [43] found that the siderophore-producing *Pseudomonas aeruginosa* was able to solubilize large amounts of Cr and Pb in soil. Dimkpa et al. [44] reported that microbial siderophores might help a plant to reduce heavy metal toxicity by increasing the supply of iron to the plant. Therefore, siderophore production in endophytic bacteria is important to reduce phytotoxicity, as well as to increase metal bioavailability. The presence of a clear orange zone around the bacterial colony in CAS agar medium indicated that isolate MN3-4 had produced siderophores. However, the isolate failed to produce the ACC deaminase enzyme or to solubilize phosphate.

The effect of isolate MN3-4 on root elongation of *B. juncea* in the absence or presence of Pb is shown in Fig. 7. The addition of Pb to the filter paper culture inhibited root elongation of uninoculated seedlings by 51–79.9% according to the concentration of metals. Inoculation with isolate MN3-4 has increased (46.25%) the root length of *B. juncea* seedlings in the absence of Pb. The maximum root length-promoting effect (161.02%) of the Pb treatment was observed after inoculation with *Bacillus* sp. MN3-4. In general, the stimulation of root elongation by the bacteria was more pronounced in Pb-treated plants compared to that in untreated plants. Statistical analysis showed significant difference between different concentration of Pb ions in both control and treated plants. The results are consistent with those of Sheng et al. [14], who reported that the endophytic bacteria *Microbacterium* spp. G16 increased the root length of Pb-treated *B. napus*. The production of IAA by isolate MN3-4 may facilitate *B. juncea* root growth. Several studies have confirmed that the IAA-producing bacteria increased the root lengths of the plants [45].

In conclusion, endophytic bacteria *Bacillus* sp. MN3-4 isolated from metal hyperaccumulator *A. firma* roots was capable of facilitating plant growth through the production of plant hormones such as IAA and siderophores. The characterization studies showed that the isolate resists high concentrations of lead via extracellular sequestration and intracellular accumulation. The observations indicate the potential role of endophytic bacteria *Bacillus* sp. MN3-4 for reduced phytotoxicity and enhanced lead phytoaccumulation. Further work will address the effect of selected bacterium on the biomass yield and bioavailability of lead in hyperaccumulators.

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