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Research Article

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Phytoextraction of Heavy Metals Induced by Bioaugmentation of a Phosphate Solubilizing Bacterium

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Abstract

BACKGROUND: Excessive metals in the soil have become one of the most significant environmental problems. Phytoremediation has received considerable attention as a method for restoring the contaminated soils. The microbes having remarkable metal tolerance and plant growth-promoting abilities could also play a significant role in remediation of metal-contaminated soils, because bioaugmentation with such microbes could promote phytoextraction of metals. Therefore, the present study was focused on evaluating the phytoextraction of heavy metals (Co, Pb and Zn) in *Helianthus annuus* (sunflower) induced by bioaugmentation of a phosphate solubilizing bacterium.
METHODS AND RESULTS: A phosphate solubilizing bacterium was isolated from metal-contaminated soils based on the greater halo size (>3 mm) with solid NBRIP agar medium containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. Isolated bacterial strain was assessed for their resistance to heavy metals; $CoCl_2.6H_2O$, $2PbCO_3.Pb(OH)_2$, and $ZnCl_2$ at various concentrations ranging from 100-400 μg/mL (Co, Pb and Zn) using the agar dilution method. A pot experiment was conducted with aqueous solutions of different heavy metals (Co, Pb and Zn) to assess the effect of bacterial strain on growth and metal uptake by *Helianthus annuus* (sunflower). The impact of bacterial inoculation on the mobility of metals in soil was investigated under laboratory conditions with 50 mL scaled polypropylene centrifuge tubes. The metal contents in the filtrate of plant extracts were determined using an atomic absorption spectrophotometer (Perkinelmer, Aanalyst 800, USA).
CONCLUSION: Inoculation with *Enterobacter ludwigii* PSB 28 resulted in increased shoot and root biomass and enhanced accumulation of Co, Pb and Zn in *Helianthus annuus* plants. The strain was found to be capable of promoting metal translocation from the roots to the shoots of *H. annuus*. Therefore, *Enterobacter ludwigii* PSB 28 could be identified as an effective promoter of phytoextraction of Co, Pb and Zn from metal-contaminated soils.
Key words: *Enterobacter ludwigii* PSB 28, Inoculation, Phytoextraction, Sunflower

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Introduction

Metal contamination of soils has become one of the most significant environmental problems today. Metal uptake by crop plants from the contaminated agricultural lands can have strong adverse impacts on human health through the food chain (Karavoltsos *et al.*, 2002). Moreover, excessive metals in the soil can result in decreased crop yield due to the inhibition of plant metabolic processes (Singh and Aggarwal, 2006). Apart from the metals with unknown biological functions (Cd, Cr, Pb, Co, Ag, Se, and Hg), essential elements (Fe, Mn, Zn, Cu, Mg, Mo, and Ni) also keep accumulating in agricultural soils by means of wastewater irrigation, animal manures and sewage sludge application, use of fertilizer and agrochemicals (Thomas *et al.*, 2012). In the toxicological point of view, the essential elements are also important, because, at higher concentrations they too can be toxic to plants as well as to dietary intake levels (Karavoltsos *et al.*, 2002).

With the continuous addition of undesirable metals into the environment, remediation of contaminated soils receives increasing attention (Cao *et al.,* 2007). However, due to the fact that metals are not easily degraded, remediation of the contaminated soils is always considered being a demanding exercise (Rajkumar *et al.*, 2008). Depending on the resource availability, severity of the problem, nature of the metals and contaminated soil, different methods have been employed in restoring the contaminated lands (Arunakumara *et al*, 2013). In this context, systematic technologies such as bioremediation, physical/chemical remediation and integrated remediation are among the widely used techniques (Luo, 2009). However, the physical and chemical methods such as physical separation, acid leaching or electrochemical processes, are considered to be ineffective because of high cost, low efficiency, and destruction of soil structure and fertility (Jing *et al.*, 2007). In contrast, phytoremediation, a method which uses plants to extract, sequester and detoxify pollutants has received considerable attention (Arunakumara, 2011). However, the wider application of the technology has been restricted due to the limitations such as low soil thickness that can be treated, low translocation rate of metals from roots to shoots, and the slowness of the treatment (Lebeau *et al.*, 2008).

The amount of heavy metals uptake in plants varies with the mobility and the concentration of metals in soil (Chen *et al.*, 2010) and the interface between soil microbes and plant roots (rhizosphere) is displayed to have a great influence on the uptake of nutrients as well as on the decrease of metal toxicity (McNear, 2013). Since soil microbes could alter the metal status of the soil (Fazal and Bano, 2010), exploitation of such microbes to reduce the metal toxicity to plants is worth investigating (Rajkumar and Freitas, 2008). In this context, some metal resistant bacterial strains were proved exceptional at enhancing the growth of the host plant through different mechanisms such as the production of plant growth promoting substances, nitrogen fixation and phosphate solubilization (Hemambika *et al.*, 2013). As reported by Rajkumar et al. (2008), heavy metal tolerance of the microbes may be attributed to one or several mechanisms including exclusion, active removal, biosorption, and precipitation or bioaccumulation of metals both in external and intracellular spaces. Therefore, microbes having remarkable metal tolerance and plant growth-promoting abilities could play a significant role in remediation of metal-contaminated soils, because bioaugmentation with such microbes could promote phytoextraction of metals (Prapagdee *et al.*, 2013). In the present study, we isolated phosphate solubilizing bacterial strains from metal-contaminated soils and the strain with the highest degree of metal resistance was employed in (i) assessing the potential of mobilization of Co, Pb and Zn, and (ii) evaluating the effects of inoculation with the selected strain on plant growth and uptake of Co, Pb and Zn by *Helianthus annuus* (sunflower).

Materials and Methods

Isolation of phosphate solubilizing bacterial strains

Heavy metal contaminated soils collected from abandoned mines of Boryeong area in South Korea were used in isolating phosphate solubilizing bacteria. Aliquots of serially diluted soil samples were inoculated on NBRIP (National Botanical Research Institute Phosphate) medium containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7 ± 0.1. The petri plates were incubated at 30^0C for 7 days. Morphologically distinct colonies with clear halos were purified by repeated sub culturing. A total

of 20 isolates were selected based on the greater halo size (>3 mm) and maintained on solid NBRIP agar medium until use.

Assay of heavy metal resistance

Isolated bacterial strains were assessed for their resistance to heavy metals using the agar dilution method (Cervantes *et al.*, 1986). Freshly prepared agar plates were amended with 3 different heavy metals; $CoCl_2.6H_2O$, $2PbCO_3.Pb(OH)_2$, and $ZnCl_2$ at various concentrations ranging from 100-400 µg/mL (Co, Pb and Zn). They were inoculated with isolated strains and heavy metal tolerance was determined by the appearance of the bacterial growth after 2 days of incubation at 30^0C. The bacterial strain showing the highest degree of metal resistance was selected for further studies.

Strain identification

The partial sequencing of 16S rRNA for the bacterial strain was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1492R (5′-GGTTACCTTGTTACGACTT -3′). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A Phylogenetic tree was constructed using CLUSTAL X program (Thompson et al., 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA4 program (Kumar et al., 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Effect of heavy metals on bacterial growth

NBRIP medium supplemented with heavy metals (Co, Pb and Zn) at the concentration of 200 mg/L was inoculated with bacterial suspension (10^6 colony forming units/ml) and incubated with continuous shaking at 30^0C. Optical density of culture supernatant was measured at definite time intervals using UV spectrophotometer at 660 nm to estimate the cell growth.

Assay of inorganic phosphate solubilization

Bacterial culture having 10^6 colony forming units/ mL (2 days old) was inoculated in sterilized liquid NBRIP medium (250 mL) supplemented with different heavy metals (Co, Pb and Zn) at the concentration of 200 mg/L and incubated with continuous shaking at 30^0C. Sterilized liquid NBRIP medium without supplemented with heavy metals was served as a control. A sample (10 ml) of each cultured and control were taken and centrifuged at 12000 ×g for 15 min. The clear supernatant was used in determining the pH and amount of phosphorous released into the medium.

Effect of bacterial strain on growth and metal uptake by H. annuus

A pot experiment was conducted under green house conditions at the College of Agriculture, Chungnam National University in October 2012. The soils collected from several locations of a nearby forest and a waste button mushroom bed in Buyeo-gun area, Chungchungnam-do, South Korea, were mixed with the ratio of 1:1, air dried and sieved (2 mm). Sterilized soil (by steaming at 100°C for three consecutive days) was amended with aqueous solutions of different heavy metals (Co, Pb and Zn) to achieve the final concentrations of 200 mg/kg soil. They were then kept for 2 weeks in a greenhouse for metal stabilization and used in filling the plastic pots (25 cm diameter, 35 cm height). Seeds of *H. annuus* were surface sterilized by immersing in alcohol (70%) for 40 s, NaClO (1.0%) for 15 min followed by rinsing several times with sterile distilled water. Seeds sown in germination trays containing sterilized non-contaminated soil were provided with 14/10 light/ dark regime and kept at 25°C for germination. Bacterial cultures grown under standard conditions for 2 days were harvested by centrifugation at 12000 ×g at 15 min. The cells were washed twice with sterile distilled water and resuspended in biological saline (0.85% KCl) to be used in inoculation. Three weeks old seedlings were carefully uprooted from the germination bed and their roots were dipped in the bacterial culture (10^9 colony forming units/mL) for 2 h. They were transplanted into the plastic pots (five plants/pot) containing 300 g of metal contaminated or non- contaminated soil and allowed to grow at 25°C and 14/10 light/dark regime. The average pH of soil at the time of planting was recorded as 6.65. Three

weeks later, the plants were carefully uprooted and cleaned the root surface thoroughly with distilled water. As growth parameters, fresh and dry biomass was measured and accumulation of metals in plant biomass was quantified as described by Freitas *et al.* (2004). Each treatment had three replicates.

Mobility of the metals in soil

The impact of bacterial inoculation on the mobility of metals in soil was investigated under laboratory conditions with 50 mL scaled polypropylene centrifuge tubes. The bacterial strain transferred into 100 mL flasks containing LB broth was cultured aerobically on a rotating shaker (150 rpm) at 30°C until reaching the final concentration of 10^6 colony forming units/mL. The bacterial cells were then harvested by centrifugation at 10000 × g for 15 min and washed in phosphate buffer (pH 7.0) twice. The bacterial pellet was washed in sterile water, re-centrifuged, and finally re-suspended in 5 mL sterile water. Artificially contaminated soil (1 g) in the centrifuge tubes was inoculated with small aliquots (up to 1 mL) of the final washed bacterial culture. After taking the weight the tubes, they were wrapped with brown paper and placed on an orbital shaker at 200 rpm at 25 °C. At the end of the period of 10 d, the weight of the tubes was recorded and 10 mL of sterile water were added to each tube to extract the soil water soluble heavy metals. The extracts were centrifuged at 10000 × g for 10 min and filtered and the metal contents in the filtrate were determined using an atomic absorption spectrophotometer (Perkinelmer, Aanalyst 800, USA). Artificially contaminated soil without inoculation with the strain served as the control after centrifugation.

Results

Isolation and identification of phosphate solubilizing bacterial strain

A total of 20 bacterial strains with the potential ability to solubilize inorganic phosphates were screened based on the greater halo size (>3 mm). They were then assessed for their resistance to heavy metals and the bacterial strain showing the highest degree of metal resistance was selected for the study. According to 16S rRNA sequence analysis, the selected strain showed close proximity with *Enterobacter ludwigii* DSM 16688. Phylogenetic tree (Fig. 1) shows the position of the isolated phosphate solubilizing bacterial strain with respect to related species.

Effect of heavy metals on bacterial growth

The growth of the strain measured as the optical density of culture supernatant at definite time intervals is given in Fig. 2. During the incubation period of 36 h, none of the metal was found to be highly toxic to the strain. However, compared to the metal free culture medium, slight reductions in bacterial growth were observed in metal supplemented media.

Inorganic phosphate solubilization

Phosphate solubilization as measured by the amount of phosphorous released into the medium is depicted in Fig. 3. As indicated by the results, the strain was shown to be capable of utilizing tricalcium phosphate as the sole source of phosphate. However, the presence of metals in NBRIP medium (200 mg/L) caused reduction in phosphate solubilizations. Compared with the control, reductions were 26, 56, and 19%, respectively for Co, Pb and Zn.

Effect of bacterial strain on growth and metal uptake by H. annuus

Inoculation with the strain resulted in increased fresh and dry biomass of *H. annuus* plants, compared to non-inoculated plants (Table 1). When non-inoculated plants were exposed to heavy metal stress, the growth was inhibited in a significant level with $p < 0.05$. For instance, Pb toxicity caused 23 and 20% reductions in fresh and dry weight of the plant, respectively. Inoculation however led to increase in plant fresh and dry weight in the presence of heavy metals. The fresh and dry weight of the plants grown in Pb contaminated soils were respectively 25 and 26% higher than those of non-inoculated plants. Similarly, in Zn contaminated soil, the percent increments were recorded as 31 and 46% respectively, and in Co contaminated soil, the corresponding figures were 33 and 49%.

The amounts of Co, Pb and Zn accumulated in the roots and shoots of *H. annuus* grown under inoculated and non-inoculated conditions are given in Table 2. Inoculation with *Enterobacter ludwigii* PSB-28 resulted in increased accumulation of metals both in the shoots and roots. The accumulations of Co, Pb and Zn in shoots were respectively 45, 79 and 27% higher than those of non-inoculated plants. The corresponding accumulations for Co, Pb and Zn in

roots were 21, 18 and 17% higher than those of non-inoculated plants. Regardless of inoculation or non-inoculation, the accumulation of metals in root system was found to be considerably higher than that of in shoots, which has been further confirmed by the low translocation factor (TF) for all the metals. However, TF of Zn was significantly higher than that of the other two metals. Similarly low bioconcentration

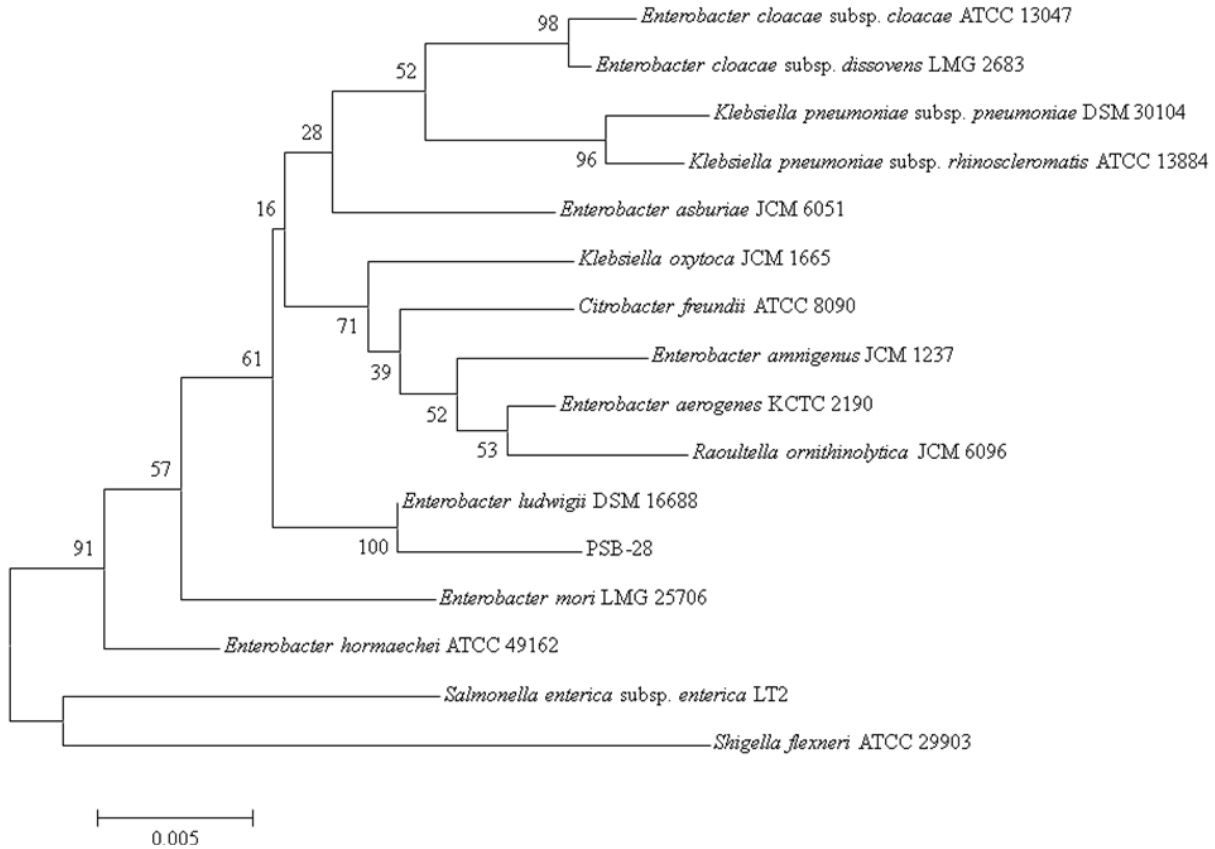

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of isolated phosphate solubilizing bacterial strain (PSB-28) with respect to related species. The scale bar indicates 0.002 substitutions per nucleotide position and accession numbers are given in parenthesis.

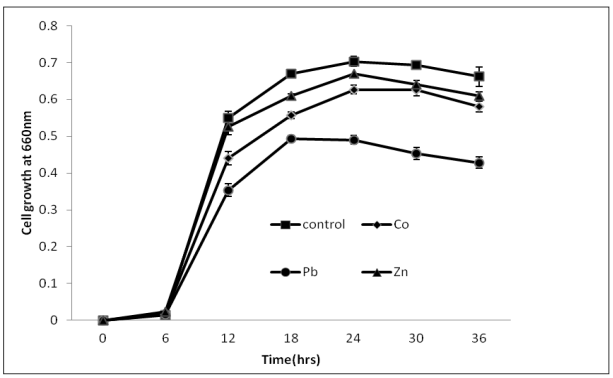

Fig. 2. Growth of *Enterobacter ludwigii* PSB 28 on sterilized liquid NBRIP medium supplemented with metals (Co, Pb and Zn) at the concentrations of 200 mg/L. Sterilized liquid NBRIP medium without supplemented with heavy metals was served as a control. Values are the means of three replicates. Error bars represent standard deviation.

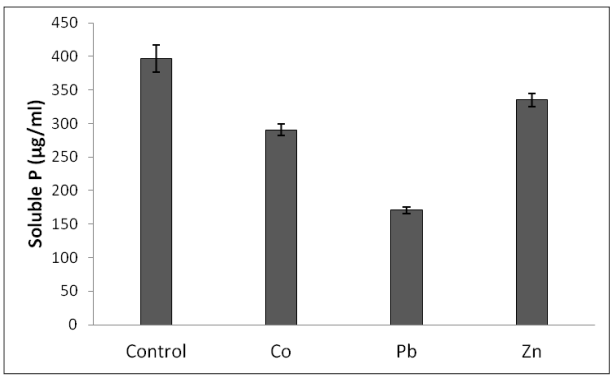

Fig. 3. Phosphate solubilization by *Enterobacter ludwigii* PSB 28 on NBRIP medium supplemented with heavy metals (Co, Pb and Zn) at the concentration of 200 mg/L. Sterilized liquid NBRIP medium without supplemented with heavy metals was served as a control. Values are the means of three replicates. Error bars represent standard deviation.

Table 1. Effect of inoculation with *Enterobacter ludwigii* PSB 28 on shoot and root weight of *Helianthus annuus*

Metal	Treatment	Fresh weight (g/plant)		Dry weight (g/plant)	
		Shoot	Root	Shoot	Root
Metal free soil	control	1.46 (± 0.031)	0.093 (± 0.006)	0.075 (± 0.003)	0.028 (± 0.002)
	with strain	1.65 (± 0.028)	0.118 (± 0.005)	0.089 (± 0.005)	0.041 (± 0.004)
Co	control	1.11 (± 0.025)	0.038 (± 0.002)	0.069 (± 0.001)	0.011 (± 0.002)*
	with strain	1.40 (± 0.033)	0.077 (± 0.004)	0.115 (± 0.002)	0.017 (± 0.003)*
Pb	control	1.05 (± 0.018)	0.084 (± 0.004)*	0.055 (± 0.002)*	0.027 (± 0.002)*
	with strain	1.31 (± 0.035)	0.105 (± 0.002)*	0.072 (± 0.003)*	0.031 (± 0.004)*
Zn	control	0.89 (± 0.041)	0.028 (± 0.003)*	0.049 (± 0.002)	0.010 (± 0.001)*
	with strain	1.17 (± 0.026)	0.035 (± 0.004)*	0.072 (± 0.003)	0.014 (± 0.003)*

Values are means ($n=3$) ± standard deviation. Within each column, means indexed by * are not significantly different at $p > 0.05$ between inoculated and non-inoculated plants according to Duncan's multiple range test.

factor (BCF) was also recorded from Co and Pb. However, the results showed a good agreement and demonstrated that inoculation of the bacterial strain led to increase both TF and BCF of the three metals distinctly.

Mobility of the metals in soil

Metal mobilization potential of the strain was assessed in a batch experiment and the results showed that inoculation of the strain could increase the contents of water soluble metals in the soil. The mobilization of Co, Pb and Zn was respectively 41, 27 and 48% higher than those of the control soil (Fig. 4).

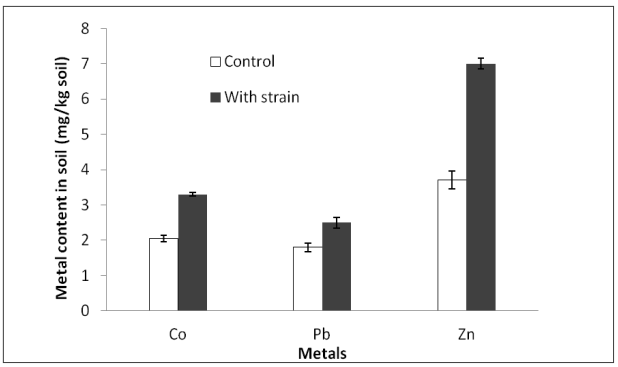

Fig. 4. Effect of inoculation with *Enterobacter ludwigii* PSB 28 on the mobilization of Co, Pb and Zn in soil. Soil without inoculation the strain served as the control. Values are the means of three replicates. Error bars represent standard deviation.

Discussion

Growth response of the present strain under metal contamination conditions is in line with Rajkumar et al. (2008) and Prapagdee et al. (2013), who observed Zn, Cu and Ni resistance in *Bacillus weihenstephanensis* and Cd resistance in *Klebsiella* sp. BAM1. Generally microorganisms isolated from heavy metals contaminated soils possess the ability to withstand against multiple pollutants as they have adapted to such environments (Pal *et al.*, 2005; Abou-Shanab *et al.*, 2007).

The effectiveness of the strain as a plant growth-promoter was assessed with *Helianthus annuus*, a species known to have an ability to accumulate biomass rapidly and take up substantial amounts of metals (Turgut *et al.*, 2004; Prapagdee *et al.*, 2013). As reported by Ouzounidou et al. (2005) and El-Tayeb *et al.* (2006), accumulation of plant biomass could be affected by excessive concentrations of heavy metals, which exert adverse impacts on growth and function of root system resulting in poor uptake of water and nutrients. As reported by Jiang *et al.* (2008), inoculation with *Burkholderia* sp. J62 led to increase shoot and root dry weights of corn and tomato plants. Inoculation with *Pseudomonas fluorescens* PsIA12 resulted in enhanced growth of *Zea mays* and its uptake of N, P and K (Egamberdiyeva et al., 2002). The content of P, K, S and Ca was reported to be increased by the inoculation of rhizobacteria in barley plant grown in metal contaminated soil (Belimov *et al.*, 2004). According to them, inoculation with rhizobacteria resulted in 42% increase in growth of the barley plant compared to the control. Based on the results, they further stated that nutrients play an important role in the detoxification of heavy metals. Their findings were in line with Lebeau *et al.* (2008), who reported

that rhizobacteria could have strong impacts on the nutritional status and the plant resistance to heavy metals. Most recently, Prapagdee *et al.* (2013) reported that growth of *H. annuus* could be enhanced by the inoculation of *Micrococcus* sp. MU1 and *Klebsiella* sp. BAM1 under Cd contaminated conditions. Belimov *et al.* (2001) also observed bacterial-assisted growth enhancement in *Brassica napus* grown in a soil contaminated with Cd. The plant growth-promoting potential of the present strain could be attributed at least partly to the phosphate solubilization ability of the strain under metal stress conditions. In this regards, Rajkumar *et al.* (2005) also reported that phosphate solubilization ability of *Pseudomonas* sp. could be contributed to the growth enhancement of the inoculated plants. Inoculation of phosphate solubilizing *Bacillus subtilis* SJ-101 resulted in higher shoot and root length and biomass with or without Ni (Zaidi *et al.*, 2006). Bacteria is reported to promote the growth of plants (i) indirectly through producing antibiotics to inhibit soil pathogens, and (ii) directly through increasing nutrient and water uptake and thereby the plant biomass (Belimov *et al.*, 2004). Through the production of siderophores, specific enzymes, and organic acids involved in phosphorus solubilization, and fixation of atmospheric N_2, bacteria could assist plants to withstand against metal toxicity (Kloepper, 2003). In this regards, Borgmann (2000) reported that *Kluyvera ascorbata* SUD165 protected *Brassica juncea* and *Brassica campestris* against Ni, Pb and Zn toxicity through the production of enzyme ACC deaminase. Plant growth promoting rhizobacteria was reported to enhance root elongation of *Brassica napus* by stimulating IAA synthesize (Sheng and Xia, 2006). In *Brassica juncea*, root elongation was reported to be enhanced by non-identified rhizobacteria (Belimov *et al.*, 2005), *Variovorax paradoxus* 5C-2 (Belimov *et al.*, 2005) and root dry weight was increased by rhizobacteria (Sheng and Xia, 2006).

Regardless of inoculation or non-inoculation, the accumulation of metals in root systems was found to be considerably higher than that of in shoots. This could primarily be attributed to the poor translocation of heavy metals from roots to shoots (Rajkumar *et al.*, 2006). However, as shown in Table 2, translocation factor of the each metal was increased with the inoculation of the strain, which was of enormous practical significance. Furthermore, metal accumulations in both shoots and roots were found to be higher in inoculated plants than those of non-inoculated plants. Similar observations were made by Rajkumar *et al.* (2008) for Zn accumulation in *H. annuus* inoculated with *Bacillus weihenstephanensis*. However, according to Wani *et al.* (2007), inoculation of *Bradyrhizobium* sp. on surface sterilized seeds of *Vigna radiate* reduced the concentration of Ni in roots, shoots and grains by 15, 19 and 22%, respectively, compared with non-inoculated plants.

As observed by Walpola and Yoon (2013), acidification of the medium could facilitate the inorganic phosphate solubilization by the PSBs. According to them, the acidification occurs mainly through the production of low molecular weight organic acids such as oxalic acid and gluconic acid. Analogous to their findings, an inverse relationship between pH and soluble phosphorus concentration from $Ca_3(PO_4)_2$ by *Enterobacter ludwigii* PSB-28 was also observed in the present study. The inter-relationships among soil pH, solubility and speciation of metals have been intensively investigated (Gadd, 2004). Bacteria such as

Table 2. Effect of inoculation with *Enterobacter ludwigii* PSB 28 on accumulation and translocation of Co, Pb and Zn in *Helianthus annuus*

Metal	Treatment	Metal content (mg/kg dry weight)		Bioconcentration Factor (BCF)[a]	Translocation Factor (TF)[b]
		Shoot	Root		
Co	control	16.05 (± 2.41)	85.12 (± 4.56)	0.426	0.188
	with strain	23.48 (± 3.06)	102.83 (± 6.98)	0.514	0.228
Pb	control	2.67 (± 0.92)*	107.14 (± 11.24)	0.536	0.025
	with strain	4.78 (± 1.27)*	126.85(± 6.37)	0.634	0.038
Zn	control	89.31 (± 6.43)	190.46 (± 13.73)	0.952	0.469
	with strain	113.39 (± 12.25)	223.73 (± 9.82)	1.119	0.506

[a]BCF, metal concentration ratio of plant roots to soil; [b]TF, metal concentration ratio of plant shoots to roots.
Values are means ($n=3$) ± standard deviation. Within each column, means indexed by * are not significantly different at $p > 0.05$ between inoculated and non-inoculated plants according to Duncan's multiple range test.

Azotobacter chroococcum (N-fixing bacteria), *Bacillus megaterium* (P-solubilizer) and *Bacillus mucilaginosus* (K-solubilizer) (Wu *et al.*, 2006) and *Bacillus* sp. RJ16 (Sheng and Xia, 2006) were reported to decrease the pH, enhancing the bioavailability of Cd, Pb and Zn (Chen *et al.*, 2005). As stated by Zaidi et al. (2006), reduction in pH from 7.5 to 4.8 with the inoculation of phosphate solubilizing *Bacillus subtilis* SJ-101 possibly created favourable conditions for the solubilization of metals and their subsequent uptake by the plants. The increased accumulation of metals in the presence of bacterial strain might be due to the increased uptake of metals under acidic soil conditions created by the phosphate solubilization (Rajkumar *et al.*, 2008). Inoculation of Cd-resistant bacterial strains to *Brassica napus* to a metal contaminated soil significantly increased the plant uptake of Cd when compared with the non-inoculated controls, as a result of pH reduction (Sheng and Xia, 2006). However, on the contrary, *Glomus caledonium* (Chen *et al.*, 2004) and *Glomus mosseae* (Citterio *et al.*, 2005) were reported to have no effect on the speciation of Cd and Zn, and Cr and Ni, thus no effect of bioaugmentation by these arbuscular mycorrhizal (AM) fungi on the rate of phytoextraction has been observed, which could be attributed to whether strong symbiotic relationships between AM fungi and host plants.

The present findings of metal mobilization are in agreement with Wu *et al.* (2006) and Prapagdee *et al.* (2012) who also reported bacteria-assisted increase in heavy metal mobilization. Generally, the low amount of metals extracted by plants from a soil is attributed mainly to the low availability of metals. As reported by several authors, the available metal content in a soil is less than 1% of the total metal content (Whiting *et al.*, 2001; Braud *et al.*, 2006). Metal availability is influenced by the nature of the metal and soil characteristics such as pH, CEC and organic matter (Kayser *et al.*, 2001; Lebeau *et al.*, 2008). Bioaugmentation could enhance metal bioavailability by increasing the concentration of the available fractions. As revealed by the present results, the release of heavy metals from the non-soluble phases to soluble phases could be facilitated by the bacterial strain. Therefore, increased accumulation of metals, in particular Zn in both the shoots and roots of *H. annuus* could be attributed to the higher water soluble metal contents in soil inoculated with bacterial strain. As reported by the results of previous studies, *H. annuus* is capable of accumulating high amounts of Pb, Cd, Cu, Zn and Co, in both the shoots and the roots (Boonyapookana *et al.*, 2005; Marchiol *et al.*, 2007). According to Braud *et al.* (2006), inoculation of *Pseudomonas aeruginosa and Pseudomonas fluorescens* has resulted in 113% increment of Pb content in the exchangeable fraction of the soil. However, the Pb concentration bound to free Mn oxides, organic matter and in the residual fraction remained stable. Abou-Shanab *et al.* (2006) observed an increase of extractable Ni with *Microbacterium arabinogalactanolyticum* by a factor up to 15. As reported by Baum *et al.* (2006), the concentrations in NH_4NO_3-extractable Cd, Cu, Pb and Zn in a soil bioaugmented with ectomycorrhizal fungus *Paxillus involutus*, were 1.22-, 1.11-, 1.33- and 1.33-fold higher than those of non-bioaugmented soil, depending on the soil composition. However, comparing and contrasting of the results of bioaugmentation studies are hard to perform, because the estimation of bioavailable fraction of metals has been done under different conditions with different extractants such as water (Di Gregorio *et al.*, 2006; Wu *et al.*, 2006), $MgCl_2$ (Braud *et al.*, 2006), NH_4NO_3 (Baum *et al.*, 2006), NH_4O-Ac (Wu *et al.*, 2006), DTPA (Di Gregorio *et al.*, 2006; Wu *et al.*, 2006), KNO_3 (Di Gregorio *et al.*, 2006) and HCl (Wang *et al.*, 2007).

Conclusion

Inoculation with *Enterobacter ludwigii* PSB 28 resulted in increased shoot and root biomass and enhanced accumulation of Co, Pb and Zn in *Helianthus annuus* plants. The strain was found to be capable of promoting metal translocation from the roots to the shoots of *H. annuus*. The beneficial effects of *E. ludwigii* PSB 28 further demonstrated by metal mobilization potential of the strain. Therefore, *Enterobacter ludwigii* PSB 28 could be identified as an effective promoter of phytoextraction of Co, Pb and Zn from metal-contaminated soils.

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