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Potential of *Pseudomonas* sp. JH 51-2 to stabilize lead in mining site soil

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Abstract

The potential of a lead (Pb)-tolerant *Pseudomonas* sp. JH 51-2 strain to promote Pb stabilization was evaluated in mining site soil. The strain was isolated from soil containing multiple heavy metals. Transmission electron microscopy (TEM) showed that cell walls were surrounded by extracellular substances and scanning electron microscope-energy dispersive spectroscopy (SEM-EDS) confirmed the presence of Pb on the surface of cell membrane. Fourier transform infrared spectroscopy (FTIR) revealed that amino acids, phospholipids and extracellular polysaccharides were involved in Pb complexation and biosorption. Sequential extraction and toxicity characteristic leaching procedure (TCLP) showed decreased Pb availability in mining site soil in the presence of *Pseudomonas* sp. JH 51-2. Results indicate that *Pseudomonas* sp. JH 51-2 is an efficient biological stabilizer of Pb in contaminated soil.

Key words

Biostabilization, Lead, *Pseudomonas* sp., Sequential extraction, Toxicity characteristic leaching procedure

Introduction

Heavy metals/metalloids like Cd, Pb, Cu, Zn and As at abandoned mining sites, can be hazardous to humans (Järup, 2003). Physical and chemical methods used to remediate and restore heavy metals-contaminated soil include soil covering, fixation, leaching and ion exchange (Zhang *et al*., 2010). These are expensive technologies and may not be cost-effective for lower levels of heavy metals contamination (Husain *et al*., 2013). An alternative approach uses heavy metals stabilization as an effective, practical and considerably lower cost approach for restoring contaminated soil (Lee *et al*., 2011a). Soil stabilization decreases heavy metals solubilization, reducing mobility and toxicity, and thereby lowering potential hazards (Lee *et al*., 2011a, b). A reduction in toxicity facilitates phytoremediation, allowing the use of plants to remove heavy metals and promote soil biological activity (Zhang *et al*., 2010). Physical and chemical stabilization methods have disadvantages because they can cause toxicity from reactions of stabilizers, secondary treatment is usually required to remove heavy metals leachate, and efficiency can vary greatly with soil properties (Choudhary and Sar, 2009). Alternatively, microorganisms can be used to reduce availability of toxic metals to environmentally acceptable levels in an economically viable and environmentally friendly manner (Salehizadeh and Shojaosadati, 2003).

Microorganisms can promote heavy metals stabilization because cell constituents or metabolites often act as efficient chelators of metals or create a micro environment in the vicinity of cell that promotes deposition or precipitation of metals. Mechanisms include simple physico-chemical binding to cellular components, extracellular molecules and metabolism-dependent intracellular transport as well as precipitation of metals (Naik and Dubey, 2013). *Pseudomonas* spp. with demonstrating this capacity include economically important bacteria such as *P. aeruginosa, P. fluorescens*, *and P. putida* (Cheung *et al*., 2007; Husain *et al*., 2013; Uslu and Tanyol, 2006). Such bacteria can be used to promote heavy metals stabilization and reduce leachate generation, mitigating environmental problems resulting from industrial production and sludge deposition. Restoration process

continues if bacterial growth can be sustained. In light of the above, the objective of the present study was to determine the potential of Pb-tolerant bacterial strain to promote Pb stabilization in soil, with a goal of restoring agricultural land near an abandoned mine.

Materials and Methods

Isolation of Pb-tolerant bacterial strain : *Pseudomonas* sp. strain (JH 51-2) was isolated from soil collected near an abandoned mining site in Hampyeong, Republic of Korea. The method of Mohite *et al*. (2010) was used to isolate bacteria from metal-contaminated soil. Dried soil (1 g) was suspended in 100 ml of minimal salt medium (containing 0.6 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl, 1 g NH_4Cl l^{-1}; 1 M $CaCl_2 \cdot 2H_2O$ and 1 M $MgSO_4 \cdot 7H_2O$). The suspension was incubated for 7 days at 35ºC with agitation at 180 rpm. To isolate single colonies, suspension was serially diluted (10^{-3} to 10^{-4}) and 100 µl of each dilution plated onto Luria Bertani (LB) agar medium (10 g tryptone; 5 g yeast extract; 10 g NaCl and 15 g agar l^{-1}) containing 500, 1000, 2000 and 3000 mg Pb l^{-1}, respectively. The plates were incubated at 37°C for 3 days and growth of bacteria was observed. Single colonies with distinct morphological characteristics were identified and stored at -80°C in LB broth containing 50% glycerol.

Minimal inhibitory concentration (MIC) : MIC was used to determine tolerance of bacterial isolates to Pb. LB broth medium was prepared in tubes and amended with various amounts of Pb using $Pb(NO_3)_2$ (extra pure grade, Deajung Chemical Inc., Shiheung, South Korea) to achieve the desired concentrations of 100-400 mg Pb l^{-1}. Tubes containing various concentrations of Pb and control tubes (without Pb) were inoculated with 100 µl (10-14 × 10^6 cells ml^{-1}) of each isolate in LB broth. The tubes were incubated at 35°C for 48 hr to promote bacterial growth, which was determined by UV-Vis spectrophotometry (Shimadzu Inc., Tokyo, Japan) based on optical density at 595 nm.

Identification of metal-tolerant bacteria : Chromosomal DNA from the isolate was extracted using bacterial DNA isolation kit (GeneAll Exgene Cell SV mini, 100P, Seoul, South Korea). Following the procedures of Locatelli *et al*. (2002), two primer sets were used for PCR amplification of ITS1 region: fPs16S/rPs23S (*Pseudomonas* specific primer) and S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 (bacterial primer). For *Pseudomonas*-specific PCR, reaction mix (HiPi PCR Pre Mix) containing 1 unit (4 units $µl^{-1}$) HiPiTM thermostable DNA polymerase in 250 mM TRIS-HCl buffer (pH 9.0), with 80 mM $(NH_4)_2SO_4$, 10% DMSO, 8.75 mM $MgCl_2$, 0.05% bromophenol blue, 12% glycerol, stabilizer with 1 fg-10 ng template, 5 pmol primers and 20 µl distilled water was used for amplification. The reactions were performed in an Applied Biosystem Veriti™ Thermal Cycler (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 3-min initial denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 30 sec at 55°C and 1 min at 74°C, then a final 5-min extension step at 74°C. PCR products were analyzed via electrophoresis in 1.3% agarose gel (Bio Basic Inc., Markham, Canada) and were visualized after staining with ethidium bromide. PCR amplification with bacterial primers was used to determine the quality of DNA extract and size of ITS1 region. The sequences were compared using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) for identification of isolate.

Cell growth and Pb removal : To determine the influence of initial Pb concentration on cell growth, JH 51-2 isolate was exposed to 0, 200 and 400 mg Pb l^{-1} for 48 hrs. Growth was determined by serially diluting the culture solution with 10 mM phosphate buffer solution (PBS), spreading over LB agar medium and counting single colonies (colony forming units; CFU) after incubating the plates at 35°C for 24 hr. Pb removal capacity of JH 51-2 strain was determined in LB broth containing initial Pb concentration of 50-400 mg l^{-1}. The strain (100 µl containing 10-14 × 10^6 cells ml^{-1}) was inoculated in LB broth medium and incubated for 48 hr. Pb concentration in medium was then determined by ICP-AES after centrifuging at 7000 rpm for 10 min.

Cell morphology and surface characteristics : Biological transmission electron microscopy (Bio-TEM; Hitachi H-7650, Tokyo, Japan) was used to determine changes on the surface of JH 51-2 cells after growing in LB broth containing 100 mg Pb l^{-1}. Scanning electron microscopy–energy dispersive spectroscopy (SEM–EDS; JEOL-64000, Tokyo, Japan) was used to confirm Pb adhesion to cell surface and bonding to functional groups on cell surface was shown by Fourier transform-infrared spectroscopy (FTIR; Perkin–Elmer, Irvine, CA, USA).

Physico-chemical analysis of mining site soil : To evaluate Pb stabilization potential of isolated strain, highly contaminated soil (2011 mg Pb kg^{-1}) was collected from a mining site at Jeongeup, Republic of Korea. The soil was air-dried at room temperature, sieved (100 mesh, <150 µm) and particle size was determined (Klute, 1986). The pH was measured in a 1:5 (w/v) mixture of soil and distilled water after equilibrating for 1 hr and electrical conductivity (EC) was measured in the same sample after filtering through Whatman No. 2 filter paper. Solution of CH_3COONH_4 and NH_4OH (1 M) were used to buffer pH at 7.0. Cation exchange capacity (CEC) was determined by the method of Sparks *et al*. (1996). To determine total heavy metals content, soil was extracted with aqua regia and ICP-AES was used for elemental analysis.

Pb stabilization and availability in inoculated soil : Jeongeup mining site soil was autoclaved for 2 hr at 121°C with high pressure sterilizer. Sterilized soil (1 kg) was inoculated thoroughly with 5 ml of 0.1 M phosphate buffer (pH 7.1) containing JH 51-2 strain (4.8 × 10^8 cells ml^{-1}) and uninoculated soil served as control. The mining site soil was stabilized for 30 days at room temperature (25 ± 3°C). The soil was then sieved (100 mesh, < 150 µm) and Pb availability and potential mobility was determined using sequential extraction (Tessier *et al*., 1979). Fraction 1 (F1)

used 0.5 M $MgCl_2$ (pH 7.2) to extract exchangeable Pb and fraction 2 (F2) used 1 M NaOAc (pH 5.0) to extract carbonate-bound Pb. Fraction 3 (F3) used 0.04 M $NH_2OH \cdot HCl$ containing 25% (v/v) HOAc to remove Pb bound to Fe and Mn oxides. Fraction 4 (F4) used 3.2 M NH_4OAc containing 0.02 M HNO_3, 30% H_2O_2 (pH 2) and 20% HNO_3 to remove Pb bound to organic matter and sulfide. Fraction 5 (F5) used 1:3 mixture of HNO_3 and HCl to extract residual Pb. Data obtained from sequential extractions were also used to assess the impact of inoculating soil with *Pseudomonas* sp. JH 51-2 on the potential mobility of Pb by calculating the mobility factor (MF) (Sun *et al*., 2007). Leachable Pb in soil (< 150 µm) also was periodically measured for 30 days, after inoculation with strain, using toxicity characteristic leaching procedure (TCLP) following USEPA Method 1311 (USEPA, 1992). Supernatant was filtered through 0.45 µm membrane filter and Pb was estimated by ICP-AES.

Statistical analyses : Statistical analyses were performed using SAS 9.1 (SAS, Cary, NC, USA). Data were subjected to analysis of variance (ANOVA) and means were compared using Tukey's test at $P < 0.05$. All experiments were repeated in triplicate and values were presented as means ±SD.

Results and Discussion

Partial DNA sequence of JH 51-2 strain exhibited high similarity with *Pseudomonas* species according to the NCBI database. JH 51-2 showed high tolerance to Pb (up to 400 mg Pb l^{-1}) in solution. Previous research had shown Pb biosorption and tolerance by various *Pseudomonas* species (Uslu and Tanyol, 2006). *P. fluorescens* bacteria have excellent biosorption properties because of their high surface-to-volume ratio and a high number of chemisorption sites due to constituents such as teichoic acid in their cell walls (Husain *et al*., 2013). Bacterial cell wall is the main site for chemical compounds capable of passively sequestering metals. The importance of any given group for biosorption of a specific metal by a certain biomass depends on factors such as number of sites, their accessibility and chemical state and affinity between site and metal. Various bacteria (such as *Pseudomonas* sp., *Bacillus* sp. and *Micrococcus* sp.) have been reported to be efficient Pb removers (Puyen *et al*., 2012; Uslu and Tanyol, 2006; Zhang *et al*., 2013).

Pseudomonas sp. JH 51-2 cell growth and Pb removal at different initial Pb concentration with incubation time are shown in Fig. 1a and b, respectively. Growth of bacteria was affected by initial Pb concentration, with decreasing growth as the initial concentration increased from 200 to 400 mg Pb l^{-1} (Fig 1a). Reduced growth at higher Pb concentrations could be attributed to heavy metal stress and toxicity. Pb removal by JH 51-2 was 93% at 50 mg Pb l^{-1}, 87% at 100 mg Pb l^{-1}, 64% at 200 mg Pb l^{-1} and 61% at 400 mg Pb l^{-1}, showing that removal efficiency depended highly on initial Pb concentration (Fig. 1b). Removal efficiency decrease at high concentration could be attributed to a smaller number of reactive sites for Pb ions when cell biomass was smaller in solution.

TEM micrographs of *Pseudomonas* sp. JH 51-2 isolate showed that cell wall was surrounded by extracellular substances, 24 hr after inoculation (Fig. 2a). After 48 hrs, the particles increased in size (Fig. 2b). SEM-EDS confirmed Pb on cell surface (Fig. 3), likely replacing Na and K (Davis *et al*., 2003). These observations are consistent with the previous research work which showed that secreted polysaccharides facilitate heavy metals adhesion to *Pseudomonas* sp. (Choudhary and Sar, 2009).

FTIR analysis was used to determine association of

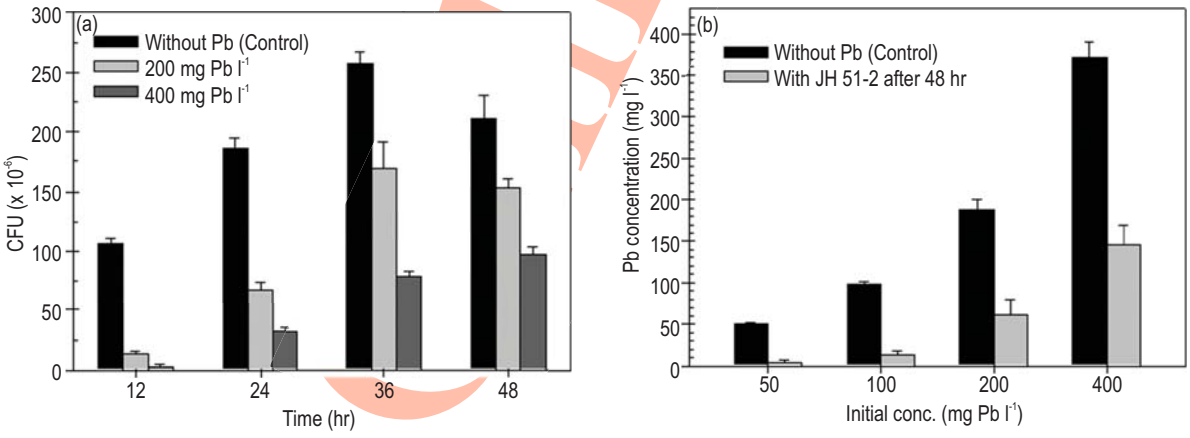

Fig. 1 : Influence of initial Pb concentration (200 and 400 mg l^{-1}) and incubation time on *Pseudomonas* sp. JH 51-2 colony forming units (CFU) (a) and Pb concentration in solution 48 hr after incubating with JH 51-2 at initial Pb concentrations of 50, 100, 200 and 400 mg l^{-1} (b). Values are mean of three replicates ±SD

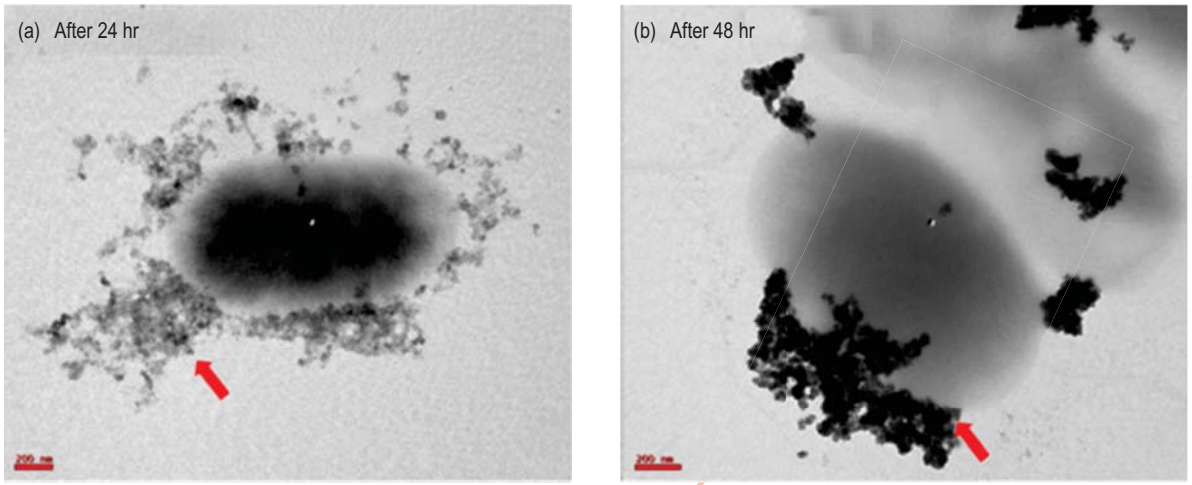

Fig. 2 : TEM images of the *Pseudomonas* sp. JH 51-2 isolate showing cell walls surrounded by extracellular substances after growth in LB medium with 100 mg Pb l^{-1} for 24 hr (a) and 48 hr (b). Arrows indicate the presence of Pb complexes.

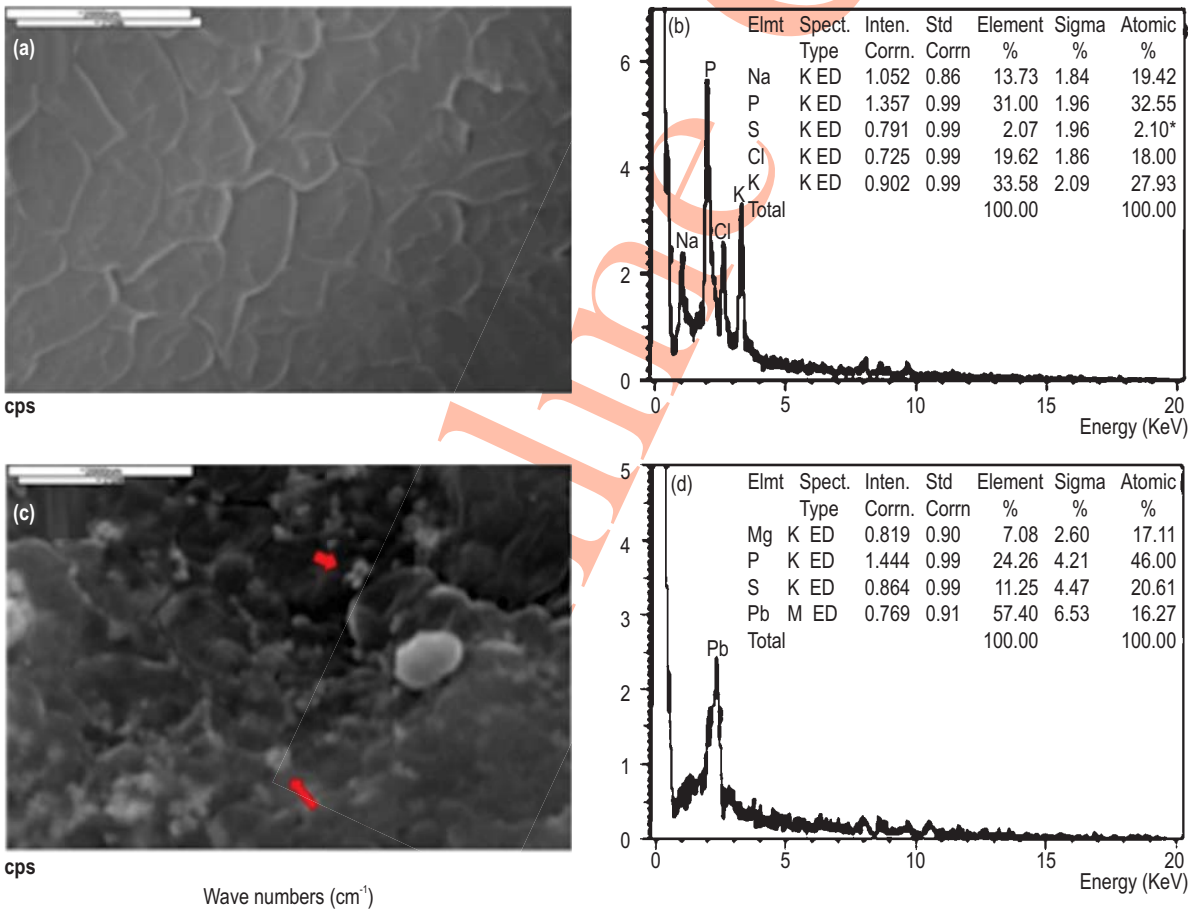

Fig. 3 : SEM images and EDS spectra of surface of *Pseudomonas* sp. JH 51-2 after growth in LB medium without Pb (a and b) and with 100 mg Pb l^{-1} (c and d). Arrows indicate the presence of Pb complexes

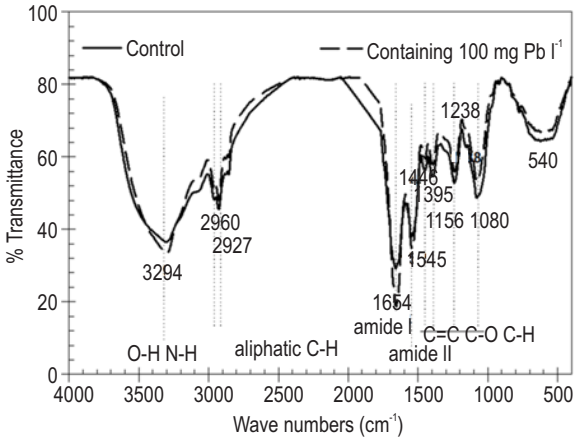

Fig. 4 : FTIR spectra of *Pseudomonas* sp. JH 51-2 grown in control media and media containing 100 mg Pb l^{-1}

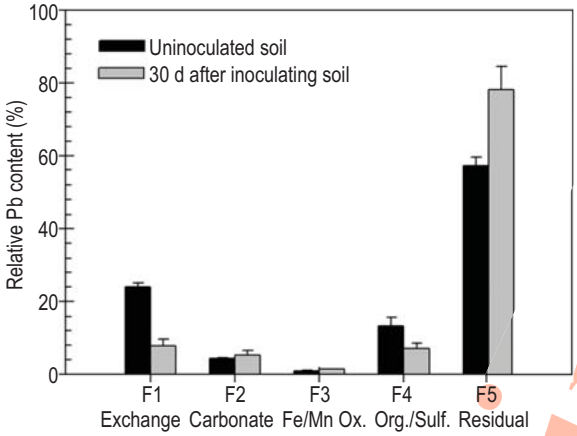

Fig. 5 : Relative amount of Pb in sequentially extracted fractions of uninoculated soil and in soil inoculated with JH 51-2 (F1 = exchangeable, F2 = carbonate-bound, F3 = Fe and Mn oxide-bound, F4 = organic matter- and sulfur-bound, and F5 = residual Pb fractions). Error bars indicate standard deviations; where absent bars fall within treatment bars. Treatments followed by same letter are not significantly different according to Tukey's test at $P < 0.05$

Table 1 : Physico-chemical characteristics of lead-contaminated soil

Parameter	Units	Value[a]
pH	-	4.12 ± 0.02
Electrical conductivity	($\mu S\ m^{-1}$)	2894 ± 0.3
Cation exchange capacity	($cmol_c\ kg^{-1}$)	92.4 ± 1.2
Texture	(%)	Sand: 70.1 ± 8.4
	(%)	Silt: 20.2 ± 3.1
	(%)	Clay: 9.8 ± 1.1
Lead concentration	($mg\ kg^{-1}$)	2010.99 ± 9.87

[a]Mean and standard deviation ($n = 3$).

functional groups on the surface of JH 51-2 with Pb biosorption (Fig. 4). FTIR spectra of microorganisms typically include six regions (Neumann *et al*., 2005). Each region provides information about different cell components: 3500-3100 cm^{-1}, hydration of bacterial cells; 3000-2800 cm^{-1}, fatty acids in bacterial cell membrane; 1800-1500 cm^{-1}, amide groups from proteins and peptides; 1500-1200 cm^{-1}, mixed region (proteins and fatty acids); 1200-900 cm^{-1}, polysaccharides and phospholipids within cell wall; and 900-500 cm^{-1}, the fingerprint region. The spectrum of control cells differed from that of Pb-exposed cells. A more intense peak at 3294 cm^{-1} indicates greater hydration of Pb-exposed cells. Sharper amide I and II peaks at 1654 and 1545 cm^{-1} can be attributed to interactions of Pb with proteins and peptides (Mecozzi *et al*., 2007) and amino acid-metal complexes (Cheung *et al*., 2007). Interactions are also due to changes in the fatty acid region and in phosphate (phospholipid) bond stretching at 1200-900 cm^{-1} (D'Souza *et al*., 2008). Pb-exposed cells also showed decrease in intensity of polysaccharide peak (1080 cm^{-1}) and at 540 cm^{-1} in the fingerprint region. These changes are consistent with previous observations reported in metal-stressed bacterial cells (Kamnev *et al*., 2006).

Sequential extraction was used to determine Pb stabilization by *Pseudomonas* sp. JH 51-2 in the mining site soil. Physico-chemical properties of the soil are given in Table 1. The soil contained 2011 mg Pb kg^{-1}. Sequential extraction showed reduced Pb availability in soil after incubation with JH 51-2, as reflected in 16.1 mg kg^{-1} (67.0%) decrease in the exchangeable (F1) fraction and 6.2 mg kg^{-1} (45.6%) decrease in the organic-bound (F4) fraction (Fig. 5). This was accompanied by 324.0 mg kg^{-1} increase (36.4%) in the least available (covalently bound residual) Pb fraction (F5). There was little change in carbonate-bound (F2) or oxide-bound (F3) Pb. Stabilization of heavy metals in soil is indicated by decrease in the exchangeable fraction (F1) and increase in the most strongly bound fraction (F5) (Lee *et al*., 2011a); this change was observed in the present study. These changes showed that *Pseudomonas* sp. JH 51-2 shifted metal distribution from easily exchangeable fraction to residual fraction where Pb availability was very low. Wu *et al*. (2006) previously reported binding of metals to bacterial cell walls and that exuded proteins, amino acids and organic acids were involved in metal stabilization or redistribution in contaminated soil.

Decrease in Pb availability in mining site soil treated with *Pseudomonas* sp. JH 51-2 was reflected in MF values, which decreased from 28.4% in uninoculated soil to 13.4% 30 days after inoculation. TCLP results showed Pb leaching from soil after inoculation with JH 51-2 (Fig. 6). After 30 days, Pb in leachate decreased by 51.7% as compared to control. This finding is consistent with the MF results and previous research showing that decrease in the readily exchangeable fraction indicated reduction in metal leaching (Lee *et al*., 2011b). This change was attributable to increases in carbonate and residual Pb after inoculation with JH 51-2.

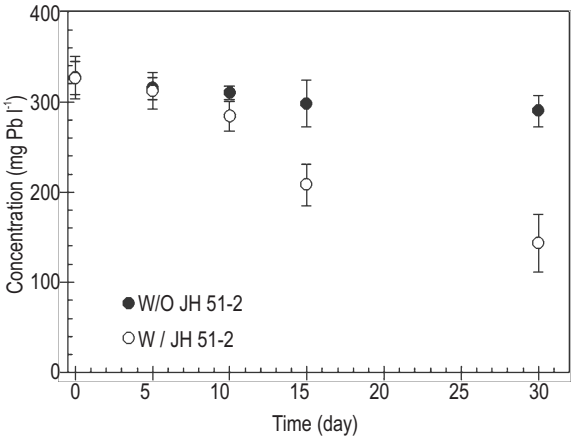

Fig. 6 : Pb in leachate from mining site soil after inoculation with *Pseudomonas* sp. JH 51-2, as determined by Toxicity Characteristic Leaching Procedure (TCLP). Error bars indicate standard deviations; where absent bars fall within symbols

These observations demonstrated the potential use of JH 51-2 to stabilize Pb in soil, which reduced solution concentrations and transport, thereby decreasing its toxicity. The results showed the potential of Pb-tolerant bacteria such as JH 51-2 to effectively decrease Pb bioavailability in soil.

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References

Cheung, H.Y., G.K.L. Chan, S.H. Cheung, S.Q. Sun and W.F. Fong: Morphological and chemical changes in the attached cells of *Pseudomonas aeruginosa* as primary biofilms develop on aluminium and CaF_2 plates. *J. Appl. Microbiol.*, **102**, 701-710 (2007).

Choudhary, S. and P. Sar: Characterization of a metal resistant *Pseudomonas* sp. isolated from uranium mine for its potential in heavy metal (Ni^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}) sequestration. *Biores. Technol.*, **100**, 2482-2492 (2009).

Davis, T.A., B. Volesky and A. Mucci: A review of the biochemistry of heavy metal biosorption by brown algae. *Water Res.*, **37**, 4311-4330 (2003).

D'Souza, L., P. Devi, M.P.D. Shridhar and C.G. Naik: Use of Fourier Transform Infrared (FTIR) spectroscopy to study cadmium-induced changes in *Padina tetrastromatica* (Hauck). *Anal. Chem. Insights*, **3**, 135-143 (2008).

Husain, R.A., A.J. Thatheyus and D. Ramya: Bioremediation of nickel using *Pseudomonas fluorescens*. *Amer. J. Microbiol. Res.*, **1**, 48-52 (2013).

Järup, L.: Hazards of heavy metal contamination. *Brit. Med. Bull.*, **68**, 167-182 (2003).

Kamnev, A.A., A.V. Tugarova, L.P. Antonyuk, P.A. Tarantilis, L.A. Kulikov, Yu.D. Perfiliev, M.G. Polissiou and P.H.E. Gardiner: Instrumental analysis of bacterial cells using vibrational and emission Mössbauer spectroscopic techniques. *Anal. Chim. Acta.*, **573**, 445-452 (2006).

Klute, A.: Methods of soil analysis. Part 1 - Physical and mineralogical methods. Second edition. American Society of Agronomy, Inc., Madison, WI (1986).

Lee, K.Y., D.H. Moon, K.W. Kim, K.H. Cheong, T.S. Kim, J.H. Khim, K.R. Moon, and S.B. Choi: Application of waste resources for the stabilization of heavy metal (Pb, Cu) in firing range soils. *Kor. Soc. Environ. Eng.*, **33**, 71-76 (2011a).

Lee, S.H., E.Y. Kim, H. Park, J. Yun and J.G. Kim: *In situ* stabilization of arsenic and metal-contaminated agricultural soil using industrial by-products. *Geoderma*, **161**, 1-7 (2011b).

Locatelli, L., S. Tarnawski, J. Hamelin and P. Rossi, M. Aragno and N. Fromin: Specific PCR amplification for the genus *Pseudomonas* targeting the 3' Half of 16S rDNA and the whole 16S–23S rDNA spacer. *Syst. Appl. Microbiol.*, **25**, 220-227 (2002).

Mecozzi, M., M. Pietroletti and R. Di Mento: Application of FTIR spectroscopy in ecotoxicological studies supported by multivariate analysis and 2D correlation spectroscopy. *Vib. Spectrosc.*, **44**, 228-235 (2007).

Mohite, B.V., R.E. Jalgaonwala, S. Pawar and A. Morankar: Isolation and characterization of phenol degrading bacteria from oil contaminated soil. *Innov. Rom. Food. Biotechnol.*, **7**, 1033-1037 (2010).

Naik, M.M. and S.K. Dubey: Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. *Ecotoxicol. Environ. Saf.*, **98**, 1-7 (2013).

Neumann, G., Y. Veeranagouda, T. B. Karegoudar, Ö. Sahin, I. Mäusezahl, N. Kabelitz and H.J. Heipieper: Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. *Extremophiles*, **9**, 163-168 (2005).

Puyen, Z.M., E. Villagrasa, J. Maldonado, E. Diestra, I. Esteve and A. Sole: Biosorption of lead and copper by heavy-metal tolerant *Micrococcus luteus* DE2008. *Biores. Technol.*, **126**, 233-237 (2012).

Salehizadeh, H. and S.A. Shojaosadati: Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. *Water Res.*, **17**, 4231-4235 (2003).

Sparks, D.L., A. Page, P. Helmke, R. Loeppert, P. Soltanpour, M. Tabatabai, C. Johnston and M. Sumner: Methods of soil analysis. Part 3 - Chemical methods. *Soil Science Society of America*, Inc., Madison, WI (1996).

Sun, L.N., C. Su, C. Lei and T.L. Sun: Effects of flooding on changes in Eh, pH and speciation of cadmium and lead in contaminated soil. *Bull. Environ. Contam. Tox.*, **79**, 514-518 (2007).

Tessier, A., P.G.C. Campbell and M. Bisson: Sequential extraction procedure for the speciation of particulate trace metals. *Anal. Chem.*, **51**, 844-851 (1979).

USEPA: Test methods for evaluating solid waste, physical/chemical methods, 3rd ed. (Vol. 1) EPA Publication SW-846. Washington, DC: Office of Solid Waste and Emergency Response, USEPA (1992).

Uslu, G. and M. Tanyol: Equilibrium and thermodynamic parameters of single and binary mixture biosorption of lead (II) and copper (II) ion onto *Pseudomonas putida*: Effect of temperature. *J. Hazard. Mater*., **135**, 87-93 (2006).

Wu, S., Y. Luo, K. Cheung and M. Wong: Influence of bacteria on Pb and Zn speciation, mobility and bioavailability in soil: A laboratory study. *Environ. Pollut*., **144**, 765-773 (2006).

Zhang, B., R. Fan, Z. Bai, S. Wang, L. S. Wang and J. Shi: Biosorption characteristics of *Baciilus gibsonii* S-2 waste biomass for removal of lead (II) from aqueous solution. *Environ. Sci. Pollut. Res*., **20**, 1367-1373 (2013).

Zhang, B.Y., J.S. Zheng and R.G. Sharp: Phytoremediation in engineered wetlands: Mechanisms and applications. *Proc. Environ. Sci.,* **2**, 1315-1325 (2010).