P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 26 No.1 January - March 2021
Page: [96-106]
Original research article

Screening of Pb-Tolerant Plant Growth-Promoting Rhizobacteria from Roots of Pb-Excluder, Grown on Pb Contaminated Soil

Jiraporn Yongpisanphop[1,*], Sandhya Babel[2]

[1]*Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok 10800, Thailand*
[2]*School of Bio-Chemical Engineering and Technology, Sirindhorn International Institute of Technology, Thammasat University, Pathum Thani 12120, Thailand*

Received 19 November 2019; Received in revised form 19 May 2020
Accepted 10 June 2020; Available online 16 March 2021

ABSTRACT

Bacteria are microbes that can be used as natural tools for phytoremediation. The present study was conducted to discover a new species of Pb-tolerant siderophore producing and non-phosphate solubilizing rhizobacteria for phytostabilization. In this study, a Pb-phytostabilizer (*Pityrogramma calomelanos*) rhizobacterium, grown on Pb contaminated soil, was isolated and characterized genotypically and phenotypically. No isolate diversity was found in a harsh environment containing high, total and extractable Pb concentrations up to 45500 and 621 mg/kg, respectively. Only the S3 isolate was found and selected. This rhizobacterial isolate showed remarkable Pb tolerance up to 1850 mg/L. Moreover, it produced siderophores and could not solubilize phosphate. Interestingly, these properties support the immobilization of Pb. This isolate was identified as a strain that is closely related to *Arthrobacter humicola* by partial 16S rRNA gene analysis. To the best of our knowledge, this is the first study proposing a new candidate, *A. humicola*, for assisting in Pb phytostabilization. The potential use of this rhizobacterial candidate should be further investigated, using a pot experiment.

Keywords: Excluder; Lead contaminated soil; *Pityrogramma calomelanos*; Plant growth-promoting trait; Rhizospheric bacteria

1. Introduction

Environmental pollution is a severe problem on a global scale, including soil pollution. The main cause of this problem results from human activities with improper management. Among pollutants, toxic heavy metal, especially lead (Pb), is of great concern as it is toxic to living organisms

***Corresponding author:** jiraporn.y@sci.kmutnb.ac.th
doi: 10.14456/scitechasia.2021.9

even at low concentrations [1]. However, Pb compounds are still indispensable for modern human life [2]. Normally, a small amount of Pb contamination in soil comes from natural sources such as weathering of minerals and underlying bedrock, and volcanic activity. However, a main source of Pb contamination in soil is anthropogenic activities (e.g., agriculture, metalliferous mining and smelting, industrial effluents, domestic sewage, and atmospheric deposition) [1-3].

Pb contamination causes negative effects on the ecosystem and on biota health. Especially in humans, Pb toxicity causes anemia, encephalopathy, weight and coordination loss, abdominal pain, vomiting, constipation, and insomnia [1]. To protect and conserve the environment for future generations, Pb must be removed as much as possible from the environment.

There are many conventional techniques for Pb remediation in soil. Physical remediation includes soil excavation replacement and isolation, vitrification, and electrokinetic remediation. Chemical remediation, for example, is coagulation-filtration, ion exchange immobilization and soil washing [3-4]. These are physicochemical methods considered rather expensive, ranging from 10 to 1000 $/m^3[5]. They also may disrupt the soil ecological and biological structure, and produce dangerous secondary waste [3-4]. Their advantages are fast and easy application [3].

Phytoremediation using metal-tolerant plants to remediate metal contaminated sites is a simple, low-cost (0.05 $/m^3), environmentally friendly, and self-sustainable technique, compared with the existing conventional techniques [4-6]. Phytoremediation has disadvantages such as (a) phytoremediation potential relies on the growing conditions of each plant; (b) it takes a long time; and (c) an increase in metal solubility over time may cause leaching and environmental damage [5]. Among phytoremediation techniques, phytostabilization using metal tolerant plants to reduce or prevent the mobility of toxic metals in soils is a common method for soil remediation [6].

The success of phytostabilization depends on plant species that can tolerate multiple stresses and detoxify heavy metals, making plant selection an important step in phytostabilization [6-7]. There are many plants from field surveys identified as Pb-tolerant. For example, *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl., *Sesbania drummondii* (Rydb.) Cory, and *Helianthus annuus* L. accumulate 24, 40, and 100 g/kg of Pb in their tissues, respectively [8-9]. Unfortunately, metal phytotoxicity can reduce the efficiency of plants to remove contaminants from the soil [10]. Efficiency of phytostabilization that is increased by microbes has been proposed as a practical technique for removing or inactivating metals in contaminated soil via increasing the tolerance and alleviating toxicity [6]. Generally, bacteria living in metal contaminated areas have the specific mechanisms to cope with the heavy metal toxicity [11]. Hence, finding bacteria that have specific properties from a harsh environment needs to be investigated.

The area of the rhizosphere which is the narrow zone of soil directly surrounding the root system [12] is useful since this area contains a large microbial population with high metabolic activity, compared to bulk soil [13]. In addition, rhizobacteria that are lodged around the plant roots [12] have received much attention since they possess several traits. These bacteria are tolerant to high metal concentrations, and can mobilize, immobilize, and transform metals [6,13].

Metal-resistant rhizobacteria can be used as an inoculant in phytostabilization via metal biosorption, oxidation-reduction, and metal-ligand complexation [4,6]. These rhizobacteria reduce metals uptake by producing various metabolites including

siderophores [6]. Many researchers have isolated metal-tolerant rhizobacteria from different sources [11, 14-17]. However, the search for new Pb-tolerant rhizobacteria is still ongoing. New bacterial candidates that can be used to detoxify Pb contaminated soils are needed. Additionally, the relationships among plants, soil, metals, and microbes are an important factor for phytostabilization success [6].

To the best of our knowledge, there is little information about Pb-tolerant bacteria that are extracted from rhizosphere soil of a Pb excluder, *Pityrogramma calomelanos* (L.) Link grown on a Pb contaminated site. This plant can be used to screen Pb-tolerant rhizobacteria due to its high Pb content (in root) of about 32633 mg/kg [18].

The aim of this study was to isolate, characterize, and identify Pb-tolerant rhizobacteria from rhizosphere soil with *P. calomelanos*, grown on Pb contaminated soil in Kanchanaburi province, Thailand.

2. Materials and Methods

2.1 Rhizosphere soil collection

Rhizosphere soil was collected by shaking the roots of *P. calomelanos*, growing in Pb contaminated soil at Kanchanaburi, Thailand (Fig. 1). The rhizosphere is defined as the volume of soil adjacent to and influenced by the root [13].

Fig. 1. Rhizosphere soil collected around roots of *P. calomelanos* from Pb contaminated soil; location represented by the red star.

2.2 Total Pb concentration of rhizosphere soil analysis

Total and extractable concentrations of Pb were analyzed. In brief, 0.5 g of sieved and soil heated at 60ºC for 3 d was digested with 10 mL of 69% HNO_3 using microwave digestion according to the method of US-EPA 3051A [19]. The operational conditions and the heating program were performed according to the manufacturer's instructions (Milestone Inc., 2015) for the digestion of soil: a ramp time of 10 min to reach 200ºC, and then a hold time of 15 min. After digestion, samples were filtered using Whatman filter paper No. 42, and the volume was adjusted to 50 mL with deionized water. Pb concentrations in the extracts were determined by flame atomic absorption spectrophotometry (FAAS).

2.3 Extractable Pb concentration of rhizosphere soil analysis

The diethylene triamine pentaacetic acid (DTPA)-extractable Pb in rhizosphere soil was extracted according to the method of Lindsay and Norvell [20]. In brief, 10 g of soil sample was sieved and air-dried for 2 d. Pb was extracted with 20 mL of DTPA solution containing 0.005 M of DTPA, 0.01 M of $CaCl_2 \cdot 2H_2O$, and 0.1 M of TEA (triethanolamine). The pH was adjusted to 7.3±0.05 with 1 N HCl. Samples were horizontally shaken at approximately 150 rpm for 2 h, and filtered through Whatman filter paper No. 42. Pb concentrations were analyzed by FAAS.

2.4 Rhizospheric bacteria isolation

The procedure was modified from Son et al. [21]. In brief, 1 g of soil was transferred to 9 mL of sterilized physiologic water (0.85% NaCl in distilled water). After rotary shaking at 180 rpm for 30 min, serial dilution was done by transferring a 1-mL aliquot with 9 mL of sterilized 0.85% NaCl. After dilution (10^{-4}), 100 µL was spread on a Luria-Bertani (LB) agar plate, and 20 and 50 mg/L Pb was added as Pb $(CH_3COO)_2 \cdot 3H_2O$. The medium without Pb served as control. All plates were incubated at 30ºC for 48 h. Single colonies with different morphologies were picked and

purified by the streak plate method twice. The isolates were observed under a microscope. The characteristics of colonies were noted for the color, size, shape, margin, elevation, and surface.

2.5 Pb tolerance and plant growth promoting (PGP) traits characterization

The ability of the isolate to tolerate Pb was determined using the agar dilution method. The isolate was grown on LB agar with increasing amounts of Pb as $Pb(CH_3COO)_2 \cdot 3H_2O$ in a range of 1800–2000 mg/L. Bacteria were incubated at 30ºC for 2 d. The lowest concentration of a metal that completely inhibited the growth of the isolates was used as the minimal inhibitory concentration (MIC value) [22]. The phosphate solubilization was investigated using the National Botanical Research Institute Phosphate (NBRIP) medium [23]. The S3 isolate was spot-inoculated on the medium and incubated at 30ºC for 7 d. The occurrence of a clear zone around the growth colony indicated a positive for phosphate solubilization. The isolate was streaked on chrome azurol S (CAS) media [24] and incubated at 30ºC for 3 d. The occurrence of an orange halo around the growth colony indicated a positive for siderophore production.

2.6 16S rRNA gene identification

The bacterial genomic DNA of the S3 isolate was extracted. The selected fresh colony was cultured in LB broth at 30ºC, 150 rpm for 12 h. A 1-mL aliquot was centrifuged. The cell pellet was washed with 400 µL of 10 mM Tris-HCl (pH 8.0). Then, they were centrifuged. The pellets were resuspended in 200 µL of 10 mM Tris-HCl (pH 8.0), and boiled at 100ºC for 15 min using a heat block. Samples were centrifuged, and 100 µL of the aqueous phase containing purified DNA was transferred to a new microcentrifuge tube and stored at -20ºC. The conditions of centrifugation were 13000g for 10 min at 4ºC. A forward primer (8F) and reverse primer (534R) were used to amplify the 16S rRNA gene [14]. DNA (1 µL) was amplified in 49 µL of the master mix containing 40.6 µL of RNase free water, 5.0 µL of 10 x PCR Buffer, 4.0 µL of 10 mM dNTP Mix, 0.1 µL of each primer (100 pmol/µL), and 0.25 µL of TaKaRa Taq. The PCR amplification was performed in an automated thermocycler (Eppendorf International, Germany) as follows: 94ºC for 5 min (initial denaturation), 30 cycles of 94ºC for 1 min (denaturation), 60ºC for 1 min (annealing), 72ºC for 1 min (extension), and a final extension step of 72ºC for 15 min. A blank that contained all the components of the reaction mixture without the bacterial genomic DNA sample was used as a negative control. A length of 500 base pairs of PCR products was examined by 1.5% (w/v) agarose gel electrophoresis. The PCR products were purified using a MinElute®PCR product purification kit (Cat. No. 28004). The purified 16S rRNA gene was sequenced by FASMAC Company, JAPAN. The obtained sequence was analyzed with 16S rRNA genes in the NCBI database using the BLAST program. The species identification of the S3 isolate was performed using phylogenetic tree analysis, implemented by MEGA 7 [25]. Some sequences showing high identity were aligned by the MUSCLE algorithm. The phylogenetic tree was inferred by the Maximum Likelihood method, based on the Tamura 3-parameter model [26].

3. Results and Discussion

3.1 Total and DTPA-extractable Pb concentrations of rhizosphere soil

The total Pb concentration was 45500±6801 mg/kg, while the extractable Pb concentration was 612±22.0 mg/kg. This is consistent with the result of Yang et al. [27] who found that the total Pb concentration was 20X higher than that of the extractable Pb concentration. The reason the extractable Pb concentration was low

may be due to the adsorption of Pb on particle surfaces, such as organic matter [28]. Moreover, plants also reduce Pb availability in rhizosphere by releasing root exudates such as oxalate, which form a complex with Pb and then immobilize Pb in soil [27].

3.2 Isolation of rhizobacteria

Rhizosphere soil of a plant grown in Pb contaminated soil is a suitable source to isolate the strain containing Pb-tolerant bacteria. After the isolation, only one culturable Pb-tolerant rhizobacterium was found, since the characteristics of colonies were not different (Fig. 2). These colonies showed characteristics of ivory-white punctiform with a diameter of less than 1 mm, raised elevation, for the entire margin and a smooth-glistening surface. Interestingly, the brown precipitate at the center of a colony was found only in the media with added Pb. This could be from a Pb tolerance mechanism. To survive in the Pb rhizosphere soil, bacteria have developed some mechanisms to cope with Pb toxicity. In this study, rhizobacteria may cause Pb precipitation, since the color of the central area of the colony changed to brown, which may be a Pb precipitate. However, the form of the Pb precipitate depends on the strain [29]. Additionally, Pb precipitation is a general mechanism to lower the concentration of free Pb^{2+} by sequestering Pb outside and inside of the cells [30].

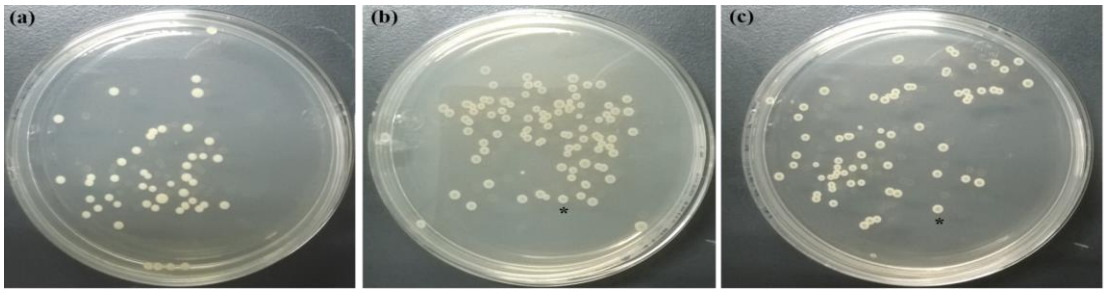

Fig. 2. Density and colony characteristics of Pb tolerant rhizosphere bacteria grown on different media: (a) LB agar; (b) LB agar with 20 mg/L of Pb; (c) LB agar with 50 mg/L of Pb. The black star mark shows Pb precipitation.

The population density of rhizobacteria averaged 50 x 10^5, 88 x 10^5, and 78 x 10^5 cfu/g grown on LB agar, LB agar with Pb 20 mg/L and 50 mg/L, respectively (Fig. 2). The density and diversity of each plate were similar. This may be because the amount of Pb added in LB-agar was low, compared to the habitat environment of this rhizobacterium.

3.3 Characterization of Pb-tolerant rhizobacteria

Only one isolate was selected and designated as "S3" for assessment of the MIC value against different Pb concentrations. The S3 isolate had a high degree of Pb tolerance with a MIC value of 1875 mg/L; in other words, the isolate could grow on media with added Pb up to 1850 mg/L. Similarly, *A. phenanthrenivorans* had a MIC value of 1200 mg/L, checked on soya agar (pH 7.0) plates containing lead as lead nitrate [31]. However, each experiment had different conditions, such as the type of Pb salt and the media used. High Pb tolerance is the ability of a bacterial strain to survive in the presence of high Pb concentrations [32]. This is supported by the result of the high total Pb concentration in rhizosphere soil, up to 45500 mg/kg from this work. Moreover, 61.6% of Pb-resistant bacterial strains can produce siderophores [33]. This

is supported by the result of this study. The S3 isolate produces siderophores, showing an orange halo (Fig. 3a).

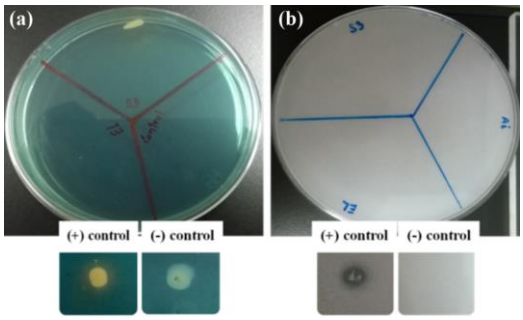

Fig. 3. Plant growth promoting traits of S3 isolate: (a) siderophore production on CAS agar, (b) phosphate solubilization on NBRIP agar.

Siderophores play a special role in metal detoxification, and thereby increase the metal tolerance in bacteria [33]. Siderophores, natural chelating agents, can form stable complexes with heavy metals such as Al, Cd, Cu, Ga, In, and Pb and can inactivate and minimize the cytological impacts of free metal ions [33-34]. This could reduce Pb toxicity. In addition, siderophores can be used for *in situ* phytostabilization and phytoextraction [35]. For example, Cr^{6+} reducing bacteria decreased Cr uptake in a green chili plant [36]. *Pseudomonas aeruginosa* improved the metal concentration and translocation factor of the metal from the root to shoot in maize grown on Cr and Pb contaminated soil [34]. Besides, siderophore-producing bacteria play a vital role in the phytoremediation success by increasing plant growth and metal uptake. They help plants to survive in contaminated soil via alleviating the metal toxicity and supplying the nutrients. They support plants to enhance metal uptake by binding with metals leading to increased metal bioavailability in the rhizosphere zone [37]. Additionally, Yu et al. [38] found that siderophore-producing bacteria (*Bacillus* sp. PZ-1) produce hydroxamate-type siderophore (32.24 µg/ml). The results from the pot experiment in this study show that siderophore increase plant biomass, Pb uptake ratio and translocation factor of *Brassica juncea* about 1.31, 3.38 and 1.12 times, respectively, in comparison with the control at 400 mg/kg of Pb in soil. These seemingly conflicting results of different siderophore-producing bacteria on metal uptake by plants suggest that the effects of bacterial siderophores in the presence of high metal concentrations are complex, and the mechanisms are still unclear [34]. Furthermore, the efficiency of siderophore-producing bacteria to either mobilize or immobilize metal from the soil depends on plant species, metal type and concentration, and soil composition [34]. These characteristics make siderophore-producing bacteria more suitable as biological amendments rather than chemical amendments, since they are degradable, less toxic, and they improve the soil structure and function [4].

However, the S3 isolate could not solubilize phosphate, as shown by no clear zone (Fig. 3b). This is consistent with previous work. Some rhizobacteria isolated from contaminated soil could produce siderophores, but not solubilize phosphate, such as *Bacillus* sp., *Arthrobacter* sp., *Microbacterium* sp., *Rhodococcus* sp, etc. [15]. Normally, phosphate-solubilizing bacteria can improve the bioavailability of metals in soil, which increases the uptake of metals by plants [34]. This is suitable for phytoextraction. In contrast to the result of their experiment, the S3 isolate could not solubilize phosphate. This indicates that the S3 isolate can be utilized for phytostabilization.

3.4 Identification of rhizobacteria

In the amplification of the 16S rRNA genes, the molecular marker is seen in lane L, and fragments of 500 bp of the S3 isolate are seen in lane S3. The remaining lanes show other genes of endophytic bacteria (Fig. 4).

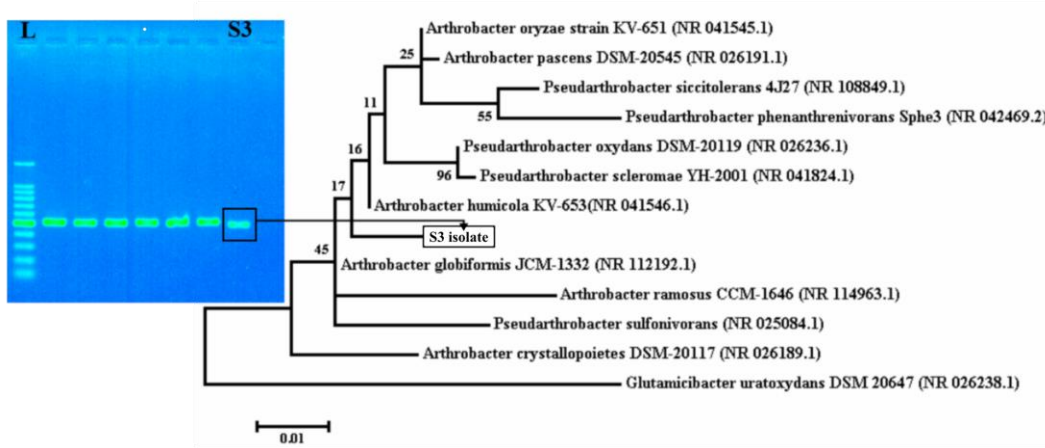

Fig. 4. PCR products obtained by gel electrophoresis showing amplified 16S rRNA partial sequence of 500 bp. Lane L: marker; Lane S3: 16Sr RNA gene of S3 isolate. The phylogenetic tree was constructed using the maximum likelihood method implemented in the MEGA 7. *Glutamicibacter uratoxydans* was used as the outgroup. Bootstrap values (based on 1000 replications) are listed as percentages at the nodes. The scale bar indicates genetic distance. The GenBank accession number is given in parentheses for each bacterium.

The S3 isolate sequence showed an identity of 98.33% *Arthrobacter humicola* (accession number NR_041546.1), compared to the NCBI database using the BLAST tool. According to the phylogenetic tree, the representative bacteria of related taxa are shown in Fig. 4. The S3 isolate strain is closely related to *A. humicola*. This is not surprising because *Arthrobacters* are considered to be ubiquitous and predominant members of culturable soil microorganisms [14]. The results from this study are consistent with previous works, which isolated *Arthrobacter* sp. from a Pb environment, including lead-zinc mine tailings [14-15,31]. Moreover, *Arthrobacter* sp. is reported as a metal-resistant microbe that causes the biotransformation of highly toxic metals into less toxic states [39].

The PGP traits of *A. humicola* from this study compared to other Pb-tolerant plant growth-promoting rhizobacteria are shown in Table 1. It is not necessary to meet all the PGP traits. These Pb-tolerant plant growth-promoting rhizobacteria are able to promote Pb phytoremediation via plant growth [40]. Thus, *A. humicola* with only siderophore production can be used for Pb phytoremediation.

Phytostabilizers reduce the mobility and inactivate toxic heavy metals through rhizospheric processes such as metal biosorption, oxidation-reduction, and heavy metal-ligand complexation [4]. Rhizobacteria may associate, which can promote the efficiency and mechanism of phystabilization. This is consistent with the properties of *A. humicola*, which can immobilize Pb in soil. Our experiments suggest that rhizobacterium can promote the ability of a Pb-excluder to immobilize Pb in soil.

4. Conclusion

In this study, a strain closely related to *Arthobacter humicola* is recommended as a suitable and eco-friendly candidate for the remediation of Pb contaminated soil. Our strain is Pb-tolerant siderophore-producing and non-phosphate solubilizing rhizosphere bacteria. It used Pb precipitation to cope with Pb toxicity. Its properties include siderophore production. It does not solubilize phosphate, but can immobilize Pb

Table 1. PGP characteristics of Pb-tolerant rhizobacteria.

Rhizobacteria	Plant growth promoting traits					Ref.
	Siderophore production	Phosphate solubilization	IAA production	Nitrogen fixation	Ammonia production	
Arthobacter humicola	+	-	not tested	not tested	not tested	This study
Bacillus proteolyticus	-	+	+	+	+	[40]
Bacillus velezensis	+	+	+	+	+	
Lysinibacillus sp.	-	-	+	-	+	
Lysinibacillus varians	+	+	+	+	+	[41]
Pseudomonas putida	+	+	+	+	+	

+ indicates the positive results; - indicates the negative results; IAA is indole-3-acetic acid.

in soil. In addition, it can tolerate Pb up to 1850 mg/L with MIC value 1875 mg/L. Therefore, this rhizobacterium, acting as the biological amendment, could be considered as an alternative strategy over chemical amendments for increasing the Pb phytostabilization efficiency. Further study, using pot and field experiments, is needed to determine the influence of *A. humicola* amendment on Pb uptake by plants. Moreover, the full length of 16S rRNA gene analysis of *A. humicola* should be tested.

Acknowledgement

The authors would like to thank the Royal Golden Jubilee (RGJ) Ph.D. program of the Thailand Research Fund (TRF) under grant number PHD/0214/2556 for financial support.

References

[1] Bahnasawy M, Khidr A-A, Dheina N. Assessment of heavy metal concentrations in water, plankton, and fish of lake Manzala, Egypt. Turk J Zool 2011;35(2):271-80.

[2] Sharma P, Dubey RS. Pb toxicity in plants. Braz J Plant Physiol 2005; 17(1):35-52.

[3] Khalid S, Shahid M, Niazi NK, Murtaza B, Bibi I, Dumat C. A comparison of technologies for remediation of heavy metal contaminated soils. J Geochem Explor 2017; 182(B):247-68.

[4] Rajkumar M, Ma Y, Freitas H. Improvement of Ni phytostabilization by inoculation of Ni resistant *Bacillus megaterium* SR28C. J Environ Manage 2013;128:973-80.

[5] Anubhuti S. Phytoremediation: new frontia. Res. J. Biotech 2013; 8(5):1-2.

[6] Ma Y, Rajkumar M, Rocha I, Oliveira RS, Freitas H. Serpentine bacteria influence metal translocation and bioconcentration of *Brassica juncea* and *Ricinus communis* grown in multi-metal polluted soils. Front Plant Sci [Internet]. [cited 2019 Nov 13]. Available from: https://doi.org/10.3389/fpls.2014.00757

[7] Zhivotovsky OP, Kuzovkina JA, Schulthess CP, Morris T, Pettinelli D, Ge M. Hydroponic screening of willows (Salix L.) for lead tolerance and accumulation. Int J Phytorem 2011;13(1):75-94.

[8] Mahmood T. Phytoextraction of heavy metals-the process and scope for remediation of contaminated soils. Soil Environ 2010;29(2):91-109.

[9] Sahi SV, Bryant NL, Sharma NC, Singh SR. Characterization of a lead hyperaccumulator shrub, *Sesbania drummondii*. Environ Sci Technol 2002;36:4676-80.

[10] Rostami S, Azhdarpoor A. The application of plant growth regulators to improve phytoremediation of contaminated soils: A review. Chemosphere 2019; 220: 818-27.

[11] Ndeddy Aka RJ, Babalola OO. Identification and characterization of Cr-, Cd-, and Ni-tolerant bacteria isolated from mine tailings. Bioremediat J 2017;21(1):1-19.

[12] Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J. King Saud Univ Sci 2014;26(1):1-20.

[13] Naees M, Ali Q, Shahbaz M, Ali F. Role of rhizobacteria in phytoremediation of heavy metals: An overview. Int Res J Plant Sci 2011;2(8):220-32.

[14] Hanbo Z, Changqun D, Qiyong S, Weimin R, Tao S, Lizhong C, Zhiwei Z, Bin H. Genetic and physiological diversity of phylogenetically and geographically distinct groups of *Arthrobacter* isolated from lead–zinc mine tailings. FEMS Microbiol Ecol 2004; 49: 333-41.

[15] Pereira SIA, Barbosa L, Castro PML. Rhizobacteria isolated from a metal-polluted area enhance plant growth in zinc and cadmium-contaminated soil. Int J Environ Sci Technol 2015; 12: 2127-42.

[16] Nissi Paul M, Triveni S, Latha PC, Chandini Patnaik M, Manohar Rao A. A. Resistance of potential plant growth promoting rhizobacteria to heavy metals (Ni, Cd, Co, Pb) isolated from polluted areas of Hyderabad. Int J Curr Microbiol Appl Sci 2018;7(8):3583-91.

[17] Tirry N, Tahri Joutey N, Sayel H, Kouchou A, Bahafid W, Asri M., El Ghachtouli N. Screening of plant growth promoting traits in heavy metals resistant bacteria: Prospects in phytoremediation. J Genet Eng Biotechnol 2018; 16: 613-9.

[18] Yongpisanphop J, Babel S, Kruatrachue M, Pokethitiyook P. Phytoremediation potential of plants growing on the Pb-contaminated soil at the Song Tho Pb Mine, Thailand. Soil Sediment Contam: An Int J 2017;26(4):426-37.

[19] United States Environmental Protection Agency (U.S. EPA). Method 3051A (SW-846): Microwave assisted acid digestion of sediments, sludges, and oils [Internet]. [cited 2015 February 7]. Available from: https://www.epa.gov/sites/production/files/2015-12/documents/3051a.pdf

[20] Lindsay WL, Norvell WA. Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 1978; 42: 421-28.

[21] Son J-S, Sumayo M, Hwang Y-J, Kim B-S, Ghim S-Y. Screening of plant growth-promoting rhizobacteria as elicitor of systemic resistance against gray leaf spot disease in pepper. Appl Soil Ecol 2014; 73:1-8.

[22] Shin M-N, Shim J, You Y, Myung H, Bang K-S, Cho M, Kamala-Kannan S, Oh B-T, Characterization of lead resistant endophytic *Bacillus* sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*. J Hazard Mater 2012;199-200:314–20.

[23] Nautiyal CS. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 1999;170(1):265–70.

[24] Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 1987;160(1):47-56.

[25] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33: 1870-74.

[26] Tamura K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol Biol Evol 1992; 9: 678-87.

[27] Yang Y, Dong M, Cao Y, Wang J, Tang M, Ban Y. Comparisons of soil properties, enzyme activities and

microbial communities in heavy metal contaminated bulk and rhizosphere soils of *Robinia pseudoacacia* L. in the northern foot of Qinling mountain. Forests [Internet]. [cited 2019 Nov 13]. Available from: https://www.mdpi.com/1999-4907/8/11/430

[28] Fleming M, Yiping T, Ping Z, McBride MB. Extractability and bioavailability of Pb and As in historically contaminated orchard soil: Effects of compost amendments. Environ Pollut 2013; 177: 90-7.

[29] Naik MM, Dubey SK. Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. Ecotoxicol Environ Saf 2013; 98: 1-7.

[30] Jarosławiecka A, Piotrowska-Seget Z. Lead resistance in microorganisms. Microbiol 2014; 160: 12-25.

[31] Banerjee G, Ray AK, Pandey S, Kumar R. An alternative approach of toxic heavy metal removal by *Arthrobacter phenanthrenivorans*: Assessment of surfactant production and oxidative stress. Curr Sci 2016;110(11):2124-28.

[32] Lima e Silva AA, Carvalho MAR, Souza SAL, Teixeira Dias PM, Silva Filho RG, Meirelles Saramago CS, Melo Bento CA, Hofer E. Heavy metal tolerance (Cr, Ag and Hg) in bacteria isolated from sewage. Braz J Microbiol 2012;1620-31.

[33] Yahaghi Z, Shirvani M, Nourbakhsh F, Peña TC, Pueyo JJ, Talebi M. Isolation and characterization of Pb-solubilizing bacteria and their effects on Pb uptake by *Brassica juncea*: Implications for microbe-assisted phytoremediation. J Microbiol Biotechnol 2018; 28(7): 1156-67.

[34] Kong Z, Glick BR. The role of plant growth-promoting bacteria in metal phytoremediation. In: Poole RK, editor. Advances in microbial physiology. Academic Press; 2017. p.97-132.

[35] Cornu J-Y, Huguenot D, Jézéquel K, Lollier M, Lebeau T. Bioremediation of copper-contaminated soils by bacteria. World J Microbiol Biotechnol [Internet]. [cited 2019 Nov 5]. Available from: https://doi.org/10.1007/s11274-016-2191-4

[36] Chatterjee S, Sau GB, Mukherjee SK. Plant growth promotion by a hexavalent chromium reducing bacterial strain, *Cellulosimicrobium cellulans* KUCr3 World J Microbiol Biotechnol 2009;25(10):1829-36.

[37] Rajkumar M, Ae N, Prasad MNV, Freitas H. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends in Biotechnology 2010;28(3):142-9.

[38] Yu S, Teng C, Bai X, Liang J, Song T, Dong L, Jin Y, Qu J. Optimization of siderophore production by bacillus sp. pz-1 and its potential enhancement of phytoextration of pb from soil. J Microbiol Biotechnol 2017; 27(8):1500-12.

[39] Mishra S, Bharagave RN, More N, Yadav A, Zainith S, Mani S, Chowdhary P. Heavy metal contamination: An alarming threat to environment and human health. In: Sobti R C, Kumar Arora N. Kothari R, editors. Environmental biotechnology: For sustainable future. Singapore: Springer; 2018. p. 103-25.

[40] Kamaruzzaman MA, Abdullah SRS, Hasan HA, Hassan M, Othman AR, Idris M. Characterisation of Pb-resistant plant growth-promoting rhizobacteria (PGPR) from *Scirpus qrossus*. Biocatal Agric Biotechnol [Internet]. [cited 2020 May 15]. Available from: https://doi.org/10.1016/j.bcab.2019.101456

[41] Pal AK, Sengupta C. Isolation of cadmium and lead tolerant plant growth promoting rhizobacteria: *Lysinibacillus*

varians and *Pseudomonas putida* from Indian Agricultural Soil. Soil Sediment Contam. An Int J 2019; 28(7):601-29.