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ISOLATION AND CHARACTERIZATION OF PHOSPHATE-SOLUBILIZING AND HEAVY-METAL TOLERANT BACTERIA FROM AGRICULTURAL FIELDS IN MATARA DISTRICT, SRI LANKA

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ABSTRACT

The capacity to solubilize inorganic phosphates by phosphate-solubilizing bacterial strains isolated from soil samples collected from different agricultural fields in Matara District was assessed. The isolated phosphate solubilizing bacterial strains were then tested for tolerance against four heavy metals such as cadmium (Cd), lead (Pb), zinc (Zn) and copper (Cu) each with three concentrations (100 mg/L, 200 mg/L, and 400 mg/L). The bacterial strains with the highest phosphate solubilization capacity and resistance to heavy metal were selected and the phosphate solubilization potential under different heavy metals was then assessed. The results showed that most of the tested isolates proved to be tolerant to the heavy metals at low concentrations. However, a subsequently significant reduction in tolerance was observed when heavy metal concentration increased. Except three isolates, all the other isolates were proved to be vulnerable to the heavy metals of Cd and Pb at the 400 mg/L concentration. Among the tested 15 isolates, PSB-14 showed the highest tolerance to 100 mg/L, 200 mg/L and 400 mg/L of Cd, Pb, Cu and Zn. The strain was identified as *Enterobacter cancerogenous* according to the 16 rRNA analysis. The bacterial strain showed very high degree of reductions in phosphate solubilization in the presence of heavy metals Cd and Pb. The order of the toxicity of the metals to strain was found to be Pb > Cd > Cu > Zn. According to the results, it could be concluded that heavy metals Cd and Pb was shown to display a major impact on phosphate solubilization while Cu and Zn had a mild effect.

Keywords: Agricultural soils, *Enterobacter cancerogenous*, heavy metals, phosphate solubilization, tolerance

INTRODUCTION

Heavy metal toxicity to various environmental niches has created environmental problems due to their potential adverse ecological effects and is a great matter of concern for environmentalists (Jiang *et al.,* 2008). Toxic effects of heavy metals such as Cd, Cu, Zn, Ni, Co, Cr, Pb and As last longer and they cannot be degraded chemically or biologically (Ahemad, 2012). Continuous and excess use of agrochemicals such as fertilizers and pesticides could directly or indirectly have negative impacts as they could release toxic materials such as heavy metals to the environment. The elevated concentration of these metals in soils has direct and detrimental effects on plant growth and healthy production (Wani and Khan, 2010). Indirectly they affect the composition of naturally occurring microbial communities and they limit the growth of naturally occurring beneficial microbial communities (Ahemad and Khan, 2012). However, there are some microorganisms including plant growth promoting bacteria (PGPB) exert various kinds of tolerance mechanisms against elevated concentrations of heavy metals (Guo and Mahillon, 2013; Mallick *et al.,* 2014). They are able to evade the toxicity generated by the various heavy metals through various mecha-

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nisms such as expulsion of metal species outside the microbial cell surface, bioaccumulation the metal ions inside the cell, biotransformation of toxic metals to less toxic forms and metal adsorption on the cell wall (Ahemad, 2012).

Phosphate solubilizing microorganisms (PSMs) are able to mobilize insoluble inorganic phosphates to the soil solution, making them available to plant uptake (Khan *et al.,* 2009). Therefore, the potential use of PSMs to increase phosphorous utilization efficiency through its application as bio-inoculants has attracted the interest among the scientific community engaged in P acquisition and P utilization. Among various domains of PSMs, bacteria belonging to the genera *Bacillus, Pseudomonas, Burkholderia, Pantoa, Enterobacter, Erwinia, Azotobacter, Rhizobium, Acinetobacter, Flavobacterium, Klebsiella*, and *Micrococcus* have been reported as efficient phosphate solubilizers (Sharma *et al.,* 2013; Paul and Sinha, 2013). Although many soil bacteria are tolerant to heavy metals it is reported that bacterial strains isolated from heavy metals polluted environments were shown to be tolerant to higher concentrations of metals than those isolated from unpolluted areas (Rajkumar *et al.,* 2010).

Therefore phosphate solubilizing bacterial strains with genetic potential for increasing tolerance to elevated levels of heavy metals are substantially improved the growth of plants implanted in heavy metal contaminated polluted soils by lowering the metal toxicity (Wani and Khan, 2010). The present study was undertaken to isolate phosphate solubilizing bacterial strains from different agricultural fields and to assess their heavy metal resistance in the presence of different heavy metals.

MATERIALS AND METHODS

Isolation of phosphate solubilizing bacterial strains

Rhizosphere soil samples were collected from different agricultural fields in Matara District for isolation of phosphate solubilizing bacterial strains. Serially diluted soil sample aliquots were inoculated on NBRIP medium (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, $(NH_4)_2SO_4$ and 16 g agar in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7. The petri plates were incubated for 7 days at 30^0C. The colonies with clear halos were considered to be phosphate solubilizing colonies. Predominant colonies were further purified by re-streaking on the fresh NBRIP agar plates at 30^oC.

Assay of inorganic phosphate solubilization

The isolated bacterial strains were grown in sterilized liquid NBRIP medium (20 ml) at 30^0C for 2 days with continuous shaking at 150 rpm. Aliquots of culture (1 ml) was then transferred to a 500 ml flask (n=3 per strain) containing sterilized liquid medium (250 ml) and incubated with continuous shaking at 30^0C. Sterilized un-inoculated medium served as a control. A sample (10 ml) of each cultured and control were taken 3 days after the inoculation and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the pH and amount of phosphorous released into the medium. The pH of the culture medium was also recorded with the pH meter equipped with glass electrode. The phosphorus availability was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

Assay of heavy metal resistance of the bacterial strains

Isolated bacterial strains were assessed for their sensitivity or resistance to heavy metals by using agar dilution method (Cervantes *et al.,* 1986). Freshly prepared tris-minimal salts medium agar plates were amended with four different heavy metals (Cd, Pd, Cu and Zn) at various concentrations (100-400 mg/l). They were inoculated with isolated strains and heavy metal tolerance was determined by the appearance of bacterial growth after 5 days of incubation at 30 ^{0}C. The experiments were carried out in triplicate.

Assay of growth and phosphate solubilization potential under heavy metals

The bacterial strain with the highest phosphate solubilization capacity and resistance to heavy metal was selected and the growth and phosphate solubilization potential under different heavy metal concentrations were assessed.

Effect of heavy metals on bacterial growth was measured by measuring the absorbance at 660 nm using spectrophotometer (Shimadzu UV-VIS). NBRIP liquid medium supplemented with heavy metals at the concentrations of 100, 200 and 400 mg/L was inoculated with bacterial suspension (10^6 CFU/ml) and incubated with continuous shaking at 30 ^oC. Samples from cultures grown in NBRIP medium were diluted 1:1 (v/v) using 1 N HCl to dissolve the residual insoluble phosphate and absorbance was recorded at 660 nm against a blank to measure the growth of the strain (Rodriguez *et al.,* 2000). Bacterial culture inoculated heavy metal free medium considered as control.

Bacterial strain was grown in sterilized liquid NBRIP medium (20 ml) at 30^0C for 2 days with continuous shaking at 150 r min^{-1}. Aliquots of culture (10^6CFU/ml) was then transferred to a 500 ml flask (n=3 per strain) containing sterilized liquid NBRIP medium (250 ml) supplemented with heavy metals (Cd, Pb, Cu and Zn) at the concentrations of 100, 200 and 400 mg/L. The flasks were incubated with continuous shaking at 30 ^0C. A sample of culture broth (10 ml) from each cultured flask was removed after 3 days and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the amount of phosphorous released into the medium. The pH of the culture medium was also recorded with the pH meter equipped with glass electrode. The phosphorus availability was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

Identification of the selected bacterial strain

The bacterial strain showing the highest degree of metal resistance was selected and partial sequencing of 16S rRNA for the 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 Bio Edit 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 (Figure 1).

RESULTS AND DISCUSSION

Isolation of phosphate solubilizing bacterial strains

The collected soil samples were plated in NBRIP medium for isolation of phosphate solubilizing bacterial strains.Fifteen bacterial strains (PSB 1 to PSB 15) which exhibited clear zones on the NBRIP agar plates were selected as phosphate solubilizing bacterial strains. They had a marked solubilizing ability of insoluble phosphate as visualized by the clear zone developed around the colony after 3 days of incubation. The isolate PSB – 14 showed the maximum phosphate solubilizing halo zone around the colony. The solubilization index (SI – Colony diameter + halo zone diameter /colony diameter) of the strain PSB – 4 was calculated at the end of the incubation

period and it was 3.25.

Assay of inorganic phosphate solubilization

The phosphate solubilizing potential of the isolated strains in NBRIP liquid media indicated that all the isolated bacterial strains efficiently solubilized inorganic phosphate in the medium containing tri calcium phosphate (Table 1). The initial turbid color of the NBRIP medium was turned to clear within 2-3 days due to the solubilization of inorganic phosphate by inoculated bacterial strains. Results showed that the isolated strains had different capabilities to release soluble phosphorus from inorganic phosphate. Among the fifteen isolates, PSB-14 was shown to release the highest content of soluble phosphorus (652 mg/L) into the medium, followed by PSB-8 and PSB-9 with 611 and 599 mg/L of soluble phosphorus, respectively.

All isolated strains lowered the pH of culture medium. The initial pH of the NBRIP medium was 7.0 and pH drop by phosphate solubilizing bacterial isolates ranged from 3.95-4.65.The inverse relationship between pH and

Table 1: Phosphate solubilizing potential of the isolated strains in NBRIP liquid media

Bacterial Strain	Phosphate solubilization mg/L	Final pH
PSB 1	379.2±15.6	4.41 ± 0.08
PSB 2	279.9±13.9	4.48 ± 0.05
PSB 3	410.2±14.2	4.65 ± 0.09
PSB 4	255.4±13.5	3.95± 0.12
PSB 5	509.7±14.6	4.21 ± 0.09
PSB 6	472.3±14.1	4.11 ± 0.16
PSB 7	506.8±15.4	4.08 ± 0.08
PSB 8	611.0±14.5	4.06 ± 0.11
PSB 9	598.7±14.6	4.22 ± 0.09
PSB 10	466.2±13.4	4.14 ± 0.11
PSB 11	502.5±12.4	4.29 ± 0.10
PSB 12	417.6±11.4	4.06 ± 0.07
PSB 13	589.4±15.6	3.96 ± 0.14
PSB 14	651.8±15.7	4.31 ± 0.13
PSB 15	431.9±14.5	4.28 ± 0.07

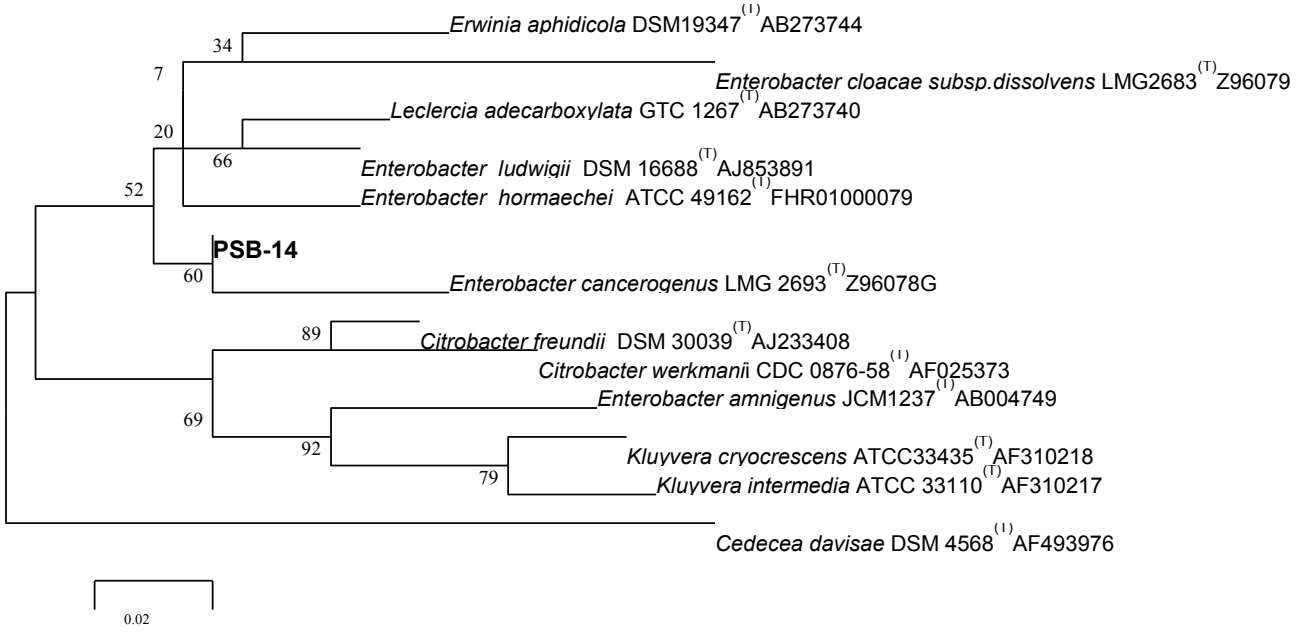

Figure 1: Phylogenetic tree based on 16S rDNA gene sequences, showing the position of *Enterobacter cancerogenus* strain with respect to related species. The scale bar indicates 0.02 substitutions per nucleotide position and accession numbers are given in parenthesis.

soluble phosphorus concentration was observed among all the isolated bacterial strains suggested that low pH of the medium could facilitate the inorganic phosphate solubilization (Sharma *et al.,* 2013). A Similar relationship between pH and phosphate solubilization were reported by other researchers (Yu *et al.,* 2011; Yasmin and Bano, 2011).

Assay of heavy metal resistance of the bacterial strains

The isolated strains were tested for tolerance against four heavy metals (Cd, Pb, Zn and Cu) at the concentrations of 100, 200 and 400 mg/L.

Soil microorganisms having higher resistance to heavy metal s are considered as the best choice for phytoremediation studies. Our results indicated that most of the tested isolates were proved to be tolerant to the heavy metals at low concentrations. However, subsequently significant reduction in tolerance was observed when heavy metal concentration increased. Out of the tested four different heavy metals most of the isolates were able to grow at 100 ppm concentration. Except three isolates (PSB-2, PSB-14 and PSB-7 for Cd and PSB-14, PSB-8 and PSB-13 for Pb), all the other isolates were proved to be vulnerable to the heavy metals of Cd and Pb at the 400 ppm concentration (Table 2). Therefore, Cd and Pb are considered as the most toxic heavy metals for isolated phosphate solubilizing organisms. Among the tested 15 isolates, PSB-14 showed the highest tolerance and the strain showed tolerance to 100 mg/L, 200 mg/L, and 400 mg/L of Cd, Pb, Cu and Zn.

Assay of phosphate solubilization potential under heavy metals

Strains showed diverse levels of phosphate solubilizing activity in the presence of different heavy metals. None of the metals was found to be highly toxic to the strain during the incubation period. However, the presence of heavy metals in NBRIP medium caused significant reductions in phosphate solubilization. Phosphate solubilization decreased with increasing concentrations of heavy metals. The bacterial strain showed very high degree of reductions in phosphate solubilization with the presence of heavy metals Cd and Pb (Figure 2, 3, 4 and 5). The order of the toxici-

Table 2: Tolerances of the isolated strains at different concentrations of heavy metals

Strain	Heavy metal concentration (mg/L)											
	Cd			Pb			Zn			Cu		
	100	200	400	100	200	400	100	200	400	100	200	400
PSB-1	+	+	-	+	+	-	+	+	+	+	+	+
PSB-2	+	+	+	+	+	-	+	+	+	+	-	-
PSB-3	+	-	-	+	-	-	+	+	+	+	+	+
PSB-4	+	+	-	+	+	-	+	+	-	+	+	-
PSB-5	+	+	-	+	-	-	+	+	-	+	+	+
PSB-6	-	-	-	-	-	-	+	+	-	-	-	-
PSB-7	+	+	+	+	+	-	+	+	+	+	+	+
PSB-8	+	+	-	+	+	+	+	+	+	-	-	-
PSB-9	-	-	-	+	+	-	+	-	-	-	-	-
PSB-10	+	-	-	+	+	-	-	-	-	+	+	+
PSB-11	+	+	-	+	+	-	+	+	+	+	+	+
PSB-12	+	+	-	-	-	-	+	+	+	+	+	+
PSB-13	+	+	-	+	+	+	+	+	+	+	+	+
PSB-14	+	+	+	+	+	+	+	+	+	+	+	+
PSB-15	+	-	-	+	-	-	+	+	-	+	+	+

(+): tolerance to the heavy metal, (-): susceptible to the heavy metal

ty of the metals to strain found to be Pb > Cd > Cu > Zn. According to the results heavy metals Cd and Pb had a major impact on phosphate solubilization and Cu and Zn had a mild negative, but significant effect on phosphate solubilization. Similar toxicity results were observed by Singh *et al.* (2016). They observed consistently slower growth rates of the isolated strains in the presence of heavy metals (Cd and Pb). It was decreased by 41%, 45% and 46% respectively for the Cd concentrations of 100 mg/L, 200 mg/L, and 400 mg/L and 44%, 45% and 50% respectively for the Pb concentrations of 100 mg/L, 200 mg/L, and 400 mg/L compared to the control. This may be due to the impairment of various metabolic activities of the organisms (Kumar *et al.,* 2010).

The reduction of phosphate solubilization in heavy metal Cu inoculated NBRIP medium was 16%, 24% and 30% respectively for the concentrations of 100 mg/L, 200 mg/L, and 400 mg/L whereas Zn inoculated medium it was 5%, 7% and 12% respectively for the concentrations of 100 mg/L, 200 mg/L, and 400 mg/L. Phosphate solubilizing microorganisms isolated from heavy metal contami-

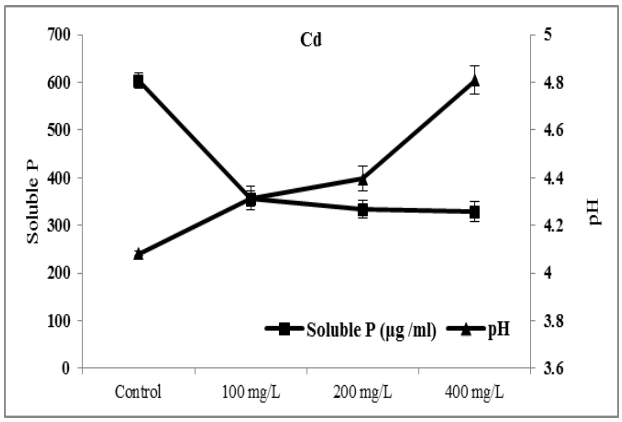

Figure 2: Effect of three different concentrations of Cd (cadmium) on phosphate solubilization and pH change by *Enterobacter cancerogenus*. Values given here are the means (n = 3) ± standard deviation.

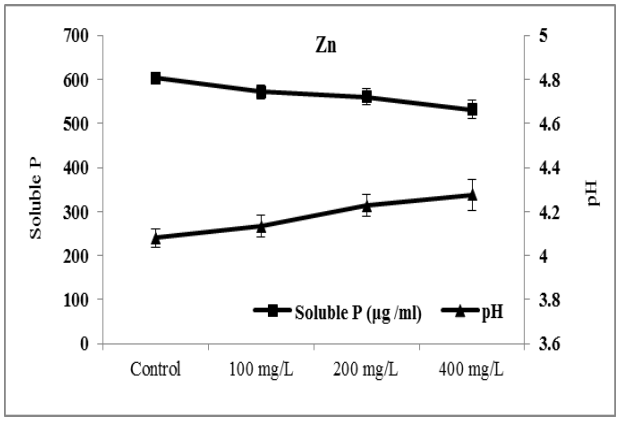

Figure 3: Effect of three different concentrations of Zn (zinc) on phosphate solubilization and pH change by *Enterobacter cancerogenus*. Values given here are the means (n = 3) ± standard deviation.

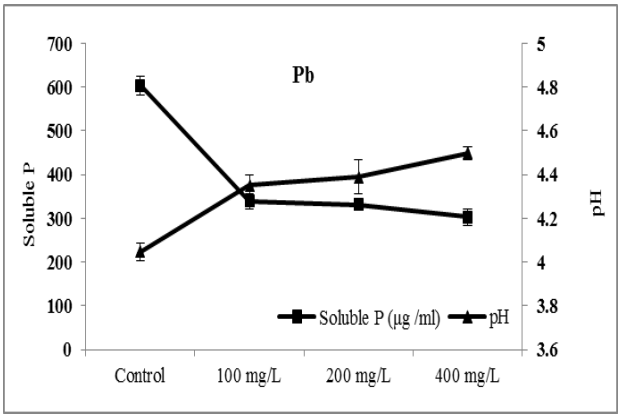

Figure 4: Effect of three different concentrations of Pd (lead) on phosphate solubilization and pH change by *Enterobacter cancerogenus*. Values given here are the means (n = 3) ± standard deviation.

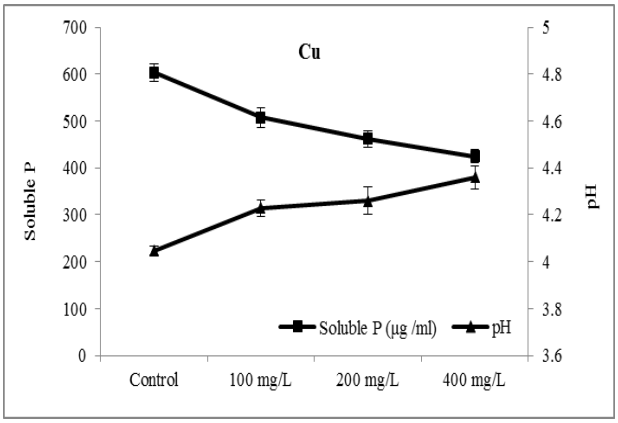

Figure 5: Effect of three different concentrations of Cu (copper) on phosphate solubilization and pH change by *Enterobacter cancerogenus*. Values given here are the means (n = 3) ± standard deviation.

nated soils have developed the mechanisms to cope with a variety of toxic metals for their survival in the environment enriched with such metals, thus adaptation of microorganisms to such a heavy metal contaminating environment may be the possible reason attributed to their withstand against heavy metals (Abou-Shanab *et al.,* 2007; Singh *et al.,* 2016). Our results are in agreement with pandy *et al.* (2011) who isolated *Bacillus sp* with phosphate solubilizing potential in the presence of metals Pb and As.

The isolation of microorganisms both metal tolerant and efficient in producing plant growth promoting properties are of particular importance of the reclamation of heavy metal polluted soils (Sinha *et al.,* 2011; Sinha and Paul, 2014). They are considered as cost effective and environmentally friendly tools for restricting heavy metal movement to plant organs by transforming metal species into immobile forms to decrease toxicity of metals (Ahemad, 2012; Glick, 2012; Paul and Sinha 2013). They can be useful to speed up the colonization process of the plant rhizosphere in heavy metal polluted soil. Therefore, further studies are needed to assess the other plant growth promoting activities of isolated phosphate solubilizing bacterial strain under heavy metal stress.

CONCLUSION

The results of the present study suggested that isolated heavy metal tolerant *Enterobacter cancerogenous* PSB-14 can be useful to remediate heavy metal contaminated sites. Use of these heavy metal tolerance phosphate solubilizing bacteria as bio-inoculants will increase the available phosphorus content in the soil and reduces heavy metal pollution. Further studies are needed to assess the other plant growth promoting activities of isolated phosphate solubilizing bacterial strain under heavy metal stress.

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