cLEAD BIOACCUMULATION BY *PSEUDOMONAS* SPECIES ISOLATED FROM PIG WASTE.

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ABSTRACT: Living organisms are exposed in nature to lead commonly in their ionized forms, which at different concentrations affect microbial population. This can have significant impact given that many microorganisms are essential parts of the decomposing food chain. Their presence in the atmosphere, soil and water, even in traces, can cause serious problems to all organisms. Microorganisms are known to interact with heavy metals through a number of mechanisms including intracellular accumulation. *Pseudomonas* species isolated from pig waste was exposed to different concentrations of lead solution within 24 hours. The percentage log survival / growth rate in the different concentrations of lead was determined periodically. Bioaccumulation of lead by the test isolate was determined in the graded lead concentrations (0, 1.10, 100. 500 µg/ml). The result showed that the growth of the isolate was progressively inhibited by lead in a dose dependent fashion. The isolate showed a potential to survive lead intoxication and accumulated the toxicant. Therefore, *Pseudomonas* species isolated from pig waste shows a promise for its use in bioremediation of lead polluted environments and can be used remedy the toxic effect of heavy metals on plants. This can be applied as organic manure together with the microorganism in heavy metal-polluted site to prevent heavy metal toxicity and to enhance the growth of plants.
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1. INTRODUCTION

There is a continuous influx of heavy metals into the biosphere from both natural and anthropogenic sources (Perelomov and Prinsky, 2003(1). This could be from industrial activities lead to substantial release of toxic metals into the environment. These heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000(2). Heavy metals, such as lead, copper, cadmium, chromium and mercury, are important environmental pollutants, particularly in areas with high anthropogenic pressure. Their presence in the atmosphere, soil and water, even in traces, can cause serious problems to all organisms. Heavy metal accumulation in soils is of concern in agricultural production due to the adverse effects on food quality (safety and marketability), crop growth (due to phytotoxicity) and environmental health (Augusto Costa and Pereira Duta, 2001(3).

Living organisms are exposed in nature to lead commonly in their ionized forms, which at different concentrations affect microbial population. This can have significant impact given that many microorganisms are essential parts of the decomposing food chain. The affected microbial population are likely to be replaced by same/other species that may be less efficient in organic matter decomposition, Nutrient recycling, soil formation etc. thereby putting a bridge to Agricultural sustenance / continuity (Yu 2005(4). Lead pollution affects a broad spectrum of species and its persistence in the environment is considered to be hazardous. It affects the human body organs and systems negatively especially the nervous system, (White et. al., 2007(5). It slows down photosynthetic processes, reduces essential nutrient and water absorption, retards plant growth and eventually plant death. Also, Grazing animals are directly affected by the consumption of forage and feed contaminated by air borne lead and somewhat indirectly by the uptake of lead through plant root which subsequently lead to reproductive failure and death (Casarett *et al*., 2007(6).

The metal ion toxicity is determined by many factors such as physio chemicals characters of metals ion including electro- negativity, reduction-oxidation potential, and etc. (Workentine et al., 2008(7). Chemical methods such as precipitation, oxidation or reduction have been widely used to remove metal ions from industrial waste water. Those methods are ineffective or expensive (Volesky, 1990(8). The activity of microorganisms is extended to environmental management, and microbes have superseded the conventional

techniques of remediation (Vidali, 2001(9). Biological methods such as biosorption and bioaccumulation provide promising alternative to chemical methods (Kapoor and Viraragharan, 1995(10). The mechanism by which microorganisms remove heavy metals can be divided into three categories; the first mechanism is the biosorption of metals ions on the cell surface, second intracellular uptake of metals ion and third chemical transformation of metal ions by microorganism (Pardo *et al*., 2003(11). Among the different technique employed for metals removal from multi elemental system, biosorption has been found to be highly selective (Knauer *et al.,* 1997(12).

Furthermore metal accumulating bacteria can be used to remove, concentrate and recover metals from industrial effluents (Malekzadeh *et al*., 2002(13) and Chowdhury *et al*., 2008(14)). The capacity of any biosorbent is mainly influenced by biomass characteristic, physiochemical properties of the target metals, and the micro environment of contact solution including pH, temperature and interaction with other ions (Chen and Wang 2007(15). Moreover once the toxic metals are adsorbed or transferred within organic materials they can be removed from waste water (Smith and Collins, 2007(16).

The present study has been able to show that microorganisms isolated from pig waste have the inherent capability of removing heavy metals from heavy metal-polluted soil. It implies that adverse effects of heavy metal on plants in heavy metal-polluted soil can be remedied using pig waste. This serves the double purpose of supplying nutrients to the plants while also removing the heavy metals from the soil.

2. MATERIALS AND METHODS

2.1. Sample preparation and isolation of lead-resistant *Bacillus*

Pig waste was collected using a clean polyethylene bag from the Department of Animal production in the School of Agriculture and Agricultural Technology (SAAT) of Federal University of Technology Owerri (F.U.T.O), lmo state, Nigeria. Two grams of the pig waste were homogenized in sterile water and serially diluted. Lead [$(PbNO_3)^2$] incorporated nutrient agar plates containing different concentrations (1, 10,100,500 µg/ml) of the lead salt were prepared and inoculated with 0.1 ml of the diluted samples. Incubation was done at 37^{o}C for 24 hours. Isolated colonies were purified by two subsequent single colony transfers. Pure colonies were specifically transferred into nutrient agar slants. The slants were incubated at 37°C for 18 - 24 h. These served as the stock cultures and were stored at 4°C in the refrigerator. The isolates were identified according to the method stipulated in Holt *et al*. (1994(17).

2.2. Preparation of stock solution of heavy metal salt

A weight of lead salt that gave 1 g of the heavy metal (metal without the salt) dissolved in 1000 ml of deionized water. It was left to stand for 30 min to obtain complete dissolution. This was followed by sterilization by membrane filtration.

2.3. Preparation of standard inoculum

A loopful of cells from the stock culture was inoculated into 100 ml sterile nutrient broth in triplicates and incubated at 37°C for 24 h with intermittent shaking. At the end of the incubation period, cells were harvested by centrifugation at 4000 rpm for 30 min and re-suspended in 100 ml sterile physiological saline. The total viable counts were carried out to estimate the number of viable organisms. During this process, the cultures were subjected to serial dilutions up to 10^6 dilutions. An aliquot (0.1 ml) from each dilution was inoculated by spread plate technique into freshly prepared nutrient agar plates, which were incubated at 37°C for 24 h. The dilutions producing between 30 - 300 colonies were chosen and served as inoculum for Percentage log survival test.

2.4. Percentage log survival test

Different concentrations of lead solution were prepared in deionized water to obtain 1.0, 10.0, 100.0 and 500.0 µg/ml. Ninety milliliters of each the different concentrations was put in 100 ml conical flask and inoculated with 10 ml of the standard culture with constant shaking. A control was set up with 90 ml of normal saline without toxicant and was inoculated with 10 ml of the standard culture. At exposure times of 0, 2, 4, 12, 24 h, 1 ml was aseptically withdrawn from each of the flasks for viable count using the spread plate technique. The percentage log survival of the isolate was calculated using the formula:

$$\text{Percentage log survival} = \frac{\log A}{\log B} \times 100$$

Where A = Count in toxicant concentration

B =Count in the control

2.5. Metal up take assay

The isolate was developed by growing in 100 ml of freshly prepared nutrient broth (pH 7.0) at 37^0C for 18-24hrs with constant shaking. Cells were harvested by centrifugation at 4000rpm for 30 min. they were washed thrice with sterile phosphate buffered saline and re-suspending in 100ml of deionized water. The viability of the cells was assessed by plating 0.1ml onto a nutrient agar plate. Stock solution of different concentrations (1.0, 10.0, 100, 500 µg/ml) of lead was prepared and adjusted to pH of 7.0 using 0.1 M sodium hydroxide and 0.1 M trioxonitrate (V) acid. From the various concentrations of the heavy metal salt, 40 ml were withdrawn using sterile pipette into duplicate set of 100 ml flask and inoculated with 10 ml of each of the standard inoculum. For the control, 40 ml of sterile normal saline was inoculated with 10 ml of the inoculum. All flasks were incubated at 25°C ± 2 for 24 h. At the end of the incubation period, cells were harvested by centrifugation at 4000 rpm for 30 min, washed thrice in sterile phosphate buffered saline, dried, weighed, digested and analyzed for heavy metal content using AAS.

2.6. STATISTICAL ANALYSES

Data obtained from this study were analyzed using a one-way analysis of variance (ANOVA) and values for P≤0.05 were considered statistically significant.

3. RESULT

The growth curve of the test organism relative to the control was calculated. This was done by measuring and plotting the absorbance as a function of time of incubation. The absorbance of the control after 24 hours of incubation was taken as the maximum growth of the test organism. The result is presented in figure 1. From the result, it was observed that the growth curve was concentration dependent. There was no significant effect of the lead on the growth curve of the organism when exposed to the toxicant up to the concentration of 100 µg/ml at P≤ 0.05. Conversely, there was a very high significant effect of the lead toxicant on the growth curve of the organism when exposed to 500 µg/ml concentrations after the incubation period. At this concentration, the organism entered the stationary phase after 16 hours of incubation.

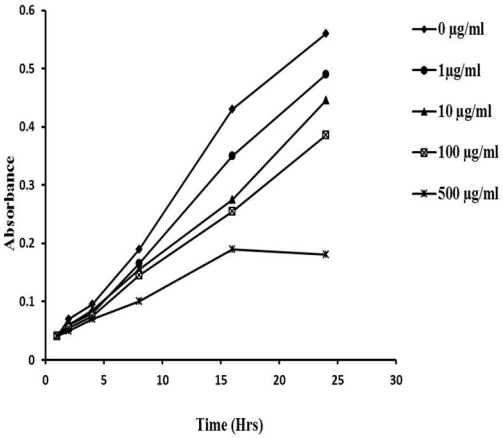

Figure 1. Growth curve of the test organism in different concentations of lead.

The result of the percentage log survival of the test organism in different concentrations of the lead toxicant and at different incubation times are presented in figure 2. The effect of lead concentrations on the percentage log survival of the test organism showed that at increasing concentrations of lead, the percentage log survival decreased with increase in time of exposure. At the initial hour of incubation, the test organism had 100% survival at all the lead concentrations. At subsequent hours of incubation, the survival rate of the test organism decreased significantly with increase in lead concentration and time of incubation (P≤0.05).

Dose response curve obtained from the plot of lead concentration (µg/ml) against the bioaccumulation of lead (mg/kg) by the test organism is presented in figure 3. The lead concentration correlated well with lead bioaccumulation with a very high R^2 value (R^2 = 0.9971). The bioaccumulation model gave a good linearization of the dose-response data. The equation of the curve is given as lead concentration (µg/ml) = 0.0725 lead bioaccumulation (mg/kg) + 2.3815. The result shows that the bioaccumulation increased significantly with increase in concentration of the lead toxicant with the highest

bioaccumulation observed in the test organism when exposed to 500 µg/ml concentrations.

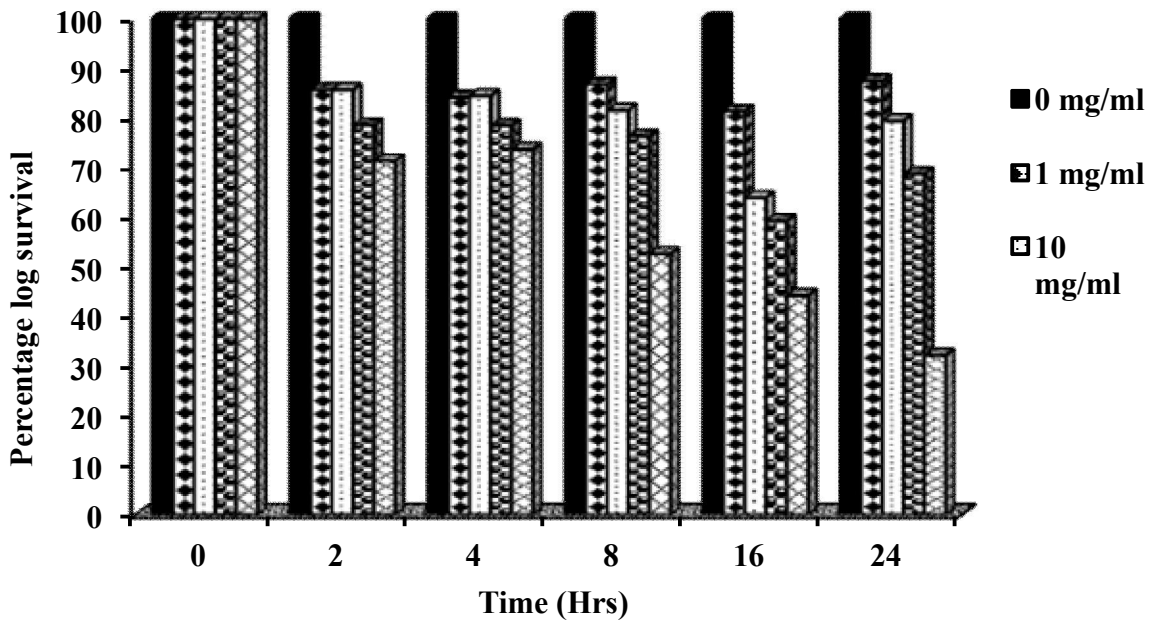

Figure 2. percentge log survival of the isolates in different concentrations of lead at different time intervals.

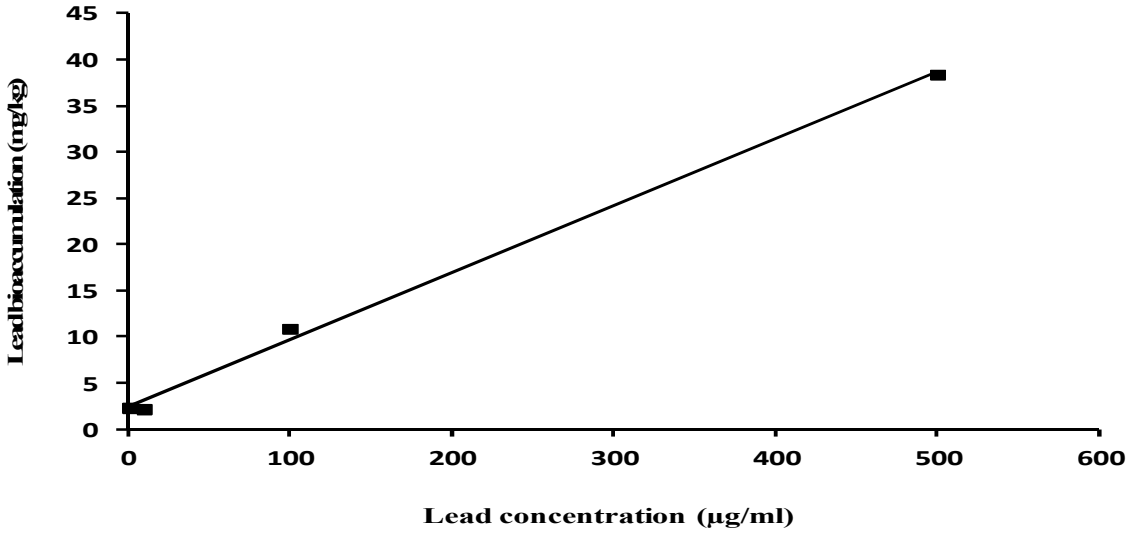

Figure 3. Lead bioaccumulation by *Pseudomonas* species in different concentrations of lead

4. DISCUSSION

Industrial activities led to substantial release of toxic metals into the environment. Heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000(2). Some metals including iron, zinc, copper and manganese are micronutrients used in the redox processes, regulation of osmotic pressure, enzymes cofactors and are also important in the maintenance of the protein structure (Vallee and Auld 1990(18). On the other hand metals including lead and cadmium do not play any known physiological role and\ are in fact toxic to cells. Lead reacts with the sulphydryl groups of protein and inhibits their function (Ron *et al.,* 1992(19). The metal ion toxicity is determined by many factors such as physio-chemicals characters of metals ion including electro- negativity, reduction-oxidation potential, etc. (Workentine *et al.,* 2008(7).

The results of the study showed that the *Pseudomonas* species is capable of surviving when exposed to various concentrations of lead salt within 24 hours exposure duration. This is in accordance with the works of Odokuma1 and Akponah (2010(20), Odokuma and Ijeomah (2003(21), Odokuma and Emedolu (2005(21). In their reports *Bacillus* sp. and *Aeromonas* sp. were shown to be resistant to the toxicity of heavy metals. The persistence of these isolates in the presence of the respective heavy metals may be as a result of the possession of heavy metal resistant plasmids (Odokuma and Oliwe, 2003). The spore forming ability of *Bacillus* sp. might also, have contributed to its ability to survive when exposed to the various concentrations of the heavy metal salt.

The result of the percentage log survival of the test organism in different concentrations of the lead toxicant and at different incubation times as presented in figure 2 revealed that at the initial hour of incubation, the test organism had 100% survival at all the lead concentrations. At subsequent hours of incubation, the survival rate of the test organism decreased significantly with increase in lead concentration and time of incubation ($P \leq 0.05$). This is in line with the works of Odokuma and Akponah, (2010) that showed that the percentage survival of their isolates decreased with increase in contact time as well as concentration when exposed to different concentrations of heavy metals. This shows that contact time is a very crucial factor in establishing the resistance of organisms to the toxic pressure of the metals.

At the initial hour of incubation, the test organism had 100 % survival in all the lead concentrations. At subsequent hours of incubation, the test organism had irregular rate of survival in the 1 µg/ml and 10 µg/ml concentrations respectively. When exposed to 100 µg/ml and 500 µg/ml, the rate of survival decreased with increase in the time of incubation. The effect of lead concentrations on the percentage log survival of the test organism showed that at high concentration lead, the percentage log survival decreased with increase in time of exposure. This is in line with the works of Odokuma1 and Akponah, (2010) that showed that the percentage survival of their isolates decreased with increase in contact time as well as concentration when exposed to different concentrations of heavy metals. This shows that contact time is a very crucial factor in establishing the resistance of organisms to the toxic pressure of the metals.

Dose response curve obtained from the plot of lead concentration (µg/ml) against the bioaccumulation of lead (mg/kg) by the test organism presented in figure 3 depicted that lead concentration correlated well with lead bioaccumulation with a very high R^2 value (R^2 = 0.9971). The bioaccumulation model gave a good linearization of the dose-response data. The equation of the curve is given as lead concentration (µg/ml) = 0.0725 lead bioaccumulation (mg/kg) + 2.3815. The result shows that the bioaccumulation increased significantly with increase in concentration of the lead toxicant with the highest bioaccumulation observed in the test organism when exposed to 500 µg/ml concentrations.

Bioaccumulation test carried out revealed that *Pseudomonas* species had an inherent capability to withstand the toxicity of lead and bioaccumulate the metal (Odokuma and Emedolu, 2005). Richard *et al.,* 2002 reported that Cu^{+2} and Pb^{+2} appear to bind to materials on the cell surface. Lead is precipitated in an insoluble form that is localized to the cell membrane or cell surface. Similar results were obtained by El-Hendawy (2009) which shows the localization of one or more metal to cell wall of *V. alginolyticus*. This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of bacterial cell wall absorb metal cations through various

mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or combination of such processes (Chojnacka *et al.,* 2005). Both dead and living cells adsorb metal ions (Ansari and Malik 2007).

Several principal sites of metal-complex formation in biological systems have been proposed (Vieira and Volesky, 2000). These processes involve a typical ion-exchange process where the metal ion is exchanged for a counter-ion attached to biomass. Bioleaching is a similar process where microbes dissolve the metals present in solid matrix into soluble form. Others include accumulation in the cell wall, carbohydrate or protein polyphosphate complexes, and complexion with carboxyl groups of the peptidoglycan in the cell wall. However, there are five basic mechanisms that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymatic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets. In the present study, it was observed that there was an increase in bioaccumulation with increase in the lead concentration. These observations suggest that metal uptake may involve diffusion phenomenon whereby, metal ions move from regions of high concentrations to low concentrations and the fact that the steeper the concentration gradient, the more raped is the movement of molecules or ions (Taylor et al., 1997) or any of the above-mentioned mechanisms. The high R^2 values obtained in the regression plot indicated that lead concentration was a strong determinant of the bacterial accumulation. The *Bacillus* species can be used, in the future, for heavy metals removal, immobilized on waste biomaterials. Input of heavy metals imposes a selective pressure that may favor the growth and activity of resistant/tolerant microbes. The development of a metal-resistant population in a contaminated soil can result from: (i) vertical gene transfer (reproduction), (ii) horizontal gene transfer (including transposons and broad host range plasmids), and (iii) selection pressures on spontaneous mutants (due to the presence of metals). Transposable elements carrying mercury resistance genes have been linked to the distribution of this trait in nature (Khosro *et al*, 2011).

The present study has been able to show that microorganisms isolated from pig waste have the inherent capability of removing heavy metals from heavy metal-polluted soil. It implies that adverse effects of heavy metal on plants in heavy metal-polluted soil can be remedied using pig waste. This serves the double purpose of supplying nutrients to the plants while also removing the heavy metals from the soil.

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REFERENCES

1. Augusto Costa AC, Pereira Duta F. Bioaccumulation of copper, zinc, cadmium and lead by *Bacillus* SP*., Bacillus cereus, Bacillus speaerecus* and *Bacillus subtillus. Braz. J. Microbiol.* 2001; 32 (1):32-50.
2. Boopathy, R. (2000). Factors limiting bioremediation technologies. *Bioresource Technology* 74: 63-67.
3. Boopathy, R. (2000). Factors limiting bioremediation technologies. *Bioresource Technology* 74: 63-67.
4. Casarett LJ, Klaassen CD. and Doull J. Biological and health effects of lead pollution. *Wat. Sci. Technol.* 2007; 29:152-161.
5. Chen, C. and Wang, J. (2007). Correlation metal ionic characteristics with biosorption capacity using QSAR model. Chemosphere. 69:1610-1616.
6. Chowdhury, S.; Mishra, M.; Adarsh, V. K.; Mukherje, A.; Thakur, A. R. and Chadhuri, S. R. (2008). Novel metal accumulator and protease secretor microbes from east Calcutta wetland. American Journal of biochemistry and biotechnology 4(3): 255-264.
7. Holt JG, Krieg NR, Sneath PHA, Staley JT, and Williams T. Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Wilkins, Baltimore, USA. 1994; 1-12.
8. Kapoor, A. and Viraragharan, T. (1995). Fungal biosorption - an alternative treatment option for heavy metals bearing waste water. Bioresource technology, 53:195-206.
9. Knauer, M.F.; Kridel, S.J.; Hawley, S.B. and Knauer, D.J. (1997). The efficient catabolism of thrombin-protease nexin 1 complex is a synergistic mechanism that requires both the LDL receptor-related protein and cell surface heparins. *J. Biol. Chem.* 272:29039–29045.
10. Malekzadeh, F.; Farazmand, A.; Ghafourian, H.; Shahamat, M.; Levin, M. and Colwell, R. R. (2002). Uranium bioaccumulation by a

bacterium isolated from electroplating effluent. World J. Microbiol. Biotechnol 18(4): 295-302.
11. Odokuma LO, Akponah E. Effect of nutrient supplementation on biodegradation and metal uptake by three bacteria in crude oil impacted fresh and brackish waters of the Niger Delta. *J. Cell Animal Biol*. 2010; 4 (1): 1-18.
12. Odokuma LO, Emedolu SN. Bacterial sorbents of heavy metals associated with two Nigerian Crude Oils. *Glob. J. Pur. Appl. Sci*. 2005; 11(3): 343-351.
13. Pardo, R.; Herguedas, M. and Barrado, E. (2003). Biosorption of cadmium, copper, lead and zinc by inactive biomass of *Pseudomonas putida. Analytical and Bioanalytical chemistry*. 376:26-32.
14. Perelomov LV, Prinsky DI (2003). Manganese, lead and Zinc compunds in Gray forest soils of the central Russian Upland, Eurasian. *Soil Sci*. 6: 610-618.
15. Richard, W.; Glenn, D. Krumholz; Matthew, S. Chval and Louis, S. Tisa (2002). Heavy metal resistance pattern of *Frankia* strains. *Applied and environ. Microbiology*. 68:923-927.
16. Ron, E.Z.; Minz, D.; Finkelstein, N. and Rosecnberg, E. (1992). Introduction of bacteria with cadmium. *Biodegradation* 3: 161-171.
17. Smith, J.L. and Colllins, H.P. (2007). Management of organisms and their processes in soils. In Soil Microbiology, ecology and biochem., ed by Eldor A.P.. Elsevier Inc., Bulington, USA pp.389-430.
18. Vallee, B.L. and Auld, D.S. (1990). Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29: 5647-5659.
19. Vidali, M. (2001). Bioremediation. An overview. *Pure Applied Chem*. 73(7):1163-1172.
20. Volesky, B. (1990). In: Volesky B. (Ed) Biosorption of heavy metals. CRC press Boca Raton. Florida.
21. White LO, Cory – Shechta DA, Giblet ME, Tiffany J, CastigIioni E, Zawia NH, Virgilian M, Ross-George A, and Lesley SM. New and evolving concept of the Neurotoxicology of Lead. *Indian J. Med. Res*. 2007; 225(1): 1-27.
22. Workentine, M. L.; Harrison, J. J.; Stenroos, P. U.; Ceri, H. and Turner, R. J. (2008). *Pseudomonas fluorescens* view of the periodic table. *Environ. Microbiol*. 10:238-250.
23. Workentine, M. L.; Harrison, J. J.; Stenroos, P.U.; Ceri, H. and Turner, R. J. (2008). *Pseudomonas fluorescens* view of the periodic table. Environ. Microbiol. 10:238-250.
24. Yu MH. Soil and water pollution: Environmental metals and metalloids. *Environ. health persp*. 2005; 115(3):472-82.

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