Journal of Research in Biology

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Bioaccumulation of lead by *Bacillus* species isolated from pig waste

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ABSTRACT:

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. Living organisms are exposed in nature to lead commonly in their ionized forms, which at different concentrations affect microbial population. Microorganisms are known to interact with heavy metals through a number of mechanisms including intracellular accumulation. *Bacillus* 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, *Bacillus* species isolated from pig waste shows a promise for its use in bioremediation of lead polluted environments. 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.

Keywords:
Bioaccumulation, lead, *Bacillus* species.

Article Citation:
Akujobi CO, Odu NN and Okorondu SI.
Bioaccumulation of lead by *Bacillus* species isolated from pig waste.
Journal of research in Biology (2012) 2: 083-089

Dates:
Received: 02 Jan 2012 /**Accepted:** 11 Jan 2012 /**Published:** 07 Feb 2012

INTRODUCTION

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). The mobilization of heavy metals into the biosphere by human activity has become an important process in the geochemical cycling of these metals. This is acutely evident in urban areas where various stationary and mobile sources release large quantities of heavy metals into the atmosphere and soil, exceeding the natural emission rates (Da Costa, 1999).

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). 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). 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).

Heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000). These metals enter the human body mainly through two routes namely: inhalation and ingestion, and with ingestion being the main route of exposure to these elements in human population. Heavy metals intake by human populations through the food chain has been reported in many countries with this problem receiving increasing attention from the public as well as governmental agencies, particularly in developing countries (Augusto Costa and Pereira Duta, 2001).

Contaminated sites can be a preferential source of those microorganisms which represent, therefore, important material for both study and applications of bioremediation for differential targets (Malik, 2004). The term bioremediation for heavy metals may seem improper, since no process can degrade and thus eliminate inorganic elements (Barkay and Schaefer, 2001); nevertheless in some cases their immobilization, performed by microorganisms, may be the only feasible means to protect groundwater and food-chain from contaminations. In these cases remedial goals can be achieved in different ways: the precipitation, and thus the immobilization, through different biological processes of inorganic contaminants, the concentration and then reduction in volume of contaminated matrices and the compartmentalization of metals to a part of the environment in which their toxicity is reduced. The importance of the bacterial effects in the removal of heavy metals is supported by many studies and Fein (2000) suggests the incorporation of bacteria in models of water–rocks interaction and contaminants transport. The measures evolved by microorganisms to respond to heavy metal stress have been reviewed (Nies, 1992, 1999; Ji and Silver, 1995; Nies and Silver, 1995) and the main processes are bioaccumulation, enzymatic reduction and complexation. Bioaccumulation can occur either by metabolism-independent (passive) biosorption or by intracellular, metabolism-dependent (active) uptake (Ledin, 2000).

The aim of this study is to isolate and characterize bacteria from pig waste, to study the heavy metals resistance pattern and the bioaccumulation potential of the selected organism.

MATERIALS AND METHODS

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 ^oC 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. Pure bacterial isolates were characterized and identified using criteria as in Holt *et al*. (1994).

Preparation of stock solution of heavy metal salt

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

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.

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 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:

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

Where A = Count in toxicant concentration

B =Count in the control

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 were 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.

Statistical Analysis

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.

RESULT AND DISCUSSION

The growth curve of the test organism relative to the control was calculated. The absorbance of the control after 24 hours of incubation was taken as the maximum growth of the test organism and was assigned the value of 100%. Based on this, the percentage growth of the test organism in the different concentrations of the lead toxicant after 24 hours of incubation was calculated. 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 1 µg/ml and 10 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 100 µg/ml and 500 µg/ml concentrations after the incubation period. The effect was so pronounced that the organism was unable to enter into the logarithmic phase of growth when exposed to these lead concentrations. The results of the study showed that the *Bacillus* 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 Odokuma and Akponah (2010), Odokuma and Ijeomah (2003), Odokuma and Emedolu (2005). 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 are presented in **Figure 2.** 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

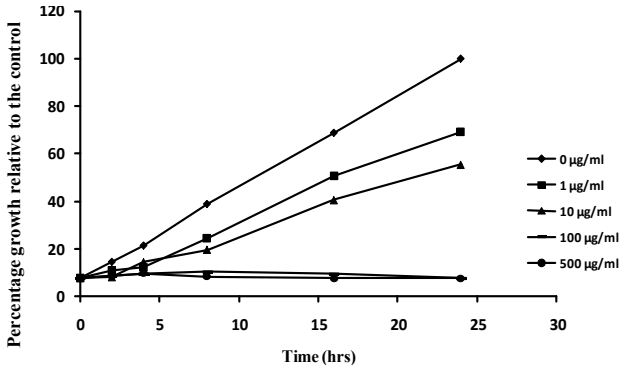

Fig.1. The growth curve of the test Organism relative to the control

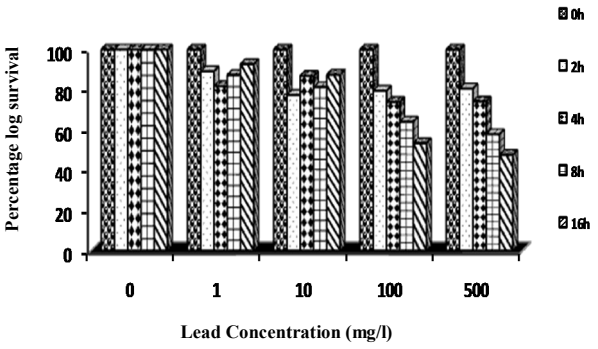

Fig. 2. Percentage log survival of the isolates in different concentrations of lead at different time intervals.

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 Odokuma and Akponah, (2010) and Buikema et al., (1982) 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 showed 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 is presented in **Figure 3.** The lead concentration correlated well with lead bioaccumulation with a very high R^2 value (R^2 = 0.9945). The bioaccumulation model gave a good linearization of the dose-response data. The equation of the curve is given as lead concentration (µg/ml) = 1.6394 lead bioaccumulation (mg/kg) + 9.2257. The result showed 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 *Bacillus* species had an inherent capability to withstand

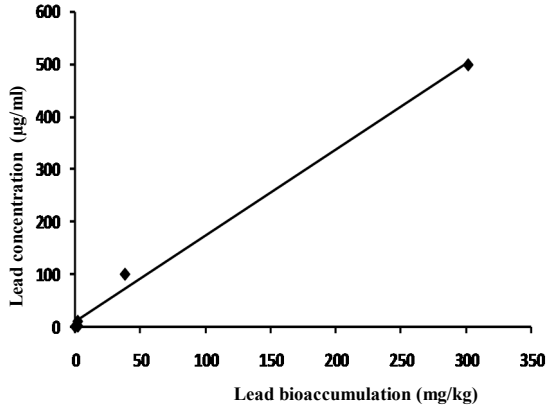

Fig.3. Lead bioaccumulation by Bacillus species in different concentrations of lead

the toxicity of lead and bioaccumulate the metal (Odokuma and Emedolu, 2005). 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 suggested 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 impose 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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