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RESEARCH ARTICLE

BIOACCUMULATION OF SOME HEAVY METALS BY METAL RESISTANT *BACILLUS THURINGIENSIS* ISOLATED FROM SOIL IN BASRA GOVERNORATE- IRAQ

[1]Raghad S. Jaafar, [1]Amin A. Al-Sulami, [2*]Asaad M.R. Al-Taee and [3]Faris J. Aldoghachi

[1]College of Education for Pure Science, Basra University, Basra-Iraq
[2]Marine Science Center, Basra University, Basra-Iraq
[3]College of Science, Basra University, Basra-Iraq

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ABSTRACT

In the present study heavy metal resistant bacteria were isolated from soil collected from the FaoUm-Qasr district in Basra governorate, South of Iraq. On the basis of morphological, biochemical, and 16S rRNA gene sequencing and phylogeny analysis, the isolate was authentically identified as *Bacillus thuringiensis*. The minimal inhibitory concentration (MIC) of the isolate against cadmium (Cd) and lead (Pb) was determined on solid medium. *B. thuringiensis* showed significant resistance to high concentrations of Pb of 1800 mgl^{-1} and 50 $mgl^{-1}$for Cd. The bioaccumulation capabilities of *B. thuringiensis* for Cd and Pb were monitored at different ion concentrations andcontact times. The transmission electron microscope study confirmed the accumulation of (Cd) and (Pb) by *B. Thuringiensis* causing morphological changes, including speculation.

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INTRODUCTION

Heavy metals have a major problem to human health and environmental issues due to the high incidence as a contaminant, low solubility in biota and classification of various heavy metals as carcinogensand mutagens (Rani and Goel, 2009). Heavy metals can produce harmful effects on human health when they are taken up in amounts that cannot be processed by the organism. In addition, these metals cannot be degraded to harmless products and hence persist in the environment indefinitely. For these reasons several methods have been designed for the treatment and removal of heavy metals in contaminated site (Akhtar *et al*., 2013). Physico-chemical methods have been used, such as electrochemical treatment, ion exchange, precipitation reverse osmosis, evaporation, and sorption (Congeevarama *et al.,* 2007). But these are economically expensive, incomplete metal removal, requiring of higher reagent energy, and generating of toxic sludge.

Corresponding author:Asaad M.R. Al-Taee,
Marine Science Center, Basra University, Basra-Iraq.

In some cases it may change the environment properties and spread contaminants from one to another would also increase the consumption of non renewable resources (Chojnaka, 2010). Bioremediation is a natural process which depends on bacteria, fungi, and plants to change pollutants as these organisms carry out their normal life functions. These organisms have the ability of using chemical contaminants as an energy source in their metabolic processes. Thus, bioremediation affords a substitute to destroy or reduce the harmful contaminants through biological activity and this method is cost effective (Salem *et al*., 2012).Bioaccumulation is the active method of metal accumulation by living cells. The capacity of living cells to remove metal ions from environment is influenced by environmental growth conditions, as temperature, pH and biomass concentrations (Abd El-Raheem *et al*., 2013). TEM is a useful technique that can help to localize and to identify metals deposited within or around microbial cells. Identification of the site of accumulation is important as it can give clues to the biochemical mechanisms driving metal accumulation. Biological materials which are largely composed of light elements such as C, N, H, O, P, and S, do not deflect the electron beam to the same degree. Thus, it is possible to

visualize metals against the faint image of a bacteria cell (Lloyd and Macaskie, 2002). *Bacillus thuringiensis* has multiple heavy metal resistant phenotypes, and considerable cell surface affinity for metal cations and the ability to express a variety of extracellular digestive enzymes (Amer, 1996). These advantageous characteristics provide promising prospects for future environmental protection studies. It seems likely that, this bacterium can be tailored for efficient growth in metal-polluted environment supplemented with inexpensive nutrients, which might include by-products and wastes, resulting in bioremediation with simultaneous secretion of commercial extracellular enzymes (El-Helow *et al*., 2000). The present study, aims to isolating *B. thuringiensis* from Basra, south of Iraq, and evaluating metals bioaccumulation ability, and also studying the effect of metals initial concentration, contact times, and determine the cellular localization of accumulated metals within this bacterium by using Transmission electron microscope.

MATERIALS AND METHODS

Isolation of bacteria

Three soil samples (30 gm each) were collected from Fao district, 90 Km south of Basra city- Iraq during January 2013. The samples were collected using a sterile plastic bag and transferred within 2hto laboratory for analysis. One gram of air dried soil sample was serially diluted using distilled water and spread over nutrient agar. The plates were incubated at 30°C for 24 h.

Bacterial characterization

Properties of the bacteria included gram stain, citrate utilization, indole production, methyl red, nitrate reduction, Voges Proskauer, catalase, dextrose, mannitol and sucrose utilization, starch hydrolysis, and gelatin liquefaction tests were determined according to Sneath *et al*. (1986).

16S rRNA based identification

The isolates were identified by sequencing of the 16S rRNA gene. To determine the identity of bacterial isolates, the amplified 16S rRNA gene PCR products obtained from total genomic DNA using primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 72.1492R (5′-GGTTACCTTGTTACGACTT-3′), (Lane *et al*., 1985) were sequenced commercially. DNA sequences obtained were compared to sequences available online in a GenBank database (http://www.ncbi.nlm.nih.gov). Homology search was performed using Bioinformatics tools available online, BLASTn www.ncbi.nlm.nih.gov/BLA (Altschul *et al*., 1997).

Determination of minimal inhibitory concentrations (MIC) for Cd and Pb

The MIC of Cd and Pb of bacteria were determined by disc diffusion method. The concentrations of Cd and Pb were between 40 - 2500 mgl^{-1}. Filter paper disks were saturated with heavy metals for 30 min, and then placed on nutrient agar plates and incubated for 24h at 30ºC.$CdCl_2$ and Pb $(NO_3)_2$ were used to prepare mother solution of these metals in sterile distilled water and were used in various concentrations. The lowest concentrations of Cd and Pb that completely prevented growth of each bacterium were considered as the MIC (Sethuraman and Kumar, 2011).

Bioaccumulation of heavy metals by bacteria

Bacteria were grown in LB broth containing different concentrations of lead of 5, 10, 25 and 50 mgl^{-1} and for cadmium 10, 20, 50 and 100 mgl^{-1} for 2, 4, 6, 24 and 48 h then incubated at 30ºC in a shaker incubator at 150 rpm. Three replicates for each concentration have been done, and one as a control. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min and suspended in 1 ml of distilled water, oven – dried and weighted. Metal concentrations were measured by atomic absorption spectrophotometer. Control was represented by the same microbial culture without heavy metals. Each metals concentration is measured with two replicates (Sprocati *et al*., 2006).

Transmission electron microscope

This work, done in the Electron Microscope Laboratory, Institute of Bioscience, University Putra MalaysiaBy centrifuging samples broth culture for 10 min at 3000 rpm, and decanting supernatant, fixing pellet with 4% gutaraldehyde for 4h at 4°C and centrifuged again, decanted fixative and adding appropriate quantity animal serum to submerge sample, and allowed serum to clot. It was washed three times with 0.1M Cacodylate buffer for 10 min. and Posted fix in 1% Osmium tetrroxide for 2 hr at 4°C. Also, it is washed again three times with 0.1M Cacodylate buffer for 10 min. Dehydrating in series of acetone of 35, 50, 75, 95, and 100% for 10, 10,10, 10 and 15 min respectively.

Finally, we make infiltration of the specimen with acetone and resin

Acetone		Resin	Time
1	:	1	1h
1	:	3	2h
		100% resin	Overnight
		100% resin	2h

Embedding: specimens were placed into beam capsule filled with resin.Polymerization: polymerize in oven at 60 °C for 24-48h.Make ultracectioning, by choosing an area of interest, then cut for ultrathin section, selected the silver section, picked up a section with a grid, then drying with filter paper. Finally the section stained with Uranyl acetate for 15 min, and washed double distills water. Lead stained for 10 min, and washed double in distilled water.

RESULTS AND DISCUSSION

Characterization and molecular identification of isolated bacteria

The selected bacterium was characterized and identified by using standard morphological, physiological and biochemical tests (Table 1). It was presumptively identified as *Bacillus* sp. The sequence of 16S rRNA gene of this bacterium was submitted to Blastn {database 16S ribosomal RNA sequences

(Bacteria and Archaea) Megablast} http://www.ncbi.nlm.nih.gov/blast. It indicated a close genetic relatedness of this bacterium with the rDNA sequence of *Bacillus thuringiensis* (Oves *et al*., 2013).

Minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the heavy metals that completely inhibited bacterial growth (Froidevaux *et al*., 2001) *B. thuringiensis* showed significant resistance to high concentrations of Pb, the MIC was 1800 mgl^{-1}, while to cadmium was 50mgl^{-1}. This result is higher than those of Oves *et al*., (2013) who observed that, *B. thuringiensis* strain OSM29 could survive at 1500mgl^{-1} of lead, but less in the case of cadmium. This reflects a strain difference and this result is supported by the fact that cadmium is one of the most powerful biological inhibitors, so the growth of bacteria was inhibited with cadmium, even at low concentrations (Qing *et al*., 2007).

Bioaccumulation

The potentiality of Pb accumulation by *B. thuringiensis* has been illustrated in Fig (1). The accumulation ability of this bacterium changes with the change of incubation period and concentrations. So, the highest accumulation was 11.95 mg/g at concentration 50mg/l for24h, while the lowest was 1.17 mgg^{-1} at a concentration 5mg/l for 2 h. Fig (2) Shows the accumulation rate of Cd by *B thuringiensis.*

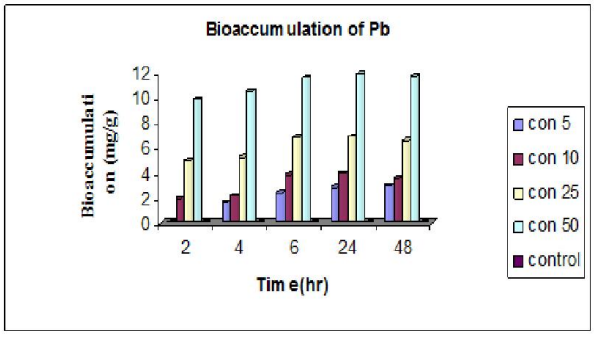

Figure 1. Bioaccumulation (mg/g) of Pb by *B. thuringiensis* during different incubation periods and different concentrations. LSD (0.0049)

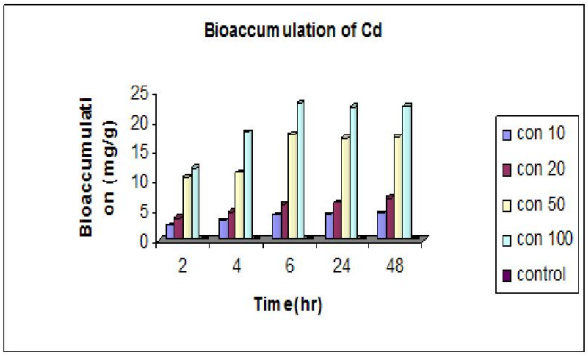

Figure 2. Bioaccumulation (mg/g) of Cd by *B. thuringiensis* during different incubation periods and different concentrations. LSD (0.0341)

Table 1. Morphological and biochemical characteristics of *B. thuringiensis*

Tests	*Characteristics observed*
Oxidase test	-
Catalase test	+
Indol formation	+
Nitrate reduction	-
Voges Proskauer	+
Citrate utilization	+
Methyl red	+
Carbohydrate utilization	
Sucrose	+
D- glucose	+
Mannitol	-
Hydrolysis of	-
Starch	+
Gelatin	+

"+"and "-" indicate positive and negative reactions, respectively

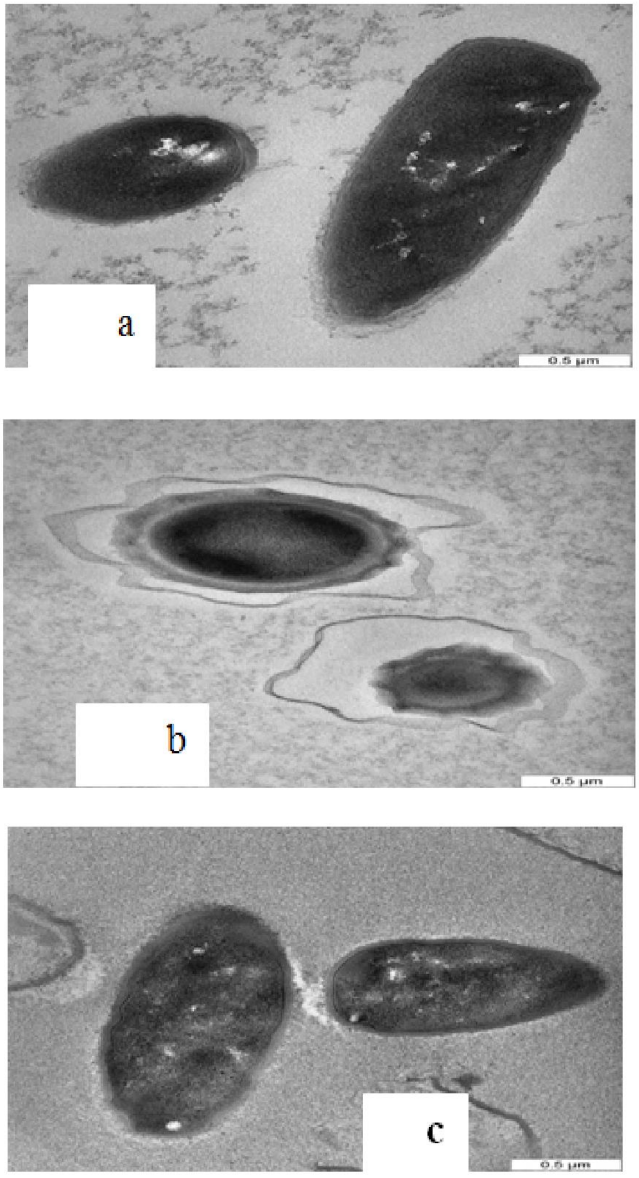

Figure 18. Transmission electron micrographs of *B. thuringiensis*, a: control, b: treated with 50mg/l of Cd for 24h, c: Treated with 50mg/l of Pb for 24h (Scale of bar 0.5µ).

The accumulation increased with the increase of both of incubation period and concentrations. The highest accumulation was 22.70 mgg^{-1} at a concentration 100mg/l for 48h.The lowest was 2.50mgg^{-1} at concentration 10mgl^{-1} for 2h. The analysis of variance of bioaccumulation of Pb and Cd between time and concentration was significant (P>0.05) in all treatments from LSD value. In this study, *B. thuringiensis* exhibited a high rate of metal accumulation, and these results agree withthe other results reported that, the strains isolated from polluted soil showed the capacity of high accumulation (Ozdemir *et al*., 2004, Xia *et al*., 2003).Azabou *et al*. (2007) demonstrated that, bacterial populations in metal polluted environments adapted to the conditions and would be suitable for remediation purposes. A similar study had been conducted by Issazadeh *et al*. (2011) where there were 1.1 $molg^{-1}$ biomass for bioaccumulation of lead by *B. licheniformis*.

And in related to effect of metals concentration and time on accumulation rate by this bacterium, the results of the present study showed that, the high accumulation rate of Pb was 11.95 mgg^{-1} in a concentration 50mgl^{-1} at 24h, and for Cd, it was 23.2 mgg^{-1} in a concentration 100 mgl^{-1} at 6h. From these results we can conclude that these bacteria have high ability to accumulate Cd than Pb, and the high accumulation occurs with high metals concentration and after a long period of exposure time.This difference in the uptake of these two metals by this bacterium as appears in the results may be due to the difference in mechanisms by which the bacteria can tolerate different heavy metals (El- Shanshoury *et al*., 2013). Also the results showed that, the time required for high accumulation differs for two metals and cell age is considered as an important factor that affects metal accumulation.This agrees with El-Shanshoury *et al.* (2013) where he reported that the maximum uptake by *Enterobacter* sp. for Cd^{+2}, Cu^{+2}, and Zn^{+2} occurred after 24 h. However, 18 and 48 h were optimum for Co^{+2}, and Pb^{+2} uptakes respectively. With theeffect of the release of metals concentration on the accumulation, the results showed that, the accumulation rate increased with increasing metals concentration; then start decreasing slightly after a specific concentration, and these results agree with the results reported by Malik (2004) who, reported that the accumulation of Zinc and copper of Zinc resistant bacteria increased progressively when the concentration of Zinc in medium increase from 0.4 to 1.6Mm. The explanation of these results depends on gradation in concentration and its importance in metal accumulation, is that the higher metals gradient the more rapid movement of ions and the decrease in accumulation which occur after that can be explained as a result of saturated bacteria with metals after specific concentration or due to the toxicity of these metals (Al-Garni, 2005).

Transmission electron microscope

Two types of samples of the same bacterium were selected for TEM ND one grown in medium without metals as a control (Fig. 3 a) and cells exposed to 50 mgl^{-1} of Cd and Pb for 24h (Fig 3 b, c). Metals occurring inside cells will thus be present as dark entities or spots as can be seen from the image (Fig. 3 b and c). Metals were mostly seen on the cell membrane and inside the cell, in addition to the morphological changes in the cells as well as spore formation.Results indicated that the cell surface morphology showed considerably changed after metals exposure. The cellular localization of the metals bound by the cells of three types of bacteria was located mainly within the cell membrane. However, some intracellular metal accumulates were also identified in the cytoplasm of the bacterial cells. These results agree with Merroun *et al*. (005), who reported that, the cellular localization of the uranium bound by the cells of three types of *Acidithiobacillus. ferrooxidans* was studied using TEM. Also, El-Helow *et al*. (2000) reported that, cell surfaces of cultures treated with cadmium chloride tended to be rough, suggesting that the cell increased its surface to improve the interaction of toxic substances with the cell surface.

Also, these results agree with Singh *et al*., (2013), who reported that, cell surface morphological changes in *Cryptococcus* sp after exposure to heavy metals, and which could be observed by the presence of shrunken and distorted cell wall in the presence of Cd and depressions in the presence of Pb and Zn.Secretion of extracellular polymeric substance by *Desulfovibrio desulfuricans* during biosorption of Zn and Cu was reported to modify its cell surface morphology (Chen et al., 2000).Similarly, El-Meleigy *et al*. (2013) reported that high dark dense cytoplasm due to Co^{+2} precipitation is partially emptied with a very thick cell wall; changing in the morphology of vegetative cells of *B. firmus* and *B. subtilis.* Results showed that this bacterium when exposed to 50 mgl^{-1} of cadmium formed spore which contributed to its ability to survive under such condition. These results agree with the results reported by Odokuma and Emedolu (2005), whom showed that, the *Bacillus* sp. resistant to the toxicity of heavy metals and the persistence of these bacteria in the presence of the respective heavy metals may be as a result of the spore forming ability under heavy metals stress condition. Sahin and O ¨ ztu ¨rk (2005) reported that, the *B. thuringiensis* has the unique ability of producing spore which is thought to effect its accumulation abilities compared to those cell forms that are vegetative only. Shukla *et al*. (2008) reported that *CyanobacteriumAnabaena doliolum* forming spore in response to Ni stress.

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