Bioremediation of soil

This book contain different studies in bioremediation of heavy metals using different species of bacteria which isolated from soil of Basra city south of Iraq. these bacteria showed excellent bioremediation ability through two routes, bioaccumulation and biosoption which in turn emphasis using advance technique such as TEM, FTIR, and , XRD. Finally from the results we can recommended to use these bacteria in remediate heavy metals in situ after increase their biomass using bioreactor.

Dr.Raghad Shubbar Jaafar specialist in bacteriology particularly in bioremediation

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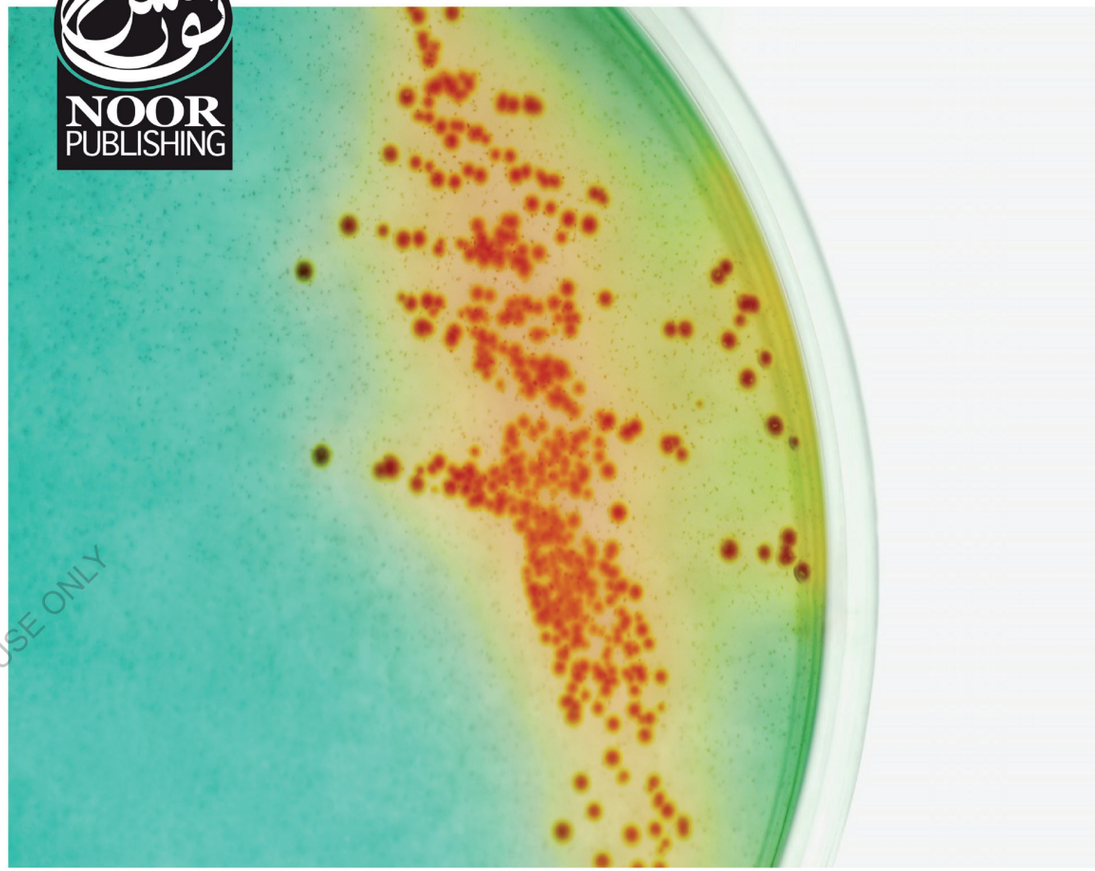

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RESEARCH ARTICLE

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

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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 tetroxide 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.*

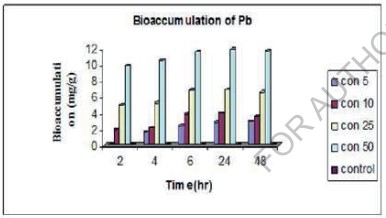

Figure 1. Bioaccumulation (mg/g) of Pb by *B. thuringiensis* during different incubation periods and different concentrations. LSD (0.0049)

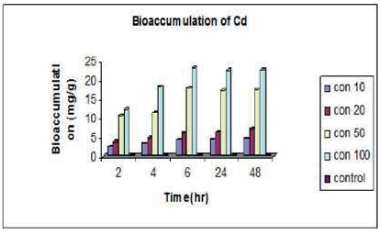

Figure 2. Bioaccumulation (mg/g) of Cd by *B. thuringiensis* during different incubation periods and different concentrations. LSD (0.0341)

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

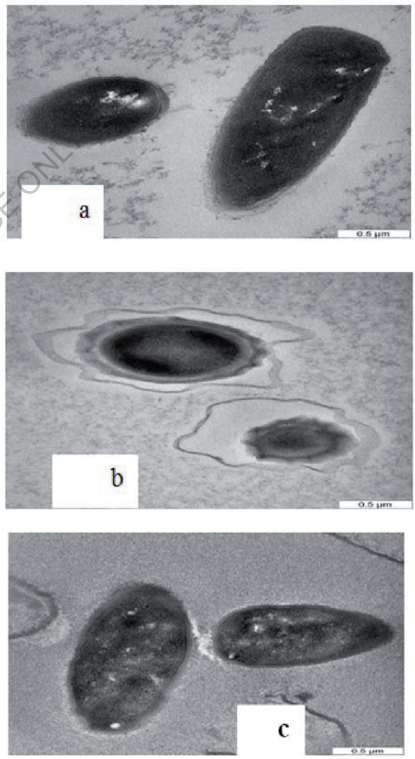

Figure 18. Transmission electron micrographs of *B. thuringiensis*, a: control, b: treated with 50mg/l of Cd for 24h, c: Treated with 50mg/l of Pb for 24h (Scale of bar 0.5µ).

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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Full Length Research Paper

Bioaccumulation of cadmium and lead by *Shewanella oneidensis* isolated from soil in Basra governorate, Iraq

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In the present study heavy metals resistant bacteria were isolated from soil collected from Al-Zubair district in Basra governorate south of Iraq. On the basis of morphological, biochemical, 16S rRNA gene sequencing and phylogeny analysis, the isolates were authentically identified as *Shewanella oneidensis* in addition to *Bacillus thuringiensis* and *Deinococcus radiodurans*. The minimal inhibitory concentration (MIC) of isolates against cadmium (Cd) and lead (Pb) was determined on solid medium. *S. oneidensis* showed significant resistance to high concentrations of Cd (1000 mgl^{-1}) and Pb (700 mgl^{-1}). The bioaccumilation capabilities of *S. oneidensis* for Cd and Pb were monitored at different ion concentrations and contact times. The transmission electron microscope (TEM) study confirmed the accumulation of Cd and Pb by *S. oneindensis* causing morphological changes.

Key words: *Shewanella oneidensis*, bioaccumulation, minimal inhibitory concentration, heavy metals, transmission electron microscope.

INTRODUCTION

Heavy metals play an important role in the metabolic processes of the biota, some of them are essential for organisms as micronutrients (cobalt, chromium, nickel, iron, manganese and zinc). They are involved in redox processes, to stabilize molecules through electrostatic interactions, as catalysts in enzymatic reactions, and regulating the osmotic balance. On the other hand, cadmium, mercury, lead, have no biological role and are harmful to the organisms even at very low concentration. However, at high levels, both of the essential and non-essential metals become toxic to the organisms (Rathnayake et al., 2010).

Cadmium is widespread and one of the most toxic soil contaminants released by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tang et al., 2006). Cadmium is poisonous to plants, animals, and humans (Gupta and Gupta, 1998) and is listed as one of the 126 priority contaminants by the USEPA and as a human carcinogen by the International Agency for Research on Cancer (IARC, 1994). Thus cadmium

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pollution is presently attracting more attention from environmentalists worldwide.

Lead (II) is a heavy metal poison which forms complexes with oxo-groups in enzymes to affect nearly all steps in the process of hemoglobin synthesis and porphyrin metabolism. Toxic levels of Pb (II) in man have been associated with encephalopathy appropriations and mental delay (Ademorati, 1996). Conventional physico-chemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis evaporation and sorption (Kadirvelu et al., 2001, 2002) have been used for removing heavy metals but are economically expensive and have disadvantages. Bioremediation is a natural process which depends on bacteria, fungi and plants to altering pollutants as these organisms perform their normal life functions. These organisms have the ability of exploiting chemical contaminants as an energy source in their metabolic processes. Therefore, bioremediation affords alternative tool to destroy or reduce the risky contaminants through biological activity with an effective cost (Salem et al., 2012).

Microbial populations in metal polluted environments become metals resistant (Prasenjit and Sumathi, 2005) so the response of microorganisms towards toxic heavy metals is of importance in view of the interest in the reclamation of polluted sites (Shankar et al., 2007). Microorganisms uptake metals either actively (bioaccumulation) and/or passively (biosorption) (Shumate and Strandberg, 1985; Anders and Hubert, 1992; Hussein et al., 2004). 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 bacterial cell (Lloyd and Macaskie, 2002).

The present study, aims at isolating *S. oneindensis* from Basra soil, 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 g each) were collected from AL-Zubair district west of Basra city- Iraq during January 2013. The samples were collected using a sterile plastic bag and transferred within 2 h to laboratory for analysis. One gram of air dried soil sample was serially diluted using sterilized 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).

S16 rRNA gene based identification

The isolates were identified by sequencing of the 16S rRNA gene. To determine the identification 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 Pb and Cd

The minimum inhibitory concentration (MIC) of Cd and Pb of bacteria were determined by disc diffusion method. The concentrations of Cd and Pb were between 40 to 2500 mgl^{-1}. Filter paper discs were saturated with heavy metals for 30 min, and then placed on nutrient agar plates and incubated for 24 h at 30°C. Pb $(NO_3)_2$ and $CdCl_2$ 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 5, 10, 25 and 50 mgl^{-1} of lead and for cadmium 10, 20, 50 and 100 mgl^{-1} then incubated for 2, 4, 6, 24 and 48 h at 30°C 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 at 80°C for 1 h 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

By centrifuging samples broth culture for 10 min at 3000 rpm, and decanting the supernatant, fixing pellet with 4% gutaraldehyde for 4 h at 4°C and centrifuged again, decanting fixative and adding an appropriate quantity animal serum to submerge sample, and allowing serum to clot. It was washed three times with 0.1 M Cacodylate buffer for 10 min. and Posted fix in 1% Osmium tetroxide for 2 h at 4°C. Also, it was washed again three times with 0.1 M Cacodylate buffer for 10 min. Dehydrating in series of

Table 1. Biochemical characteristics of *S. oneidensis* isolate from soils.

Tests	Characteristics observed
Oxidase test	+
Catalase test	+
Indole formation	-
Nitrate reduction	-
Production of H_2S	+
Gelatin liquefaction	+
Fermentation of	
Sucrose	+
Fructose	+
D-glucose	+

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

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	1 h
1	: 3	2 h
100%		Overnight
100%		2 h

Embedding: Specimens were placed into beam capsule filled with resin. Polymerization: polymerized in oven at 60°C for 24 h. Make ultrasectioning, by choosing an area of interest, then cut for ultrathin section, selecting the silver section, picking up a section with a grid, then drying with filter paper. Finally staining with Uranyl acetate for 15 min, and washed double distills water. Lead stained for 10 min, and washed twice in distilled water. This work was done at the Electron Microscope Laboratory Institute of Bioscience, University Putra Malaysia.

RESULTS AND DISCUSSION

Characterization and molecular identification of isolated bacteria

The selected bacterium was characterized and identified by using conventional morphological, physiological and biochemical tests (Table 1). It was presumptively identified as *Shewanella* sp (Holt et al., 2005). The sequence of 16S rRNA gene of this bacterium was submitted to Blastn database 16S ribosomal RNA sequences (Bacteria and Archaea) Megablast http://www.ncbinlm.nih.gov/blast. It indicated a close genetic relatedness of this bacterium with the rDNA sequence of *Shewanella oneidensis* (Holt et al., 2005).

Minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the heavy metals that completely inhibited bacterial growth (Froidevaux et al., 2001) *S. oneindensis* showed significant resistance to high concentrations of Pb and Cd (700 and 1000 mgl^{-1}) respectively. This may be considered new finding, that the other studies showed different results. Chihomvu et al. (2014) recorded MIC for Pb by *Shewanella* (4 Mm), while MIC was 0 for Cd. Francis and Dodge (1988) and Toes et al. (2008) demonstrated that, the tolerances inhibited growth of different *Shewanella* strains completely at 150 µM Co, 150 - 400 µM Zn, 75 - 150 µM Cd, and 150 µM Cu when cultivated aerobically in 10% LB broth. The effect of the medium on metal toxicity was demonstrated in a study by Toes et al. (2008) where higher tolerances of Cu by *Shewanella* between 75 and 750 µM in more nutrient rich media and the presence of manganese oxides also reduce the toxicity of Cu.

Bioaccumulation

S. oneidensis as sulfate reducing bacteria has the potential to enhance metal retention via extracellular binding, cellular uptake and accumulation of metals, oxidation/reduction processes, and surface mediated mineral precipitation (Burkhardt, 2010). From results of the present study, *S. oneindensis* was able to accumulate Cd than Pb (26.77 and 3.98 mgg^{-1}) at 48 h and at concentrations 50 and 100 mgl^{-1} respectively (Figures 1 and 2). The differences in this accumulation ability for these two metals may be related to different toxicity of these metals to this bacterium. From the results, the accumulation of both metals increases with increasing the time. Varghese et al. (2012) showed that, with increasing time, the biomass of the bacterial strains increased. Likewise, with an increase in biomass, the heavy metals bioaccumulation also increased. The results of the present study showed that the high amount of accumulation occurs with high metals concentration (50 and 100 mgl^{-1}). These results agree with the results reported by Odokuma and Akponah (2010), where they concluded an increasing uptake pattern observed in the respective test isolates as the initial concentration of the various heavy metal salts were increased. These observations suggested that metal uptake may involve diffusion phenomenon, whereby metal ions move from regions of high to low concentrations.

Transmission electron microscope

Cells were evaluated by TEM to observe the locations of precipitate of metals in relation to the *S. oneidensis* cells. In order to differentiate whether extracellular or intracellular reduction occurred, the cells were stained with uranyl acetate. Figure 3 has shown the cells before being exposed to the metals (a). Dark precipitate can be seen around the inside of the cell membrane, indicating

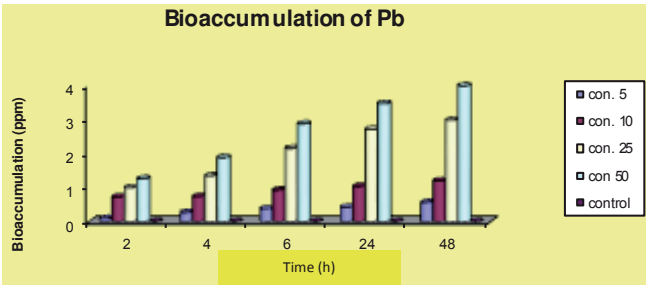

Figure 1. Bioaccumulation of Pb by *S. oneidensis* during different incubation periods and different concentrations.

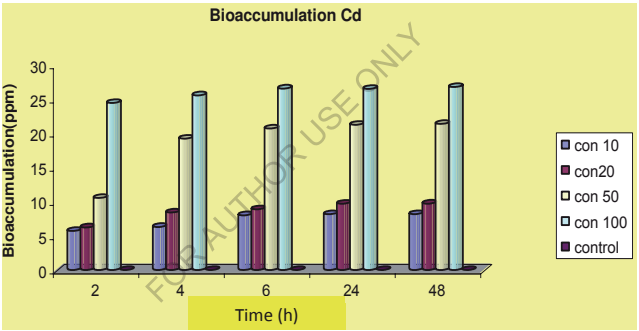

Figure 2. Bioaccumulation of Cd by *S. oneidensis* during different incubation periods and different concentrations.

intracellular Cd and Pb reduction has occurred (b and c). Also, from Figure (3b and c) there were changes in size and shape of cells and some cells have been lysed. These results could add to the toxicity of the substance, and ultimately results in cell death.

The cell surface morphology considerably changed after metals exposure. The cellular localization of the metals bound by the cells of the bacterium was located mainly within the cell membrane. However, some intracellular metal accumulates were also identified in the cytoplasm of the bacterial cells. Merroun et al. (2005) 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, Singh et al. (2013), reported 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 *Bacillus firmus* and *Bacillus subtilis.*

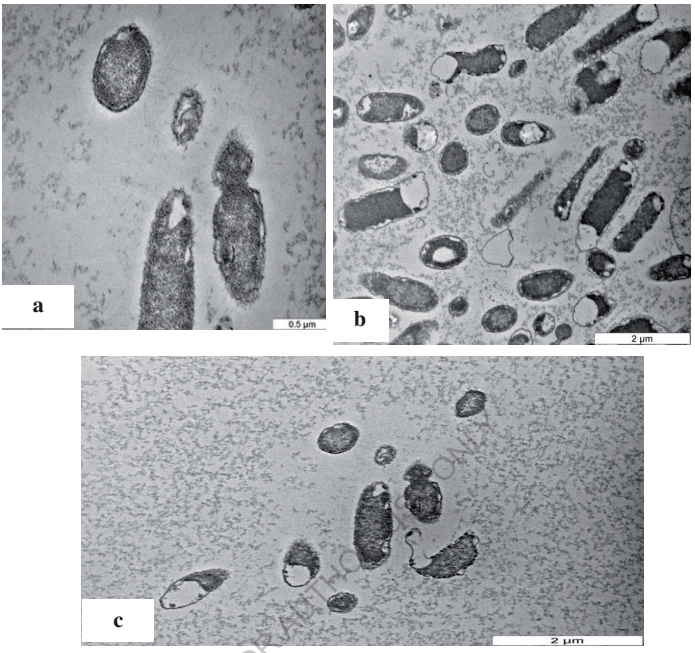

Figure 3. Transmission electron micrographs of *S. oneidensis* a: control, b: treated with 50 mgl^{-1} of Cd for 24 h, c: Treated with 50 mgl^{-1} of Pb for 24 h (Scale of bar 0.5 and 2 µm).

Conflict of interests

The authors have not declared any conflict of interests.

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Research Article Open Access

Biosorption of some Heavy Metals by *Deinococcus radiodurans* Isolated from Soil in Basra Governorate-Iraq

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Abstract

The bacterium *Deinococcus radiodurans* has been isolated from Basra soil. On the basis of morphological, biochemical, 16S rRNA gene sequencing and phylogeny analysis, the isolates were authentically identified as *D. radiodurans*. The biosorption capabilities of *D. radiodurans* for cadmium (Cd^{+2}) and lead (Pb^{+2}) were monitored at different concentrations and contact times. The characterization of heavy metals around the cells of bacterial strains was observed using the Fourier Transform Infrared (FT-IR) spectrophotometer, X-ray powder diffraction analysis (XRD) and transmission electron microscope (TEM).

Keywords: *Deinococcus radiodurans*; Biosorption; Fourier Transform Infrared; X-ray powder diffraction; Transmission electron microscope

Introduction

Bioremediation is a natural process which depends on bacteria, fungi, and plants to alternating pollutants as these organisms carry out their normal life functions. Thus, bioremediation offers a substitute tool to destroy or reduce the harmful pollutants through biological activity with an effective cost. In the early 1980s, certain microorganisms were found to accumulate metallic elements at a high capacity [1,2].

There are a lot of studies about the bioremediation ability of *D. radiodurans* and its genetic engineering, for cleaning up heavy metals in nuclear waste contaminated sites [3-7]. The development of bioremediation strategies using *Deinococcus* spp is therefore vital for the cleanup of contaminated site with radioactive waste. Additional advantages of deinococci are that they are vegetative, easily cultured, and nonpathogenic. As these sites rarely contaminated by a single chemical, it is necessary to bio remediating strain to be multi resistant to various toxic agents. The present work describes the use of a combination of spectroscopic and microscopic methods to characterize the heavy metals around the cells of bacterial strains isolated from extreme habitats as well as to elucidate the interaction mechanisms of these bacteria with these metals.

Materials and Methods

Source of bacterial isolate

The tested isolates, *D. radiodurans* used in this study was isolated previously from the Um - Qasr district, south of Basra city- Iraq. The isolate was identified by biochemical tests and sequencing 16S rRNA gene and comparing the sequences online with GenBank database (www.ncbi.nlm.nih.gov).

Biosorption experiment

The equilibrium, kinetics data of the biosorbent *D. radiodurans* were obtained by performing batch experiments

Characterization study

FTIR analysis: The Fourier Transform Infrared (FT-IR) analysis was done with Perkin Elmer spectrometer model 100 series (sample preparation UATR). This analysis was done in the Chemistry department, University Putra Malaysia.

X-ray powder diffraction analysis (XRD): The powder X-ray diffraction analysis was performed using a Shimadzu diffractometer model XRD 6000. The diffractometer employed Cu-Kα radiation to generate diffraction patterns from powder crystalline samples at ambient temperature. The Cu-Kα radiation was generated by Philips glass diffraction, X-ray tube broad focus 2.7KW type. The crystallite size D of the samples was calculated by using the Debye–Scherer's relationship. Where D is the crystallite size, λ is the incident X-ray wavelength, β is the (FWHM) Full Width at Half-Maximum, and θ is the diffraction angle, Deby -Scherrer equation: $D= K \lambda / \beta \cos \theta$. This analysis was done in the Chemistry department, University Putra Malaysia.

Transmission Electron Microscope: By centrifuging samples broth culture for 10 min at 3000 rpm, and decanting the supernatant, fixing pallet with 4% gutaraldehyde for 4 h at 4°C and centrifuged again, decanting fixative and adding an appropriate quantity of animal serum to submerge sample then allowing serum to clot. Washing three times with 0.1M Cacodylate buffer for 10 min. Postng fix in 1% Osmium tetroxide for 2h at 4°C. Washing again three times with 0.1M Cacodylate buffer for 10 min. dehydrating in series of acetone at 35, 50, 75, 95, and 100% for 10, 10, 10, 10 and 15 min respectively. (Table 1).

Embedding: Specimens were placed into the beam capsule fill with resin and polymerized in oven at 60 °C for 24-48h. Ultra sectioning was realized by choosing an area of interest, for ultrathin section, i.e.

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

Table 1: Finally infiltrating the specimen with acetone and resin.

selecting the silver section, picking up sections with a grid, then drying with filter paper. Finally the sections were stained with Uranyal acetate for 15 min, and washed by double distilled water. Lead sections were stained for 10 min, washed twice with distilled water. This analysis was done in the Electron Microscope Laboratory, Institute of Bioscience, University Putra Malaysia.

Results and Discussion

FT-IR

One of the most important characteristics of a biosorbent is the presence of its surface functional groups, which are largely characterized by the FTIR spectroscopy method. This technique can only provide a qualitative description for biosorbent functional groups. The studies of FTIR spectra on *Deinococcus* provided the basis to interpret the results with *Fucus vesiculosus* [8], as shown in Figure 1 there was a shift in the bands corresponding to carboxyl (COOH) groups after the biosorption process. Following metal binding, the asymmetric carboxyl stretching band shifted from 1630 to 1636 and 1632 Cm^{-1} for Cd and Pb respectively [9], and there was an increase in the distance between this band and the symmetric stretching of the same groups at 1418 Cm^{-1}, to lower wave numbers after biosorption, indicating that chelating complexes were formed [10,11]. Therefore, chelation was another important mechanism involved in the biosorption of Cd and Pb with *Deinococcus*; as reported by Sheng et al. [12] for *Sargassum* with FTIR spectroscopy studies. These changes in the FTIR spectra have also been observed in other biosorption studies [13,14]. Also the peak at 1387 attributed to C-O bond shifted to 1392 and 1390 Cm^{-1} when exposed to Cd and Pb respectively.

Other studies stated that cadmium biosorption was achieved by the formation of ionic bridges between the metal and two carboxyl groups or a bidentate chelating complex with one carboxyl group [15,16]. The symmetric carboxyl-stretching band's intensity decreased after Cd and Pb binding. The bacteria formed stronger bonds with these two metals. Carboxyl groups are the most abundant functional groups and are the main functional groups involved in the biosorption of heavy metals with *Deinococcus* and other biomasses [17-19]. Cadmium biosorption with *Deinococcus* reduced after blocking these groups, the majority of these groups are located in the *Deinococcus* cell wall and their negative charge can attract metal cations [20,21].

XRD

X-ray diffraction is a non-destructive technique used to provide detailed information on the crystallographic structure of materials. This method offers several advantages e.g., non-destructive, high accuracy, capability to detect single crystals, polycrystalline or amorphous materials. Moreover, standards are readily accessible for thousands of material systems. Due to its versatility, XRD has been widely employed to assist in the characterization of biosorbent and in the verification of heavy metals biosorption mechanisms [22]. Figure 2 shows the biosorbent of *D. radiodurans* un-loaded and loaded with Cd (II) and Pb (II). The XRD profile of the unloaded biosorbent shows typical diffraction peaks. Broad peaks were obtained instead of sharp peaks

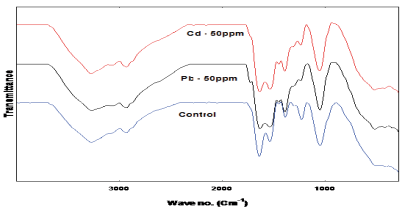

Figure 1: The FTIR Spectra of *D. radiodurans* with Pb (II), and Cd (II) loaded and unloaded.

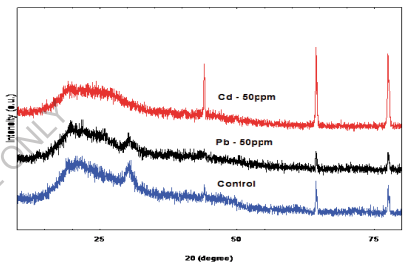

Figure 2: XRD analysis of *D. radiodurans* biosorbent before and after Pb and Cd biosorption.

indicating the sample was poorly crystalline. The peaks at 2-theta 30.2 44, 64.2, and 77.5°corresponding to (201), (114), (641), and (811) planes The XRD spectrum is compared with the exited spectrums of contro that have been published by the Joint Committee on Powder Diffractio Standards (JCPDS file no. 00-001-0166). The XRD spectra of Cd (II) an Pb (II) exhibit strong peaks at 2-theta value 29.9, 44.1, 64.4 and 77.8 corresponding to (200), (114), (640) and (822) planes, respectively for Cd (II), whereas, the value 44.2, 64.2 and 77.8° corresponding t (112), (211), and (422) for Pb (II). The XRD spectrum is compared wit the exited spectrums of control that have been published by the Join Committee on Powder Diffraction Standards (JCPDS file no. 00-002 097) and (JCPDS file no. 00-002-085) respectively.

The average crystal size of the un-loaded control, loaded with Cd (II) 50 mgl^{-1}, and Pb (II) 50 mgl^{-1} nanoparticle estimated from th highest peak by using the Debye-Scherrer Eq. is 20.0, 27.8, and 14.(respectively. The results of XRD patterns of *D. radiodurans* show three sharp peaks with a decrease in the intensity of the these peak for un-loaded biosorbent after exposing the bacteria to Pb (II) an Cd (II), which could explain the immobilization process. This is i agreement with the results reported in the literatures of [23-25]. An this may suggest that Pb (II) and Cd (II) immobilized on surface of *D radiodurans* [26].

Transmission electron microscope

One of the most important of TEM techniques has revealed th internal structure of the bacteria, especially when embedding bacteri in a resin and staining thin sections of the resulting block with urany

acetate. Figure 3a shows the cells of *D. radiodurans* before interaction with metals while Figure 3b,3c show the bacteria after exposure to Pb and Cd solutions. Metals were mostly seen on the cell wall, looking almost like a crust around the cell in addition to the change in the size and the shape of cells. The cellular localization of the metals bound by the cells of study bacteria was located mainly within the cell wall. However, some intracellular metal accumulates associated were also identified in the cytoplasm of the bacterial cells [27]. Reported that, the cellular localization of the uranium bound by the cells was located mainly within the cell wall of three types of *A. ferrooxidans* using

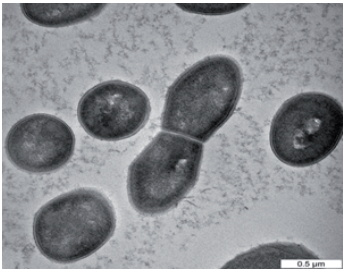

Figure 3a: Transmission electron micrographs of *D. radiodurans* control.

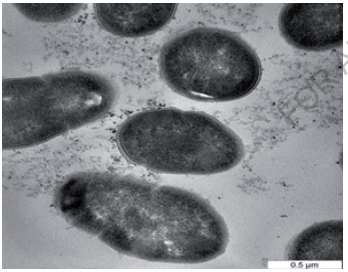

Figure 3b: Transmission electron micrographs of *D. radiodurans* treated with Pb.

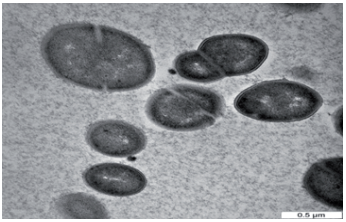

Figure 3c: Transmission electron micrographs of *D. radiodurans* treated with Cd showing some unequal cell products (daughter cells) (Scale of bar 0.5µ).

TEM. Electron microscopic observation carried out by Panak et al. [28] revealed the presence of Ag^{+2} as discrete particles at or near the cell wall of both gram positive and gram negative bacteria. Sinha and Mukherjee [29] showed that, *Bacillus* sp. cell wall components with phosphate residues i.e polysaccharides, teichoic and teichuronic acids or phospholipid layers of the membranes can bind U. While cellular functional groups can be responsible for the extracellular association of [30] showed localized intracellular site of accumulated Cd^{+2} by *P. aerugenosa* and showed electron dense grains in the cytosol and towards the cell envelope. Also, Sultan et al. and Singh et al. [31,32] reported that, the 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. The results of the present study also indicate morphology and size changes of bacteria exposed to heavy metals, such changes may due to cell surface changes as a result of heavy metals exposure reported that, the cell surface morphological changes in *Cryptococcus* sp. after exposing to heavy metals, appeared as shrunken and distorted cell wall in the presence of Cd and depressions in the presence of Pb and Zn. Various factors may be responsible for such alterations in cell surface morphology of microbial biomass in the presence of heavy metals. Secretion of extracellular polymeric substance by *Desulfovibrio desulfuricans* during biosorption of Zn and Cu was reported to modify its cell surface morphology [34] Similarly, Murray [35] reported high dark dense cytoplasm due to Co^{+2} precipitations; partially emptied with a very thick cell wall; changing in the morphology of vegetative cells of *B. firmus and B. subtilis.*

Conclusion

Deinococcus radiodurans isolated from polluted soils in Basra city south of Iraq, showed high tolerance for Pb and Cd with the possibility of being exploited in bioremediation in two routes, bioaccumulation and biosorption. *D. radiodurans* survive the adverse effect of high concentration of Pb and Cd as expressed in morphological changes.

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Research Article Open Access

Biosorption and Bioaccumulation of Some Heavy Metals by Deinococcus Radiodurans Isolated from Soil in Basra Governorate- Iraq

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Abstract

The bacterium Deinococcus radiodurans has been isolated from soil. On the basis of morphological, biochemical, 16S rRNA gene sequencing and phylogeny analysis revealed that, the isolates were authentically identified as D. radiodurans.

D. radiodurans showed significant resistance to high concentrations of Pb and Cd, but it was more tolerant to Cd than Pb. Minimum inhibitory concentration was 400 mgl^{-1} for Pb, while it was 600 mgl^{-1} for Cd. The potent bacterium has the optimal bioaccumulation capacity differ according to metal type, concentration, and contact time. In bioaccumulation experiment, the results showed the highest increase in accumulation of Pb in the concentration 50 mgl^{-1} at 6 h of incubation (0.33 mgg^{-1}), while the lowest accumulation was in concentration 5 mgl^{-1} (0.029 mgg^{-1}) at 2h of incubation. For Cd the results showed maximum accumulation at 24h for concentration 100 mgl^{-1} then decreased at 48 h.

The results of biosorption experiment showed that D. radiodurans has a good ability to absorption both Pb and Cd in considering to the metals concentrations and times. This which can be clarified from the elevated percentage of Pb absorption (63.46%) in concentration 50 mgl^{-1} and during 2h. For biosorption of Cd the was decreased with the increasing time and the high biosorption noticed during 2h at concentration 50 mgl^{-1} (31.23%).

Keywords: Deinococcus radiodurans ; Heavy metals; Minimum inhibitory concentration; Bioaccumulation; Biosorption

Introduction

D. radiodurans is one of the most known radiation resistant organisms. It can live in cold, dehydration, vacuum and acid, and is therefore known as a polyextremophile. It is found in habitats rich in organic materials, such as soil, feces, meat, or sewage, in addition to that, it's isolated from dried foods, room dust, medical instruments and textiles [1].

The manufacturing of energy from nuclear power factories, uranium mining, nuclear weapons production and nuclear accidents are the major cause of release of radionuclide's into the environment. The nuclear wastes typically contain inorganic and organic contaminants that include radionuclide's, heavy metals, acids/base and solvents. The high radiation level, in combination with the chemical hazards cause intensive damage to ecosystem and living organisms. The clean –up of nuclear waste by physiochemical methods is impractical and the cost is prohibitive. As a result a less costly in situ bioremediation technology is being investigated as a potential substitute method for treating such contaminated sites. The development of bioremediation strategies using Deinococcus spp, is therefore vital for the clean up of contaminated sites with radioactive waste. As these sites rarely contaminated by a single chemical, it is necessary to developed strain to be multi- resistant to various toxic agents [2].

Bioremediation is a natural process which depends on bacteria, fungi, and plants to altering pollutants as these organisms perform their normal life functions. These organisms have the ability of exploiting chemical contaminants as an energy source in their metabolic processes. Therefore, bioremediation affords alternative tool to destroy or reduce the risky contaminants through biological activity and this method has an effective cost [3].

Among the different methods, bioaccumulation and biosorption are potential for the removal of metals [4]. The active mode of metal accumulation of living cells is in most cases referred to bioaccumulation which relys on intrinsic biochemical and structural properties, physiological and genetic adaptation, environmental modification of metal specification, availability and toxicity [5]. Bioaccumulation is defined as the uptake of toxicant by living cells and transports it into the cell [6] and is a growth dependent process mediated only by living biomass [7]. The process of biosorption is possible by both living and dead biomasses [8]. The ability of certain species of bacteria in accumulating heavy metals was investigated inclusively [9,10]. Hence the natural organisms, either indigenous or extraneous can be used for bioremediation of heavy metals [11].

The present study aimed to isolate Deinococcus radiodurans from soil and identifying it biochemically and molecularly, in addition to determining their ability to remediation Pb and Cd through bioaccumulation and biosorption processes.

Materials and methods

Isolation of bacteria

Three soil samples (30 gm each) were collected from Um- Qasr

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district, south of Basra city- Iraq during the January (2013). The samples were collected using a sterile plastic bag and transferred within 2 h to laboratory for analysis. For isolation of Deinococcus radiodurans, one gram of air dried soil sample was serially diluted using distilled water and spread over Tryptone Glucose Yeast extract (TGY) medium (0.5% tryptone, 0.1% glucose and 0.3% yeast extract) agar solid plates. The plates were incubated at 30°C for 24 h.

Bacterial characterization

Properties of the isolates that included gram reaction, citrate utilization, indole production, nitrate reduction, catalase, fermentation of D- glucose, arginine, lactose and mannose, hydrolysis of casien, and gelatin liquefaction tests were determined according to Murray [12].

16S rRNA based identification

Bacterial samples were identified by sequencing of the 16S rRNA gene. To determine the identity of bacterial samples, the amplified 16S rRNA gene PCR products obtained from total genomic DNA using primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), [13] were sequenced commercially. DNA sequences obtained were compared to sequences available online in the Gene Bank database (www.ncbi.nlm.nih.gov). Homology search was performed using Bioinformatics tools available online, BLASTn www.ncbi.nlm.nih.gov/BLA [14].

Determination of minimum inhibitory concentrations (MIC) for Cd and Pb The MIC of Cd and Pb of bacteria were determined by disc diffusion method [15]. The concentrations of Cd and Pb were between 100 to 1000 mgl^{-1}. Filter paper disks were saturated with heavy metals for 30 min, and then added to nutrient agar plates which incubated for 24h at 30°C. The salts of $CdCl_2$ and Pb $(NO_3)_2$ were used to prepare mother solution of these metals in sterile distilled water.

Bioaccumulation of heavy metals by Bacteria

Bacteria were grown in TGY broth containing different concentrations of lead (5, 10, 25, 50) mgl^{-1} and for cadmium 10, 20, 50, 100 mgl^{-1} for 2, 4, 6, 24 and 48 h and incubated at 30°C in a shaker incubator at 180 rpm. Two replicates for each concentration have been done plus, 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 weighed. The dried biomass were then digested as follow: 100 ml beaker containing the dried cells, 5 ml concentrated nitric acid was added; the beaker was placed on a hot plate, stirred continuously, and heated initially at a medium rate for 5 min. Then, the beaker was heated on maximum setting until nitrogen oxide fumes were given off for a short time and a white residue was left. The beaker was left to cool for about 2 min and digestion was repeated with an additional 2 ml of concentrated nitric acid; this time it was heated until brown nitrogen oxide fumes almost ceased to appear. The beaker was cooled again for about 2 min and then 2 ml of 1:1 hydrochloric acid (35- 37%) was added. The mixture was heated at a medium rate for 3 min. After that it was cooled to room temperature and made up to 25 ml or bigger volume with distilled water.

Metal concentrations were measured by an atomic absorption spectrophotometer (Thermo Scientific ICE 3000 Series AA Spectrometer, USA) [16]. The metals accumulation then calculated using this equation:

E. Con. = A*B/D

Where E. Con = the concentration of heavy metals in (mg/g)

A= Concentration of heavy metals from calibration curve

B= final concentration of sample (mg/l)

D= dry weight of sample (gram)

Biosorption experiment

The equilibrium, kinetics data of the biosorbent D. radioduran were obtained by performing batch experiments. The experiment were carried out in 250 ml flasks to which 100 ml of single ion metal solution of Cd and Pb, and l ml of biomass (exponential phase) wa added. The mixture was stirred at 180 rpm at 30°C and 15 ml of sampl was collected at interval times (2, 4, 6, 24, and 48 h) centrifuged a 6000 rpm for 15 min in a centrifuge. The remaining concentration o metals was analyzed by the atomic absorbance spectrophotometer. A experiment was carried out twice and the mean values were reported The difference between the initial metal ion concentration and th final metal ion concentration was considered as metal bound to th biosorbent [17].

Statistical analysis

The data obtained on the Bioaccumulation and biosorption o different metals at different concentration and time interval, by th bacterium D. radiodurans subjected to Statistical analysis usin the SPSS program (SPSS Inc., Chicago, IL Version 15.0). The dat were analyzed through analysis of variance (ANOVA). To detect th statistical significance of differences ($P<0.05$) between means.

Results and Discussion

Characterization and molecular identification of isolate bacteria

The selected bacterium was characterized and identified by usin morphological, physiological and biochemical tests (Table 1). Bacteri were presumptively identified as Deinococcus sp. According t Chaturvedi [18]. Also the bacteria were subjected to 16S rRNA gen sequence analysis. The sequence of 16S rRNA of bacteria was submitte to Blastn (database 16S ribosomal RNA sequences (Bacteria an

Tests	Characteristics observed
Morphology	
shape	Cocci
pigment	pink
Gram reaction	+
Biochemical reaction	
Oxidase test	+
Catalase test	+
Indol formation	+
Citrate utilization	+
Gelatin liquefaction	+
Nitrate reduction	-
Fermentation	
D-glucose	+
Arginine	+
Lactose	-
Mannose	+
Casien	+
"+"and "-" indicate positive and negative reactions, respectively	

Table 1: Morphological and biochemical characteristics of D. radiodurans.

Archaea); Megablast) (www.ncbi.nlm.nih.gov/blast). That indicated a close genetic relatedness of bacteria with the rRNA sequence of D. radiodurans.

Minimum inhibitory concentration

The MIC is the lowest concentration of the heavy metals that completely inhibited bacterial growth [19]. D. radiodurans showed significant resistance to high concentrations of Pb and Cd, but it was more tolerant to Cd than Pb. MIC was 400 mgl^{-1} for Pb, while it was 600 mgl^{-1} for Cd. Chaturvedi [18] reported different results in his study, he reported that the different isolated of Deinococcus (GrK2, Grk4, GrK5 and DR1) were sensitive to Cd^{+2}, but they exhibited varying levels of tolerance. Grk2 was being extremely sensitive to Cd^{+2}, DR1 is moderately tolerant, while Grk4 and Grk5 showed comparable tolerance, and also he mentioned that the toxicity of Cd affected by growth state, with stationary phase cells being more sensitive than the exponential phase. Hua et al. [20] reported that D. radiodurans R1 does not exhibit strong resistance to heavy metal Hg (II), Ag (I), Cr (VI) and Pb (IV), and recorded MIC (300 , 1600 300 and 3200 μM) for these metals respectively.

From the results of the present study there is a difference in results recorded in comparison with other studies results recorded previously, and this can be explained as the isolated strains was different and the growing conditions such as growth stage also different. In addition to the high concentration of heavy metals in the study soil, which enhance the ability of tolerant to these bacteria. Those can be supported by the result of Chaturvedi [18], who reported that, the growth phase depending differences in tolerance. Qi and Hulett [21] reported that, the tolerance of growing B. thuringiensis DM55 cells has been shown to vary at different growth stages, supporting the existing evidence that the structural features of gram positive bacterial cell walls are affected by the developmental state of the cell. Another reason for these results differences is the variance in design of experiment, such as growth media composition as clarified by Kumar et al. [22]. Complex media such as LB precipitate Pb as a result of sequestration with organic moieties thus reduce the bioavailability of Pb to the bacterial strains [23].

Bioaccumulation study

From the results shown in Figure 1 there is an increasing in the accumulation of Pb with the increasing of the concentration. The highest accumulation occurs in the concentration 50 mgl^{-1} after 6h of incubation was 0.33± 0.0007 mgg^{-1}, while the lowest was 0.029 ± 0.268 mgg^{-1} after 2h. In addition to that, the rising of incubation period may reduce the accumulation for all concentrations.

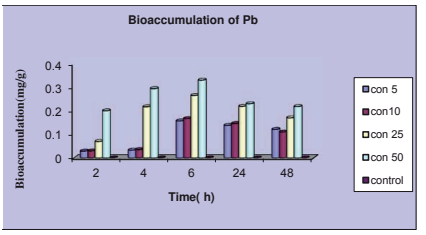

Figure 1: Bioaccumulation of Pb by D. radiodurans at different incubation period and different concentrations. LSD (0.0049).

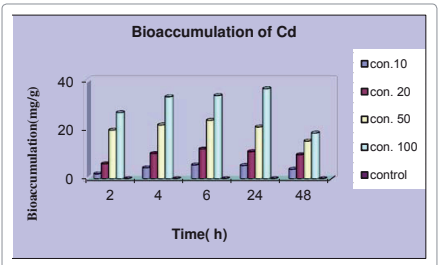

Figure 2: Bioaccumulation of Cd by D. radiodurans at different incubation period and different concentrations. LSD (0.0341).

The accumulation of Cd increase in parallel with the increasing of concentration for all studying time (Figure 2). The figure clarifies increasing in Cd accumulation with the times (2, 4 and 6 h) for concentrations 10, 20 and 50 mgl^{-1}, then decrease during 24 and 48h. For concentration 100 mgl^{-1}, accumulation increase within 2, 4, 6, and 24h, then decrease with 48h. The analysis of variance of bioaccumulation of Pb and Cd between time and concentration was significant ($P<0.05$) in all treatments as show by LSD value.

The results of Pb and Cd accumulation showed that, this bacterium was able to accumulate high amounts of Cd 36.86 ± 0.007 mgg^{-1} in comparison to 0.33 ± 0.0007 mgg^{-1} for Pb. This may be due to difference in toxicity of these metals to this bacterium and to its intrinsic properties which help it in occupying varying tolerance, also the history of pollution of the soils from which bacterium isolated by these metals play critical tool in development ability of these bacteria to deal with such contaminations, this which agrees with the results of the present study where it show high MIC value for Cd than this for Pb for this bacterium.

From the results the accumulation increase with time, then start decreasing over specific time. This agrees with Ray et al. [24] who reported that, the accumulation of Pb by B. cereus increased with time, then starts decreasing. It may be concluded that, metal binding sites became saturated after specific time. The maximum accumulation of both of metals by this bacterium occurs in the highest concentration of metals then start decreasing with increasing of the time, this observed by Tunali et al. [25] who reported that, the amount of metal ions accumulated per unit mass of Bacillus sp. (ATS-1) increased first with increasing of the initial metal ion concentration and reached to a saturation value. Then the value changes with the initial metal ion concentration change. Also Ozdemir et al. [26] reported that, the accumulation of chromium (VI), cadmium (II) and copper (II) by Pantoea sp. TEM18 increase with high initial metals concentration, then reach to saturate so the accumulation decrease. Table 2 shows a comparison between different microorganisms and D. radiodurans in the present study.

Biosorption study

The results of the present study (Table 2) showed that D. radiodurans has a good ability to absorption Pb and Cd. The highest percentage of Pb absorption was 63.46% in concentration 50 mgl^{-1} for 2h. Biosorption of Pb increased with the increase of time for concentration 5, 10 and 25 mgl^{-1}, then start decreasing with increase time for concentration 50

Biomass	Heavy Metals	Bioremediation capacity (mgg^{-1}) or %	Heavy Metals	Reference
Saccorhiza polyschides	Cd	95	Cd	Loderio et al. [32]
Enterobacter sp.	Pb	50	Pb	Lue et al. [33]
Bacillus cereus	Pb	36.71	Pb	Babak et al. [34]
Micrococcus sp.	Zn	84.27	Zn	Hussein et al. [35]
Aspergillus niger	Cr	133	Cr	Goyal et al. [36]
D. radiodurans	Cd	36.86	Cd	Present study
	Pb	0.33	Pb	
D. radiodurans	Cd	31.23%	Cd	Present study
	Pb	63.46%	Pb	

Table 2: Comparison of different microorganisms based on their maximum capacity for bioremediation of different heavy metals in comparison to D. radiodurans in the present study.

Concentration (mg/l)	% Biosorption of Pb at different times (h.)				
	2	4	6	24	48
5	22.72± 0.007	22.85± 0.141	23.50± 0.007	27.91± 0.007	30.43± 0.014
10	28.15± 0.007	30.32± 0.021	30.40± 0.014	30.43± 0.007	30.45± 0.007
25	41.8± 0.007	43.20± 0.141	44.00± 0.141	44.20± 0.070	44.33± 0
50	63.46± 0.007	59.97± 0.007	39.33± 0.007	33.40± 0	31.89± 0.070
LSD= 0.001					
Concentration (mg/l)	% Biosorption of Cd at different times (h)				
	2	4	6	24	48
5	18.97± 0.014	18.57± 0.014	17.65± 0.014	16.10± 0.707	15.58± 0.007
10	22.80± 0.070	22.76± 0.007	21.00± 0	20.71± 0.007	20.14± 0.007
25	27.64± 0.014	27.60± 0.070	27.58± 0.021	26.57± 0.014	26.52± 0.014
50	31.23± 0.014	31.12± 0.014	30.75± 0.021	30.64± 0.014	30.32± 0.014
LSD= 0.024					

Table 3: Biosorption (%) of Lead and Cadmium at different period of incubations and different concentrations by *D. radiodurans.*

mgl^{-1}. Explanations for these results is that at first three concentrations, the increase in biosorption with the time due to the sorbent sites were not saturated, but in concentration 50 mgl^{-1}, the concentration was sufficient to saturate these sites, so the maximum absorption occurs in the first two hours. Tarangini [27], reported that, biosorption of arsenic by mixed cultures of Pseudomonas aeruginosa and Bacillus subtilis was increased with the increase time.

In related to the effect of Pb concentration, results showed sorption increase with the increasing Pb concentration. Taty-Cortodes et al. [28], showed that, the initial ion concentration exhibits quite an interesting effect on the equilibrium sorption capacity of the Pinus sylvestris for Cd (II) and Pb (II). At a fixed biosorbent dose, pH and temperature, the equilibrium sorption capacity, improved with higher initial ion concentration. The ion removal was highest concentrated dependent. The increase in the biosorbents loading capacity as a function of metal ion concentration was believed to be due to a high driving force for mass transfer.

The results of the present study of Cd sorption showed decrease of biosorption with increasing time (Table 2). The high sorption noticed after 2h at concentration 50 mgl^{-1}. This is similar to Anzeze et al. [29], who reported that, the rate of adsorption of Cd by Eichhornia crasippes was very fast at first and over 95 % of total biosorption o Cd (II) ions occurs in the first 5 minutes and thereafter it proceed at a slower rate and finally no further significant adsorption is note beyond 20 minutes of contact time. For effect of metal concentrati on biosorption, results show increases in Cd sorption with increasin metal concentration. The initial concentration provides an importan driving force to overcome all mass transfer resistance of metal betwee the aqueous and solid phases [30]. The increasing amount of meta adsorbed by the biomass will be increased with initial concentratio of metals. Optimum percentage of metal removal can be taken at hig initial metal concentration. Thus, at a given concentration of biomass the metal uptake increases with increase in initial concentration [31].

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Biosorption of some Heavy Metals by Metal Resistant *Bacillus thuringiensis* Isolated from Soil in Basra Governorate- Iraq

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Abstract
In present study heavy metal resistant bacteria were isolated from soil collected from Fao district in Basra governorate South of Iraq. On the basis of morphological, biochemical, 16S rRNA gene sequencing and phylogeny analysis revealed that, the isolates were authentically identified as *Bacillus thuringiensis*. The minimal inhibitory concentration (MIC) of isolates against cadmium (Cd) and lead (Pb) was determined on solid medium. *B. thuringiensis* showed significant resistance to high concentrations of Pb (1800 mg/l) and Cd (50 mg/l). The biosorption capabilities of *B. thuringiensis* for Cd and Pb were monitored at different ion concentrations and contact times. The functional groups of bacterial surface were determined using Fourier transform infrared, and X-ray powder diffraction analysis.
Key Words: *Bacillus thuringiensis*, Minimal Inhibitory Concentration, Biosorption, Fourier transform infrared, X-ray powder diffraction

Introduction

Heavy metals play an important role in the metabolic processes of the biota, some of them are essential for organisms as micronutrients such as (cobalt, chromium, nickel, iron, manganese and zinc). They are involved in redox processes, to stabilize molecules through electrostatic interactions, as catalysts in enzymatic reactions, and regulating the osmotic balance. On the other hand, cadmium, mercury, lead, has no biological role and are harmful to the organisms even at very low concentration. However, at high levels, both of the essential and non-essential metals become toxic to the organisms (Rathnayake *et al*., 2010).

Cadmium is widespread and one of the most toxic soil contaminants released by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tang *et al*., 2006). Cadmium is poisonous to plants, animals, and humans (Gupta and Gupta, 1998) and is listed as one of the 126 priority contaminants by the US-EPA and as a human carcinogen by the International Agency for Research on Cancer (IARC, 1994). Thus, cadmium pollution is attracting more attention from environmentalists worldwide.

Lead (II) is a heavy metal poison which forms complexes with oxo-groups in enzymes to affect nearly all steps in the process of hemoglobin synthesis and porphyrin metabolism. Toxic levels of Pb (II) in man have been associated with encephalopathy appropriations and mental delay (Ademorati, 1996). Conventional physico-chemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu *et al*., 2001; Kadirvelu *et al*., 2002) have used for removing heavy metals, but are economically expensive and have disadvantages.

Microbial populations in metal polluted environments become metals resistant (Prasenjit and Sumathi, 2005), so the response of microorganisms towards toxic heavy metals is of importance in view of the interest in the reclamation of polluted sites (Shankar *et al*., 2007). Microorganisms uptake metal either actively (bioaccumulation) and/or passively (biosorption) (Shumate and Strandberg, 1985; Anders and Hubert, 1992; Hussein *et al*., 2003). Biosorption exploits various certain natural materials of biological origin including bacteria, fungi, yeast, algae, etc. It can effectively sequester dissolved metal ions out of dilute complex solutions with high efficiency and quickly, therefore it is a suitable candidate for the treatment of high volume and low concentration complex heavy metal wastes (Wang and Chen, 2006). The present study, aims to isolating *Bacillus thuringiensis* from Basra south of Iraq, and evaluating metals biosorption ability, and also studying the effect of metals initial concentration, contact times, and determined the functional groups of bacterial surface using Fourier transform infrared, and X-ray powder diffraction analysis.

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 2h to 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 reaction, citrate utilization, indole production, methyl red, nitrate reduction, Voges Proskauer, catalase, dextrose, mannitol and sucroseutilization, 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 1492R (5′-GGTTACCTTGTTACGACTT-3′), (Lane *et al*., 1985) were sequenced commercially. DNA sequences obtained were compared to sequences available online in a Gen Bank 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 (Wistreich and Lechtman, 1980). 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 added to nutrient agar plates which 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).

Biosorption experiments

The equilibrium, kinetics data of the biosorbent *B. thuringiensis* were obtained by performing batch experiments. The experiments were carried out in 250 ml flasks to which 100 ml solution of either Cd or Pb, and l ml of biomass from exponential phase were added. The mixture was stirred at 180 rpm at 30 ̊C and 15 ml of sample was collected at interval times (2, 4, 6, 24 and 48 h), centrifuged at 3000 rpm for 10 min. The remaining concentration of metals was analyzed by the flame atomic absorbance spectrophotometer (Thermo Scientific ICE 3000 Series AA Spectrometer USA). Each experiment was carried out twice and the mean values were reported. The difference between the initial metal ion concentration and final metal ion concentration was considered as metal bound to the biosorbent (Sethuraman and Kumar, 2011).

Effect of contact time on biosorption

Experiments to determine the equilibrium time required for biosorption was performed using 1 ml of cell biomass from the initial metal concentration (50 mgl^{-1}) of either Cd or Pb in 100 ml of metal solution at pH 6 for Cd and Pb, at 30 °C. and were taken at the desired interval time of 2, 4, 6, 24 and 48 h. and subsequently centrifuged at 3000 rpm for 10 min. The heavy metal concentration in the supernatant was analyzed by flame atomic absorption spectroscopy.

Effect of initial metal concentration

The experiments of the effect of initial concentration of Cd and Pb were performed at different concentrations (5, 10, 25, 50) mgl^{-1} at optimum temperature and pH for each metal, Aliquots of 1ml of cells of *B. thuringiensis* were added to 100 ml solution of either metal at 5, 10, 25 and 50 mgl^{-1} and incubated for 24h on orbital shaking incubator at 180 rpm. Aliquots of 15 ml were collected, centrifuged at 3000 rpm for 10 min and analyzed as mentioned in biosorption exp.

FTIR analysis

The Fourier transform infrared (FT-IR) analysis was done with PerkinElmer spectrometer model 100 series (sample preparation UATR) (UPM-Malaysia).

X-ray powder diffraction analysis (XRD)

The powder X-ray diffraction analysis was performed using a Shimadzu diffractometer model XRD 6000 (UPM-Malaysia). The diffractometer employed Cu-Kα radiation to generate diffraction patterns from powder crystalline samples at ambient temperature. The Cu-Kα radiation was generated by Philips glass diffraction, X-ray tube broad focus 2.7KW type. The crystallite size D of the samples was calculated using the Debye–Scherrer's relationship. Where D is the crystallite size, λ is the incident X-ray wavelength, β is the Full Width at Half-Maximum (FWHM), and θ is the diffraction angle.

The Scherrer equation can be written as:

$D = K \lambda / \beta \cos \theta$

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 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 rRNA sequence of *Bacillus thuringiensis.* This genus represents a common soil bacteria and have been reported as soil inhabitants (Oves *et al.,* 2013).

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

Tests employed	Characteristics observed
Morphology	
shape	Rod
pigment	-
Gram reaction	+
Biochemical reaction	
Citrate utilization	+
Indole	+
Methyl red	+
Nitrate reduction	-
Oxidase	-
Voges Proskauer	+
Catalase	+
Carbohydrate utilisation	
Glucose	+
Mannitol	-
Sucrose	+
Hydrolysis	
Starch	+
Gelatin	+

(+) and (-) represent positive and negative reaction respectively

Minimum inhibitory concentration

MIC is the lowest concentration of the heavy metals that completely inhibited bacterial growth (Froidevaux *et al*., 2001). *B thurirngiensis* 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).

Effect of Contact Time

Fig. (1) shows the effect of contact time on Cd and Pb uptake by this bacterium. As the rate of metal ion biosorption of Cd and Pb ions per unit mass of sorbent increased sharply up to 2h and then slightly decreased in case of Pb gradually, and equilibrium reached after 24h for both of metals.

Contact time is one of the important factors of biosorption process, and the explanation of the highest rate of metal ion biosorption in the beginning is due to the high affinity of free metal ion binding sites on absorbent to

the active sites on the surface of cell become saturated by metal ion within 2h. The order of biosorption rate was Pb > Cd. These indicate the equilibrium time at which an equilibrium metal ion concentration is presumed to have been attained and it seems that the affinity for Cd binding is greater than Pb. In this context, Zoubolis *et al* (2004)) and Volesky (1990) observed that, the initial shortest time period of sorption process is important for a high rate of metal sorption. Similar results have also determined by Gabr *et al* (2008) for Ni and Pb biosorption. Marandi (2011) showed biosorption of Mn and Cu by *B. thuringiensis* where increased sharply up to 30 minutes and then slowed gradually, as a result of the availability of active metal binding sites at the beginning of the experiment. After 50 minutes metal uptake became very slow for both metal ions and equilibrium reached after 120 and 150 minutes for Mn and Cu respectively. Giri (2012) mentioned that, the percentage removal of living cells of *Bacillus cereus* biomass was found to increase from 50.11% to 90%, 45.33% to 85.32% and 43.16% to 80.11% for 5 min to 60 min of contact time, for initial chromium (VI) concentration of 1 mgl^{-1}, 5 mgl^{-1} and 10 mgl^{-1} respectively. The change in the rate of removal might be due to the fact that initially all sorbent sites are vacant and also the solute concentration gradient was high. .

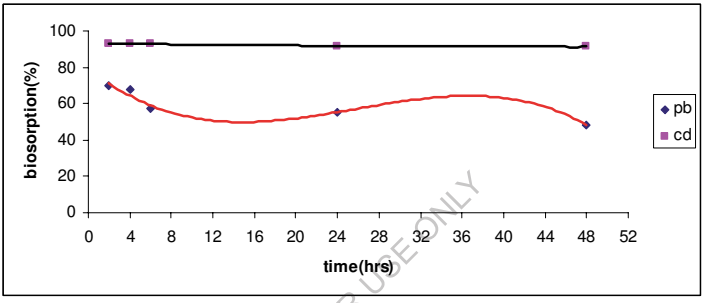

Figure 1: Effect of contact time on the absorption of Lead and Cadmium (II) ions with initial concentration 50 mg/l.

Effect of Initial Metal Ion Concentration

From Fig (2) the absorption capacity of this bacterium for Cd increases by increasing initial metals concentration, while there's fluctuating for sorption of (Pb) with the increasing of metal ion concentration, it was slow when the concentration increased from 5 to 10 mgl^{-1}, but it increased with concentration 25 mgl^{-1} and then reduced with concentration 50 mgl^{-1}. The differences between these two results of removing ions by the same bacterium may be ascribed to variance in a viability of the active site suitable for both metal ions on the surface of the cell. Initial concentration of metal ions, an important factor to be measured for more effective absorption. Higher amounts of metal ions increased the contact probability between the ion and active binding sites on the surface of the biosorbent and subsequently enhanced the metal removal, and thus explain the increase of removal of Cd by bacteria with concentration increase (Marandi, 2012). On the other hand, in case of Pb, the greater uptake of metal by the absorbent materials at the lowest concentration could probably be due to a rapid metal absorbing ability of the bacterial biomass. In contrast, at higher metal concentrations metal ion diffuses into the biomass surface by intraparticle diffusion and therefore, the hydrolyzed ions are likely to diffuse very slowly (Horsfall and Spiff, 2005).

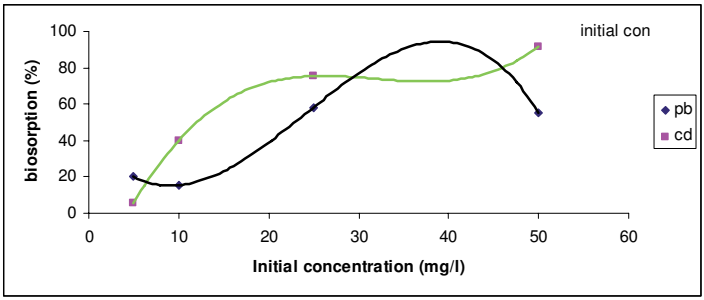

Figure 2: Effect of initial concentration on the biosorption of Lead and Cadmium (II) ions after 24h.

FT-IR spectral analysis

One of the important characteristics of a biosorbent is the presence of its surface functional groups, which are largely characterized by the FTIR spectroscopy method. This technique can only provide a qualitative description. The FT-IR spectra for *Bacillus thuringensis* is given in Fig. (3), they are done in order to characterize the biosorbent. In order to discover which functional groups are responsible for the biosorption process, The FTIR spectra of Pb (II) and Cd (II) loaded and unloaded biosorbent in a range of 280–4000 Cm^{-1} were analyzed. FT-IR spectra for biosorbent, showed the difference between the loaded and unloaded Pb (II) and Cd (II) metal ion (Fig. 3), in all biosorbent. It has an intense absorption band around 3500–3100 Cm^{-1}, which represents the stretching vibrations of amino (N-H) and hydroxyl (O-H) groups in table (3) which clearly states the vibration peak. The spectra of biomass also display absorption peaks at 2925 Cm^{-1} corresponding to stretching of the C-H bonds in the methyl group present in the cell wall structure. (Sethuraman and Kumar, 2011). The absorption band characterization, including C-H in CHO group peak was assigned at 2850 Cm^{-1}, whereas, carbonyl group (C=O) of amide groups at 1646 Cm^{-1}, (COO^{-1}) of the carboxylate groups appeared at 1544 Cm^{-1} (stretching), the band located at 1238 and 1398 Cm^{-1} represent (C-N) and (C-O) respectively. Furthermore, the peak located at 1080 Cm^{-1} was indicative of organic phosphate group P–O of the ($C–PO_4^{-3}$).

The FT-IR spectra of the loaded biomass varied with the metal species Cd and Pb. A stretching of bands appearing at 1070 and 1074 Cm^{-1} was revealed in the FT-IR spectrum, which was attributed to the interaction of sorbed metals Cd and Pb with phosphate groups, respectively. In addition, shifting of bands observed in 1646–1644 Cm^{-1} (Cd and Pb) after biosorption could be due to the involvement of carboxyl groups. Similarly, stretching of bands from 1396 to 1387 Cm^{-1} was due to the involvement of hydrogen bonds as reported by (Sar *et al*., 1999). The bands located between 3484 and 3283 Cm^{-1} however, verified the interaction of hydroxyl and amine groups. The transmittance of the peaks in the loaded biomass was substantially lower than the unloaded bacterial biomass. These changes suggest that bond stretching occurs to a lesser degree due to the presence of metals and therefore, peak transmittance is consequently reduced. In agreement with our findings, numerous workers have also reported similar results (Tunali *et al*., 2006; Lodeiro *et al*., 2006; Gabr *et al*., 2008; Giotta *et al.,* 2011). Conclusively, the formation of varying spectra following adsorption of metal ions on the bacterial biomass validated the contribution of functional groups in metal binding. However, it is difficult to pinpoint the exact mechanism as to how metals are adsorbed onto the microbial biomass due to some unidentified peaks appearing in this experiment.

Table 3: Assignments of Infrared absorption bands

Wave numbers (Cm-1)	Intensity shape	Assignment
3500-3750	Sharp	O-H stretching
3100-3500	Strong-broad	N-H stretching
2850-2950	Variable	C-H stretching
1400-1660	Variable	N-H bending
1280-1430	Variable	C-H bending
1160-1420	Variable	O-H bending
900-1350	Variable	C-N stretching
900-1380	Variable	C-O stretching

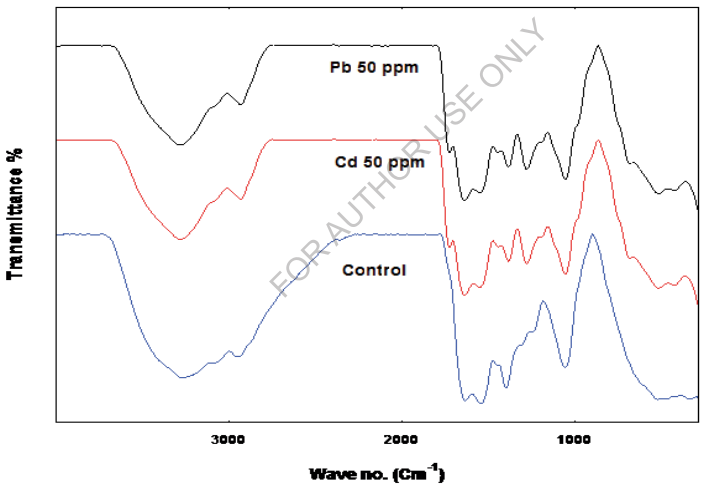

Figure 3: The FTIR Spectra of *B. thuringiensis* in Pb(II), with Cd (II) loaded and without metal loaded.

X-ray powder diffraction analysis (XRD)

The XRD spectra were used to confirm the crystalline nature of the biosorbent (un-loaded *Bacillus thuringiensis)* and loaded with Cd (II) and Pb (II) ions nanoparticles and the pattern is exhibited in Figure (4).The XRD spectrum of Cd (II) and Pb (II) nanoparticales exhibits strong peaks at 2-theta value of 29.9, 37.8, 44.1, 64.4 and 77.8° corresponding to (200), (420), (114), (640) and (822) planes, respectively. The XRD spectrum is compared with the excited spectrums of control that have been published by the Joint Committee on Powder Diffraction Standards (JCPDS file no. 00-002-097). The average crystal size of the un-loaded control, loaded with Cd (II) 50ppm, and Pb (II) 50ppm nanoparticle is estimated from the broadening plane (114) by using the Debye-Scherrer Eq. (1). $D= K \lambda / \beta \cos \theta$.

Where D is the average crystal size, k is the Debye constant (0.9), λ is the X-ray wave length (0.15438nm), β is the full width at half maximum of the peak (FWHM), and θ is the diffraction angle. The average size of the particles was around 54,55,45nm, for Cd (II) 50 mgl^{-1}, Pb (II) 50mgl^{-1} and control respectively.

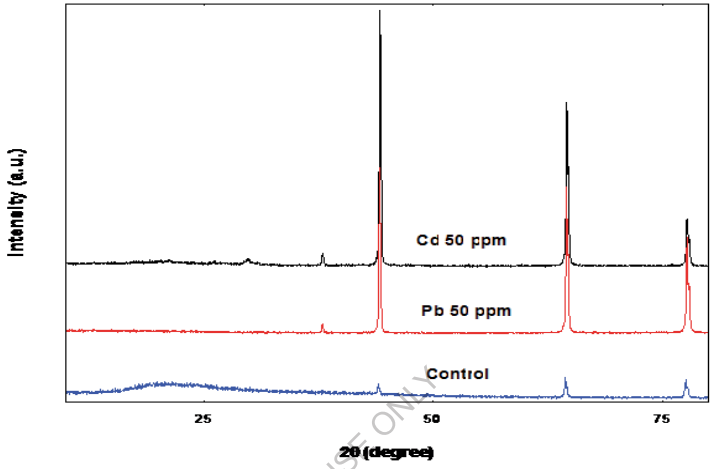

Figure 4: XRD analysis of *B. thuringiensis* biomass before and after Pb and Cd biosorption

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Effect of Quarterly Changes on the Concentration of Heavy Metals in Al-Zubaidi Fish (*Pampus argenteus*) collected from Iraqi marine coasts

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Abstract –

In the present study concentrations of some heavy metals were calculated in three tissues (Liver, gills, and muscles) of Al-Zubaidi fish *(Pampus argenteus*) which date back to the family Stromateidae, that collected from Iraqi marine coasts, during December 2014 to March 2015.The concentrations of the studied heavy metals (Cobalt, Iron, Nickel, Manganese, Copper, and Cadmium)were measured by Flame Atomic Specrtophotometer), and results showed that their highest concentrations were in the liver (16.71, 98.9, 88.13, 10.99, 30.22,and 12.69)µg/g respectively, while the lowest concentrations appear in white muscles (1.16, 5.2, 2.87, 40.68, 3.69, 55.23) µg/g. In general in the studied period, concentrations of the heavy metals were higher in liver than that in the muscles and gills. The order of seasonal concentration of heavy metals in the muscles were Summer> Spring> Autumn> Winter.

***Key Words**: Al-Zubaidi fish, heavy metals, Iraqi marine coasts, biological accumulation*

Introduction

Aquatic ecosystems are exposed to many toxic pollutants that affect living organisms at different levels, especially non-degradable pollutants, which cause serious problems, such as heavy metals [1]. Organisms require trace amounts of certain heavy metals such as calcium, copper, iron, manganese, molybdenum, vanadium, strontium and zinc: For example concentration of "copper, manganese, zinc, chromium, nickel and cadmium" in fish plays an important role in the function of different body tissues within certain concentrations [2]. Although these metals tend to accumulate in the bodies of these organisms, this depends on the type of the metals, the organism, and the object and here lies the seriousness [3].

Many fish species are among the top spent of trophic pyramids in aquatic ecosystem [4] and [5]. In consequence, they are endangered by diet-borne pollutants e.g. "heavy metals" which transferred through the food chain, and accumulate in different body organs,causes dangerous for the fish and in turn, they led to serious problems in both man and animals [6] [7].

Fish are an important source of protein in the world, meaning "fish can be used as the sole source of protein in the diet".In addition omega-3 fatty acids present in fish are very important for normal growth. [6] [7] [8]. Therefore, fish should be maintained from all form of pollution, even in small proportions.

As humans consume large amount from fish, which may concentrate high amount of metals present in aqua environment, so it is important to appointed the heavy metals concentration, which their consume lead to range of diseases including "central nervous system disorders, sclerosis, paralysis, tremor, Alzheimer's disease, heart disease, and immune disorders [9]. Accordingly in order to develop the freshwater fish culture industry, it is important to maintain water standards and quality and monitor the accumulation levels of these metals regularly [10].

Fish can be used to determine the state of water pollution and are therefore an excellent marker in the identification of contaminants including heavy metals in the water ecosystem [11]. The current study goal to study the seasonal changes in the concentrations of some heavy metals in the Al-Zubaidi fish assembled from the Iraqi marine coasts, and to compare them with previous

studied so that set referential study about the status of heavy metals pollution in the marine area through the fish lives there.

Material and Methods

To study heavy metals accumulation by the tissues of Al-Zubaidi fish,20 samples from this fish were used for each season,through the period from December 2014 to March 2015. The method mentioned in ROPME (1982) [12] was adopted for digestion the fish samples and as flow : 0.5 g of lyophilized and milled fish tissues samples (gills, liver, white muscle), were taken (three replicates),then digested in 3 ml of 1:1 ($HCLO_4$, and HNO_3) in glass tubes,tubes were placed in a 70 ° C water bath for 30 minutes, after that they were transfered to the heating plate to complete digestion process (until the mixture becomes clear). The leachate was taken and the volume was completed with deionized water to 25 ml, finally samples were kept in sealed plastic bottles until they were examined in the atomic absorption spectrometer. Statistical program (SPSS) was adopted for the statistical analysis of the results. The differences between the averages were tested using the least significant difference (LSD) at a significant level (0.05), as mentioned by Al-Rawi and Khalaf Allah (2000) [13].

Results

Figure1. shows that the concentration of cobalt during winter and spring is the lowest in the three parts. The concentration in the liver (11.99, 10.13, 5.22 and 16.71) µg/gm dry weight, while concentration in gills (16.12, 15.62, 11.13 and 19.30) µg/gm dry weight, and in the muscles were as follows (4.78, 2.87, ND and 5.54) µg/gm dry weight. Statically no significant differences were founded at the level of probability ($P < 0.05$) between the three tissues but at the same level of probability significant difference was found between cobalt, manganese and cadmium on the one hand and the rest of the metals on the other hand.

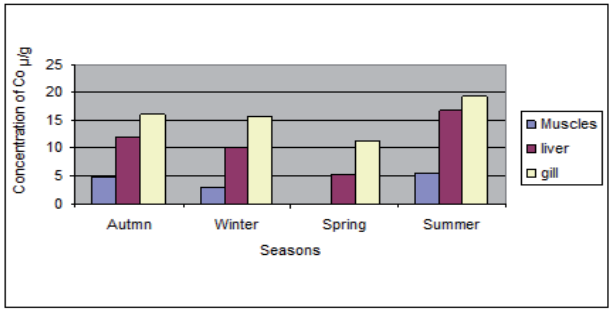

Figure 1. Concentration of Cobalt (µg/gm) dry weight in the muscles,liver,and gills,during four seasons

Figure (2) shows that the concentration of the Iron was high during the study seasons, the concentration in the liver were (98.9, 55.8, 71.75 and 94.65) µg/gm of dry weight. Also, and the concentrations of the metal in the gills were high (63.4, 50.5, 60.18 and 83.91) µg/gm dry weight. In the muscles, the concentrations were (41.35, 40.68, 45.07 and 72.1) µg/gm dry weight respectively. The results also showed a meaning difference in the level of probability ($P < 0.05$) between iron and the other metals.

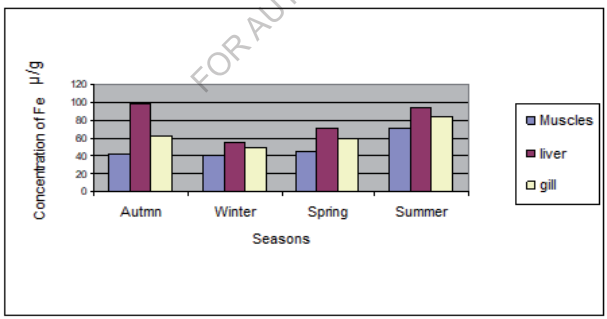

Figure 2. Concentration of Iron (µg/gm) dry weight in the muscles,liver,and gills, during four seasons.

The concentration of nickel was also high in the three studied parts of the fish body as in Figure (3), as it was in the liver (88.13, 70.45, 88.13 and 70.45) µg/gm dry weight,and in the gills (72.34, 78.91, 77.34 and 78.91) µg/gm dry weight, either in the muscles (55.23, 59.23,

65.23 and 69.23) µg/gm dry weight. A significant difference was found at the level of probability (P <0.05) between the nickel, iron and the other metals at the same level of probability. There was also a significant difference between winter and summer in muscles on the one hand and in liver and gill on the other.

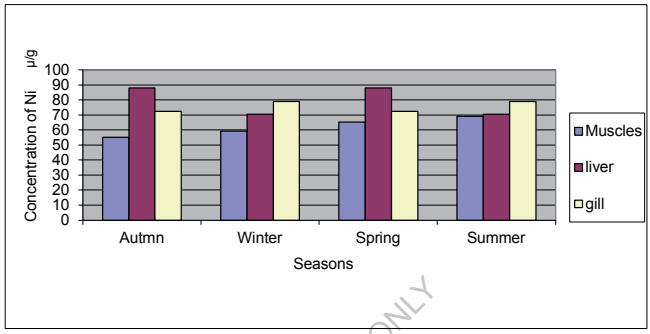

Figure 3. Concentration of Nickel (µg/gm) dry weight in the muscles,liver,and gills tissues. during four seasons.

Figure (4) shows the lowest values of Manganese in liver, gills and muscle (9.23, 8.00, 10.99 and 6.01), (13.62, 7.21, 8.97 and 7.00), (3.69, 3.69, 5.72 and 3.82) µg/gm dry weight respectively. There were no significant differences at the level of probability (P <0.05) between the three tissues.

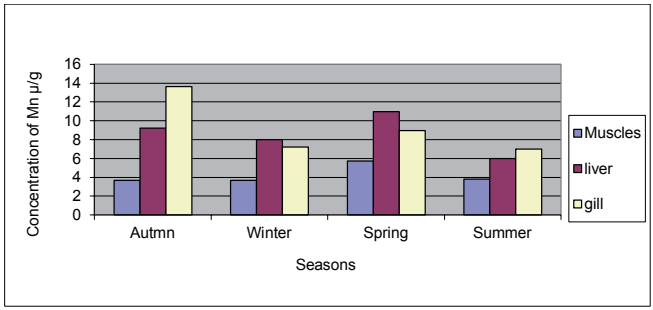

Figure 4. Concentration of Manganese (µg/gm) dry weight muscles,liver,and gills, during four seasons

The copper component recorded the following concentrations, as shown in Figure (5). Concentrate in the liver (20.1, 22.4, 28.43 and 30.22) µg/gm dry weight, while in gills (11.3, 16.3, 20.41 and 23.61) µg/gm of dry weight, and the muscles recorded (5.20, ND, 9.56 and 14.34) µg/gm dry weight, respectively. The results showed significant differences at the level of probability (P <0.05) between the spring and the rest of the seasons.

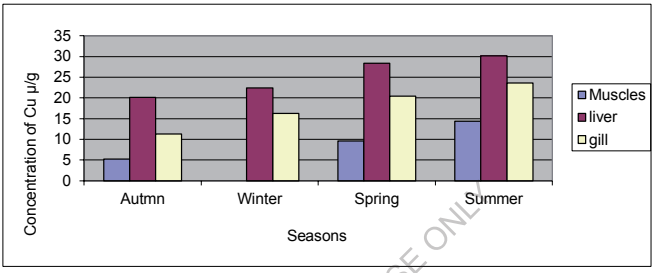

Figure 5. Concentration of Copper (µg/gm) dry weight in the muscles,liver,and gills, during four seasons

Concentrations of cadmium were volatile as they are shown in Figure (6). In the liver were (6.5, 7.2, 7.39 and 12.69) µg/gm dry weight, and in gills (9.34, 10.22, 8.82 and 8.51) µg/gm dry weight, and in the muscles (1.16, 2.32, 2.89 and 1.52) µg/gm dry weight, respectively, and significant difference was found at the level of probability (P <0.05) between autumn,winter,spring and summer and at the same level of probability significant difference was found between the muscles on one hand, and liver on the other.

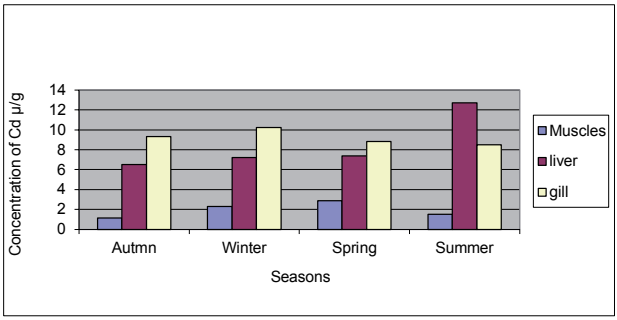

Figure 6. Concentration of Cadmium (µg/gm) dry weight in the Muscles,Liver,and gill tissues,during four seasons and

Discussion

Measuring the concentrations of metals in the fish play an important role in the life system, because it is necessary for humans who feed on these fish, some of which may be toxic and may cause serious problem to the human health [1]. Results of the present study indicate that there is variance in the concentration of heavy metals in the liver, gills and muscles. The levels of accumulation in different fish organ/tissue vary according to their "physiological role". There are other factors that can affect the level of accumulation,such as regulator ability, behavior and feeding habits differences [7],what is important here is their concentration in the muscles because this part is good to eat.When the present results were compared with previous works, the measured concentrations were found to be much less than these recorded by [3], where they recorded the concentration of cadmium, cobalt and copper in the muscles (4.57, 18.22 and 13.05) µg/gm respectively. Mohamed *et al*. (2009) [14] has found that the concentration of copper was (23.16, 78.46), and this is far less than the concentration of copper in the current study accordingly the results of the present study are within internationally permissible limits compared to the limits recommended by FAO/WHO (2004) [15]. This can be attributed to the high levels of salinity in the marine area, which helps to adsorb heavy metals on the suspended minutes in the water and thus deposition to the bottom, In addition, the fish under study is fed on jellyfish which lives in the surface of the water where low concentrations of heavy metals occure.

In relation to the distribution of metals in the studied tissues, results showed that the accumulation of metals was mainly in both liver and gill, and this may be due to the fabric nature of liver and its physiological composition, in addition to its location in the circulatory system, and the presence of enzymes that have the ability to combine with heavy metals and remove them outside the body [16]. This is consistent with the Mediha *et. al.* (2007) [17], where they founded that the highest concentration of iron, copper and nickel in the liver and the lowest concentration was found in the muscles of Cyprinidae species. In the case of gills, the high concentration of heavy metals recorded in them due to the association of metals with the mucus layer forming "complexities that are difficult to remove from the gill lamellae when preparing the samples for analysis [6].The adsorption of metals onto the gills surface, as the first target for pollutants in water, could also be an important influence in the total metal levels of the gill [18] The nature of the tissue cells, as well as the site of ion exchange in the tissues of the gill and existing enzymes, which have the ability to hold the metals. The concentration of metals in gill also differs by varying the concentration of metals in the water. Most fish are able to concentrate heavy metals in their bodies through their diet or through gills [19]. Target organs, such as liver and gills, are metabolically active tissues and accumulate heavy metals in higher levels, as shown in many species of fish in different areas: like in *M. cephalus* in the Mediterranean Sea [20], *Cyprinus carpio* and *Tinca tinca* from Lake Beysehir Turkey [21],and this is also consistent with the study of [22] and [23], where they indicated that the concentration of the metals is recorded heavily in the liver and gills and lower in the muscle for different kinds of fish.

From the result of the present study we can observe that it is very difficult to compare the concentrations of heavy metals even between two tissues of the same type of fish, and this is because of differences in the type of tissue and its anatomical and physiological composition, and this led to presence different link locations to the metals within the tissue, in addition to difference in the body reaction toward the heavy metals and the differences within the same type in the absorption of metals and to the type and level of water pollution, this is consistent with the study of Al-Najare *et. al*. (2013) [24] in their studied on the *Acanthopagrus latus* fish from the Iraqi waters. Muscles, in the present study contained the lowest levels of heavy metals, this result agree with many authors who reported that the muscles is not an active tissue in accumulation heavy metals [5] [6].

Present result also indicates that the highest concentrations of the metals was in the liver and the lowest concentration found in the muscle tissue except the metals Cadmium, and Cobalt have the highest concentration in gills and this due to feeding habits [25], environmental needs and metabolism [26], age, size and length of fish [27].

As far as the relation between the seasons and heavy metals accumulation in fish tissues, results show that the highest values of metals were recorded during spring and egg laying season, this is consistent with the findings of [14]. In this period, the proportion of fats in the body's to proteins and carbohydrates increase, and heavy metals usually concentrated in fat [28]. Ansari *et. al*. (2004) [21] reported that variations of the metals concentration at given site may often be due to seasonal changes of the organisms tissues weight rather than to the variability in the absolute metal content of the organism, also noticed that fish with large lengths have a lower concentration of heavy elements than fish of lower lengths, this is due to the proportion of body size and the development of organs. The amount of contaminants in large fish may be larger than small fish in terms of total concentration, but its concentration in the unit area is less. The seasonal variations of heavy metals in fish were reported by many authors [5] [29] [30], and this also depends on the ready element in the environment during the season [31]. The minerals accumulated in living tissue can exceed their water content by 102 times for iron, 103 for cadmium and 105 times for zinc and manganese. There is a special relationship between the accumulation of heavy elements and their concentration in body parts [32].

Conclusions

This survey permitted to assess the degree of temporal and spatial contamination by heavy metals (Co, Fe, Ni, Mn, Cu, Cd) in Al-Zubaidi fish *(Pampus argenteus*) that collected from the Iraqi Marian water, and from the results we can conclude the following.

1. Al-Zbede fish accumulate trace metals specifically in liver and gills;while muscle contains the lowest concentration of trace metals compared to that recorded in the liver and gills.
2. The fish tissues are not contaminated that the accumulated metals were in the tissues within international limits and it does not show any danger for human consumption. Although the concentration of the metals in both gills and liver is within the permissible limits, it is not recommended to take them because it may be harmful to health.

3. The tendency of the metals to accumulate significantly in both the gills and the liver indicates that the source of those metals is artificial and it has entered the water and may be diluted by water but concentrated in the tissues of the fish being inclined to accumulate inside it.
4. The accumulation of the metals in the fish tissues can reflect the amount of pollution in the aquatic environment and the diversity of its sources and therefore its use as vital evidence of pollution in environmental monitoring programs.
5. The observation of the accumulation of metals in different parts of the fish indicates the presence of those metals in a free and ready image in the water that the fish can be taken directly.

Recommendation

1. It is recommended to intensify research on aquatic organisms, especially fish of all kinds and genotypes, in order to determine the extent of their contamination with various heavy metals in order to prevent their access to the food chain.
2. Conduct further studies on the different age stages of this species of fish.
3. Follow-up of environmental studies in the region and on the coasts area with the study of pollutants at the highest tide to determine the amount of pollutants that water brings with them.

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تأثير التغيرات الفصلية على تركيز المعادن الثقيلة في أسماك الزبيدي (*Pampus argenteus*) التي تم جمعها من السواحل البحرية العراقية

الخلاصة

درست تراكيز بعض العناصر الثقيلة (Cd, Cu, CO, Fe, Mn, Ni) في ثلاثة انسجة (كبد, غلاصم, عضلات) من جسم اسماك الزبيدي للفترة من كانون الاول 2014 الى اذار 2015, قيست تركيز العناصر بواسطة جهاز مطياف الامتصاص الذري Flame Atomic Absorption Spectro photometer , اظهرت النتائج ان اعلى القيم لتراكيز العناصر المدروسة في الكبد اذ بلغت (88.13, 10.99, 98.9, 11.99, 30.22, 12.69) مايكروغم/غم على بنفس الترتيب في الكبد في حين كانت اقل القيم للعناصر في العضلات البيضاء هي (55.32, 6.01, 55.8, 2.87, 0, 1.16) مايكروغم/غم في الاسماك .وبصورة عامة كان تركيز العناصر في الكبد خلال فترة الدراسة اعلى منة في العضلات والغلاصم وان ترتيب الفصول في تركيز العناصر الثقيلة في العضلات كان كالتالي صيف> ربيع> خريف> شتاء.

الكلمات المفتاحية: اسماك الزبيدي , المعادن الثقيلة, السواحل البحرية العراقية

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