academicJournals

Vol. 7(23), pp. 2875-2884, 4 June, 2013
DOI: 10.5897/AJMR12.1352
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Uptake of some heavy metals by metal resistant *Enterobacter* sp. isolate from Egypt

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Accepted 8 March, 2013

The bacteria displaying a high growth rate were isolated from polluted industrial waste water. The bacteria *Enterobacter* sp. had variable resistant to heavy metals such as Cd, Cu, Co, Zn and Pb. Out of which the minimal inhibitory concentrations were 0.4, 1.0, 1.0, 2.0 and 3.0 mM, respectively. The potent bacterium had optimal biosorption capacity raised according to the metal, incubation temperature, pH of the solution and contact time. Under the optimal conditions, the bacterium was capable of taking up the heavy metals Cd, Cu, Co, Zn and Pb at 2.69, 1.87, 3.56, 4.3 and 5.6 mg/g dry weight respectively. Transmission electron microscopy showed accumulation of Pb within and external to bacterial cells. The mechanism of heavy metal tolerance in *Enterobacter* sp. was plasmid encoded, the occurring band marked at RF had values of about 2700 Kbp.

Key words: Heavy metals uptake, heavy metals resistance, bacterial biosorption, plasmid, *Enterobacter* sp.

INTRODUCTION

Mobilization of heavy metals in the environment due to industrial activities is of serious concern due to the toxicity of these metals in humans and other forms of life. Removal of toxic heavy metals from industrial waste waters is essential from the standpoint of environmental pollution control (Puranik and Pakniker, 1999; Guangyu and Thiruvenkatachari, 2003). Heavy metals mercury, nickel, lead, arsenic, zinc, cadmium, aluminum, platinum, copper and cobalt are trace metals with a density of at least five times that of water, they are stable elements (meaning they cannot be metabolized by the body) and bio-accumulative (passed up the food chain to humans). These include: mercury, nickel, lead, arsenic, zinc, cadmium, aluminum, platinum, copper and cobalt. Heavy metals have function in the body and some heavy metals can be highly toxic for human health (Parry, 2009; Hornung et al., 2009). Toxicity of metallic ions could be the result of competition with or replacing a functional metal as well as causing conformational modification, denaturation, and inactivation of enzymes and disruption of cellular and organelles integrity (Blackwell et al., 1995).

Remediation technologies using microorganisms are feasible alternatives to the physical cleaning of soil or the concentration of metals in polluted water by physical or chemical means (Valls and de Lorenzo, 2002; Zooboulis et al., 2007; Abou zeid et al., 2009; Adewole et al., 2010). Metal tolerance reflects the ability of an organism to survive in an environment with high concentration of metals or to accumulate high concentration of metal without dying. Metal exposure also leads to the establishment of tolerant microbial populations, which are often represented by several Gram positives belonging to *Bacillus*, *Arthrobacter*, and *Corynebacterium*, as well as Gram negatives as *Pseudomonas*, *Alcaligenes*, *Ralstonia* and

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Burkholderia (Kozdro and Van Elsas, 2001; Ellis et al., 2003; Ajaz, et al. 2010). In contaminated sites, these populations may be involved in the alteration of mobility of metals through their reduction, accumulation, and in situ immobilization by extracellular precipitation (Roane, 1999). Different microorganisms such as fungi, yeast and bacteria were tested for the availability and biosorption potential to bind heavy metals (Volesky and Holan, 1995). There are at least three types of microbial processes that can influence toxicity and transport of metals and radionuclide's: biotransformation, bioaccumulation and biodegradation. However, microorganisms can interact with these contaminants and transform them from one chemical form to another by changing their oxidation state through the addition of (reduction) or removing of (oxidation) electrons. Several authors have reported high capability of heavy metals bioaccumulation by Gram negative bacteria (Noghabi et al., 2007; Choi et al., 2009; Elshanshoury et al., 2012). Bacterial resistance to heavy metals might be chromosomal or plasmid mediated (Raval et al., 2000; Zouboulis et al., 2003). Zolgharnein et al. (2007) reported that the frequency of occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria.

The main objective of this study was to obtain a local bacterium resistant to heavy metals, in order to use for remediation of metal ions in polluted habitats in Egypt. In this connection, the potential of *Enterobacter* to absorb and uptake Cd, Cu, Co, Zn, and Pb was focused, with special emphasis to Pb. The mechanism and the form of Pb accumulation by the bacterium are discussed.

MATERIALS AND METHODS

Source of bacterial isolate

The tested isolate used in this study was isolated previously from location in Egypt polluted with heavy metals. *Enterobacter* sp. (accession no. GQ360072.1, GQ478272.1, GQ478258.1) was isolated from industrial waste water. The isolate was identified by sequencing their 16S rRNA genes and comparing the sequences with database library using analysis software. The program Blast was use to assess the DNA similarities, and multiple sequence alignment and molecular phylogeny was performed using BioEdit software (Hall, 1999).

Samples collection

Samples were collected from 3 different polluted locations: a lathe and Motors manufacturing workshops in Tanta, El-Gharbia Governorate, and industrial wastewater of Industrial Area in Quesna, El-Monofeya Governorate. The samples included dusts containing metal filings, grinding metals and industrial wastewater.

Isolation of bacteria

The isolation of bacteria was carried out on nutrient agar medium adjusted at pH7.3 ± 0.2. One gram of each dry contaminated dust was dissolved in 50 ml sterile distilled water, and then 250 µl from the resultant suspension were spread on the surface of nutrient plates. The plates were incubated for 24 h at 35°C. Growing colonies were investigated for their morphological characteristics, purified and kept at 5°C as slant cultures.

Isolation and selection of metals resistance bacteria

All the isolated bacteria were checked for their metal tolerance against five selected metals; Cadmium, Copper, Cobalt, Zinc and Lead. Aqueous solutions of the metal salts; $CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2$, $ZnSO_4.7H_2O$, and Pb $(NO_3)_2$ were prepared in de-ionized water and 0.1 mM/L were added to the nutrient agar medium. Media were sterilized, dispensed in Petri plates and then inoculated with bacteria; the plates were incubated at 35°C for 24 h. The tolerance was checked on the basis of growth observed within 24 h. The growing colonies were used for detection of minimal inhibitory concentrations (MICs) for each metal. The most highly tolerant bacterium (with highest MICs) was selected for identification and further study.

Preparation of bacterial culture

One liter of nutrient broth medium free of metals was prepared and sterilized by autoclaving. Medium was inoculated with 24-h-old culture previously prepared. Culture was then grown to the end of exponential phase for *Enterobacter* sp. (growth curve data not shown).

Determination the minimum inhibition concentration (MIC)

The minimum inhibition concentration was checked for their metal tolerance against five selected metal salts $CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2$, $ZnSO_4.7H_2O$, and Pb $(NO_3)_2$. Aqueous solutions of these salts were prepared in de-ionized water, the pH values of the solutions were adjusted to the optimum values 7. The flasks were incubated at 35°C with *Enterobacter* sp. for 24 h. The growing colony was using minimum inhibitory concentrations (MICs) detected for each metal separately by increasing the concentration of each metal salts gradually in media till MIC was achieved as visualized by cessation of growth according to the study of Chowdhury et al. (2008). The highly tolerant isolate (with the highest MICs values) were selected for characterization, identification and further experiments.

Electron microscopy

The highly uptake isolate (of the five metal ions mg/g dry wt), (with following order: $Pb^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Cu^{2+}$) were selected for characterization, and identification. Before and after the treatment with $Pb(NO_3)_2$, cell of isolate was examined by scanning electron microscope (SEM) to detect any change in the morphology of the cells as a result of metal treatment. The control and treated cultures were also examined by Transmission electron microscopy (TEM) in order to identify the location of lead particles within the cells (Chowdhury et al., 2008). Cells of control and treated cultures (as described before) were centrifuged, washed twice and fixed in 2.5% buffered glutaraldehyde in 0.1 M PBS (phosphate buffer solusion) pH:7.4 for 24 h at 4°C, washed three times with PBS for 10 min each time and then centrifuged. These steps were followed by post fixation in 1% osmic acid for 30 min. Dehydrated in a series of ethyl-alcohol (30– 100%) infiltrated with acetone each concentration for 30 min.

Transmission electron microscopy (TEM)

After dehydration, samples were embedded in Araldite resin. The plastic molds were cut at 850 nm thicknesses in a LEICA Ultracut ultra-microtome, and stained with 1% toluidine blue. After examination of semi-thin sections, ultrathin sections were cut at thickness of 75 nm, stained with uronyl acetate for 45 min, then counter stained with lead citrate and examined. Images were taken using a JEOL, JEM-100 SX electron microscope. All the isolated bacteria that were able to grow on media supplemented with different salt decreased gradually by increasing the concentration of each metal salt. All isolates were low tolerant to all metals except one isolate which showed higher tolerance, in comparison to other isolates. The highly tolerant organism for most metals and they were thus selected for characterization, identification and further experiments.

Sequencing of 16S-rRNA gene and phylogenetic analysis

The bacterium selected as the most resistant isolate to all five heavy metals was identified and confirmed using Biolog automated system Bochner (1989). The selected isolate was identified by sequencing of 16Sr-RNA gene. Bacterial genomic DNA was extracted from the cells by using Qiagen kit. The DNA is used as template for PCR using universal primers. The forward primer is 5'-AAC TGG AGG AAG GTG GGG AT-3', while the reverse primer is 5'-AGG AGG TGA TCC AAC CGC A-3'. The purified product of the PCR is used as template in cycle sequencing using (3130 X / Genetic Analyzer, Applied Biosystems, Hitachi, Japan), in which Big dye terminator cycle sequences technique, developed by Sanger et al. (1977). After making gel electrophoresis, the sequencing reaction was performed with four fluorescent labels identified ddNTPs. The PCR products were sequenced by using the same PCR primers.

The products were purified using special column. Ten mucroliter (10 µl) of PCR product after thermal were taken and 10 µl of high dye formamide were added (1:1 V/V) and run in 95°C for 5 min for denaturation, shock on ice, then the sample became ready for sequencing in 3130 X DNA sequencer and analysis. This consensus sequence was then compared with a database library by using analysis software. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). Well-known database of 16S rRNA gene sequences that can be consulted via the World Wide Web to detect the sequence homologies is GenBank.

Plasmid isolation

The plasmid DNA of the selected isolate was extracted, purified and separated using agarose gel electrophoresis according to the method employed by Manniatis et al. (1982), for isolation and screening plasmid. The developing bands were compared with DNA marker.

Optimization of metal uptake

Effect of different incubation temperature

One milliliter of the aliquots of *Enterobacter* sp. selected isolate suspension 6 h. old (exponential phase) were inoculated in 100 ml nutrient broth media containing sub-MICs concentrations of $CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2$, $ZnSO_4.7H_2O$, and Pb $(NO_3)_2$ respectively. After the addition of metal solutions Media was adjusted at pH=7 by using 0.1 N NaOH and 0.1 N HCl and (0.1 N HNO_3 with Pb $(NO_3)_2$. The cultures were incubated at different temperatures (25, 35 and 45°C) for 24 h. The incubated cultures were centrifuged at 5000 rpm for 20 min. The supernatants were used for the determination of the residual metal ion contents by using atomic absorption spectrophotometer (Perkin Elmer 2380) with hollow cathode lamp at specific wavelength for each metal. Control cultures without the inoculation of bacteria were prepared to detect the initial metal concentration.

Effect of different pH values

To test the pH values of nutrient broth, media containing the metal solutions were adjusted at different pH values (2, 5, 7, 8 and 9). All the cultures were incubated at 35°C for 24 h. The initial and the residual metal concentrations were measured.

Effect of contact times

Media containing metal solutions adjusted at pH=7 and inoculated with selected isolate was incubated at 35°C for different periods (12, 18, 24 and 48 h). The initial and the residual concentrations were measured as mentioned before.

Determination of metal uptake by the resistant bacteria

The uptake of Cd, Cu, Co, Zn and Pb metals in (mg/g dry wt.) were detected. According to each metal, bacterial culture (6 h old) was adjusted at the optimal pH, incubated temperatures and optimal periods of time. The cultures were centrifuged at 5000 rpm for 20 min. The supernatants were discarded and the residual bacterial pellets were washed with sterilized distilled water and then the bacterial biomasses were transferred to known weight. The supernatants were used for the determination of the residual metal ions contents in mg/l. The initial metal ions contents in mg/l were determined in control without bacterial cell. Supernatants were passed through bacterial filters (0.22 Mm diameter) in each case by using Atomic Absorption spectrophotometer (model Perkin Elmer 2380) (Abou Zeid et al., 2009). The metal uptake in (mg/g dry wt.) was calculated according to the equation of Volesky and May-Phillips (1995):

Metal uptake (mg/g) $=V (C_I - C_F) / w$

Where, C_I is the initial metal concentration (mg/l); C_F is the final metal concentration (mg/l); V is the volume of reaction (l); W is the total biomass (g).

RESULTS AND DISCUSSION

The pure isolated strain obtained from the polluted location was studied. The morphological characters of the isolated strain was found to be tolerant to all the five metal salts at concentration equal to 0.1 mM/L giving growth after 24 h of incubation. Different concentrations of each metal solution were prepared, the minimum concentration of each metal added was 0.1 mM/L and the concentration was gradually increased till MIC was achieved. The isolated strain was found to give low tolerance with $CdCl_2$ and was found to be highly tolerant to $Pb(NO_3)_2$. The MICs of Cd^{2+}, Cu^{2+} Co^{2+}, Zn^{2+} and $Pb^{2+}$was 0.4, 1.0, 1.0, 2.0 and 3.0 mM/L respectively.

This varying response of tested bacteria might be due to variation in resistance mechanisms (Abou Zeid et al.,

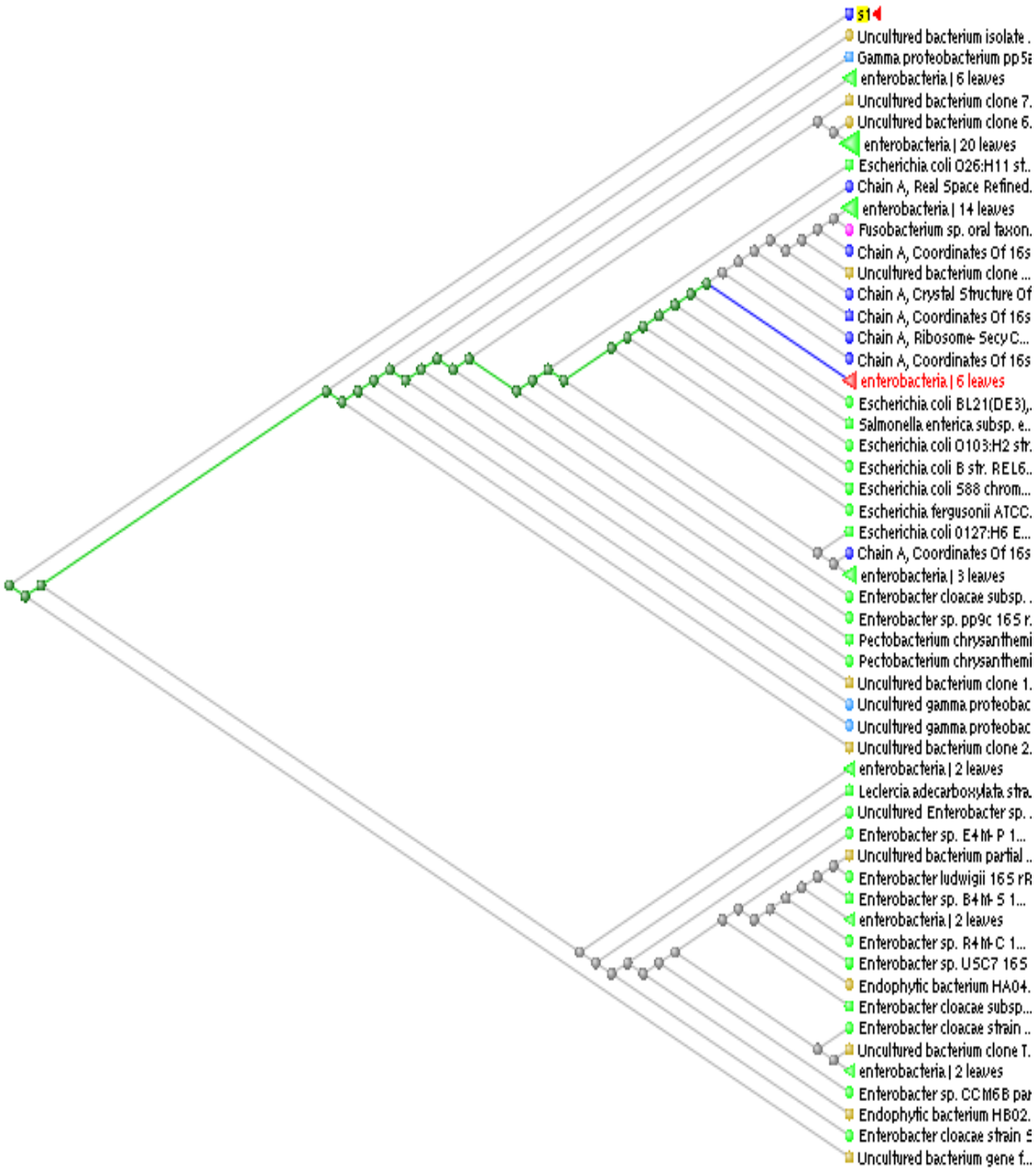

Figure 1. Phylogenetic tree based on 16S-rRNA gene partial sequences obtained from the respective band match to the national center for biotechnology information (NCBI) nucleotide sequence database (GenBank).

2009). Cellular morphology of isolated strain was observed, the cells shape are large bacilli, white, slightly raised, opaque, Gram negative strain. The phylogenetic analysis of the 16Sr-RNA gene partial sequence of isolated strain revealed close similarity to *Enterobacter* sp. (95% similarity) (Figure 1). For phylogenetic analysis, the 16S rRNA gene sequence of a single band was compared with those retrieved from GenBank database with accession numbers GQ360072.1, GQ478272.1 and GQ478258.1. According to Figure 1 the sequences have high similarity or are even identical to cultivable bacterial organism. A single DNA band of MW (~ 320 bp) was obtained (Figure 2). Resistance to heavy metals might be chromosomal or plasmid mediated (Gupta et al., 1999). Zolgharnein et al. (2007) reported that the frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria. Figure 3 reveals the presence of plasmid DNA in the extracts of

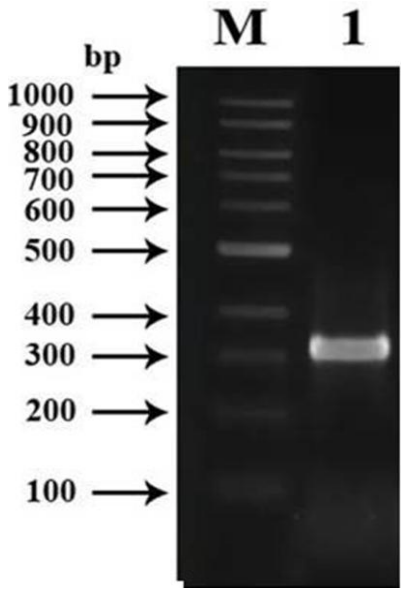

Figure 2. Agarose gel electrophoresis for PCR products of 16S-rRNA gene analysis M: 1 kbp DNA marker, lane 1: PCR product of *Enterobacter* sp.

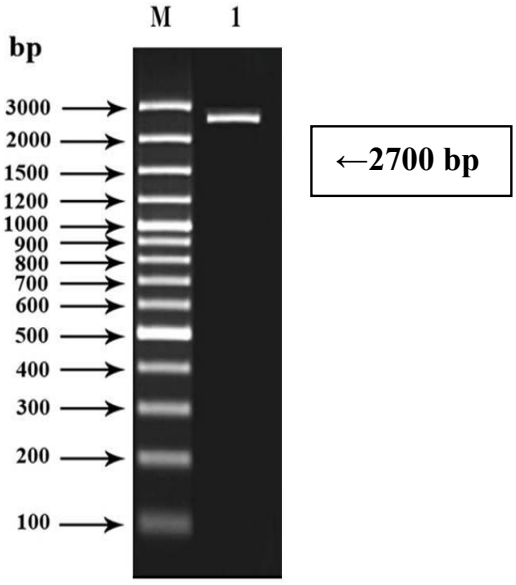

Figure 3. Agarose gel electrophoresis of plasmid profile for *Enterobacter* sp. M: DNA marker (100 bp), lane 1: Plasmid of *Enterobacter* sp.

Enterobacter sp. In agreement, Silver (1996) reported that bacterial plasmids encoded resistance systems for several toxic metal ions including Ag^{+}, As^{2+}, Cd^{2+}, Cr^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Sb^{3+}, Te^{2+} and Zn^{2+}. Resistance to heavy metals might be mediated by genes encoded on chromosomes, plasmids or transposons (Tenover and McGowan, 1996; Ghosh et al., 2000). These plasmids carried genes responsible for resistance to high levels of toxic heavy metals (As^{2+}, Cr^{2+}, Cd^{2+} and Hg^{2+}) as well as ampicillin antibiotic. The correlation between resistance to Cd^{2+}, Zn^{2+} and Pb^{2+} and the presence of plasmid in *Enterobacter* sp. was examined. The ability of bacteria to grow in the presence of $Cd^{2+}$and Zn^{2+} was plasmid encoded, whereas the ability to grow in the presence of Pb^{2+} appeared to chromosome encoded. Wasi et al. (2008), revealed the presence of one DNA band in plasmid profile of metal multi resistant *Enterobacter* sp. The occurring band marked at RF values of about 2700 Kbp.

The capacity of living cells to remove metal ions from aqueous solutions is also influenced by environmental growth conditions, as temperature, pH and biomass concentrations (Chen and Ting, 1995). In this present study the growth and metal uptake capability of the resistant *Enterobacter* sp. were affected by the different environmental conditions (incubation temperature, pH value and contact time). The effect of different incubation temperatures on the uptake of the five selected metals (Figure 4) revealed that 35°C was the optimum tempera-ture for Cd^{2+}, Co^{2+} and pb^{2+} while 25°C was optimum for Zn^{2+} uptake, and this is in agreement with the study of Sag and Kutsal (1995) who mentioned that, maximum biosorption rates for Ni^{2+} and Cu^{2+} by *Zooglea ramigera* could be obtained at 25°C, an activated sludge bacterium could biosorb copper at 25°C. The best temperature for Ni^{2+} uptake by the bacterium *Bacillus thuringiensis* was found at 35°C (Öztürk, 2007). The temperature effects are confined to metabolism-depended metal accumulation (Norris and Kelly, 1977). Also, 25 -35°C was optimum for Cu^{2+} uptake by *Enterobacter* sp. This result agrees with the study of Sag and Kutsal (1995) who reported that the maximum Cu^{2+} uptaking by *Z. ramigera* was between 25–45°C. Furthermore, *Arcanobacterium bernardiae* and *B. amylolikuefaciens* achieve their maximum capacity for lead uptaking at 35°C (Aksu et al., 1991; Tohamy et al., 2006; Jackson et al., 2011).

The pH value is one of the main factors in the biosorption efficiency and binding to microorganisms (Babich and Stotzky, 1985; Lopez et al., 2000; Jalali et al., 2002; Pardo et al., 2003). Results indicate that pH=8 and range 7-8 are the optimum for Cd^{2+} and Cu^{2+}uptake (Figure 5), which agrees with that of Ozdemir et al. (2003) in which the optimum adsorption pH value of Cd^{+2} by *Ochrobactrum anthropi* and *Enterobacter* sp. were 8. The maximum removal capacity of Cd^{+2} was obtained at pH=7 wherepH=8suppressivevaluefor*Pseudomonasmendocina* (Abou Zeid et al., 2009). Congeevaram *et* al. (2007), found the optimal pH value for the bioaccumulation of Cr^{6+}andNi^{2+}byheavymetalresistantbacterium*Micrococcus* sp. was pH=7. Silva *et al.* (2009) revealed the chromium level sorbed by *P. aeruginosa* AT18 biomass with 100% removal was in pH range of 7-7.2. At low pH, the Cd^{2+} accumulation decreased and caused increased competition between hydrogen and cadmium ions for binding sites on the cell surface or by an increase in metal efflux

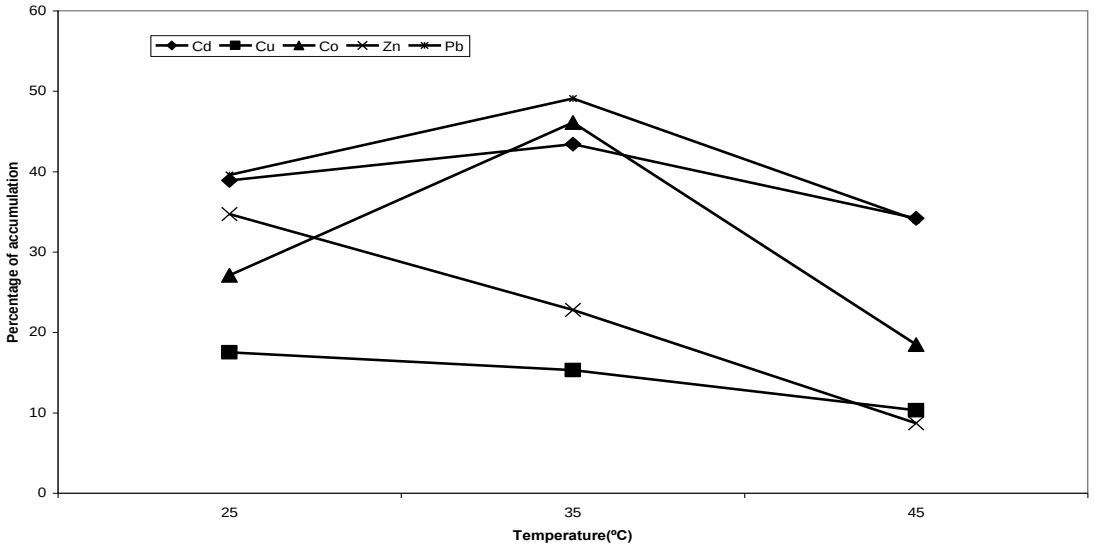

Figure 4. Effect of different temperatures on heavy metal accumulation by *Enterobacter* sp.

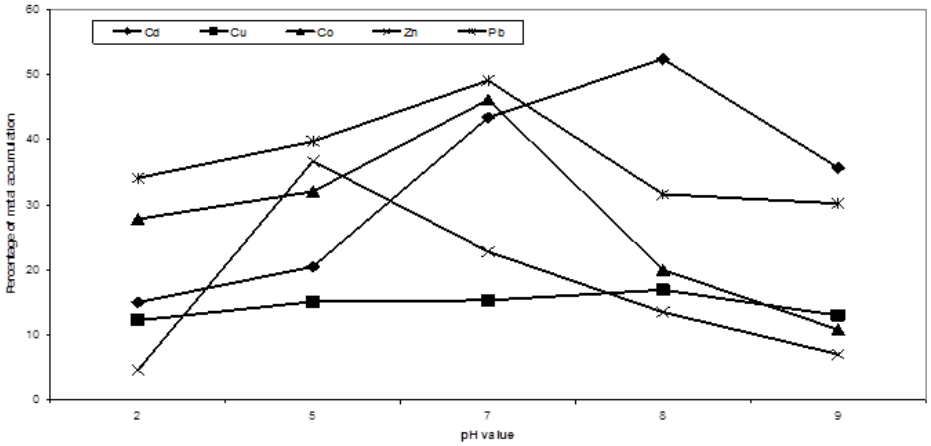

Figure 5. Effect of different pH values on heavy metal accumulation by *Enterobacter* sp

pump activity due to an increase in the proton gradient that drives the efflux pump. The pH=7 was optimum for Pb^{2+} and Co^{2+} accumulation by *Enterobacter* sp., while pH 5 was optimum for Zn^{2+} uptake. It was reported that pH 4.5 was optimum for biosorption of lead by *Citrobacter* strain MCM B-181 and pH < 3 or > 5 resulted in lower biosorption efficiency of lead (Puranik and Pakniker, 1999; Guangyu and Thiruvenkatachari, 2003). Highly acidic pH (<3) lead ions compete with H on the binding sites of microbial cells. However, at higher pH (>5) solubility of lead was lowered (Change et al., 1997). The optimum pH value for accumulation of lead by *Chryseomonas luteola* was 7 (Abou Zeid et al., 2009), while at higher (alkaline) pH, hydroxyl groups dominate in the solution and complex metal cations, these hydroxide complexes reduce the free metal ions available for sequestration preventing their attachment to cell wall surface (Wang and Chen, 2006). The variation in external pH can also affect the degree of protonation of potential ligand that contribute to metal binding (Tobin et al., 1984).

The contact time between the bacterial cells and the metal solutions is an important factor affecting the metal uptake. Figure 6 shows the maximum uptake for Cd^{2+},

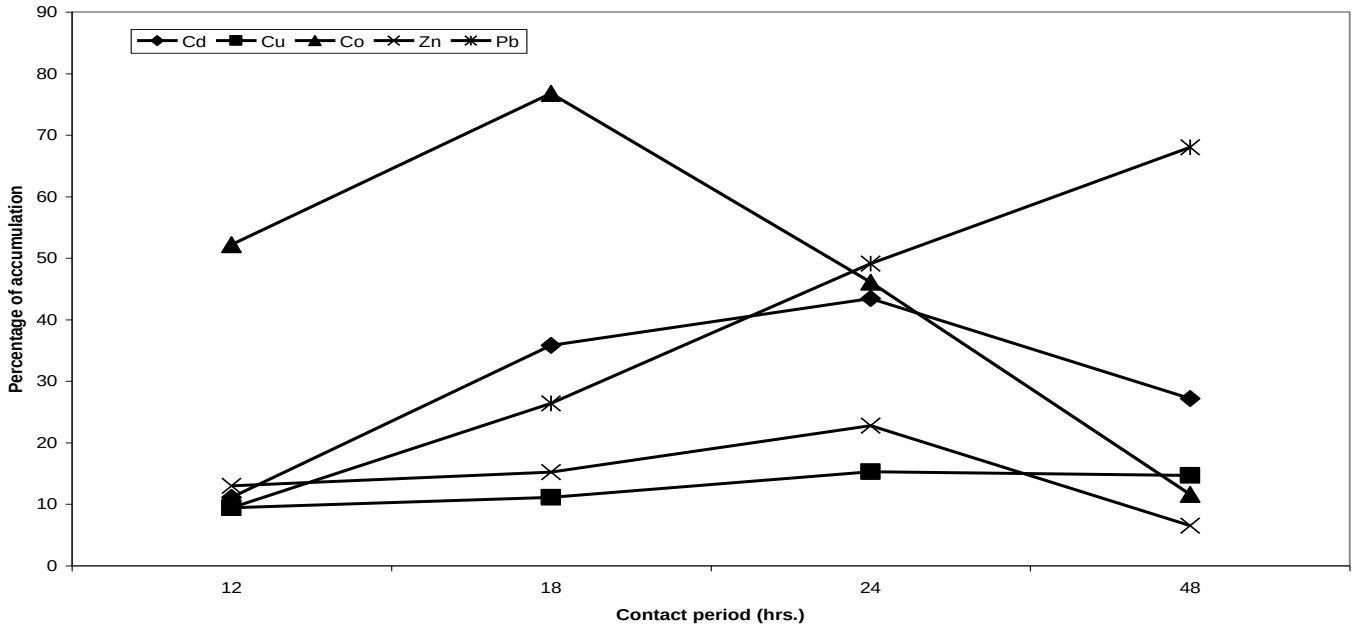

Figure 6. Effect of different contact periods on heavy metal accumulation by *Enterobacter* sp.

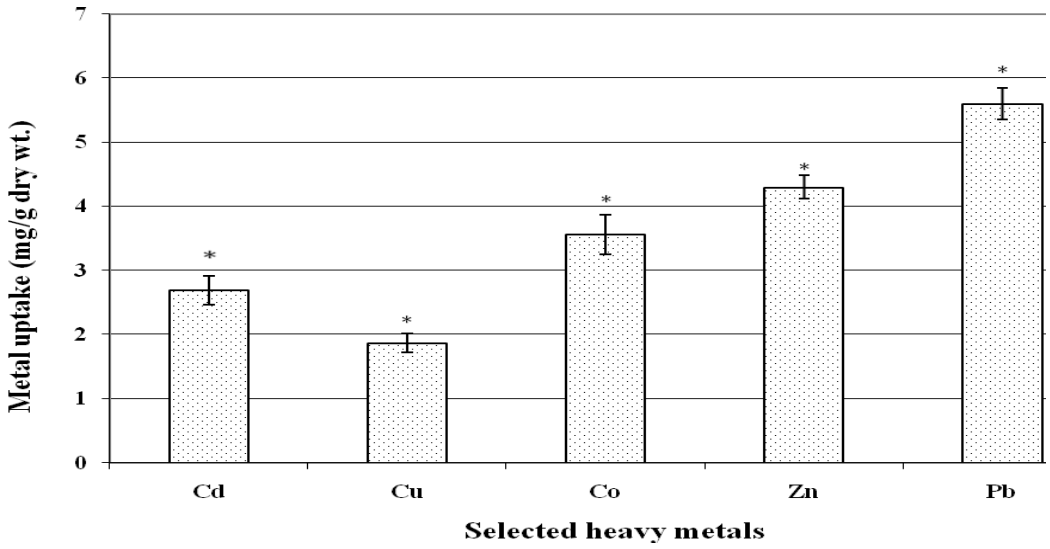

Figure 7. Metals uptake by *Enterobacter* sp. under the optimum conditions.

Cu^{2+} and Zn^{2+} occurred after 24 h. However 18 and 48 h was optimum for Co^{2+} and Pb^{2+} uptake respectively. Cell age is considered as an important factor that affects metal accumulation. During the detection of metal uptake with *Enterobacter* sp. as illustrated in Figure 7, it was found that Pb^{2+} was the most highly uptake than Cu^{2+}. The uptake of the five metals by *Enterobacter* sp. was in the following order Zn^{2+}> Pb^{2+} > Co^{2+} > Cd^{2+} > Cu^{2+} with different uptake values of 1.87, 2.69, 3.56, 4.3 and 5.6 mg/g dry weight, respectively. This difference in the uptake may be due to the difference in mechanisms by which the bacteria can tolerate the different heavy metals.

The synthesis of Pb nanoparticles by *Enterobacter* sp. was detected by examining the cells of *Enterobacter* sp. before and after treatment with 0.4 mM of $Pb(NO_3)_2$, with TEM. The nanoparticles accumulation on the cells of *Enterobacter* sp. was found. An important finding was detected, the bacterium was able to synthesize nanostructure particles from Pb in Figure 8, it was clear that these nanoparticles were synthesized intracellularly within the periplasmic space of *Enterobacter* sp. cells and then released outside when the cell wall decayed as a result of lead exposure. The X- ray powder diffraction (XRD) analysis of the dried $Pb(NO_3)_2$-treated cells indicated

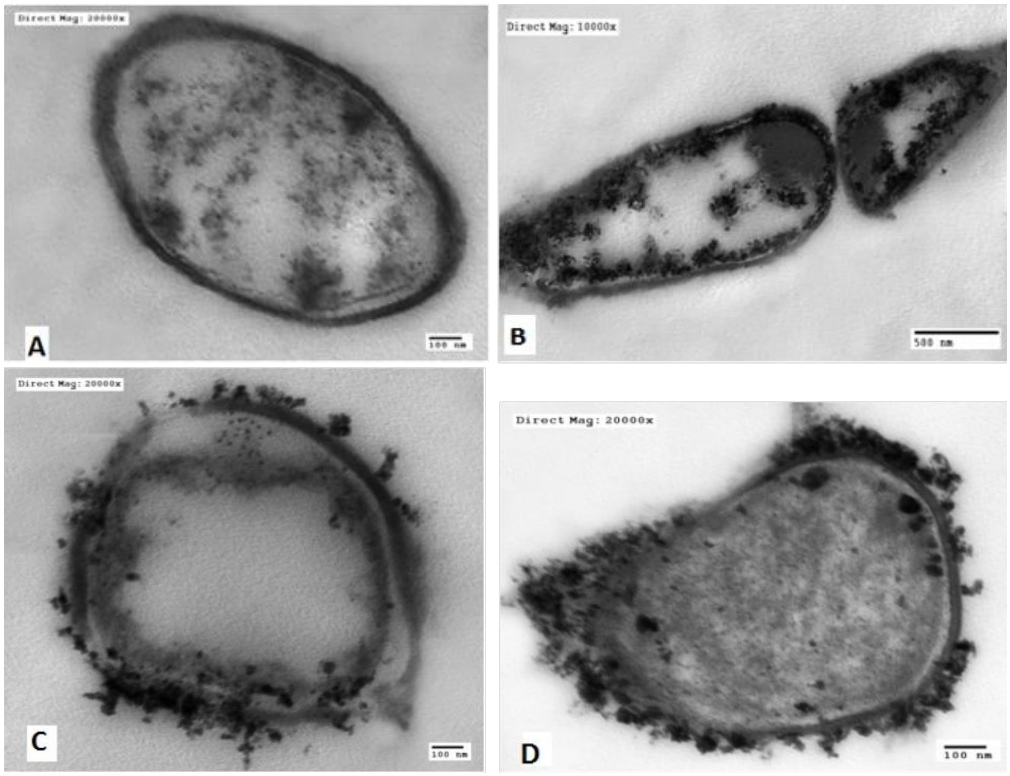

Figure 8. Transmission electron micrographs of *Enterobacte*r sp., **A**: untreated cell; B,C&D: Pb-treated cells; **B:** showing intracellular accumulation of nanoparticles within periplasmic space; **C&D**: the nanoparticles getting out of the cell as a result of cell wall decay.

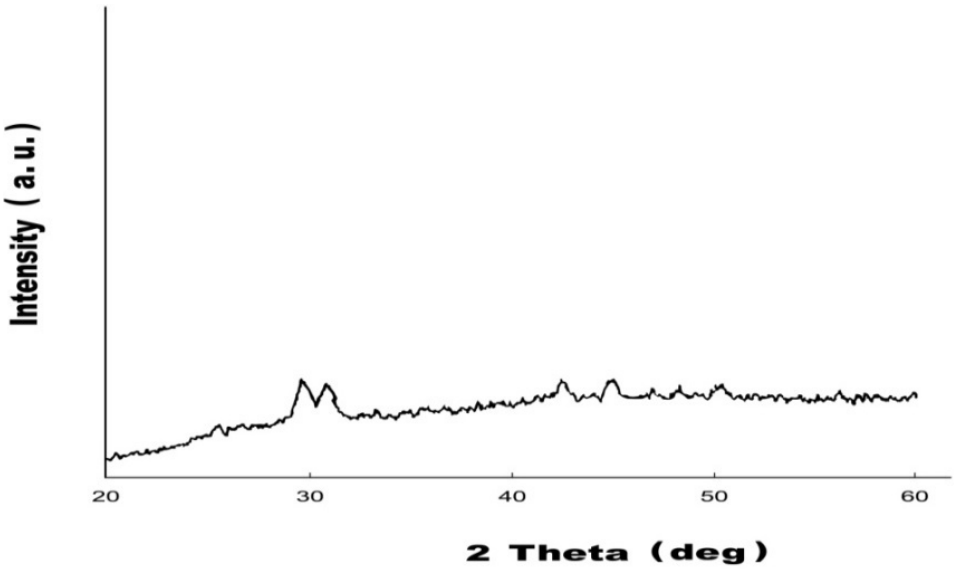

Figure 9. X–ray diffractogram of PbO nanoparticles synthesized by *Enterobacter* sp.

the synthesis of lead oxide (PbO) nanoparticles by *Enterobacter* sp. (Figure 9). The suggested mechanism for the formation of PbO nanoparticles by *Enterobacter* sp. is an aerobic denitrification of $Pb(NO_3)_2$ to PbO by nitrate reductase. This enzyme could be secreted by all members of Enterobacteriaceae except *Pseudomonas* sp. usually under anaerobic conditions (Rusmana, 2003). This is correlated with the activity of enzyme is expressed under aerobic growth conditions (Bell et al., 1990; Jackson et al., 2011). This finding supports the presence of the

nanoparticles within the periplasmic space of *Enterobacter* sp.

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