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RESEARCH ARTICLE

REMOVAL OF HEAVY METALS FROM RIVERS IN BASRA GOVERNORATE USING *BACILLUS SPP.*

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ABSTRACT

The present study aims to isolation, identification and characterization of heavy metal resistant bacteria from six rivers in Basra governorate-Iraq, during October and November, 2013. Three species of *Bacillus* (*B. licheniformis, B. badius* and *B. megaterium*) were isolated on the basis of morphological and biochemical tests and selected based on high levels of heavy metal (lead, copper, cadmium and nickel) resistance. The concentrations of dissolved heavy metals (Pb, Cu, Cd and Ni) in rivers were determined. The minimal inhibitory concentration (MIC) of isolates against Pb, Cu, Cd and Ni, was determined in solid media after 72 h incubation. All isolates were resistant to Pb (2000-2800 mg L^{-}), Cu (200-550 mg L^{-}), Cd (200-400 mg L^{-}) and Ni (300-400 mg L^{-}). In this work we have used living biomass of *B. licheniformis, B. badius* and *B. megaterium* to removal of heavy metal ions at different concentrations (25, 50,100) mg L^{-} from aqueous metal solutions. The best concentration to removal was 25 mg L^{-}after 72 h at 120 rpm. % removal efficiency of *B. licheniformis* was 33.6 for Cd, for *B.megaterium*was 28.14 for Pb and for *B. badius* was 53.1%, 34.46% for CU and Ni respectively.

INTRODUCTION

Environmental pollution has been increased gradually because of human activities and natural resources (Ahemad, 2012). Heavy metals Pollution is considered a serious world wide problem, for the toxicity of these metals even at low concentrations (Nasrazadani *et al*., 2011). Some elements (cobalt, manganese, nickel and zinc) are essential for the growth of microorganisms, but the higher concentrations have toxic effects. Other heavy metals are non- essential for the growth of organisms (cadmium, mercury, lead, etc.) is harmful even in lowest concentration (Issazadeh *et al*., 2013). However, the essential and non-essential metals become toxic to the organisms at high levels. These heavy metals affecting on the growth, morphology, metabolic activities and resulting in reduced biomass and diversity (Roane and Pepper, 2001). Heavy metals are not biodegradable and be want to accumulate in organisms causing many diseases and disorders (Ozer and Pirincci, 2006). In order to live on under metal-stressed conditions, bacteria have developed several kinds of mechanisms to resist the uptake of heavy metal ions. These mechanisms include the sequester of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and decrease the toxicity of heavy metals (Nies, 1999). Some traditional technologies have been used for removing heavy metals such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery but, its found ineffective and very costly (Volesky, 1990). So the biological methods, bioaccumulation and biosorption have been demonstrated to be an alternative to the traditional methods for he removal of metals, because it can be used in the site of contamination, environmentally non-threatening and they are cost effective (Volesky and Holan, 1995; Malik, 2004). Different bacteria have been suggested to use in removal of heavy metals from water (Waisberg *et al*., 2003). The objective of this study is to determine the heavy metals pollution in the inner rivers of the Basra governorate especially with cadmium, copper, nickel and lead, isolation, identification and characterization of heavy metal resistant bacteria, MIC, and exploitation these bacteria to remove heavy metals.

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MATERIALS AND METHODS

Sampling

A total of 12 samples of water from six rivers (Abu Al-Khaseeb, Al-Khora, Al-Saraji, Al-Ashar, Al-Khandak and Al-Rubat) in Basra governorate (Fig. 1) were collected during October and November, 2013. The samples for bacteria were collected according to Standard Methods for Examination of Water and Wastewater (APHA, 1995), while the samples for heavy metals were collected using 1 Liter polyethylene bottles. All samples transferred to an ice box and transported to the laboratory.

Isolation and identification of bacteria

In the present study heavy metals resistant isolates were isolated from rivers water, by serial dilution and streaking method using nutrient agar. The isolates were incubated at 37ºC for 24h. They were characterized morphologically and on the basis of biochemical reactions which including: Gram stain, catalase, indol, endospore location, starch hydrolysis, Vogas-Proskauer, growth with 6.5% NaCl, growth at 55 ºC, citrate utilization, nitrate reduction, mannitol fermentation and acid from glucose (Claus and Berkely, 1986).

Determination of Heavy Metals concentration in water

Twenty five ml of sample was placed in a 250 ml digestion tube and 5ml of concentrated $HNO_3$was added. The sample was heated for 120 min at 80-90ºC,and then temperature was increased to 150 ºC at which the sample was boiled for at least 4huntil a clear solution was obtained. Concentrated $HNO_3$was added to the sample (5ml was added three times) and digestion occurred until the volume was reduced to about 1ml. After the digestion was completed, and the sample cooled, 5ml of 1% $HNO_3$was added. The solution was filtered with What man No.42 filter paper. It was transferred quantitatively to a 25ml volumetric flask by adding distilled water. The concentration of Cd, Ni, Cu and Pb in the final solutions were determined by anatomic absorption spectrophotometers (AAS) (phoenix-986, Biotec.UK) (APHA, 1995).

Heavy Metal Resistance Test

The ability of isolates to tolerated different concentrations of Cd, Ni, Cu and Pb were determined, using the agar diffusion method (Hassan *et al*., 2008). Isolates were grow non LB agar supplemented with different concentration of heavy metals. The bacteria were incubated for 72h at 37ºC.

Determination of minimal inhibitory concentration (MIC)

In present study, the maximum resistance of isolates against increasing concentrations of Cd, Ni, Cu and Pb on LB agar plates were assessed until the strains unable to grow colonies on the agar plates. The initial metal concentration used 0.1 mM which prepared from 1M stock solution. The stock solution of $Cd(NO_3)_2.4H_2O$,$Cu(NO_3)_2.3H_2O$,$Ni(NO_3).6H_2O$ and $Pb (NO_3)_2$ was prepared in sterile deionized water and sterilized by autoclaving at 121ºCfor 15 min (APHA,1999). The culture grows at a given concentration were then transferred to the next concentration. The MIC was done at 37ºCfor 72h. All the experiments were carried out in triplicates and the results indicates the mean values.

Removal of heavy metal ions by *Bacillus spp.*

The living biomass of *Bacillus spp.* was used for removal studies of Cd, Ni, Cu and Pb. Bacterial isolates were grown on nutrient bro that 37º C for 24h, Cells were harvested by centrifugation at 6000rpm for 15min,then washed with normal

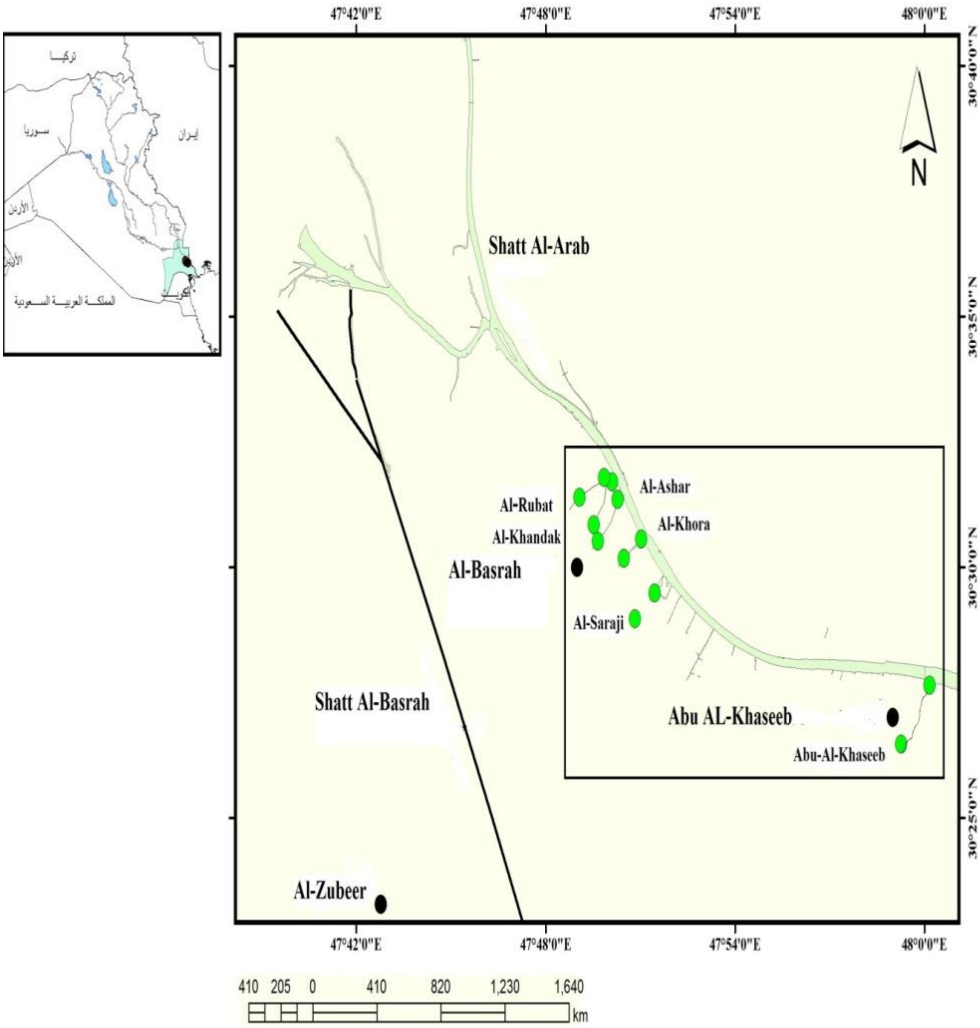

Fig. 1. Location of inner rivers in Basra governorate-Iraq

saline triplicates. 50mg of cells were mixed with 50ml of nutrient broth containing various metal concentrations (25mg L^-, 50mg L^- and 100mg L^-) for each heavy metals (Cd, Ni, Cu, Pb). These cultures were incubated at 30ºC for 24,48 and 72hin, shaker incubator, at 120 rpm, and PH7 (Tsuruta, 2005; Stefanescu *et al*., 2011; Ray *et al*., 2005). The cells were removed for each time by centrifugation at 6000 rpm for 15min, and the supernatant prepared for measuring by AAS (Muneer *et al*., 2013; Philip *et al*., 2000). Removing of metal by bacterial cells was calculated as the ratio of ions removal % R (%) = $(C_0 - C_1) / C_0 \times 100$

Where is the R = Removal Ratio (%)

C_0=concentration of heavy metal ions in the original solution (mg L^-) and C1 = concentration of heavy metal ions in the treated solution (mg L^-) (Qin *et al*., 2006).

RESULTS AND DISCUSSION

Heavy metals contamination

In present study,12 water samples were taken from 6 rivers in Basra governorate, during the period October and November 2013.The heavy metal concentrations were measured and found to be Pb>Cu>Ni>Cd.

Lead

The mean concentration of Pb in the six rivers averaged 0.338mg $L^-$and ranged from less than 0.219 mg L^- in Al-Khandak river to the highest value in Abu Al-Khaseeb river was 0.439 mg L^- (Table 1).

These values are higher than the USEPA of 0.05mg L^- and the guidelines for drinking water (WHO) concentration of 0.01 mg L^-.

Table 1. Heavy metal concentrations in the rivers mg L^-

Sample Name	Pb	Cu	Ni	Cd
Abu Al-Khaseeb	0.439	0.026	0.089	0.047
Al-Saraji	0.384	0.020	0.068	0.033
Al-Khora	0.329	0.030	0.075	0.034
Al-Ashar	0.384	0.067	0.071	0.023
Al-Rubat	0.274	0.028	0.064	0.030
Al-Khandak	0.219	0.320	0.046	0.009
Average	0.338	0.082	0.069	0.029
USEPA,1993	0.05	1	ND	0.005
WHO, 2004	0.01	2	0.02	0.003

Copper

The mean Cuvalue of all rivers is 0.082 mg L^-.The lowest value was 0.02 mg L^- in Al-Saraji river while the highest value was 0.32 mg L^- in Al-Khandak river. These values are less than the USEPA which was 1mg L^- and 2mg L^- for WHO.

Nickel

Nickel concentration of the rivers averaged 0.069 mg L^-.The lower detection limit was 0.046 mg L^- in Al-Khandak river while the highest detection limit was 0.089 mg L^- in Abu Al-Khaseeb river. The average value of Nigreater in three times of WHO which was 0.02 mg L^-.

Cadmium

The mean concentration of Cadmium in the rivers was 0.029 mg L^-.Lower value was 0.009 mg L^- in Al-Khandak river and higher value was 0.047 mg L^- in Abu Al-Khaseeb river. The average value was higher than USEPA 0.005 mg L^- and 0.003 mg L^- for WHO. The main reason for the increased in the concentration of heavy metals in these rivers as a result of their use for the disposal of sewage and throwing the solid waste since 1988, in addition to the lack of water level. The data show that, based on USEPA and WHO, there is a risk to exploiting these rivers for drinking water or plants irrigation.

Isolation of heavy metal Resistant bacteria

The present study revealed that all isolated bacteria belongs to three species of *Bacillus* which including *B.licheniformis, B. badius* and *B. megaterium* according to Claus and Berkely (1986) (Table 2).

Table 2. Biochemical Characterization of isolated bacteria

Test	*B.licheniformis*	*B.badius*	*B.megaterium*
Gram stain	+	+	+
Shape	Rod	Rod	Rod
Endospore	Central	Terminal	Central
Catalase	+	+	+
Indol	-	-	-
Motility	+	+	+
Starch hydrolysis	+	-	+
Vogas-Proskauer	+	-	-
Acid from glucose	+	-	+
Mannitol fermentation	+	-	+
Citrate utilization	+	-	+
Nitrate reduction	+	-	-
Growth on 6.5% NaCl	+	-	-
Growth on 55ºC	+	-	+

+:Positive -: Negative

Heavy Metal Resistance Test

Metal resistance studies showed that *B.licheniformis* is more resistant to Cu, Ni, Cd and Pb than other species (Table 3). *B.licheniformis* has shown no growth at 550mg L^-, 400mg L^-, 400mg L^- and 2800mg L^- respectively, whereas *B.badius* showed no growth at 400mg L^-, 300mg L^-, 400mg $L^-$and 2400 mg L^- respectively. *B.megaterium* showed no growth at 200mg L^-, 300mg L^-, 200mg L^- and 2000 mg L^- respectively after.

Table 3. MIC of heavy metals against *Bacillus spp.* after 72h of incubation

Heavy metals	*B.licheniformis*	*B.badius*	*B.megaterium*
Copper	550 mg/l	400 mg/l	200 mg/l
Nickel	400 mg/l	300 mg/l	300 mg/l
Cadmium	400 mg/l	400 mg/l	200 mg/l
Lead	2800 mg/l	2400 mg/l	2000 mg/l

The high levels of resistance and the widespread tolerance that was found among the isolates is probably attributed to the high metal contents in the water (Abou-Shanab *et al.,* 2007). All the isolates were tolerant to multiple metal ions.

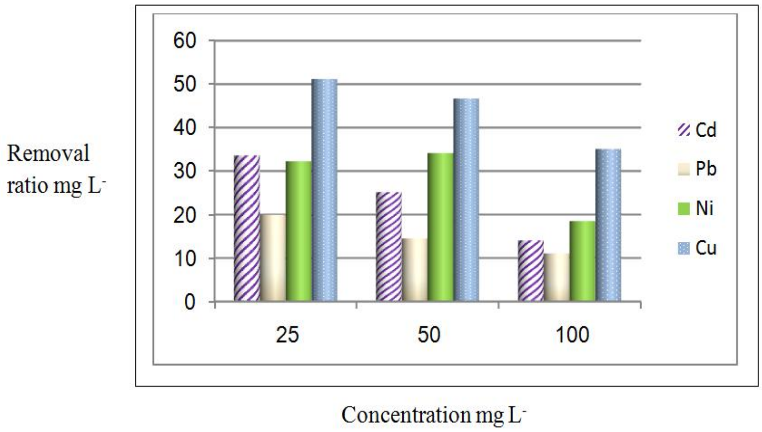

Figure 2. Heavy metal uptake by *Bacillus licheniformis* in different concentrations

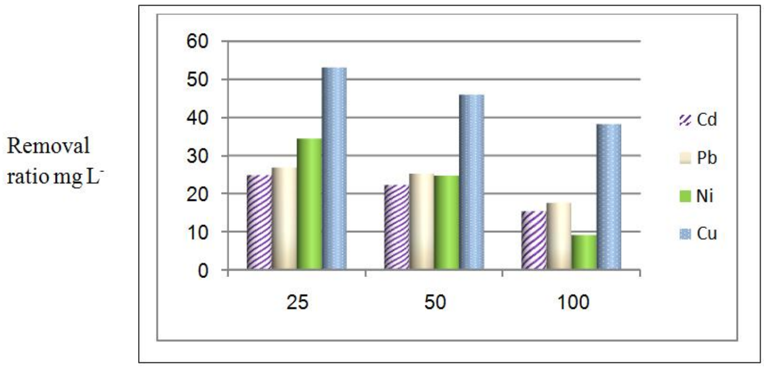

Figure 3. Heavy metal uptake by *Bacillus badius*in different concentrations

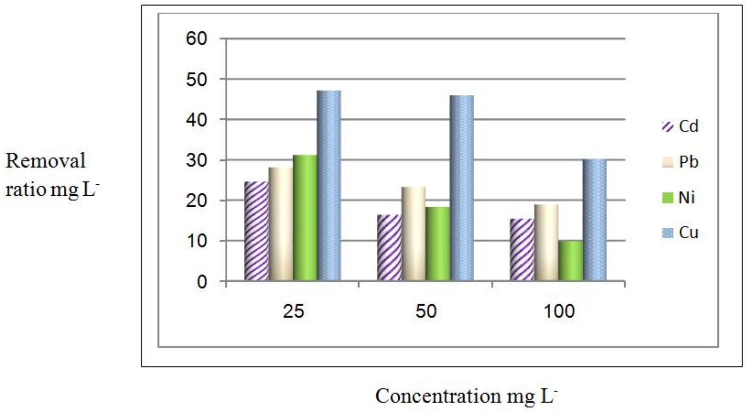

Figure 4. Heavy metal uptake by *Bacillus megaterium* in different concentration

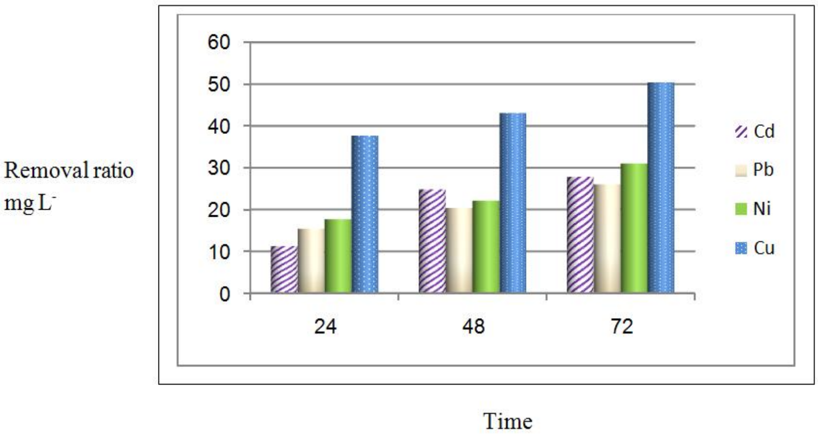

Figure 5. Heavy metal uptake by *Bacillus spp.* in different time

However, the patterns of resistance variations may be due to the difference in the concentration of the different heavy metals in the environment. The site from which the samples was taken has been polluted with high levels of heavy metals for many years, perhaps giving a diverse range of bacteria the chance to adapt to the environment, either by convergent evolution of resistance mechanisms or by transferring the resistance genes via a plasmid. Resistance to heavy metals, including cadmium, copper, lead, and nickel, is most often carried by bacteria on plasmids or transposons and it has been theorized that this allows for lateral transfer in the environment (Silver, 1992).

Removal of heavy metal ions by *Bacillus spp.*

The capability of bacteria to grow in the presence of high concentration of heavy metals may be helpful in the wastewater treatment, because bacteria are contributed in the decomposition of organic matter in biological processes for wastewater treatment (Filali *et al*., 2000). The ability of *B.licheniformis* to remove heavy metals was found to be Cu (51.21%) >Cd (33.6%) >Ni (32.27%)>Pb (19.94%)at concentration 25mg L^{-} (Fig 2). Whereas *B.badius* was Cu (53.1%)>Ni(34.46%)>Pb(26.85%)> Cd(24.83%) (Fig 3). While *B.megaterium* was Cu(47.16%)>Ni(31.16%)> Pb (28.14%)>Cd(24.62%) (Fig.4).

In the present study, the *Bacillus spp*. has the ability to biosorption heavy metals in 25mgL^{-} more than 50 and 100 mg L^{-} for all ions studied. Also the biosorption has increased with time interval (Fig. 5) and reached maximum in 72h incubation period. These results are accordance with Burke and Pfister (1986) who found that, here is an inverse relationship between tolerance and metal uptake. In addition to that may researchers attribute the reason of biosorption of heavy metals to the cell wall of bacteria, especially peptidoglycan, teichoic acid and other amino acids which find in gram positive bacterial cell wall (Beveridge *et al*., 1982; Bruins *et al.,* 2000; Gupta and Mahapatra, 2003; Schaffer and Messner, 2005).

Conclusion

From results obtained, the use of live cells for biosorption of heavy metals may be more efficient than conventional methods, and *Bacillus spp.* which used are eco-friendly. More than these bacteria can be used in situ bioremediation after increasing it by construction of large scale bioreactor.

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