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Bioaccumulation of heavy metals by *Vibrio alginolyticus* isolated from wastes of Iron and Steal Factory, Helwan, Egypt

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ABSTRACT

The isolation of bacteria resistant to heavy metals is a topic of interest in the field bioremediation of contaminated water, soil and sediments. We report here the isolation of bacteria that is resistant to high concentration of a mixture of heavy metals namely cadmium, cupper, lead and zinc. The bacterial isolate was obtained from a site receiving heavy metal waste from the iron and steal factory; a major factory located in El-tebeen, south Helwan. The isolate was identified as *Vibrio alginolyticus* using the API system**.** The maximum tolerable concentration was 2.5 mM, 4 mM, 2.5 mM and 3.5 mM for cadmium, copper, lead and zinc respectively. Transmission electron micrograph of *Vibrio alginolyticus* grown in nutrient broth containing a mixture of the four tested heavy metals, showed bioaccumulation of heavy metal(s) on the bacterial cell wall. At the same time, there was an over all reduction in the concentration of heavy metals in culture supernatant; the percentage reduction was 20% for cadmium, 31% for cupper 40% for lead and 45%for zinc. The reduction occurred after 4 hrs incubation at 30°C for all metals, cupper, lead, and zinc while cadmium required 24 hrs incubation were required to achieve maximum reduction. This isolate could be used to accelerate the *in situ* bioremediation of sites contaminated by loads of mixed metals.

Keywords:

INTRODUCTION

Industrial activities led to substantial release of toxic metals into the environment. Heavy metals constitute a major hazard for the human health and ecosystem (Boopathy, 2000).

The Iron and Steal Factory was constructed in 1947 and is major factory with estimated sales 1.8 million pounds / year.

According to Kaiser (1980), heavy metals are defined as ions with partially or completely filled *d*-orbital. Some metals including iron, zinc, copper and manganese are micronutrients used in the redox processes, regulation of osmotic pressure, enzymes cofactors and are also important in the maintenance of the protein structure (Vallee and Auld 1990). However even essential metals such as zinc and copper are toxic at high concentration.

On the other hand metals including lead and cadmium do not play any known physiological role and are in fact toxic to cells. Lead reacts with the sulphydryl groups of protein and inhibits their function; cadmium is extremely toxic and was shown to induce DNA breakage (Ron *et al.,* 1992). The metal ion toxicity is determined by many factors such as physio-chemicals characters of metals ion including electro- negativity, reduction-oxidation potential,……etc. (Workentine *et al.,* 2008).

Chemical methods such as precipitation, oxidation or reduction have been widely used to remove metal ions from industrial waste water. Those methods are ineffective or expensive (Volesky, 1990). The activity of microorganisms is extended to environmental management, and microbes have superseded the conventional techniques of remediation Vidali (2001). Biological methods such as biosorption and bioaccumulation provide promising alternative to chemical methods (Kapoor and Viraragharan, 1995).

The mechanism by which microorganisms remove heavy metals can be divided into three categories; the first mechanism is the biosorption of metals ions on the cell surface, second intracellular uptake of metals ion and third chemical transformation of metal ions by microorganism (Pardo *et al*., 2003). Among the different technique employed for metals removal from multi elemental system, biosorption has been found to be highly selective (Knauer *et al.,* 1997). Furthermore metal accumulating bacteria can be used to remove, concentrate and recover metals from industrial effluents (Malekzadeh *et al*., 2002 and Chowdhury *et al*., 2008).

The capacity of any biosorbent is mainly influenced by biomass characteristic, physiochemical properties of the target metals, and the micro environment of contact solution including pH, temperature and interaction with other ions (Chen and Wang 2007). Moreover once the toxic metals are adsorbed or transferred within organic materials they can be removed from waste water (Smith and Collins, 2007).

The aim of this study was to isolates and characterizes bacteria from sites receiving heavy metals pollutants, to study the heavy metals resistance pattern and the bioaccumulation potential of the selected organism.

MATERIALS AND METHODS

Sample collection and total bacteria count

Water samples receiving waste from the Iron and Steal Factory, El-Tebeen, Helwan, Egypt were collected, and three replicates were considered. The initial pH was determined at the site of collection Samples were kept in ice and sent to lab for heavy metal analysis. For total count samples were stored at 4°C. Then 0.1 ml of the water sample was inoculated into nutrient agar plates. Plates were incubated at 30°C for 24 hrs.

Heavy metals analysis

The heavy metal content of the water sample was determined according to Cunningham and Lundie (1993) ; where 1 ml nitric acid was added, after over night incubation the result liquid was diluted, the concentrations of Cd^{+2}, Cu^{+2} Pb^{+2}, Zn^{+2} and Fe^{+3} were determined using the atomic adsorption spectrophotometer 3100 Perkin- El-MER, Central Laboratory Ain Shams University.

Dertermination of MTCs (maximum tolerable concentration)

To test the heavy metals resistance pattern, the heavy metals Cd^{+2},Cu^{+2}, Pb^{+2} and Zn^{+2} used as $(CdNO_3)_2.4H_2O$,$CuSO_4.7H_2O$,$C_4H_6O_4Pb.3H_2O$ and Zn SO_4. $7H_2O$ were added to nutrient agar media at concentrations covering the range from 0.1mM to 4.0 mM, plates were then spot inoculated and incubated at 30°C for 24hrs. The maximum tolerable concentration (MTC) of heavy metals was designated as the highest concentration of heavy metals that allowed growth after 24 hrs (Schmiatt and Schlegel, 1994). The most tolerable isolate was selected.

Bacterial characterization

The most tolerable bacterial isolate was characterized using analytical profile index (API system) biochemical test kit KB002 Hi Assorted Hi media, India.

Metals reduction measurements:

Bacteria were grown on 100 ml nutrient broth for 24 hours. Cells were harvested by centrifugation and suspended in 1 ml 0.08% saline solution. Cell pellets were transferred into nutrient broth media containing a mixture of heavy metals. The mixture contained 3mM Cd^{+2}, 1.1mM Cu^{+2}, 1mM Pb^{+2} and 1.1mM Zn^{+2} (Mergeay *et al*., 1985). At time intervals the metal content was determined in the cell free supernatant using atomic adsorption spectroscopy (Gainji and Page 1974).

Electron microscopy

Electron microscopy was performed through the electron microscope facility, at Ain Shams University. Pellet of 24 hrs cultures grown on media with and without heavy metals were examined. Briefly cells were fixed in 2.5% (v/v) glutaraldhyde, the sample was post fixed in osmium tetraoxide then dehydrated in ethanol. Thin sections were prepared and examined using Jeol-JEM 1200 EX II transmission electron microscope. Japan (Crooks *et al.,* 1986).

RESULTS

The initial pH of sample was 1.9. The heavy metals content of the water sample from which the bacteria was isolated was estimated as: 0.05 mg/l cadmium, 0.024 mg/l copper, 0.32 mg/l lead, 18.1 mg/l zinc and 1.13 mg/l iron.

The colony forming units was found to be 125x10. Based on colonial morphology, nine distinct colonies were, isolated, purified, and recognized. The isolate that tolerated high concentration of heavy metal (2.5mM Cd^{+2}, 4 mM Cu^{+2}, 2.5mM Pb^{+2} and 3.5mM Zn^{+2}) was selected for identification and used for further studies.

Accordingly to the cell morphology, Gram reaction and biochemical characterization tests (Table1) the selected isolate was identified as *Vibrio alginolyticus*.

In nutrient broth containing a mixture of heavy metals, *V. alginolyticus* was able to reduce the concentration of all tested metals the percentage reduction was 20% for cadmium, 31% for copper, 40% for lead and 45% for zinc. Maximum reduction was achieved at 30°C after 4 hrs incubation for all heavy metals except cadmium were 24 hrs incubation were required to attain maximum reduction .

Table 1: Biochemical characterization tests of the selected isolate.

Test	Result
Citrate utilization	-ve
Lysine decarboxylation	+ve
Ornithine decarboxylation	variable
Urease	-ve
Phenyl alanine deamination	-ve
Nitrate reduction	+ve
H_2S production	-ve
Glucose	+ve
Adonitol	-ve
Lactose	-ve
Arabinose	-ve
Sorbitol	-ve

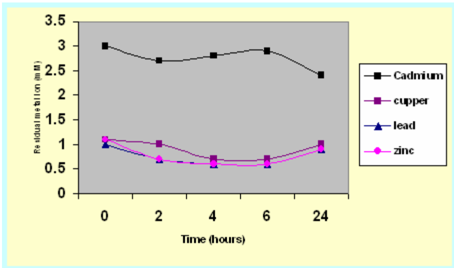

Fig.1: Metal reduction as a function of time

As shown in (Fig. 2) *V. alginolyticus* was grown on nutrient broth containing a mixture of heavy metal, electron microscopic analysis revealed localized areas of heavy metals at the cell surface indicating possible accumulation by binding to the cell wall.

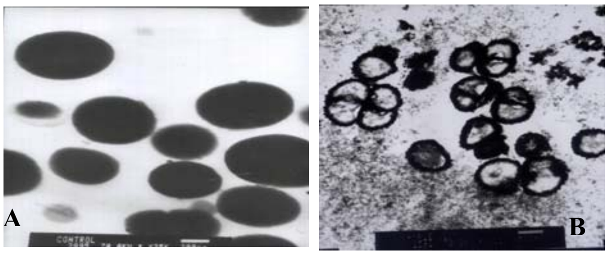

Fig. 2: Transmission electron micrographs of *V. alginolyticus* grown for 24 hrs at 30 °C. (A) Cells grown on nutrient broth.(B) Cell grown on nutrient broth containing a mixture of heavy metals. Cells showing localized precipitation of heavy metal(s) on the cell surface. Bar 1 µm.

DISCUSSION

According to the standards permitted by the Ministry of Environmental Affairs in Egypt, the water sample obtained from wastes of Iron and Steal Factory contained above the permitted amounts of cadmium, lead and zinc. There was 10 times more Cd^{+2}, 16 times more Pb^{+2} and 3 times more Zn^{+2} in the water sample, leakage of the waste water would cause heavy metal contamination of the ground water. Among the four tested heavy metals cadmium is considered the most toxic metal. Cadmium is more mobile than other heavy metals because of the low affinity between soil particles to cadmium (Cunninngham and Lundi, 1993).

Resistance of toxic metals in bacteria probably reflects the degree of environmental contamination with these metals (Aiking *et al*., 1984 and Malik and Jaiswal 2000).

According to Malik and Jaiswal, 2000 there is no acceptable concentration of metal ions which can be used to distinguish metal resistant and metal sensitive bacteria.

The presence of metal resistant microbes was reported by many authors. Hetzer *et al.* 2006 isolated members of the Genus *Geobacillus* that were all considered resistant to cadmium at concentrations ranging between 0.4mM to 3.2 mM. In this study *V. alginolyticus* was resistant to 2.5mM Cd^{+2}. Moreover Dressler *et al.,* 1991 reported that *Alcaligenes denitrificans* tolerated copper up to 4 mM, in this study *V. alginolyticus* was resistant to 4mM Cu^{+2}.

Richard *et al.,* 2002 reported that Cu^{+2} and Pb^{+2} appear to bind to materials on the cell surface. Lead is precipitated in an insoluble form that is localized to the cell membrane or cell surface (Aiking *et al.,* 1985; Levinson *et al.,* 1996; Roane 1999) similar results were obtained from this study showing localization of one or more metal to cell wall of *V. alginolyticus*. This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of bacterial cell wall absorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or combination of such processes (Chojnacka *et al.,* 2005).Both dead and living cells adsorb metal ions (Ansari and Malik 2007).

Pardo, *et al.,* 2003 reported that the percentage removal of Cd^{+2}, Cu^{+2}, Pb^{+2} and Zn^{+2} from aqueous solution by *Pseudomonas putida* was 80% , in this study the percentage reduction by *V. alginolyticus* was 20% Cd, 31% Cu^{+2}, 40% Pb^{+2} and 45% Zn^{+2}. Further investigation are needed to increase the rate of bioaccumulation by *V. alginolyticus* .Due to the metal up-taking ability of *V. alginolyticus* it could be used either as pure culture, in mixed consortium to treat industrial effluents before release to the environment or it could be genetically manipulated to increase the rate and efficiency of metal removal capability.

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ARABIC SUMMARY

التراكم الحيوي للمعادن الثقيلة بواسطة بكتريا الفيبريو الجينوليتكس(*Vibririo alginolyticus*)المعزولة من مخلفات مصنع الحديد والصلب بحلوان

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يعتبر عزل البكتريا المقاومة للمعادن الثقيلة موضوع ذو أهمية بالنسبة للتطبيق في مجال التحلل الحيوي في المياه ، والتربة والرواسب الملوثة. وقد تم في هذه الدراسة عزل بكتريا تقوم التركيزات العالية من خليط من المعادن الثقيلة وهي الكادميوم والنحاس والرصاص والخارصين وقد تم عزل هذه البكتريا من أماكن يتم فيها صرف مخلفات مصنع الحديد والصلب بحلوان. وقد تم تعريف هذه البكتريا بإستخدام كواشف API على أنها بكتريا الفيبريو الجينوليتكس(Vibrio alginolyticus). وقد وجد أن هذه البكتريا تتحمل حتى 2.5 ملي مول و4 ملى مول و 2.5ملي مول و 3.5 ملي مول من الكادميوم والنحاس والرصاص والخارصين بالترتيب.

وبفحص البكتريا النامية على وسط يحتوي على خليط من المعادن الثقيلة وبإستخدام الميكروسكوب الإلكتروني النافذ لوحظ وجود تجمع من المعادن الثقيلة حول جدار البكتريا. في نفس الوقت وجد أنه يوجد إنخفاض في كمية المعادن الثقيلة الموجودة بالبيئة التي ينمو بها الكائن وقد كانت نسبة الإنخفاض هي 20% بالنسبة للكادميوم، 31% بالنسبة للنحاس، 40% بالنسبة للرصاص، و45% بالنسبة للخارصين وقد حدث هذا الإنخفاض بعد أربع ساعات في حالة النحاس والرصاص والخارصين بينما تطلب الكادميوم 24 ساعة من التحضين حتى يتم خفض نسبته.

وعليه فقد يستخدم هذا الكائن في التعجيل بالتحلل الحيوي الميكروبي في المواقع التي تتعرض للتلوث بالمعادن الثقيلة المختلطة.