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Full Length Research Paper

Cadmium resisting bacteria in Alexandria Eastern Harbor (Egypt) and optimization of cadmium bioaccumulation by *Vibrio harveyi*

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Cadmium resisting bacteria (CRB) were present in all water and sediments (samples) collected from Alexandria Eastern Harbor, Egypt. The occurrences of CRB in sediments samples were higher than in water samples and reached up to 77.22% of total counts. Five isolates were selected to be the most resistant to cadmium with minimal inhibitory concentration of 60 ppm. The most potent isolate that accumulates a maximum Cd^{2+} concentration of 23.3 mg Cd^{2+}/ g dry cells was identified as *Vibrio harveyi*. Medium components affecting metal removal by *V. harveyi* were explored based on the application of Plackett-Burman design. The growth of *V. harveyi* in the optimized culture conditions, recorded cadmium biosorption of 1.52 fold increase. Immobilized living cells of *V. harveyi* on hazelnut shells increased the uptake of cadmium to 49.35 mg Cd^{2+}/g dry cells and removal efficiency up to 84%. Transmission electron microscope study confirmed the accumulation of Cd^{2+} by *V. harveyi* cells.

Key words: Cadmium resisting bacteria, *Vibrio harveyi*, bioaccumulation, Plackett-Burman design.

INTRODUCTION

A variety of microbes inhabit extreme environments (Satyanarayana *et al*., 2005). Extreme habitats lie outside the range of conditions in which most of organisms live (Oarga, 2009). Extreme environments include high and low temperature, pH, pressure, salt concentration, nutrient concentration and water availability and also, conditions having high levels of radiation, harmful heavy metals and toxic compounds (organic solvents) (Satyanarayana *et al*., 2005). The release of heavy metals into our environment is still large and causes an environmental pollution problem because of their unique characteristics (Soltan *et al*., 2008). Contamination of the aquatic environment by toxic metal ions is a serious pollution problem (El-Deeb, 2009). Heavy metals may reach watercourses either naturally through a variety of geochemical processes or by direct discharge of municipal, agricultural and industrial wastewater (Semerjian, 2010; Srinivasa-Rao *et al*., 2010), to a lesser extent, from natural weathering (Higham et al., 1985). At elevated concentrations, soluble metal compounds can be deleterious to human health as well as to aquatic and marine environments (Semerjian, 2010; Srinivasa-Rao *et al*., 2010).

Cadmium is one of the heavy metals considered to be toxic to humans and aquatic life. Chronic exposure to cadmium can affect the nervous system, liver, cardio-vascular system and may lead to renal failure and death in mammals and humans (Semerjian, 2010). Cadmium, arsenic, mercury, lead and chromium have been known to be extremely toxic at low concentration (El-Sersy and El-Sharouny, 2007), although, they have no significant biological function so far reported. Cadmium causes reduced growth rate, long lag phase, lower cell density and may even cause death of bacteria at levels below 1 ppm (Shapiro and Keasling, 1996; Sinha and Mukherjee, 2009).

The bioremediation of heavy metals using micro-organisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its

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potential application in industry (Hussein *et al*., 2004). Studies on the accumulation and biotransformation of cadmium by bacteria are of special interest, as the final chemical form of cadmium is likely to dictate its relative toxicity (Higham *et al*., 1985). The response of micro-organisms towards toxic heavy metals is of importance in view of the interest in the reclamation of polluted sites. Microorganisms uptake metal either actively (bioaccumulation) and/or passively (biosorption) (Johncy *et al*., 2010). Many microorganisms have developed chromosomally- or extra chromosomally-controlled detoxification mechanisms to overcome the detrimental effects of heavy metals (Ehrlich, 1997).

The Eastern Harbor presents a natural laboratory site to study the effect of pollutants on the ecosystem. This Harbor receives about 230×10^3 m^3 of untreated domestic sewage daily, beside minor wastes input from a local shipyard (Emara *et al*., 2008).

The present study aimed to document the occurrence of native cadmium resisting bacteria (CRB) in the Eastern Harbor. In addition, optimizing Cd^{2+} accumulation conditions by the most potent strain via applying Plackett-Burman design. The study extended to evaluate the efficiency of immobilized cells on agriculture waste.

MATERIALS AND METHODS

Samples collection

A total of 10 samples were selected along the west of Alexandria Eastern Harbor, Egypt. Sampling was performed according to the World Health Organization manual for recreational water and beach quality monitoring and assessment (WHO, 1995) during Autumn, 2009. Total viable count (TVC) and cadmium resistance bacteria (CRB) in surface sea water and sediments samples were estimated.

Preparation of metal solution

Cadmium salt (CdCl2. 2½H2O) was added in equivalent weight to certain volume of deionized water to reach final concentration of 100 ppm. This stock solution was sterilized by filtration through a flow pore filter of 0.22 µm pore size and further used for the preparation of the different metal concentrations in the study.

Isolation of cadmium resisting bacteria

Ten grams of sediments samples were suspended in 100 ml sterilized aged sea water to dissociate the adhered bacterial population. Water and sediment samples from different locations were plated by pour plat method onto sea water nutrient agar (SWNA) with the following composition gl^{-1}: peptone (Oxoid) 5.0, yeast extract (Oxoid) 3.0, agar (Oxoid) 15 g, aged sea water 500 ml; distilled water 500 ml; pH 7.2 amended with 20 and 40 ppm Cd^{2+} (as $CdCl_2$). Stock solution of metal chloride was added after sterilization of the medium. Plates were incubated at 30°C for 48 h. Total viable counts (TVC) from each sample were enumerated by plating aliquots on SWNA without adding cadmium chloride. Triplicates were used for each sample.

Screening for cadmium resisting bacteria

Determination of minimal inhibitory concentration (MIC)

In this preliminary screening for cadmium resisting bacteria, colonies showing resistances to cadmium at 40 ppm were selected for further screening process. A number of morphologically different colonies were randomly picked and isolated after successful purification process on the same medium. The levels of the cadmium resistance for the specific cadmium isolated strains were tested individually by subculturing on SWNA medium supplemented with different concentrations of Cd^{2+} (30, 40, 50 and 60 ppm). Growth was monitored with respect to the control which contained no metal. From these, strains were chosen for its specific metal resistance ability then, the isolated strains undergo the biosorption process.

Cadmium removal assay

For biomass production, cells were allowed to grow in the absence of cadmium, At A_{550} = 1.4, aliquots (0.015 g fresh cells) were harvested by centrifugation for 10 min. at 5200 × g and washed by sterile glass distilled water. Cell samples of each strain were resuspended in 10 ml solution of 8 ppm Cd^{2+} and incubated at 30°C on a rotary shaker at 150 rpm for 1 h of contact, after which the cells were centrifuged, the biomass was dried at 60°C until constant weight and the supernatants were analyzed for residual Cd^{2+} (El-Helow *et al*., 2000). After appropriate dilution of cell free supernatants with deionized water, residual Cd^{2+} was determined as mg/l by atomic absorption spectrophotometer (Sabry *et al*., 1997). The amount of metal absorbed was calculated as mg/l (ppm) and the metal uptake (mg Cd^{2+}/g dry wt.) was calculated according to the equation;

Metal uptake = V(IC -FC)/W

Where, IC, initial metal concentration (mg/l), FC, final metal concentration (mg/l), V, volume of reaction (l) and W, total biomass (g) (Abou Zeid *et al*., 2009).

Unless stated otherwise, all experiments were performed in duplicates and the averages of the observation were presented.

Molecular identification of the promising isolate

The identification was carried out at Mubarak City for Scientific Research and Technology Applications, Arid Land Institute, Molecular Plant Pathology Department, New Borg El Arab City, 21934, Alexandria, Egypt.

Effect of increasing Cd^{2+} concentrations on *Vibrio harveyi* uptake

Aliquots of fresh bacterial biomass were kept in contact with different concentrations of Cd^{2+} solutions (8, 10, 12, 14 and 16 ppm) at 30°C on a rotary shaker at 150 rpm for 1 h and the residual Cd^{2+} was determined as previously described .

Plackett-Burman design and optimization

A fractional factorial Plackett-Burman (Plackett and Burman, 1946) is a technique devoted to evaluate the relative importance of various medium components to approach a near optimal response region of medium composition that improve biomass for maximal Cd^{2+} uptake in liquid culture, under metal stressed conditions. Two

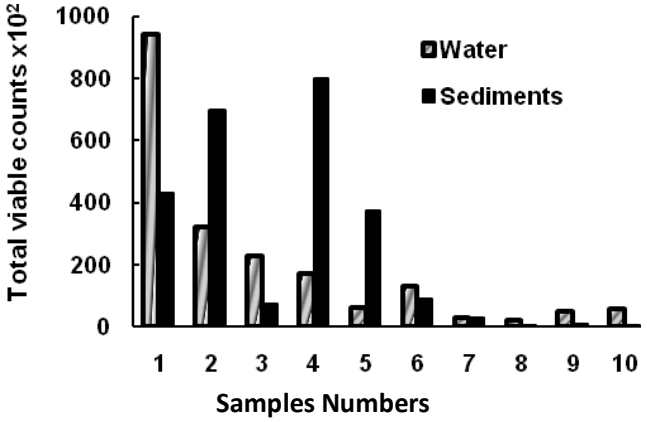

Figure 1. Total viable counts in water (CFU/ml) and sediment (CFU/g) samples.

additional components, namely KH_2PO_4 and K_2HPO_4, were added to SWNB formula. Consequently, the medium contained seven different variables, each variable was examined in 4 trails at low level (-1) and in 4 trials at high level (+1). Eight different trials and the basal control were performed in duplicates. The main effect of each variable was calculated as the difference between the average of measurements made at the high setting (+1) and the average measurements observed at the low setting (-1) of that factor. The main effect of each variable was calculated for determining whether variations in the observation sets are the result of examined factors or experimental errors. Statistical t-values for equal unpaired samples were calculated (Cochran and Snedecor, 1989) using Microsoft excel to determine the variable significance. From main effect results an optimized medium was predicted.

Verification experiment

A verification experiment was carried out in duplicates, the predicted optimum levels of the independent variables were examined and compared to the basal conditions setting and the average bioaccumulation of Cd^{2+} was calculated.

Electron microscopy

Cells of *V. harveyi* were contacted with solution of 8 ppm Cd^{2+}, for 1 h and the blank was in absence of Cd^{2+}, centrifuged, washed with deionized water and fixed for about 2 h at room temperature in 5% glutaraldehyde, buffered with 0.1 M phosphate, pH 7. Sections (80 nm thick) were cut using an LKB2208-180 ultramicrotome and stained with saturated solution of uranyl acetate. Electron micrographs were taken using a transmission electron microscope. (JEM-100XJoel). Electronic Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Immobilization of microbial cells

Hazel nuts shells were used as support material for immobilization. The shells were broken in pieces of approximately 5×5 mm, washed twice with diluted HNO_3, three times with distilled water and dried at 90°C until constant weight. Then, they were cooled and kept in desiccator for subsequent uses. Bacterial cells were immobilized on the shells surface by immersing the shell pieces into a growing bacterial culture for 48 h at 30°C. The immobilization is basically a film adhesion process. After incubation, the shells were filtered and washed with distilled water aseptically in order to remove the immobilized cells from the shell's surfaces. The removal of cadmium from aqueous solution by living immobilized cells of the selected isolates was determined with initial Cd^{2+} concentration of 8 ppm at different time intervals. Immobilized cells as well as free nuts shells were examined by scanning electron microscopy (Buyukgungor, 2000).

Adsorption efficiency

Adsorption efficiency was expressed as a percentage of adsorbed metal compared to initial metal concentration, using the following equation:

Cd^{2+} removal efficiency (%) = (IC – FC) x100 /IC.

Where, IC is the initial metal concentration (mg/l) and FC is the final metal concentration (mg/l) (Semerjian, 2010).

RESULTS

Occurrence of cadmium resisting bacteria in water and sediments samples

The total viable counts in water samples ranged from 2.0×10^3 to 9.4×10^4 colony forming unit per ml (CFU/ml). While sediments samples contain bacterial counts ranged from 6.5×10^2 to 8.0×10^4 CFU/g, (Figure 1).

Cadmium resisting bacteria were present in all water and sediments samples and tolerated 20 ppm of Cd^{2+}. In terms of percent of TVC, the occurrence of cadmium resisting bacteria in water samples ranged from 1.91 to 25.83%, while in sediments samples CRB increased and their occurrence ranged from 4.64 to 77.22% (Figure 2). The abundance of CRB that tolerate 40 ppm of Cd^{2+} were lower and no occurrence of cadmium resisting bacteria were in half of the samples, the occurrence in the remaining samples ranged from 0.11 to 4.39% in water samples and from 0.46 to 10.81% in sediments samples (Figure 3). It was noticed that, the occurrences of CRB in sediments samples were higher than that in water samples.

Isolation and selection of cadmium resisting bacteria (minimum inhibitory concentration)

Nineteen bacterial isolates have been isolated from growing colonies during enumeration of CRB at 40 ppm (eight isolates from sea water and eleven isolates from sediments). The nineteen isolates were screened for their metal resistance (from 30 to 60 ppm). Table 1 showed that, although, all these bacterial isolates were obtained from media containing 40 ppm Cd^{2+}, few of them could not grow again on media containing the same concentration. At 50 ppm of Cd^{2+}, only twelve isolates were able to grow. Sea water nutrient agar supplemented by 60

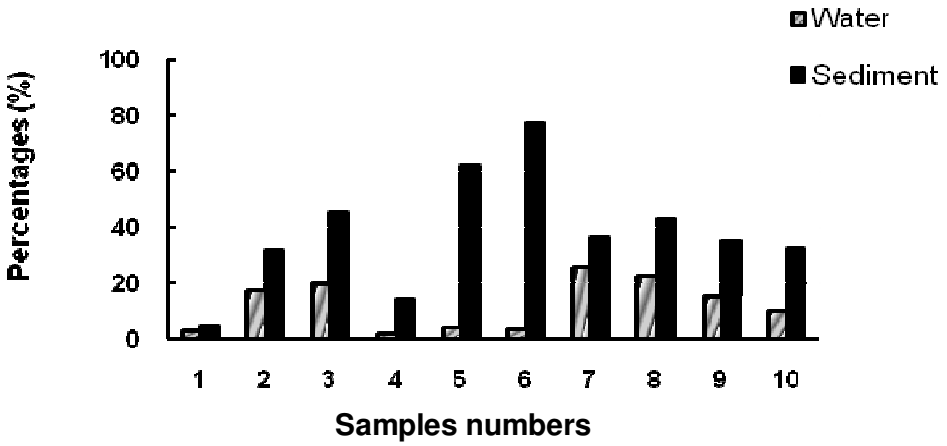

Figure 2. Percentages of cadmium resisting bacteria in water and sediments samples at 20 ppm cadmium.

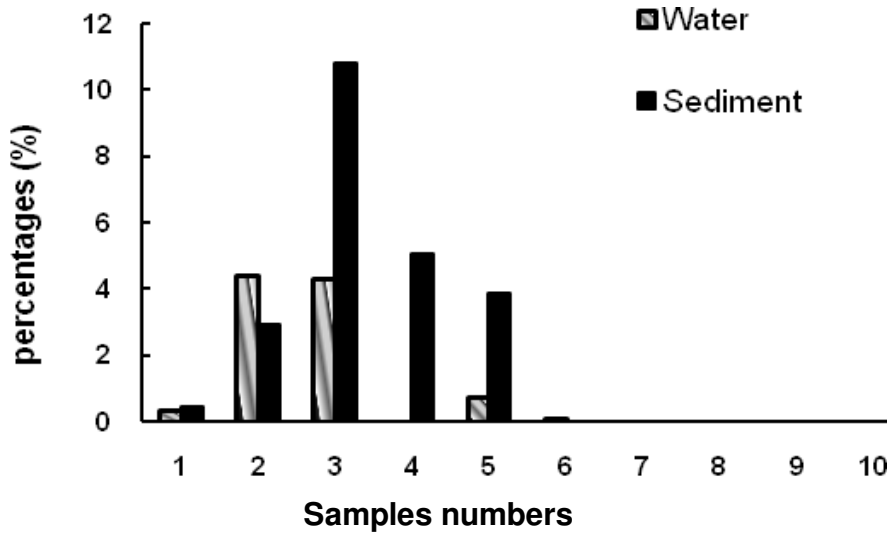

Figure 3. Percentages of cadmium resisting bacteria in water and sediment samples at 40 ppm cadmium.

ppm Cd^{2+} inhibited the growth of all bacterial isolates except five isolates, one from water (5W-1) samples and four from sediments samples (1S-1, 2S-2, 3S-1 and 5S-2), so that the MIC for all isolates was 60 ppm except these five isolates which resisted this concentration of Cd^{2+}. Bacterial isolates from sediments samples were more tolerable to the same concentration of Cd^{2+} than those isolated from sea water.

Bioaccumulation of cadmium by the most potent isolates

The five bacterial isolates, which resisted the highest Cd^{2+} concentration (60 ppm), were chosen to be tested for their potentiality to accumulate Cd^{2+}. The data in Figure 4 revealed that, the efficiency of bacterial isolates to accumulate Cd^{2+} varied according to the bacterial isolates. Wide range of Cd^{2+} accumulation capacities were found, where isolate number 5S-2 was more efficient to accumulate the maximum concentration of Cd^{2+} (23.3 mg Cd^{2+}/g dry cells) and isolate number 2S-2 accumulated the lowest concentration (9.1 mg Cd^{2+}/g dry cells).

Identification and nucleotide sequence accession number

DNA of the promising isolate was extracted and the

Table 1. Growth of cadmium resisting bacteria on different cadmium concentrations.

Isolates code	Cadmium concentrations (ppm)			
	30	40	50	60
1W-1	+	+	+	–
1W-2	+	+	+	–
2W-1	+	+	+	–
2W-2	+	+	+	–
2W-3	+	+	+	–
3W-1	–	–	–	–
3W-2	–	–	–	–
5W-1	+	+	+	+
1S-1	+	+	+	+
1S-2	+	+	–	–
2S-1	+	+	+	–
2S-2	+	+	+	+
3S-1	+	+	+	+
4S-1	+	–	–	–
4S-2	+	+	–	–
4S-3	+	–	–	–
4S-4	+	+	–	–
5S-1	+	+	+	–
5S-2	+	+	+	+

W, Isolated from water samples; S, isolated from sediments samples; +, can growth at this cadmium concentration; –, can not growth at this cadmium concentration.

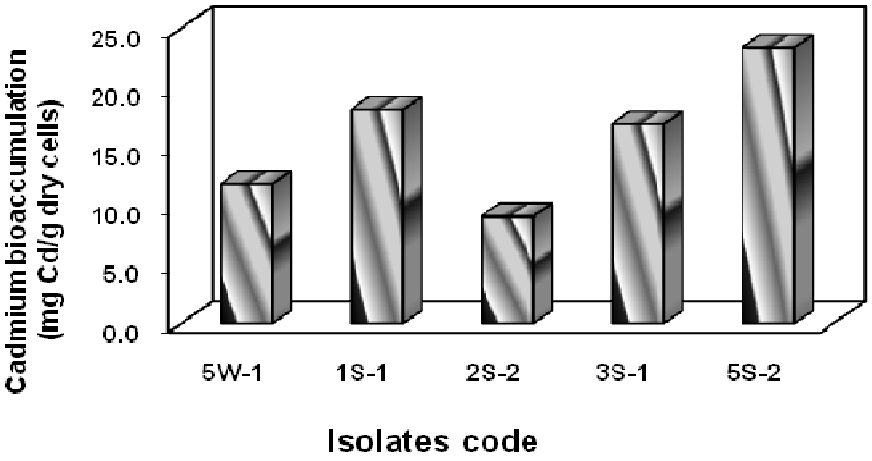

Figure 4. Bioaccumulation of Cd^{2+} by different isolates.

extracted 16S rRNA gene was amplified, the produced amplicons was analyzed using agarose gel electrophoresis as shown in Figure 5 and phylogenetic tree in Figure 6. The GenBank accession number for the 16S rDNA sequences is GQ907040.1 the isolate was identified as *V. harveyi* with similarity percentage 100%, its classification under (Division: Proteobacteria).

The effect of Cd^{2+} concentration on Cd^{2+} bioaccumulation by *V. harveyi*

The effect of Cd^{2+} concentrations on bioaccumulation capacity by *V. harveyi* were examined at different concentrations from 8 to 16 ppm. As revealed in Figure 7, the maximum bioaccumulation of Cd^{2+} was observed at 8

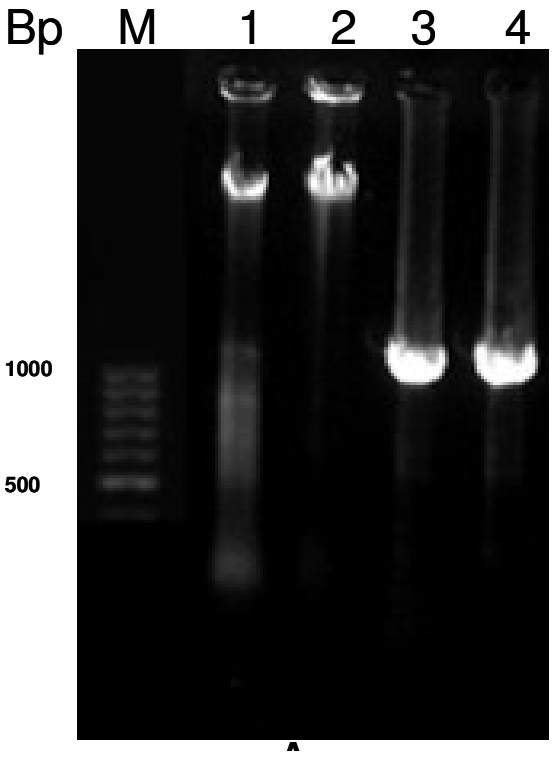

Figure 5. Agarose gel electrophorases of amplified 16S rRNA gene of the isolate under study. M: DNA marker 100bp, Lane 1, 2 DNA for isolates, 3, 4 PCR products of 16S rRNA for markers.

ppm, where *V. harveyi* accumulated 21.96 mg Cd^{2+}/g dry cells. The bioaccumulation of Cd^{2+} decreased gradually as the concentration of Cd^{2+} was increasing from 10 to 16 ppm.

Evaluation of nutritional requirements affecting Cd^{2+} bioaccumulation by *V. harveyi*

Plackett-Burman design has been employed to evaluate the significant effect of SWNB components with extraneous supply of KH_2PO_4 and K_2HPO_4 on accumulation of Cd^{+2} by *V. harveyi*. Examined concentration of media components at basal control, low level (-1) and high level (+1) are shown in Table 2. The applied Plackett- Burman experimental design for seven cultural variables and their Cd^{2+} bioaccumulation results after 1 h of contact are illustrated in Table 3. The main effect of each constituent on the Cd^{2+} bioaccumulation, as well as t-values was estimated for each variable as shown in (Table 4). Main effects results showed that inoculum size, peptone and yeast extract had a highly positive main effect which positively affected the increase of Cd^{2+} bioaccumulation, also, culture volume and KH_2PO_4 negatively affected the increase of cadmium bioaccumulation (Figure 8). The statistical analysis by calculating t-test, confirmed also the main effect results, peptone and inoculum size had a significant effect on the bioaccumulation of cadmium. Figure 9, illustrates the interaction effect between peptone concentration (g/l) and inoculum size (ml) on the cadmium bioaccumulation. This figure explained that the high concentration of peptone with the low volume of inoculum size (ml) will highly increase the bioaccumulation of cadmium.

Based on these results, a medium of the following formula (gl^{-1}) was predicted to be near optimum for cadmium bioaccumulation: KH_2PO_4, 1.0; K_2HPO_4, 0.5; yeast extract, 5.0 and peptone, 7.0; inoculum size, 1.5 ml and culture volume 25 ml, aged sea water must be omitted from the fermentation medium.

Verification experiment

A duplicate of experiment was performed to verify the optimization result to validate the developed optimized medium. The Growth of *V. harveyi* on the optimized medium, recorded a higher cadmium biosorption, (41.26 mg Cd^{2+}/g dry cells), than those of the basal by 1.52 fold increase. This result confirmed the validity of the optimized medium.

Transmission electron microscope study

Thin section of Cd^{2+} stressed and unstressed *of V. harveyi* cells were compared by transmission electron microscope examination (Figure 10). Dark grain densities condensed in the cells and towards the cell envelope were observed in the presence of Cd^{2+} reflecting that cells have anionic aspects with high metal binding capacities.

Effect of immobilization on cadmium accumulation by *V. harveyi*

It was observed that, metal ions were effectively removed by immobilized living cells of *V. harveyi* on hazelnut shells (Figure 11). The amount of Cd^{2+} accumulated by immobilized cells and hazelnut shells gradually increased with the increase in contact time and the maximum uptake and maximum removal efficiency were (49.35 mg Cd^{2+}/g dry cells and 84%, respectively) after 60 min, although, about 73% of Cd^{2+} accumulated after 40 min only. The immobilization of *V. harveyi* cells on the outer and inter surfaces of nuts shells were examined by scanning electron microscopy (Figure 12).

DISCUSSION

Contamination of the aquatic environment by toxic metal ions is a serious pollution problem (El-Deeb, 2009).

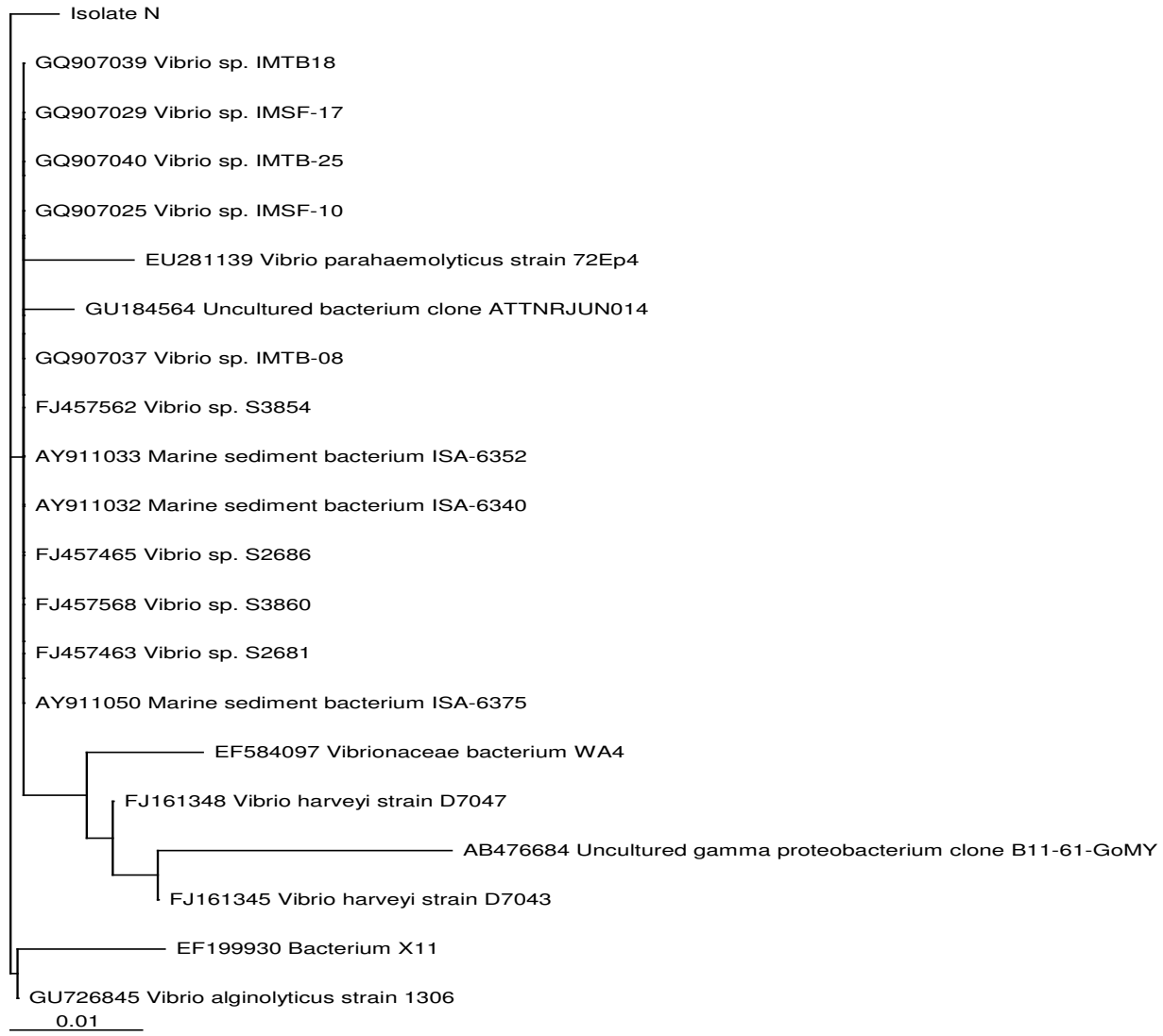

Figure 6. Phylogenetic relationships among representative experimental strain and the most closely related *Vibrio* species. The dendogram was generated using tree view program.

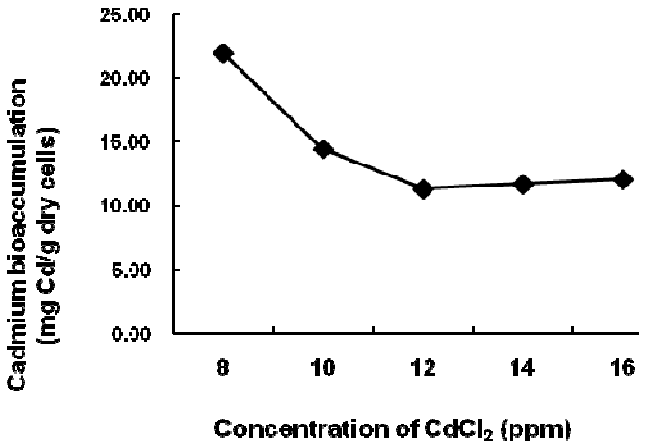

Figure 7. The effect of Cd^{2+} concentration on Cd^{2+} accumulation by *V. harveyi*.

Microorganisms undergo selection pressures in the presence of toxic compounds and develop resistance. Enumeration of this resistant group from different geographical locations has shown that, these groups are ubiquitous (Mudryk *et al*., 2000; De Souza *et al*., 2006). Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal tolerance and/or resistant (Johncy Rani *et al*., 2010). Measurement of microbial tolerance is a powerful tool in the managements of contaminated natural environments because it serves to predict the potential of an ecosystem to assimilate heavy metals, as well as to indicate past and present exposures (Liebert and Barkay, 1988). So sampling environment, that contain elevated concentrations of heavy metals was a potential source for toxic metal-tolerant bacteria. It is likely that such environment foster adaptation and selection

Table 2. Screening for growth factors affecting Cd^{2+} accumulation by V. harveyi and their levels in Plackett-Burman design.

Factor (g/l)	Symbol	Level		
		-1	0	+1
KH_2PO_4	KH	1.0	1.3	1.7
K_2HPO_4	K_2	0.0	0.2	0.5
Aged sea water	SW	0	50%	100%
Yeast extract	YE	1	3	5
Peptone	P	2	5	7
*Inoculum size (ml)	IS	0.5	1	1.5
Culture volume (ml)	CV	25	50	75

*Inoculum size was added (ml) of 24 h culture (A_{550}= 1.2).

Table 3. The applied Plackett- Burman experimental design for seven cultural variables and their Cd^{+2} bioaccumulation results.

Trial	Factor*							Cadmium bioaccumulation (mg Cd^{2+}/g dry cells)
	KH	K_2	Na	YE	P	IS	CV	
1	-1	-1	-1	1	1	1	-1	33.70
2	1	-1	-1	-1	-1	1	1	15.14
3	-1	1	-1	-1	1	-1	1	12.80
4	1	1	-1	1	-1	-1	-1	14.23
5	-1	-1	1	1	-1	-1	1	12.51
6	1	-1	1	-1	1	-1	-1	14.14
7	-1	1	1	-1	-1	1	-1	21.90
8	1	1	1	1	1	1	1	21.97
9	0	0	0	0	0	0	0	27.17

*See Table 2 for explanation of factor symbols.

Table 4. Statistical analysis of the Plackett- Burman experimental design results.

Variable	Cadmium bioaccumulation (mg Cd^{2+}/g dry cells)	
	Main effect	t-value*
KH_2PO_4	- 2.55	-0.432
K_2HPO_4	0.155	0.025
Aged sea water	- 0.03	-0.006
Yeast extract	5.905	1.073
Peptone	6.01	1.095
*Inoculum size	11.057	2.789
Culture volume	- 4.08	-0.708

*t-value significant at the 1% level = 3.70; t-value significant at the 5% level = 2.45; t-value significant at the 10% level = 1.94; t-value significant at the 20% level = 1.37.

for heavy metal resistance (Hussein *et al.,* 2004). Cadmium resistant bacteria are widely distributed and quite ubiquitous in nature (De Jaysankar and Ramaiah, 2006). The present results showed that, resistant isolates are more abundant in sediments than in water samples, also the occurrence of cadmium resisting bacteria (at 20 ppm Cd^{2+}) reached to 25.83 and 77.22% of total viable count (TVC) in water and sediments, respectively, while at 40 ppm Cd^{2+} their occurrence decreased to 4.39 and 10.81% in the same samples. While De Jaysankar and Ramaiah (2006) mentioned that, heavy metal resisting bacteria contributed to over 20% of TVC in surface water, 12% at 100 m, 35% at 500 m and a staggering 49% at 1000 m depth. The fact that a major portion of the natural culturable bacterial flora was metal resistant, to the global nature of heavy metal pollution and their high occurrence might signify the already prevalent adverse impact of heavy metals on the metabolic performance of heterotrophic micro flora and its role as a contaminant, as an indicator of pollution and as a member of the bacterial community in a highly polluted area (De Jaysankar and Ramaiah, 2006; Soltan *et al.,* 2008).

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 (Abou Zeid *et al*., 2009). The effect of increasing concentrations (30, 40, 50 and 60 ppm) of

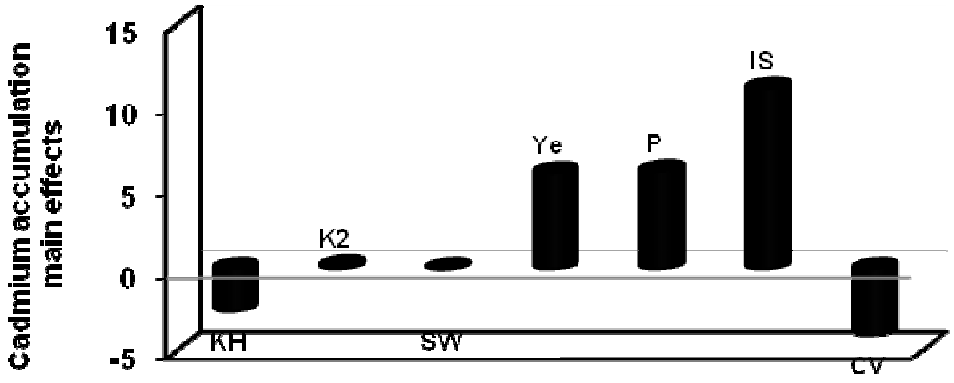

Figure 8. Cadmium bioaccumulation main effects of the medium constituents after applying Plackett-Burman experimental design.

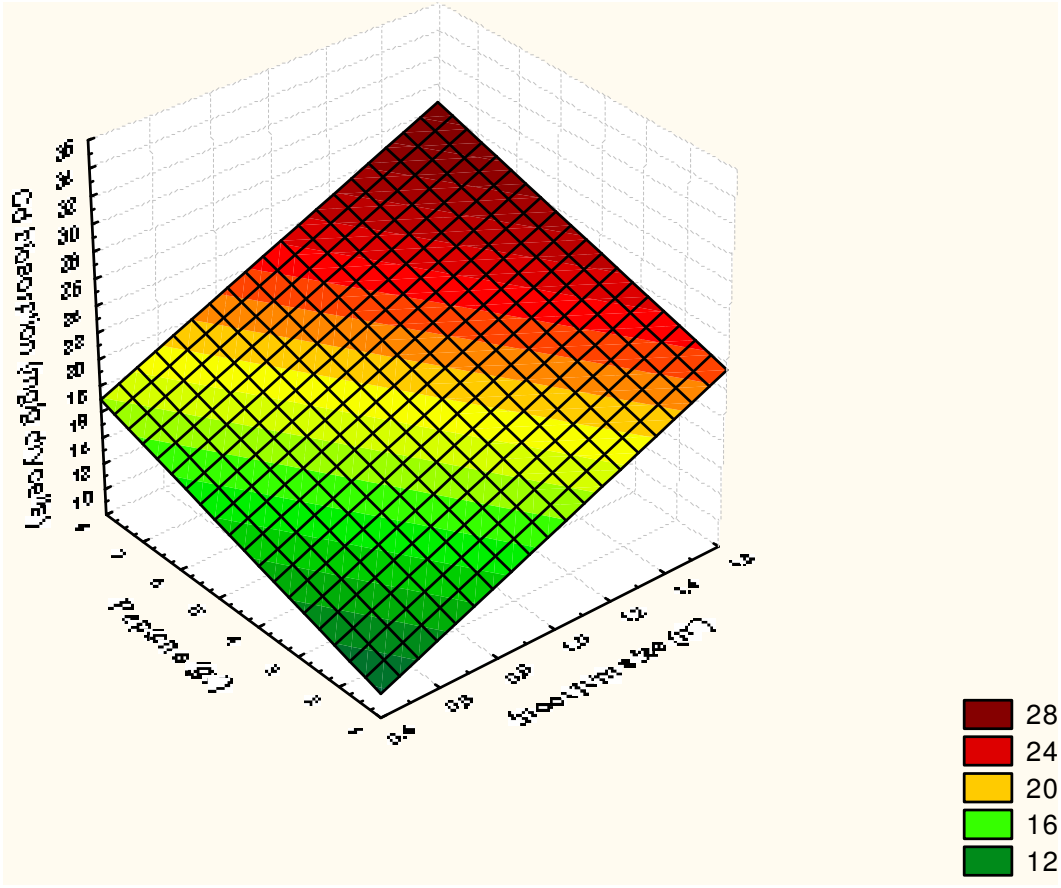

Figure 9. Interaction effect between peptone concentration (g/l) and inoculum size (ml) on cadmium bioccumulation (mg Cd^{2+}/g dry cells).

Cd^{2+} on viability of the chosen isolates observed that, as the concentration of Cd^{2+} increased the number of cadmium tolerating bacteria decreased and also, bacterial isolates from sediments samples were more tolerable to the same concentration of Cd^{2+} than those isolated from sea water. Previous study confirmed the same observation (Abd El-nabby, 2005).

Wide range of Cd^{2+} uptake capacities was found by different bacterial isolates. The efficiency of bacterial isolates to accumulate Cd^{2+} varied according to the bacterial isolates. This varying response of tested bacteria might be due to differences in cell wall composition of

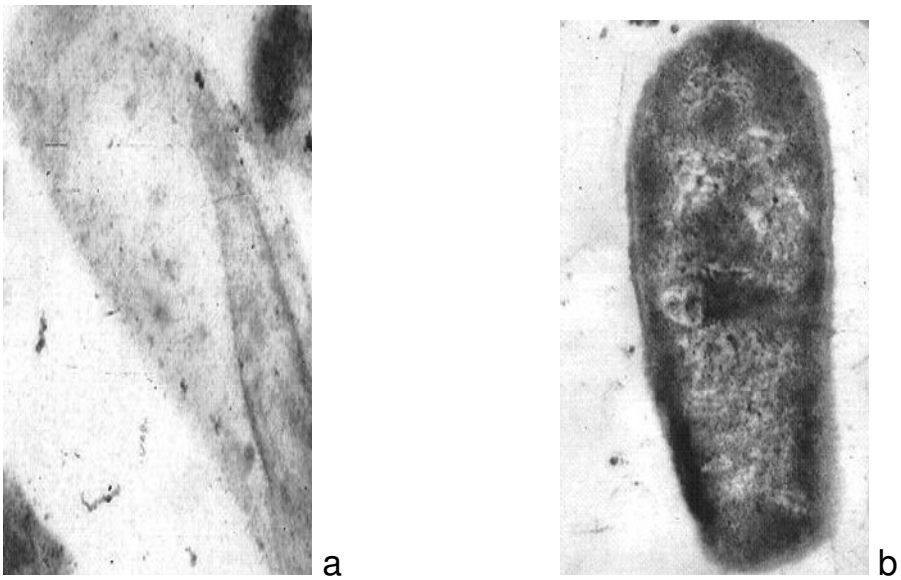

Figure 10. Transmission Electron micrographs of *V. harveyi* cells in absence of Cd^{2+} (a) and in presence of 8 ppm Cd^{2+} after 1 h. contact (b).

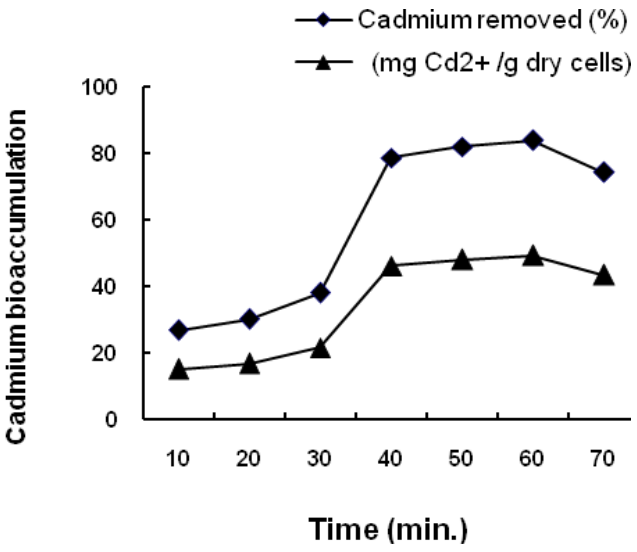

Figure 11. Cadmium accumulation by immobilized cells of *V. harveyi* on hazelnut shells in relation to contact time.

different bacterial strains or due to variation in resistance mechanisms (Abou Zeid *et al*., 2009).

Several authors have reported the high capability of heavy metals bioaccumulation by gram-negative, heterotrophic marine bacteria (Abou Zeid *et al*., 2009; Vogel and Fisher, 2010). *Vibrio* sp. has been listed and documented as a potential microorganism for bioaccumulation of heavy metals (Vogel and Fisher, 2010). The maximum uptake of Cd^{2+} by *V. harveyi* was observed at 8 ppm Cd^{2+}. Abou Zeid *et al.* (2009) mentioned that, the maximum uptake of Cd^{2+} by *Pseudomonas mendocina* was observed at concentration 2.5 ppm, while El-Helow *et al.* (2000), found that the highest Cd^{2+} uptake by *Bacillus thuringiensis* was at concentration 0.25 mM Cd^{2+}. Statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner (El-Sersy and El-Sharouny, 2007). One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or physical factor) or sign (whether contributes positively or negatively) (Youssef and Berekaa, 2009).

Factors affecting cell surface affinity for metal cations monitored by means of Cd^{2+} binding capability are investigated. The optimization results indicated the importance of phosphate for efficient metal binding by the cells. This observation was in agreement with another study on *B. subtilis* 168 (Mera *et al*., 1992). This observation can be interpreted mainly as an effect of phosphate functional groups on medium pH as well as cell wall composition and consequently, metal bioaccumulation capabilities of biomass. The presence of the two potassium phosphates in the medium resulted in a buffered medium with a pH of 6.9, which magnifies net negative charge on outer surfaces of the gram-positive bacterial wall (El-Helow *et al*., 2000). In the present study, peptone and yeast extract had a highly positive main effect which, positively affect the increase of Cd^{2+} accumulation by *V. harveyi* while, KH_2PO_4 had low positive effects on Cd^{2+} removal. In contrast, another study on accumulation of Cd^{2+} by *B. thuringiensis* (El-Helow *et al*., 2000) mentioned that, peptone is a main growth factor in the medium, showed a relatively low

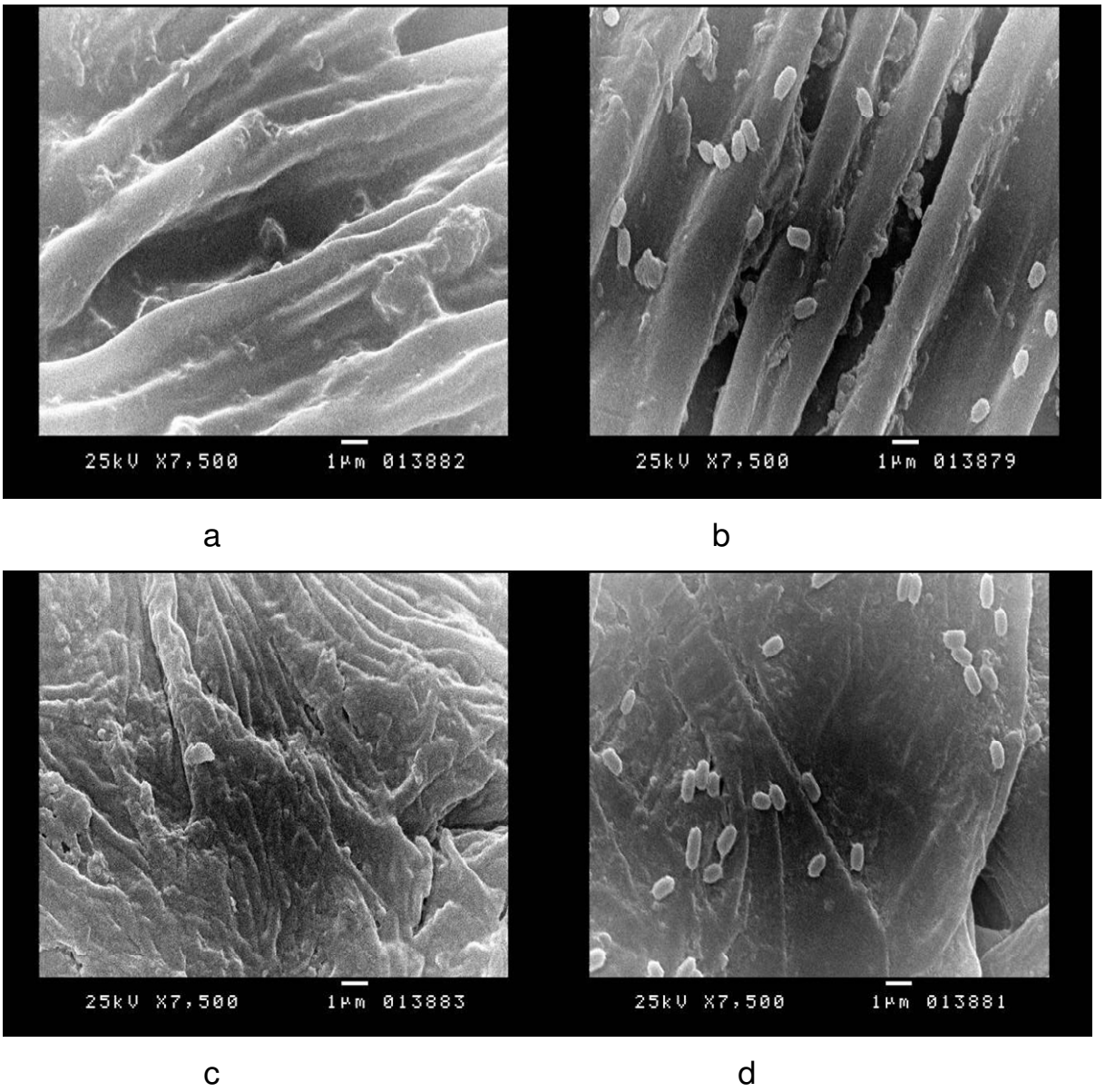

Figure 12. Scanning electron microscopy of (a) the outer surface of hazelnut shells, (b) immobilized cells of *V. harveyi* on the outer surface of hazelnut shells, (c) inner surface of hazelnut shells and (d) immobilized cells of *V. harveyi* on the inner surface of hazelnut shells.

negative main effect with no significant t-value, also moderate negative effects of K_2HPO_4 and yeast extract on the removal of Cd^{2+} was also observed. Growth of *V. harveyi* cells on optimizing medium enhanced the bioaccumulation to 41.26 mg Cd^{2+}/g dry cells, that agreed with El-Helow *et al*. (2000) who found that, optimized culture conditions allowed *B. thuringiensis* cells grown in the presence of 0.25 mM $CdCl_2$ to remove about 79% of the metal ions within 24 h with a specific biosorption capacity of 21.57 mg/g of biomass.

The resistance mechanisms take several forms, such as extracellular precipitation and exclusion, binding to the cell surface and intracellular sequestration (Ehrlich, 1997). Transmission electron microscopy studies showed that. *V. harveyi* cells are encased in anionic cell wall polymers that can immobilize discrete aggregates of cations. This agree with Sinha and Mukherjee (2009) who localized the intracellular site of accumulated Cd^{2+} by *P. aerugenosa* and showed electron dense grains in the cytosol and towards the cell envelope. Also, El-Helow *et al.* (2000) and Soltan *et al.* (2008) 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.

In most natural environments association with a

surface, in a structure known as biofilm, is the prevailing microbial live style (Watnick and Kolter, 2000). The advantages of using immobilized microbial cells to enhance metal uptake and recovery are multiple. Although, whole cell immobilization is achieved by various methods of chemical coupling and cell entrapment, the only method that has been applied to large-scale is via biofilm formation on the surface of a solid carrier. The highest removal capacity and removal efficiency of metal ions by immobilized bacterial cells was observed in many studies. In addition, immobilized bacterial cells have better mechanical stability (Abd El-nabby, 2005). In the present study the free cells of *V. harveyi* accumulate 41.26 mg Cd^{2+}/g dry cells after 60 min of contact, while immobilized cells accumulated 46.20 mg Cd^{2+}/g dry cells after only 40 min that is; immobilization technique increased the efficiency of cadmium uptake by 12% in a reduced time.

For large scale systems, rigid supports are preferred. Microbial colonization and growth on the support surface produces a layer of self-immobilized cells held in contact with the perfuming flow. In this study, food waste hazelnut shells, was a potential low cost support surface for industrial applications.

Conclusion

The results in this study indicate that, monitoring and assessment of microbial tolerance is powerful tool in the managements of contaminated natural environments because it serves to predict the potential of an ecosystem to assimilate heavy metals, as well as to indicate past and present exposures, also, the isolated *V. harveyi* could be a good candidate for heavy metal removal of polluted environments.

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