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Arsenate and Selenate Reduction by Some Facultative Bacteria in the Nile Delta

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Abstract: Facultative anaerobic bacteria capable of reducing arsenate and selenate oxyanions were isolated from water and sediment samples collected from sites contaminated with industrial and agricultural wastes from Kafr el Dawar and Edko (Behera Governorate) and from kafr el Zayat (Gharbeya Governorate). Microbiological analysis and heavy metals determination of the collected samples was carried out. Three of the obtained isolates were identified by morphological and biochemical tests, these were *Neisseria mucosa* and *Rahnella aquatilis* (both are arsenate reducers) and *Hafina alvei* (a selenate reducer). The effect of some physical and chemical parameters on the growth and transformation efficiency of the three isolates was studied. Among the electron donors tested in the medium Na-lactate was preferred by *Neisseria* yielding the best growth, while Na-acetate and Na-formate were preferred by *Hafnia* and *Rahnella*, respectively. Selenate transformation by immobilized Hafnia cells was found to be positive and scanning electron microscope examination of the immobilized bacteria and precipitated selenium nanocrystals were observed. Differentiation and comparison of the three identified strains and six other bacterial isolates reducing selenate and arsenate was carried out by using restriction fraction length polymorphism (RFLP) technique and the similarity coefficient was determined for grouping the similar strains.

Key words: Arsenate % Selenate % Facultative bacteria % Nile Delta

INTRODUCTION

Microorganisms are involved in a variety of element transformation including a change in valance (i.e. oxidation/reduction) and many of these transformations are key steps in biochemical cycles, Element transformation may be the result of assimilatory processes in which an element is incorporated into cell biomass, dissimilatory processes in which transformation results in generation of energy, or detoxification [1].

Arsenic and selenium are two elements whose significance in microbial ecology and as environmental toxins has recently been recognized [2-3]. Both have been called essential toxins because they are required in trace amounts for growth and metabolism but are toxic at high concentrations. They have similar modes of toxicity and are used as antagonist [4-5]. Their toxicity and mobility in soil or water are dependent on their chemical state. Selenium most oxidized forms (Selenate SeVI or Selenite SeIV), are highly water soluble and toxic to biological system at relatively low concentrations (ppm) while reduced elemental form (Se o) is insoluble and has minimum toxicity [6]. The opposite occurs for arsenic, where the reduction of arsenate [As (V)] to arsenite [As (III)] represents the formation of a more toxic mobile species [7].

Arsenic forms a very small percentage of earth crust, however it can become enriched in soil and aquatic environments as a result of dissolution and weathering [8]. These occurrences are augmented by anthropogenic sources [9]. This toxic element has a complex biogeochemical cycle that is in a part mediated by microorganisms including both oxidation and reduction reactions involving arsenite and arsenate, respectively [3]. Certain bacteria can use arsenate as respiratory electron acceptor for oxidation of organic substances or H_2 (electron donors) forming arsenite this phenomenon is known as dissimilatory arsenate reduction DAsR [2, 10]. Some arsenate reducing bacteria have been isolated and held in pure culture some belong to archaebacteria and others to gram negative proteobacteria or gram-positive *Bacillus* sp. and or *Clostridium* [11].

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Selenium is commonly biologically found as selenocysteines providing selenium in glycine reductase and other dehydrogenase enzymes [12-13]. Selenium oxyanions, however, represent a health hazard when present in high concentration, such as in seleniferous-rich sediments [14-15]. Its initial mobilization can be a result of human activity as a direct consequence of irrigation. Several different mechanisms are known for detoxification of selenium, as methylation via methyl transferase in prokaryotes [16]. Another way of detoxification is the reduction of selenite to elemental selenium, which may be deposit intracellularly [17], in the periplasm or extracellularly [18-19]. The use of selenium oxyanions, primarily selenate, as terminal electron acceptors is also widespread among prokaryotes with some species of Crenarchaeota or Eubacteria as *Bacillus selenitireducens*, *Bordetella petrii* and *Citrobacter* [20-22].

It is obvious that arsenic and selenium are important in microbial ecology. Events such as loss of wildlife in Keterson National Wildlife Refuge due to selenium contamination [23] and the continuing devastation to people of Bangladesh due to arsenic in their drinking water [24] have promoted the investigation of the role microorganisms play in mobilization and speciation of these two elements [25-26].

The Endeavour of the present work is to study microbial reduction of arsenic and selenium oxyanions in sediments and water samples from the Nile Delta. Isolation and identification of some bacteria capable of transformation of these elements will be also fulfilled and the effect of some physiological conditions and cell immobilization on transformation will be studied.

MATERIAL AND METHODS

Sampling Locations in Nile Delta: Isolation of arsenate and selenate reducing bacteria was done from water and sediment samples collected from selected sites of water streams receiving agricultural irrigation or industrial and domestic waste waters.

Sampling Was Carried out from Agricultural or Industrial Water Drains from the Following Sites: 1-Samples from Kafr El Dawar (Beheira Governorate): Site 1 (Kom difisho, Madkhal El Magaber; Agricultural waste drain), Site 2 (Kom difisho, Ezbet El Gadida, Agricultural and Industrial waste drain), Site 3 (Kom difisho, Lozomborg, Industrial waste drain), Site 4 (Bridge 5 near El-Harier El senai Misr Company, Industrial waste drain) and Site 5 (Water drain receiving industrial waste of El Beada Dyes Company).

Samples from Kafr El Zayat (Gharbiea Governorate): Site 6 (Water drain canal receiving industrial wastes from Salt and Soda factory and El- Maliya El Aama) and Site 7 (Nile boarder near Kafr El Zayat Bridge).

Samples from Edko (Beheira Governorate): Site 8 (El-Bosiely), Site 9 (Halg El-Gamal), Site 10 (Barsig), Site 11 (Zarkon), Site 12 (Edko Lake), Site 13 (El-Salhia) and Site 14 (Omoum 1).

Different wastewater samples were collected in sterile glass blue cap bottles and sediment samples in sterile containers. The samples were kept cool in an icebox after collection and were transferred to the laboratory for carrying out isolation and other analysis.

Isolation and cultivation of selenate and arsenate bacteria: For isolation of facultative anaerobic bacteria from the collected samples an enrichment basal medium was use. It was composed of (g/l): KH_2PO_4, 0.225; K_2HPO_4, 0.225; NaCl; 0.45; $(NH_4)_2SO_4$ 0.225; $MgSO_4.7H_2O$, 0.1; $NaHCO_3$, 4.2; $Na_2S.9H_2O$, 0.06; trace element solution, 10 ml and vitamin solution, 10 ml were added.

The medium was supplied with sodium lactate (2.25g) as electron donor and either sodium arsenate (5mM) or sodium selenate (20mM) as electron acceptors [27].

The pH of the medium was adjusted to 7.3 before inoculation. This basal medium was dispensed (20ml) in tight cup bottles and inoculated with 2ml of sediment suspension or water sample. Inoculation was carried out at 37 °C for a week. Sample showing good growth were selected and 2ml were transferred to a fresh medium and the procedure was repeated 3 times, then the samples were plated in a solid medium of the same composition and supplemented with the corresponding electron acceptor.

Estimation of Cell Growth: Bacterial growth was measured as increase in turbidity of the culture by measuring optical density at 600nm using a spectrophotometer.

Estimation of Selenate Reduction: Selenate reduction was estimated gravimetrically in some experiments by estimating the dry weight of selenium precipitated in the cultures [27].

Microbiological Analysis of Water Samples: Total bacterial count were determined using nutrient agar inoculated with different dilutions of the water samples. Total coliform and fecal coliform were determined according to the methods described in Standard Methods of Water and Wastewater Analysis "Microbiological Analysis Section" [28].

Identification of Selected Selenate and Arsenate Reducing Bacterial Isolates: The identification of 3 selected bacterial isolates was carried out using biochemical and morphological properties according to Bergey's manual [29], this was done in the fermentation biotechnology and Applied Microbiology (Ferm-BAM) center, El-Azhar University by Prof. Dr. Samir El-Louboudy.

Estimation of Heavy Metals in Sediment Samples: The concentration (μg/g) of Cu, Cr, Pb, Ni, Cd, Zn, As and Se in the collected samples, was detected using atomic absorption in the central lab. Facility of Mubarak City for Scientific Research.

Restriction Fragment Length Polymorphism (RFLP): RFLP analysis was carried out to differentiate and compare between some selected potent arsenate and selenate reducing bacteria isolated from Nile Delta.

The Genomic DNA was isolated from the bacterial cells [30]. 16S mRNA was amplified by PCR using primers (16SF 1500 and 16SR 1300).

The PCR products of the different isolates was purified and different volumes of DNA were completely digested using restriction enzymes Msp1 and Dpu1. The digested DNA was visualized by transilluminator after loading to agarose gel electrophoresis. The results were analyzed and a similarity coefficient determined. The analysis was carried out in the central laboratory of Mubarak City for Scientific Research.

Effect of Electron Donors: The effect of some electron donors in different concentrations on the growth and reduction efficiency of the three identified arsenate and selenate-reducing bacteria was studied. The electron donors tested were sodium acetate, sodium lactate, sodium citrate and sodium formate.

Effect of Immobilization: Immobilization of the selenate reducing bacteria *Hafnia alvei* was carried out on sodium alginate [31]. The alginate gel beads (10ml) entrapping *Hafnia alvei* cells were used to inoculate selenate medium (25ml) in sealed bottles. Growth and selenium precipitation were compared to those of free cells.

Scanning Electron Microscopy (SEM): For SEM examination of immobilized (alginate entrapped) bacteria. The gel beads were sectioned and dried in a desiccator for three days and then prepared for SEM by spraying with 5nm gold by a JFC- 1100 E Ion Sputtering device. The samples were examined at the electron microscope unit, Faculty of Science, Alexandria University, with a Joel SM-5300 scanning electron microscope [32].

RESULT AND DISCUSSION

Enrichment and Isolation of Arsenate and Selenate Reducing Bacteria: It was planned to isolate anaerobic facultative bacteria reducing arsenate and selenate to less or non toxic forms. Positive arsenate transformation was indicated by the presence of a yellow color or precipitate formed of arsenic sulfide (orpiment) by reaction at arsenite and sulfide ions. On the other hand, a positive selenate transformation was indicated by the presence of a red precipitate formed of elemental selenium.

The results indicate generally that water samples were lower in growth and reduction capacity of both arsenate and selenate compared to sediment. All sediment cultures (Table 1) were capable reducing selenate with variable degrees. The reduction of this electron acceptor was weak in samples 3, 5, 6 and 9. On the other hand, a very good growth and precipitation of elemental selenium as a bright red layer was obtained in samples 2, 4, 8 and 12 followed by samples 7 and 10 which showed a moderate reduction of selenium. In the case of arsenate, it was used best as an electron acceptor in samples 2, 3 and 4 showing a very good growth and formation of yellow precipitate (orpiment) these were followed by sample No.5. All the other tested samples showed a very weak or weak growth and reduction of arsenate.

Isolation was carried out from the most potent samples. It was found that most common bacteria isolated from samples 3, 8 and 12 capable of reducing selenate were gram positive cocci while those isolated from 2 and 4 were gram negative short rods. On the other hand, it was difficult to obtain arsenate-reducing colonies on agar plates and only samples 2 and 4 showed gram-positive cocci, while samples 1 and 3 gram- negative short rods gave a good growth.

Table 1: Bacterial growth and arsenate or selenate reduction in the collected sediment samples from Nile Delta

	Electron acceptor added			
	Sodium Arsenate		Sodium Selenate	
Site Number	Growth grade	Reduction (color and PPT formation)	Growth grade	Reduction (color and PPT formation)
1	++	Faint	++	Red
2	++++	Yellow	++++	Bright red
3	++++	Deep yellow	++	Orange
4	++++	Deep yellow	++++	Orange
5	+++	Yellow	++	None
6	+	Faint	++	None
7	+	Faint	+++	Orange
8	++	None	++++	Bright red
9	+	None	+	Faint
10	+	None	+++	Red
11	++	Yellow	++	Red
12	++	Yellow	++++	Bright red

C *Growth: + = Very weak ++ = Weak +++ = Moderate ++++ = High*

C *Colors Intensity and precipitation are related to reduction.*

Table 2: Some microbiological analysis of water samples collected from Nile Delta

			Fecal coliform (CFU/ml)	
Site number	Total bacterial count (x10^4 CFU/ml)	Total coliform bacteria (x10^3 CFU/ml)	*E. coli*	*Fecal streptococci*
1	9.6	3	900	800
2	4.4	25	11300	1200
3	15.6	70	27000	11000
4	22.4	10	1500	500
5	11.1	14	2500	700
6	14.2	9	100	3400
7	6.8	11	12	700
8	17.3	2	1600	200
9	8.4	10	5200	20
10	26.2	4	1400	900
11	24.2	22	1600	220
12	23.1	15	2100	1400

Water Microbiological Analysis: Bacterial counts in water samples (Table 2) indicate the presence of a high load of organic matter contamination in most of the collected samples. The number of total coliform in samples 2, 3 and 5 was 25X10^3, 70 X10^3 and 14X10^3 CFU/100ml, while in Edko sample the number of total bacteria was a slightly higher.It was also noticed that high counts of fecal streptococci were recorded in the collected samples.

Heavy Metal Analysis in the Collected Samples: Analysis of heavy metals in sediment samples showed that the concentration of these metals in the samples ranged for Cu (from 3 to 35 µg/g dry soil), Cr (From 2 to 10 µg/g), Pb (From 2 to 17 µg/g), Ni (From 2 to 6 µg/g), Cd (From 0.3 to 13 µg/g) and Zn (From 2 to 100 µg/g). Samples from Edko showed relatively higher concentrations of all the tested heavy metals. It was noticed that As was not detected in any of the tested samples, however, [11] showed that prokaryotes with the ability to reduce or oxidize inorganic or other forms of arsenic and selenium are widespread in nature and not confined to contaminated environments.

Identification of Isolated Strains: Because of the great similarity between colonies in previous steps in the present part of the work three of the bacteria capable of selenate and arsenate reduction were selected for the identification because of their high growth and reduction efficiency. These bacterial isolates were from sediment samples collected from Kafr El Dawar. The strains were identified as *Hafnia alvei* (With selenate reducing activity), *Neisseria mucosa* and *Rahnella aquatilis* (Both are arsenate reducing bacterial activity).

Table 3: Effect of PH on the growth of the selenate and arsenate reducers *Hafnia alvei*, *Neisseria mucosa* and *Rahnella aquatilis*

	Growth (OD at 600 nm)		
PH value	*Hafnia alvei*	*Neisseria mucosa*	*Rahnella aquatilis*
6	0.138	0.239	0.136
7	0.316	0.789	0.431
8	0.297	0.480	0.389
9	0.256	0.338	0.334
10	0.243	0.340	0.283

Table 4: Effect of different electron donors on the growth of the selenate and arsenate reducing bacteria

			Growth on electron donor (OD at 600 nm)			
Bacteria	Incubation time (day)	Electron donor concentration (mM)	Acetate	Citrate	Lactate	Formate
Neisseria mucosa	3	50	0.385	0.390	0.385	0.101
		100	0.395	0.455	0.450	0.335
		200	0.480	0.238	0.580	0.319
	7	50	0.150	0.182	0.123	0.162
		100	0.165	0.160	0.151	0.258
		200	0.144	0.120	0.190	0.143
Hafnia alvei	3	50	0.090	0.041	0.059	0.800
		100	0.178	0.101	0.070	0.099
		200	0.137	0.041	0.103	0.107
	7	50	0.315	0.276	0.110	0.125
		100	0.413	0.150	0.210	0.223
		200	0.513	0.125	0.351	0.210
Rahnella aquatilis	3	50	0.119	0.161	0.385	0.463
		100	0.129	0.165	0.380	0.497
		200	0.211	0.281	0.215	0.390
	7	50	0.617	0.137	0.186	0.184
		100	0.703	0.505	0.217	0.170
		200	0.176	0.288	0.175	0.129

Hafnia alvei was grouped with gamma proteobacteria of the Enterobacteriacae. A strain of *Hafnia alvei* 5-5 isolated from soil litter in California was found to be resistant to 30mM Ni^{2+} or 2mM Co^{2+} [33]. *Rahnella aquatilis* was a gram-negative rod, which is frequently isolated, it was a member of Enterobacteriacae and its natural habitat was water. *Neisseria mucosa* is believed to be a non-pathogenic species of the genus *Neisseria* that belongs to the Proteobacteria, class beta Proteobacteria, family Neisseriaceae. Most arsenate and selenate reducing bacteria as a terminal electron acceptor were also reported to belong to Enterobacteria and Proteobacteria [3, 11].

Effect of pH on the Growth of the Selected Arsenate and Selenate Reducing Bacteria: The effect of different initial pH value of the medium used for transformation was studied. The arsenate reducing bacteria *Neisseria mucosa* and *Rahnella aquatilis* were grown in a medium containing sodium arsenate (5mM) as electron acceptor and sodium lactate as electron donor, while *Hafnia alvei* was grown in a medium containing sodium selenate as electron acceptor. The results (Table 3) indicated that the three tested isolates grow better at pH 7. It was also observed that they where able to grow up to pH 10, however the growth decreased to about 43% for *Neisseria*, 67.2% for *Rahnella* and 76.9% for *Hafnia*. It was also noticed that *Neisseria* showed the best growth followed by *Rahnella* and *Hafnia* in the same order.

The three organisms were also test for their optimum temperature of incubation, which was found to be around 33-37C°. It was recorded that the most majority (90%) of microbial species capable of reducing metal oxyanions, as arsenate and selenate were neutrophiles and few species were alkaliphilic growing at pH 10 [34].

Effect of Different Electron Donors on the Growth of the Selected Arsenate and Selenate Reducing Bacteria: In the present experiment different electron donors were tested each at several concentrations. Each tested organism was cultivated in a medium containing its respective electron acceptor. The results in Table (4)

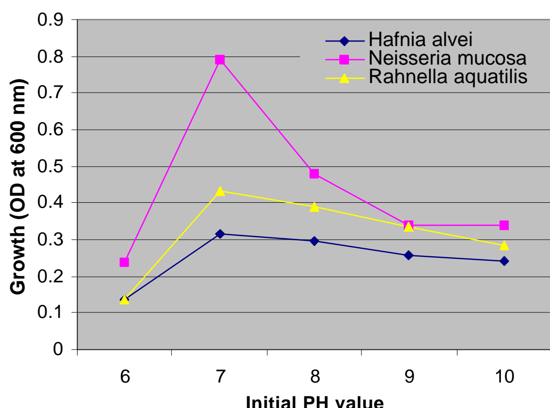


Fig. 1: Effect of PH on the growth of the tested bacteria

indicated that *Neisseria mucosa* preferred sodium lactate as electron donor showing its higher growth after 3 days of incubation when lactate was used at a concentration of 200mM; it was followed by sodium acetate, sodium formate and sodium citrate in the same order.

On the other hand, *Rahnella* and *Hafnia* showed their highest growth in cultures containing sodium acetate as an electron donor after 7 days of incubation. Among the tested bacteria *Rahnella* showed the highest growth value, which was 1.7-fold and 1.2- fold that recorded for *Hafnia* and *Neisseria*. In case of *Hafnia* a red precipitate was found with different intensities and quantities related to growth, the weight of red elemental selenium precipitated ranged from 15 to 50mg/100ml.

These results collectively are in agreement with other workers [27, 35] in testing the effect of different hydrogen donors on selenate and arsenate reduction.

Comparison Between Some Selected Selenate and Arsenate Reducing Bacteria Using Restriction Fragment Length Polymorphism (RFLP): In the present part of the work RFLP was carried out to differentiate and compare between arsenate and selenate reducing bacteria isolated from Nile Delta. Nine isolates were selected including *Neisseria mucosa* (No.1), *Rahnella aquatilis* (No.2) and *Hafnia alvei* (No.8), which were used for comparison. Isolates numbers (1-5) were all arsenate-reducing bacteria while those numbers from 6 - 9 were selenate reducers. The DNA of the bacteria was isolated and treated as shown in materials and methods. The results in Figure (2 and 3) represent the restriction bands pattern of each organism formed after restriction enzymes digestion.

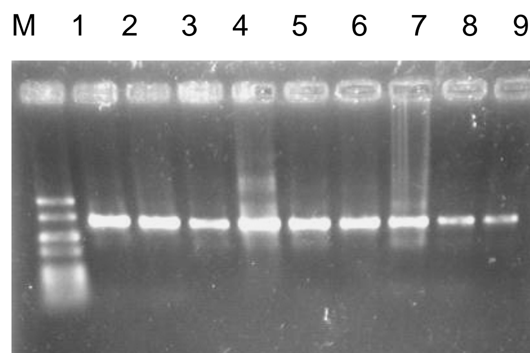


Fig. 2: PCR product of 16S rRNA amplification of different isolates using primer set 16SF1300/16SR1300 and M refer to the marker ladder and the numbers are corresponding to the isolates

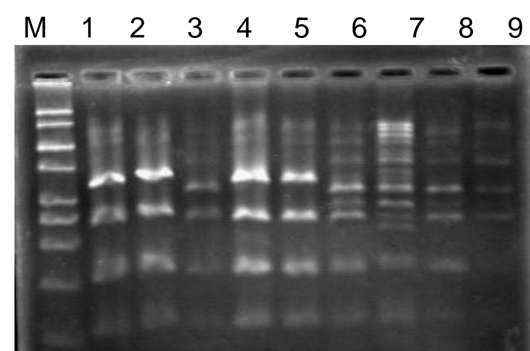


Fig. 3: Restriction digestion of the 16SrRNA PCR products of all isolates and M refer to the marker ladder and the numbers are corresponding to the isolates

The similarity coefficient was calculated for the tested bacteria and it was found that isolates number 1 (*Neisseria mucosa*), number 2 (*Rahnella aquatilis*), number 4 and number 5 may be grouped together because of their high similarity and this group included arsenate reducing bacteria.

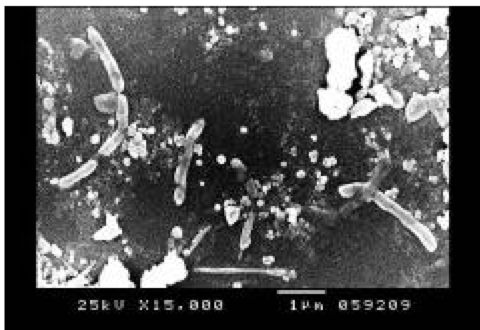
A second group included *Hafnia alvei* and isolate number 3, which were also highly similar and both were selenate reducers and organism number 6 was nearly related to this group, but with less similarity. The last two isolates numbers 7 and 9 were also moderately similar to each other but much far than all the other tested isolates.

Reduction of Selenate by Immobilized *Hafnia Alvei* Cells: In this part of the work it was aimed to test the possibility of using immobilized microbial cells for the transformation of arsenate or selenate and compare to free cells. Accordingly, *Hafnia alvei* cells were selected for immobilization by entrapment in calcium alginate beads.

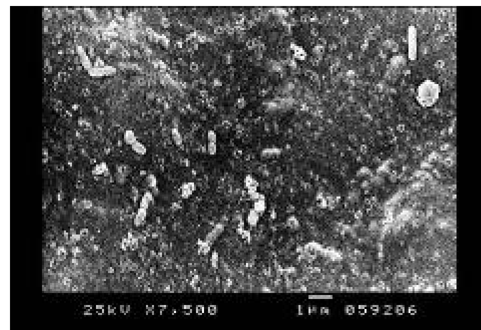
a) Free and immobilized bacterial isolates in medium with selenate

b) Immobilized beads at zero time.

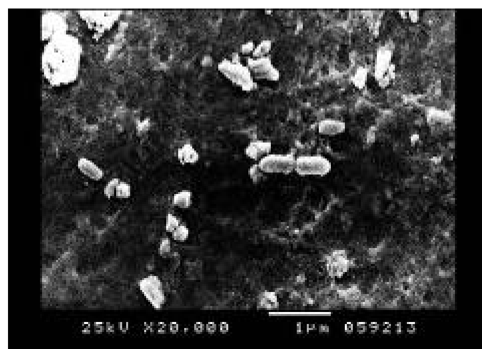
Fig. 4a,b: a) Free and immobilized bacterial isolates in medium with selenate, b) Immobilized beads at zero time



a) Free cells of *Hafnia alvei* and selenium crystals.



b) Immobilized cells of *Hafnia alvei* at zero time.



c) Immobilized cells of *Hafnia alvei* after cultivation with selenium.

Fig. 5a-c: a) Free cells of Hafnia alvei and selenium crystals, b) Immobilized cells of Hafnia alvei at zero time, c) Immobilized cells of Hafnia alvei after cultivation with selenium

The beads were incubated in arsenate minimal medium for 3-7 days at 37C°. The results shown in Fig. 4 (a and b).

Show that immobilized cells were capable of reducing selenate and a red precipitate was formed. However, it was noticed that the gel beads become fragile mostly because of the presence of a high phosphate or sulfate concentration in the medium, however this may be overcome by using other entrapment gel materials as agar, K-carageenan or polyacrylamide.

Scanning electron microscope observations of the free and immobilized cultures was carried out for *Hafnia* cells (Fig 5 a, b and c).

The cells in the immobilization material were a little bit smaller in size and dense in certain locations forming pockets. The electron microscope showed also the formation of precipitated selenium nanocrystals around the cells formed as a result of selenate reduction these particles range in size from 200 to 500 nm.

It was found by other investigators that the microbially formed nanoparticles of elemental selenium might have certain characteristics, which may not be achieved by current methods of chemical synthesis [36-37]. The present results show that selenate can serve as electron acceptor for free or immobilized cells forming distinct nanocrystals of elemental selenium.

The present work indicates the possibility of isolating a relatively large number of arsenate or selenate reducing bacteria from Nile Delta. Application of these bacteria and specially as immobilized form to contaminated sites may be offering a good way of bioremediation and a way of eliminating such toxins as much as possible from the contaminated regions.

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