

# Isolation, screening and identification of the best selenium-reducing bacteria and study on the sorption mechanism

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## Abstract

Selenium is an essential dietary trace element. However, it is toxic at micro-molar concentrations. Natural sources and anthropogenic activities have resulted in raised levels of selenium. Methods of remediation of polluted areas include various physico-chemical techniques, which are expensive and are not effective to efficiently reduce metal and metalloids concentrations. Nevertheless, it is well known that a large number of microorganisms can aerobically reduce selenite or selenate to Se (0). For this reason, microorganisms could be potentially used for bioremediation of polluted sites, as they have developed broad-range resistance mechanisms, such as oxidation, reduction, volatilization and methylation which mediate the natural cycling of these oxyanions and cause biological transformation of selenium oxyanions from one form to another. Among available technologies bacterial reduction of selenate to elemental Se (Se 0) has potential because elemental selenium is insoluble and has the lowest toxicity for biological systems. Moreover, it can be removed by some techniques such as filtration. It is worth noting that isolation of selenate-reducing bacteria has been reported from many different environments. Therefore, the aims of this research were isolation and identification of selenate-resistant bacteria, which may be applied for bioremediation purposes. In the first stage, serial dilutions of samples were cultured on modified Luria-Bertani agar medium (plus 0.2 mM sodium selenate). In order to limit the number of isolated bacteria and to achieve to the most and best resistant isolates, resistance threshold was studied by minimum inhibitory concentration, minimum bactericidal concentration and disc diffusion methods at 32.5 to 1200 mM. Of the isolated bacteria, one isolate from more resistant bacteria was selected and used to perform comprehensive studies. Biosorption and metal removal efficiency of the best isolate were investigated using metabolically active and inactive biomasses (using autoclave and drying by oven). Identification was performed using morphological (e.g. microscopic shape and colony features on agar medium) and biochemical tests (e.g. catalase/oxidase activity, utilization of maltose/sucrose and urea) and molecular methods. Among the isolates, only one isolate had high resistance to selenate; it showed minimum inhibitory concentration and minimum bactericidal concentration equal to 1200 mM and >1200 mM, respectively. Biosorption studies indicated that among different treatment methods, resting cells treated by autoclaving have the highest biosorption capacity (1550.3 mg g<sup>-1</sup>) and metal removal efficiency (12.76%). Finally, the isolate was identified as a species of the *Klebsiella* genus. Overall, comparison of this study with other published work (especially reported results from Iranian studies) showed that this isolate has potential for industrial application on environment cleanup activities.

**Key words:** biosorption, isolation, microorganisms, resistance mechanism, selenate.

**Abbreviations:** C<sub>p</sub>, final metal concentration; C<sub>i</sub>, initial metal concentration; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; mLBA, modified Luria-Bertani agar; OD, Optical density; %RE: metal removal efficiency; S, total biomass; V, volume of reaction.

## Introduction

Several contaminants are important drinking-water pollutants, including selenates, perchlorates, arsenates, nitrates, chlorates, chromates, and pesticides like dibromochloropropane (Chung et al. 2007). Among different toxic heavy metals and metalloids that may be introduced into environment, selenium (Se) is a naturally occurring trace element with considerable biological interest (Maiers et al. 1988; Pierru et al. 2006). It is the 67<sup>th</sup> most abundant element with a concentration of 50 ppb in the earth's crust, 5 ppm in soil and 0.2 ppb in sea water (Burra 2009). The concentration of selenium in air is 1 ng

per cubic meter of air, commonly existing in a methylated form (such as CH<sub>3</sub>SeCH<sub>3</sub>) (Burra 2009).

There are four inorganic oxidative states of Se. Two high valence forms, i.e. SeO<sub>4</sub><sup>2-</sup> (the most oxidized form) and SeO<sub>3</sub><sup>2-</sup> cause chronic and acute toxicity for organisms inhabiting aquatic environments because of high solubility of Se (VI) and Se (IV) (Burra 2009). Se (0) is insoluble and therefore is non-toxic. However, Se (-II) is the most reduced type with high toxicity and reactivity, but because it is readily oxidized to insoluble elemental selenium in the presence of air, it is seldom a biological threat (Maiers et al. 1988; Barasa 2008; Burra 2009; Lenz, Lenz 2009). Toxicity and availability of these Se forms strictly depends on their

chemical form and water solubility. Therefore, because selenate and selenite are the most water soluble forms, they are most harmful for living organisms (Antonioli et al. 2007; Barasa 2008).

In trace amounts selenium plays an important biological role in the form of Se-containing amino acids (selenomethionine and selenocysteine), which are similar to sulfur-containing cysteine and methionine. Moreover, it has different cellular roles including protection of cell membranes from oxidative damage, and reducing the toxicity of metals such as mercury in biological systems in the form of glutathione peroxidase, which has antagonist activity (Lenz, Lens 2009). This key biologically element is found in all main domains of life (*Bacteria*, *Archaea*, and *Eukaryota*), as well as in viruses (Oremland et al. 2004). Selenium deficiency in humans can be resolved by its uptake in the form of dietary supplements such as sodium selenate and sodium selenite. Anticancer activity of selenium and the correlation between selenium deficiency and anemia have been reported (Burra 2009). When the amount of Se exceeds optimum concentration, adverse effects will affect different organs of body such as the nervous system (Burra 2009). As Se is essential for living cells at low concentrations and since it is toxic at high dose, it is also called an "essential toxin" (Lenz 2008). Because of the conductive properties of Se, it is used in various industrial products such as light meters, photocopiers, and solar cells, and in different industries producing products containing selenium (pesticides, pigments, glass etc.), and its emission to the environment can cause problems, for example, bird malformations (Chung et al. 2007).

The occurrence of toxic levels of Se concentrations in the environment is due to both natural (e.g. fossil fuels and igneous rocks, severe weathering of minerals and rocks, and release of chemicals from volcanic eruptions) (Zawadzka et al. 2006; Burra 2009) and anthropogenic (e.g. mining and industrial wastewaters, use of hazardous chemicals for different activities) phenomena (Burra 2009; He, Yao 2010). Because this, humans and animals life are threatened with many toxic effects of this metalloid. Because of a dual role of selenium, studies on Se speciation and presence in the environment are important (Burra 2009). In this regard, various treatment methods are being used to reduce

pollution of different environments to selenium oxyanions.

Conventional treatment methods (including some types of physico-chemical methods) are expensive and in some cases are not effective in removing these compounds from wastewaters. In contrast, there is an emerging method that uses natural property of microorganisms, metal resistance capability (Roux et al. 2001), called bioremediation (Belzile et al. 2006). Many microorganisms can perform a variety of Se oxyanion transformations (for example, dissimilatory and assimilatory reduction, alkylation-dealkylation, oxidation, bio-induced precipitation, disproportionation), which can be used to reduce its negative biological effects. Microorganisms that can tolerate or resist to high toxic levels of selenium are not restricted to specific areas and are not limited to any specified taxonomic groups (Watts et al. 2005). Some of these microbial genera include: *Acinetobacter*, *Aeromonas*, *Bacillus*, *Candida*, *Cephalosporium*, *Citrobacter*, *Flavobacterium*, *Fusarium*, *Neurospora*, *Penicillium*, *Pseudomonas*, *Salmonella*, *Scopulariopsis*, and *Selenomonas* (Maiers et al. 1988; Lortie et al. 1992; Lenz, Lens 2009).

These microorganisms have developed multiple detoxification strategies by which even high concentrations of toxic elements can not damage cells (Stolz et al. 2002). Some examples of these mechanisms (which may either in a form of intra- or extracellular mechanisms) are: reduced sensitivity of inner targets against metallic ions, metabolic diversity for using these elements, using of some metals in specific (i.e., cytoskeleton) or functional (i.e., an enzyme catalytic site) structures. These functions result from assimilatory or dissimilatory processes (Stolz et al. 2002). In the field of Se detoxification, reduction of selenium oxyanions by microbial cells plays an important role in selenium transformation in the biosphere (Dridge et al. 2007). Utilizing this mechanism, some bacteria can use oxidized forms of Se (selenate and selenite) as respiratory substrates and produce elemental selenium. The elemental selenium readily precipitates and is mostly unavailable for biological systems. Therefore the potential toxicity of selenium oxyanions is reduced (Dridge et al. 2007).

The main goals of this study were isolation and identification of selenate-resistant bacteria for further use for bioremediation of contaminated sites. In addition, a possible sorption mechanism was investigated.

**Table 1.** The number of isolated bacteria from primary screening of selenate-resistant bacteria

| Sample code | Site of sampling                     | The number of isolated bacteria |
|-------------|--------------------------------------|---------------------------------|
| CBW         | Carbon-black waste                   | 14                              |
| CBS         | Carbon-black sludge                  | 9                               |
| FAW         | Farsit Waste                         | 1                               |
| AMW1        | Amir-Kabir sugarcane waste (site 1)  | 2                               |
| AMS1        | Amir-Kabir sugarcane sludge (site 1) | 17                              |
| AMW2        | Amir-Kabir sugarcane waste (site 2)  | 18                              |
| AMS2        | Amir-Kabir sugarcane sludge (site 2) | 12                              |

## Materials and methods

### *Isolation and screening of selenate-resistant strains*

Three wastewater and sediment samples were collected from industrial plants (namely, Amir-Kabir, Farsite and Carbon-Black) of Khouzestan province, Iran (Table 1). These samples were kept on ice without freezing and immediately transferred to the laboratory (Mortazavi et al. 2005). Thereafter, in the period from 24 to 48 h after sampling, for direct culturing, subsamples (0.1 mL) were serially diluted in the range  $10^{-1}$  to  $10^{-5}$ . Aliquots (0.1 mL) from each dilution were spread onto modified Luria Bertani (mLB) agar medium, pH 7.5. The ingredients of the mLB agar medium (per L) were: 5.0 g yeast extract, 10.0 g bacto-tryptone and 15.0 g agar. Because of the sensitivity of selenate to high temperature during autoclaving, 0.2 mM of  $\text{Na}_2\text{SeO}_4$  was added to the medium as filter-sterile solution after autoclaving the medium. Incubation was performed aerobically at 30 °C for 3 days. To obtain pure cultures from isolated colonies, discrete colonies were sub-cultured on nutrient agar medium until only one colony with unique features appeared on the medium (Chovanová et al. 2004; Mortazavi et al. 2005; Zolgharnein et al. 2007; Ghosh et al. 2008; Mishra et al. 2009).

### *Qualitative analysis of selenate resistance levels*

In order to decrease the number of isolated bacteria for the next stage, three qualitative tests were used, including growth inhibition zone assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. Selection of the best isolate was performed comparing the results from these tests (Ruanchaiman et al. 2009). All assays at this stage were performed in triplicate.

### *Toxic effect of Se (VI) on the isolated strains*

The disc diffusion method was selected for analysis of the toxic effect of selenate on isolated strains. For this purpose, 50 µL of previous isolate grown on mLB medium was inoculated into mLB agar plates by lawn culture. The discs were impregnated with different concentrations of  $\text{Na}_2\text{SeO}_4$  (at the range of 32.5 to 1200 mM), applied to the surface of the medium and incubated at 30 °C for 24 h (Soltan et al. 2008; Prapagdee, Watcharamusik 2009).

### *Determination of MIC and MBC*

MIC is defined as the lowest concentration of inhibitor that prevents the growth of the selected microorganism (Richards et al. 2002; Soltan et al. 2008). In this study, MIC was determined in mLB medium with various concentrations of selenate salt solution ranging from 32.5 to 1200 mM. Inoculation was performed using 100 µL of fresh and pre-grown bacterial suspension (set to McFarland Standard No. 0.5; Mortazavi et al. 2005; Sarin, Sarin 2010; Nasrazadani et al. 2011). After incubation at 30 °C for 48 to 72 h at 150 rpm, the tested suspensions were assayed by

measurement of optical density (OD) at 600 nm. Culture medium with a respective concentration of sodium selenate (without any inoculation of bacteria) was used as a blank for the OD measurement. Each test tube without noticeable growth was used for MBC determination. For this purpose, 50 µL of sample was streaked onto mLB medium without any metal challenge (Abdelatey et al. 2011; Nasrazadani et al. 2011).

### *Growth profile of selected isolates*

Growth studies were performed in flasks containing 50 mL mLB medium in triplicate with two subsets: supplemented with and without 100 mM  $\text{Na}_2\text{SeO}_4$  (Raja et al. 2009). Inoculum (1 mL) from an overnight culture was inoculated to each flask (Adarsh et al. 2007; Abdelatey et al. 2011). At predefined intervals, growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer, until the stationary phase was reached (Raja et al. 2009). The final growth curve was based on the average of three replicates.

### *Quantitative analysis of bio-removal ability by resting bacterial cells*

The best strain was selected and cultivated aerobically in mLB broth at 30 °C and 150 rpm. With reference to the growth curve, medium was centrifuged for 15 min at 4 °C, 6000 rpm, and cells were harvested by washing twice with double-distilled water (Ozdemir et al. 2004; Tarangini 2009). Cell samples of the strain (0.4 g dry mass) were re-suspended in 40 mL Se (VI) solution (100 mM) and incubated at 30 °C on a rotary shaker at 150 rpm until equilibration was reached (Abd-Elnaby et al. 2011). All assays at this stage were performed in triplicate.

### *Study of the removal mechanism by test isolate*

The capability of the cells to remove Se (VI) from solution was studied with both living and dead resting cells of the selected microorganism. In order to determine the biosorption mechanism of the isolate, both metabolically active and inactive biomass was used. Inactivation was performed by two heat treatment methods: autoclaving at 121 °C for 15 min and overnight treatment at 100 °C. Each type of biomass was challenged with selenate aqueous solution in triplicate, as described above. After the set contact time between metal solution and bacterial biomass, biomass and metal solution was separated by centrifugation at 6000 rpm for 15 min at 4 °C (Andreoni et al. 2003; Bai et al. 2008).

### *Analysis of residual metal concentration*

The residual selenate concentration in the supernatant was analyzed using an atomic absorption spectrophotometer (Savant AA A7104, Australia) (Kasra Kermanshahi et al. 2007; Bai et al. 2008; Sinha, Mukherjee 2009). The amount of biosorbed metal was calculated according to the equation:

$$\text{Metal uptake} = V (C_i - C_f) / S,$$

**Table 2.** Inhibition zone (mm) assay of nine bacterial isolates. All results represent an average of three experimental replications

| Strain  | Concentration (mM) |      |      |      |      |      |      |
|---------|--------------------|------|------|------|------|------|------|
|         | 0                  | 32.5 | 75   | 150  | 300  | 600  | 1200 |
| CBW-S1  | -                  | 9    | 9.3  | 10.7 | 16.3 | 20   | 26.3 |
| AMW2-S2 | -                  | -    | -    | 9.7  | 9.7  | 10.7 | 11.7 |
| CBW-S3  | 9.3                | 9.7  | 11.3 | 11.3 | 15.3 | 19   | 21.7 |
| CBW-S4  | 8.3                | 9    | 11.3 | 17.7 | 23   | 29   | 33   |
| AMW2-S5 | -                  | -    | 9    | 10   | 12   | 16.3 | 19.7 |
| AMW2-S6 | -                  | 8    | 8.3  | 8.7  | 9.3  | 10   | 11   |
| AMS1-S7 | -                  | -    | 8.3  | 10.7 | 14   | 17   | 22   |
| AMS1-S8 | -                  | -    | 9.3  | 12.7 | 17   | 21.3 | 25.3 |
| CBS-S9  | -                  | -    | -    | -    | 9    | 9.3  | 10.3 |

where  $C_i$  is an initial metal concentration ( $\text{mg L}^{-1}$ ),  $C_f$  is a final metal concentration ( $\text{mg L}^{-1}$ ),  $V$  is a volume of reaction (L) and  $S$  is a total biomass (g) (Pierru et al. 2006; Yee et al. 2007). The mass of dried biomass was determined after drying at 100 °C overnight. A standard curve of selenate sorption was produced using metal solution containing 80, 200 and 500 ppm of selenate anion.

#### Removal efficiency

Removal efficiency was expressed as the percentage of adsorbed metal compared to initial metal concentration. This parameter was calculated using the equation:

Se (VI) removal efficiency (%) =  $(C_i - C_f) \times 100 / C_i$ , where  $C_i$  is the initial metal concentration ( $\text{mg L}^{-1}$ ) and  $C_f$  is the final metal concentration ( $\text{mg L}^{-1}$ ) (Abd-Elnaby et al. 2011).

#### Characterization of the selected isolate

Cellular morphology (including gram reaction, cellular shape, presence/absence of spores) was determined by light microscopy on an Olympus BX51 microscope.

The isolate was tested and characterized using several physiological key conventional tests for basic differentiation of bacteria. Some tests applied for identification of the strain were: utilization of different sugars like maltose and sucrose; growth features in SIM medium and others

(Chovanová et al. 2004).

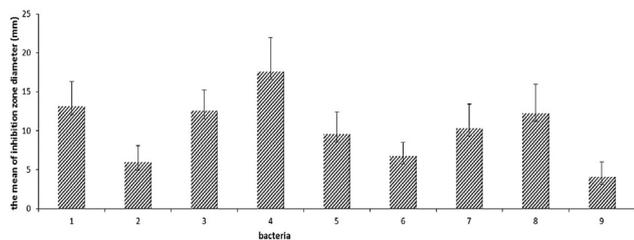
Genomic DNA of the test bacterial strain was prepared using a pure culture grown from a single colony. DNA extraction was performed according to a DNA extraction kit (Choudhary, Sar 2009; Raja et al. 2009). After assurance from purity and amount of extracted genome, bacterial 16S rRNA was amplified using universal 16S rRNA primers F and R with the corresponding sequences:

5'-CCGAATTCGTCGACAACAGAGTTT GATCCTG GCTCAG-3' (F-primer sequence), 5'-CCCGGGATCCAA GCTTACGGTTACCTTGTTACGACTT-3' (R-primer sequence).

The PCR mixture (25  $\mu\text{L}$ ) contained 1  $\mu\text{L}$  template, 2.5  $\mu\text{L}$  10  $\times$  Taq DNA polymerase buffer, 1  $\mu\text{L}$   $\text{MgCl}_2$  (50 mM), 1  $\mu\text{L}$  10 mM dNTP, 0.3  $\mu\text{L}$  1.5 units Taq polymerase, 0.5  $\mu\text{L}$  of each primer (25  $\mu\text{mol}$ ). PCR was carried out according to Jiang et al. (2008). The thermal cycling of applied PCR program was as follows: 94 °C for 3min, 30 cycles of 94 °C for 30 s, 61.3 °C for 30 s and 72 °C for 1 min; final extension was performed at 72 °C for 5 min. PCR products were analyzed by agarose gel electrophoresis using 1% agarose gel in TAE buffer and running at 90 V for 30 min (Raja et al. 2009). The sequence obtained was subjected to nucleotide BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). The novel sequence was subjected to the GenBank. Phylogenetic analysis was performed by neighbor joining method

**Table 3.** Optical density of nine isolates at the stage of MIC determination. All results represent an average of three experimental replications

| Strain  | Concentration (mM) |        |        |        |        |        |        |
|---------|--------------------|--------|--------|--------|--------|--------|--------|
|         | 0                  | 32.5   | 75     | 150    | 300    | 600    | 1200   |
| CBW-S1  | 1.3412             | 1.9666 | 1.5087 | 1.3703 | 0.5628 | 0.1718 | 0.1572 |
| AMW2-S2 | 1.5246             | 3.1750 | 2.2241 | 0.9776 | 0.1382 | 0.0902 | 0.0994 |
| CBW-S3  | 1.4179             | 2.091  | 1.8139 | 1.9181 | 1.1458 | 0.1573 | 0.1059 |
| CBW-S4  | 1.4962             | 2.1835 | 1.6800 | 1.4494 | 1.2550 | 0.1661 | 0.0954 |
| AMW2-S5 | 0.9226             | 1.4305 | 1.3590 | 0.3940 | 0.1060 | 0.1182 | 0.0959 |
| AMW2-S6 | 0.1284             | 1.7458 | 1.6895 | 1.5681 | 0.8157 | 0.2723 | 0.1229 |
| AMS1-S7 | 1.2912             | 1.1631 | 0.5953 | 0.4859 | 0.2542 | 0.1129 | 0.0957 |
| AMS1-S8 | 1.4957             | 2.2525 | 2.2374 | 2.0042 | 1.1784 | 0.0904 | 0.1094 |
| CBS-S9  | 0.9731             | 1.7578 | 1.8849 | 1.3101 | 0.1834 | 0.1262 | 0.0817 |



**Fig. 1.** Statistical comparison between inhibition zone diameter of all selenate-resistant isolates ( $p < 0.05$ ). 1, CBW-S1; 2, AMW2-S2; 3, CBW-S3; 4, CBW-S4; 5, AMW2-S5; 6, AMW2-S6; 7, AMS1-S7; 8, AMS1-S8; 9, CBS-S9.

using MEGA 4.0 software (Adarsh et al. 2007). The novel sequence of the 16SrRNA gene sequence was deposited as accession JQ965666 in the GenBank database.

### Statistical analysis

The significance in differences was determined using one-way ANOVA, SPSS software, version 19. Distribution of MIC, MBC, %RE and biosorption were examined by Chi-Square test (0.95 confidence level).

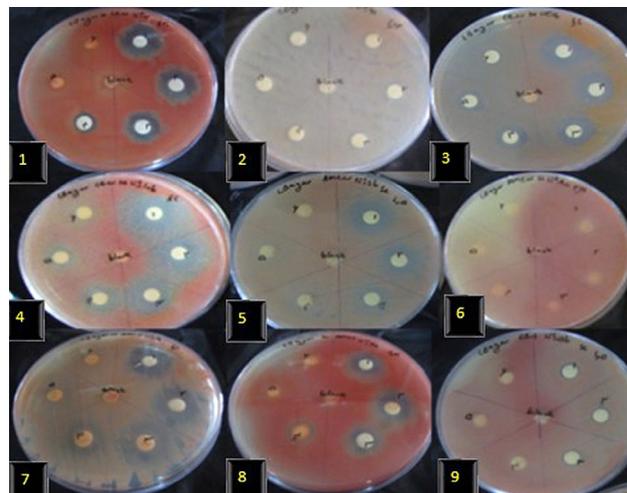
## Results

### Selenate reduction

Among the first 73 isolated bacteria (Table 1), nine isolates could tolerate as high as 160 mM of selenate in the mLBA medium at pH 7.5. These isolates were investigated for formation of reddish-colored colonies on agar plates supplemented with selenate (data not presented), a clear indication that strains reduced selenate to elemental red selenium [Se(0)]. The code name of each isolate was assigned according to the site of isolation.

### Selenate toxicity

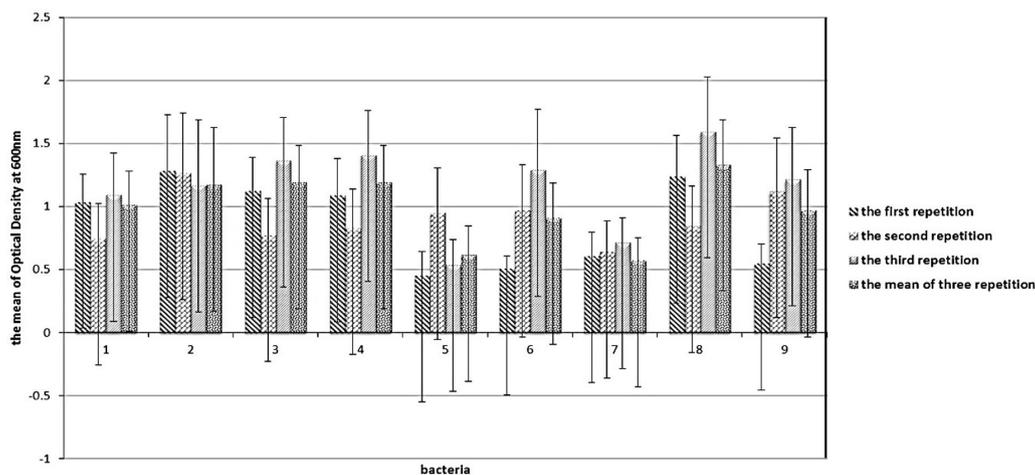
The results of plate sensitivity assay and optical density of the



**Fig. 2.** Production of red color on agar medium in the presence of Se (VI). 1, CBW-S1; 2, AMW2-S2; 3, CBW-S3; 4, CBW-S4; 5, AMW2-S5; 6, AMW2-S6; 7, AMS1-S7; 8, AMS1-S8; 9, CBS-S9.

nine isolates are shown in Table 2 and 3. Statistical analysis of results from growth inhibition zone assay showed that there was no statistically significant differences between isolates ( $p > 0.05$ ; SE 0.5501; SD 7.5622) (Fig. 1). For this reason, the best isolate was selected by the production of red color on plates as an indication of bioreduction capacity of Se (VI) to Se (0) (Fig. 2).

There were no significant differences in  $OD_{600}$  between the replicates ( $p > 0.05$ ; SE 0.0637; SD 0.8748) (Fig. 3). Moreover, for isolate CBW-S1, MIC exceeded 1200 mM and MBC was equal to 1200 mM, which is an unusual level of resistance (Table 4). These results were confirmed using the Chi-square test and indicated that the CBW-S1 isolate significantly differed from the other isolates ( $X^2_{0.05,2} > 5.991$ ). Therefore this isolate was selected for further studies. The optical density curve of the selected isolate in the presence of different selenate concentrations is shown in Fig. 4.



**Fig. 3.** Statistical comparison of  $OD_{600}$  results among all studied strains at MIC determination stage ( $p < 0.05$ ). 1, CBW-S1; 2, AMW2-S2; 3, CBW-S3; 4, CBW-S4; 5, AMW2-S5; 6, AMW2-S6; 7, AMS1-S7; 8, AMS1-S8; 9, CBS-S9.

**Table 4.** MIC and MBC of nine bacterial isolates. All data represent an average of three experimental replications

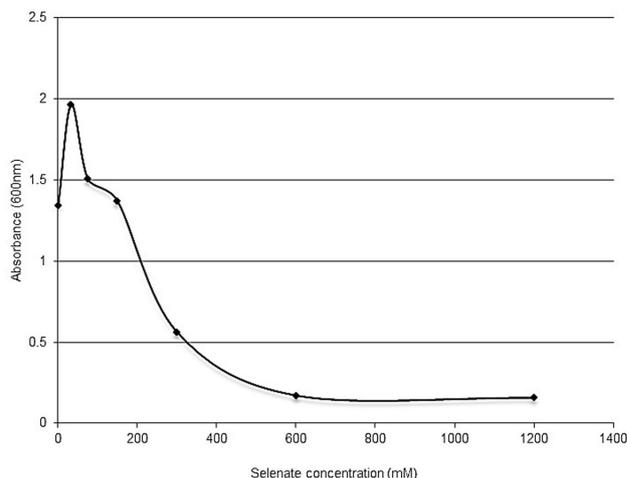
| Isolate | MIC (mM) | MBC (mM) |
|---------|----------|----------|
| CBW-S1  | 1200     | >1200    |
| AMW2-S2 | 300      | >1200    |
| CBW-S3  | 600      | 600      |
| CBW-S4  | 600      | 600      |
| AMW2-S5 | 300      | 300      |
| AMW2-S6 | 600      | 600      |
| AMS1-S7 | 150      | 150      |
| AMS1-S8 | 600      | 1200     |
| CBS-S9  | 300      | >1200    |

**Growth of selected isolate**

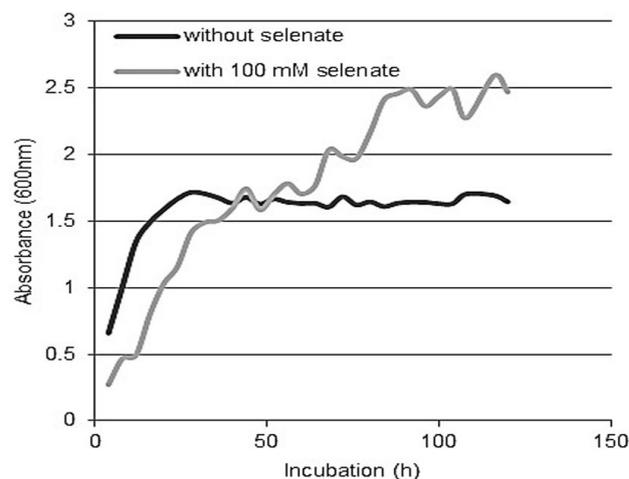
Growth was monitored at predefined intervals until the stationary phase was reached (Fig. 5).

**Biosorption studies**

In this study, 100mM of selenate solution was applied to pre-grown mid-exponential bacterial cells. The biosorption and metal removal efficiency are shown in Table 5 and Fig. 6. This bacterium preferred the biosorption mechanism (rather than bioaccumulation) for removal of selenate anions, which is a good property in industrial applications. During comparison of the three different treatments methods, Chi-square test analysis of sorption capacity showed that the cells treated by autoclaving had more biosorption efficiency rather than living cells and the cells treated by heating at 100 °C, respectively. There was a significant difference between cells treated by heating at 100 °C and living cells ( $X^2_{0.05,2} = 10.5909$ ) and between



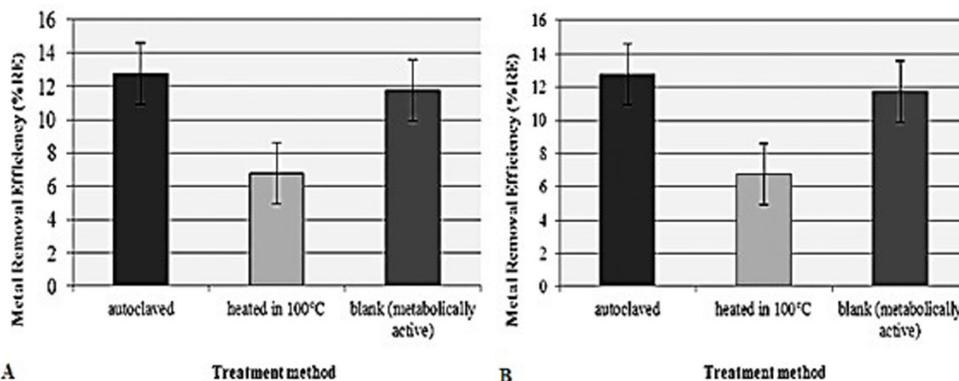
**Fig. 4.** Optical density curve of CBW-S1 isolate.



**Fig. 5.** Growth studies of selected bacteria.

**Table 5.** Adsorption capacity and metal removal efficiency of the selected strain. All data represent an average of three experimental replications

| Parameter  | Cell type            |                              |                                    |
|--|----------------------|------------------------------|------------------------------------|
|  | Blank (living cells) | Cells treated by autoclaving | Cells treated by heating at 100 °C |
| SeO <sub>4</sub> <sup>2-</sup> adsorption capacity (mg g <sup>-1</sup> ) | 1482.1               | 1550.3                       | 1130.5                             |
| Metal removal efficiency (%)   | 11.73                | 12.76                        | 6.80                               |



**Fig. 6.** Selenate sorption capacity (A) and selenate removal efficiency of active and inactive biomass (B) of CBW-S1 isolate

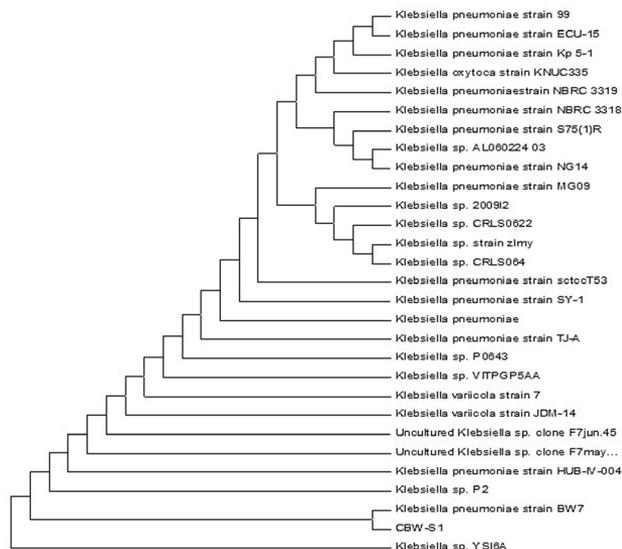
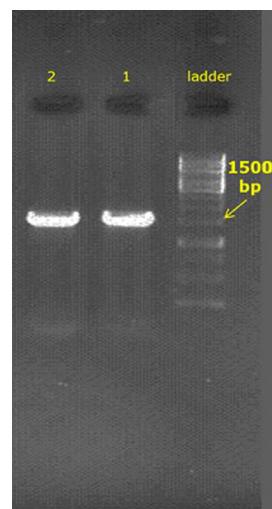
**Table 6.** Morphological and biochemical characteristics of CBW-S1

| Characteristic              | Response                         |
|-----------------------------|----------------------------------|
| Catalase activity           | +                                |
| Oxidase activity            | -                                |
| Oxidative/Fermentative (OF) | +/+                              |
| Urease activity             | +                                |
| Utilization of citrate      | +                                |
| Utilization of glucose      | +                                |
| Utilization of xylose       | +                                |
| Utilization of lactose      | +                                |
| Utilization of arabinose    | -                                |
| Utilization of sorbitol     | +                                |
| Utilization of mannitol     | +                                |
| Utilization of maltose      | +                                |
| Growth feature on MacConkey | Lactose positive                 |
| Reaction in SIM medium      | Motility indole-H <sub>2</sub> S |

cells treated by autoclaving and treated by heating at 100 °C ( $X^2_{0.05,2} = 6.7634$ ). There were no significant differences between three replicates of %RE results for all treatment methods ( $X^2_{0.05,2} < 5.991$ ).

#### Morphological and biochemical characterization of the selected isolate

One bacterial isolate (CBW-S1) was isolated from carbon-black industry wastewater of Ahwaz, Iran. The preliminary characterization of this isolate was done on the basis of its morphology and gram stain. This isolate was characteristic Gram-negative short bacilli. Biochemical characterization was conducted based on different biochemical abilities according to Bergey's manual (Table 6).

**Fig. 8.** Phylogenetic relationships among representative experimental strain (CBW-S1) and the most closely related *Klebsiella pneumoniae*. The dendrogram was generated using MEGA 4.0 program..**Fig. 7.** Electrophoresis result of PCR products 1, positive control; 2, CBW-S1 isolate. In the first column from right, ladder is shown.

#### Molecular characterization

DNA of the promising isolate was extracted and amplified. Thereafter, the produced amplicons were analyzed using agarose gel electrophoresis, as shown in Fig. 7. The BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to make sequence comparisons and a neighbor-joining tree was constructed with MEGA 4.0 software (Fig. 8).

#### Discussion

Metal-resistant bacteria can survive in contaminated habitats and can be isolated and selected for potential application in the bioremediation of contaminated sites (Jiang et al. 2008). In the present study, 73 bacterial isolates with possible resistance to selenate were isolated. Based on the results of MIC, MBC and disc diffusion method tests, one isolate was selected as the most resistant strain for further experiments.

There have been various reports on the heavy-metal resistance (especially resistance to selenium oxyanions) among bacteria. Additionally, there are great differences among the resistance and tolerance thresholds in the literature. For example, comparison of the present study with other reported data showed our isolate to have the highest MIC (1200 mM) and MBC (up to 1200 mM) to selenate.

Various mechanisms including oxidation/reduction, mineralization, methylation etc. are used by microorganisms to detoxify Se oxyanions (Hunter, Manter 2009). There are two possible pathways for microbial selenate metabolism: immobilization of Se as elemental Se, and assimilation of selenate, which leads to the formation of volatile selenium (Souza et al. 2001). In this study, the increased optical density from the 12<sup>th</sup> hours of incubation, which was observed in flasks containing selenate, was due to the contribution of

the red elemental selenium formed during reduction as a path of detoxification. Similar results were observed in some other studies (Lortie et al. 1992).

Many publications have reported high tolerance to selenite among isolated bacteria. Zolfaghari and Khalilian (2013) characterized two bacterial strains among the 263 isolates, which had MIC equal to 550 and 500 mM of selenite, respectively. In a study of Li et al. (2014) the *Rhodopseudomonas palustris* strain N, which showed ability of growth in presence of selenite at concentrations 1, 2, 4 and 8 mM, showed formation of red color after 2 days of cultivation. This strain had MIC equal to 8.0 mM of sodium selenite and after 72 h of growth, the end of log growth was reached in the presence of selenite (Li et al. 2014). Khalilian et al. (2015a) isolated a resistant *Bacillus* sp. from selenium-contaminated sites in Qom, Iran, which showed MIC equal to 550 mM concentration of selenite (by testing the MIC in the range of 100 to 600 mM). Moreover, regarding the formation of red color in cultured media containing 10 mM selenite (as a sign for formation of less toxic elemental selenium), it was reported that this isolate could be used for bioremediation purposes.

On the other hand, usually the ability of isolated microorganisms for growth in the presence of selenium oxyanions is very low. For example, in the study of Fernández Llamosas et al. (2016), *Azoarcus* sp. CIB showed ability of anaerobically growth in the presence of up to 8 mM selenite. They also reported that the formation of orange or red color in the growth medium after 72 h of cultivation (in the presence of 1 mM selenite) is a significant feature that reflects the capability of bacteria to convert the toxic selenium oxyanions to elemental selenium. Ashengrogh and Saedi (2016) isolated a lactic-acid bacteria that had high resistance to selenite. In their study, among 24 resistant bacteria isolated, only four isolates had resistance to concentrations of selenite up to 50 mM. Using the agar-dilution method, they found that this isolate had the highest MIC (110 mM) and MBC (140 mM) and the lowest extent of inhibition (with average inhibitory effect of 26.6 mm) in the medium containing 10 to 150 mM sodium selenite. After 72 h of incubation and contact between resting cells of selected isolate and selenite, it was found that this bacteria reduced selenite from 10 to < 1 mM and removed 90.2% of selenium oxyanion from the medium (Ashengrogh, Saedi 2016). In our study, the introduced isolate removed 11.73% of selenate from medium contaminated with 100 mM selenate.

Ghosh et al. (2008) isolated bacterial strains with MIC values ranging from 650 to 750 mM of selenate. Hunter and Manter (2009) isolated a *Pseudomonas* sp. CA5 from soil that was highly resistant to both selenite and selenate. This bacteria had high selenite resistance (150 mM), but at concentrations of 50, 100 and 150 mM of this oxyanions, bacterial growth was inhibited by 28, 57 and 66%, respectively. On the other hand, this bacteria

could grow at 64 mM selenate without any adverse effects on growth. In the presence of 64 mM selenate, colonies developed reddish-orange centers as a detoxification mechanism of selenate (Hunter, Manter 2009). Khalilian et al. (2015b) found a *Proteus* strain showing the highest MIC (760 mM) in medium supplemented with 100 to 800 mM selenate. However, bioremoval capacity of this isolate was tested in the presence of 1000  $\mu\text{g L}^{-1}$  of selenate, which is not comparable with our isolate, because in our study biosorption capacity was evaluated in medium containing 100 mM sodium selenate.

In the field of bioremediation of heavy metals and metalloids, there are two known biosorption mechanisms: inactive (biosorption) and active (bioaccumulation) (Al-Garni et al. 2010). It is obvious that when using various microorganisms (including bacteria, fungi, yeast and algae) for bio-removal of heavy metals, a broad range of behaviour in respect to bioremediation can be seen. For example, Gabr et al. (2008) reported that using dead (lyophilized or heat-treated bacteria) and living cells of *Pseudomonas aeruginosa* ASU 6a for lead and nickel removal ability, dead cells showed higher biosorption capacity for both metals (Gabr et al. 2008). In a study by Li et al. (2010) it was shown that dead cells of *Streptomyces ciscaucasicus* CCNWHX 72-14 have more biosorption capacity (54 mg of  $\text{Pb}^{2+}$  per g) than live cells (42.75 mg per g). With the advantage of high desorption and biosorption capacity, dead cells have the ability to become promising tools and effective biomaterials for sorption and recovery of heavy metals (Li et al. 2010).

The order of efficiency of four strains of *Bacillus* for biosorption of Cu, Ni and Cr was by immobilized > dead biomass (dried in oven at 105 °C) > living cells was reported (Al-Daghistani 2012). This order was also reported in the case of Cu, Cd and Pb (Rani et al. 2010). Preference of biosorption instead of bioaccumulation was also reported by Huang et al. (2013). It was concluded that dead cells of *Bacillus cereus* RC1 accumulated more  $\text{Cd}^{2+}$  (31.95 mg per g) than living cells (24.01 mg per g). Moreover, the highest sorption capacity was achieved after 30 min for dead cells against 50 min for living cells of the studied bacteria (Huang et al. 2013). This behaviour was observed also by Weirzba (2014) who found that using dead and living cells of *Yarrowia lipolytica* maximum biosorption capacity was 14.31 and 12.56 mg per g for each type of cells, respectively. Since dead biomass is low-cost and a more efficient biosorbent, it can be used as an alternative for the treatment of polluted waters (Weirzba 2014).

In contrast, greater efficiency of untreated or live microbial cells than dead or treated cells has been reported in some studies. In the case of *Saccaromyces cerevisiae*, living cells were more efficient than dead cells treated by autoclaving (Suh et al. 1998). This was explained by changes in structure and features of the cell wall. Junlian et al. (2010) used transmission electron microscopy to show that cells treated with either diluted HCl or heated at 100 °C had

minimum damage of surface structure. Therefore, besides using untreated biomass, these two treatment methods are more efficient than NaOH, H<sub>2</sub>SO<sub>4</sub> and concentrated HCl treatments (Junlian et al. 2010). Living non-growing cells of *Kocuria palustris* and *Micrococcus luteus* showed identical behaviour for biosorption of Cd<sup>2+</sup> (Machalová et al. 2015). Yaghoobizadeh et al. (2016) isolated a strain of *Enterobacter* sp. (with MIC equal to 600 mM for selenate). It was reported that the order of biosorption capacity of bacteria was: active biomass (558.9 mg g<sup>-1</sup> DM) > biomass inactivated by autoclaving (506.2 mg g<sup>-1</sup> DM) > biomass dried at 100 °C (Yaghoobizadeh et al. 2016). Isolated *Enterobacter ludwigii*, which was resistant to Hg (II), had also the same trend (Yaghoobizadeh et al. 2017). Living cells of *Bacillus cereus* S5 strain were more efficient than dead cells (autoclaved) in biosorption of Cd<sup>2+</sup> (Wu et al. 2016). With increasing level of cadmium in solution, the efficiency of these types of cells became nearly the same (Wu et al. 2016).

The differences between the bio-removal capacity by living, non-living and immobilized microorganisms can be explained by differences in biosorption of heavy metal ions due to interaction between cations and anionic groups commonly occurring on the cell surface (Junlian et al. 2010). It is known that the bacterial cell surface is mainly composed of peptidoglycan, phospholipids, and proteins. Some negatively-charged groups, such as carboxyl and phosphodiester groups, attached to peptidoglycans, phospholipids and proteins, provide the main binding sites for heavy metal ions. Strong treatment factors destroy cell structure leading to the loss of some negatively-charged groups. Therefore, significant destruction of cell surface structure is commonly accompanied by loss of heavy metal adsorption capacity (Junlian et al. 2010).

The present isolate had high tolerance to selenate (MIC 1200 mM and MBC up to 1200 mM) in contrast to many studies. Due to its capability for reduction of selenate to elemental selenium after 12 h of cultivation in medium containing 100 mM selenate, this strain can be potential candidate for bioremediation of contaminated environments. In this study the higher biosorption capacity of autoclaving treated cells may be explained by the importance of the cell surface structural integrity. This is important, because in bioremediation, heavy metal concentrations can be toxic to living cells. Moreover, use of dead biomass does not require supply of nutrients for growth, and metal uptake by dead biomass occurs by chemical functional groups on outside of the cells (Al-Daghistani 2012). It can be concluded that the following factors may affect bioremoval ability of microorganisms: the origin of selected strains (wastewaters, sediments, slurry), the ability of selected microorganism to established resistance mechanisms and production of unique compounds (e.g. exopolysaccharides). Thus, it is very important to perform comprehensive studies to investigate various aspects of biosorption and compare different parameters that can

affect bioremoval capacity. Comparison of various types of microorganisms (including microalgae, bacteria, fungi and yeasts) can provide valuable data for introduction of the most efficient strains for utilization in this field.

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