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To cite this article: Saima Javed, Arslan Sarwar, Mohsin Tassawar & Muhammad Faisal (2015) Conversion of selenite to elemental selenium by indigenous bacteria isolated from polluted areas, *Chemical Speciation & Bioavailability*, 27:4, 162-168, DOI: [10.1080/09542299.2015.1112751](https://doi.org/10.1080/09542299.2015.1112751)

To link to this article: <https://doi.org/10.1080/09542299.2015.1112751>



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Published online: 27 Dec 2015.



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Conversion of selenite to elemental selenium by indigenous bacteria isolated from polluted areas

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ABSTRACT

The present research work is on the biotic transformation of highly soluble and toxic selenite to less toxic elemental selenium using bacterial strains *Bacillus subtilis*, *Exiguobacterium* sp., *Bacillus licheniformis*, and *Pseudomonas pseudoalcaligenes*. The conditions were optimized by changing different physical parameters such as pH, temperature, different Selenium (Se) concentration levels (200, 400, and 600 $\mu\text{g ml}^{-1}$), aeration, and incubation time for increased selenite reduction. The Se reduction rate increased with increase in pH. On average, 28–90% selenite reduction was observed at pH 9, while 15–33% at pH 3. To check the optimum temperature, all strains were cultured at three temperatures 32, 37, and 42 °C. Selenite reduction was observed at various temperatures and the results show that each strain has different preferences for optimum Se reduction. Selenite reduction was also monitored using different initial selenite concentrations and the results showed that at lower initial concentration (200 $\mu\text{g ml}^{-1}$) maximum Se was reduced. This study showed that selenite-reducing bacteria can remediate Se in both aerobic and anaerobic environments, as well as their reduction ability decreases with increase in incubation time.

ARTICLE HISTORY

Received 8 July 2015
Accepted 18 October 2015

KEYWORDS

Selenite reduction; *Bacillus subtilis*; *Exiguobacterium* sp.; *Bacillus licheniformis*; *Pseudomonas pseudoalcaligenes*

Introduction

Environmental pollution by heavy metals is considered to be lethal for all forms of life.[1] Trace amounts of selenium are important for humans, animals, and plants but high concentration results in detrimental effects.[2] The difference between productive and noxious levels of selenium is pretty narrow making both selenium deficiency and selenium pollution a public health issue worldwide.[3] A number of microorganisms have the ability to reduce Se oxyanions.[4] They play a crucial role in recycling and transformation of selenium through redox and methylation reactions. The biomethylation of selenium in seleniferous environments is readily carried out by bacteria and plants and is thought to be an important detoxification mechanism in these organisms.[5]

Selenium is a metalloid which can exist as reduced form (selenide, Se^{2-}), as an element form (Se^0), and as water-dissolved form (selenite SeO_3^{2-} /selenate SeO_4^{2-}). Bioaccumulation of selenium as an agricultural drainage and effluents is the main cause of selenium contamination.[6] Moreover, waste discharge from industries like tanneries, glass production industry, plastic industry, paint and pigment industry, oil refineries, and power

stations are sources of water-soluble selenium contamination to our aquatic life form.[7]

Microorganisms living in selenium-rich environments often equipped with certain mechanisms enable them to convert inorganic selenium compounds into volatile compounds.[8] Those microorganisms were reported to secrete certain methyltransferases which stimulate the emission of DMSe but its mechanism is still unknown.[9] The present study was conducted on bacterial strains capable of transforming toxic SeO_3^{2-} (IV) into elemental Se(0) so that they can be used in future for remediating toxic selenium oxyanions from soils.

Materials and methods

Bacterial strains and culture conditions

Four selenium-resistant bacterial strains *Bacillus subtilis*, *Exiguobacterium* sp., *Bacillus licheniformis*, and *Pseudomonas pseudoalcaligenes* previously isolated were used in the present investigation.[10] These strains were routinely maintained at a concentration of 100 $\mu\text{g ml}^{-1}$ of selenium. These strains were isolated from polluted environment and were identified by morphological, biochemical, physiological, and 16S rRNA gene sequencing.

Bacterial selenite reduction experiments

Selenite content determination

All of selenite reduction experiments were carried out in nutrient broth and under aerobic conditions. For measuring the selenite reduction potential of different isolates, sodium selenite was added in sterilized nutrient broth at a concentration of $100 \mu\text{g ml}^{-1}$ (until otherwise stated concentration). A known quantity of fresh bacterial inoculum was given in each flask except control. After 48-h incubation, 1 ml of culture was taken in a microfuge tube and centrifuged at 14,000 rpm for 5 min. Selenite content (elemental selenium) was determined using spectrophotometer using modified method of Brown and Watkinson.[11] First, 10 ml of 0.1 M HCL, 0.5 ml of 0.1 M EDTA, 0.5 ml of 0.1 M NaF, and 0.5 ml of 0.1 M disodium oxalate were mixed in a glass tube. 250- μl sample was added and then 2.5 ml of 0.1% 2,3-diaminonaphthalene in 0.1 M HCL was added. After the contents were mixed, the tubes were incubated at 40°C for 40 min and then cooled to room temperature. The selenium 2,3-diaminonaphthalene complex was extracted with 6 ml of cyclohexane by shaking the tubes vigorously for about 1 min. The absorbance at 377 nm of the organic phase was determined, whereas absorbance was made zero for cyclohexane.[12] All manipulations were done in the dark.

Effect of various pH on selenite reduction

Nutrient broth was prepared in 500-ml flasks. Three flasks for each strain were prepared and supplemented with $200 \mu\text{g ml}^{-1}$ of sodium selenite. pH was adjusted to 3, 5, 7, and 9 in different flasks and labeled properly. After inoculation, all the flasks were incubated at 37°C for 48 h. Selenite content was estimated in the supernatant after 48 h of incubation. Optical densities were plotted on the calibration curve to measure the selenite concentration. Negative control (uninoculated nutrient broth) supplemented with $200 \mu\text{g ml}^{-1}$ was also estimated for selenite content after 48 h.

Effect of various temperatures on selenite reduction

In order to determine the effect of temperature on reduction of sodium selenite, three different temperatures (32°C , 37°C , and 42°C) were selected as a temperature range on which these strains can grow. Nutrient broth was prepared in flasks and supplemented with $200 \mu\text{g ml}^{-1}$ sodium selenite. Three flasks were prepared for each strain for all the temperatures (32°C , 37°C , and 42°C) and labeled properly. A negative control was also prepared. Afterward, inoculation was given in the same manner as already described. Flasks were incubated at 32°C , 37°C , and

42°C for 48 h and then checked the reduction in the selenite as described above.

Effect of aeration on selenite reduction

It is important to know the aeration requirement for selenite reduction by these strains at the laboratory level before going to large-scale studies. To study the effect of aeration on selenite reduction, two sets of flasks were prepared. One set was incubated on the shaking incubator, while the other set was kept in the still incubator at 37°C for 48 h. Reduced selenite was checked as described above.

Effect of different initial selenite concentrations

Three different sets of flasks were prepared for each strain which were supplemented with 200, 400, and $600 \mu\text{g ml}^{-1}$ of sodium selenite. Negative controls for each concentration were also prepared. Inoculum was given and flasks were incubated at 37°C for 48 h.

Effect of incubation times on selenite reduction

For this purpose, flasks containing $400 \mu\text{g ml}^{-1}$ of sodium selenite were incubated for various time periods (24, 48, and 96 h) and selenite reduction was monitored after these time intervals.

Effect of other metals on selenite reduction

In the environment, selenite is present along with other metallic salts such as chromium, copper, arsenic, and cadmium. Therefore, these toxic metals would have some influence on selenite reduction by the bacterial strains. For this purpose, cultures were prepared containing $400 \mu\text{g ml}^{-1}$ of sodium selenite along with $50 \mu\text{g ml}^{-1}$ of chromium and copper salts each and were incubated at 37°C for 48 h.

Effect of supernatant on selenite reduction

Bacterial cultures were grown in nutrient broth and were incubated for 24 h. After 24 h, cultures were centrifuged at 13,000 rpm and the supernatant was taken. To this supernatant, $200 \mu\text{g ml}^{-1}$ of sodium selenite was added and then monitored for selenite reduced.

Results

Bacterial strains and culture conditions

These bacterial strains (*B. subtilis*, *Exiguobacterium* sp., *B. licheniformis*, and *P. pseudoalcaligenes*) were isolated from selenium-polluted area and were characterized and identified by 16S rRNA sequencing.

Selenium reduction

Selenite reduction was checked by varying different environmental factors such as pH, temperature, aeration, selenite concentration, and incubation times.

Effect of various pH on selenite reduction

At pH 3, all of the strains showed less selenite reduction, while at pH 5, 7, and 9, reduction percentage was increased. At a higher pH, more selenite reduction was observed with all strains but maximum reduction was observed with strain *B. subtilis* which was 85% (Figure 1). But at acidic pH, bacteria reduce selenium not so efficiently (Figure 1). Formation of red precipitates is an indication that reduction has taken place.

Effect of various temperatures on selenite reduction

Three different temperatures (32, 37, and 42 °C) were selected to check the ideal temperature which supports maximum selenite reduction. It was observed that in each strain the temperature preferences for optimum selenite reduction were different (Figure 2). For *B. licheniformis*, the optimum temperature for maximum selenite reduction was 37 °C, while for strain *P. pseudoalcaligenes*, it was 32 °C (Figure 2).

Effect of aeration on selenite reduction

In shaking conditions, more reduction of selenite was observed as compared to stationary cultures (Figure 3). On average, there was 22–81% selenite reduction potential of strains in shaking condition, while it was 13–30% in stationary cultures (Figure 3). Surprisingly, less selenite reduction was recorded in strains *B. licheniformis* and *Exiguobacterium* sp.

Effect of various selenite concentrations on selenite reduction

At all initial selenite concentrations (200, 400, and 600 µg ml⁻¹), all strains showed reduction. At lower concentration such as 200 µg ml⁻¹, all strains supported higher reduction than at higher concentrations (600 µg ml⁻¹) (Figure 4). Rate of selenite reduction decreased at high initial concentration 600 µg ml⁻¹. Selenite reduction in flasks initially supplemented with 200 µg ml⁻¹ ranged from 43 to 87% after 48 h. With an increase in the concentration, there was a decrease in reduction of selenite. The relation of concentration is inversely proportional to the reduction potential of selenite. Maximum selenite was reduced by strains *B. licheniformis* and *B. subtilis* at lower initial selenite concentrations.

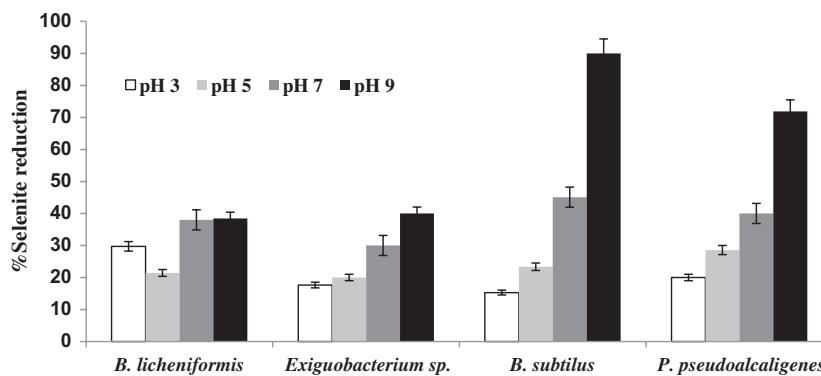


Figure 1. Percentage selenite reduction by bacterial strains at various pH (3, 5, 7, and 9) after 48 h of incubation.

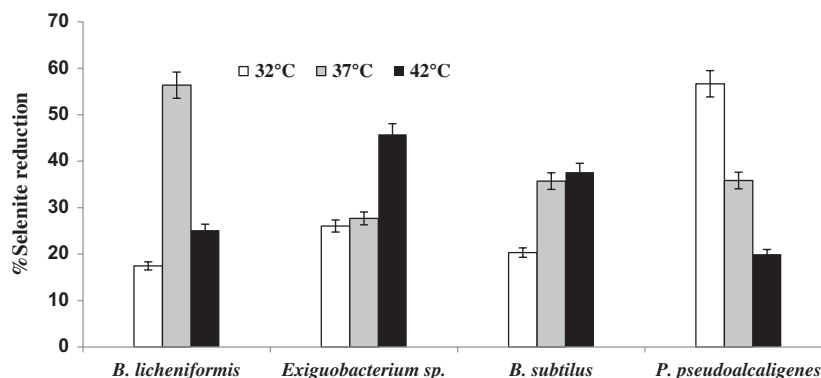


Figure 2. Percentage selenite reduction by bacterial strains at various temperatures (32, 37, and 42 °C) after 48 h of incubation.

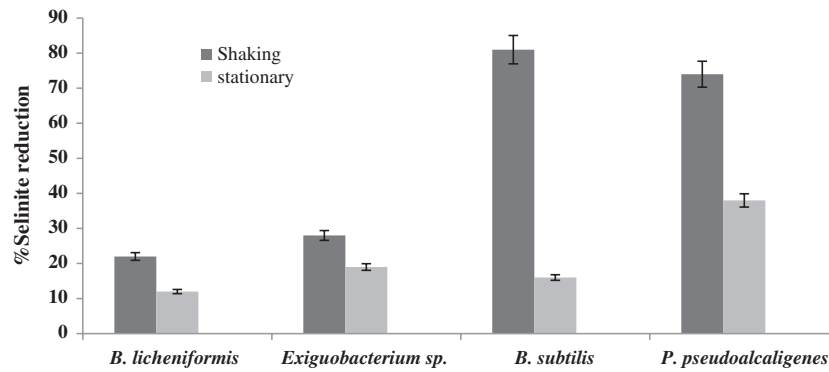


Figure 3. Percentage selenite reduction by bacterial strains at stationary and shaking condition after 48 h of incubation at pH 7 and temperature 37 °C.

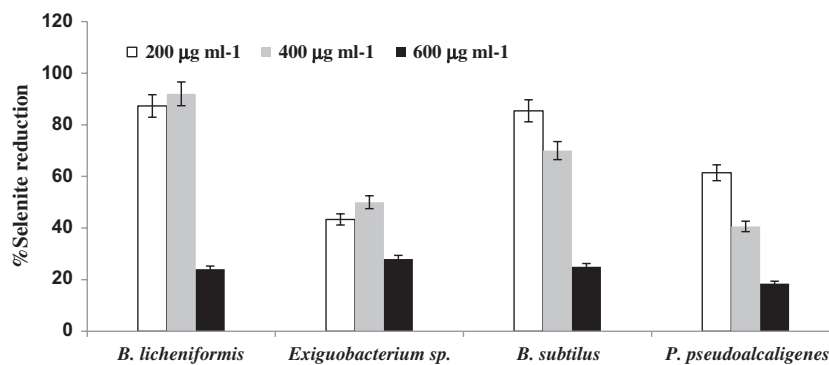


Figure 4. Percentage selenite reduction by bacterial strains at various concentrations of selenite (200, 400, and 600 µg ml⁻¹) after 48 h of incubation at pH 7 and temperature 37 °C.

Effect of various incubation times on selenite reduction

In order to check the effect of time duration on selenite reduction, bacterial cultures supplemented with selenite (400 µg ml⁻¹) were incubated for different incubation times and selenite was estimated on regular time. If incubation time is less, there was less reduction of selenite. All the strains showed maximum reduction at all three incubation times i.e. 24, 48, and 96 h, but maximum reduction was observed at 48 and 96 h, respectively (Figure 5).

Effect of different metals on selenite reduction

Results indicated that *B. licheniformis* reduce selenite in the presence of copper and chromium. On average, chromium had 37–67% reduction potential, while copper had range between 35 and 56% and selenite between 40 and 90% (Figure 6) after 48-h incubation period at 37 °C.

Effect of supernatant on selenite reduction

With an increase in incubation time, the amount of selenite reduction also increased. After 30 min of culture incubation, all the strains showed reduction between 20 and 30%, while at 120, 480, and 720 min, all the strains showed higher reduction potential (Figure 7).

Discussion

Due to the human activities, selenium contamination is considered to be the main environmental pollution found almost everywhere (soil, water).[13] Selenium has widespread uses in industrial and agricultural processes, which are responsible for its high toxic levels in the environment.[14] The present research work focused on the biotic transformation of highly soluble and toxic selenite to elemental selenium by bacterial strains (*B. subtilis*, *Exiguobacterium sp.*, *B. licheniformis*, and *P. pseudoalcaligenes*). Biotransformation of selenate and selenite to elemental selenium is known as dissimilatory selenate/selenite reduction.[15] In the contaminated environment, pH and temperature are the most important factors. Selenite reduction pattern was studied with respect to pH, temperature, aeration, selenite concentration, incubation times, comparison with other metals, and in bacterial supernatant. More selenite reduction was observed with the shaking cultures in comparison to stationary ones. In certain anaerobic microorganisms, selenium in the form of Se (VI) and Se (VI) can be used as a terminal electron acceptor, [16] while few aerobic and phototrophic bacteria can catalyze the selenium oxyanions to insoluble Se(0).[17,18] Few facultative anaerobes were also discovered which precipitate the Se(0) particles by utilizing a unique oxygen-sensing

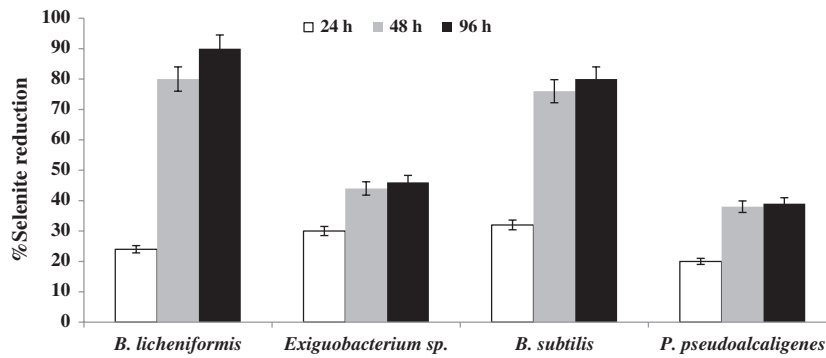


Figure 5. Percentage selenite reduction by bacterial strains at various incubation times (24, 48, and 96 h) at pH 7 and temperature 37 °C.

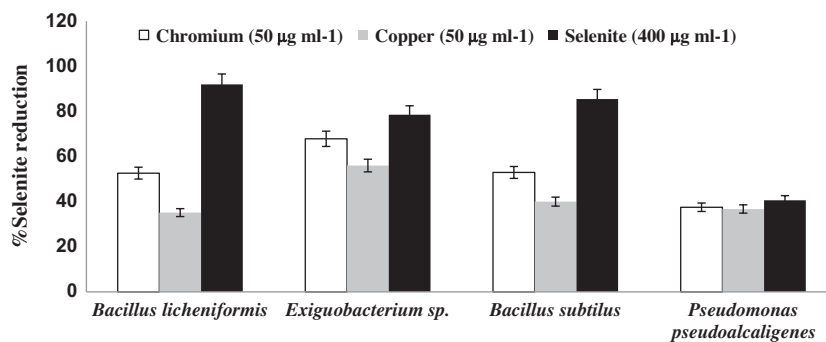


Figure 6. Effects of two metallic salts Cr (50 µg ml⁻¹), Cu (50 µg ml⁻¹), and Se (400 µg ml⁻¹) by bacterial strains after 48 h of incubation at pH 7 and 37 °C.

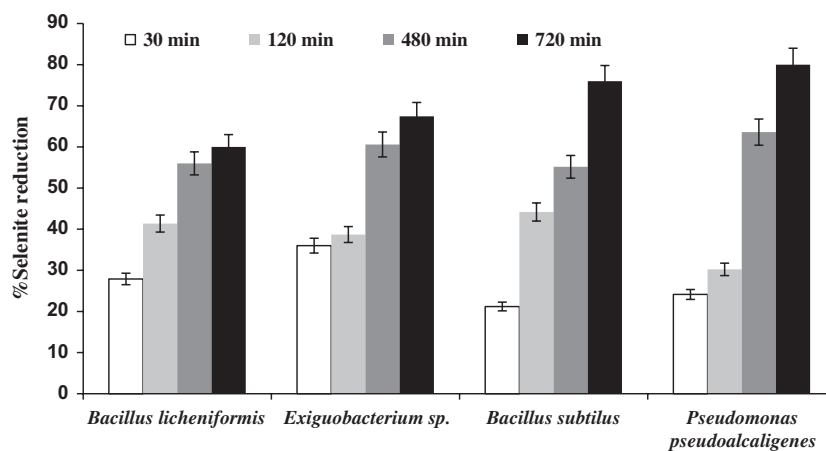


Figure 7. Percentage selenite reduction by bacterial supernatant after various time intervals (30, 120, 480, and 720 min) at pH 7 and 37 °C.

protein under low oxygen levels. Among microorganisms, *Enterobacteriaceae* family play an important role in selenium reduction due to the growth in low oxygen availability, profusion in soil, and metabolic tractability. [19] Recently, many other bacterial strains are reported which convert selenite to elemental selenium.[20–22]

Effect of initial selenite concentration on selenite reduction potential of bacterial strains showed that more selenite is reduced at lower initial selenium concentration. The higher toxicity of the selenite ion may be responsible for the decrease in the rate of selenite reduction at higher selenium concentrations in *Pseudomonas stutzeri*. [23]

With the increase in incubation time, more selenite was reduced. Incubation time had a direct relation with selenite reduction. Greater the incubation time, greater will be the reduction potential. After 96 h of incubation on average, there was 92% reduction, while at 48 and 24 h of incubation, it was 81 and 63%, respectively. It has been shown in one of the previous works that Se(IV) reduction occurred only in late stationary phase under aerobic conditions in *Shewanella oneidensis*. [24] In the present study, rapid reduction occurred after 48 h of incubation. Selenite reduction is not an independent process since a number of other metals are also present in the

environment and that can either inhibit or enhance the reduction process when we try to use selenite-reducing strain for bioremediation purposes. Chromium and copper are the major heavy metal which are also used in many industries along with selenite. So in the present study, we also used both these metals in combination with selenite to check the bacterial strains as successful bioremediation candidates. *Exiguobacterium* sp. shows maximum reduction of chromium and copper i.e. 67 and 56%, respectively, along with selenite reduction. *B. licheniformis* shows maximum selenite reduction (90%) in the presence of chromium and copper stress. On the whole, selenium was more reduced as compared to other metals (chromium and copper). At high concentration of metal, microbial cells temporarily stop their activities but the gene responsible for metal resistance provides protection against these metal stresses and these genes can reside both on plasmid and chromosome.[25] In order to check whether these bacterial strains reduced selenite inside the cell or in the external environment by releasing reductase protein, another experiment was designed in which the bacterial supernatant (cell-free extract) was used. Results showed a very high level of selenite reduction by these supernatants which confirm that these strains release reductase protein which reduced selenite outside the cell.

Conclusion

Selenium exists in natural environment in trace quantity but the anthropogenic activities such as mining and agricultural chemicals have increased the risk to the ecosystem. However, in the current study, we used microorganism having the potential to bioremediate the toxic form of selenium into less toxic form in a cost-effective way.

Disclosure statement

No potential conflict of interest was reported by the authors.

Notes on contributors

Muhammad Faisal did his PhD in Environmental Microbiology and currently working as assistant professor in University of the Punjab, Lahore, Pakistan. He has published widely in the field of Environmental Microbiology. He has completed a couple of research projects and guided BS, MS and PhD students in Microbiology.

Saima Javed, Arslan Sarwar and Mohsin Cheema are PhD scholars doing their research work.

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