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ORIGINAL ARTICLE

Screening, identification and experimental design to optimization of the selenite bioremediation by new isolated *Bacillus* sp. Selena 3 in water

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ABSTRACT

Background and Objectives: Heavy metals pollution is one of the most important concerns in the world. Selenium is one of the most important elements for the life, but if the absorption of this element in cells increases, it acts as a toxic element.
Materials and Methods: In this study, bacterial isolates were screened and isolated from selenium-contaminated soil and water. Twenty -five out of 42 isolates were able to reduce Selenite. Also, the response surface method (RSM) was used to evaluate and optimize the biological reduction of selenite by Selena 3. Factors of bacterial inoculation percentage, time, and amount of selenium oxyanion salt concentration were studied at five levels of -α, -1, 0, +1, and +α.
Results: *Bacillus* sp. Selena 3 was able to reduce 80 mM sodium selenite in less than 4 hours compared to other bacterial isolates. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sodium selenite *Bacillus* sp. Selena 3 was reported as 160 and 320 mM, respectively. The results showed that with increasing duration, the percentage of selenite reduction by bacteria increases and the percentage of bacterial inoculation does not have much effect on its reduction.
Conclusion: Due to the ability of *Bacillus* sp. Selena 3 for rapid reduction in significant concentration of selenium oxyanion (SeO_3^{2-}), this bacterium can be used as an efficient candidate in removing selenite from the environment.

Keywords: *Bacillus*; Metalloids pollution; Selenite bioremediation

INTRODUCTION

Pollution of the environment with heavy metals and non-metallic compounds is one of the world's most troubling problems. These compounds are durable and are not easily destroyed by chemical, light, and temperature reactions (1). One of the biological pollutants is soil and water pollution with selenium and its oxyanions (2). Due to the existence of natural and human producer resources of these compounds, polluted effluents cause environmental pollution. Selenium is an essential element in cellular activity that is required in certain cellular processes (protein synthesis) and the activity of some enzymes. This element, in doses higher than cellular hemostatic, is harmful and dangerous and has been described as an essential toxin (3). In nature, selenium has four oxidation states: selenate (Se VI), selenite (Se IV), elemental selenium (Se), and selenide (Se II) (4). Environmental pollution by selenium and its oxyanions (selenite and selenate) is the result of human or natural activities. This element is used in the com-

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position of rubber, paint, chemical catalysts, and the composition of agricultural chemical fertilizers in soils poor in selenium. Environment by releasing selenium from mining (mining), activities related to nuclear activities, field drainage, fossil fuels, insecticides, glass industry, and optical cells, as well as naturally due to weathering Selenium-containing rocks and rocks as well as volcanic interactions can be contaminated (5, 6). In humans, if selenium intake is less than 40 micrograms per day, the person is deficient in selenium, and if more than 400 micrograms of selenium per day are absorbed, the person suffers from selenium poisoning. Chronic selenium poisoning occurs in people who consume about 5 mg of selenium daily (2, 4). Studies have shown that decreasing or increasing the amount of selenium causes more than 20 kinds of clinical manifestations in humans, include growth retardation, endemic diseases, defective bone metabolism, and risk of diabetes. Selenium poisoning has also been reported in humans. At levels higher than the hemostatic requirement, selenium is toxic and acts as a carcinogen (liver and prostate), a cytotoxic factor (inhibiting cell cycle and inhibiting cell growth), and a genotoxic (effect on DNA). High consumption of this element in the food chain causes digestive disorders and skin jaundice due to liver dysfunction (7, 8). Due to its chemical similarity to sulfur, selenium not only participates in competitive substitution in the assembly of proteins but also shows its toxic effect concerning oxidative stress (9). Selenium derivatives, especially its inorganic derivatives, react with thiols to produce oxygen free radicals, which cause selenium toxicity in cells. Inorganic forms (mainly selenite and selenate) are present in water and show greater toxicity than organic forms (10). These oxyanions are highly toxic due to their high solubility and stability in the environment as well as their potential mobility in natural environments. Therefore, it is necessary to remove selenium contamination in geographical areas that are contaminated with this element and its oxyanions (11).

Pollution removal in the environment is done physically, chemically, and biologically. Physical and chemical methods of decontamination impose constraints because they produce misplaced products and are also not cost-effective. But biological methods of decontamination and purification of contaminants by organisms have benefits. The bioremediation technique itself includes sub-processes that require interdisciplinary studies, including botany, physics, and chemistry. The phenomenon of bioremediation is done by micro and macro organisms, the main purpose of this technique is to remove and eliminate environmental pollutants such as organic, inorganic, radioactive pollutants, etc. Due to the rapid reproduction of microorganisms, the variety of selected pathways in the metabolism of materials, they are preferable to physical and chemical methods (12). Microbial reduction of selenium oxyanions to insoluble elemental selenium plays an important role in removing selenium oxyanions from the environment. Approximate reduction of selenium oxyanions or soluble selenium to insoluble elemental selenium by microorganisms is an important part of the biogeochemical cycle of selenium in nature. Therefore, microorganisms that can play a role in the reduction of selenium oxyanions are useful for the bioremediation of selenium-contaminated environments (13). *Bacillus* sp. STG-83 Strain was isolated from contaminated water and reported to have the ability to regenerate both oxy anion selenite and selenate. In the mechanisms of detoxification of microorganisms, there are specific enzymes, but in different conditions, due to the ability of microorganisms, it has been seen that the detoxification of some compounds can have similar pathways. For example, bacteria may reduce selenite instead of nitrate, so it is important to consider reducing selenite as well as nitrate. This study is carried out with the aim of screening and identifying environmental bacteria that have the potential for bioremediation of selenite in water and soil contaminated with this oxyanion. Also, with the increment of activities that cause more accumulation of selenite in the environment and also the need to remove this oxyanion from the environment, the response surface method (RSM) was carried out to evaluate and optimize the effective factors on the bioremediation process of selenite.

MATERIALS AND METHODS

Sampling, screening and isolating bacteria from samples of contaminated water and soil. For the initial screening of bacteria, contaminated agriculture water and soil sampling (surface and depth at 30 cm from the soil surface) were performed in 3 adjacent areas of different silica and iron mines. In short, sampling was performed from the surface and a depth

of 30 cm from the surface of the contaminated soil. Also, water samples were collected in a volume of 20 cc in sterile water bottles under standard water sampling conditions. These areas were located in 3 areas of Azandarian, Khondab, and Kahkadan. Azandarian has located 40 km from Malayer city (Hamadan province, Iran), which has a latitude of 34.5 and a longitude of 48.6. Also, the geographical characteristics of Khondab and Kahkadan are 65 and 25 km of Malayer city, respectively, which are located at 34.49, 34.14 degrees north, 49.22, and 48.65 degrees east.

Evaluation of the selenium amount in the samples. The samples were sent to the reference laboratory of the Atomic Research Organization to evaluate the amount of selenium. The water and soil samples (after hydrolysis) were examined using the Inductively Coupled Plasma (ICP) test method. ICP analysis showed that selenium in soil and water samples was less than 0.2 mg/kg and less than 100 mg/kg, respectively.

Screening and isolating bacteria from the samples. To screen and isolate the bacteria, dilutions of 10^{-1} to 10^{-4} were prepared from the collected water samples (according to standard protocols with standard number 2347). Also, dilutions of 10^{-1} to 10^{-4} were prepared from the collected soil samples. First, 1 g of soil was weighed and mixed in 9 ml of 0.9% saline solution. After settling of large soil particles, the supernatant was filtered and the supernatant was used to prepare dilutions. Each dilution was cultured on the nutrient agar medium, and the bacteria were incubated at 28-35° C for 24-48 hours.

Investigation of the reduced ability of sodium selenite by bacterial isolates. To evaluate the ability to reduce sodium selenite of isolates, LB broth medium (NaCl (5 g), trypton (10 g) and yeast extract (5 g) per 1000 ml of distilled water and adjust the pH 2 / 7-7) with 10 mM sodium selenite was used at 30°C in aerobic condition and the isolates were examined daily. Sodium Selenite are a salt of the elements selenium that are toxic. Due to the reduction of selenite to selenium, the color of the culture medium turns red, which confirms the reduction and production of elemental selenium. Therefore, resistance of bacterial cultures to oxyanions was determined by appearance of growth and reduction of selenite to their respective elemental forms, which was detected by color change of the broth.

Identification and determination of biochemical and morphological characteristics of isolated bacteria. To survey the morphological features (macroscopic and microscopic of the colonies) as well as the biochemical characteristics of the isolated bacteria, the selected isolates were cultured by standard dilution plating method on nutrient agar medium at 30°C overnight (14). After Gram-staining, differential biochemical tests including oxidase, catalase, hemolysis on blood agar, analysis of glucose, sucrose and lactose, citrate, SIM, TSI, VP test, nitrate reduction, scolin, presence or absence. The presence of capsules, examination of EMB and McConkey media, and the presence of spores were performed to identify and detect bacteria. Finally, the closest genus to the isolated bacteria was determined using Bergey's manual of systematic bacteriology book (15). The isolated bacteria were stored in 80% glycerol and kept at -70°C for future studies. At the end of the study, 25 out of 42 isolates showed the ability to reduce sodium selenite and 17 isolates did not grow. The criteria for selecting the preferable isolate (isolates) was the growth of the isolate as well as the rate of the color change of the environment in a shorter time than other isolates. After selecting the preferable isolate, additional studies were performed.

Determination of MIC and MBC of selected isolates by microdilution broth against sodium selenite. The microdilution method in the microtiter plate was used to determine the MIC of sodium selenite on the growth of the resistant isolated bacteria. The *Bacillus subtilis* PTCC 9372 was used as quality control and the minimum growth inhibitory concentration (MIC) was determined according to the standard method M100-S22 CLSI. Briefly, after overnight incubation of the bacteria at 30°C, several colonies were selected and incubated overnight in 5 ml of liquid LB medium at 30°C and 150 rpm. Using sterile liquid LB medium, a fine bacterium equal to 0.5 McFarland was prepared from the studied bacteria. The obtained dilutions were diluted 150 times using the sterile physiological serum. 100 microliters of sterile LB liquid culture medium was added to each 96 wells of microliter plate, and a series of 0.62-320 mM dilutions of the desired solution was prepared from sodium selenite solution with a concentration of 1280 mM plate (10 wells). Then a 0.5 McFarland suspension, diluted

150 times, was added to each well with a volume of 100 .l. Wells 11 and 12 were considered as bacterial growth control (positive control) and culture medium sterility control (negative control), respectively. After incubation (for 24 hours at 30°C, aerobic condition), the growth of bacteria in the wells was examined. The lowest dilution of sodium selenite solution, which inhibited bacterial growth, was considered as MIC.

To determine the minimum bactericidal concentration of bacteria, 10 µl from turbidity-free wells were cultured on LB agar medium and incubated at 30°C for 24 hours and in aerobic condition.

Investigation of nitrate reduction ability by selected isolates. The isolates were incubated in nitrate broth medium overnight (at 30°C and aerobic condition) and after the incubation period using reagent A (acetic acid 5). N and sulfanilic acid) and reagent B (5 N acetic acid and alpha naphthyl amine) were evaluated for nitrate reduction. After nitrate reduction studies, out of 25 isolates, one did not perform nitrate reduction and performed selenite reduction better than the other isolates, and was selected as the selective isolate and Was drawn its growth curve at 30°C for 10 hours. Considering that the metabolic pathway of selenite and nitrate is the same in bacteria, we selected strains that were not able to reduce nitrate. Among the isolates, only one showed this characteristic, which was selected and molecularly identified.

Molecular identification of selected bacteria. For molecular identification, the isolate was incubated in an LB broth at 30°C overnight and 150 rpm. The cells were harvested by centrifugation and the genomic DNA was extracted using DNPTM kit (Sinaclone, Iran). The amplification of 16S rDNA fragments was performed by thermal cycles as an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. Primers used for determining nucleotide sequences of 16S rDNA gene from *Bacillus* sp. Selena 3 genomic DNA are 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGCTACCTTGTTACGACTT). Product PCR was ~1500 bp.

PCR products were then extracted from the gel by ambiclean kit (Vivantis, Taiwan) and sequenced (Macrogen, Korea). The 16S rDNA sequence gene was aligned with multiple sequences from databases in NCBI Database using Blastn. Among the selected isolates, *Bacillus* sp. Selena 3 (MG814041.1) was selected for further studies. The phylogenetic tree was constructed using neighbor-joining algorithms (Mega 7).

Experimental design and optimization of the effective factors on bioreduction of selenium. In this study, the RSM method was used to evaluate the process of bioreduction of selenite by *Bacillus* sp. Selena 3 and their optimization. Three variables including bacterial inoculation percentage, duration, and amount of selenium oxyanion salt concentration in five levels of -α, -1, 0, +1, and +α were studied as Table 1. Based on the variables and their values, a series of 17 experiments were designed using Central Composite Design. For statistical analysis of data, statistical software Design-Expert 7.0 was used (Stat-Ease, Inc., Minneapolis, MN, USA). In this study, *Bacillus* sp. Selena 3 was cultured in Nutrient Broth medium containing sodium selenite ($Na_2SeO_3.5H_2O$) under aerobic conditions and 30°C. At regular intervals, samples were taken from the bacterial culture medium and the concentration of soluble selenium in the samples was analyzed by ICP-Optical Emission Spectroscopy.

RESULTS

Sampling, screening and isolating bacteria from samples of contaminated water and soil. In this study, in the initial screening, 42 isolates were isolated from water and soil samples. Twenty five out of 42 isolates were able to reduce sodium selenite and 17 isolates showed no growth in the culture medium containing sodium selenite.

The process of the sodium selenite reduction by 25 isolates was evaluated qualitatively. The criterion for qualitative evaluation was based on the duration of selenite reduction by the studied bacteria (the criterion for reduction is the change of color of the culture medium to red brick) (Fig. 1). Fig. 2 shows the results of a qualitative study of the reduction of sodium selenite by different isolates.

Biochemical and morphological properties of selenite reducing bacteria (25 isolates). The results of examining the biochemical and morphological characteristics of 25 isolates that had the ability to reduce sodium selenite showed that 70% of the isolated bac-

Table 1. Central composite design method variables and coded surfaces of each

Variables	Symbol	Surfaces				
		-α	**-1**	**0**	**+1**	**+ α**
Bacterial inoculation percentage	A	5	6	7.5	9	10
Time (hours)	B	4	23	52	80	100
Selenium oxyanion salt concentration (mM)	C	1	2.8	5.5	8	10

Fig. 1. Qualitative evaluation selenite reduction by the bacteria. The criterion for reduction is the change of color of the culture medium to red brick. Flask number 11 shows the lack of selenite reduction, the culture medium containing the grown bacteria is yellow in color, and the other flasks have a brick red color, which indicates the reduction of selenite by different isolates.

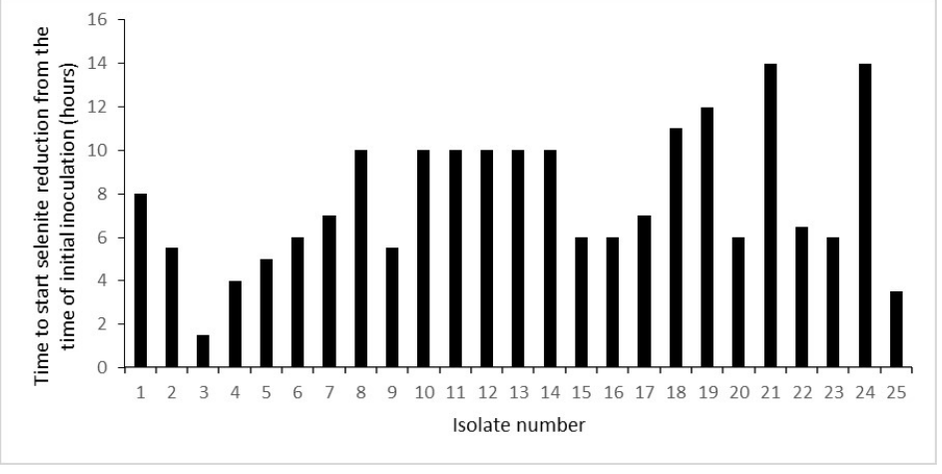


Fig. 2. Qualitative evaluation of sodium selenite reduction by selected isolates. According to the diagram, isolate 3 performed sodium selenite reduction in the shortest time (1.5 hour) and isolates 21 and 24 performed the longest time (14 hours). Isolate 3 started to deduct selenite in 90 minutes after initial inoculation under incubation conditions, which was confirmed by changing the color of the culture medium to brick red. This result shows that Isolate 3 had the most activity to reduce selenite.

teria were Gram positive and 30% were Gram negative. The dominate genus in Gram-positive bacteria was *Bacillus*, and its genus in Gram-negative bacteria was belonged to the group of *Pseudomonas*.

Results of biochemical, morphological, and molecular properties of *Bacillus* sp. Selena3. Results of morphological and differential biochemical tests (oxidase, catalase, hemolysis, analysis of glucose, sucrose, and lactose, citrate, SIM, TSI, VP test, nitrate reduction, scolin, presence or absence of capsules, study of media EMB and McConkey, presence or absence of spores) the *Bacillus* sp. Selena3 had the characteristics listed in Table 2.

Table 2. Results of biochemical tests of selective isolate

Properties	Selena3 strain
Gram stain	+
Shape	Rods
Spore	+
Motility	+
Pigmentation	cream/dark cream
Enzyme activity of:	
Oxidase	-
Catalase	+
VP test	+
Indole	-
H_2S formation	-
Hydrolysis of:	
Esculin	-
Citrate	-
Nitrate reduction	-
Acid formation of:	
Glucose	+
Sucrose	+
Lactose	-
Hemolysis	+
Capsule	+

Based on the biochemical properties, *Bacillus* sp. Selena 3 strain showed >75% similarity to *Bacillus* species. Also, the results of PCR and sequencing were evaluated for molecular identification of selective isolate with universal primers (Fig. 3). For the precise identification of *Bacillus* sp. Selena 3, the 16S rDNA sequence was determined and analyzed using Blastn. Homology studies revealed that the 16S rDNA gene of the strain Selena 3 showed sequence identities of 94% with *Bacillus sonorensis* strain SXYC17, *Bacillus* sp. Hb51, *Bacillus lichonoformis* strain F_1, *Bacillus spongiae* strain DN-5, *Bacillus lichonoformis* strain QT201, *Bacillus* sp. XZ-1, *Bacillus paralichonoformis* strain B52-023, *Bacillus* sp. XAAS.x502.

The phylogenetic tree was constructed using the neighbor-joining method based on the comparative sequence analysis of 16S rDNA (Fig. 4). Based on the evaluation of biochemical, morphological, and physiological characteristics and 16S rDNA sequence analysis, it is concluded that strain Selena 3 belongs to the genus *Bacillus* and is named *Bacillus* sp. Selena 3. The nucleotide sequence of the 16S rDNA of *Bacillus* sp. Selena 3 was submitted to GenBank (accession number MG814041.1).

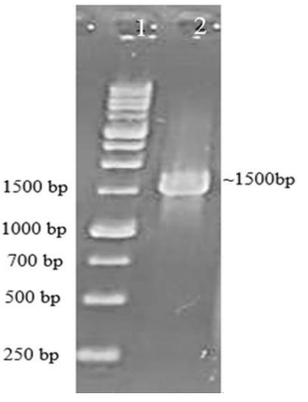


Fig. 3. PCR product 16S rDNA gene amplification from *Bacillus* sp. Selena 3. Lane 1, molecular weight standard (1 kbp, Fermentase, Lithuania).; Lane 2, *Bacillus* sp. Selena 3.

Determination of the sodium selenite MIC and the sodium selenite MBC for isolates. The isolated results of MIC and MBC 25 in concentrations of 320 to 0.62 mM of sodium selenite using Broth microdilution method and the ability to reduce sodium selenite in the studied concentrations are shown in Table 3. Based on the results of Table 2, isolate 3 has the highest ability to grow and reduce selenite compared to other isolates. Isolate 3 has MIC and MBC of 160 and 320 mM, respectively. The results showed that isolate 3 did not have the ability to decrease in concentrations of more than 80 mM sodium selenite, while other isolates did not show the ability to decrease in concentrations of more than 40 mM sodium selenite.

Nitrate reduction ability by selective isolates. According to nitrate reduction test, all isolates except *Bacillus* sp. Selena 3, reduced nitrate. This isolate showed a decrease in selenite at a concentration of 80 mM. This isolate was chosen as a selective isolate for the purpose of designing an experiment to evaluate the factors affecting the process of selenite reduction by *Bacillus* sp. Selena 3 and its optimization, isolated growth curve was drawn (Fig. 5).

Experimental design to evaluate the effective factors on the process of selenite reduction by *Bacillus* sp. Selena 3 and its optimization. In this study, the response surface methodology was used to evaluate the process of selenite bioreduction by *Bacillus* sp. Selena 3 and its optimization. Three variables including bacterial inoculation percentage, duration, and amount of selenite salt concentration were studied at five levels of -α, -1, 0, +1, and +α. Based on the vari-

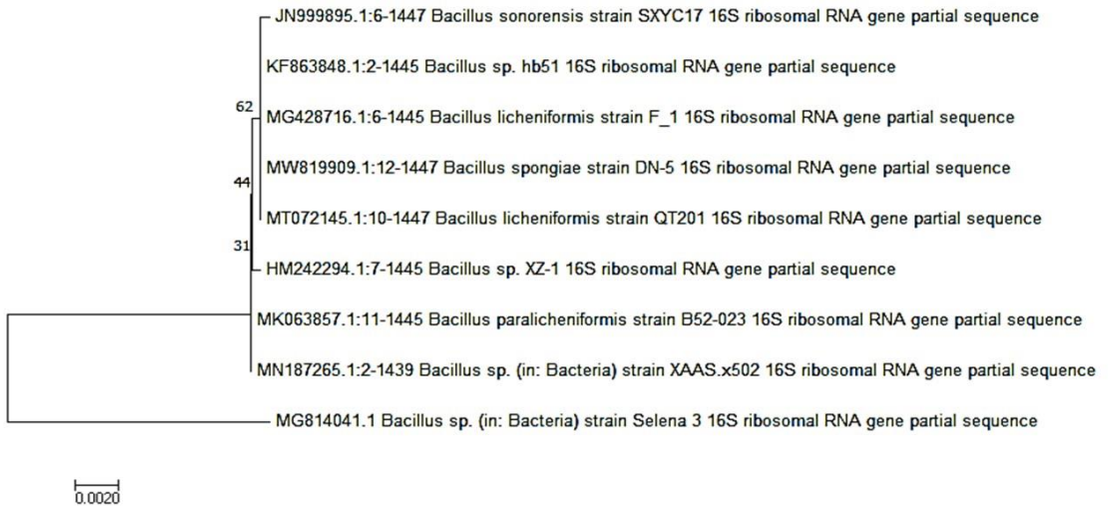


Fig. 4. Phylogenetic analysis based on the 16S rDNA sequences from *Bacillus* sp. Selena 3 and eleven other bacteria. Neighbour-joining model was employed for the tree construction, and bootstrap values were obtained with 500 repetitions. The bar labeled 0.002 indicates 2 base changes per 1000 nucleotides.

Table 3. Results of MIC, MBC, and reduction of sodium selenite 25 selective isolates using microdilution Broth method.

Isolate number	MIC	MBC
1	80 mM	80 mM
2	5 mM	5 mM
3	160 mM	320 mM
4	20 mM	20 mM
5	20 mM	80 mM
6	80 mM	80 mM
7	20 mM	20 mM
8	80 mM	160 mM
9	40 mM	40 mM
10	80 mM	160 mM
12	20 mM	60 mM
13	40 mM	40 mM
14	40 mM	40 mM
15	80 mM	160 mM
16	80 mM	80 mM
21	40 mM	160 mM
22	40 mM	80 mM
23	80 mM	80 mM
25	80 mM	160 mM
29	80 mM	80 mM
31	80 mM	160 mM
32	80 mM	80 mM
34	40 mM	40 mM
36	20 mM	20 mM
40	40 mM	40 mM

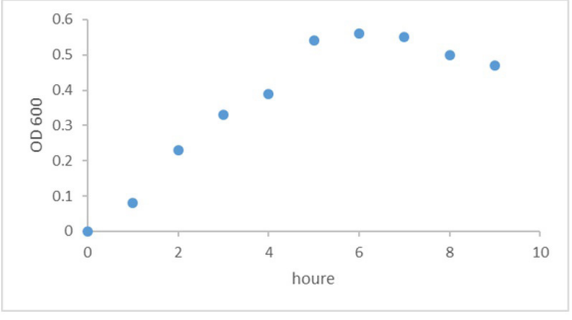


Fig. 5. Growth curve of selective isolate *Bacillus* sp. Selena 3

ables and their values, a series of 17 experiments were designed using Central Composite Design (CCD). Table 4 shows values of variables and laboratory responses of CCD.

Variance analysis was performed to confirm the effectiveness of the main effects and the interaction of the variables. In general, the value of P is less than 0.05 and the value of F in the variance analysis table indicates the greater effect of variables with a 95% confidence level. The values of P and F of the proposed model are equal to 0.0027 and 7.72, respectively, which indicates the accuracy of the proposed model. Also, the values of R2 are equal to 0.8225, which indicates that the proposed model is well able to predict the experimental values. As shown in Table 5, variable A (percentage of bacterial inoculation) has the statistically least effect (p-value >0.05) on selenite reduction. Two factors B (time) and C (selenite salt concentra-

Table 4. Values of variables and laboratory responses of the designed experiments using Central Composite Design in Response surface methodology

Experiments	Variable A (bacterial inoculation percentage)	Variable B (h- Time)	Variable C (selenite salt concentration)	Percentage of reduced selenite
1	6.013491	80.54097	8.175716	34.99547
2	7.5	100	5.5	97.27059
3	7.5	52	5.5	65.12739
4	8.986509	23.45903	8.175716	8.611111
5	6.013491	23.45903	2.824284	97.87097
6	8.986509	80.54097	8.175716	27.45342
7	7.5	52	10	14.31111
8	7.5	52	5.5	59.20177
9	5	52	5.5	29.15254
10	7.5	52	1	87.9798
11	7.5	52	5.5	62.31061
12	10	52	5.5	54.72527
13	8.986509	80.54097	2.824284	90.42735
14	7.5	4	5.5	4.140127
15	6.013491	23.45903	8.175716	8.611111
16	6.013491	80.54097	2.824284	95.75949
17	8.986509	23.45903	2.824284	98.42795

The model proposed by the software is defined as follows:
% = +89.27361+4.04778 * Dose+0.29052 * Time-15.73527 * Concentration-0.039572 * Dose * Time-0.086955 * Dose * Concentration+0.090580* Time * Concentration

Table 5. Variance analysis for Response Surface of the model parameters

Reference	p-value	F value	Mean square	Df	Sum of squares
model	0.0027	7.720792	2749.984	6	16499.9
A	0.6693	0.193641	68.97098	1	68.97098
B	0.0205	7.55805	2692.019	1	2692.019
C	0.0001	37.43233	13332.61	1	13332.61
AB	0.8064	0.06331	22.54955	1	22.54955
AC	0.9597	0.002687	0.956958	1	0.956958
BC	0.3243	1.074732	382.7969	1	382.7969

tion) have the most statistical effect (p-value <0.05) on the selenite reduction by *Bacillus* sp. Selena 3.

The three-dimensional surfaces of the responses that result from the interaction of the three variables of bacterial inoculation percentage, duration, and selenite salt concentration are shown in Figs. 6-8. As shown in the figures, the amount of selenite reduction is strongly affected by the concentration of salt in the culture medium, so that the higher the amount of selenite salt in the culture medium, the lower the percentage of selenite reduction. Time is also directly related to the selenite reduction. As can be seen in the results, the percentage of selenite reduction increases over time. Also, the results showed that the percentage of bacterial inoculation in these experiments had little effect.

The results suggested that *Bacillus* sp. Selena 3, under optimal conditions proposed by Design-Expert 7.0 software (7.69% bacterial inoculation, duration 97 hours, and 2.5 mM selenite salt concentration) can reduce 100% of the culture medium selenite.

Analysis showed that with increasing the bacterial inoculation percentage from 5 to 10 under optimal conditions, the percentage of selenite reduction does

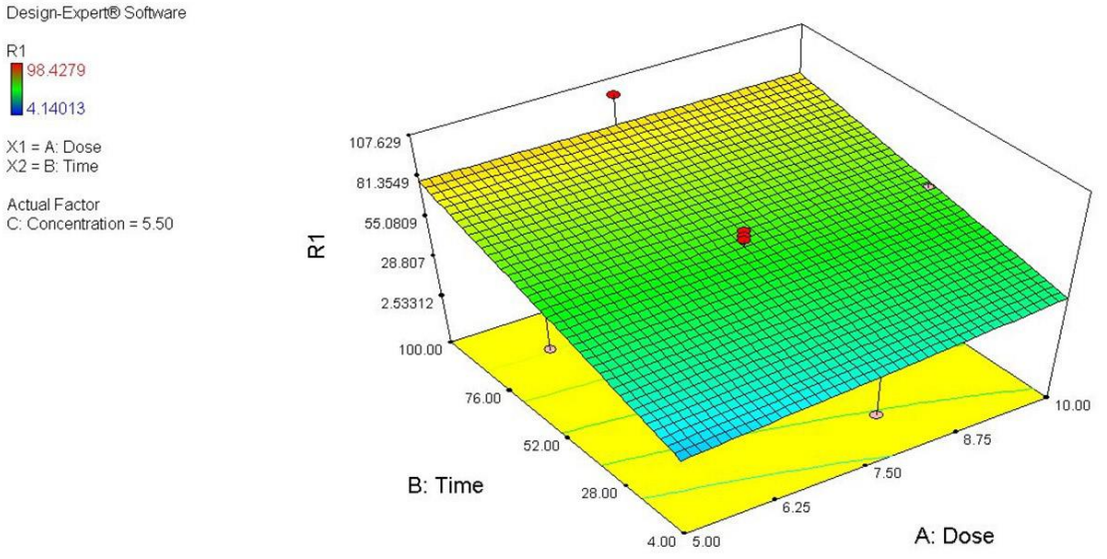


Fig. 6. Three-dimensional surfaces plot for interaction of bacterial inoculation percentage (Variable A) and duration Time (Variable B) in selenite reduction by *Bacillus* sp. Selena 3.

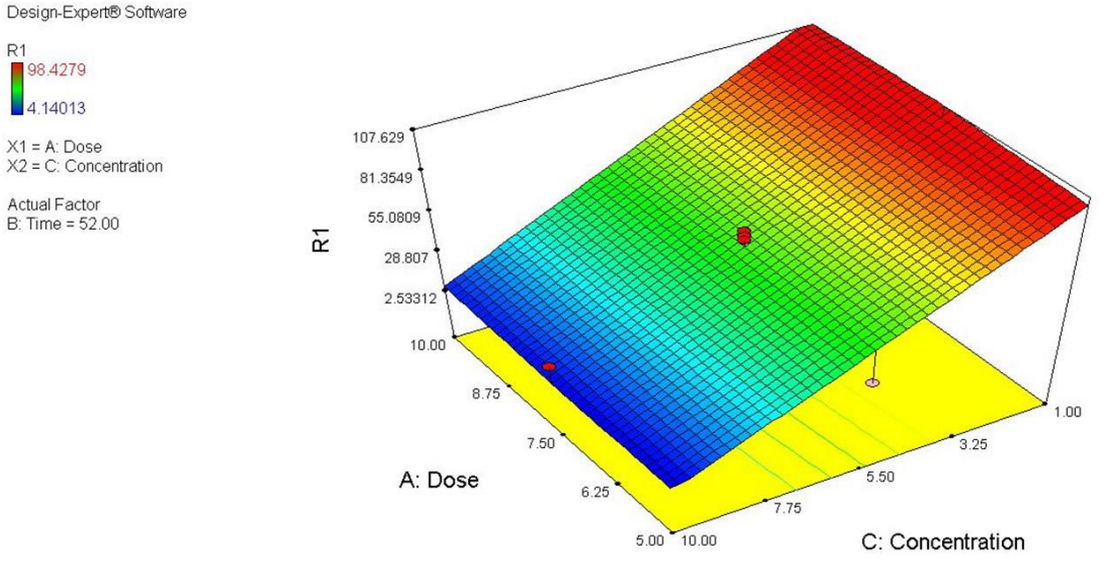


Fig. 7. Three-dimensional surfaces plot for interaction of bacterial inoculation percentage (Variable A) and selenite salt concentration (Variable C) in selenite reduction by *Bacillus* sp. Selena 3.

not change significantly. Also the analysis showed that with increasing time from 4h to 100h under optimal conditions, the percentage of selenite reduction increased from 80.24% to 100%. But, with increasing the selenite salt concentration from 1 mM to 10 mM under optimal conditions, the percentage of selenite reduction was reduced from approximately 100% to 42.89% (Fig. 9).

DISCUSSION

According to the principles of green chemistry, protective and preventive measures to reduce the release of harmful metallic and non-metallic compounds such as selenium, first, require attention and then evaluation of treatment technologies (16). Se-

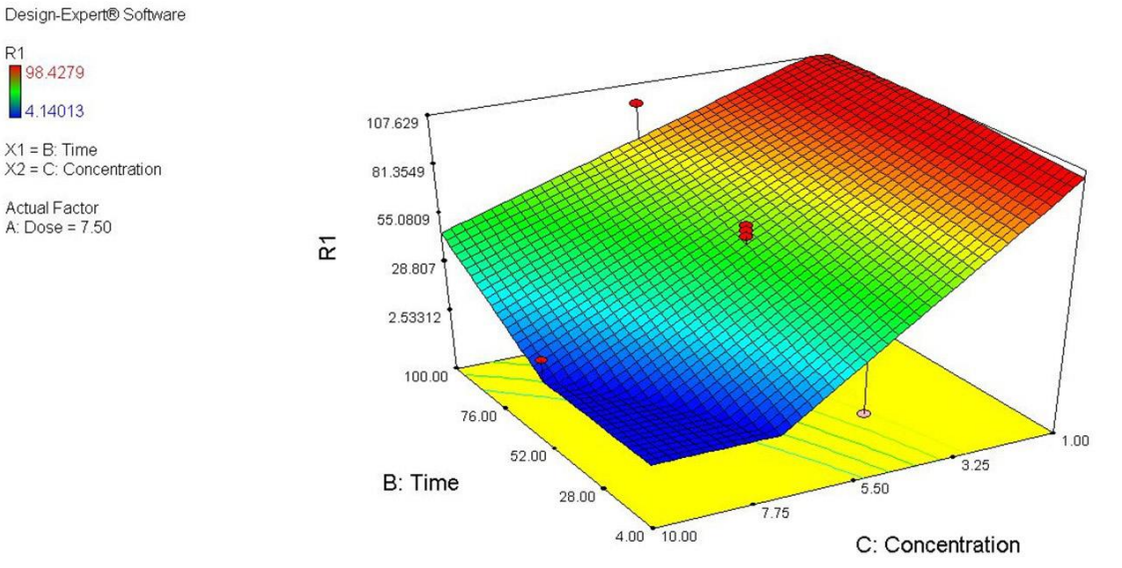
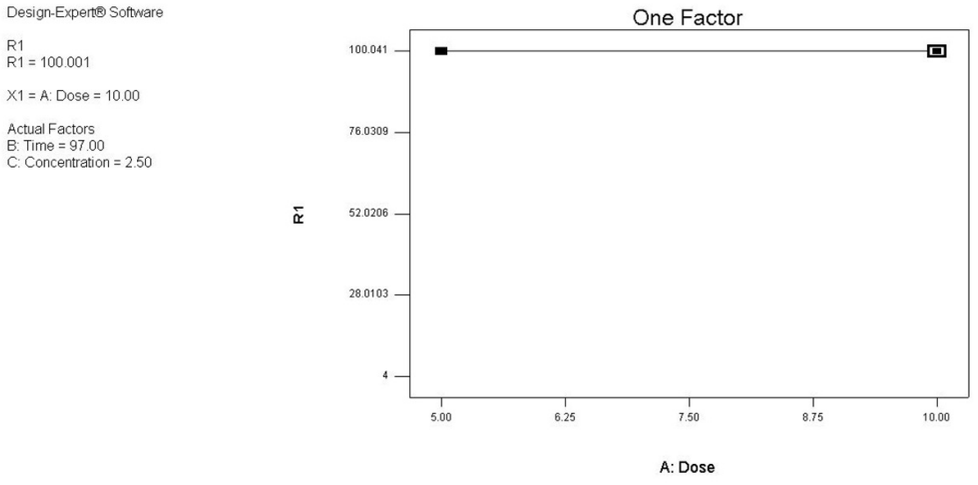
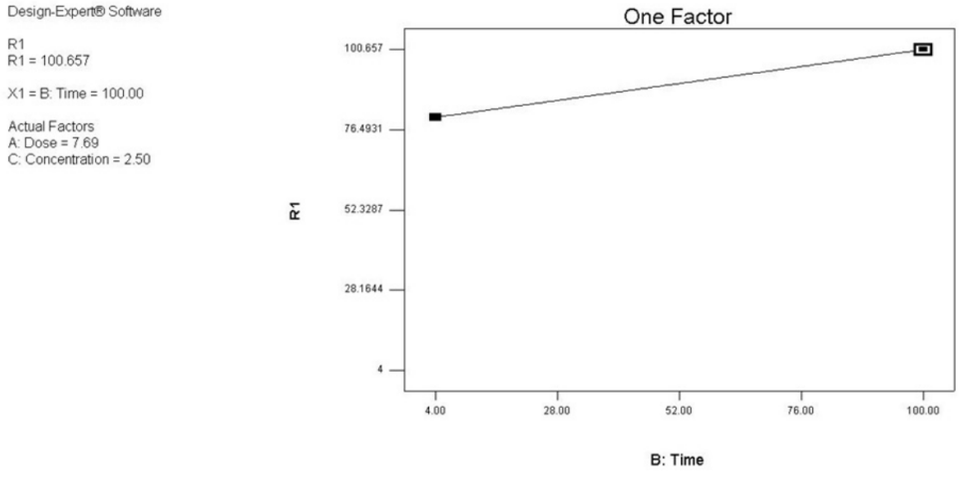


Fig. 8. Three-dimensional surfaces plot forinteraction of interaction of selenite salt concentration (Variable C) and duration time (Variable B) in selenite reduction by *Bacillus* sp. Selena 3.

lenium can be eliminated and removed physically, chemically, and biologically. The use of physical and chemical methods is often difficult and expensive, so biological methods are considered more because of their high potential and the lowest cost of selenium removal. These methods are environmentally friendly because fewer chemicals are needed and also allow the researcher to investigate in situ (17). Selenium metabolism is found in all domains of life, including bacteria, archaea, eukaryotes, and viruses. Therefore, biological techniques can be divided into phytoremediation and bioremediation by microorganisms, which are the most acceptable technologies that have been considered (18-20). The use of plants in the phytoremediation process is one of the biological methods to remove environmental pollutants. Plants readily absorb Se through sulfur (S) transporters and biochemical pathways and can also discharge methylated Se. Plants can be used to provide dietary Se in areas with Se deficiency, as well as to eliminate Se pollution from selenium-rich areas (21). Due to the ability of plants to remove selenium from the environment (soil and water), studies have shown that there is an accumulation of selenate (85%) and selenite (70%) in the aerial parts of the plant. Due to the nutrition of humans and animals from the plant aerial parts, the possibility of increasing the dose of selenium and selenium oxyanions in the diet increases and this is a kind of health risk (22).

Another useful way to remove contaminants from the environment is to use microbes. Approximate reduction of selenium and soluble selenite to insoluble elemental selenium by microorganisms is an important part of the biogeochemical cycle of selenium in nature. Therefore, microorganisms that can play a role in the reduction of selenium oxyanions are useful for the bioremediation of selenium-contaminated environments (21). In this study, bacteria that were isolated in the initial screening of selenium contaminated soil and water samples and were able to reduce selenium oxyanions were in the groups of *Panebacillus*, *Bacillus*, *Micrococcus.* The results showed that *Bacillus* had a high potency in reducing sodium selenite. In the present study, aerobic and Gram-positive bacteria *Bacillus* sp. Selena3 was more potent in reducing sodium selenite than other isolated bacteria. The results indicated that the sodium selenite MIC and sodium selenite MBC of *Bacillus* sp. Selena3 are 160 and 320 mM, respectively. These results are consistent with the other observations. Studies have shown that microorganisms that can metabolize selenium are mostly in the groups of *Crenoarchaeota*, Gram-positive bacteria with a high percentage of C + G, *Halanaerobacter*, and protobacteria β, γ, and ε (23). For example, in 2014, Zheng et al. isolated an absolute aerobic bacterium called *Comanonas testosterone* S44 from soils contaminated with heavy metals, which could reduce selenite and also produce





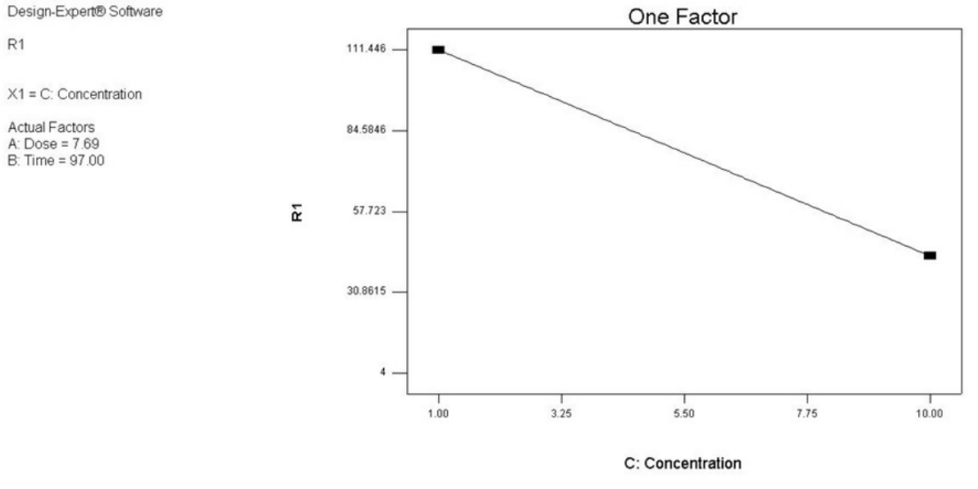


Fig. 9. Diagram of the factors changes effect on the selenite reduction by *Bacillus* sp. Selena 3 under optimal conditions.

selenium nanoparticles. The selenite MIC of isolated strain was reported to be 100 mM (4). But, Soudi et al. in 2009, *Bacillus* sp. Strain STG-83 was isolated that showed the ability to reduce both oxyanion selenite and selenate. That isolate could tolerate concentrations of 640 mM selenite and 320 mM selenate (24). Also, according to a report by Zhang et al. in 2019, two selenium-resistant strains were isolated from selenium-contaminated soils in southern Anhui, China, identified as *Lysinibacillus xylanilyticus* and *Lysinibacillus macrolides*. They showed selenite MICs 120 and 220 mM / L, respectively (3). In this study, the effect of bacterial inoculation percentage, duration, and selenite salt concentration on selenite reduction by *Bacillus* sp. Selena 3 was investigated and optimum point of independent variables illustrated. As the results, the amount of selenite reduction is strongly decreased by increasing the concentration of salt. Time is also directly related to the selenite reduction. The percentage of selenite reduction increases over time. Also, the results showed that the percentage of bacterial inoculation in these experiments had little effect. Remediation by microorganisms is classified as metabolism-dependent and metabolism independent process. Bioreduction is in category metabolism dependent process. Metabolism-dependent bioremediation is performing by only viable (live) microbial cells (25). The results indicated that increasing the selenite salt concentration probably reduced the maximum attainable cell concentration and suggesting that selenite is a stress factor for *Bacillus* sp. Selena 3. Similar results were reported by KESSI et al. (26). The results also showed that the amount of selenite reduction by *Bacillus* sp. Selena 3 increased with the growth of the strain over the time. Similar results have been observed by others (24- 26).

The results also suggested that the *Bacillus* sp. Selena 3 under optimal conditions, 7.69% of bacterial inoculation, duration of 97 hours, and 2.5 mM selenite salt concentration can reduce 100% of selenite. In this design, the percentage of bacterial inoculation has little effect on the ability of bacteria to reduce oxyanions.

CONCLUSION

In this study *Bacillus* sp. Selena 3 showed high resistance to selenium oxyanions compared to other bacteria isolated from selenium-contaminated water and soil. The results showed that this bacterium has a high potential at sodium selenite bioremediation and can reduce 100% of 2.5 mM selenite salt duration 97 hours. This feature is one of the most significant features of this bacterium that has made it an efficient candidate for selenium bioremediation in polluted environments. The results of this study also showed that the response surface method can be used as an efficient method for designing experiments and optimizing the bioremediation of selenium oxyanions. So that by using this method, the parameters affecting the process can be performed effectively with a smaller number of experiments.

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