

Assessment Of Selenite And Chromate Reduction Potential In *Bacillus safensis* And *Bacillus altitudinis* Isolated From Industrial Sites

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ABSTRACT

This study investigates the selenite- and chromate-reducing capabilities of *Bacillus safensis* and *Bacillus altitudinis* strains isolated from industrially contaminated soil. These heavy metals pose significant threats to human health and ecosystems. The strains of *B. safensis* and *B. altitudinis* were isolated, purified and subjected to a series of biochemical tests and analytical strategies, including bacterial growth assays and spectrophotometric methods to determine the reduction potential of these strains. The results clearly emphasized the natural abilities of these strains to reduce selenite and chromate efficiently. Notably, *B. safensis* strains S1 and S9 reduced selenite by 46% and 53%, respectively, while *B. altitudinis* strain S2 achieved a 69.5% reduction. Physiological parameters such as temperature, pH, salinity, and metal ion concentration were optimized to enhance reduction efficiency. Optimal conditions were identified at 37°C and pH 9, under which the highest reduction rates were observed; the lowest reductions occurred at 25°C and pH 5. Taxonomic identification was performed using 16S rRNA gene sequencing, and a phylogenetic tree was constructed to confirm species classification.

The findings support the potential use of *B. safensis* and *B. altitudinis* in bioremediation, specifically for detoxifying selenium and other heavy metals in polluted environments.

Keywords: Selenite, Chromate, Bioremediation, Reduction Potential, Heavy Metals, Phylogenetic Tree

Introduction:

Heavy metals tend to be the elements with high atomic weights and densities and are characterized by their potential toxicity and persistence in the environment. These naturally occurring elements basically include Selenium (Se), Lead (Pb), Cadmium (Cd), Arsenic (As), Chromium (Cr), and Nickel (Ni) and other include pollutants found in soil, air, water and biota due to both natural phenomena and human activities [1]. Different synthetic and natural sources, such as mechanical release, car debilitation, and mineral extraction are responsible for the release of these metals into the environment.

Certain forms of heavy metals are not dispensable and tend to accumulate in the living body as well as in the environment [2]. To avoid their hazardous effects, reasonable strategies have to be set up for their effective expulsion from the environment. Heavy metals have threshold values, and when they are used above tolerable changes, they are damaging to both humans and environment, e.g. Lead is a toxic element, known for its lethal effects on human health. The World Health Organization (WHO) has determined a threshold limit for Lead in drinking water to be 10 µg/L.

Different factors such as metal species, concentration, chemical forms and exposure duration define the harmful effects of heavy metals [3]. As they have an ability to assemble in the cells and tissues of living organisms eventually by the process of biomagnification, they adversely affect both their physiological and biological functions in many ways [4]. Many studies propose that they hinder the growth processes in plants, pose damage to cell organelles and inhibit the process of photosynthesis [5]. This is concerning because heavy metals can potentially contaminate food substances at the soil-root interface.

Selenite (Se) and Chromate (Cr (VI)) are harmful pollutants usually found in soil and pose serious environmental threats and health risks because of their toxicity and abundant occurrence in contaminated areas. Various anthropogenic activities such as agriculture, inefficient waste disposal, industrial processing and mining are the main reasons for the origin of these contaminants [6]. The soluble form of selenium, i.e., selenite, is specifically hazardous at elevated levels and is commonly present in compounds like sodium selenite and ends up taking the form of SeO_2^{-3} ion [7]. In the case of Selenite, the oxidation state of selenium is +4 and is known to have a pyramid shape because of its energy directing abilities.

On the other hand, the hexavalent form of Chromium is deadly due to its

high solubility, which is capable of causing serious health problems such as cancer and mutations upon exposure [8]. Activities like electroplating, leather processing and metal shaping are major sources for contamination of chromate. One of the distinguishing characteristics of chromate is its solubility in water, which facilitates its migration through soil and groundwater. Cr (VI)-induced toxicity is more serious than Cr (III) because of its high mutagenicity, carcinogenicity and teratogenicity [9].

Bacteria possess a remarkable potential of detoxifying heavy metals due to their metabolic flexibility and adaptive mechanisms [10]. Many bacterial species possess specialized metal-binding proteins like metallothioneins and phytochelatins, allowing them to sequester metals within their cells. Additionally, certain bacteria enzymatically convert toxic metal ions into less harmful forms, reducing their toxicity and bioavailability [11]. Extracellular substances such as exopolysaccharides and siderophores aid in the precipitation as well as the immobilization of heavy metals in soil [12]. Bacterial species can be utilized for heavy metal bioremediation by the selection and optimization of biotechnological methods such as bioaugmentation, bioleaching and phytoremediation. Bioaugmentation entails introducing metal-tolerant or metal-resistant bacteria to contaminated sites to enhance microbial activity. Similarly, bioleaching employs bacteria to solubilize metals from solid matrices, facilitating their subsequent removal [13]. Phytoremediation utilizes plant-bacteria symbiotic relationships to remediate metal-contaminated soils, with bacteria aiding in metal uptake, translocation and detoxification within plant tissues [14]. Effective implementation of these strategies requires the consideration of environmental factors, microbial community dynamics and engineering principles. Optimizing parameters such as pH, temperature and nutrient availability can enhance bacterial activity and metal removal efficiency. Furthermore, genetic engineering techniques can be utilized to intensify the metal-binding capacity and metal tolerance of bacterial strains, thereby improving their effectiveness in remediation applications.

Materials and Methods:

The media and equipment were autoclaved at 15psi for 15 minutes and the temperature was set at 121°C. Before and after dealing with the cultures, disinfectant was utilized.

Isolation of Selenite and Chromate Resistant Bacterial Strains and Culture Conditions:

The defile soil sample was taken for the isolation of selenite and chromate resistant Bacterial strains from the working zone of Industrial estate. The number of possible microorganisms was reduced with the serial dilutions of soil sample. Dilutions of 10^{-1} , 10^{-5} , and 10^{-9} were uniformly spread on N-agar plates supplemented with 1500 μL of Na_2SeO_3 to assess selenite resistance. Similarly, for the growth of chromate-resistant colonies, the above mentioned serial dilutions were spread directly on chromate

supplemented N- Agar plates. Incubation at 37°C was given to all the plates for 24 hours and after the incubation, particular isolated colony was selected, streaked on Nutrient agar plates and finally proceeded towards another incubation at 37°C for 24 hours.

Culture Characteristics and Cellular Morphology:

After incubation, morphology of bacterial colonies was observed on the basis of color, elevation, size, pattern, margins, form and texture for their characterization. These colonies were then isolated by a sterile inoculating loop, quadrant streaked on N-agar media and incubated for 24 hours at 37°C to obtain purified colonies.

The purified bacterial colonies were then subjected to gram staining [15]. After gram staining, the gram-positive rods were proceeded towards spore staining to visualize whether strains were spore formers or not. Malachite green and Safranin dyes were used in this methodology. The observed bacterial strains were non-spore formers.

Biochemical Tests:

Catalase test was used to distinguish microorganisms on the basis of their ability to produce catalase [16]. All the bacterial strains were catalase positive as they showed bubbles formation within 1-2 minutes. Oxidase test was used to identify the presence of oxidase enzyme in the strains and tetra-methyl-p-phenylenediaminedihydrochloride was used as oxidase reagent. The blue color indicated positive results [17]. Methyl Red test was used to clarify microbe's capacity to carry out mixed acid fermentation. 10 ml media incorporated with few drops of methyl red was added in test tubes and incubated for 24 hours at 37°C. Red color indicates positive results and yellow color indicates negative results. Vogues Proskauer test was used to check the ability of strains to undergo glucose fermentation. Test tubes containing VP reagent A and B were incubated for 2 days at 37°C. The strains showing pink color indicate positive results, whereas yellow color indicates negative results. Lastly, starch hydrolysis test was performed to differentiate bacterial strains on the basis of their ability to utilize starch by producing amylase enzyme. N- Agar plates incorporated with 1g starch were streaked and placed for incubation at 37°C for 48 hours. No clear zones were shown by the bacterial strains.

Protocol for Selenite Reduction Test:

Selenite reduction was assessed to quantify selenite concentration in each bacterial strain. For this purpose, 10 mL of N-broth containing 1% sodium selenite was prepared and added to test tubes with the respective strains. The tubes were incubated at 37°C for 2 days. Following incubation, the cultures were centrifuged at 5000 rpm, and the supernatant was discarded. To aid in the removal of un-metabolized selenite, 1 M NaCl was added. Optical density was then measured using a spectrophotometer.

Protocol for Chromate Reduction Test:

In this test, bacterial strains showed different growth patterns by varying concentrations of chromate. N-broth was poured into test tubes, which were autoclaved and then Potassium dichromate was added to the media at different concentrations ranging from 200-800 µg/ml. Test tubes were then placed in incubator at 37°C for 48 hours. Optical density was determined at 600nm after incubation. Few drops of DPC reagent were also added in all samples after centrifugation to access the remaining concentration of chromate.

Results:

Culture Characteristics and Cellular Morphology:

Colony morphology was studied by observing color, height, texture, shape and edges of colonies. All the strains exhibited distinctive features with minor variations in colony morphology when cultivated on N-agar plates. Cellular characteristics were studied by performing gram staining and endospore staining. The strains were found to be gram positive rods and were non-spore formers.

Biochemical Tests:

All the strains showed bubble formation within 1-2 minutes and were reported as catalase positive. The strains showed blue color in oxidase test and were observed to be oxidase positive. All the three strains S1, S2 and S9 showed red coloration in Methyl Red test and yellow coloration in Vogues Proskauer test. So they were positive for MR test and negative for VP test. Lastly in the starch hydrolysis test, all the strains exhibited negative amylase activity.

Selenite and Chromate Reduction Test:

For Selenite reduction test, 200-2000µg/ml range of concentration was provided to check the reduction ability of strains. The optical densities were taken at 600nm with the help of spectrophotometer. Bacterial strain S2 (*Bacillus altitudinis*) showed maximum growth up to 1000 µg/ml. Further increase in selenite concentration up to 2000 µg/ml resulted in less growth as shown in Table 3.3. *Bacillus safensis* Strain S1 showed maximum growth at 1000 µg/ml and strain S9 at 400 µg/ml. Both these strains showed minimum growth at 1400 µg/ml as shown in the table 3.3.

For Chromate reduction test, all isolated strains were grown at various chromate concentrations ranging from 200-800 µg/ml and for these strains optical densities were taken at 600nm with the help of spectrophotometer. All three strains i.e. *Bacillus safensis* (S1, S9) and *Bacillus altitudinis* (S2) showed maximum optical densities at 600 µg/ml after centrifugation, whereas *Bacillus safensis* strain S1 showed least growth at 800 µg/ml and *Bacillus altitudinis* strain S2 at 200 µg/ml before centrifugation, as shown in the table 3.3.

Table 3.3: Optical density measurements recorded after 24 hours of incubation with varying concentrations of sodium selenite and potassium dichromate.

Strains	Optical density at different sodium selenite concentrations									
	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml	1200 µg/ml	1400 µg/ml	1600 µg/ml	1800 µg/ml	2000 µg/ml
<i>Bacillus safensis</i> S1	0.430 ±0.112	0.571 ±0.111	0.621 ±0.106	0.555 ±0.286	0.636 ±0.117	0.601 ±0.101	0.266 ±0.057	0.308 ±0.212	0.291 ±0.086	0.334 ±0.208
<i>Bacillus safensis</i> S9	0.967 ±0.152	0.989 ±0.200	0.833 ±0.288	0.951 ±0.133	0.831 ±0.148	0.572 ±0.062	0.376 ±0.067	0.451 ±0.133	0.398 ±0.197	0.582 ±0.173
<i>Bacillus altitudinis</i> S2	0.378 ±0.069	0.419 ±0.129	0.478 ±0.168	0.787 ±0.280	0.89 ±0.085	0.878 ±0.069	0.878 ±0.069	0.472 ±0.144	0.377 ±0.107	0.38 ±0.170
Strains	Optical density taken before and after centrifugation at different potassium dichromate concentration									
	200 µg/ml	200 µg/ml	400 µg/ml	400 µg/ml	600 µg/ml	600 µg/ml	800 µg/ml	800 µg/ml		
	Before	After	Before	After	Before	After	Before	After		
<i>Bacillus safensis</i> S1	0.36 ± 0.05	0.741 ± 0.001	0.543 ± 0.002	1.242 ± 0.003	0.138 ±0.003	1.622 ± 0.05	0.116 ± 0.005	1.594 ± 0.004		
<i>Bacillus safensis</i> S9	0.349 ± 0.009	0.215 ± 0.005	1.088 ± 0.05	0.207 ± 0.007	0.288 ± 0.05	1.915 ±0.003	0.649 ±0.004	1.437 ± 0.076		
<i>Bacillus altitudinis</i> S2	0.225 ± 0.104	0.173 ± 0.05	0.228 ± 0.007	1.604 ± 0.05	0.541 ±0.001	1.937 ± 0.003	0.317 ± 0.060	0.959 ± 0.050		

Temperature and pH Stability of Bacterial Strains:

The growth patterns of bacterial isolates were recorded at different temperature regimes i.e. 25°C, 37°C and 45°C [18]. Optical density was measured for all the strains at 600nm and the highest optical densities for the selenite and chromate reduction were observed at 37°C-45°C in the strains, where *Bacillus safensis* showed strong activity at 37°C and *Bacillus altitudinis* (S2) at 45°C as shown in Table 3.4. These observations align with the findings that mesophilic temperatures (30°C-45°C) are optimal for bacterial growth and metal reduction. [11].

The strains were also cultured at different pH ranging from 5 to 9. Optical densities of bacterial strains were determined at 600nm for the solutions of sodium selenite and potassium dichromate. Strains S1 and S2 showed heavy metal reduction at pH 7 for selenite concentration, whereas strains S9 and S2 showed maximum reduction potential at pH7 for chromate concentration as shown in Table 3.4. These results align with the findings that pH range from neutral to slightly alkaline favors the bacterial bioremediation efficiency [8].

Table 3.4 Optical density measurements recorded under varying pH values for sodium selenite and potassium dichromate concentrations.

Strains	Optical density for selenite			Optical density for chromate		
	20°C	37°C	45°C	20°C	37°C	45°C
<i>Bacillus safensis</i> S1	0.072 ±0.001	0.184 ±0.002	0.957 ±0.001	0.468 ±0.003	0.744 ±0.002	1.421 ±0.001
<i>Bacillus safensis</i> S9	0.040 ± 0.002	1.823 ± 0.001	0.410 ± 0.001	0.410 ± 0.001	0.210 ± 0.002	0.778 ± 0.004
<i>Bacillus altitudinis</i> S2	0.066 ± 0.003	0.021 ± 0.001	0.570 ± 0.006	1.205 ± 0.005	0.181 ± 0.001	1.827 ± 0.007
Strains	Optical density for selenite			Optical density for chromate		
	pH5	pH7	pH9	pH5	pH7	pH9
<i>Bacillus safensis</i> S1	0.037 ± 0.00	0.433 ± 0.001	0.024 ± 0.001	1.4 ± 0.1	0.116 ± 0.0015	0.325 ± 0.001
<i>Bacillus safensis</i> S9	0.04 ± 0.01	0.976 ± 0.001	0.48 ± 0.01	0.382 ± 0.001	0.645 ± 0.001	0.584 ± 0.001
<i>Bacillus altitudinis</i> S2	0.031 ± 0.001	0.382 ± 0.001	0.03 ± 0.001	0.304 ± 0.001	0.735 ± 0.001	0.436 ± 0.001

Effect of salinity on Growth of Bacterial Strains:

The amount of salt present in the environment is referred to as Salinity [19]. Different salt concentrations were used to depict the effect of salinity. These strains were also grown in the presence of chromate along with NaCl. Maximum growth was shown by all strains at 0.5M NaCl concentration as shown in Table 3.5. The results match the observations that moderate salinity favors bacterial activity but higher salinity inhibits growth and bioremediation capacity [19].

Table 3.5 Optical density values recorded under varying saline conditions for sodium selenite and potassium dichromate.

Strains	Optical Density for Selenite					
	0.5 M	1M	1.5 M	N-Broth	Selenite	Selenite NaCl +
<i>Bacillus safensis</i> S1	0.547 ± 0.047	0.073 ± 0.023	0.067 ± 0.017	0.603 ± 0.103	1.017 ± 0.117	0.296 ± 0.096
<i>Bacillus safensis</i> S9	0.528 ± 0.128	0.130 ± 0.003	0.101 ± 0.051	0.688 ± 0.188	0.075 ± 0.025	0.490 ± 0.009
<i>Bacillus altitudinis</i> S2	0.430 ± 0.013	0.277 ± 0.077	0.129 ± 0.079	0.634 ± 0.134	0.911 ± 0.111	0.299 ± 0.099
Strains	Optical Density for Chromate					

	0.5 M	1M	1.5 M	Chromate	Chromate + NaCl
<i>Bacillus safensis</i> S1	0.025 ± 0.015	0.018 ± 0.013	0.036 ± 0.026	0.018 ± 0.013	0.037 ± 0.027
<i>Bacillus safensis</i> S9	0.069 ± 0.019	0.009 ± 0.003	0.005 ± 0.001	0.018 ± 0.003	0.049 ± 0.004
<i>Bacillus altitudinis</i> S2	0.032 ± 0.0017	0.012 ± 0.002	0.011 ± 0.002	0.028 ± 0.004	0.048 ± 0.005

16SrRNA Sequencing for Identification of Bacterial Isolates:

Recent studies have revealed that 16S rRNA sequencing is the most reliable method for the identification of bacterial strains and classification of bacterial taxonomy [20]. Microbiologists are required to identify the bacteria in order to analyze their clinical diagnosis and antimicrobial therapies. Bioinformatics tools were utilized in this research for the phylogenetic analysis of microbial strains and eventually a phylogenetic tree was generated comprising of the bacterial strains' species and genera. Next Generation Sequencing techniques have widely modernized the concept of sequencing 16S rRNA gene in the field of microbial ecology. NGS has an ability to relate the environmental parameters with its host at both specie and genera levels [21]. Quantitative analysis of bacterial dynamics has been possible due to the advantageous method of NGS which results in the accurate taxonomic identification as shown in Figure 4.

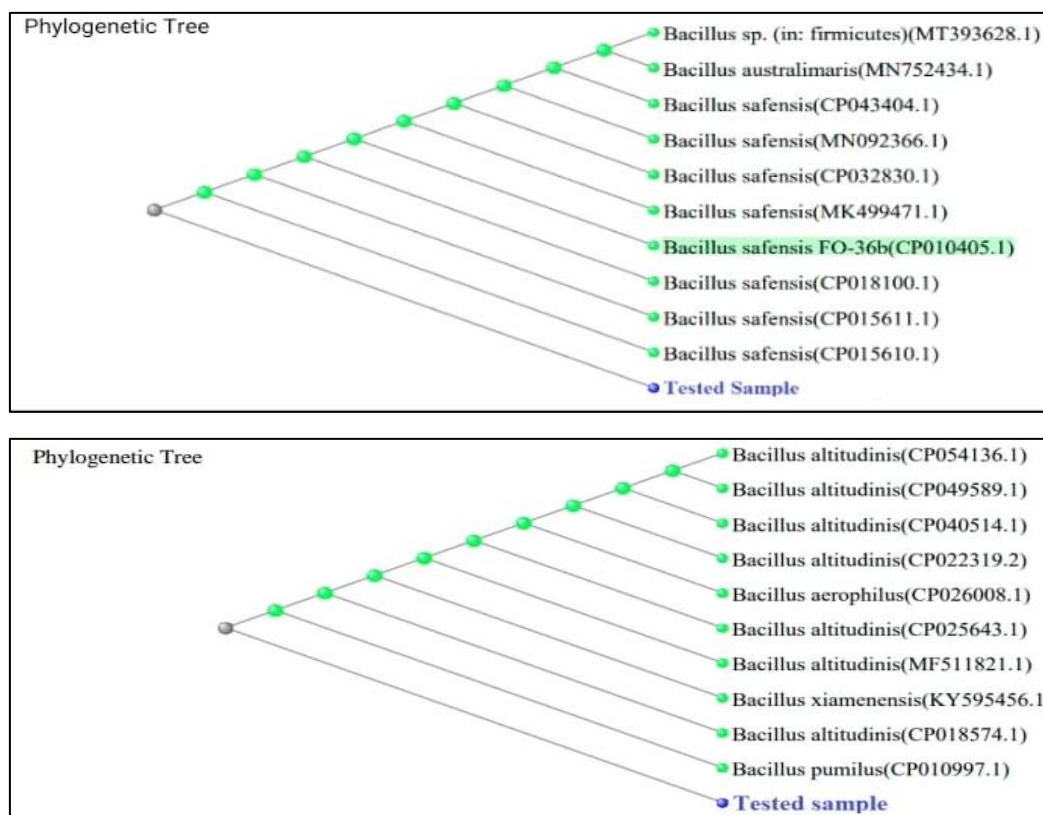


Figure 4: Phylogenetic trees of *Bacillus safensis* strains S1, S9 and *Bacillus altitudinis* strain S2 obtained by 16SrRNA sequencing

Discussion:

Heavy metals are present in minute quantities in the environment and are lethal to the human health at low concentrations [22]. They can become extremely harmful if not metabolized by the human cells. These metals include cadmium, chromium, cobalt, zinc, arsenic and selenium [23]. Microorganisms, especially bacteria play a very crucial role in bioremediation by degrading pollutants such as heavy metals.

In this study, the soil samples contaminated with selenium and chromate were collected from the site of Ali Ago fertilizer in Lahore. The serial dilutions (10^{-1} , 10^{-5} , and 10^{-9}) of these samples were prepared and further plated on N-agar plates comprising of appropriate amounts of sodium selenite and potassium dichromate. These plates were incubated at 37°C for 24 hours. Distinct colonies were selected and streaked on fresh N-agar plates to obtain pure isolates. Gram staining was performed to categorize the isolates and all the strains were found to be Gram positive rods in purple color. These results settle in with the interpretation that gram positive bacteria always retain dark purple color and Gram negative bacteria appear pink or red in color [15]. These gram positive rods were then subjected to endospore staining with malachite green and were observed to be non-spore forming.

A series of biochemical reactions was carried out to fully understand the

isolated strains. The results showed that all the strains were catalase positive. In oxidase test, the color change to blue within 15-30 seconds, indicated positive results [17]. Some strains gave MR test results positive by giving red coloration, whereas some strains showed negative results by giving yellow coloration. Yellow coloration was observed in case of VP test in all strains. All the strains showed negative amylase activity.

Different concentration of sodium selenite (200-2000 µg/ml) and potassium dichromate (200-800 µg/ml) were used to evaluate the reduction capabilities of the isolates. Strain S2 showed optimal growth at 1000 µg/ml, with a decrease beyond this concentration, for sodium selenite. The study of *Bacillus megaterium* strain also reveal an inverse relationship between selenite concentration and reduction efficiency, as the efficiency peaked at low concentrations (200-400 µg/ml), with a decline at high concentrations [24]. For chromate reduction test, all strains were observed to have optimal growth at 600 µg/ml. Post centrifugation analysis revealed that strain S1 had least growth at 800 µg/ml.

Optical density measurements at 600nm showed that all the strains grew best at 37-45°C temperature. Strains S1 and S9 exhibited maximum selenite and chromate reduction at 37°C, while strain S2 gave optimal growth at 45°C

These results suggested optimal metabolic activity at mesophilic temperatures, as the strains of *Bacillus megaterium* also show efficient selenite reduction at pH 7 along with temperature of 37° C which are gram positive bacterial strains [24]. The pH tolerance assays revealed that the selenite reduction for the strains S1 and S2 was highest at pH 7, while chromate reduction peaked at same pH for strains S9 and S2, indicating the neutral pH best for the growth of these strains as compared to the strain of *Bacillus firmus* from industrial effluent which shows that the Cr (VI) reduction is best at at pH 8 to 10 and temperature at 35°C [25]. Salinity tolerance of these strains was also tested using varying NaCl concentrations. Maximum growth for all three strains was observed at 0.5M, NaCl concentration, confirming that moderate salt concentrations support the optimal bacterial growth and heavy metal reduction. The results settle in with the study of *Bacillus safensis* strain PM22 which shows the maximum growth at 0.5M NaCl but decreases at higher salinity levels [26].

Finally, the identification of the bacterial strains was confirmed by 16SrRNA sequencing which confirmed the strains S1 and S9 to be of *Bacillus safensis* and strain S2 as of *Bacillus altitudinis*. Phylogenetic trees (as shown in Figure 4) were generated using bioinformatics tools. These results confirm the efficient potential of isolated strains in selenite and chromate reduction. Overall this study highlights the potential of indigenous soil bacteria for bioremediation of selenium and chromate contamination, under the promising influence of environmental factors such as pH, temperature, pollutant concentration and salinity form maximal reduction.

Conclusion:

In conclusion, the purified strains had exceptional reduction ability for both selenite and chromate. All the strains proved that an increase in salt concentration decreases the ability of microbes to reduce the heavy metals. Reduction of heavy metals by using industrial protocols is an expensive approach, however in this research study, microorganisms have been applied for the potential bioremediation of toxic selenite and chromate in a cost-effective way. This study potentially enhances the concept of heavy metals reduction by the natural abilities of microorganisms to perform this task especially, bacteria.

Future Perspective:

In the field of environmental microbiology, this research study provides principle understanding of how *Bacillus safensis* and *Bacillus altitudinis* strains can play a pivotal role in alleviating harmful environmental contaminants. Looking ahead, future perspectives may involve a deeper exploration of the underlying molecular mechanisms responsible for selenite and chromate reduction by these bacteria, clarifying the essential enzymes and significant pathways. Furthermore, investigating the potential use of these bacteria in remediation strategies for polluted industrial sites could lead to sustainable and environmental friendly solutions. By incorporating genomic and metagenomics approaches, there's an opportunity to enhance the major role of microbial communities in bioremediation, ultimately guiding the optimization of microbial consortia to improve pollutant degradation efficacy.

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Conflict of interest

Authors have no conflict of interest

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