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Bioprocessing of seleno-oxyanions and tellurite in a novel *Bacillus* sp. strain STG-83: A solution to removal of toxic oxyanions in presence of nitrate

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ABSTRACT

Bioremediation of toxic nonmetal and metalloid oxyanions is of great interest. In this study, among 148 bacterial isolates from two types of polluted water, strain STG-83 showed maximum oxyanion reduction and resistance ability. Sequencing of the 16S rDNA gene of STG-83 showed that the strain is closely related to *Bacillus pumilus* and morphological and biochemical tests confirmed the result. The strain was nitrate negative, but it could reduce half of tellurite in solution containing 1-mM concentration and completely reduced selenite and selenate in solutions containing 1-mM concentrations. Both reduction to elemental form and volatilization occurred in case of all oxyanions tested, according to hydride generation atomic absorption spectroscopy and proton induced X-ray emission analytical methods. The strain was able to tolerate remarkably high concentrations of selenite (640 mM), selenate (320 mM), and tellurite (1250 μM); and tolerance to tellurite increased in presence of selenite and selenate. Biochemical tests and zymogram of extracted culture solutions on gel electrophoresis showed that the strain was nitrate negative and therefore nitrate did not interfere with reduction of other oxyanions. Thus, the strain opens up good opportunities for the bioremediation of polluted waters in natural environment, since nitrate usually inhibits or decelerates reduction of the mentioned toxic oxyanions.

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1. Introduction

Selenium is found in fossil fuels, shale, alkaline soils and as a constituent in over 40 minerals [1]. Selenate [Se(VI)] and selenite [Se(IV)] are the predominant species in aqueous environments, and occur as soluble oxyanions. Selenium is also found in ground water which may be used as a source of drinking water in many developing countries [2]. Under certain conditions, Se(VI) and Se(IV) can be reduced to insoluble elemental selenium [Se(0)] in natural environments. Se(0) is the dominant species of Se in anoxic sediments [1], where the transformation of Se in nature occurs primarily by biotic processes [3].

In contrast to selenooxyanions, tellurite [Te(IV)] is generally not found in biological systems. It is more toxic to living organisms than elemental tellurium [Te(0)] and tellurate [Te(VI)], with toxicity being related to its activity as a strong oxidant [4].

Although low Se status is associated with several chronic diseases in limited regions [5], the main problem in many areas of the world is assigned to increased concentrations of the related oxyanions in a range of μg/mL concentrations which are released in environment due to anthropogenic activities such as mining, fossil fuel combustion, and agricultural activities specially in arid areas. Most of the oxyanionic forms of Se and Te are toxic to living organisms and bacteria as well as many other organisms. Microorganisms apply their metabolic capacity in different ways to transform the oxyanions to other non-toxic chemicals, and also possess a high capacity for detoxifying and metabolizing of Se- and Te-oxyanions.

In contrast to Se, less work has been carried out on Te interactions with microorganisms. Te compounds can be found in considerable concentrations near sites of waste discharge, and their toxicity to living organisms, particularly to gram negative bacteria, is well established [6].

Apparently, microbes that can reduce Se(VI) and Se(IV) are not restricted to any particular group/subgroup of prokaryotes and examples are found throughout the bacterial and archaeal domains [7]. Although microbial reduction of Se(IV) to Se(0) and selenide [Se(II)] has been widely reported [8], reports of Se(VI) being reduced to Se(IV), Se(0), or Se(II) are less numerous [8]. Several bacteria isolated from different environments are capable of reducing Se(VI) to Se(0), These include *Wolinella succinigenes*, *Pseudomonas stutzeri*, *Bacillus* sp. SF-1, *Bacillus selenitireducens*, *Citerobacter freundii*, *Citerobacter braakii*, *Enterobactercloacae*, *Enterobacter taylorae*, *Sulfurodpirillum barnesii* and *Thauera selenatis* [9]. Reduction of the mentioned oxyanions is in relation with reduction

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of nitrate. Nitrate reductase is an enzyme responsible for Se(IV), and Se(VI) reduction and Te(IV) reduction in many other known bacteria [6,10,11]. Yet, there are a number of bacteria with known enzymes specifically reducing a defined type of oxyanion [12].

Bacterial reduction of Se(VI) to Se(0) is an important biogeochemical process in an aquatic environment [13,14]. In this system, Se(VI) can be used in microbial respiration or dissimilatory Se(VI) reduction, as a terminal electron acceptor for growth and metabolism.

Biodiversity of the microorganisms harboring the oxyanion-reducing capability attracts attention of researchers leading to the elucidation of new pathways or specific novel enzymes with unique and superior activities or different substrate specifications. The reported degree of reduction is lower for Te(IV) and Se(VI) than for Se(IV). Thus, bacteria with increased capacity for Te(IV) and Se(VI) reduction are of special interest. Results of such studies may find applications in biotechnology, for use in industry and environment.

This research introduces a new gram positive bacterium with unique specificities regarding novel enzymatic properties, in addition to diversity and extent of oxyanions which can be reduced.

2. Materials and methods

2.1. Isolation, screening and selection

Water samples were collected from different areas of the Anzali lagoon (in the southern coastal region of Caspian Sea, Gilan province, Iran) and Neidasht spring (in Mazandaran province, Iran), were enriched in Trypticase Soy Broth and cultured on agar plates to obtain separate colonies as described previously [15]. The broth cultures were incubated aerobically at 30 °C in a shaking incubator (150 rpm) and pH 7.0 for 72 h. Initially, 148 bacterial isolates were obtained and cultured in microtiter plates containing Luria Bertani medium (LB) plus 1-mM concentration of each of Na_2SeO_3, Na_2SeO_4 (Sigma) and 0.1-mM K_2TeO_3 (Merck) from filter-sterilized stock solutions to screen for the more resistant isolates. Subsequently, and to carry out the minimum inhibition concentration (MIC) test, over night cultures of selected bacterial isolates with a defined optical density and at 0.1% concentration, were used to inoculate LB medium (2× concentration). Inoculated LB medium was added in 50 μL aliquots to each well previously filled with 50 μL of an appropriate dilution of oxyanion solutions.

Cultures were examined daily within 14 days after incubation. Resistance of bacterial cultures to oxyanions was determined by appearance of growth and/or reduction of oxyanions to their respective elemental forms, which was detected by color change of the broth. The isolates were cultured on Trypticase Soy Agar (TSA) without oxyanions to ensure that the red [Se(0) released] or black [Te(0) released] colors were not due to the presence of bacterial pigment. In each case, the MIC was defined as the lowest concentration of Na_2SeO_4, Na_2SeO_3, or K_2TeO_3 that inhibited growth.

The viability of cells following exposure to high concentrations of Se(IV), and Se(VI) (each 80 mM) and Te(IV) (1 mM) was determined by incubation in LB plus related oxyanions for 72 h.

To select the strain with the highest capacity of tolerance to Se(IV), Se(VI) and Te(IV), 9 out of 148 isolates which passed the above primary screening procedure, were cultured in LB plus various concentrations of Se(IV), Se(VI), and Te(IV). One isolate showed remarkable resistance to these compounds in comparison to others.

2.2. Bacterial identification

The most resistant strain was tentatively identified as *Bacillus* sp. according to morphological and biochemical properties and was named strain STG-83. Genomic DNA was extracted and purified according to Sambrook and Russell [16] and its purity was assessed by the spectrophotometric reading at A_{260}/A_{280}. Universal 16S rDNA PCR forward primer (5′-AGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-GGC/TTACCTTGTTACGACTT-3′) were used in the amplification of 16S rDNA gene [17,18]. A thermal cycler (Eppendorf) was used with the following program: (1) an initial denaturation temperature of 94 °C for 5 min, (2) a run of 30 cycles with each cycle consisting of 45 s at 94 °C, 45 s at 48 °C and 90 s at 72 °C and (3) 5 min at 72 °C to allow for the extension of any incomplete products. The amplification products were purified using DNA purification kit (Fermentas) followed by DNA sequencing on both strands directly by SEQ-LAB (Germany) according to super long run procedure. Multiple sequence alignment was carried out using 16S rDNA sequences from 20 *Bacillus* species obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and hyper-variable multiple sequence alignment was performed with Clustal W version 1.852 [19]. Morphological and biochemical tests were performed according to Bergeys Manual of Systematic Bacteriology [20].

2.3. Growth measurement

Absorbance at 620 nm was measured spectrophotometrically (Jenway 6505 UV/Vis) using 2-mm path-length cuvettes and undiluted culture samples.

The total protein content of the cell cultures was determined using modified Lowry [21]. The absorbance at 660 nm of the copper-protein complex was determined using a blank containing all of the reagents plus uninoculated LB instead of test samples. To measure the protein content of the culture, at first step a 250-μL aliquot of sample was withdrawn and mixed with 750 μL of reacting solution in a microtube, and left undisturbed for 10 min. This resulted in cell lysis and degradation of protein contents into peptides and aminoacids. Insoluble material especially elemental form of metalloids was removed by a spin in a microfuge (15,000 rpm). All measurements were done in triplicate. Bovine serum albumin was used as the standard.

2.4. Reduction and volatilization assays

Studies on Se(IV), Se(VI) and Te(IV) reduction were carried out in 500-mL serum bottles containing 250-mL steam sterilized LB medium supplemented with filter sterilized Se(IV), Se(VI) and Te(IV). The pH was adjusted to 7.0 by the addition of 0.1 M HCl or 0.1 M NaOH. Culture bottles were equipped with stainless steal plates with built-in inlet and outlet for entering and exiting gases plus silicon O-rings to seal the plastic caps of each bottle and prohibit any outward and inward leakage. Meanwhile, culture bottles were shaken in a shaking incubator all through the incubation period. The culture media were inoculated with bacterial cells from an overnight culture and incubated at 30 °C. Following inoculation, 20-mL samples were taken at 8-h intervals, the biomass separated by centrifugation at 15,000 × *g* for 15 min at 4 °C, washed twice with 10 mL ddH_2O and finally dried overnight at 100 °C. The contents of the oxyanions in the supernatant and dried biomass were measured using analytical methods as mentioned below. All experiments were performed in triplicate under aerobic conditions with uninoculated controls run simultaneously.

Culture bottles were aerated with normal air at a rate of 6.5-L air per liter of broth per minute, using an electrical air pump. The system was gas-tight and membrane filters with 0.45-μm pore size were used to sterilize entering air and exiting gases and inhibit contaminations. To trap volatile metalloid gases, the same gas-tight containers as those described for culture bottles, other than their

smaller sizes, were used to trap gases. Short tubing was used to transfer gases from culture bottle into trap bottles which were connected serially and exiting gas from culture was passed into first trap bottle and from the first trap into second trap and so on.

Although we used normal air for aeration, it is clear that exiting gas from culture bottle is not normal air since it contains products of fermentation, including carbon dioxide, organometalloids and other gaseous and volatile products of fermentation, in addition to intact oxygen remaining after respiration. At any rate, the oxidization of a negligible trace amount of organometalloids is inevitable.

Volatile Se and Te produced by cultures were trapped in gas washing bottles, which contained 200 mL of alkaline peroxide (40 mL of 30% H_2O_2, 160 mL of 0.05 M NaOH). Samples of the alkaline peroxide trap solutions were collected at different time points during the course of the bacterial growth. The samples were heated at 92 °C for 30 min in a water bath, 5 mL of concentrated HCl was added, and the samples were heated again at 92 °C for 30 min in tightly closed bottles [22]. The Se and Te contents were determined by hydride-generation atomic absorption spectroscopy (HGAAS).

2.5. Analytical methods

Total soluble selenium [Se(IV) and Se(VI)] was determined by sample centrifugation (15,000 × *g*) and analysis of the supernatant was carried out by HGAAS. Se(IV) content in solutions was quantified colorimetrically at 420 nm using 3,3′-diamino-benzidine (DAB). The amount of Se(VI) ion was measured by subtracting the total soluble selenium content from Se(IV) content. Analytical standards containing different concentrations of Se(IV) and Se(VI) were used to draw standard curves [23].

Te(IV) in solution was determined using diethylthiocarbamate (DDTC) method in which 100–400 μL aliquot of Te(IV) containing broth was mixed with 100 μL of diethylthiocarbamate reagent. Absorbance of this mixture was read at 340 nm [24]. Se- and Te-contents in pellet (bacterial cells) were measured using HGAAS method after the acidic digestion of biomass in each case.

To further confirm the results obtained from the above quantitative assays, PIXE (Proton induced X-ray emission) method was used.

2.6. Extraction of the reductase

Bacillus sp. STG-83 cells (22 g wet weight) were suspended in 210 mL of PBG (20 mM potassium phosphate pH 7.0, 4 mM β-mercaptoethanol and 5% glycerol) buffer which contained 0.1 mM PMSF. Unless otherwise stated, all steps were carried out at 4 °C, and PBG buffer was used throughout the purification protocol. The suspension was subjected to sonic disruption at 0 °C and the cell debris were discarded by centrifugation at 18,000 × *g* for 15 min. Cold streptomycin sulphate solution (2% final concentration) was then added to the supernatant to precipitate the nucleic acids. After 30 min on ice with occasional shaking, the precipitated material was pelleted at 15,000 × *g* for 30 min [25].

2.7. Enzyme assays

Electrophoresis under non-denaturing conditions was carried out as described by Avazeri et al. [6]. A separating gel with 7.5% acrylamide containing 0.1% Triton X-100, and a stacking gel (3% acrylamide containing 0.1% Triton X-100) were prepared from a stock solution (30% acrylamide, 0.8% bis-acrylamide). The buffer system was 25 mM Tris (pH 8.5), 192 mM glycine, 0.02% Triton X-100 [6].

The enzyme activities were also visualized on non-denaturing polyacrylamide gels [26,27]. The gels were incubated in 40 mM Tris/HCl buffer (pH 7.6) containing 0.6 mM methyl viologen reduced with $Na_2S_2O_4$. Enzyme activity was visualized by the appearance of a clear band(s) on the gel, degassed and impregnated with reduced methyl viologen (which results in a dark blue-stained gel), after introduction of different substrates including (in 5-mM concentration): KNO_3, K_2TeO_3, Na_2SeO_4 and Na_2SeO_3.

3. Results

Out of 148 bacterial isolates with different microscopic and macroscopic morphologies, 78 bacterial isolates were able to reduce Se-oxyanions. In this study, processing of oxyanions by selected bacteria was assessed. All data presented here are arithmetic mean of three tests. The error bars were less than 0.05 and thus not shown here.

The ability to resist increased concentrations of Na_2SeO_4, Na_2SeO_3 and K_2TeO_3 varied among isolates, and highly tolerant isolates could grow and reduce considerable amounts of oxyanions, as shown in Table 1. All except STG-5 and STG-83 were sensitive to the presence of Te(IV) as the sole added oxyanion, but Te(IV) reduction occurred in the presence of Se-oxyanions. Strain STG-83 showed maximum oxyanion reduction and resistance ability. This strain grows in presence of 640 mM Se(IV) and reduces a part of it to Se(0). However, reduction of Se(IV) in other known bacteria is not more intensive compared to that of our strain. This spore-forming, gram-positive and rod-shaped bacterium was selected for further studies.

Sequence of the 1451-bp fragment from 16S rDNA obtained by PCR was determined. The phylogenic tree was constructed by neighbor-joining method (Fig. 1). Multiple alignment and phylogenic tree showed that the isolate STG-83 is closely related, and has 99% homology to *Bacillus pumilus*. A more definitive classification in the future, however, should take additional characteristics, especially DNA–DNA hybridization data, into account [28]. The obtained 16S rDNA sequence has been deposited in GeneBank under accession no. EF051255 for *Bacillus* sp. strain STG-83.

This bacterium was shown to be catalase-positive, oxidase-negative and capable of using sodium citrate as the sole carbon source. The selected isolate was also able to grow in salt-containing nutrient media at different concentrations up to 10% (w/v); however, lecithinase activity and nitrate reduction were negative. This facultative aerobic bacterium was shown to grow at temperatures as high as 50 °C and produce acid from glucose, mannitol, xylose, and arabinose. The combination of morphological, physiological, and biochemical data confirmed that the STG-83 isolate was a *Bacillus* species.

In absence of oxyanions, the growth curve based on the protein content of the bacterial cells paralleled the growth curve obtained from the turbidity measurements (data not shown here).

Growth of STG-83 in oxyanion-free medium was compared to those of Se-oxyanions and Te(IV) containing media. As shown in Fig. 2, the growth was variably influenced by the presence of the tested oxyanions.

Fig. 2 shows the result of the protein assay in the cultures of Bacillus sp. STG-83 in medium containing 1-mM concentration of different oxyanions and also in the absence of added oxyanions as control. Protein content in the presence of Se(IV) is nearly twofold higher than that of control. Also, 39% increase in protein content was shown in presence of Se(VI). But in case of Te(IV), total protein content of the culture decreased due to toxic effects of the oxyanion on Bacillus sp. STG-83. In mid-log phase of growth, a rate of 6.84, 27.9 and 17.35 μg protein/mL h was recorded in control, Se(IV) and Se(VI) containing media, respectively. STG-83 is highly resistant and protein content of the culture shows that selenite in

Table 1
Maximum concentrations of oxyanions which can be tolerated by bacterial strains isolated from Anzali Lagoon or Neidasht spring.

Bacterial isolate	Se(IV) reduction (mM)	Se(VI) reduction (mM)	Te(IV) reduction (μM)	Te(IV) reduction[a] (μM)	Te(IV) reduction[b] (μM)	NO_3^- reduction
STG-1	640	160	–	1250	600	–
STG-2	160	160	–	1250	600	+
STG-3	160	160	–	1250	600	+
STG-4	160	160	–	2500	1250	+
STG-5	160	320	600	2500	2500	–
Bacillus sp. STG-83	640	320	1250	2500	2500	–
STG-7	320	320	–	2500	2500	+
Bacillus sp. MGG-83 [15]	80	160	–	2500	1250	+
GT-83	160	160	–	1250	1250	+

[a] In presence of 20 mM Se(IV).
[b] In presence of 20 mM Se(VI).

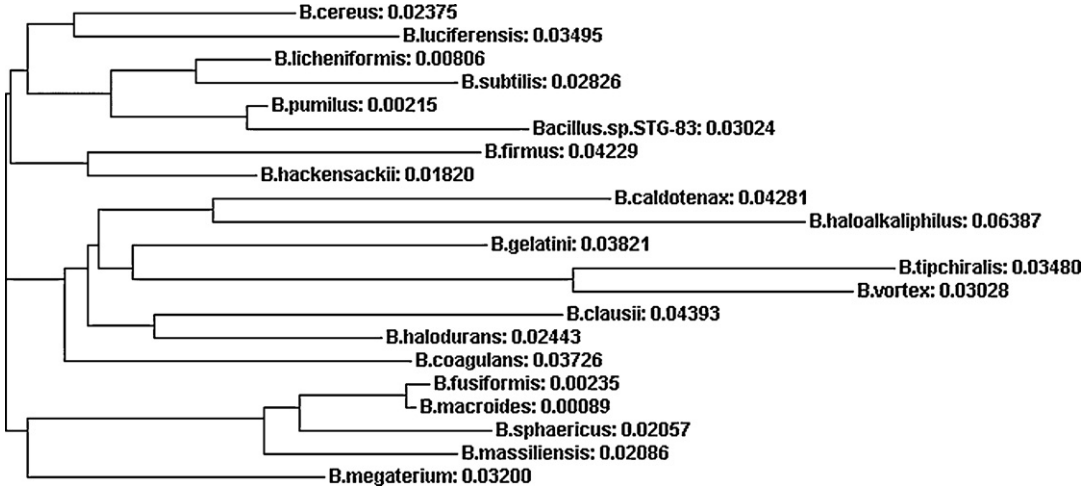

Fig. 1. Phylogenetic tree of strain STG-83 and related *Bacillus* spp. inferred from sequence of 16S rDNA.

concentrations as low as 1 mM does not exert toxic effects on the growth of the bacterium. The release of metalloids did not interfere with protein measurement, since metalloids were separated during the protein measurement procedure. Furthermore, the time course of increase in protein content and formation of elemental forms were not exactly parallel.

As shown in Fig. 2, in the presence of Te(IV), the protein content of the culture decreased compared to those of control and the sole Se-oxyanion containing media.

The decrease in the Se(IV) concentration during growth is shown in Fig. 3. Typically, there was a rapid initial reduction of Se(IV) to Se(0) which was almost completed within 32 h after inoculation.

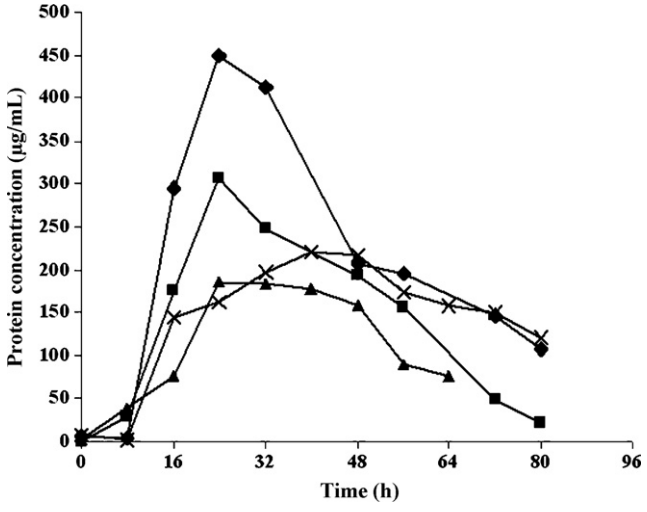

Fig. 2. Time course of changes in protein contents in whole culture of *Bacillus* sp. strain STG-83 grown in LB medium in presence of 1-mM concentration of different oxyanions: Se(IV) (♦), Se(VI) (■), Te(IV) (▲), and control (×).

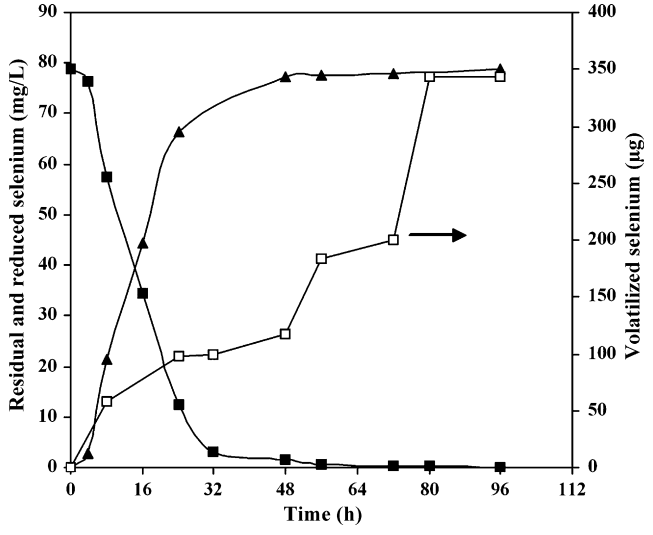

Fig. 3. Time course of transformation of Se(IV) in LB medium by *Bacillus* sp. STG-83: residual Se-oxyanions in solution (■), precipitated Se (▲), and volatilized Se (□). Arrow shows relation of the curve to right axis.

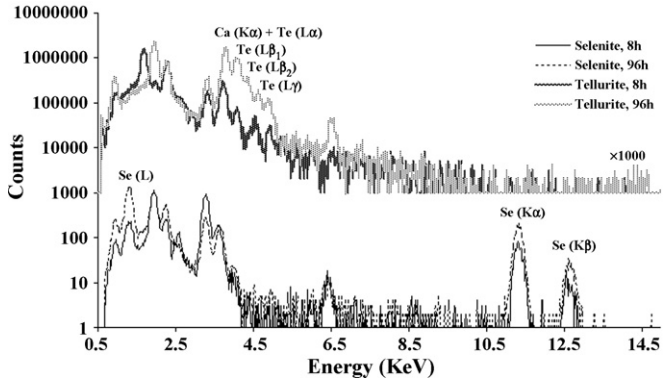

Fig. 4. Characterization of X-ray of dried pellet obtained after centrifugation of culture broth at two different times (8 and 96 h). Increasing counts of energy in PIXE analysis of pellet samples from early and late phases of culture indicates precipitation of related elements (Se and Te). The graphs were obtained by PIXE analysis using 3 MeV protons at 70 nA beam intensity.

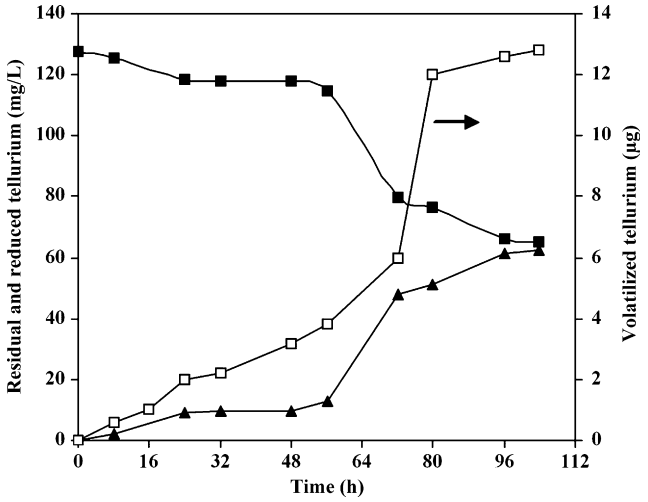

Fig. 6. Reduction of 1 mM K_2TeO_3 in LB medium by *Bacillus* sp. STG-83. Te(IV) concentration in liquid phase (■), precipitated Te (▲), volatilized Te (□). Arrow shows relation of the curve to right axis.

The amount of Se volatilized by the *Bacillus* sp. STG-83 increased concurrently with the growth of the strain over the time (Fig. 3). *Bacillus* sp. STG-83, which grew well in the liquid medium containing Se(IV), volatilized 344 μg Se. The ability of *Bacillus* sp. STG-83 to withstand extremely high Se and salt concentrations was tested and compared to control conditions. The *Bacillus* sp. STG-83 could tolerate 10% NaCl and 640 mM Se(IV) concentrations. Lag time of growth increased as higher concentrations of oxyanions were used.

PIXE was used to characterize the precipitates of Se in culture medium after reduction processes. PIXE is a nondestructive, analytical technique used to simultaneously trace several elements and very powerful with minimum detection limits (MDL) between 0.1 and 50 μg/mL depending on the element and host matrix [29]. An analysis by PIXE for Se precipitates in pellets obtained from Se(IV) containing culture medium in 8 and 96 h after inoculation is shown in Fig. 4.

In preliminary experiments, it was demonstrated that strain STG-83 was able to reduce Se(VI). Fig. 5 shows typical results of Se(VI) reduction tests. The medium turned densely red during the Se(VI) reduction, suggesting formation of amorphous Se(0). The results of HGAAS analysis of the digested pellet indicated that almost all of the soluble Se removed from supernatant was converted to insoluble Se. There was a rapid initial reduction of Se(VI) to Se(IV) which was completed within 24 h after inoculation. When 90% of the Se(VI) was reduced to Se(IV), the reduction of the Se(IV) and precipitation of the resulting Se compound had begun. *Bacillus* sp. STG-83 removed completely 1 mM Se(VI) within 96 h after inoculation.

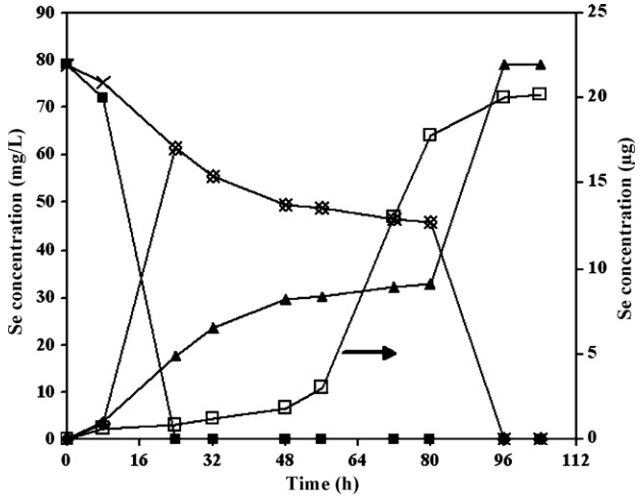

Fig. 5. Time course of reduction of 1 mM Se(VI) in LB medium by *Bacillus* sp. STG-83: total residual Se-oxyanions in solution (×), Se(IV) (◊), Se(VI) (■), precipitated selenium (▲), and volatilized Se (□). Arrow shows relation of the curve to right axis.

Analysis of the deposits by PIXE confirmed the conversion of Se(VI) into insoluble Se. Furthermore, *Bacillus* sp. STG-83 was active in the production of volatile Se compounds from Se(VI). Amounts of volatilized Se compounds was determined to be 20 μg Se (Fig. 5).

Black precipitate was produced in liquid LB medium containing 1 mM Te(IV). Fig. 6 shows that about 50% of the added Te(IV) (1 mM) had precipitated after 104 h of incubation. Amounts of volatilized Te compounds were equal to 13 μg Te. The PIXE analysis for precipitates of Te in culture medium is shown in Fig. 4.

Propagation of cells from strain STG-83 in LB medium containing increased concentrations of each of Se(IV), Se(VI) and Te(IV) revealed that the bacterium is able to tolerate and reduce all oxyanions simultaneously (Table 1).

A zymogram test was performed to show the catalytic activity of the extracted solution, to confirm the substrate specificity of the enzyme(s) against Se-, Te-oxyanions and nitrate, and to indicate the presence or absence of an induction mechanism in reducing-enzyme production by the strain STG-83.

While extracts from bacterial cells grown in different oxyanion containing media did not produce any band in the presence of nitrate, colorless bands due to oxidation, in dark blue context of reduced methyl viologen, were visualized in the presence of each of Se(IV), Se(VI), or Te(IV). These bands appeared at identical distance from the loading point. This indicates production of one or perhaps more than one related enzyme (showing similar gel migration properties) capable of reducing all tested oxyanions other than nitrate.

The results not only confirmed that nitrate is not a substrate for the reducing enzyme, but also showed that the enzyme expression does not need to be induced by each of the other three oxyanions (Fig. 7).

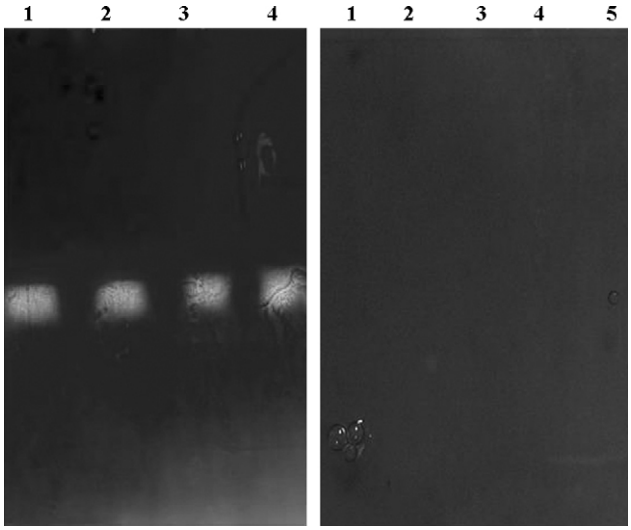

Fig. 7. Zymogram of oxyanion-reductive enzyme(s) in cell extract from *Bacillus* sp. STG-83 grown in LB media plus different oxyanions: 1 (Na_2SeO_4), 2 (Na_2SeO_3), 3 (K_2TeO_3), 4 (without oxyanion additive), 5 (plus KNO_3). Gels were dyed with methyl viologen in presence of K_2TeO_3 (left), and KNO_3 (right). The similar results were obtained when the gels were exposed to selenite or selenate solutions instead of tellurite.

4. Discussion

4.1. Diversity of reducing bacteria and water resources

Microorganisms possess a high capacity for detoxifying and metabolizing Se(IV), Se(VI), and Te(IV). Biodiversity of microorganisms harboring this capability attracts attention of researchers to look for novel microorganisms, elucidate new pathways or specific enzymes with superior oxyanion-metabolizing activity or different substrate specificity [30]. Thus, introduction of a newly isolated bacterial strain and characterization of its oxyanion-processing capabilities were considered the goal of this research.

In addition to natural water resources [31], agricultural and industrial wastewaters have usually been used to isolate various microorganisms with desired traits [9,32]. Neidasht mineral water springs is a rich source of many types of inorganic oxyanions; and Anzali lagoon also has increased levels of different organic and inorganic chemicals coming from agricultural and industrial wastes. As shown in our primary results, more than half of the isolates were able to tolerate and reduce Se(IV). Therefore, finding novel oxyanion-metabolizing microorganisms from both water sources seemed promising.

4.2. Isolation of a new strain

While Se(IV) reducing bacteria are relatively abundant, only a scanty number of Se(VI)-, and Te(IV)-reducing bacteria has been reported previously [9,11]; and bacteria highly tolerant to all of Se(IV), Se(VI), and Te(IV) with simultaneous reducing capability are rare. While 52% of the isolates in this research were able to tolerate and reduce the Se(IV), only 11% of Se(IV)-reducing bacteria showed Se(VI)-reducing activity. Among the latter, the strain STG-83 was of great interest, due to the ability of this strain to tolerate and reduce Se(IV), Se(VI), and Te(IV) all oxyanions in higher levels of concentration.

Results of bacterial identification procedures showed that strain STG-83 is related to *B. pumilus*. This species is introduced as Se-oxyanion and Te(IV) reducing organism for the first time in this research, although several other oxyanion-reducing bacteria from this genus such as *Bacillus* sp. SF-1 [33], *Bacillus subtilis* [34], and *Bacillus stearothermophilus* V [25] were previously introduced.

A number of *Bacillus* spp., among many other genera of bacteria, are capable of respiring with selenate, arsenate and nitrate, and other species of oxyanions as terminal electron acceptors under anaerobic condition [10,35,36,37]. Yet, there are other strains of *Bacillus* spp., as well as strain STG-83, that reduce these oxyanions under aerobic conditions [38].

4.3. Tolerance and reduction capabilities

According to Fig. 2, in the presence of Se(IV) the strain produced a protein concentration approximately twofold higher than that of control. But, in the presence of Se(VI), the culture showed only 39% increase in protein content. This indicates that the bacterial cells initially and actively respond to presence of Se(IV) and secondarily to the presence of Se(VI). It is clear that the related oxyanion reductive enzyme, as the detoxifying agent, is responsible for at least a part of the additional increase in protein content. This was while the protein content of culture decreased in the presence of Te(IV) (as examined in 1000 μM), pointing to the more toxic effect of Te(IV) on *Bacillus* sp. STG-83 than that of Se(IV), and Se(VI).

In contrast to Te(IV), Se(IV) and Se(VI) induce protein synthesis at low concentrations, and this should probably be considered as a more potent response for detoxification. This detoxification is a quick process in our strain, the increase in protein synthesis and reduction of oxyanions taking place in less than 24 h, while in many other bacteria such as *Stenotrophomonas maltophilia* [39] the reactions continue in a long run. Furthermore, concomitant presence Te(IV) with Se(IV), or Se(VI) increase the tolerance to Te(IV) and higher amounts of Te(IV) was reduced. The same effect has not been studied in many microorganisms characterized previously. This interaction has not been reported in case of other *Bacillus* spp. [33,40]; *Bacillus* sp. strain SS [38] reduces Se(IV), Se(VI), and Te(VI), but not Te(IV); and in case of recently characterized *Shewanella oneidensis* [41] reduction of Se(IV) and Te(IV) has been known as completely distinct processes.

The strain is able to tolerate and reduce Se(VI) and Te(IV) at 320-mM and 1250-μM concentrations, respectively. This is while the amount of Se or Te removal is much lower in many other microorganisms presented in the literature including articles cited here.

4.4. Volatilization

The strain is able to volatilize all tested oxyanions [Se(IV), Se(VI), Te(IV)]. As our results indicate, the rate of reduction and total amount of reduced Se-oxyanions into Se(0) and Te(IV) into Te(0) are much higher than that of volatilized amount, similar to results obtained, for example, in case of *Rhodobacter sphaeroides* [42]. In contrast, the amount of volatilization is limited compared to some other previously introduced strains [22]. Thus, the use of *Bacillus* sp. STG-83 in bioremediation of the toxic oxyanions should be considered since volatilized Se is recycled more rapidly in the environment than its less toxic and biologically less available elemental form [43,44].

4.5. Capacity for use in bioremediation

Reduction of Se(IV), Se(VI) and Te(IV) to their elemental forms occurs by the aid of a probable single enzyme, an enzyme complex or more enzymes occupying the same place on gel, as a single band formed on polyacrylamide gel after electrophoresis. This is likely to be a constitutive enzyme since it is produced both in the absence and presence of Se-oxyanions and Te(IV). Moreover, the enzyme lacks nitrate reductase activity as shown in biochemical test as well

as methyl viologen method for qualitative assay of reductases under anaerobic condition. This is an interesting finding and a promising opportunity for bioremediation, since nitrate reduction interferes with reduction of many other oxyanions [45], as well as Se(VI), and Se(IV) and Te(IV); adversely affecting bioremediation processes and hence the removal of the mentioned oxyanions in natural environment. Nitrate or nitrite may inhibit reduction of Se(IV), Se(VI), and Te(IV) [33,41,45,46]; and this may precede reduction of Se(VI) [47] and may not be constitutive in nitrate-grown cells [10]. Researchers attempt to solve the problem in different ways [9,48].

Finally, in addition to the valuable capabilities of this strain to reduce Se and Te oxyanions as well as the major advantage of being nitrate-negative, a further characteristic of this novel strain that deserves a mention is a tolerance to a relatively high concentration of salt (10% NaCl). This is especially useful for bioremediation of Se-contaminated drainage water in evaporation ponds with high salt levels.

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