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Biosynthesis and recovery of selenium nanoparticles and the effects on matrix metalloproteinase-2 expression

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Today, green synthesis of nanoparticles is attracting increasing attention. In the present study, the *Bacillus* sp. MSh-1 was isolated from the Caspian Sea (located in the northern part of Iran) and identified by various identification tests and 16S ribosomal DNA analysis. The reduction time course study of selenium ion (Se^{4+}) reduction by using this test strain was performed in a liquid culture broth. Then, the intracellular NPs (nanoparticles) were released by the liquid nitrogen disruption method and thoroughly purified using an n-octyl alcohol water extraction system. Characterization of the separated NPs on features such as particle shape, size and purity was carried out with different devices. The energy dispersive X-ray and X-ray diffraction patterns showed that the purified NPs consisted of only selenium and are amorphous respectively. In addition, the transmission electron micrograph showed that the separated NPs were spherical and 80–220 nm in size. Furthermore, the cytotoxicity effect of these extracted biogenic selenium (Se) NPs on the fibrosarcoma cell line (HT-1080) proliferation and the inhibitory effect of the Se NPs on MMP-2 (matrix metalloproteinase-2) expression were studied using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay and gelatin zymography. Biogenic Se NPs showed a moderately inhibitory effect on MMP-2 expression.

Introduction

An essential part of nanotechnology concerns the arrangement of materials at the atomic stage to achieve nanoscale matter with unique characteristics [1]. There is a noticeable extension in different physical and chemical procedures for the synthesis and assembly of nanoscale materials, but the fabrication of NPs (nanoparticles) using biological entities can be described as a novel technique for NP assembly [2]. Biosynthetic methods, as simple substitutes for conventional protocols, have various benefits and applications [3]. Among the biosynthetic methods, green synthesis of NPs by using micro-organisms such as bacteria can compete with other methods due to its clean, non-toxic and ecofriendly features [4].

Selenium (Se) is a micronutrient metalloid with extensive applications. It is well known that dietary Se compounds have an important role in human health, including antioxidant effects, immune health, cancer prevention and antiviral activities [5–8]. All of the oxidation states of Se (–2, 0, +2, +4, +6) are regularly created in Nature except for Se^{2+}. Insoluble Se^0 is prepared at the nanoscale by reduction of higher oxidation states to many allotropic forms [9,10]. Se NPs caught the attention of researchers due to the mineral's outstanding biological roles [11]. The ability of different micro-organisms (i.e. *Enterobacter cloacae* SLD1a-1, *Rhodospirillum rubrum*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*) to reduce selenium ions has been reported previously [12–15]. There have been no reports on the isolation, purification and characterization of biogenic Se NPs. Conversely, some micro-organisms have good potential to reduce different metal ions to NPs, which can accumulate inside the bacterial cells, but the separation and recovery of these NPs from the whole cells need further study. To the best of our knowledge, and according to a survey of the literature, little research

Key words: *Bacillus* sp. MSh-1, gelatinase-A, green synthesis, selenium nanoparticle, two-phase system, zymography.

Abbreviations used: EDX, energy dispersive X-ray; MIC_{99}, minimal inhibitory concentration for 99% of input cells; MMP-2, matrix metalloproteinase-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NP, nanoparticle; rDNA, ribosomal DNA; SEM, scanning electron microscope; TEM, transmission electron microscope; XRD, X-ray diffraction.

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The nucleotide sequence data reported for the *Bacillus* sp. MSh-1 16S rDNA sequence (1415 bp) will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number GU183144.

on extracting and employing biogenic Se NPs for various applications has been reported.

Furthermore, the physiochemical stability of metalloid NPs, which are biologically formed by micro-organisms, is higher than that of NPs obtained by ordinary synthetic methods [16].

On the other hand, MMPs (matrix metalloproteinases), a family of zinc-dependent endopeptidases, are critical enzymes in several physiological events owing to the enzymes' ability to remodel and destroy the extracellular matrix [17]. The great interest in MMPs is due to their involvement in certain pathological states such as inflammatory disease and tumour invasion, metastasis and angiogenesis in cancer [18]. Some Se compounds such as methylselenol and sodium selenite, excluding the biogenic Se^0, have been studied for their inhibitory potential in MMP-2 production [19,20].

In the present study, we screened different samples from the Caspian Sea to isolate a Se NP producer micro-organism with high Se reduction ability. The isolated strain was identified and employed to biosynthesize Se NPs. We applied a liquid–liquid partition method to separate and purify Se NPs from the whole cell lysate of the isolates. The first cells containing intracellular Se NPs were disrupted by the liquid nitrogen method. Finally, Se NPs were purified from cell debris using a liquid–liquid phases partitioning method and characterized by different devices. Subsequently, the cytotoxicity and anti-invasive effects of pure Se NPs were investigated by using an *in vitro* cytotoxicity assay and gelatin zymography.

Materials and methods

Isolation of Se NP-producing bacteria

Freshwater samples were collected in sterile bottles from the Caspian Sea (located in the northern part of Iran). The samples were serially diluted with sterile NaCl solution (0.9%) and spread on agar plates containing Nutrient Agar (Merck, Germany) supplemented with Se^{4+} ions (100 mg · l^{-1}; which is equal to 1.26 mM SeO_2) by a filter-sterilized stock solution of the selenium dioxide (Merck). The plates were incubated aerobically at 30 °C, and after 24 h, a single red colony was observed on one plate. Red is the colour of precipitated Se^0 NPs and thus serves as a provisional marker that a bacterial colony is reducing the Se^{4+} ions [16]. To make sure the red colour was not an organic bacterial pigment (i.e. *prodigiosin*), the colony was restreaked on the plain Nutrient agar plates without SeO_2, and all plates were macroscopically examined for red organic pigment production during the incubation period (48 h) at different temperatures (25, 30 and 37 °C). The organism was constantly conserved on the Nutrient agar plate supplemented with Se^{4+} ions (100 mg · l^{-1}).

Identification of the isolate

The phenotypic and physiological characterization of the isolate was carried out by the methods described in Bergey's *Manual of Determinative Bacteriology* and 16S rDNA (ribosomal DNA) sequence analysis [21,22]. To obtain genomic DNA, bacterial cells were harvested from overnight cultures, washed three times with sterile distilled water and boiled for 10 min to release the DNA. The suspension was centrifuged at 11 000 ***g*** for 5 min and then the supernatant was used as the DNA template for PCR amplification of the 16S rDNA gene. The PCR amplification included an initial denaturation at 94 °C for 180 s, 30 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 45 s and extension at 72 °C for 90 s and a final extension at 72 °C for 300 s [23].

A large fragment of the 16S rDNA gene was amplified using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′), which are located in 16S rDNA of *Escherichia coli* at nucleotide positions 8–27 and 1492–1513 respectively [24]. The amplified DNA fragment was purified from 1% agarose gel using the QIAquick Gel Extraction kit (Qiagen) according to the supplier's instructions and was sent for automated sequencing using the primers described above (Takapouzist). Sequence similarity searches were done with the BLAST database (National Center for Biotechnology Information), and the sequence was submitted to the GenBank® Nucleotide Sequence Database.

Determination of the Se^{4+} ion concentration

For indirect spectrophotometric determination of the Se^{4+} ion concentration, a modified version of the method of Somer and Kutay [25] was used. Based on this selective method, the Se^{4+} ions were reduced by the Fe^{2+} ions in acidic conditions. The reaction between Se^{4+} and Fe^{2+} is an equilibrium reaction given by the equation

$$(Se^{4+} + 4Fe^{2+} + 6H_3O^+ \rightarrow Se + 4Fe^{3+} + 9H_2O)$$

By adding SCN^- ions, the Fe^{3+} ions produce a blood red complex that has maximum absorption at 480 nm [25]. Different concentrations of Se^{4+} ions (1–100 mg · l^{-1}) with deionized water were prepared with SeO_2. These solutions were standardized by a back titration method with 0.02 M potassium permanganate and 0.02 M sodium oxalate. Then 1 ml of Se^{4+} solutions was separately transferred to test tubes, and 1 ml of the 0.16 M $FeSO_4$ freshly prepared with 5 M HCl was added. The mixtures were allowed to stand at 25 °C for 20 min, and then 1 ml of 5 M potassium thiocyanate solution was added. After the reagents were mixed, 100 μl of the mixture was diluted with 900 μl of deionized water, and the absorbance (*A*) was measured at 480 nm with a UV–visible spectrophotometer (UVD-2950; Labomed). The calibration curve was set up by plotting

the measured absorbance at 480 nm against the Se^{4+} ion concentration. In all absorption measurements, 1 ml of deionized water was subjected to the same steps and was employed as the blank. These procedures were repeated three times on different days, and the mean of the absorbances was used to draw a suitable standard curve.

General procedure for growth experiments and Se ion reduction

The agar dilution method was used for MIC_{99} (minimal inhibitory concentration for 99% of input cells) determination of SeO_2 [26]. A set of growth experiments was carried out to study the ability of the isolate to convert the Se^{4+} ions into Se NPs in Nutrient broth medium (pH 7). Sub-MIC_{99} concentrations of the Se^{4+} ions (100 and 200 mg $\cdot$ l^{-1}; which are equal to 140.5 and 281 mg of SeO_2 per litre respectively) were prepared in sterile Nutrient broth by adding these ions from a filter-sterilized stock solution of SeO_2. Approx. 100 ml of these media was transferred to sterile 500 ml Erlenmeyer flasks and were inoculated with 1 ml of the fresh inoculums [attenuance at 600 nm (D_{600}) of 0.1]. For aerobic cultivation, the flasks were plugged with cotton and were incubated for 36 h at 30 °C in a shaker incubator (150 rev. $\cdot$ min^{-1}). In a similar trial, uninoculated media containing the Se^{4+} ions and inoculated media without Se^{4+} ions were used as the control. Samples were withdrawn periodically in aseptic conditions to measure D_{600} and to perform analysis for the Se^{4+} ions remaining in the media. Before the Se analysis, cells and NPs were removed from the culture by centrifugation at 11000 ***g*** for 10 min with a Mikro 200 centrifuge (Hettich).

For spectrophotometric detection of the remaining Se^{4+} ions, 1 ml of the supernatant from the inoculated media containing Se^{4+} ions and 1 ml of the supernatant from the inoculated media without Se^{4+} ions were separately used as the test and the blank respectively. These experiments were reiterated three times on various days. Moreover, to investigate the Se^{4+} reduction ability of the cell-free supernatant, 100 ml of sterile Nutrient broth was inoculated by fresh inoculums of bacterial isolate (D_{600} of 0.1) and, after 24 h, the cells were separated by centrifugation at 4000 ***g*** for 10 min. The supernatant was filtered aseptically in a sterile 500 ml Erlenmeyer flask by using a filter (0.22 μm), and Se^{4+} ions were added (final concentration 100 mg $\cdot$ l^{-1}). The flasks were plugged with cotton and were incubated at 30 °C in a shaker incubator (150 rev. $\cdot$ min^{-1}) and checked periodically for Se NP formation.

Preparation of Se NPs

A bacterial isolate from the Caspian Sea was used for intracellular biosynthesis of the Se NPs. In a typical experiment, sterile Nutrient broth medium supplemented with Se^{4+} ions (100 mg $\cdot$ l^{-1}) was prepared, and 100 ml of these media was transferred to 500 ml Erlenmeyer flasks. The medium was inoculated with 1 ml of the fresh inoculums (D_{600} of 0.1) and incubated aerobically at 30 °C in a shaker incubator (150 rev. $\cdot$ min^{-1}). After 14 h, the bacterial cells and Se NPs were removed from the culture medium by centrifugation at 4000 ***g*** for 10 min. The pellets were washed with 0.9% NaCl solution by centrifugation and were transferred to a mortar. By adding some liquid nitrogen, the pellets were frozen and then disrupted by a pestle. The resulting slurry was ultrasonicated at 100 W for 5 min and was washed three times by sequential centrifugation (10000 ***g***, 5 min) with 1.5 M Tris/HCl buffer (pH 8.3) containing 1% SDS and deionized water respectively.

The pellets were suspended in deionized water, and the resulting suspension containing Se NPs and cell debris was collected. Approx. 4 ml of these suspensions was separately transferred to test tubes, and 2 ml of n-octyl alcohol was added to each tube. Then the mixtures were shaken vigorously. The two mixed phases were completely separated by centrifugation at 2000 ***g*** for 5 min and were stored at 4 °C for 24 h. After this period of time, the generated Se NPs could be observed at the bottom of the tubes. The lower and upper phases were discarded, and settled NPs were washed with chloroform, ethyl alcohol and distilled water respectively. The cleaned NPs were then resuspended in deionized water and stored at 4 °C. To ensure that all cell debris was removed, the purified Se NPs were resuspended in the liquid–liquid phase system and were cleaned again. A stock solution of Se Nps was prepared (1 mg $\cdot$ ml^{-1}) and used for further biological experiments.

Characterization of Se NPs

The Se NPs were characterized using different techniques. The NPs were tested for their optical absorption property by using a UV–visible spectrophotometer (UVD-2950; Labomed) operated at a resolution of 1 nm. The particle size distribution patterns of the whole cell debris suspension containing Se NPs and purified NPs were obtained by using the Zetasizer MS2000 (Malvern Instruments). For transmission electron microscopy, an aqueous suspension containing the Se NPs was dispersed ultrasonically, and a drop of the suspension was placed on carbon-coated copper TEM (transmission electron microscope) grids and dried under an IR lamp. Micrographs were obtained using a TEM (Zeiss 902A) operated at an accelerating voltage of 80 kV.

To observe the NP surface features and to determine the elemental composition of NPs, an SEM (scanning electron microscope) equipped with an EDX (energy dispersive X-ray) microanalysis attachment was employed. For SEM observation, NPs were mounted on specimen stubs with double-sided adhesive tape and coated with gold in a sputter coater device (model SCD 005; Bal-Tec). Samples were analysed by using an SEM (Philips XL30)

operated at 16 kV, and the EDX spectrum was recorded by focusing on a cluster of NPs. The crystalline structure of the Se NPs was checked by the XRD (X-ray diffraction) technique using an X-ray diffractometer (Philips PW1710) with CuK_{α} radiation ($\lambda = 1.5405$ Å) over a scanning range of Bragg angles from 20° to 80°.

The cytotoxicity assay

The human fibrosarcoma cell line (HT-1080) was purchased from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, Iran). The cell line HT-1080 was seeded on 96-well tissue culture plates. Cells were maintained in RPMI 1640 medium that was supplemented with 5% (v/v) FBS (fetal bovine serum), 100 units · ml^{-1} penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin, at 37°C in a CO_2 incubator (5% CO_2 and 95% relative humidity). For the cytotoxicity assay, 10000 cells in the exponential phase of growth were incubated for 24 h with various amounts of Se NPs (0, 10, 20, 50 and 100 $\mu g \cdot ml^{-1}$). After incubation, the corresponding supernatants of cultured cells were collected for gelatinase zymography. Then 100 μl of RPMI 1640 medium containing 0.5 mg · ml^{-1} of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] was added to each well and incubated for 3 h. The medium was discarded and replaced with 100 μl of DMSO, and the density of the purple colour due to formazan formation was read at 570 nm.

Gelatinase zymography

We performed the zymography based on our previous study [27]. Briefly, aliquots of the treated media were loaded on to an SDS/polyacrylamide gel containing 2 mg · ml^{-1} gelatin. Electrophoresis was carried out in the presence of SDS under non-reducing conditions at a constant voltage of 80 V. After electrophoresis, to remove the SDS the gels were gently washed three times with 2.5% Triton X-100 solution. The gel slabs were then incubated at 37°C overnight in 0.1 M Tris/HCl gelatinase activation buffer (pH 7.4) containing 10 mM $CaCl_2$. Gel staining was performed with 0.5% Coomassie Brilliant Blue R250 (Sigma) followed by destaining. The MMP-2 proteolysis areas became visible as clear bands against a blue background. A UVI pro gel documentation system (GDS-8000) was employed for quantitative evaluation of both the surface and the intensity of the bands, based on the grey levels. Results were compared with non-treated control wells and expressed as the relative expression of MMP-2.

Results

Identification of the micro-organism

Although different water samples were collected from the Caspian Sea, only one sample from Bandar-e Anzali contained a bacterium with the ability to convert Se^{4+} ions into Se^0. In morphological studies, the isolated strain showed up as a motile, rod-shaped Gram-positive and endospore-forming bacterium with yellowish white colonies on the Nutrient agar media. Based on these results and other biochemical tests (Table 1), the isolated bacterium was inferred to be a strain of the *Bacillaceae* family. A BLAST search of the 16S rDNA sequence against the NCBI (National Center for Biotechnology Information) GenBank® Nucleotide Sequence Database confirmed the morphological and biochemical studies. Alignment results containing 180 characters revealed 99% identity with several members of the genus *Bacillus*. The *Bacillus* sp. MSh-1 16S rDNA sequence (1415 bp) was submitted to the NCBI GenBank® Nucleotide Sequence Database (accession number GU183144).

Table 1 Some characteristics of *Bacillus* sp. MSh-1

Characteristics	Result
Catalase production	+
Oxidase activity	−
The Voges–Proskauer test	−
pH in the Voges–Proskauer broth <6	+
Methyl Red test	+
Acid from	
D-glucose	+
L-arabinose	−
D-xylose	−
D-mannitol	−
Hydrolysis of	
Casein	+
Gelatin	+
Starch	+
Utilization of citrate	−
Nitrate reduced to nitrite	+
Formation of	
Indole	−
Dihydroxyacetone	−
Growth in NaCl	
2%	+
5%	+
7%	−
10%	−
Growth at	
10°C	−
30°C	+
40°C	+
50°C	−

Se^{4+} ions reduction to Se NPs

The spectrophotometric measurement of the Se^{4+} ions showed good linearity between the absorbance obtained at 480 nm and serial Se^{4+} concentrations (1–100 mg · l^{-1}). Determination of the MIC_{99} value showed that the *Bacillus* sp. MSh-1 does not grow at SeO_2 concentrations above 3.16 mM (Se^{4+}, 250 mg · l^{-1}). Therefore sub-MIC_{99} concentrations were used for Se^{4+} ion reduction

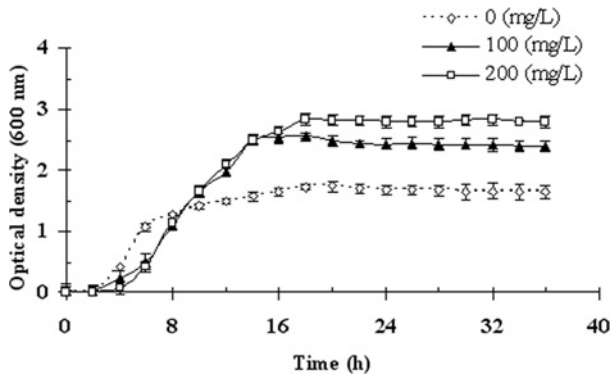

Figure 1 Growth profile of *Bacillus* sp. MSh-1 in the presence of Se ions

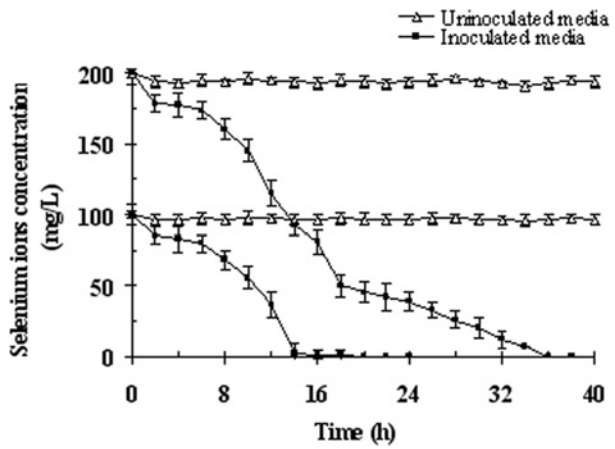

Figure 2 Reduction pattern of Se ions in either inoculated or uninoculated culture media, determined by an indirect spectrophotometric method

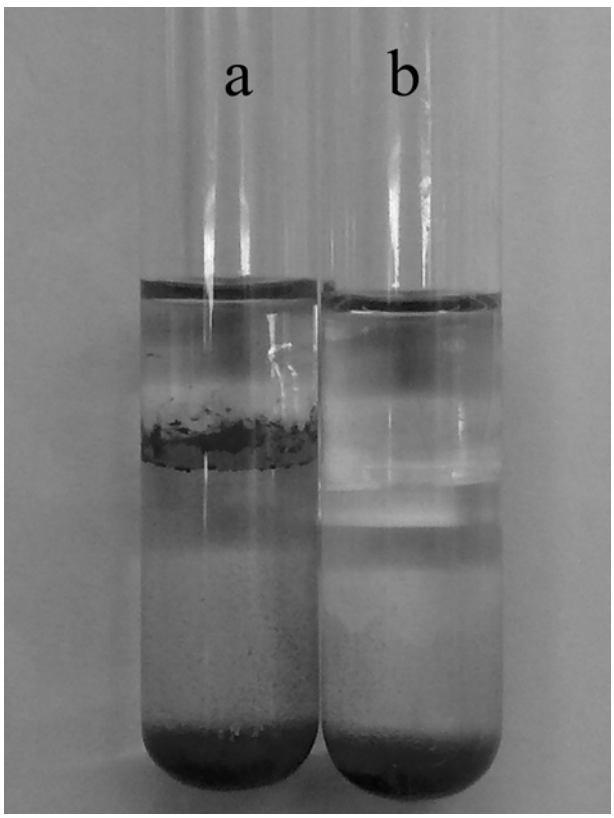

Figure 3 Isolation of Se NPs after (a) one and (b) two extractions by using the liquid–liquid two-phase system

experiments. Figure 1 shows the effect of the sub-inhibitory concentrations of Se^{4+} (100 and 200 mg · l^{-1}) on the growth of *Bacillus* sp. MSh-1. The time course study of the Se^{4+} ion reduction by the isolate revealed that the reduction process took place with different profiles in the presence of different concentrations of Se^{4+} ions (100 and 200 mg · l^{-1}). The selenium ions were completely reduced in the Nutrient broth culture supplemented with 100 mg · l^{-1}; we could not detect Se^{4+} in either culture medium after 14 h. In contrast, in the culture broth containing 200 mg · L^{-1} of Se^{4+}, total reduction of ions was observed after 36 h (Figure 2). No reduction of Se^{4+} was detected in the uninoculated Nutrient broth (Figure 2) and reduction was not observed for the 24 h culture supernatant of *Bacillus* sp. MSh-1 at the mentioned incubation time (results not shown).

Se NPs preparation and characterization

Figures 3(a) and 3(b) show the Se NPs at the bottom of the test tubes. These nanoparticles were separated from the cell debris during extraction by n-octyl alcohol. Cell debris materials got dissolved in the n-octyl alcohol/water system or remained at the interface of the aqueous and alcoholic phases in the first run of the solvent extraction (Figure 3a). But after the solvent was extracted twice, there was no visible cell debris between the two liquid phases (Figure 3b). The UV–visible spectrum of the NPs is shown in Figure 4. This spectrum is attributed to the Se NPs [9]. Figures 5(a) and 5(b) are the corresponding size distributions of Se NPs measured by laser light scattering before and after partitioning by the liquid–liquid two-phase system respectively. Thus, for purified Se NPs, one modal peak was clearly revealed in the range between 142 and 255 nm, and NPs 220 nm in size had the most frequency. The ζ potential value for Se NPs measured by a Zetasizer was −16.3 mV.

According to Figure 6, which shows the TEM image, Se NPs have a spherical shape. Size distribution measured from manual counting of 200 individual particles from different TEM images showed that most of the Se NPs were in the range between 80 and 220 nm. The SEM image of the purified Se NPs showed that the spherical particles created some aggregates of various lengths, and

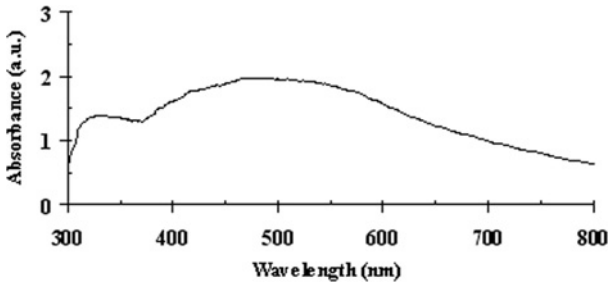

Figure 4 UV–visible spectrum of the biogenic Se NPs synthesized by using *Bacillus* sp. MSh-1 isolated from the Caspian Sea

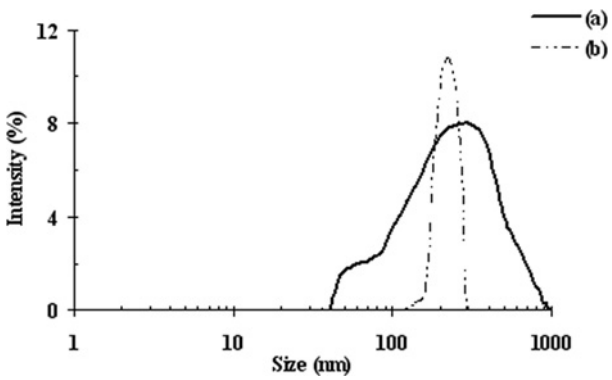

Figure 5 Size distribution patterns of particles (a) before and (b) after partitioning by using the liquid–liquid two-phase system (n-octyl alcohol/water)

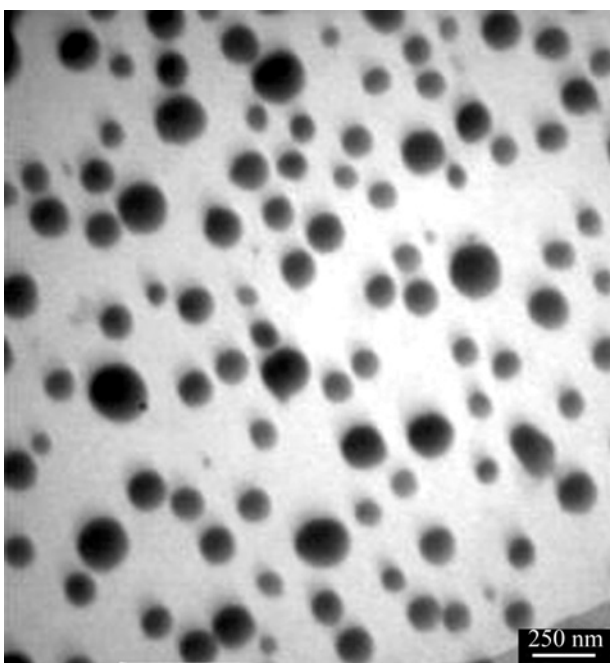

Figure 6 Transmission electron micrograph of Se NPs that were synthesized by using *Bacillus* sp. MSh-1 and purified using the n-octyl alcohol/water extraction system

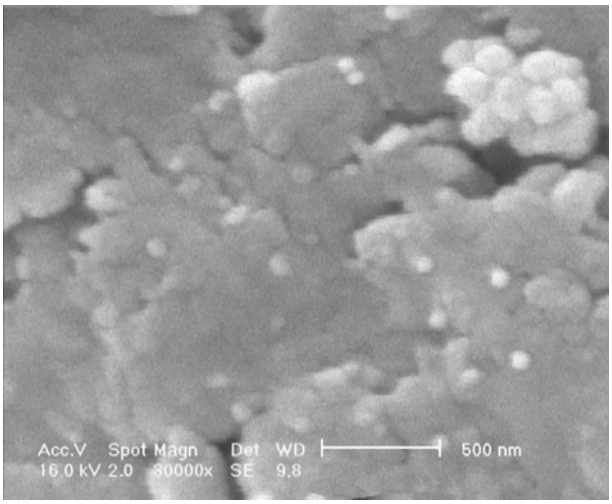

Figure 7 SEM image of purified Se NPs

single spheres were occasionally seen (Figure 7). EDX microanalysis of the purified NPs exhibited Se absorption peaks consisting of SeLα, SeKα and SeKβ at 1.37, 11.22 and 12.49 keV respectively (Figure 8). Elemental composition analysis showed the presence of strong signals from the Se atoms with wt% equal to 100 without other atom signals. The XRD pattern of Se NPs showed the presence of broad peaks without any clear lattice parameters (results not shown).

Cytotoxicity and MMP-2 activity

The cytotoxicity of the NPs prepared by *Bacillus* sp. MSh-1 was evaluated *in vitro* against the fibrosarcoma cell line (HT-1080) at four doses: 10, 20, 50 and 100 $\mu g \cdot ml^{-1}$. Our cytotoxicity analysis showed a direct dose–response result with the Se NPs; the higher the concentration, the higher the toxicity (Figure 9). The presence of 100 $\mu g \cdot ml^{-1}$ of NPs decreased the viability percentage of the cell line to 50%, whereas lower dose levels (10 $\mu g \cdot ml^{-1}$) showed a small amount of cytotoxicity with a viability percentage of more than 80%. As a final point, the anti-invasive property of the Se NPs was examined at the four doses of 10, 20, 50 and 100 $\mu g \cdot ml^{-1}$ (Figure 9). The inhibitory effect of the Se NPs on the invasion of HT-1080 cells is presented in Figure 9. As shown, the invasion of cells was significantly inhibited at concentrations >20 $\mu g \cdot ml^{-1}$.

Discussion

A *Bacillus* sp. designated as MSh-1 was isolated from the Caspian Sea. The ability of this isolate to form Se NPs was investigated. The detoxification potential of different strains of the *Bacillaceae* family for Se oxyanions has previously

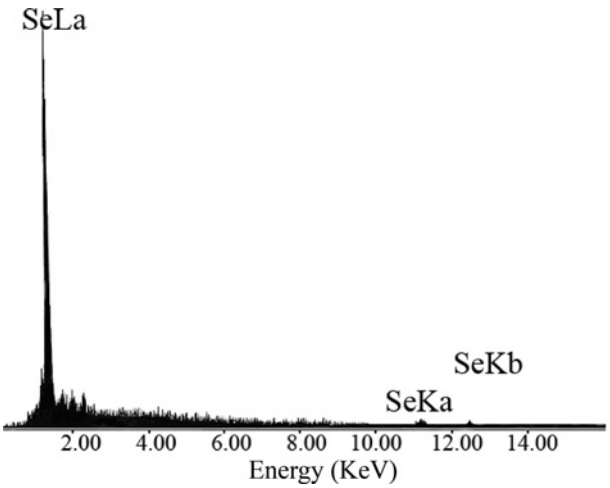

Figure 8 Energy dispersive X-ray spectrum of Se NPs that were extracted from *Bacillus* sp. MSh-1 and purified by using the n-octyl alcohol/water extraction system

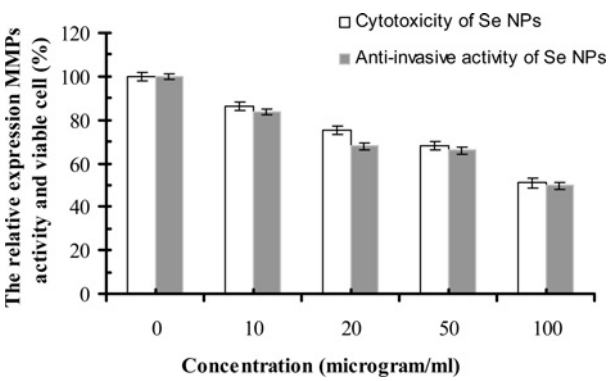

Figure 9 Cytotoxicity effects and MMP-2-inhibitory effects of purified Se NPs on the fibrosarcoma cell line (HT-1080)

been reported [16,28]. Kashiwa et al. [29] reported that the *Bacillus* sp. SF-1 reduces the selenium oxyanions to an elemental state by Se respiration and the Se particles are accumulated inside and outside the bacterial cells. The researchers used the *Bacillus* sp. SF-1 to remove the high concentrations of selenate from industrial wastewater but did not pay attention to the extract and used biogenic Se NPs [29]. We have recently reported the biosynthesis of Se NPs from selenium chloride by using *Klebsiella pneumonia* from the Enterobacteria group [30]. Nevertheless, the Se ions were not completely reduced by this bacterium during the 48 h incubation period. In our experience, *K. pneumonia* cannot reduce all of the Se ions (100 mg $\cdot$ l^{-1}) to metalloid form in the best optimized conditions [30]. Increasing the absorbance of two inoculated Se^{4+}-supplemented culture media compared with inoculated plain culture broth (without Se^{4+}) contributed to red Se NP accumulation in the cell biomass (Figure 1). Our present study shows that the Se^{4+} bioreduction rate is reduced by increasing the initial concentration of Se^{4+} in the culture broth (Figure 2). Thus it seems that the closer to the minimum inhibitory concentration, the lower the biosynthesis rate of NPs.

One of the most essential steps in the microbial synthesis of NPs is recovery of bionanoparticles. It has been proved that biphasic systems have great potential for efficient partitioning of bioproducts and NPs [31,32]. Based on the lipophilic nature of the cell debris and insolubility of Se NPs in the aqueous phase, we used an organic–aqueous partitioning system. Some of the Se NPs were tightly bound to cell debris and remained between two phase (Figures 3a and 3b). In addition, according to the EDX results (Figure 8), the NPs just composed the Se atoms. Figures 5(a) and 5(b) clearly show that the n-octyl alcohol water partitioning system can be successfully used for removing different soluble or insoluble impurities from Se NPs.

A broad absorption band in the UV–visible spectrum was observed for the extracted Se NPs (Figure 4). Yang et al. [9] reported that the expansion of the surface plasmon absorption band in the UV–visible spectrum is a sign of aggregations between Se NPs [9]. Differences between the size distribution pattern measured by the laser light scattering technique and the TEM images are due to some NP aggregation, which has been discussed as the reason for broadening of the UV–visible spectrum [9]. The Se NPs prepared by other synthetic methods were smaller than our biogenic NPs [9,33]. In future studies, it might be possible to achieve more size uniformity by optimization in *Bacillus* sp. MSh-1 culture conditions.

The apoptosis induction activity of Se NPs has been recently reported, but there is no report to show the anti-invasive effect of Se NPs against cancerous cell lines [34]. Yoon et al. [20] reported that low concentrations of selenite ($\leqslant$0.38 $\mu g \cdot ml^{-1}$) can stimulate cell proliferation of HT-1080 cells and a further increase in the concentration of selenite decreased cell viability. We found that Se NPs did not show an increasing effect on the proliferation of HT-1080 cells at the concentrations of 10, 20, 50 and 100 $\mu g \cdot ml^{-1}$. However, the observed cytotoxicity for biogenic Se NPs at the mentioned concentrations was lower than that of selenite and selenate for HT-1080 cells [20]. In addition, based on several *in vivo* studies, the lower toxicity of Se NPs has previously been established [11,35].

MMP-2 was also assessed in the present study. Some studies found that MMP-2 was overexpressed in tumour cells such as colorectal adenocarcinomas and weakly expressed in normal colon mucosa [36]. As a result, inhibition of MMP expression can be valuable in preventing tumour metastasis. Treatment of human trabecular meshwork cells with methyl

seleninic acid (1–10 μM) causes a dose-dependent decline in the secretion of MMP-2 [37]. Additionally, treatment of HT-1080 cells with selenite (Se^{4+}) caused a decrease in MMP-2 expression [20]. Our results showed that the Se NPs have a low inhibitory effect on MMP-2 expression (Figure 9). In addition, selenate ions (Se^{6+}) have been reported to show no potent inhibitory effect on the MMP-2 expression [20]. At present, the reason for this difference is not known and merits further investigation. It is possible that the oxidative states of selenium compounds play an important role in its inhibitory effect on MMP-2 expression by fibrosarcoma cells.

Conclusion

We screened and employed *Bacillus* sp. MSh-1 as a cost-effective and green synthetic method for the biosynthesis of Se NPs. After purifying and characterizing the NPs, we investigated the effect of Se NP MMP-2 expression. Comparing our results with other published reports suggests that biogenic Se NPs have lower toxicity than other oxidative states of Se. In addition, the Se NPs show a moderately inhibitory effect on the expression of the MMP-2 in the human fibrosarcoma cell line (HT-1080) at the higher concentration tested.

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