

Original Research

Isolation and characterization of a fungus for extracellular synthesis of small selenium nanoparticles

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Abstract

Objective(s): The use of biogenic selenium nanoparticles for various purposes is going to be an issue of considerable importance; thus, appropriate simple methods should be developed and tested for the synthesis and recovery of these nanoparticles.

Materials and Methods: In this study, a fungus was isolated from a soil sample, identified as *Aspergillus terreus* and used for extracellular synthesis of selenium nanoparticles (Se NPs). UV–Vis spectroscopy and energy dispersive X-ray spectrum studies were carried out to confirm Se NPs formation within 60 min. Dynamic light scattering and scan electron microscopic methods were also used to characterize both size and shapes of the Se NPs.

Results: The results show that spherical particles with average size of 47 nm were formed by adding a culture supernatant of *A. terreus* to selenium ions solution.

Conclusion: This approach appears to be an easy and appropriate method for extracellular synthesis of small Se NPs. Extracellular synthesis of small Se NPs has not been reported yet.

Keywords: *Aspergillus terreus*, Extracellular, Selenium nanoparticle, Soil, Synthesis

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Introduction

Metal nanoparticles (MNPs) have found many applications in medicine, electronics and other industries (1-3). During past decades, many reports have been published on the preparation of different MNPs by biological and chemical processes (4-8). Among these methods, there is an increasing interest to use biological techniques to produce different MNPs (6, 9, 10). Biogenic MNPs show frequently lower toxicity and exhibit better stability comparing to those nanoparticles (NPs) which fabricated by chemical methods (11). Different microorganisms can reduce toxic metal ions to elemental NPs (5, 12, 13). These toxic metal ions are often absorbed into the cells, reduced and deposited in intracellular space of microorganisms (4). Isolation of these NPs needs to release of NPs from the cells and set up an appropriate extraction method to purify them for further applications (14). During extraction method, liquid nitrogen is needed for disruption of the cells and some organic solvents (i.e. n-octanol, chloroform, ether) should be used for purification of generated MNPs from cell debris (5, 14). These mentioned processes are time consuming and may be tedious. Few papers have been recently published which report the syntheses of MNPs using cell-free supernatants of many bacterial or fungal cultures (15, 16). In this strategy the reduction of metal ions take place in the presence of the metabolites secreted in culture broth by living microorganisms. So no expensive treatments such as cell disruption method or solvent extraction are required to isolate the generated NPs (15, 17). In current investigation, different soil samples were collected from the around of Tehran, Iran and screened for finding a fungus to convert selenium (Se) ions to selenium nanoparticles (Se NPs). Se NPs have been exhibited different biological activities in cell culture or animal models which make this NPs candidate for different therapeutic purposes (18-21). In the basis of our literature survey, the extracellular synthesis of Se NPs by cell-free culture broth has not been investigated so far and this is the first report to describe the

synthesis of Se NPs using culture supernatant of a fungus strain isolated from a soil sample.

Materials and Methods

Screening procedure

Different soil samples were collected from different parts of the around of Tehran (Iran). All samples were milled, suspended in sterile normal saline and subjected to screening process. Sabouraud dextrose agar (SDA) culture medium was prepared, sterilized and supplemented with solution of SeO_2 (10 g/l) to final concentration equal to 100 mg/l SeO_2 . 1 ml of above suspensions which prepared from the collected soil samples was 10 times diluted and spread on the surface of the Se supplemented SDA plates (100 mg/l). The plates were incubated aerobically at 30°C, and after one week, all red-colored colonies which observed on the Se supplemented SDA plates, were picked up and transferred to Se free SDB medium. In next step, all SDB media were further incubated for 10 days at 30°C in an incubator (150 rpm). During incubation periods several samples (0.5 ml) were aseptically withdrawn from culture flasks, centrifuged at 4000×g for 20 min and added to 4.5 ml Se^{+4} ions solution (100 mg/l). After 60 min, the formation of red colloid was checked in all reaction vessels. Red is the color of generated elemental Se NPs and thus serves as a provisional marker to identify a culture supernatant of an isolate is capable to form Se NPs or not.

Identification of the isolate

The identification of the isolate was carried out by 28s ribosomal deoxyribonucleic acid (rDNA) sequence analysis (22). To earn genomic DNA, fungal cells harvested from 72 hrs culture mediums, washed three times with sterile distilled water. The cells were disrupted by grinding with liquid nitrogen and the slurry was subjected to phenol-chloroform DNA extraction procedure. An equal volume of TE-saturated phenol: chloroform (1:1) added to the sample contained in a 1.5 ml micro tube and vortex for 20 seconds. The sample was centrifuged for 5 minutes at room temperature

to separate the phases. The upper, aqueous layer was passed to a new tube. DNA materials were precipitated by adding cold absolute ethanol. The sample centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant decanted, and drained by inverting on a paper towel. For more purification, ethanol (80%v/v) added (corresponding to about two volume of the original sample), incubated at room temperature for 5-10 minutes and centrifuged again for 5 minutes, and decanted and drained the tube, as above. The DNA materials was re-suspended in diethyl-pyrocyanate (DEPC) solution in water bath (60°C) and subjected to 28s rDNA polymerase chain reaction (PCR) amplification.

The PCR amplification included an initial denaturation at 94°C for 180 sec, 30 cycles of denaturation 94°C for 60 sec, annealing 52°C for 45 sec and extension 72°C for 90 sec and a final extension 72°C for 300 sec. A fragment of the 28s rDNA gene was amplified using forward primer 5'-AAGCATATCAATAAGC-GGAGG-3' AND reverse primer 5'-GGTCC-GTGTTC AAGACGG-3'. The amplified DNA fragment was purified from agarose 1% gel using the QIAquick Gel Extraction Kit (Qiagen, USA) according to the supplier's instructions and was sent for auto-mated sequencing using the above primers (Cinagen Co., Iran). Sequence similarity searches were done with the BLAST database (National Center for Biotechnology Information), and the sequence was submitted to NCBI GenBank Nucleotide Sequence Data-base (accession number KC145152).

Preparation of Se NPs and their characterization

Fungus isolate from the soil sample was used for extracellular synthesis of the Se NPs. Sterile Sabouraud dextrose broth (SDB) medium was prepared, and 100 ml of this medium was transferred to a sterile 500-mL Erlenmeyer flask. The medium was inoculated with 1 ml of the fresh inoculums (OD600, 0.1) and incubated aerobically at 30°C in a shaker incubator (150 rpm). After 7 days, the fungal cells were removed from the culture medium

by centrifugation at 4,000 ×g for 10 min. The supernatant was collected and filtered through a 0.22 μm filter (Millipore, USA). Subsequently 20 ml of filtered supernatants was added to 80 ml of Se⁴⁺ ions solution (100 mg/ml) and reaction mixture was incubated at room temperature for 60 min to complete the Se NPs formation. The isolation of Se NPs from culture supernatant was carried out by high speed centrifuging process (20000g, 10 min). In next step, the red sediments was dispersed in distilled water, centrifuged at above mentioned condition and washed for three times. The collected Se NPs were re-suspended in distilled water to form a colloid and characterized using different techniques. The Se colloid tested for their optical absorption property by using a UV-Vis spectrophotometer (UVD-2950, Labomed, USA) operated at a resolution of 1 nm. The particle size distribution patterns of generated NPs were also obtained by the Zetasizer MS2000 (Malvern Instruments, UK). The morphology of the prepared Se NPs was studied using Scanning Electron Microscopy (SEM). For this purpose, above Se colloid was further dispersed by ultrasonication method, and a drop of suspension was located on carbon-coated SEM grid. Subsequently grid was dried under an infrared lamp and further coated by gold. Micrographs were achieved using a SEM (vega tescan, Czech) operated at an accelerating voltage at 15 kV. To determine the elemental composition of the NPs, energy dispersive X-ray spectrum (EDX) micro-analysis (vega tescan, Czech) was also performed.

Results and Discussion

Among more than 50 fungus strains which were isolated from soil samples and cultivated in SDB media only the culture supernatant of a fungus isolate could produce exo-metabolites to convert Se⁺⁴ ions to Se NPs. BLAST search of the amplified 28S rDNA sequence against the NCBI Nucleotide database was conducted to identify isolate. Alignment results show some characters which had identity with several members of the genus *Aspergillus*

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terreus. The isolate was designated as *A. terreus* BZ1 and its 28S rDNA sequence was submitted to the NCBI nucleotide database and registered with accession number of KC145152.

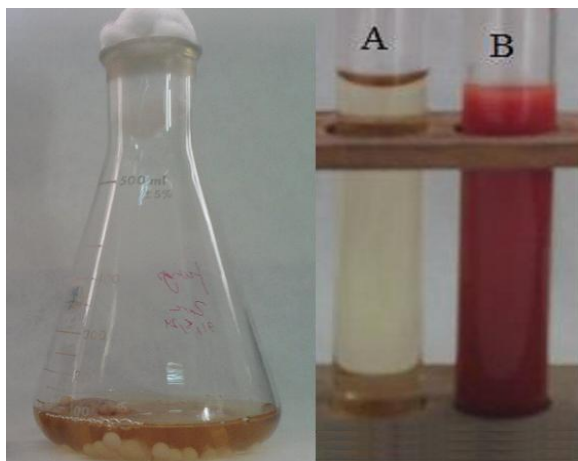


Figure 1. Left illustration shows a flask contains normal culture of *A. terreus* BZ1 in the plain SDB. Right illustration demonstrates test tubes contain Se^{4+} ions before (a) and after (b) addition of culture supernatant of *A. terreus* BZ1.

As mentioned in materials and methods part, this isolate was cultivated in liquid media (SDB) and its filtered supernatant was used for preparation of Se NPs. Left illustration in Figure 1 shows normal culture broth of *A. terreus* incubated for 1 week. Collected supernatant of this isolate has been demonstrated in right illustration of Figure 1 before (tube A) and after addition to Se ions solution (tube B). The UV-visible spectrum of the prepared Se NPs is shown in Figure 2. A band observed in the spectrum, corresponding to surface plasmon resonance and occurred at 245 nm, indicating the formation of Se NPs (23). Figure 3 shows the corresponding size distribution of Se NPs measured by laser light scattering method. For separated Se NPs, one modal peak was clearly revealed in the range between 18 and 105 nm, and NPs in the size of 47.5 nm had the most frequency. The zeta potential value for fabricated Se NPs was also measured by Zetasizer and reported as -22.9 mV. According to SEM image demonstrated in Figure 4, the prepared Se NPs show spherical shapes with particle size of ≤ 100 nm.

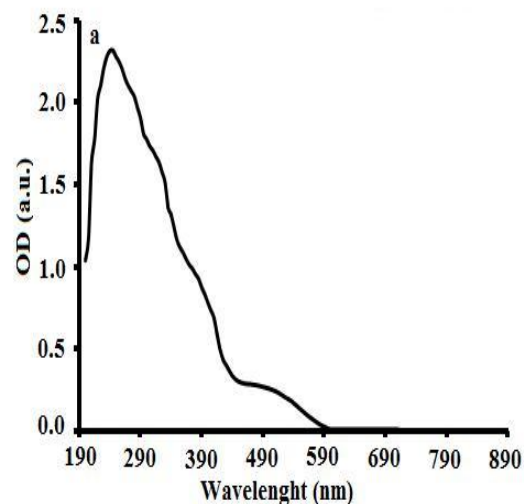


Figure 2. UV-visible spectrum of the Se NPs synthesized by culture supernatant of *A. terreus* BZ1.

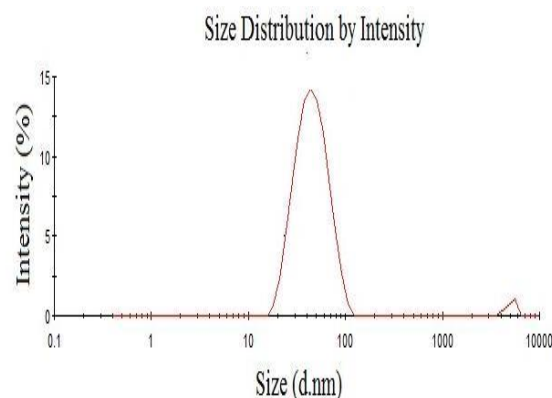


Figure 3. Size distribution pattern of Se NPs synthesized by culture supernatant of *A. terreus* BZ1.

Furthermore, EDX microanalysis of the separated NPs exhibited Se absorption peaks consisting of $\text{SeL}\alpha$, $\text{SeK}\alpha$ and $\text{SeK}\beta$ at 1.37, 11.22 and 12.49 keV, respectively (Figure 5) and confirms the presence of Se in the samples.

Elemental composition analysis also showed the presence of signals from the gold which are related to the gold which has been used in grid coating process MNPs such as Se NPs and silver NPs have found much attention in different branches of science and technology (1, 24, 25). Both of these NPs are important elements in healthcare issue (19, 26). Zhang and his co-workers showed that Se NPs has a size-dependent effect in directly scavenging free radicals *in vitro* (20).

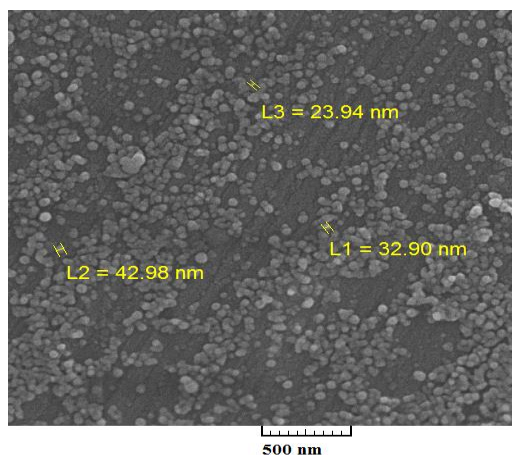


Figure 4. SEM image of the Se NPs synthesized by culture supernatant of *A. terreus* BZ1.

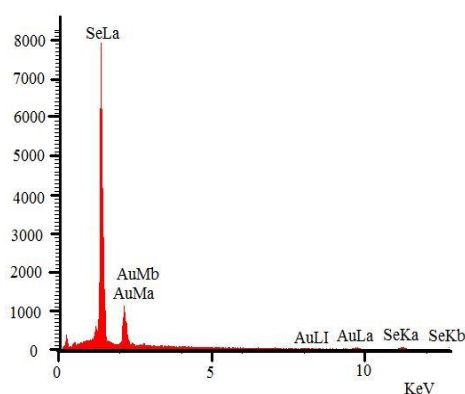


Figure 5. EDX spectrum of the Se NPs synthesized by culture supernatant of *A. terreus* BZ1.

Nano-Se has comparable efficacy in up-regulating selenoenzymes but is less toxic compared to selenite (20, 27). Nano-elements such as Se NPs can be produced intracellular or extracellular by different microorganisms. Although the intracellular biosynthesis of Se NPs using different bacteria and their isolation and purification have been recently reported in literature and well described [28-30] but there is no report on the synthesis of Se NPs using cell-free culture supernatant obtained from a fungus strain. In contrast, the syntheses of Ag NP using culture supernatants of some bacterial or some plant extracts have been published in literature (16, 31). To obtain a culture supernatant for extra-cellular biosynthesis of Se NPs, in this research, we screened many soil samples and isolated a fungus which identified as *A. terreus*. Culture

supernatant of this fungus convert Se^{4+} ion to red Se NPs with spherical shape and mean size diameter of 47 nm. The size of Se NPs which have been previously prepared in intracellular spaces of *Bacillus Sp.* were greater than 100 nm so other alternative microorganisms should be explored for fabrication of Se NPs with smaller size (30). This study is the first report showing the extracellular biosynthesis of small Se NPs using culture supernatants of *A. terreus*.

Conclusion

Recently there is an increasing interest to prepare Se NPs for different medical and industrial purposes. The synthesis of Se NPs using a culture supernatant of *A. terreus* appears to be simple and an appropriate method for synthesis of Se NPs with particle size less than 100 nm. Also this approach would be suitable for developing a biotechnological process for large-scale production of small Se NPs.

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