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Biosynthesis and Characterization of Selenium Nanoparticles Produced by Terrestrial Actinomycete *Streptomyces microflavus* Strain FSHJ31.

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Research Article

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ABSTRACT

During last decades, study on the development of eco-friendly processes for the production of selenium nanoparticles (Se NPs) have received much attention due to hazardous effects of chemical compounds used for nanoparticle preparation. The present study was designed to screen actinomycete strains able to produce Se NPs. Among isolated bacterial strains, a terrestrial actinomycete strain which was tolerant to Se^{4+} ions (200 µg/ml of) was launched as Se NPs producer. Morphological and biochemical characteristics as well as 16S rDNA gene analysis of the selected strain introduced it as *Streptomyces microflavus* strain FSHJ31. The biologically synthesized Se NPs was then purified using *n*-Octyl alcohol/water extraction system and characterized by UV-vis spectroscopy, transmission electron microscopy (TEM), energy dispersive X-ray (EDX) and Fourier transform infrared spectroscopy (FTIR) techniques. Analysis of the particle size distribution pattern of biogenic Se NPs via laser light scattering method demonstrated the size range of 28–123 nm for Se NPs with the 48 nm NPs being the most frequent particles.

INTRODUCTION

The remarkable physicochemical advantages of materials at nanoscales compared to that of bulk materials has influenced this field as one of the most intriguing trends over the last two decades [1,2]. Metal nanoparticles such as Fe_3O_4 magnetic nanoparticles (MNPs), silver and gold have been widely applied in diverse areas such as chemistry, physics, biomedicine and material sciences [3,4,5]. Some exclusive physical characteristics of selenium (Se), one of the most important semiconductor elements, such as thermo-conductivity, anisotropy and high photoconductivity have made Se a key element for industrial manufacturing of photocells and photographic exposure meters [6]. Furthermore, the presence of Se in the structure of seleno-enzyme and glutathione peroxidase which protect the lipids, lipoproteins, and DNA from oxidative damage in animal cells has introduced this metalloid as an essential trace element for biological systems [7,8,9]. However, the dose and chemical form of selenium derivatives play an important role both in their bioavailability and biological activities [4]. *In vivo* and *in vitro* studies

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showed that Se NPs not only represents lower toxicity but also exhibits excellent biological activity compared to selenite (SeO_3^{2-} or Se^{4+}) and selenate (SeO_4^{2-} or Se^{6+}) which are the most abundant forms of Se [10,11].

The time-consuming and costly nature of physicochemical techniques in addition to environmental hazardous effect of the organic solvents applied for nanostructures' synthesis have persuaded investigators to replace these methods with biological approaches [12]. Microorganisms (bacterial and fungal strains) [3,13], enzymes, and plants or plant extracts [14] are among the biological resources applied for the synthesis of Se NPs.

Actinomycetes comprise a diverse group of filamentous bacterial strains found in different habitats from high mountains to deep seas. Actinomycetes, and the *Streptomycetes* genus in particular, are shown to have an almost unlimited capacity for the production of secondary metabolites (such as antibiotics, antiviral and immunomodulators) with diverse chemical structures and biological activities as well as valuable enzymes [15]. The ability of actinomycetes for the biosynthesis of metal nanoparticles such as silver and gold has previously been reported [16,17]. However, the production of Se NPs using these valuable microorganisms has not been well documented. Given this, the current study attempted to screen terrestrial actinomycetes able to synthesize Se NPs. Purification and characterization of biologically synthesized Se NPs were also performed.

MATERIALS AND METHODS

Chemicals

The consumed Selenium dioxide (SeO_2), nutrient broth, tryptone and *n*-Octanol were available from Merck chemicals (Darmstadt, Germany). Calcium cloride and casein were provided by Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Screening for Se NPs-producer actinomycete strains

In order to isolate actinomycete strains with Se^{4+} reducing activity, thirty agricultural soil samples were collected from various locations in Kerman (57° 3' 36" N, 30° 17' 24" E), Iran and allowed to dry at room temperature. Twenty ml of 0.9% NaCl sterile solution containing Tween 80 (0.05%, v/v) was then added to each soil sample (1 g), followed by shaking the samples and passing them through filter paper (Whatman No. 1). Thereafter, each soil extract was diluted on a gradient, and 100 µl of each filtrate was spread on a casein glycerol agar (CGA) plate [containing (g/l) casein, 0.3; glycerol, 10; NaCl, 2; KNO_3, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01 and agar 18] supplemented with SeO_2 (1.26 mM). The plates were then incubated at 30 °C until the actinomycete colonies with Se^{4+} ions reducing activity appeared. Reduction of Se^{4+} ion into Se^0 (Se NPs) changes the color of reducing colony to red and thus serves as a provisional marker for isolation of targeted bacterial colonies. Positive bacterial strains (red colonies) were then picked up and repeatedly subcultured to obtain axenic culture. This was followed by culturing the selected isolates on CGA plates without SeO_2 to ascertain the absence of red pigment production (false positive) in the isolated bacterial strains. In order to select the most potent bacterial strain (able to tolerate higher concentration of SeO_2), a set of MIC (minimal inhibitory concentration) determination experiments were performed using the agar dilution method. Selected isolates were then conserved in a nutrient broth medium containing glycerol (15%) and maintained at –80 °C.

Identification of the isolate

Morphological and biochemical characteristics of the selected isolate were determined according to the Bergey's Manual of Determinative Bacteriology [18]. Molecular identification (16S rDNA gene sequencing) was also performed as follows. In order to obtain genomic DNA, the isolate was grown in Luria-Bertani (LB, tryptone, 10 g/l; yeast extract, 5 g/l; and NaCl, 10 g/l) medium at 30 °C and 150 rpm for 6 days followed by harvesting the produced biomass through centrifugation (10000 rpm for 5 min) and three times-washing with distilled water. The genomic DNA of actinomycete was then extracted using the phenol-chloroform extraction method [19]. A primer pair with 27F (5'-AGAGTTTGATCCTGGCTCAG-3') as the forward primer and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') as the reverse primer [20] was used to amplify a 1422 bp fragment of the 16S rDNA gene. PCR amplification was performed in a Primus 96 advanced thermal cycler (PEQLAB, Erlangen, Germany) programmed as follows: a) initial denaturation at 94 °C for 3 min; b) 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 45 sec and synthesis at 72 °C for 90 sec; and c) the final extension at 72 °C for 90 sec. To compare the sequence of amplified DNA with the sequences in GenBank, the basic local alignment tool known as BLAST was employed.

Purification of biologically synthesized Se NPs

Two plugs of freshly-cultivated actinomycete on CGA plate were transferred into a 500-ml Erlenmeyer flask containing 100 ml CG broth medium supplemented with sub-MIC concentration of Se^{4+} ions (100 µg/ml) and incubated at 30 °C and 150 rpm for 5 days. The produced biomass was then harvested via centrifugation (10000 rpm for 5 min) and washed three times with 0.9% NaCl solution. In the next step, actinomycete biomass was disrupted by grinding in a mortar and pestle after addition of some liquid nitrogen. The resulting slurry was

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ultrasonicated at 100 W for 5 min and washed three times by sequential centrifugation (10000 rpm, 5 min) with 1.5 M Tris/HCl buffer (pH 8.3) containing 1% sodium dodecyl sulphate (SDS) and deionized water, respectively. The pellets were then resuspended in deionized water, and the resulting suspension containing Se NPs and cell debris was collected. *n*-Octanol (2 ml) was consequently added to 4 ml of obtained suspensions and the mixtures were shaken vigorously. Later, the resultant mixed phases were separated by centrifugation at 5000 rpm for 5 min and stored at 4 °C for 24 h. Following 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 precipitated NPs were washed with chloroform, ethyl alcohol and distilled water, respectively. The purified NPs were then resuspended in deionized water and stored at 4 °C before being used for characterization.

Characterization of Se NPs

A Shimadzu UV-Vis Double Beam PC Scanning spectrophotometer (UV-1800, Shimadzu CO, USA) was used to record the UV-Visible spectrum of the purified Se NPs. Samples for examination by transmission electron microscopy (TEM) were prepared by placing one drop of formed Se NPs on carbon-coated copper TEM grids exposed for slow evaporation at room temperature. Transmission electron microscopy was performed using a Zeiss Supra 55 VP TEM (operated at 100 Kv) equipped with an EDX (energy dispersive X-ray) microanalyzer. The patterns of particle size distribution were determined using a Zetasizer MS2000 (Malvern Instruments Inc.). The FTIR spectrum of dried powder of Se NPs (in KBr pellet) was recorded by a Perkin Elmer instrument at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

Screening and identification of Se NPs-producer bacterial strains

Table 1: Biochemical characteristics of *Streptomyces microflavus* strain FSHJ31.

Characteristics	Results
Catalase production	+
Oxidase activity	-
Voges-Proskauer test	-
Methyl red test	-
Acid from	
D-Glucose	+
D-Fructose	-
D-Sucrose	-
D-Maltose	+
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	
5˚C	-
30˚C	++
40˚C	+
50˚C	-

Out of 45 actinomycete colonies obtained from soil samples, five colonies with ability to reduce Se^{4+} ions to Se^{0} (red color) were isolated. Determination of the MIC for mentioned isolates in the presence of SeO_2, introduced isolate J31 as the most potent strain able to tolerate high concentration of Se^{4+} ion (200 µg/ml). Cultivation of the selected strain in CGA plate (Figure 1a) and CG broth medium containing Se^{4+} ion (Figure 1d) turned the color of the culture media into red in a time-dependent manner. Lack of such color change in the culture media without SeO_2 (Figure 1b and Figure 1c) confirmed the ability of the selected strain to biosynthesize Se NPs.

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The J31 isolate was a Gram-positive and filamentous bacterial strain represented a smooth appearance but developed a weft of aerial mycelium which either appeared as floccose and powdery or a velvety colony. The results of biochemical characteristics are summarized in Table 1. Both morphological and biochemical properties of the selected isolate, candidate it as *Streptomycete* strain. Alignment of the amplified 16S rDNA gene sequence of J31 isolate against the present sequences of GenBank using BLAST tool represented 99% identity of the obtained gene to *Streptomyces microflavus*. The 1442 bp sequence was then submitted to GenBank under accession number of KC626004.

Figure 1: Cultivation of *S. microflavus* strain FSHJ31 on CG agar plates a) with and b) without SeO_2. Culture flasks of the selected isolate in c) absence and d) presence of Se^{4+} ions after 5 days incubation at 30 °C.

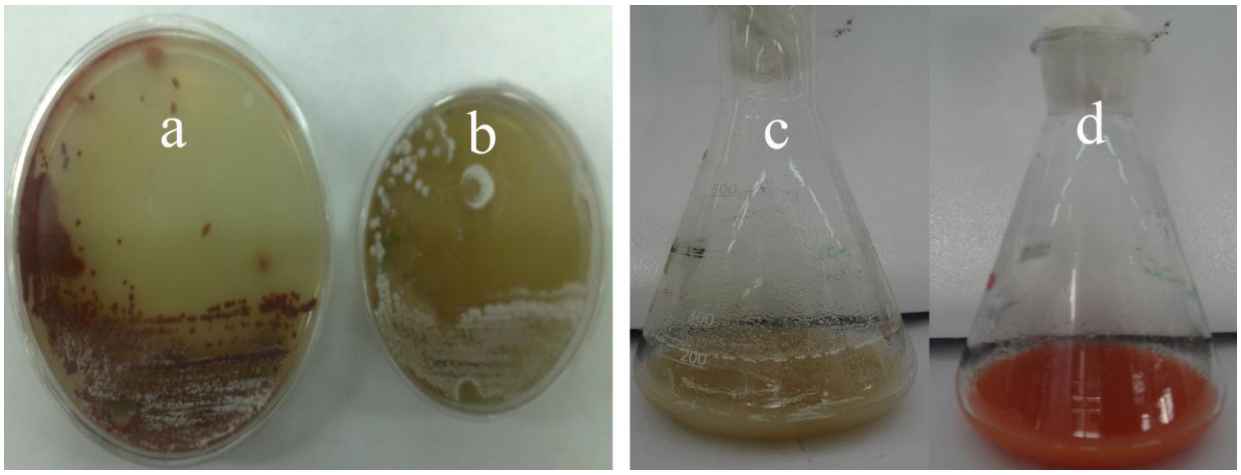

Figure 2: UV–visible spectrum of the biogenic Se NPs purified from *S. microflavus* strain FSHJ31.

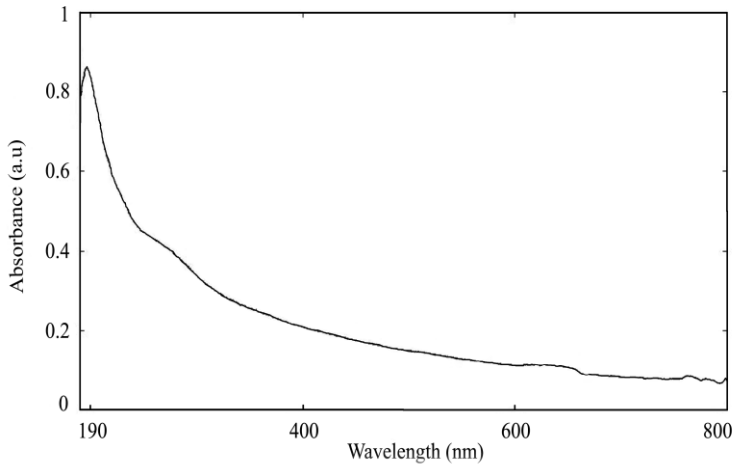

Figure 3: Transmission electron micrograph of the Se NPs synthesized by *S. microflavus* strain FSHJ31 and purified using the *n*-Octanol/water extraction system.

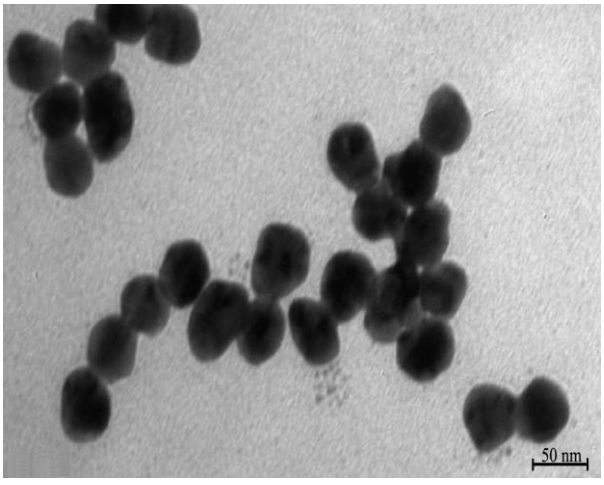

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Figure 4: Size distribution patterns of the biogenic Se NPs recovered from *S. microflavus* strain FSHJ31.

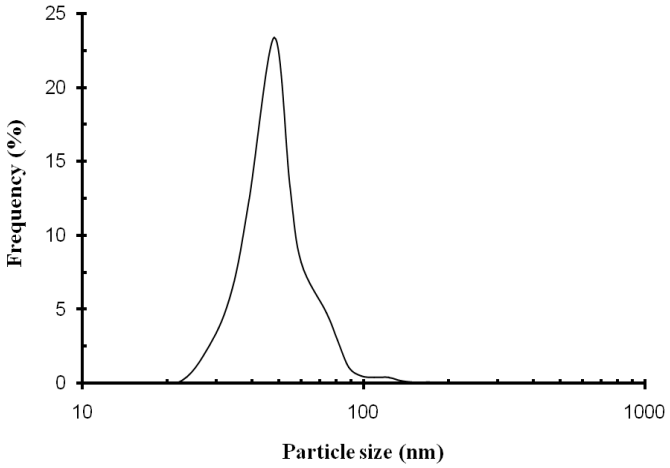


Figure 5: Energy dispersive X-ray spectrum of biogenic Se NPs produced by *S. microflavus* strain FSHJ31.

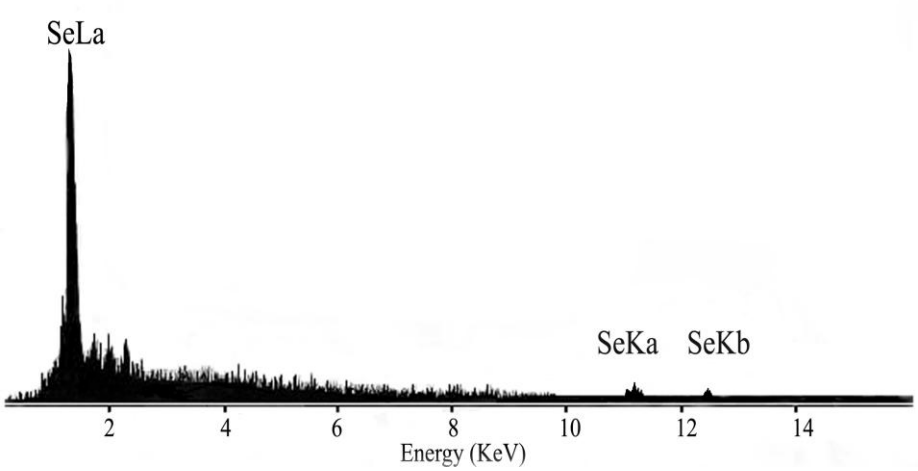
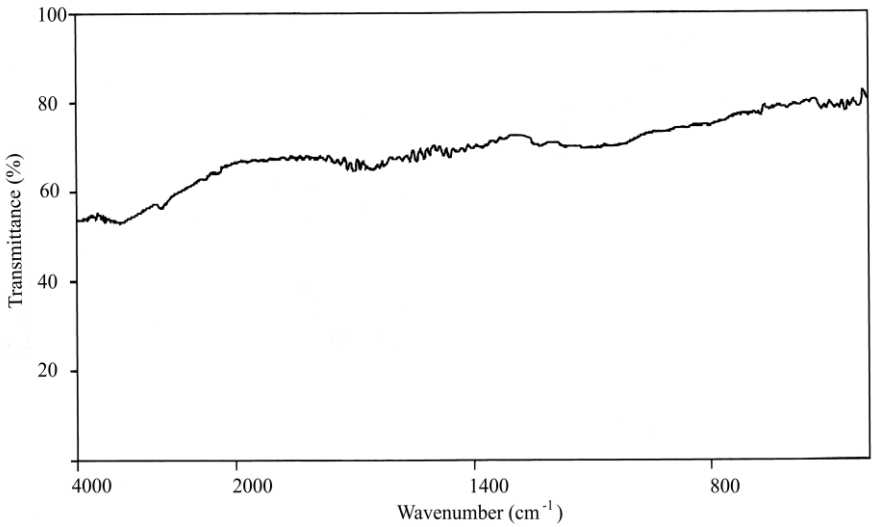


Figure 6: FTIR spectra of the purified biogenic Se NPs synthesized by terrestrial actinomycete *S. microflavus* strain FSHJ31.



The eco-friendliness of the bacterial strains- assisted biosynthesis of metal nanoparticles, as an alternative for physicochemical methods, has recently been discussed in the literature. For instance, Zhang et al. [6] isolated *Pseudomonas alcaliphila* able to synthesize spherical selenium particles with diameters range of 50 to 500 nm. A

halotolerant bacterial strain, *Bacillus megaterium*, which could tolerate 7% NaCl was isolated from Bhitarkanika mangrove soil. This Gram-positive bacterial strain efficiently reduced selenite (up to 0.25 mM) to Se NPs after 40 h of incubation [12]. Application of the culture supernatant of *Aspergillus terreus* towards SeO_2 (final concentration of 100 µg/ml) led to formation of Se NPs with average size of 47 nm [13]. The study carried out by Yazdi et al. [21] introduced *Lactobacillus plantarum* as a probiotic strain capable of producing Se NPs (with particle size less than 250 nm) after 72 h incubation.

Se NPs purification and characterization

The liquid-liquid extraction method applied in the present study could efficiently purify biogenic Se NPs from actinomycete biomass. The UV-vis spectrum of the NPs is illustrated in Figure 2. Same absorbance spectrum observed by Zhang et al. [6] applied *Pseudomonas alcaliphila* for the synthesis of Se NPs. Oremland et al. [22] reported that the Se NPs biosynthesized by Se-respiring bacteria such as *Selenihalanaerobacter shriftii,* exhibited the most unusual and broadest absorption spectrum at wavelengths greater than 600 nm. They reported that the biologically formed Se nano-spheres had very different spectral properties than the chemically formed Se NPs [22]. Yang et al. [23] who synthesized Se NPs by reduction of H_2SeO_3 using the UV-irradiated tungstosilicate acid solution ascribed the expansion of the surface plasmon absorption band in the UV-vis spectrum of Se NPs to the aggregations between nano particles.

As demonstrated in Figure 3 (the TEM image of the purified Se NPs), it is be clearly inferred that the well-dispersed and spherical shape Se NPs were biosynthesized by *S. microflavus*. The obtained results of particle size distribution pattern of the purified Se NPs (determined by light scattering method) are illustrated in Figure 4. A typical one modal peak was observed in the range between 28 nm and 123 nm, and NPs in the size of 48 nm were the most frequent particles (Figure 4). Broadening of the acquired peak in size distribution curve (Figure 4) in the present study might be attributed to NPs aggregation which is the probable reason for expansion of the UV-vis spectrum of biogenic Se NPs (Figure 1). In general, the Se NPs prepared by chemical methods are shown to be smaller than the biogenic Se NPs [23,24]. 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 5). Furthermore, the elemental composition analysis indicated the presence of strong signals from the Se atoms with a weight percent equal to 100 without signals of other elements. Thus, the *n*-Octanol/water partitioning system could be successfully applied to remove different soluble or insoluble impurities of biologically synthesized Se NPs.

FTIR spectrum of the biogenic Se NPs (Figure 6) did not show any typical and strong absorption band which confirmed the absence of functional groups on the surface of purified Se NPs. Generally, formation of the nanoparticles assisted by microorganisms led to insertion of some unknown compounds on the surface of nanoparticles which is evident from their FTIR spectra [2,3]. The obtained results of our previous study revealed that the Se NPs produced by *Bacillus* sp. MSh-1 represented different functional group such as hydroxyl and carbonyl group on their surface [3]. Same results were reported for other metalloid element such as tellurium NPs synthesized by *Bacillus* sp. BZ [25]. However, results of the present study revealed the absence of any functional group on the surface of biogenic Se NPs formed by *S. microflavus* strain FSHJ31. The reason of this observation was not clear and merits further investigatins.

CONCLUSION

A Se NPs-producing actinomycete was isolated from soil samples and identified as *S. microflavus* strain FSHJ31 based on 16S rDNA sequencing analysis. The biologically synthesized Se NPs were then purified and characterized. Further investigations need be conducted to optimize nanoparticles production.

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