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Extracellular biosynthesis of silver nanoparticles using cell filtrate of *Streptomyces* sp. ERI-3

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Abstract In this study, we present a biological method for synthesis of silver nanoparticles (AgNPs) using *Streptomyces* sp. ERI-3 cell-filtrate. AgNO₃ solution (1 mM) was added to the cell-free culture supernatant and the mixture was incubated at 28 °C for 48 h in the dark in an orbital shaker. The AgNPs were characterized using UV–visible spectroscopy, X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). The nanoparticles exhibited maximum absorbance at 430 nm in UV–Vis spectroscopy. The XRD spectrum exhibited 2 θ values corresponding to the silver nanocrystals. TEM and SEM micrographs revealed the extracellular formation of spherical nanoparticles in the size range of 10–100 nm. AgNPs formed flower-like self-assembled structures after three months incubating at room temperature in the dark. The study provides the evidence that the factors in the cell-free culture supernatant facilitate synthesis of AgNPs. This study is the first report on the biosynthesis of AgNPs using supernatant of *Streptomyces* sp. ERI-3 bacterium.

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1. Introduction

The synthesis of metal nanoparticles and nanostructure materials are attractive due to their unusual optical, chemical, photoelectrochemical and electronic properties [1]. This is particularly important for noble metals such as Au and Ag, which have strong surface plasmon resonance oscillations [2]. Nanocrystalline silver particles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnosis, antimicrobials and therapeutics, catalysis, sensors, micro-electronics and filters [3,4]. Physical methods, such as attrition and pyrolysis, were previously utilized for synthesis of metallic nanoparticles. Chemical methods of nanoparticle synthesis are the most widely and traditionally used [5,6].

Generally, the physical methods have low yields and the chemical methods cause contamination due to precursor chemicals, use of toxic solvents and the generation of hazardous by-products [7]. Hence, there is a great need to develop high yield, safe, reliable, clean and eco-friendly methods for the preparation of nanoparticles. Biosynthesis methods, employing microorganisms, have emerged as a simple, clean and viable alternative to chemical and physical methods. A vast array of biological resources available in nature, including bacteria, fungi, yeasts, algae and plants, can be used for the synthesis of nanoparticles. Prokaryotic bacteria have received the most attention in this area. One advantage of using bacteria for synthesis of nanoparticles is ease of handling and their genetic manipulation without much difficulty [8]. The studies have indicated that culture supernatants of some bacterial genera, like *Bacillus*, *Arthrobacter*, *Pseudomonas* and *Escherichia*, could induce the synthesis of silver nanoparticles [9–11]. Nevertheless, the number of microbial culture supernatants evaluated so far for their ability to induce nanoparticles is limited and needs wider study. The use of fungi in the synthesis of nanoparticles is a relatively recent addition to the list of microorganisms. The use of fungi is potentially exciting since they secrete large amounts of enzymes. However, the genetic manipulation of eukaryotic organisms is much more difficult than that of prokaryotes. The *Streptomyces* species, members of the bacterial order *Actinomycetales*, are found worldwide in soil. Already, biologists have classified this genus in the class of fungi. Later, based on

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molecular methods, it was found that the *Streptomyces* species belonged to the prokaryotic class and have some similar characteristics of fungi [12]. Therefore, the *Streptomyces* species will be a suitable candidate for producing nanoparticles. This report presents data on the generation of AgNPs using the culture supernatant of *Streptomyces* sp. ERI-3 for the first time.

2. Materials and methods

2.1. Materials

AgNO₃ was obtained from Sigma–Aldrich, USA. All other chemicals were purchased from Merck, Germany. Freshly prepared doubly distilled water was used throughout the experimental work.

2.2. Microorganism

The soil samples were collected from the Songon copper mine in north–west Iran in sterile falcon tubes. They were serially diluted and spread onto starch casein agar plates (starch 10 g, casein 0.3 g, CaCO₃ 0.02 g, FeSO₄ 0.01 g, K₂HPO₄ 2 g, KNO₃ 2 g, MgSO₄ 0.05 g, NaCl 2 g, agar 15 g in 1000 ml sterile distilled water, pH 7) to isolate the genus *Streptomyces* [13]. To minimize the fungal and bacterial growth, actidione (20 mg/l) and nystatin (100 mg/l) were added to the cultures. The plates were then incubated at 28 °C for 7 days.

2.3. Characterization of the isolate

The morphological and physiological characterization of the isolate was performed according to methods described in Bergey's manual of determinative bacteriology [14]. The cultures were maintained at 4 °C in starch casein agar plates by continuous sub-culturing every 14 days.

2.4. Biosynthesis of silver nanoparticles

Inocula were prepared by transferring 5 ml of 1 McFarland standard of bacterial suspension, prepared from a 7-day old starch casein agar culture, into 250-ml Erlenmeyer flasks containing 45 ml of a sterile MGYB broth medium (malt extract 3 g, glucose 10 g, yeast extract 3 g and peptone 5 g per in one liter of distilled water and pH 7) at 28 °C in an orbital shaker set at 200 rpm. After 48 h, when the culture OD at 600 nm was in the range of 1.5–2, the culture supernatant was used for the generation of nanoparticles. For this purpose, the culture was centrifuged at 6000 g and the cell pellet was recovered. The cell pellet was then washed thrice with 50 mM phosphate buffer (pH 7) by centrifugation at 5000 g. Then, the cell pellet was suspended in distilled water and incubated at 28 °C in an orbital shaker set at 200 rpm for an extra 48 h. Later, the cell-free supernatant was recovered by centrifugation at 7500 g. 50 ml of AgNO₃ solution (1 mM) was added to 10 ml of the cell-free culture supernatant in 500-ml Erlenmeyer flasks, which were incubated at 28 °C in an orbital shaker set at 200 rpm for 48 h in the dark. Formation of AgNPs was characterized using UV–visible spectroscopy, X-Ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and scanning microscopy (SEM).

2.5. Evaluation of maximum nanoparticle synthesis

Five samples for UV–vis spectroscopy were prepared as described in Section 2.4. The absorption spectra of the samples

were taken using a UV–vis spectrophotometer (Shimadzu, UV Pharma spec 1700 with a resolution of 0.72 nm) from 300 to 800 nm at different time intervals of incubation (12, 24, 36 and 48 h).

2.6. X-Ray Diffraction (XRD) measurement

XRD measurements of the dried powder of silver nanoparticles were done by a Phillips PW 1800 instrument.

2.7. TEM measurements

The morphology and size of AgNPs was studied by TEM. For this purpose, an aliquot of an aqueous suspension of AgNPs was transferred onto a carbon coated copper grid and allowed to be dried [15]. The grid was then scanned using a Phillips EM 208S transmission electron microscope operated at a voltage of 100 kV.

2.8. SEM measurements

For SEM measurements, the suspension from the maximum time-point of production of silver nanoparticles was air-dried and subjected to SEM, using a Phillips XL 3000 scanning electron microscope [15,16].

2.9. Molecular identification

The genomic DNA of the isolate was extracted by methods described in earlier reports [17]. The 16S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and primers 27f (51 AGT TTG ATC CTG GCT CAG 31) and 1492 (51 ACG GCT ACC TTG TTA CGA CTT 31). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for four minutes, followed by 30 cycles at 94 °C for one minute, primer annealing at 52 °C for one minute, and primer extension at 72 °C for one minute. At the end of the cycling, the reaction mixture was held at 72 °C for 10 min and then cooled to 4 °C. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystems, USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at <http://www.ncbi.nlm.nih.gov/>.

2.10. Statistical analysis

The experiments for each sample were performed 5 times and the final values were presented as the mean ± Standard Deviation (SD). The statistical software, SPSS.10 (one way ANOVA), was used to estimate the statistical parameters.

3. Results and discussion

3.1. Visible observation of silver nanoparticles biosynthesis

The pure colonies were obtained and characterized as *Streptomyces* sp. ERI-3 based on the microscopic, biochemical and molecular results [14,17]. Addition of 10 ml of the cell-free culture supernatant of *Streptomyces* sp. ERI-3, which was grown to an OD of 600 nm in the range of 1.5–2, to 50 ml of 1 mM aqueous silver nitrate, changed the colourless silver nitrate solution to a reddish brown colour within 12 h, characteristic of AgNPs (Figure 1).

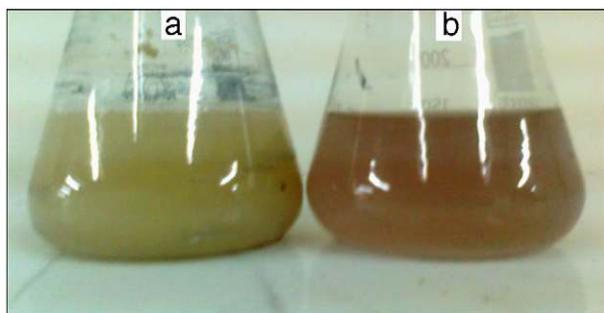


Figure 1: Visible observation of AgNPs biosynthesis. (a) ErlenMeyer flask with *Streptomyces* sp. ERI-3 cell-free supernatant and after exposure to AgNO_3 solution (1 mM) for a few minutes (no color change), and (b) ErlenMeyer flask with *Streptomyces* sp. ERI-3 cell-free supernatant and after exposure to AgNO_3 solution (1 mM) for 48 h (reddish-brown color).

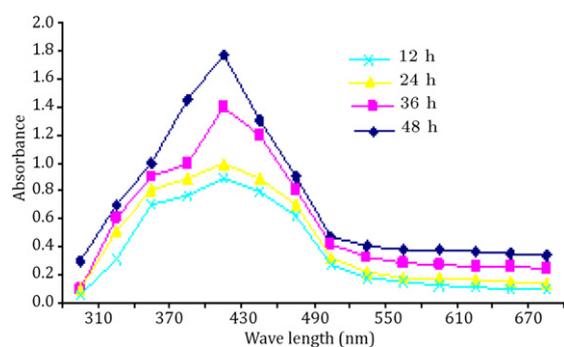


Figure 2: UV-visible absorption spectra of produced silver nanoparticles in 430 nm wavelength using culture supernatant of *Streptomyces* sp. ERI-3 at different incubation times.

3.2. Characterization of AgNPs by UV-visible spectroscopy

The UV-Vis spectroscopy indicated that the samples have a maximum absorption at 430 nm, attributable to the Surface Plasmon Resonance band (SPR) of silver nanoparticles [18]. Maximum absorbance at 430 nm increased with the time of incubation of the silver nitrate with the cell-free supernatant. The statistical analysis demonstrated a significant difference ($P = 0.0022$, one way ANOVA) in the production of silver nanoparticles. As illustrated by Figure 2, a high production of silver nanoparticles was achieved after 48 h of incubation.

3.3. Characterization of AgNPs by XRD

The XRD pattern of the silver nitrate-treated sample (Figure 3) corresponds to that of silver nanoparticles. The XRD pattern shows four intense peaks in the whole spectrum of 2θ values, ranging from 30 to 80. It is important to know the exact nature of the formed silver particles and this can be deduced from the XRD spectrum of the sample. XRD spectra of pure crystalline silver structures and pure silver nitrate have been published by the Joint Committee on Powder Diffraction Standards (file nos. 04-0783 and 84-0713). A comparison of our XRD spectrum with the standard sample confirmed that the silver nanoparticles had been formed in the form of nanocrystals, as was evidenced by the peaks at 2θ values of

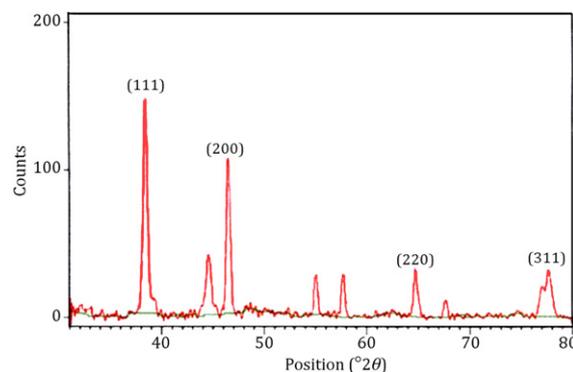


Figure 3: X-ray diffraction pattern of silver nanoparticles. Silver nanoparticles were synthesized from 1 mM silver nitrate-treated *Streptomyces* sp. ERI-3 cell-free supernatant at 28 °C. The samples were harvested, sonicated, air-dried and the XRD pattern was observed.

38.25°, 46.37°, 64.60° and 77.62° corresponding to 111, 200, 220 and 311 planes for silver, respectively.

3.4. Characterization of AgNPs by transmission electron microscopy and scanning electron microscopy

TEM and SEM are powerful methods to determine the morphology and size of nanostructures. TEM and SEM micrographs of the synthesized silver nanoparticles revealed the formation of spherical nanoparticles with a size range of 10–100 nm extracellularly (Figure 4).

The extracellular formation of AgNPs would be of great advantage to industry, since it would overcome steps involved in the purification of AgNPs.

Thus far, TEM micrographs of the following bacteria: *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Arthrobacter kerguelensis*, *Arthrobacter gangotriensis*, *Pseudomonas antarctica*, *Pseudomonas proteolytica* and *Escherichia coli* [9–11] have been shown to produce extracellular AgNPs. Therefore, the present study adds to this list a novel genus, which has never been tried earlier. Similar to other studies, in the present study, it was also observed that AgNPs formed in darkness [19,20], but it is still not known how darkness influences the formation of AgNPs. It has been suggested that DNA [21], sulfur-containing proteins [22] and NADH-dependent nitrate reductase [16,23] are involved in the synthesis of AgNPs by the bioreduction of silver ion to metallic silver. For example, in one of the biomimetic approaches towards the generation of nanocrystals of silver, the reduction of silver ions has been carried out using *Bacillus licheniformis*. The reduction mediated by the presence of the specific enzyme α -NADPH-dependent nitrate reductase in the microorganism has been found to be responsible for the synthesis. This enzyme is induced by nitrate ions and reduces silver ions to metallic silver [24]. In the present study, cell-free supernatant could induce the synthesis of silver nanoparticles. Thus, it is implied that the factors involved in the biosynthesis of AgNPs are present in the cell-free supernatant of *Streptomyces* sp. ERI-3. Characterization of the factors would be the focus of our future research. The representative TEM and SEM images of silver nanoparticle morphology and size distribution analysis are shown in Figure 4.

Also, Figure 5 shows the SEM micrograph of the synthesized silver nanoparticles after approximately three months maintained at room temperature. The self-assembly of nanoparticles with a formation of flower-like structures is clearly observed.

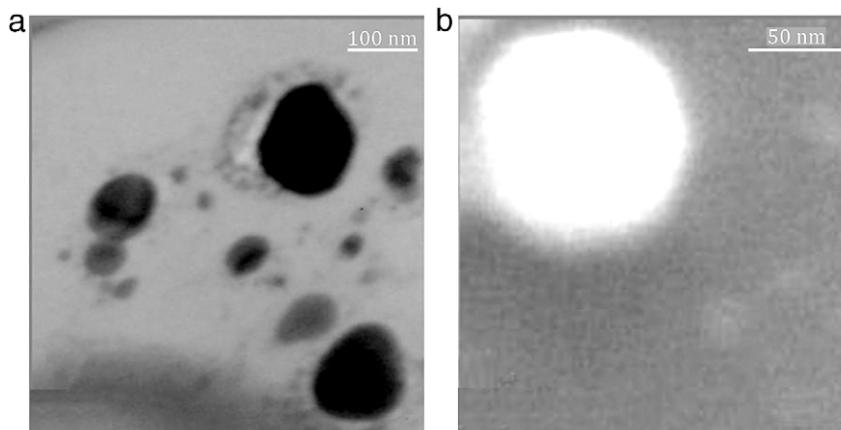


Figure 4: Electron microscopy images of produced silver nanoparticles using culture supernatant of *Streptomyces* sp. ERI-3 at 48 h of incubation. (a) TEM image of silver nanoparticles, and (b) SEM image of silver nanoparticles.

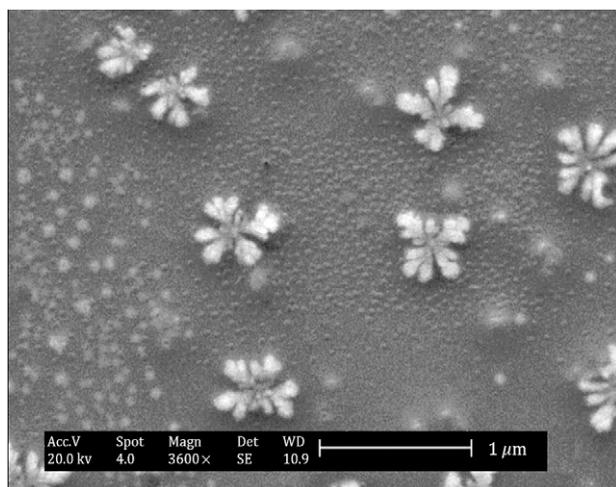


Figure 5: SEM image of self-assembly of produced silver nanoparticles by culture supernatant of *Streptomyces* sp. ERI-3.

It is unknown whether or not self-assembled structure formations depend on temperature, light or darkness; this must be investigated.

The directed-self-assembly of nanostructures into larger structures through nanoscale interaction is an important phenomenon in the synthesis of novel nano or microstructure materials [25,26]. However, determining possible steps and factors involved in the formation of such self-assembled structures requires further investigation.

4. Conclusions

This study indicated that the cell free supernatant of *Streptomyces* sp. ERI-3 started the synthesis of AgNPs after 12 h of incubation, but the maximum rate of synthesis was achieved after 48 h of incubation with formation of a characteristic peak at 430 nm at all incubation times. The silver nanoparticles exhibited a uniform morphology of a spherical nature with sizes of 10–100 nm. This report presents data for the first time on the ability of the cell-free culture supernatant of genus *Streptomyces* to convert silver nitrate to AgNPs and subsequent self-assembly.

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