Screening of soil fungi in order to biosynthesize AgNPs and evaluation of antibacterial and antibiofilm activities

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Abstract. The biosynthesis of nanoparticles (NPs) has recently attracted a lot of research attention due to its being an eco-friendly and economical method. NPs are formed under normal temperatures and pressures. The shape and size of NPs can be controlled by choosing a suitable pH and temperature. In this study, 24 strains of fungi isolated from desert soils were screened for AgNP synthesis. The MS17 isolated was chosen as the superior strain capable of rapidly synthesizing monodisperse AgNPs. The optimum conditions for AgNP synthesis were investigated. AgNPs were characterized by UV–visible spectrophotometry, dynamic light scattering, X-ray diffraction, transmission electron microscopy and Fourier-transform infrared. The NPs produced were found to be in the form of Ag/AgCl with a size range of 5–15 nm. Then, the NPs were capped by proteins and carbohydrates, which play an important role in NP stability. The NPs were capable of antimicrobial activities against the standard bacterial pathogens, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 1431 and the multidrug-resistant P. aeruginosa B52 and P. aeruginosa 48.

Keywords. Soil fungi; AgNPs; antimicrobial activity.

1. Introduction

Historically, silver has been widely used for several purposes, such as fabricating jewellery and food containers and also in medicine [1,2]. Silver nanoparticles (AgNPs) can be applied in antibacterial agents, biosensors, composite fibres, refrigerator superconductors, cosmetics and electronics [3]. There are three general ways for the synthesis of NPs, including physical, chemical and biological methods. While chemical methods use several toxic solvents and produce a degree of hazardous materials, physical methods are energy-consuming [4]. Biological approaches have been developed in response to the increasing request for the high-efficiency, low-cost, non-toxic and biocompatible construction of metal NPs. Biological resources, including plants, plant products, algae, fungi, yeasts, bacteria, actinomycetes and viruses, are capable of producing different types of NPs [5]. Today, fungi are regarded as a nano-factory for the bio-synthesis of NPs. Biosynthesizing NPs by fungi has some advantages over other biological resources because fungi produce high levels of reducing agents, such as proteins, to reduce metallic ions to a less toxic form [6]. Various fungi have recently been used for biotechnological processes, and using the residual mycelium of these fungi has been proposed as a potential cost-effective solution [7].

The present study was conducted to screen some desert-soil fungi in terms of AgNP synthesis. The biosynthesis of AgNPs from the superior strain was optimized and then characterized. The antibacterial and antibiofilm activities of the produced NPs were then assayed.

2. Materials and method

2.1 Screening of fungi for the synthesis of AgNPs

The synthesis of NPs of 24 fungal isolates was investigated. Twenty-three out of 24 belong to Aspergillus and the other one belongs to Fusarium achieved from the microbial bank of Alzahra University which was collected from the desert soil of Khabr National Park (Kerman, Iran). Fungi were cultured on potato dextrose agar and kept at room temperature for 8 days to produce sufficient conidiospores. Then, 1 ml of the conidiospore suspension with a concentration of 10⁶ conidiospores per ml was inoculated into the 250 ml flask containing 100 ml liquid culture medium of malt glucose yeast peptone agar and comprising 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1% glucose and then incubated at 27–30°C in 120 rpm, for 4 days. After 4 days, fungi were filtered by the sterilized Whatman paper
After selecting the superior strain, the synthesis conditions, such as the effect of pH (6, 8, 10 and 12), temperature (80, 50°C and room temperature (25–27°C)) and silver nitrate concentrations (1–5 mM), were optimized and characterized.

2.3 Characterization of AgNPs

The preliminary characterization of AgNP synthesis was visually done through the observation of colour changes. The UV–vis spectroscopy measurements were performed on a SPEKOL 2000 spectrophotometer in the range of 350–700 nm. The hydrodynamic diameter, polydispersity index (PDI) and distribution of AgNPs were examined by a NanoPhox 90-246V instrument (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The biosynthesized AgNP solution was dropped in a cast on a carbon-coated copper grid for transmission electron microscopy (TEM) analysis (Philips CM30). The distribution of AgNP size was calculated using Digimizer software version 4.1.1.0. The X-ray diffraction (XRD) patterns were obtained on a Rigaku instrument, operating at 40 kV and at a current of 30 mA. The Fourier-transform infrared (FTIR) spectra of AgNPs were recorded by TENSOR 27 spectrometer in the range of 400–4000 cm⁻¹.

2.4 Mechanisms involved in AgNP biosynthesis

2.4a Determination of reducing biomolecules: To determine the effective agents in biosynthesis of AgNPs, the biomolecules in the fungal extract were investigated. The phenol sulphuric acid method was used to determine the carbohydrate content of fungal extract [9]. For this reason, 200 µl of phenol solution (5% w/v) with different concentrations was added to the 400 µl of fungal extract. Immediately, 1 ml of sulphuric acid (97%) was added. After 10 min, each sample was vortexed for 30 s. After 20 min, the absorption was obtained at 490 nm. Glucose was applied as a standard sugar to illustrate the standard curve.

\[
\text{%Scavenging} = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100.
\]  

(1)

2.4b Nitrate reductase assay: The quantitative assay of nitrate reductase of the fungi extract was performed by the Harley method [12]. In this method, nitrate as a substrate was used, and the activity of enzyme is determined after 1 h through measuring the produced nitrite. Afterwards, 5 ml of extract of superior strain and 5 ml of assay medium (potassium nitrate 30 mM and propanol 5% (v/v) in phosphate buffer (0.1 M, pH 7.5)) were mixed and incubated in dark at room temperature for 1 h and then placed in a hot water bath. After cooling the tubes at room temperature, 2.5 ml of sulphanilamide solution (sulphanilamide 1% (w/v) in HCl 25% (v/v)) 2.5 ml of 0.1% N-(1-naphthyl)ethylenediaminedihydrochloride solution were added into each tube and mixed. After 20 min, the absorption of each sample was surveyed in 540 nm. The activity of enzyme was calculated considering the produced nitrite per hour for the initial sample (5 ml) and was expressed as nmol nitrite h⁻¹ ml⁻¹.

2.4c Determination of free radical scavenging of fungal extract using the DPPH method: The antioxidant activity of fungal extract was evaluated through the free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [13]. Different volumes of fungal extract (25–55 ml) were diluted with methanol up to 1 ml and the mixture was added to 2 ml of 0.1 mM DPPH methanolic solution. This mixture was thoroughly mixed and kept in the dark for 1 h. The control tube contained DPPH and methanol. In this assay, 50 µg ml⁻¹ of ascorbic acid was used as standard. After 1 h, the absorbance of the samples was obtained at 517 nm. The antioxidant activity of the sample was calculated according to equation (1):

2.4d SDS-PAGE gel electrophoresis: To evaluate the protein content of the fungal extract, SDS-PAGE gel electrophoresis was performed by the Laemmli method [14]. The fresh fungal extract was frozen at −70°C over night and lyophilized. The produced dried powder was solved in distilled water and diluted in a 5× buffer (containing 0.125 M Tris-HCl, pH 6.8, glycerol, beta-mercaptoethanol and bromophenol blue.
as the tracking dye) and boiled for 10 min. The colloidal AgNPs, which were centrifuged and collected, were boiled in the presence of SDS 1% for 10 min. Then, the sample was centrifuged for 10 min at 10000 rpm, and its supernatant used for electrophoresis [6]. All molecular weight markers, fungal extract and AgNP samples were run at room temperature with the constant voltage of 100 V for 3 h. Finally, the gel was stained with Coomassie Brilliant Blue dye.

2.5 Antimicrobial activity of mycosynthesized AgNPs

2.5a Minimum inhibitory concentration (MIC) of AgNPs: In this study, the MIC of AgNPs was determined using the broth microdilution method [15]. Four standard strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 1431 and *Bacillus subtilis* ATCC 6633 and antibiotic-resistant strains collected from burns and sputum, *P. aeruginosa* 48 and *P. aeruginosa* B52, were applied to study the antimicrobial activity of AgNPs. Two dilution series of AgNPs and fungal extract were prepared using Mueller-Hinton broth and 100 μl of inoculum with diluted concentration of (1:150) the first tube of McFarland added into each well of microtiter plate and incubated at 37°C for 20 h. The lowest concentration, which completely inhibited the growth of microorganisms, was selected as MIC.

2.5b Minimum bactericidal concentration (MBC) of AgNPs: To determine the MBC of AgNPs, 100 μl from samples that did not show any bacterial growth from the microtiter plate wells was collected and spread them on the surface of the nutrient agar plate. Then, the plates were incubated at 37°C for 24 h [15].

2.5c Minimum biofilm inhibitory concentration (MBIC) of AgNPs: To determine the MBIC of AgNPs, the *P. aeruginosa* 48 and *P. aeruginosa* B52 strains were used and the test was carried out according to the CLSI protocol, 2012 [15]. Serial dilution of AgNPs and the fungal extract were prepared using trypticase soy broth containing 0.2% glucose. Then, 100 μl of bacterial suspensions containing the concentration of bacteria equivalent to the first McFarland tube was added into the wells. Microtiter plates were incubated at 37°C for 24 and 40 h for *P. aeruginosa* 48 and *P. aeruginosa* B52, respectively. The crystal violet colorimetric method was used to detect the MBIC.

3. Results and discussion

3.1 Screening of soil fungi for the synthesizing of AgNPs

Soil fungi are one of the diverse sources of extracellular metallic NP production [16]. Studies have also shown that microorganisms have various defensive mechanisms to help them adapt to adverse environmental conditions, such as unendurable and stressful conditions. One of the well-known mechanisms for overcoming environmental stresses in desert fungi is to produce high amounts of melanin and various carotenoid pigments. Fungal antioxidants play a protective role against the long-range part of UV radiation [17]. The present study, therefore, examines microorganisms from a stressful environment. These fungi were isolated from the soil in Khabr National Park and kept at Alzahra University’s microbial bank and screened for the production of AgNPs. All of the 24 fungi isolates (23 isolates of *Aspergillus* and one isolate of *Fusarium*) showed an ability to produce AgNPs. Three factors, including synthesis rate, dispersion and size of AgNPs, were taken into consideration when selecting the superior isolate. In nine out of the 24 isolates, the synthesis of AgNPs was completed in less than 2 h due to the results of the UV–vis spectra and the surface plasmon resonance (SPR) peak of the synthesized AgNPs (figure 1). The SPR peak of AgNPs offered useful information about their size, shape and dispersion. When the size of AgNPs is increased, the band gap energy between the capacity band and the conduction band decreases, and the SPR spectrum is thus transmitted to higher wavelengths. The peak width of SPR is directly related to the dispersion size of AgNPs [18,19]. Among the 24 strains of fungi screened, the MS17 strain (i.e., *Aspergillus fumigatus*) was selected. The criteria for this selection included producing the highest amount of AgNPs in less than 1 h, producing small-sized AgNPs compared to other fungi (λ<sub>max</sub> = 420 nm) and yielding low-sized dispersity (i.e., a narrow absorption peak).

3.2 Optimization of AgNP synthesis

Environmental conditions, such as silver nitrate concentration, pH and temperature, affect the size, shape and stability of NPs. AgNPs were synthesized in this study using the superior strain with various concentrations of silver nitrate, pHs and temperatures. The UV–vis absorption spectra of the AgNPs and their stability were then monitored for 2 weeks. Based on the results of the UV–vis spectra of the synthesized AgNPs in various concentrations of silver nitrate, the AgNPs that were produced in 1 mM of silver nitrate had a lower size dispersion, a smaller size and a higher stability. In acidic pH (pH = 6), the AgNPs synthesized by the superior strain showed a very low stability, and an aggregation of AgNPs was observed immediately after synthesis. From all the pH values (pH = 6, 8, 10 and 12), the pH of 8 was selected since it produced AgNPs that possessed a higher stability and mono dispersion. The results were consistent with the findings of previous studies [16,20]. Temperature also plays an important role in the production and stabilization of AgNPs. The results of a study by Sahoo et al [21] showed that, at temperatures below 30°C, the size of AgNPs is 30 nm, and at higher temperatures, the size...
increases [21]. In the present study, room temperature was considered the optimal temperature for synthesizing more stable AgNPs with a lower dispersion. Room temperature, pH of 8 and 1-mM concentration of AgNO₃ were thus considered the optimal conditions for synthesizing AgNPs and further analyses were conducted for these conditions.

In this research, the change in the colour of the extract from yellow to pale yellowish-brown occurred just 1 h after incubation with 1 mM of silver nitrate (figure 2) without the presence of fungal mycelium, but in the other study, Aspergillus niger collected from the forest soil sample produced AgNPs in 12 h [16]. Aspergillus flavus NJP08 was separated from the soil of iron-rich Rajasthan region in India and showed the brown colour of the produced AgNPs after 72 h [6].

3.3 Characterization of AgNPs

In many cases, colour alteration is the first sign of AgNP production [22]. The brown colour, formed under optimum conditions, represents the production of AgNPs. Nonetheless, under the same conditions, no colour alteration was observed in the first control group (i.e., the silver nitrate solution) and the second one (i.e., free mycelium fungal extract solution) compared to the initial sample.

The UV–vis spectra of the synthesized AgNPs were measured in the range of 350–700 nm. A special SPR is the reason for the unique optical phenomenon of AgNPs. The absorption peak occurring at 420 nm due to the SPR of AgNPs was observed in the UV–vis spectra (figure 3). AgNPs possessed an absorption peak at visible wavelengths due to the vibration of the surface plasmon within the visible area (about 450–380 nm), which explains the yellowish brown colour of AgNPs [23].

The dynamic light scattering (DLS) analysis was used to investigate the size and dispersion of NPs. The X₅₀ parameter, which was equal to the statistical index of the mode, showed that 50% of the NPs had a larger size and the other 50% had a smaller size. The surface mean diameter and volume mean diameter are parameters that show the average diameter, and when the ratio of these parameters tends towards 1, the particles become spherical. The PDI was measured as 0.01 for the monodispersed particles up to 0.5–0.7 for the polydispersed particles. According to
figure 4a, the AgNPs synthesized by the superior strain under optimal conditions showed a spherical shape with the average hydrodynamic diameter of 75.36 nm, and the PDI of the obtained particles was 0.27, indicating a mediocre dispersion. The XRD analysis revealed the crystalline nature of the produced AgNPs. The appearance of peaks at 2θ = 38° and 64° indicated the presence of Ag0 NPs, and the appearance of two peaks at 2θ = 32° and 46° indicated AgCl NPs in the samples (figure 4b).

Based on the Scherrer equation and the data obtained from the XRD analysis, the average crystal size of Ag0 and AgCl NPs was 20 and 10 nm, respectively. The difference in the average size that was obtained from both the DLS and XRD analyses can be attributed to the fact that the hydrodynamic diameter of the NPs (AgNPs with surface-bound agents) is measured in the DLS analysis, whereas only the size of AgNP crystals, regardless of the capping agents, is measured in the XRD analysis.

The TEM analysis was also used to determine the size and shape of the AgNPs. As for the images obtained from the TEM, the size of the AgNPs was determined using Digimizer software. The TEM images showed that the produced AgNPs possessed a spherical shape with a medium-sized dispersion and an average size of 5–20 nm. With high resolutions, the pale beads can be observed around AgNPs’ TEM micrographs (figure 4c). This halo is probably related to the capping agent on the surface of AgNPs that affects their stability and the colloidal solution of synthesized AgNPs under optimum conditions and can be preserved for a month, and the sedimentation was not observed.

The biomolecules that capped the AgNPs were investigated by FTIR spectroscopy. AgNPs and dried fungal extract were analysed by FTIR and the results were reported in the range of 400–4000 cm⁻¹. The 670.95 and 1648 peaks represent alkene and carboxylic acid, respectively. The 1045.65 peak indicates aliphatic amine. The 1362.45, 1396.41 and 2923.78 peaks are related to the methyl group and the 3445.58 peak is for primary amine and 3675.88 for alcoholic and phenolic O–H. The present findings are consistent with the results of the study by Gajbhiye et al [24], who synthesized AgNPs by the extracellular extract of *Alternaria alternata* and reported that proteins can link to NPs via free amines or cysteine residues or by the creation of an electrostatic interaction through the carbocyclic groups in enzymes, such that NPs are stabilized by proteins. The presence of methyl, O–H and alkenyl groups indicates the presence of carbohydrates in the fungal extract. The presence of primary amines and carboxylic acid groups is also the result of amino acids and proteins in the samples.

3.4 *The mechanisms involved in the synthesis of AgNPs*

Biological systems are able to synthesize AgNPs due to their reducing and stabilizing agents. Studies show that enzymes, proteins, amino acids and carbohydrates play an important role in the AgNP biosynthesis [25–27]. The concentration of carbohydrate, protein and amino acids in the fungal extract was 35.6, 37.7 and 2.6 μg ml⁻¹, respectively.

3.4a *Nitrate reductase assay:* The presence of nitrate reductase enzyme in the fungal extract of the superior strain was confirmed only by the qualitative measurement method. The results of quantitative measurements of nitrate reductase showed that all of the nitrate reductase enzyme substrate was consumed by the enzyme which exists in the fungal extract. Then, the nitrite was reduced to N2 by the other enzymes that exist in the extract.

3.4b *DPPH scavenging of the fungal extract:* The antioxidant capacity of the fungal extract was assessed by the evaluation of the scavenging of free radical of DPPH. According to the results, the fungal extract possesses 41% antioxidant activity.

3.4c *The SDS-PAGE analysis:* The obtained results by the Bradford and FTIR analysis exhibited the presence of proteins not only in the fungal extract, but also in the cap of the NP surface. In order to determine the proteins involved in the synthesis and stability of the NPs, the SDS-PAGE pattern obtained from the fungal extract and AgNPs was investigated. The pattern of fungal proteins exhibited almost 8 bands in the range of 10–75 kD (figure 5b). These proteins can also affect the synthesis and the stability of NPs. To identify the proteins linked to NPs, the synthesized NPs were treated by 1% SDS and boiling water. SDS leads to denature the proteins and distinct them from AgNP surface. AgNPs treated by SDS showed two bands (figure 5c) that could be indicated to both of these proteins. They functionalized as a capping agent on the protein surface and affect the stability of NPs. These findings are also compatible with the FTIR results which had exhibited the relationship of the fungal extract proteins on NPs. Furthermore, the broad band between the stacking gel

![Figure 3. UV–vis spectrum of biosynthesized AgNPs of the superior strain after 24 h.](image-url)
and separating gel (figure 5d) that linked to the untreated AgNPs showed a strong linkage between proteins and AgNPs, so that it prevents the migration of proteins to the separating gel.

3.5 Antibacterial activity of AgNPs

MIC is the minimum concentration that inhibits the bacterial growth. The results of the inhibitory effect of AgNPs on the growth of bacteria are represented in table 1. Each experiment was repeated three times. As it can be seen, the combination of extract and nitrate (as a control) has no effect on the bacterial growth. The results showed that \textit{P. aeruginosa} ATCC 27853 was the most sensitive to the NPs and the multidrug-resistant strain isolated from burns; \textit{P. aeruginosa} B52 was the most resistant to the synthesized AgNPs.

The results are completely compatible with previous studies \cite{16}. The studies have also shown that Gram-positive and Gram-negative bacteria represent a different sensitivity to AgNPs because of their structural differences in cell membranes and cell walls. Regarding the results of Gram-positive and Gram-negative bacteria exposed to the AgNPs, \textit{B. subtilis} ATCC 6633 and \textit{S. aureus} ATCC 1431 showed a higher MIC and MBC than the Gram-negative bacteria. Obviously, the high resistance of \textit{P. aeruginosa} B52 against AgNPs is probably due to the defensive mechanisms which bacteria have gained during the evolution to resist against antibiotics and antimicrobial agents.

Biofilms formed by pathogenic bacteria are an important factor in the development of the chronic and reversible infections. AgNPs have a unique ability to eliminate the biofilm of the pathogenic bacteria \cite{28}. The AgNPs synthesized by superior strain possess an inhibitory effect on the biofilms of both bacteria (figure 6). AgNPs in
concentrations of 26.92 and 13.46 μg ml⁻¹ possess an inhibitory effect on the biofilm formation of P. aeruginosa P48 and P. aeruginosa B52 strains, respectively. Also, the combination of fungal extract and nitrate in all dilutions did not affect the biofilm formation, indicated to the anti-biofilm property which was exclusively caused by AgNPs. The obtained results were consistent with the research of Markowska et al [29]. They showed that AgNPs with the size range of 2–32 nm can prevent the biofilm formation of P. aeruginosa ATCC 10145.

4. Conclusion

This study examined the synthesis of AgNPs by fungi isolated from desert soils in Khabr National Park, as a harsh environment. A simple, rapid, affordable and environment-friendly method was used for synthesizing AgNPs. These NPs had the same nature as AgCl/Ag⁰ with a size of 15 nm, were spherical in shape and had a medium-sized dispersion. AgCl/Ag⁰ NPs also encompassed a notable stability at room temperature because of the stabilizing biomolecules existing in the fungal extract. The NPs, biosynthesized in this study showed antibacterial and antibiofilm effects. Further studies are recommended on the molecular identification of the superior strain for optimizing the conditions for biosynthesizing AgNPs by the superior strain so as to study more effective factors of the fungal extract, AgNPs and cytotoxicity of NPs on healthy and cancerous cells.

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Table 1. MIC and MBC of biosynthesized AgNPs.

|                | P. aeruginosa ATCC 27853 | E. coli ATCC 25922 | B. subtilis ATCC 6633 | S. aureus ATCC 1431 | P. aeruginosa P48 | P. aeruginosa B52 |
|----------------|--------------------------|-------------------|----------------------|---------------------|------------------|------------------|
| MIC (μg ml⁻¹)  | 1.68                     | 3.37              | 3.37                 | 6.74                | 3.37             | 13.48            |
| MBC (μg ml⁻¹)  | 3.37                     | 3.37              | 3.37                 | 6.74                | 6.74             | 13.48            |

Figure 5. SDS-PAGE (separating gel 12%): (a) molecular weight marker, (b) fungal extract, (c) AgNPs boiled in 1% SDS and (d) untreated AgNPs.

Figure 6. MBIC analysis of AgNPs. In each plate the wells of A1–A9 and F1–F9 are serial dilutions of AgNPs and fungal extract, respectively and the wells of columns 10, 11 and 12 are positive control, medium control and negative control, respectively: (a) P. aeruginosa P48 and (b) P. aeruginosa B52.
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