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Green Synthesis of Silver Nanoparticles Using *Aspergillus niger* and Its Efficacy Against Human Pathogens

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ABSTRACT

Antibiotic resistance is one of the world's most pressing public healthcare problems. People who become infected with drug-resistant microorganisms usually spend more time in the hospital and require a form of treatment that uses two or three different antibiotics and is less effective, more toxic, and more expensive. Silver nanoparticles (AgNPs) are attractive option because they are non-toxic to the human body at low concentrations and have broad-spectrum antibacterial actions. The biosynthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost-effective and environmentally friendly technologies for nano-materials synthesis. In this report, silver nanoparticles (AgNPs) were synthesized using a reduction of aqueous Ag^+ ion with the culture supernatants of Aspergillus niger. The reaction occurred at ambient temperature and in a few hours. The bioreduction of AgNPs was monitored by ultraviolet-visible spectroscopy, and the AgNPs obtained were characterized by transmission electron microscopy and X-ray diffraction. The synthesized AgNPs were polydispersed spherical particles ranging in size from 1 to 20 nm and stabilized in the solution. Furthermore, the antimicrobial potential of AgNPs was systematically evaluated. The synthesized AgNPs could efficiently inhibit various pathogenic organisms, including bacteria and fungi. The current research opens a new avenue for the green synthesis of nano-materials and AgNPs have the potential to serve as an alternative to antibiotics and to control microbial infections such as those caused by multidrug- resistant pathogens.

Keywords: silver nanoparticles; *Aspergillus niger*; Antimicrobial activity

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INTRODUCTION

Antibiotic resistance is one of the world's most pressing public healthcare problems. In recent decades, almost every variant of bacteria has become stronger and less vulnerable to antibiotic treatment, threatening new strains of infectious disease or super-strains that are both more expensive to treat and more difficult to cure. Drug-resistant bacteria are emerging pathogens whose resistance profiles present a major challenge for containing their spread and their impact on human health. Currently, over 70% of bacterial infections in the United States are resistant to one or more of the antibiotics traditionally used to eliminate them. People who become infected with drug-resistant microorganisms usually spend more time in the hospital and require a form of treatment that uses two or three different antibiotics and is less effective, more toxic, and more expensive [1]. Nanoparticles (NP) are usually clusters of atoms in the size range of 1–100 nm. It is understood that the properties of a metal NP are determined by its size, shape, composition, crystallinity, and structure. As an important metal, silver nanoparticles (AgNPs) have a number of applications, from electronics [2] and catalysis to infection prevention [4] and medical diagnosis [5]. For example, AgNPs could be used as substrates for Surface Enhanced Raman Scattering (SERS) to probe single molecules [6], and also useful catalysts for the oxidation of methanol to formaldehyde [7]. AgNPs has been known as excellent antimicrobial and anti-inflammatory agents, and thus were used to improve wound healing [8]. To date, a number of physical and chemical strategies were employed for the synthesis of AgNPs [9]. However, concern has

been raised on the toxicity of chemical agents used in AgNPs synthesis. Thus, it is essential to develop a green approach for AgNPs production without using hazardous substances to the human health and environment.

Compared with the traditional synthetic methods, biological systems provide a novel idea for the production of nano-materials [10]. Up to now, several microorganisms from bacteria to fungi have been reported to synthesize inorganic materials either intra- or extracellularly, and thus to be potentially utilized as eco-friendly nanofactories [11]. *Pseudomonas stutzeri* AG259, isolated from silver mines, has been shown to produce silver nanoparticles [12], and the bioreduction of Ag was also reported in *Bacillus licheniformis.* Recently a further advancement in the biological synthesis approach was shown by demonstrating that the shape of Ag nanoparticles could be tuned from nanospheres to nanoprisms by controlling the growth kinetics of a silver resistant bacteria *Morganella psychrotolerans* [13]. Moreover, the same research group also demonstrated that all the members of the genus *Morganella* were capable of synthesizing extracellular Ag nanoparticles, which was correlated to silver resistance machinery operating in these organisms [14] Compared with bacteria, fungi have been known to secrete much higher amounts of bioactive substances, which made fungi more suitable for large-scale production [15]. In addition, the extracellular biosynthesis using fungi could also make downstream processing much easier than bacteria. An interesting example of the biosynthesis using fungi was that the cell-associated biosynthesis of silver using *Fusarium oxysporum* was demonstrated by Ahmad *et al.*, and the particles were overall quasi-spherical with size range between 5 and 15 nm [16]. There also have been several reports on the biosynthesis of AgNPs using fungi, including *Fusarium acuminatum* [17]. Despite these impressive results, the origins of fungi having the ability for AgNPs synthesis were still limited, and the detailed mechanism was still not well elucidated. Previous reports have shown that a large number of active substances secreted by fungi played important roles as reducing agents and capping agents in the reaction [19]. Therefore, it was of great significance to explore novel fungi strain for synthesizing AgNPs based on the biodiversity. More importantly, it could also facilitate the deeper understanding of molecular mechanism for AgNPs biosynthesis.

Herein, we investigated the biosynthesis of AgNPs using *Aspergillus niger* and its underlying mechanism. The properties of obtained AgNPs were characterized by ultraviolet-visible spectroscopy, transmission electron microscopy (TEM) and X-ray diffraction (XRD) techniques. This work provided a potential for the production of AgNPs without the involvement of toxic chemicals and radiation. AgNPs have the potential to serve as an alternative to antibiotics and to control microbial infections such as those caused by multidrug- resistant pathogens.

MATERIALS AND METHODS

2.1 Materials

A. niger was isolated from soil, and maintained on potato dextrose agar (PDA) medium at 28°C. The isolated fungus was identified using morphological characteristics and mitochondrial cytochrome *b* gene analysis. Three kinds of bacteria were tested for their susceptibility for AgNPs: *S. aureus, E. coli* and *P. aeruginosa*. Six kinds of fungi were all tested for its antifungal effect: *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *A. fumigatus* and *A. flavus*. *A. niger*. Potato dextrose agar and potato dextrose broth were purchased from BD (Becton, Dickinson and company Co., Sparks, MD, USA). The chemical silver nitrate (AgNO3) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and used as received.

2.2. Biomass Preparation

A. niger was grown in potato dextrose broth (PDB) at 28°C on a rotary shaker (120 rpm) for 96 h. The biomass was harvested by filtration using Whatman filter paper No. 1, followed by washing with distilled water to remove any components of the medium. The biomass (25 gm) wet weight was placed in individual flasks containing 100 mL Milli-Q water and incubated as described above for 24 h. The biomass was filtered, and the cell filtrate was collected and used for biosynthesis of AgNPs.

2.3. Biosynthesis of AgNPs

For biosynthesis of AgNPs, 50 ml of cell filtrate was mixed with 10 ml AgNO3 solution (10 mM) and reaction mixture without AgNO3 was used as control. The prepared solutions were incubated at 28 °C for 24 h. All solutions were kept in dark to avoid any photochemical reactions during the experiment. The AgNPs were purified by centrifugation at 10,000 rpm for 10 min twice, and collected for further characterization.

2.4. Characterization of AgNPs

The bioreduction of Ag^+ in aqueous solution was monitored using an ultraviolet-visible spectrophotometer (Shimadzu UV-2550) from 240 to 750 nm, at a resolution of 1 nm. The dried reaction mixture embedded with AgNPs was used for XRD analysis. XRD patterns were recorded on RINT2000 vertical goniometer operated at a voltage of 50 kV and current of 200 mA with Cu Kα radiation (λ = 1.5405 Å), and the diffracted intensities were

recorded from 30° to 80° 2θ angles.
For TEM analysis, a drop of aqueous solution containing AgNPs were placed on the carbon coated copper grids and dried by allowing water to evaporate at room temperature. Micrographs were obtained using a Tecnai F20 S-Twin (USA) operating at 200 kV. The sizes of AgNPs were estimated from the Debye-Scherrer Eq by determine the width of the (111) Bragg reflection [2], and size distribution of the resulting nanoparticles was also estimated on the basis of TEM micrographs.

2.5. The Antimicrobial Activity Analysis of AgNPs

The antimicrobial activity of AgNPs was investigated against *P. aeruginosa*, *S. aureus*,*E. coli*, *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *A. fumigatus* and *A. flavus* using disk diffusion assay. The disk diffusion assay was carried out using the Oxford cup method. Each strain was swabbed uniformly onto individual plates, and a concentrated solution of AgNPs was poured into each cup (20 µg per cup) on all the plates. After incubation at 37 °C or 28 °C for 24 h, the diameter of inhibition zone was measured using caliper. AgNO3 (10mM) was used individually as control. The assays were performed in triplicate.

RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of AgNPs Using *Aspergillus niger*

In this study, AgNPs were synthesized using a reduction of aqueous Ag^+ with the culture supernatants of *Aspergillus niger* at room temperature. It was generally recognized that AgNPs produced brown solution in water, due to the surface plasmon resonances (SPR) effect and reduction of AgNO3 [20]. After the addition of AgNO3 solution, the cell filtrate of *A. niger* changed from light yellow to brown in a few hours, while no color change was observed in the culture supernatant without AgNO3(Figure 1).

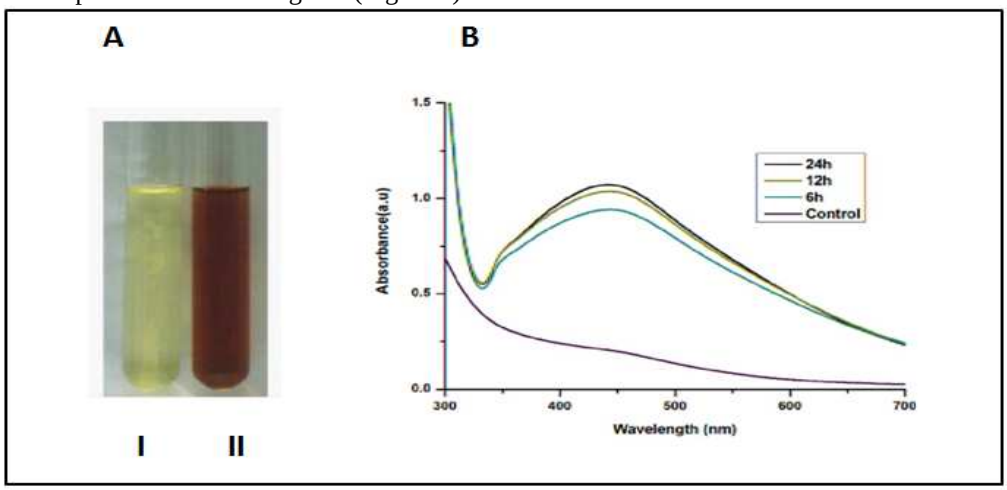

Figure 1: (A) The crude cell filtrate of *Aspergillus niger* mixed without AgNO3 (**I**) and with AgNO3 (**II**) after 24 h.(**B)** The UV-Vis spectra recorded for the reaction of fungal cell filtrate with AgNO3 solution.

Thus, color change of the solution clearly indicated the formation of AgNPs. The color intensity of the cell filtrate with AgNO3 was sustained even after 24 h incubation, which indicated that the particles were well dispersed in the solution, and there was no obvious aggregation.All these reactions were monitored by ultraviolet-visible spectroscopy of the colloidal AgNPs solutions. The ultraviolet-visible spectra of the cell filtrate with AgNO3 showed a strong broad peak at 440 nm which is surface Plasmon resonances (SPR band), which indicated the presence of AgNPs (Figure 1B). These results were consistent with the reports of Naik *et al.* and Verma *et al.* [21,22]. The intensity of the SPR band steadily increased from 6 h to 24 h as a function of time of reaction. It was also observed that the AgNPs formed were quite stable in the supernatant of *A. niger*.

The application of AgNPs was highly dependent on the chemical composition, shape, size, and monodispersity of particles [23]. To broaden the application scope, the AgNPs obtained were systematically characterized using TEM and XRD analysis. Through the TEM analysis, the particles were spherical and polydisperse with an average size of 4.3 nm (1–20 nm), and the majority of the particles were less than 10 nm (Figure **2A, 2B**).

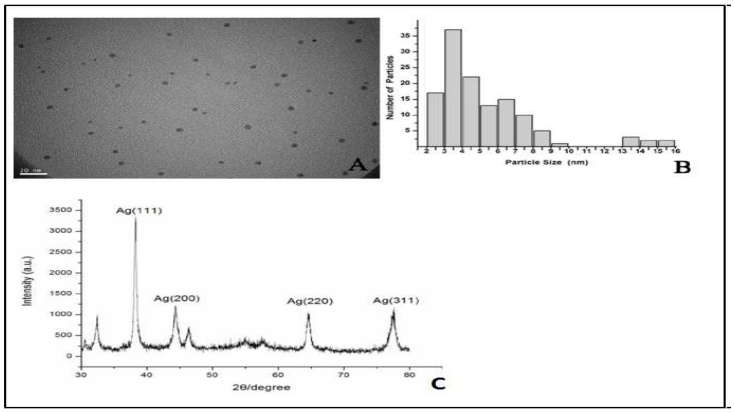

Figure 2: (**A**) Representative images of AgNPs synthesized by the reduction of AgNO3 solution with the crude cell filtrate from *Aspergillus niger*; (**B**) Size distribution of the AgNPs from TEM analysis, X-ray diffraction patterns of AgNPs (a.u. = arbitrary units).

For the crystalline nature of the AgNPs, intense XRD peaks were observed corresponding to the (111), (200), (220), (311) planes at 2θ angles of 38.28°, 44.38°, 64.54°, and 77.64°, respectively (Figure 2C). This was in good agreement with the unit cell of the face centered cubic (fcc) structure (JCPDS File No. 04-0783) with a lattice parameter of a = 4.077 Å. Some intense diffraction peaks at 2θ angles of 32.05°, 46.05°, 54.6° and 57.3°, might be related to AgCl which was owing to the chloride ions involved during preparation of the cell filtrate. Because of the biomass residue, other crystallographic impurities were also observed in the XRD profile. The size of AgNPs according to the XRD was about 5.2 nm. This result was consistent with the TEM study.

Table 1. Size of the inhibition zone for AgNPs synthesized by *Aspergillus niger* against the tested microorganisms.

Tested Pathogenic Organisms	Mean Size of Inhibition Zone (mm)	
	Control	Test (AgNPs)
Candida albicans	9	16 ± 1
Candida krusei	10	14 ± 2
Candida parapsilosis	9	13 ± 1
Candida tropicalis	10	14 ± 1
Aspergillus flavus	9	13 ± 2
Aspergillus fumigates	9	14 ± 2
Staphylococcus aureus	9	16 ± 1
Pseudomonas aeruginosa	9	12 ± 1
Escherichia coli	10	13 ± 1

3.2. Antimicrobial Activity Analysis of AgNPs

The antimicrobial activity of AgNPs against various pathogenic organisms including bacteria and fungi was investigated. Compared with the control, the diameters of inhibition zones increased for all the test pathogens (Table 1). The AgNPs produced could inhibit three different typical pathogenic bacteria, including *Staphylococcus aureu*, *Pseudomonas aeruginosa* and *Escherichia coli*, as previously described [21]. Thus, AgNPs could be considered as excellent broad-spectrum antibacterial agents. More importantly, the AgNPs produced by *A. niger* exhibited potent antifungal activity against *Candida* species, which were the most important pathogenic fungi. Additionally, the AgNPs showed good inhibition activity towards two kinds of filamentous fungus, which were naturally resistant to the common antifungal agent Fluconazole. Since the biosynthesized AgNPs showed considerable antifungal activity, they could be potential to be widely used in clinical applications.

CONCLUSION

In this study, AgNPs were synthesized extracellularly by *A. niger* at room temperature. The AgNPs were quite stable without using any toxic chemicals as capping agents. The spherical AgNPs ranged in size from 1 to 20 nm, and showed promising broad-spectrum antimicrobial activity. The ability to synthesize AgNPs as potential anti-microbial agents using *A. niger* is highly promising for the green, sustainable production of nano-metals, and also enhances its widespread application as an important strategy.

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