



Biogenic synthesis and antibacterial activity of silver nanoparticles (AgNPs) produced by *Phomopsis* sp. strain GFPA2

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Abstract

Silver nanoparticles (AgNPs) have many applications in different fields such as electronic industries, chemical industries, medical diagnostics and detergent products. In this research AgNPs were produced by *Phomopsis* sp. GFPA2 isolated from zinc mineral rocks, and were successfully synthesized by using fungal culture broth. The biogenic crystals were investigated and identified by field emission scanning electron microscopy (FE-SEM) equipped with energy dispersive X-ray spectroscopy (EDS) and X-ray powder diffraction (XRD). The spectra confirmed that the biogenic crystals were AgNPs, and they are mostly spherical in shape. The crystals were also tested for their antibacterial activity against human pathogenic bacteria such as *Escherichia coli* ATCC 27853, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 6633 and *Staphylococcus aureus* ATCC 25623. The result showed that the biogenic AgNPs can inhibit the growth of both Gram positive and Gram negative pathogenic bacteria. Therefore, these biogenic AgNPs could be used to treat the infection caused by human pathogenic bacteria.

1. Introduction

Nanomaterials have become common materials used in various industries such as cosmetics, data storage, food, space, optical, electrical, medicinal and chemical industries [1,2]. Metal nanoparticles are synthesized by physical, chemical and biological methods. Normally, physical and chemical processes have been used to synthesize metal nanoparticles, but these processes can cause damage to the environment and human health because physical and chemical methods involve high temperatures, high pressures, and toxic chemicals [3]. Therefore biological processes have been increasingly used because they have benign reaction and are environmental friendly [4,5]. Filamentous fungi are reported as potential candidates for the production of various metal nanoparticles such as zinc, silver, copper and lead [6-8]. Moreover, metal nanoparticles could be produced by plant extracts [2,9].

Silver and silver nanoparticles (AgNPs) have been known to have high potential as antimicrobial agents. However the use of biological synthesis by fungi, bacteria and plant extracts for the production

of AgNPs is more acceptable for medical and pharmaceutical applications [8]. Many recent reports have emphasized the ability of fungi to synthesize metal nanoparticles. The native physiological processes of the fungi can lead to the precipitation of metal nanoparticles at the extracellular environment [6]. Thus, the objective of this research was to study the biological synthesis of AgNPs by using the filamentous fungus *Phomopsis* sp. and to evaluate the potential of their antibacterial activity against human pathogenic bacteria.

2. Materials and methods

2.1 Fungal isolation and identification

Phomopsis sp. was isolated from zinc mineral rock collected from a zinc mine in Tak province, northern Thailand [10]. For molecular genetic identification, *Phomopsis* sp. was inoculated into 100 ml of potato dextrose broth (PDB; 4 g/l potato starch, 20 g/l dextrose) and incubated at 28°C for 7 days. The mycelium of the fungus was harvested and extracted with the CTAB method to obtain

fungal DNA following the standard protocols. Mycelial biomass was scraped into a 2.0 ml tube containing zirconia balls and transferred to a container with liquid nitrogen. The mycelium of *Phomopsis* sp. was pulverized by using a homogenizer (Retsch Mixer Mill MM400, Hann, Germany) for 30 s and then 700 µl of CTAB solution (2% Cetyltrimethylammonium bromide, 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 0.5 % beta-mercaptoethanol, 1.4 M NaCl) was added to the tube. The reaction was homogenized for 30 s and incubated at 65°C for 1 h and then 700 µl of chloroform : isoamyl alcohol (24:1) was added. After that, the tube was mixed and centrifuged at 15,000 rpm for 8 min in the room temperature (25°C). The supernatant was removed to another tube. The fungal DNA was precipitated by adding an equal volume of isopropyl alcohol and keeping the tube at -20°C for 15 min. Finally, the reaction was centrifuged at 8,000 rpm for 10 min and the pellet of fungal DNA was washed with 500 µl ethanol (70%). The DNA pellet was dissolved in 100 µl of TE buffer (1 mM EDTA, 10 mM Tris-HCl (pH 8)).

For polymerase chain reaction (PCR) amplification, the internal transcribed spacer region was amplified by using primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') [11,12], and the expected product size was approximately 700 bp. The optimal conditions for PCR amplification followed the condition of Sutjaritvorakul et al. [12]. The conditions for PCR were as follows: initial denaturation for 1 min at 94°C, with subsequent denaturation for 1 min at 94°C, primer annealing for 1 min at 51°C, and extension for 1 min at 72°C for a total of 35 cycles. The final extension for 5 min at 72°C preceded a constant incubation at 4°C until analysis by electrophoresis. PCR products were checked on a 1.5% agarose gel staining with ethidium bromide run at 100 V for 45 min. All of PCR products were purified and sequenced by using the same primers. The homology studies of the sequences were compared with the sequences of known species in the National Center for Biotechnology Information (NCBI).

2.2 Biosynthesis of AgNPs

Seven day old cultures on potato dextrose agar (PDA; 4 g/l potato starch, 20 g/l dextrose, 15 g/l agar) of *Phomopsis* sp. were inoculated into 100 ml

of potato dextrose broth (PDB) in Erlenmeyer flasks and incubated at 28°C for 3 days. After incubation period, the fermentation broth was filtered using filter paper (Whatman No. 1). Silver nitrate (AgNO₃) of 1 mM concentration was added to the fermentation broth to promote the formation of AgNPs. The ratio of fermentation broth to AgNO₃ was kept at 1:9 (v/v), and the reaction mixture was incubated at 28°C for 48 h [5].

2.3 Characterization of biogenic AgNPs

The biosynthesized AgNPs were isolated by centrifugation at 10,000 rpm for 10 min, washed twice with sterile distilled water [13]. The crystals were dried and were identified by X-ray diffraction (XRD, Bruker AXS: D8-Discover). The morphology and elemental composition of the biogenic AgNPs were investigated using a field emission scanning electron microscopy (FE-SEM) equipped with energy dispersive X-ray spectroscopy analysis (FE-SEM-EDS, JEOL: JSM-7610F-Oxford: X-MAXN) [10,13].

2.4 Evaluation of antibacterial activity

The antibacterial activity of biogenic AgNPs was evaluated against human pathogenic bacteria. The tested bacteria were two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25623 and *Bacillus cereus* ATCC 6633) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 27853). The antibacterial activity was evaluated by the agar well diffusion method. The tested bacteria (0.5 McFarland turbidity standard) were spread on Mueller Hinton Agar (MHA; 2.0 g/l beef extract, 17.5 g/l casein hydrolysate, 1.5 g/l starch, 17 g/l agar), and then 3 mm holes were punched with a sterile glass capillary [14,15]. The different concentrations of AgNPs solution (20, 40, 60 and 80 µg/ml) were added to the agar well [7,8]. All the MHA plates were incubated at 37°C for 24 h. The magnitude of antibacterial activity was assessed by the diameter of inhibition zone relative to those of the positive control (*Streptomycin*) [16].

3. Results and discussion

The molecular identification of *Phomopsis* sp. based on 18S rRNA was identified as *Phomopsis* sp. strain GFPA2. The morphological characteristic of

biogenic crystals was examined by field emission scanning electron microscopy (FE-SEM). The scanning electron micrograph as shown in Figure 1, which was present a smooth and mostly spherical in shape. The nanoparticles were identified and analyzed by X-ray diffraction (XRD) and energy dispersive X-ray spectroscopy analysis (EDS). XRD patterns of biogenic synthesis crystals are presented in Figure 2 and they revealed that silver nanoparticles were produced by *Phomopsis* sp. GFPA2. Mycogenic crystals synthesized by *Phomopsis* sp. GFPA2 broth extract exhibited diffraction peak at 2θ values 38.11° , 44.27° , 64.42° and 77.47° , corresponding to (111), (200), (220) and (331) respectively. The spectrum of energy dispersive X-ray spectroscopy analysis demonstrated the chemical composition of the biogenic crystals. The spectrum showed a strong silver (Ag) signal at 3 KeV. Moreover, the silver elemental mapping confirms their presence in the biogenic nanoparticles (Figure 3). *Phomopsis* sp. GFPA2 was isolated from zinc mineral rock and exhibited high potential to produce AgNPs by reducing silver nitrate (AgNO_3). Nitrate reductase is an extracellular enzyme produced by many filamentous fungi and plays a key role in AgNPs biosynthesis [14]. The fungi were collected from polluted sites, where the fungi are already resistant

to high metal concentration and include species of *Aspergillus* and *Penicillium*, these fungi are excellent producers of metal nanoparticles at high metal concentration [6]. Many filamentous fungi are reported to have the ability to synthesize AgNPs including *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., *Fusarium* spp. and *Trichoderma* spp. [6,7,17-19]. However, it has not been reported that a *Phomopsis* sp. isolated from zinc mineral rock could synthesize AgNPs.

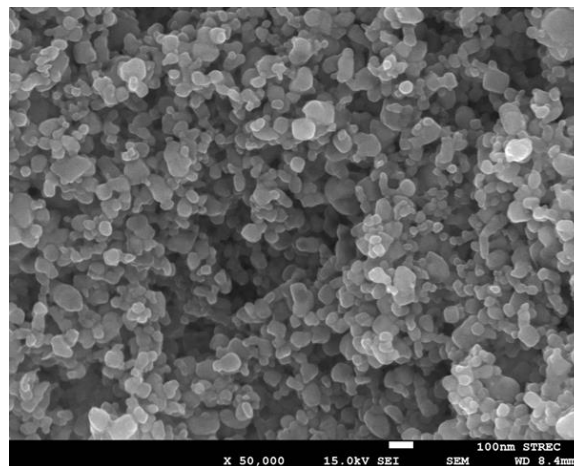


Figure 1. Scanning electron micrograph of biogenic crystals synthesized by *Phomopsis* sp. GFPA2 corresponds to 100 nm scale bar.

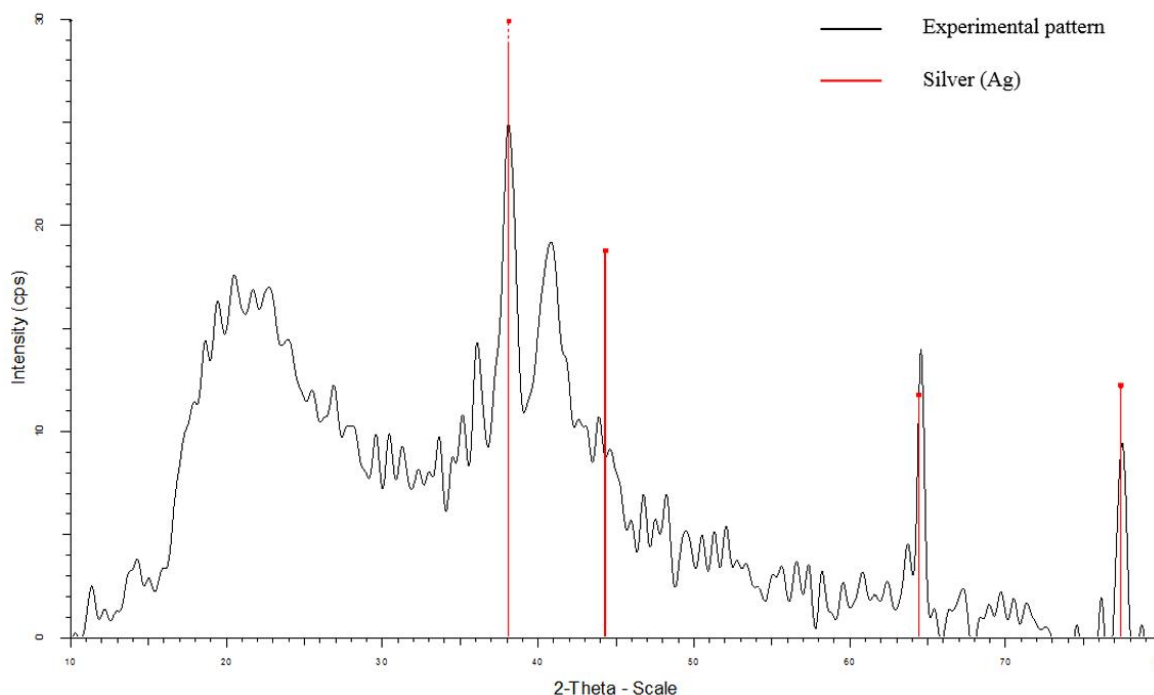


Figure 2. XRD pattern of biogenic AgNPs synthesized by *Phomopsis* sp. GFPA2.

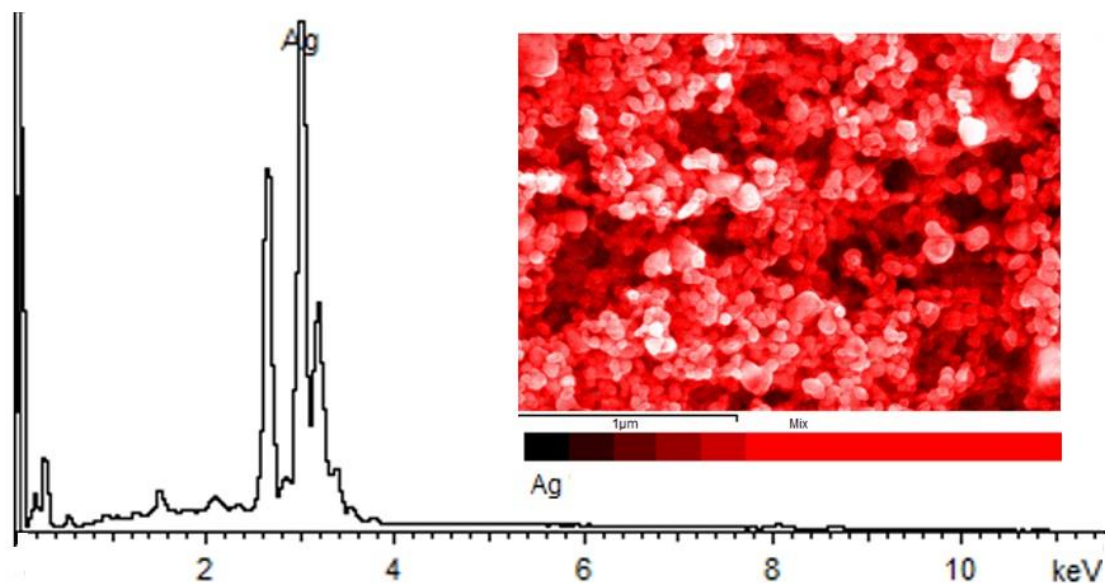


Figure 3. Spectrum analyzed by FE-SEM-EDS and elemental mapping analysis of biogenic AgNPs synthesized by *Phomopsis* sp. GFPA2

Table 1. Antibacterial activity of AgNPs against human pathogenic bacteria.

Bacterial strains	Gram	Inhibition zone diameter (mm)			
		20 $\mu\text{g/ml}$	40 $\mu\text{g/ml}$	60 $\mu\text{g/ml}$	80 $\mu\text{g/ml}$
<i>Staphylococcus aureus</i>	Positive	++	+++	+++	+++
<i>Bacillus cereus</i>	Positive	+++	+++	+++	+++
<i>Pseudomonas aeruginosa</i>	Negative	++	++	+++	+++
<i>Escherichia coli</i>	Negative	++	++	+++	+++

(-) no clear zone, (+) ≤ 5 mm, (++) ≤ 10 mm, (+++) > 10 mm

The antibacterial activity of biogenic AgNPs was investigated against various human pathogenic bacteria by the agar well diffusion method (Table 1). The result showed that the biogenic AgNPs can inhibit the growth of both Gram positive and Gram negative pathogenic bacteria. Maximum of halo zone (> 10 mm) was observed with all bacterial strains at 60 $\mu\text{g/ml}$, and *B. cereus* was more susceptible than other tested pathogenic bacteria. There are many reports for the antimicrobial activity of AgNPs. It has been reported that AgNPs exhibited excellent activity against both Gram positive and Gram negative bacteria [20]. The Gram positive and Gram negative bacteria have different susceptibility to AgNPs, probably because differences in their cell wall components and membranes [21]. AgNPs may attack with bacteria in several ways. For instance, AgNPs could release silver ions, these ions could affect the bacterial cell wall and disruption of membrane bound enzymes and lipid causing cell damage; denaturation of essential enzymes for adenosine triphosphate (ATP) synthesis and silver ions may interact with thiol

groups in protein, which induce the inactivation of the bacterial proteins [14,21,22]. Furthermore, the antimicrobial potential of AgNPs is influenced by size and shape, due to higher diffusion rate of AgNPs in the agar medium [2].

4. Conclusions

In conclusion, AgNPs were synthesized by *Phomopsis* sp. GFPA2. They were characterized and identified by field emission scanning electron microscopy (FE-SEM) equipped with energy dispersive X-ray spectroscopy (EDS) and X-ray powder diffraction (XRD). The results confirmed that the biogenic crystals were AgNPs, and they are mostly spherical in shape. The antibacterial activity of biogenic AgNPs revealed potential activity against *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli*. Therefore, the filamentous fungi mediated biosynthesis of AgNPs could be used as antibacterial agent against human pathogenic bacteria and also for the development of nanobiotechnology in future.

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