

Biosynthesis of Silver Nanoparticles from *Schizophyllum Commune* and *In-vitro* Antibacterial and Antifungal Activity Studies

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Abstract: *Silver possesses a good antimicrobial effect, which has been long used for treatment related to bacterial infections. The antimicrobial effects of silver in its nanoparticles form have not been clearly understood but it is expected to exhibit better effects than in its pure form. The use of microorganisms in the synthesis of silver nanoparticles emerges as an eco-friendly and sustainable approach. In this study, the antimicrobial effects of silver nanoparticles synthesised through reduction of silver nitrate with mycelia and culture supernatant of *Schizophyllum commune* is reported. The antimicrobial activities of silver nanoparticles are tested against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Aspergillus niger* and *Candida albicans*. Results showed that silver nanoparticles synthesised by interaction silver nitrate with mycelia fungus, when treated with *Staphylococcus aureus* and silver nanoparticles synthesised by interaction of silver nitrate and culture supernatant treated with *Staphylococcus epidermidis*, gave the largest inhibition area. However, no inhibition area was identified when silver nanoparticles were interacted with *Aspergillus niger*. Minimum inhibitory concentration and minimum bactericidal concentration/minimum fungicidal concentration are also conducted in this study. This study significantly showed that silver nanoparticles are powerful antibacterial and antifungal agent against various pathogens.*

Keywords: Silver nanoparticles, biosynthesis of particles, antibacterial, antifungal study, disc diffusion method

1. INTRODUCTION

The term bionanotechnology refers to the intersection of nanotechnology and biology.¹ In recent years, the application of bionanotechnology is gaining a tremendous impetus. One major area in bionanotechnology is the biosynthesis of nanoparticles such as Ti/Ni bimetallic nanoparticles,² alginate,³ magnesium, gold⁴ and silver.^{5–8} These noble nanoparticles serve as a platform for drug, gene delivery⁹ and cancer treatments¹⁰ for biomedicine applications. The characteristics and functionality of noble nanoparticles are closely related on their size, shape and controlled on monodispersity. Typical top-down synthesis methodologies involve complex chemical processes that use high temperature,

high pressure and finally the release of toxic pollutant to environment. Consequently, bottom-up biological nanoparticles synthesis has been explored as an alternative in developing environmentally friendly nanoparticles synthesis processes.¹¹

Among these noble nanoparticles, silver nanoparticles have been shown to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. With the emergence and increase of infectious diseases caused by various pathogenic microbial organisms which resist multiple antibiotics,¹² the interest of synthesising silver nanoparticles is acknowledged.¹³ For instance, silver nanoparticles have been studied to control bacterial growth in a variety of applications including dental work,¹⁴ catheters,¹⁵ prostheses¹⁶ and wound healing.¹⁷ It was also reported that silver nanoparticles have shown to be powerful biocides against bacteria,¹⁸⁻²¹ fungi^{22,23} and virii.²⁴⁻²⁷

Researchers have shown that silver nanoparticles like their pure form exhibit effective antimicrobial agent against various pathogenic microorganisms.^{18,28-32} Although antimicrobial effects of silver are renowned, bactericidal and fungicidal mechanism are still undetermined. It has been proposed that bactericidal mechanism happens due to the release of silver ions which generates reactive oxygen species (ROS)^{29,33,34} and causes the deposition of silver sulfur granules on the microbial cell wall,³⁵ whereas others reported that the interaction of silver ions (Ag^+) interact with vital enzymes such as NADH dehydrogenases resulting in the uncoupling of respiration from ATP synthesis hence causing their inactivation.^{36,37} It was also reported that exposure of Ag^+ to bacterial cells would suffer morphological changes such as cytoplasm shrinkage and detachment of cell wall membrane, DNA condensation and cell membrane degradation.³⁵ Although antifungal³⁸ and antiviral^{26,39} activities have been reported, the mechanistic action of silver on fungi and viruses are still unverified.

This study involves biosynthesis of AgNPs in three modes: intracellular, extracellular secretion from fungus mycelium and through culture-free supernatant of *Schizophyllum commune*. This study also relates the identification of antibacterial and antifungal properties of AgNPs produced by filamentous fungus strain *Schizophyllum commune*. Although there are literatures reporting on successful biosynthesis of AgNPs, however biosynthesis of AgNPs using fungi strains is limited. Filamentous fungi are of particular interest because they are able to secrete large amount of enzymes for the synthesis of AgNPs, and capable to produce highly stable nanoparticles.⁴⁰

As reported, biosynthesis of metallic nanoparticles by downstream process is much easier and is also environmentally friendly.⁴¹ The reduction of silver nitrate into silver nanoparticles is clearly visible when the sample solutions

change its colour from colourless to brown with increasing intensity.⁴² The brown colour of the solution was due to the excitation of AgNPs in surface plasmon vibration. This study would reveal the antibacterial and antifungal activities of AgNPs produced by white rot fungus and eventually enhance the utilisation fungi in improving the quality of life.

2. EXPERIMENTAL

2.1 White Rot Fungi

The fungus, *Schizophyllum commune* isolated from Malaysian rainforest provided by Forest Research Institute of Malaysia (FRIM) was used in this study. It was maintained and sub-cultured in malt extract agar at 4°C.

2.2 Preparation of Culture and Supernatants

60 mm diameter mycelium mat grown on agar surface was transferred aseptically into 250 ml deionised water supplemented with 0.1% Tween 80 as cell inoculum. Next, 10% cell inoculum was inoculated into 250 ml Erlenmeyer flask, containing 50 ml of complex medium composed of yeast extract (10 g l⁻¹), malt extract (10 g l⁻¹) and glucose (20 g l⁻¹). It was then allowed to grow for 96 h at 30°C (200 rpm). After 96 h of cultivation, mycelia pellets were separated from the culture broth by centrifugation at 4500 rpm, 10°C for 15 min. The pellets were washed thrice with deionised water.

2.3 Biosynthesis of Silver Nanoparticles

One-percent (w/v) of washed pellets and 1% (v/v) of culture supernatant were brought in contact with 100 ml aqueous silver nitrate (10⁻³ M) in 250 ml Erlenmeyer flask for synthesis of silver nanoparticles. The mixtures were then incubated in a rotary shaker at 200 rpm in the dark at 30°C. The bioreduction of silver nitrate into AgNPs was identified by the change of the colourless liquid to brown. Samples were collected mainly from 3 production modes: (i) bioreduction of silver ion by the tested fungi-secreted proteins in culture supernatant (CS); (ii) bioreduction of silver ion by absorption of silver atom on the mycelia pellet (MP); and (iii) bioreduction of silver ion from the mycelia pellet which was released into the silver nitrate (SN) solution.

2.4 Characterisation of Silver Nanoparticles

Change in colour from colourless to brown was observed in the silver nitrate solution. The UV-visible spectra, which represent surface resonance

absorption band of AgNPs was recorded using UV-visible spectrophotometer (Shidmazu UV-2550, US). The surface plasmon resonance spectra of AgNPs in samples were measured at resolution of 1 nm between 200–800 nm wavelengths.

Particle size analysis is done to determine the size distribution of synthesised AgNPs. AgNPs collected during the five days of incubation with silver nitrate were used for antimicrobial hence were taken for particle size determination. AgNPs collected were ultrasonicated (Transsonic Digital T490 DH, Elma, Singe, Germany) before being analysed by Zetasizer using dynamic light scattering non-invasive back scatter (NIBS®, Zetasizer Nano ZS, Malvern Instruments, Southborough, UK). The size and morphology of AgNPs were examined using a transmission electron microscopy (EFTEM, Zeiss Libra ® 120 Plus, US). The sample suspension was sonicated for 15 min to separate the agglomerated particles and make the solution homogeneous. Immediately after sonication, a drop of the suspension was sampled using a micropipette and the drop is placed on a film on a support grid. The sample was ready for examined under TEM after the solvent has completely evaporated.

AgNPs concentrations were analysed on a Shimadzu atomic absorption spectrophotometer (AA-6650). The light source used was a Hamamatsu Ag-hollow cathode lamp working at 10 mA current. Calibration curve between absorbance and concentration was prepared using 1N HNO₃ from 1 to 10 ppm. Concentrations of AgNPs were identified using the same method as experiment conducted on calibration curve.

2.5 Antibacterial and Antifungal Activities

Three bacteria strains used were *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Stock cultures were maintained on nutrient agar at 4°C. Other fungal strains, namely *Aspergillus niger* and *Candida albicans* were also used in this study and stock cultures were maintained on potato dextrose agar (PDA). All tested microorganisms were obtained from Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia (USM). *In-vitro* antibacterial and antifungal activity studies were carried out using Mueller Hinton media and malt extract media, respectively. The inocula used for this experiment were bacterial/fungal strains of 24-h old and the initial turbidity was adjusted with sterile medium to 0.5 MacFarland standards.

Samples from CS and SN were filtered using 0.2 µm membrane filters and subsequently sterilised by autoclaving at 121°C for 15 min. For MPS, mycelia were re-suspended in phosphate buffer saline (pH 7.4) and homogenised using a sonicator at a frequency of 8.5 Hz for 5 min before undergoing filtration and sterilisation as mentioned. AgNPs in three cases were collected after 5 days

of bioreduction between silver nitrate and the particles size were ranged 25.3–29.8 nm, 35.2–50.8 nm and 39.2–52.9 nm respectively for extracellular, culture-free supernatant and intracellular AgNPs synthesis.

Antibacterial and antifungal activities of silver nanoparticles synthesised by *Schizophyllum commune* were tested using disk diffusion method against pathogen: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Escherichia coli*. 20 µl of silver nanoparticles of different concentrations were transferred to 6 mm sterile blank disc and the disks were allowed to dry. Subsequently, the silver nanoparticles impregnated discs were placed on bacterial/fungal-cultured agar plate. Bacterial cultured agar plates were then incubated for 24 h at 37°C while fungal-cultured agar plates were incubated at 30°C for 48 h.

Determination of the minimum inhibitory concentration (MIC) was carried out using microdilution method. The MIC values were determined on 96-well microtitre plates. 100-µl silver nanoparticles of known concentration of different production mode produced throughout sampling period were transferred to broth in 96 well microtitre plates containing 100 µl of Mueller Hinton broth for bacterial or 100 µl Malt Extract for fungal assay. Later, 100 µl of tested microorganisms were inoculated and the microtitre plates were incubated for 24 h (bacteria) and 48 h (fungi). These tests were performed by the two-fold serial dilution method. After incubation period, optical density of cultures was measured at 595 nm. The MIC is defined as the lowest concentration of material that inhibits the growth of an organism⁴³ is represented by the absorbance. The higher the inhibition is, the lower absorbance that will be recorded.

Solution inside wells of microtitre plate in MIC study which did not show any growth of the bacteria or fungi after the stated incubation period were then inoculated into Mueller Hinton Agar and Malt Extract Agar for bacteria and fungi respectively. Plates were then incubated at 37°C for 24 h (bacteria) and 30°C for 48 h (fungi). The MBC and MFC were recorded as the minimum concentration of silver nanoparticles that did not tolerate any visible growth of tested microorganisms.⁴⁴

3. RESULTS AND DISCUSSION

Today, the development of environmental friendly nanoparticles has recently been of increasing interest to both academic and industrial sectors. Silver and its compound, which have been used as an antimicrobial agent in the past have recently received renewed interest. This is because some of bacterial strains have demonstrated resistance towards antibiotics. Hence, synthesising AgNPs

using biological method, which has higher antimicrobial activity are of particular concern. It was reported that extracts from bio-organisms such as enzymes/proteins, amino acids, polysaccharides and vitamins may act as reducing and capping agent in the AgNPs synthesis, making the synthesis is more environmentally friendly than chemical synthesis.^{45,46}

UV-visible spectra of silver nitrate solutions challenged with the fungus in 3 modes. A characteristic surface plasmon absorption band of AgNPs were observed at 420 nm. Apart from the AgNPs absorption band at 420, another absorption peak was observed above 280 nm in culture-free supernatant (CS) synthesis. This may be due to the tryptophan and tyrosine residue present in the protein to maintain AgNPs stability.⁴⁷ Proteins secreted during cultivation into the culture broth and residues attached to mycelium fungus. Hence, proteins peak above 280 nm were observed.

One of the important characteristics of AgNPs that determine the biocides activity is the particles size synthesised. As reported by Marambio-Jones and Hoek et al.,⁴⁸ smaller particles possessed larger surface area to volume ratio and hence efficient antimicrobial activity. Besides particle size, AgNPs concentration also plays an important role in determining the antimicrobial power. Table 1 shows the particle size of AgNPs and AgNPs concentration collected after 5 days of incubation with silver nitrate using dynamic light scattering technology and absorption atomic spectrophotometer respectively.

Table 1: Particle size and concentration of AgNPs synthesised by fungus *S. commune*.

Samples	Particle size (nm)	Concentration ($\mu\text{g ml}^{-1}$)
Extracellular, SN	25.3–29.8	26.87–46.55
Culture-free supernatant, CS	35.2–50.8	29.16–58.01
Intracellular, MPS	39.2–52.9	5.38–6.85

It was observed that AgNPs synthesised through SN gave the smaller average particle size of 25.3 nm followed by CS and MPS synthesis with average particle size of 35.2 nm and 39.2 nm respectively. From atomic absorption spectrophotometer analysis, it was identified that concentration of AgNPs synthesised through SN and CS are also higher than those MPS. Pal et al.⁴⁹ have discussed in their studies that bactericidal effect of nanoparticles is much dependent on the concentration of nanoparticles. The biocides power of respective size and concentration of AgNPs synthesised will be determined in antimicrobial study. In aqueous suspension, the mean hydrodynamic diameter of the nanoparticles is expected to be on the same range as TEM.⁵⁰ However, in this study the measured particle size using DLS is higher compared to the size measured by TEM. This is possibly due to agglomeration of AgNPs in water.

Figure 1 showed a TEM micrograph capture for AgNPs through SN synthesis. It further confirmed the agglomeration of AgNPs presence during the synthesis. However, it was identified that the average particles size of AgNPs was 32.08 nm. From TEM micrograph also, it was noticed that AgNPs synthesised were spherical in shape.

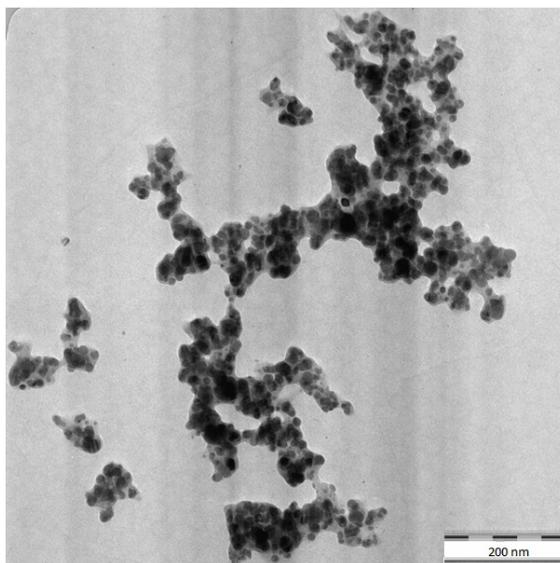


Figure 1: TEM micrograph for AgNPs synthesised through bioreduction of silver ion from the mycelia pellet, which was released into the silver nitrate solution (SN).

The present work of antimicrobial study of AgNPs mostly emphasised on pathogenic microorganisms: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. The results of inhibition study showed in Figure 2 that AgNPs produced through SN has the largest inhibition against *Staphylococcus aureus* which further support the work done by Shahverdi and co-workers that concluded AgNPs have an antimicrobial effect on *S. aureus*.³¹

It was also found that biosynthesis of AgNPs through CS has equal ability to inhibit *S. epidermidis* (20 mm). As shown in Table 2, AgNPs-synthesised MPS was unable to inhibit the growth of *Staphylococcus aureus*. Also, *Aspergillus niger* showed no antimicrobial effects towards AgNPs synthesised by *Schizophyllum commune* either SN, MPS or CS. Antimicrobial action of nanoparticles may differ according to the size of nanoparticles produced, and different species of bacteria or fungal produces varying sizes of nanoparticles.^{51,52} Negative control was also conducted with all tested pathogens

and the results revealed that AgNPs synthesised were the key element that acting as antibacterial and antifungal agents. According to Song *et al.*, the susceptibility of MRSA is due to the cell wall plasmolysis or inhibition of bacterial cell wall synthesis.⁵³

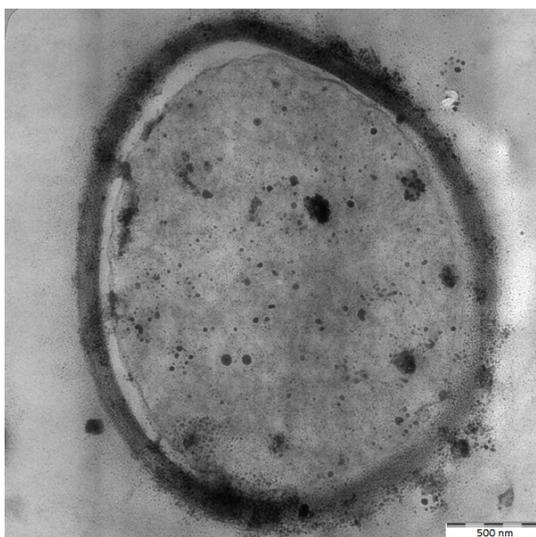


Figure 2: TEM micrograph for AgNPs synthesised through bioreduction of silver ion by absorption of silver atom on the mycelia pellet (MP).

Besides disc diffusion method, antibacterial activity of AgNPs was tested using the MIC assay and minimum bactericidal concentration (MBC). AgNPs collected from 3 modes of production were tested with *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, whereas minimum fungicidal concentration (MFC) were tested with *Candida albicans* and *Aspergillus niger*. Figure 3 shows an example of MIC and MBC/MFC assay using 96 well microtitre plate.

As reported by Sondi and Salopek-Sondi,²¹ as concentration of nanoparticles increased to MIC of the respective strains, no growth was observed. Table 2 also shows that the MIC for AgNPs were between 1.6–47, 0.25–7.03, 0.4–30 and 0.4–4.7 $\mu\text{g ml}^{-1}$ while MBC/MFC were between 10.5–37.63, 6.25–31.25, 8–30 and 6.63–18.75 $\mu\text{g ml}^{-1}$ for *S. aureus*, *S. epidermidis*, *E. coli* and *C. albicans* respectively. Similar results indicating that gram positive bacteria, *S. aureus*, which is more resistant to silver nanoparticles compared to gram negative *E. coli* is obtained.¹⁸

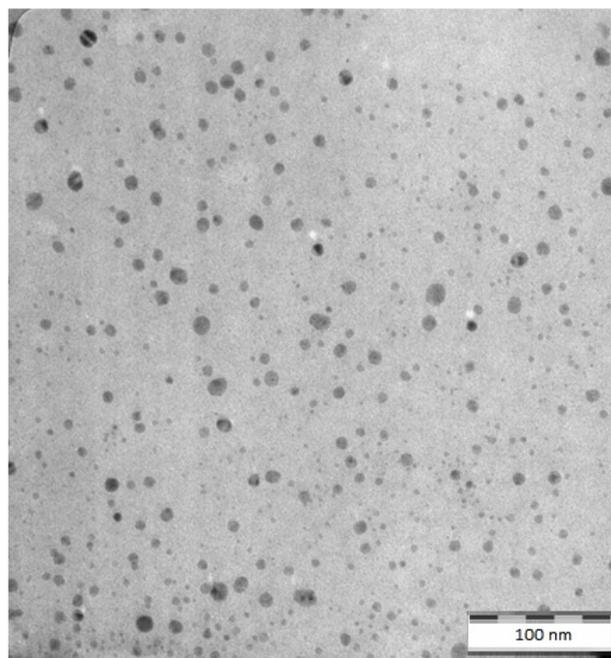


Figure 3: TEM micrograph for AgNPs synthesised through bioreduction of silver ion by the tested fungi-secreted proteins in culture supernatant (CS).

In agreement to the results published by various researchers, the size of AgNPs plays an important role in their antimicrobial activity.^{51,54} For instance, a more powerful biocide activity was observed in AgNPs through SN synthesis measuring 25.3–29.8 nm but not in those synthesised through CS and MPS synthesis. Martínez-Castañón et al.⁵⁵ reported that AgNPs of 29 nm showed MIC of $17\mu\text{g ml}^{-1}$ against *S. aureus*. In this study, we demonstrated approximately a two-fold increase in the activity against the same strain ($9.5\mu\text{g ml}^{-1}$). It was reported by Kvitek et al.⁵⁶ MIC of standard strains and strains isolated from clinical material are from $1.69\text{--}13.5\mu\text{g ml}^{-1}$. Although as reported by Gan,⁵⁷ agglomeration of AgNPs may have affected the bactericidal efficiency. In this study, the MIC of certain strains falls in the range as reported. Hence, the results showed that AgNPs have great bactericidal and fungicidal activity and comparable to the other AgNPs and nano-sized silver powders produced in the market.

SN and CS synthesis of AgNPs has shown to be powerful antimicrobial effects than AgNPs through MPS synthesis. It was reported that fungal culture supernatant can be used as an approach for monodisperse nanoparticles biosynthesis. Ability to form monodisperse nanoparticles is of concern for better biocides power. In future, several factors should take into consideration for nano-

scaled silver toxicity towards pathogen. Besides particles size, particle stability, particle shape and water chemistry are also important factors. It was reported that non-stable nanoparticles will tend to form aggregates and thus reducing the surface area ratio.⁵⁶ However, introducing high dosage of stabiliser would also reduce the surface of contact as most stabilisers are polymers and tend to form micelle around the nanoparticles. Morones et al.⁵¹ reported that particles with shapes <111> facets tend to have strongest antibacterial properties as <111> contain larger atom densities for better interaction. Particle suspension, solubility, particle size distribution and bacterial ability to face environmental stresses are much dependent on water chemistry. Hence, study on water chemistry is also important for better toxicity towards pathogen.

Although no sufficient information is available on the adverse effects of AgNPs on human health and environment, many AgNPs are small enough to penetrate into human skin into organs as well as deposition of metal on biological species such as fish, microorganisms etc.⁵⁸ Hence, assessment of environmental risks and human health associated with AgNPs is required before they are utilised in various applications.

Susceptibility of different pathogenic cultures against AgNPs synthesised in 3 modes was performed in this study. The results showed that AgNPs have great promise as antimicrobial agent against *S. aureus*, *S. epidermidis*, *E. coli* and *C. albicans*. In disc diffusion study, all culture except *A. niger* have superior antimicrobial action. MIC and MBC/MFC studies further suggested that AgNPs synthesised by *Schizophyllum commune* has comparable antimicrobial action with commercial antibiotics. The results here concludes that the production of silver nanoparticles using the Malaysian white rot fungi has a potential to be used in place of chemical-based antibiotics and because the mechanism of its anti-microbial properties is purely physical there are less chances of pathogenic bacteria developing a resistance against silver nanoparticles.

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