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Characterization of Ag Nanoparticles Produced by White-Rot Fungi and Its *in vitro* Antimicrobial Activities

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Abstracts

Biosynthesis of Ag nanoparticles (AgNPs) with diameter ranging 50 to 80 nm is achieved using the white-rot fungi, *Schizophyllum commune* and *Pycnoporus sanguineus*. AgNPs were formed when the fungal mycelia and the supernatant reacted with $AgNO_3$ after 5 days of incubation period. The synthesized nanoparticles were determined using analytical tools such as UV-vis spectrophotometer, and transmission electron microscopy. Results indicated that the UV-visible spectrum of the aqueous medium for *S. commune* and *P. sanguineus* showed a peak at 420 nm, which corresponded to the plasmon absorbance band of AgNPs. The antimicrobial properties of the synthesized AgNPs against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Aspergillus niger* and *Candida albicans* were also investigated using disc diffusion assay. Minimum inhibition concentration, minimum bacterial concentration and minimum fungicidal concentration are also identified using 96-well microtitre plate. It was found that AgNPs synthesized by the Malaysian white-rot fungi has the ability to act as an effective antibacterial agent.

Keywords: Ag nanoparticles; antimicrobial properties; disc diffusion assay, *Pycnoporus sanguineus*, *Schizophyllum commune*

Introduction

Bionanotechnology is a paradigm shift where emerging technologies in biology, chemistry, physics, mathematics, and engineering are integrated to explore benefits of the nano-world towards the betterment of the society (1). In recent years, the application of bionanotechnology is gaining tremendous impetus. Research in bionanotechnology has shown to provide reliable, eco-friendly processes for synthesis of noble nanomaterials. One major area in bionanotechnology is the biosynthesis of nanoparticles such as Ti/Ni bimetallic nanoparticles (2), alginate(3), magnesium, gold (4) and silver(5). Biological syntheses of nanoparticles using various biological systems such as yeast, bacteria, fungi, and plant extract have been reported. Among them, biosynthesis of silver nanoparticles (AgNPs) has been extensively studied due to its antimicrobial properties (5, 6-13) .

Besides bacteria, fungi are also utilized for biosynthesis of AgNPs such as *Aspergillus fumigates* (12), *Cladosporium cladosporioides* (14), *Fusarium oxysporium* (13)*Pleutorus sajor caju* (15), *Penicillium brevicompactum* (16) and *Clostoridium versicolor* (17). Recently, filamentous fungi are of concern as they possessed distinctive advantages over the bacterial strains. Filamentous nature of fungi results in high tolerance towards metals, high wall-binding capacity and intracellular metal uptake capabilities compared to the bacterial strains (18). Efficacy of AgNPs biosynthesis was reported due to reductase action or by electron shuttle quinones or both (19). In fact, it is shown that the presence of hydrogenase and nitrate reductase are the essential elements for metal reduction (19,20) .

It is well known that silver possess good antimicrobial activities. The use of silver is well documented in scientific literatures and it is known to have been used by the Persians, by ancient Phoenicians, Greeks, Romans and Egyptians for

the treatment related to bacterial infections (21-22). Recent reports designated that not only bacteria were susceptible to AgNPs, but also fungi (23) and HIV virus (24). Elechiguerra *et al.* (24) reported that AgNPs of size 1-10 nm have the capability to prevent virus from attaching to the host tissue cells. Hence, the high antibacterial, antifungal and antivirus activity of AgNPs in comparison with silver and its compounds is believed to have arisen from their high surface to volume ratio of nanoparticles size. Although the antimicrobial effects of silver are renowned, the mechanistic action is still undetermined. Several proposals have been developed to elucidate the inactivation effects of silver on bacteria. It has been reported that inactivation of cells is due to the reaction of the heavy metal with proteins that have thiol (-SH) group (25). It is also discovered that the release of silver ion generated reactive oxygen species (ROS) (26-28), and caused the deposition of silver granules on microbial cell wall.

In the present investigation, we explore the potential of white rot fungi *S. commune* and *P. sanguineus* to synthesize AgNPs and their antimicrobial activities against various pathogens. The antimicrobial effects are quantified based on the inhibition zone measured in disc diffusion assay. Minimum inhibition concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) are also determined in microtitre plates.

Materials and Methods

Biosynthesis of silver nanoparticles

Malaysian white-rot fungi *S. commune and P. sanguineus* used in the current study were obtained from the Forest Research Institute of Malaysia (FRIM), Kepong, Selangor, Malaysia. Cultures were cultivated in 50 ml of nutrient media comprised of (w/v): 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.01% $(NH_4)_2SO_4$, 0.06% yeast extract and 1% glucose at 30^{o}C, 200 rpm (Labwit ZHWY-1102 C Incubator Shaker), for 3 days. The pH of the media is adjusted to 5.6$\pm$0.2 using 1M HCl. The harvested mycelia were separated by centrifugation at 4500 rpm (Eppendorf Centrifuge 4702 R) for 15 minutes. Mycelia pellets were washed twice with deionized water before inoculated into 0.001M of $AgNO_3$ solution. Later, 1% (w/v) mycelia pellets and 1% (v/v) culture supernatant obtained were thereafter inoculated into 0.001M of $AgNO_3$, and incubated at 30^{o}C, 200 rpm for 5 days in darkness. A control containing 0.001 M of silver nitrate was also prepared. All experiments are carried out in triplicates and samples were drawn daily throughout the 5 days of incubation period.

Three modes of bioreduction of silver nanoparticles are monitored, namely (i) bioreduction of silver ion by fungus-secreted proteins in culture supernatant (CS), (ii) bioreduction of silver ion by absorption silver atom on mycelia pellet (MPS), and (iii) bioreduction of silver ion with mycelia pellet released into silver nitrate solution (SN). Samples obtained from MPS are re-suspended in deionized water and homogenized using a probe-top sonicator (Misonix Sonicator 3000) at 8.5 Hertz for 5 minutes. The mixtures of cell debris and silver nanoparticles (AgNPs) were centrifuged at 4500 rpm (Eppendorf Centrifuge 4702 R) for 20 minutes. Supernatants containing AgNPs (MPS) were used for further analysis. All samples are sterilized at 121^{o}C for 15 minutes before being used for antimicrobial studies.

Antimicrobial assay

Three bacteria strains namely *E. coli, S. aureus* and *S. epidermidis* and two fungal strains *A. niger* and *C. albicans* were obtained from the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia. All stock cultures were maintained in nutrient agar and potato dextrose agar for bacteria and fungi strains respectively. Antibacterial and antifungal assay were carried out using Müeller Hinton and malt extract medium. Both bacteria and fungi inoculums were prepared to 0.5 McFarland standards before performing antimicrobial assay.

Disc diffusion assay

AgNPs synthesized by *S. commune* and *P. sanguineus* were tested for antimicrobial activity by disk diffusion assay against the pathogenic bacteria *E. coli*, (Gram-negative) *S. aureus* and *S epidermidis* (Gram-positive), yeast *C. albicans*, and fungi *A. niger*. The pure cultures of bacteria and fungi were sub-cultured on nutrient agar, and were swabbed uniformly onto the individual plates using sterile cotton swabs. 20µL of AgNPs solution from SN and MPS were impregnated onto 6 mm sterile blank disc and allowed to dry before placing onto the culture plate. Bacterial-cultured plates were incubated at 37^{o}C for 24 hours, while the fungal-cultured plates are incubated at 30^{o}C for 48 hours, accordingly. Antibacterial and antifungal activities were measured by the diameter of inhibition zone. Antibiotics such as Amoxicillum, Cephalosporin, Doxycyline, Metronidazole, Ketoconazole and Tetracyline were also used to evaluate the antimicrobial activities of antibiotics as compared to AgNPs.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) assay was carried out using the microdilution method with slight modifications. 100µL of AgNPs of known concentration produced throughout sampling period were transferred into 96 well microtitre plates containing 100µL of Müeller Hinton broth for bacterial or 100µL of Malt Extract broth for fungal assay. Dilutions were performed by the two-fold serial dilution method. Later, 100µL of tested microorganisms were inoculated to all wells and the microtitre plates were incubated at 30^{O}C, 48 hours and 37^{O}C, 24 hours for fungi and bacteria, respectively. After the incubation period, optical densities of cultures are measured at 595 nm using a microplate reader (Biorad Model 680). The minimum inhibitory concentration was determined as the lowest concentration of AgNPs that inhibits the growth of microorganism (29) which is represented by the absorbance.

Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) assay

The inhibition of bacterial growth was evaluated by comparing the optical density (OD) of samples with normal growth in the control wells that were not treated with AgNPs. According to Dulger (30), minimum bactericidal concentration (MBC) refers to the minimum concentration yielding negative subculture . MBC was determined by taking samples that did not show any growth of bacteria from the microtitre wells and inoculating them into Müeller Hinton agar and incubated at 37^{O}C for 24 h. Similarly, minimum fungicidal concentration (MFC) was determined by inoculating samples that did not show visible growth to malt extract agar and incubated at 30^{O}C for 48 h. The lowest concentration where no growth is observed is recorded as MFC.

Characterization

The bioreduction of Ag^{+} in sample solutions SN, CS and MPS were monitored using UV-visible spectrophotometer (Shimadzu UV-2550, US). The surface plasmon resonance spectra of AgNPs in samples were measured at resolution of 1nm between 200-800 nm wavelengths. The morphology such as size and shape of AgNPs were identified using transmission electron microscope (EFTEM, Zeiss Libra® 120 Plus, US). The samples were dropped onto 300 mesh of carbon coated copper grid and allowed to air dry prior to measurements. Intracellular synthesis of AgNPs was identified by using ultramicrotome to cut the mycelia into nano-sized pieces before viewing under the TEM. Fixation was performed using method as described by McDowell and Trump (31).

Particle size analysis was performed to determine the size distribution of synthesized AgNPs. AgNPs from SN and MPS were ultrasonicated (Transsonic Digital T490 DH, Elma, Singe, Germany) and filtered using 0.2µm PTFE membrane syringe filter. The average particle size of suspended nanoparticles was obtained by dynamic light scattering non-invasive back scatter (NIBS®) technology (Zetasizer Nano ZS, Malvern Instruments, Southborough, UK).

AgNPs concentration was analyzed on a Shimadzu atomic absorption spectrophotometer (AA-6650). The light source used was from a Hamamatsu Ag-hollow cathode lamp working at operating current 10 mA. Calibration curve was prepared using standard silver in 1N HNO_3 solutions with different concentrations ranging from 1 to 100 ppm. The solutions are aspirated into an air/acetylene flame and the absorbance versus concentration (ppm) was plotted. Concentrations of AgNPs produced were quantified using the same method as experiment conducted to perform the calibration curve.

Results and Discussion

Characterization of AgNPs

The formation of AgNPs can be primarily distinguished after the bioreduction of $AgNO_3$ with mycelia (MPS, SN) and culture supernatant (CS) through visible observation of colour change from colourless to pale yellow or brown. Earlier work has shown that the formation of yellowish brown solution was due to the presence of AgNPs as a result of excitation of the surface plasmon vibration (32). It has been deduced that, the bioreduction of silver ions occurred due to the presence of reducing agents such as enzyme reductase, and electron shuttle quinines (20,33).

According to Henglein [34], UV-vis is commonly used as an analytical tool in analyzing AgNPs because free electrons in metal nanoparticles gave rise to surface plasmon resonance absorption bands. Moreover, it was also being reported that ultraviolet region is the region of absorption for bulk plasmon frequency in various metal nanoparticles. Hence, bioreduction of AgNPs can be identified using UV-vis spectrum. In fact UV-vis spectral analysis has shown to be capable in identifying the size evolution of AgNPs based on localized surface plasmon resonance band exhibiting at different wavelengths (34-36). **Figure 1** showed surface plasmon absorption band for AgNPs synthesis by both (a) *P. sanguineus* and, (b) *S. commune* which occurred at 420 nm.

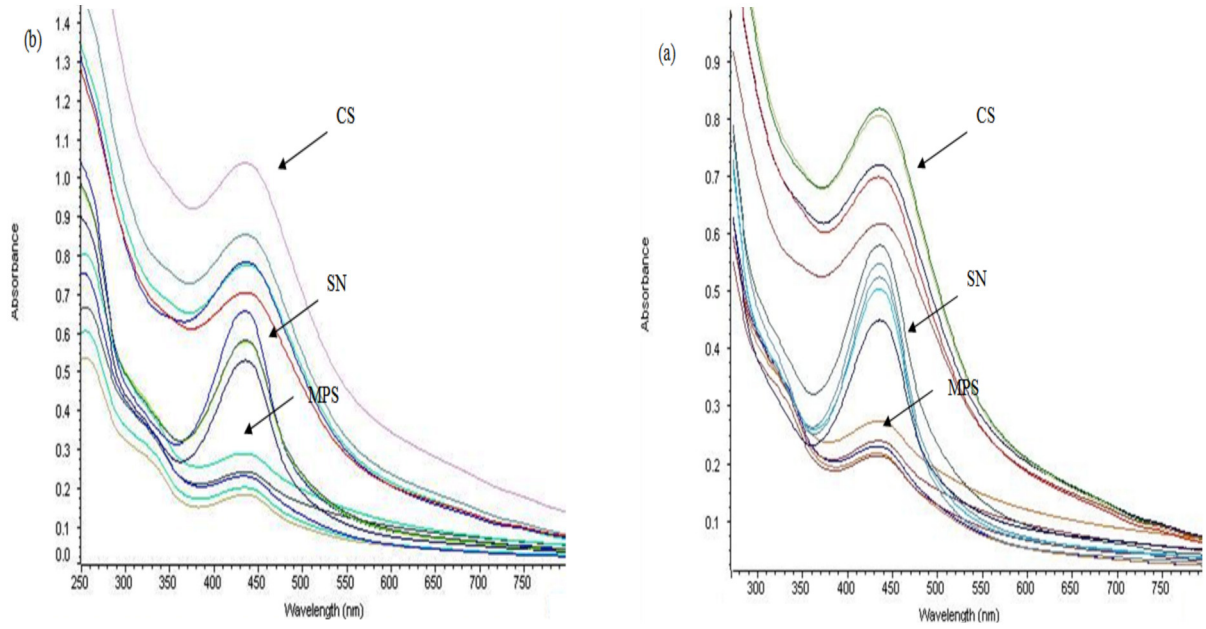

Figure 1. UV-vis absorption spectrum of AgNPs produced by (a) *Pycnoporus sanguineus* (b) *Schizophyllum commune.*

It was observed that as the incubation time increases, absorbance for AgNPs in both *P. sanguineus* and *S. commune* also increases. The peak observed for AgNPs in SN was also observed to be more pronounced than peak for AgNPs in MPS and CS, showing extracellular secretion is more pronounced than intracellular synthesis and synthesis via culture supernatant. Kaviya *et al.*(37) reported that the nanoparticles formation was mainly due to particles nucleation and growth mechanism. Thus showing that nucleation of AgNPs from $AgNO_3$ was more enunciated. As the peak increased, concentration of AgNPs increased as shown in **Table 1.** When absorbance increases, more AgNPs were produced hence corresponding to the concentration of AgNPs.

Transmission electron microscopy (TEM) images of AgNPs synthesized by *S. commune* and *P. sanguineus* were recorded with EFTEM, Zeiss Libra® 120 Plus microscope. Intact AgNPs having an average particles size of 15.8 nm and 20.9 nm for *P. sanguineus* (**Figure 2a**) and *S. commune* (**Figure 2b**) were observed, respectively. Intracellular secretion of AgNPs by *P. sanguineus* and *S. commune* were shown in Figure 3 (a) and (b) respectively. It was noticed that AgNPs are found at the circumference of the fungi with average sizes of 61.0 nm and 30.1 nm for *P. sanguineus* and *S. commune* respectively. The particles size of AgNPs produced were further confirmed using the particle size analyzer Zetasizer Nano ZS. It was also observed that AgNPs produced are slightly elon-

Table 1. Characteristics of AgNPs produced by Pycnoporus *sanguineus* and *Schizophyllum commune* over 5 days of incubation period.

Fungi	Sample	Particle size (nm)	Concentration (µg/ml)
P. sanguineus	SN	50.8 - 59.43	11.27 – 12..09
	CS	70.21 - 88.61	11.42 – 12.60
	MPS	39.5 - 46.34	4.26 – 6.22
S. commune	SN	25.3 – 29.8	26.87 – 46.55
	CS	35.2 – 50.8	29.16 - 58.01
	MPS	39.2 – 52.9	5.38 – 6.85

SN, MPS and CS denoted silver nanoparticles produced extraceullularly, intracellularly and through culture supernatant respectively.

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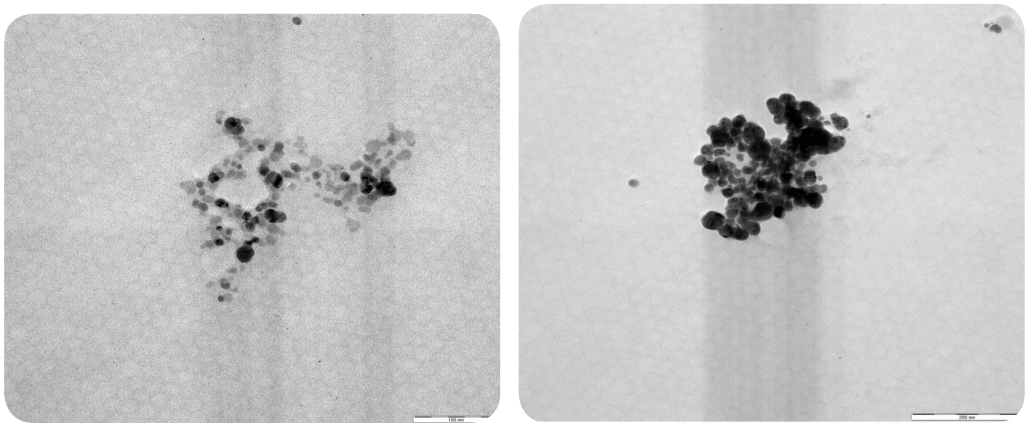

Figure 2. HRTEM micrograph of AgNPs synthesized extracellularly by (a) *P.sanguineus* (b) *S. commune*

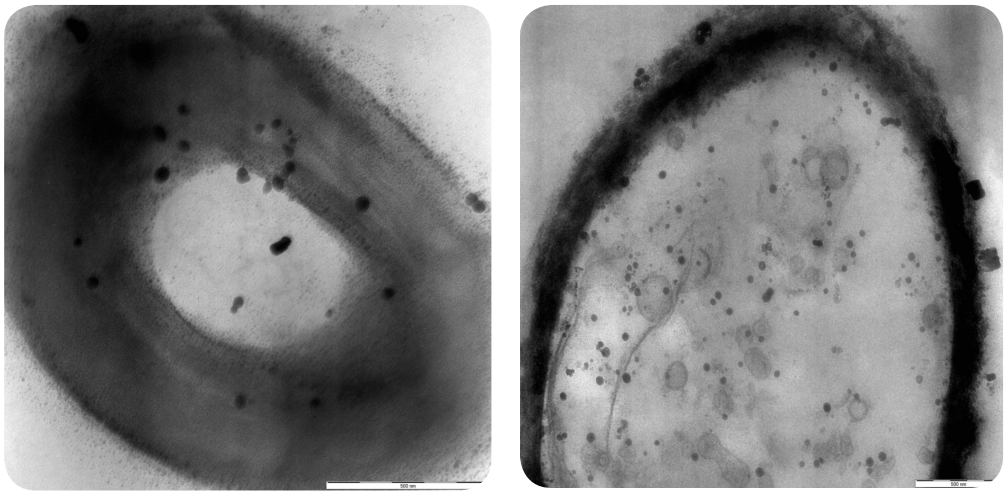

Figure 3. HRTEM micrograph of AgNPs synthesized intracellularly by (a) *P.sanguineus* (b) *S. commune*

gated in shape and tend to agglomerate. However the rate of agglomeration of AgNPs produced by *S. commune* was shown to be slower. **Table 1** showed the average particles size and silver concentration of samples SN, CS and MPS for the tested white rot fungi. It was observed that the average particle size obtained using the Zetasizer was similar with that obtained from TEM. AgNPs produced by *S. commune* extracellularly has the smallest diameters (25.3 nm – 29.8 nm) compared to those synthesized intracellularly (39.2 nm – 52.9 nm) and through culture supernatant (35.2mm -50.8mm) in all cases. This was probably due to agglomeration of AgNPs on the fungi surface.

Antimicrobial and antifungal activity

Silver has an important antimicrobial effect and has been used since the Roman times. These antimicrobial effects were reported to be dependent on superficial contact hence inhibiting the enzymatic respiratory system of microbes and altering DNA synthesis (38-39). However with the aid of nanotechnology in generating AgNPs, the antimicrobial effects of AgNPs are enhanced, and there is a resurgence of AgNPs as a powerful antimicrobial agent. The antimicrobial effects of AgNPs produced by white rot fungi were conducted. The Gram positive bacteria (*S. aureus* and *S. epidermidis*), Gram negative bacteria (*E. coli*) and fungi (*A. niger* and *C. albicans)* were used to test the antimicrobial activities of AgNPs. According to a few researchers, AgNPs were capable to inhibit *E.coli* (41) *S. aureus* (40) MRSA, and MRSE (42)

In the present study, the antimicrobial activities of the AgNPs were tested using disc diffusion assay. Results showed that antibacterial activity of AgNPs produced by MPS of *S. commune* against *S. aureus* was most effective with 2.0 cm inhibition zone, followed by SN 1.8 cm, SN and MPS from *P. sanguineus* with inhibition zone 1.7 cm and 1.4 cm, respectively. Inhibition ability against *S. epidermidis* was the highest in AgNPs produced in SN followed by those made by MPS and CS. A similar trend was observed in inhibition studies of *E. coli.* The susceptibility of *S. aureus*, *S. epidermidis* and *E. coli* may be due to wall plasmolysis, separation of cytoplasm from their cell wall or inhibition of bacterial cell wall synthesis

Table 2. Mean zone of inhibition against tested pathogens.

Pathogens	Mean zone of inhibition (mm)							
	Antibiotics					Samples	White rot fungi	
	A	C	D	K	T		SC	PS
Staphylococcus aureus	2.1	1	0.8	ND	0.7	SN	2.0	1.4
						MPS	Nil	Nil
						CS	1.8	1.7
Staphylococcus epidermidis	1.1	0.6	0.6	ND	0.6	SN	1.0	0.8
						MPS	1.9	1.5
						CS	2.0	1.8
Escherichia coli	1.2	1.2	0.6	ND	1.0	SN	1.1	1.8
						MPS	0.9	0.8
						CS	1.8	1.6
Candida albicans	1.5	0.9	0.9	0.9	0.7	SN	1.9	0.8
						MPS	1.9	0.9
						CS	1.2	1.0
Aspergillus niger	ND	ND	ND	Nil	ND	SN	Nil	Nil
						MPS	Nil	Nil
						CS	Nil	Nil

Table 3. MIC, MBC/MFC (µg/ml ±SD) of AgNPs showing antimicrobial activities.

Fungi	Sample	MIC (µg/ml + SD)					MBC/MFC ((µg/ml + SD)				
		Fungi		Bacteria			Fungi		Bacteria		
		AN	CA	SE	SA	EC	AN	CA	SE	SA	EC
PS	SN	0.04+0.0	0.27+0.3	0.03+0.0	4.27+0.2	0.09+0.2	11.27+0.3	2.845+0.2	12.6+0.0	11.68+0.3	0.18+0.1
	MPS	11.2+0.3	5.69+0.5	1.42+0.2	1.47+0.2	1.41+0.3	Nil	11.65+0.2	12.09+0.0	11.27+0.	11.86+0.3
	CS	1.41+0.0	1.43+0.1	1.43+0.0	0.37+0.1	0.71+0.0	Nil	4.28+0.2	6.22+0.0	0.55+0.2	0.09+0.0
SC	SN	Nil	0.4+0.2	1.17+0.1	1.6+0.2	2.6+0.1	Nil	6.63+0.1	9.38+0.2	10.5+0.2	10.5+0.2
	MPS	Nil	4.7+0.3	7.03+1.2	9.5+0.5	30+1.2	Nil	18.75+0.8	31.25+0.5	37.63+2.3	30+0.2
	CS	Nil	3.13+0.2	0.25+0.0	47+2.1	0.4+0.0	Nil	15+0.6	6.25+0.2	47+1.5	8+1.0

Fungi: AN, *A. niger*; *CA, C. albicans.*
Bacteria: *SE, S. epidermidis, SA, S. aureus; EC, E.coli*

(43). However, in antifungal activity assay, it was identified that *A. niger* was not susceptible to AgNPs. This is probably due to the filamentous and spore producing nature of the fungus. However, the AgNPs showed good antifungal effect against *C. albicans* and the results were similar with findings from Sadhasivam *et al.*(44). According to Tyagi (45) such condition may be attribute to the disruption of plasma lemma and structural disorganization of the cytoplasm caused by deposition of AgNPs. **Table 2** showed that AgNPs synthesized by both *P. sanguineus* and *S. commune* has comparable antimicrobial effects of commercial antibiotics. It was also observed that AgNPs produced extracellularly and through culture supernatant have better antimicrobial effects compare to AgNPs synthesized intracellularly.

White rot fungi *SC*, *S. commune* and *PS*, *P. sanguineus*

Antibiotics A, C, D, K and T refer to antibiotics amoxicillin (25µg/ml), cephalexin (25µg/ml), doxycyline (10µg/ml), ketoconazole (20µg/ml) and tetracyline (25µg/ml) respectively. ND, not determined; Nil, not positive results shwon.

MICs for AgNPs synthesized by *P. sanguineus* and *S. commune* were shown in **Table 3.** It was observed that the antimicrobial activity of AgNPs produced in SN was more effective than those made in CS and MPS. The results also showed that AgNPs synthesized by the fungi are more effective antimicrobial agent as the MIC values lay between 0.03 – 47_µg/ml and MBC/MFC values of 0.09 – 47 µg/ml in all cases. This was in agreement with the results obtained by Kim (46) who indicated that the Gram positive bacteria, *S. auerus* was more resistant to silver nanoparticles as compared to Gram negative *E. coli*. Thus, it showed that the tested white rot fungi produced AgNPs have great bactericidal and fungicidal activities.

Conclusion

Malaysian white rot fungi have been effectively used for the biosynthesis of AgNPs using an eco-friendly approach. The AgNPs produced were characterized by UV-vis spectroscopy, TEM and Zetasizer analysis, which support the stability of AgNPs. Consequently, AgNPs also showed potent antibacterial and antifungal activities against *S. aureus*, *S. epidermidis*, *E. coli*, and *C. albicans* in disc diffusion and MIC, MBC/MFC tests. It is implicit that the inactivation of bacteria or fungi may be due to deposition of silver granules on microbial cell wall caused by generation of reactive oxygen species (ROS). However the actual inhibition mechanism of AgNPs against microbes is still unknown and needs to be further elucidated.

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