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Biosynthesis and structural characterization of Ag nanoparticles from white rot fungi

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ABSTRACT

Five species of white rot fungi were screened for their capability to synthesize Ag nanoparticles (AgNPs). Three modes of AgNP bioreduction were developed. *Pycnoporus sanguineus* is found as a potential candidate for the synthesis of AgNPs with a yield at 98.9%. The synthesized AgNPs were characterized using UV–vis spectroscopy, DLS, FTIR, TEM, and SEM. Results showed that AgNP absorption band was located at a peak of 420 nm. Both the SEM and TEM confirmed that the formation of AgNPs were mainly spherical with average diameters of 52.8–103.3 nm. The signals of silver atoms' presence in the mycelium were observed by SEM-EDS spectrum.

1. Introduction

Since the last decade, white-rot fungi have been used intensively for bioremediation as they have the capability to transform or mineralize a wide range of environmentally hazardous compounds [1,2] through oxidative enzymatic mechanisms. The discovery of ligninolytic enzymes such as lignin peroxidase (LiP), manganese-peroxidase (MnP), horseradish-peroxidase (HrP) and laccase from white-rot fungi has triggered a biochemical research on lignin biodegradation [3–7]. However, the mechanistic action of these fungal secreted enzymes is non-specific, non-stereospecific and extracellular in nature [8]. Although the application of these fungi involved mainly lignin degradation, it is known to degrade a wide range of hydrocarbons such as poly-aromatic hydrocarbons (PAHs) [9], chlorinated aromatic hydrocarbons (CAHs) [10], polycyclic aromatics, polychlorinated biphenols [11], polychlorinated dibenzo-p-dioxins, pesticides DDT, lindane and azo dyes [5,6]. As stated by a few researchers, the most common white-rot fungi used in bioremediation are *Phanerochaete chrysosporium* [10], *Schizophyllum commune* [12] and *Pycnoporus sanguineus* [13,14].

Although the white rot fungi are commonly used in bioremediation, it was reported that these fungi can also serve as a platform for the bioreduction of Ag nanoparticles (AgNPs) [15]. Bioreduction is reported to be a biomimetic synthesis which utilized natural or biological principle, and implementing it into engineering [15]. The process involved absorption of metal ions onto the microbial surface by functional groups on the cell wall, and indirectly reduced by reducing sugars from hydrolysate of polysaccharides of the biomass into metal atoms [16]. Similar concept is applied on the adsorption of metal ions by fungal strain where adsorption of metals can be classified as: extracellular accumulation, cell surface sorption and intracellular accumulation [17,18]. Duran et al. in 2005 in their research mentioned that the action of enzyme reduction or electron shuttle quinones in various microbes was responsible for the biomimetic process to occur. The role of the microbes in the bioreduction of metal is to provide a multitude of nucleation for establishing a highly dispersed nanoparticle system, slow down agglomeration, and provide a viscous medium [19]. In fact, the white-rot fungus, *P. chrysosporium* which is commonly used in bioremediation is reported to be an effective bioreduction agent for the synthesis of AgNPs. Thus showing that the fungi have the ability to reduce silver into AgNPs.

Recently, AgNPs serve as noble metals and have drawn a considerable attention. Decreasing particle dimensions into nanosizes has manifested on the physical properties over bulk materials [20]. It is well known that silver has been historically recognized as a powerful biocide against bacteria, viruses and fungi. The particles are highly dispersed and posed a higher surface area which indirectly intensified antimicrobial properties and served as an effective antimicrobial agent [21]. In order to prepare these nanoparticles which serve as an effective antimicrobial agent over antibiotics, controlling the sizes and shapes as well as the stability is important [22]. Among various microorganisms screened, the white rot fungi namely *Pleurotus sajor caju* [23], *Clostridium versicolor* [24] and *P. chrysosporium* [15] produced stable AgNPs when challenged with silver nitrate in an aqueous medium. Though the capability of *P. sanguineus* for its AgNP production is less reported, it could be a potential route to produce an effective bioreduction agent using this fungus. This paper examines the capabilities of the Malaysian white rot fungi (*P. sanguineus*, *S. commune*, *Lentinus sajor caju*, *Trametes feei*, and *Trametes pocas*) that are isolated from the Malaysian rainforest to exhibit AgNPs in order to produce particles of smaller sizes. The differences in the modes of AgNP bioreduction and its characterization will also be investigated.

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2. Experimental

2.1. White rot fungi

Five Malaysian white-rot fungi, namely *P. sanguineus*, *S. commune*, *L. sajor caju*, *T. feei*, and *T. pocas* are used in this study. They are obtained from the Forest Research Institute of Malaysia (FRIM), Kepong, Selangor, Malaysia. Cultures were maintained in the malt extract agar medium.

2.2. Biosynthesis of silver nanoparticles

The mycelial mat of the tested white rot fungi were mixed with 0.1% (v/v) Tween 80 before they were transferred aseptically into the cultivation flasks containing 50 mL of nutrient media. Cultivation media comprised (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. The pH of the media was adjusted to 5.6 ± 0.2 using 1 M HCl and 1 M NaOH. The mixture was then incubated at 30 °C, 200 rpm for 3 days. The harvested mycelia and culture broth were separated by centrifugation at 4500 rpm for 15 min; the supernatant and the pellet were then used for the synthesis of AgNPs.

The mycelia pellets were washed thrice with deionized water. The washed pellets (1%w/v) and the culture supernatant (1%v/v) were then treated with a 0.001 M silver nitrate solution separately. Both mixtures were thereafter incubated at 30 °C in darkness at 200 rpm for 5 days. Three modes of AgNP bioreduction were conducted namely, (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 solution (SN), respectively. A control experiment containing only 0.001 M of silver nitrate solution was also performed. All experiments were carried out in triplicates and samples were drawn everyday throughout the 5 days of incubation.

Samples obtained from the MP mode were re-suspended in deionized water and homogenized using a probe-top sonicator at 8.5 Hz for 5 min. The mixture of cells debris and AgNPs containing mixtures was centrifuged at 4500 rpm for 20 min. Supernatants containing AgNPs (MPS) were then used for further analysis.

2.3. Determination of protein concentration

Alkaline copper reagent was freshly prepared by mixing 1 mL of 1% (w/v) $CuSO_4$ and 1 mL of 2% (w/v) Na tartrate with 98 mL of 2% (w/v) Na_2CO_3 in 0.1 M NaOH. 1.2 mL of protein sample was added into 6 mL of alkaline copper reagent. The mixture was mixed vigorously and the reaction was allowed to take place for 10 min. Later, 0.3 mL of Folin–Ciocalteu reagent was added by swirling, and then let it stand for another 30 min. The absorbance of the sample was recorded at A_{500}.

2.4. Characterization of silver nanoparticles

The bioreduction of Ag^+ ion in sample solutions CS, SN and MPS was monitored by absorbance measurement using a double beam UV–vis spectrophotometer (Shimadzu UV-2550, US). The spectra of the surface plasmon resonance of AgNPs in the samples were measured at a resolution of 1 nm between 200 and 800 nm wavelengths. Subsequently, the average sizes of AgNPs in the sample solutions were measured using dynamic light scattering (DLS) a non-invasive back scatter (NIBS®) technology (Zetasizer Nano ZS, Malvern Instruments, Southborough, UK). All sample solutions were ultrasonicated (Transsonic Digital T 490 DH, Elma, Singe, Germany) and filtered using a 0.2 μm PTFE membrane syringe filter before analyses. 0.001 M of $AgNO_3$ solution was used as the blank for both UV–vis and nanosize analyses. AgNP concentrations were analyzed on a Shimadzu atomic absorption spectrophotometer (AA-6650). The light source used was a Hamamatsu Ag-hollow cathode lamp working at 10 mA current. Concentrations of AgNPs were identified using the same method as the experiment conducted on calibration curves. Concentrations of AgNPs were used to calculate the yield of the AgNPs produced based on Eq. (1) as shown.

$$\text{Yield} = \frac{[\text{AgNPs produced}]}{[\text{AgNPs predicted}]} \times 100\% \quad (1)$$

where

[AgNPs produced] is obtained from atomic absorption spectroscopy, and

[AgNPs predicted] is obtained based on stoichiometry calculation.

The morphological properties of AgNPs produced by the tested fungal species were examined using a field emission scanning electron microscopy (FESEM) equipped with an energy dispersive X-ray (EDX) spectrometer (Zeiss Supra® 35VP, US), and a transmission electron microscopy (EFTEM, Zeiss Libra® 120 Plus, US). The micrographs of a spotted area were recorded and their corresponding EDX spectra were recorded by focusing on a cluster of particles. To examine the formation of AgNPs on the mycelial mats, freeze-dried mycelial mats were mounted on specimen stubs and coated with gold/palladium using a Bio-Rad Polaran Division SEM sputter coater, and examined under SEM operated at 10 kV at a magnification of 10,000×. For TEM, sample solutions CS and SN were dropped onto 300 mesh of carbon coated copper grid, and then allowed to dry prior to measurements. While fresh mycelial mats (MP) after culture were fixed using a McDowell–Trump fixative prepared in a 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h. Later, it was post-fixed in a 1% (v/v) osmium tetraoxide for 2 h. The mycelia were dehydrated through a graded series of ethanol (50%, 75%, 95% and 100%; 95% and 100% levels were applied twice for 15 and 30 min, respectively) and 100% acetone twice for 10 min. After dehydration, the mycelium was infiltrated in a Spurr's mix resin in a rotator overnight. Infiltration in a new change of the Spurr's mix resin was performed for an interval of 6 h for 3 days. After the third day, samples were embedded at 60 °C. Subsequently, an ultramicrotomy process was performed on the embedded blocks using a Sorvall Ultramicrotome MT 5000 before they are being observed under the EFTEM.

The infrared (IR) spectra of *P. sanguineus* and *S. commune* after the bioreduction of silver were obtained using the Fourier Transform Infrared Spectrometer (Shimadzu, IRPrestige-21). Reflectance technique was used in identifying the IR spectra. Potassium bromide (KBr) discs were prepared by grinding mycelium of *P. sanguineus* and *S. commune* with KBr in a ratio of 1:100 and compressing the mixture into a transparent disc of 13 mm in diameter. The transparent discs were directly placed into the infrared spectrometer in the wave number range of 400–4000 cm^{-1}.

2.5. Antimicrobial activity

Minimum inhibitory concentration (MIC) assay was carried out using the microdilution method as described by Qi et al. [25], with a slight modification. The Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and Gram positive bacteria (*Staphylococcus epidermidis* and *Staphylococcus aureus*) were used as the tested microorganisms. 100 μL of AgNPs of known concentrations were transferred into 96 well microtiter plates containing 100 μL of Müeller Hinton broth. Dilutions were performed by the two-fold serial dilution method. Later, 100 μL of the tested bacteria was inoculated to all wells and the microtiter plates were incubated at 37 °C for 24 h. After the incubation period, the 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