RESEARCH PAPER | Nanoscience | Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

Biosynthesis, characterisation and anti-bacterial effect of *Pleurotus florida*-mediated silver nanoparticles

KEYWORDS: Sliver nanoparticles, *Pleurotus florida*, antimicrobial activity

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ABSTRACT *Microbial synthesis of nanoparticles is becoming a widely prevalent area of research due to the affordability and ecological advantages of the method over traditional ones. In the present study, production of metal nanoparticles by using edible basidiomycete Pleurotus florida was performed. The cell free extract of P. florida was able to reduce silver ions in aqueous system to silver hydrosol in a single-step method, at room temperature using diffused light as sole source of energy. The synthesized silver nanoparticles exhibited the characteristic absorption peak for surface plasmon resonance lying between 450 to 500 nm of wavelength. Transmission Electron Micrography of the Ag hydrosol showed spherical nanoparticles capped with a gelatinous matrix, with size ranging from 10-50 nm. However, Dynamic Light Scattering showed the hydrodynamic diameters of the nanoparticles to be in the range of 85-226 nm. Functional group characterization by Fourier Transform Infra Red spectroscopy confirmed the presence of proteins in silver sol. The changes or shifts in absorption peaks of the proteins indicated attainment of novel conformations in proteins on capping the nanoparticles. The antimicrobial efficacy of the synthesized silver sols was tested against various gram negative and positive pathogenic bacteria, which indicated the sols to be moderately effective.*

Introduction:

Metallic silver has long been recognized as strong antimicrobial agent (Liau *et al* 1997 Jiang *et al* 2004, Klasen, 2000). Silver-based compounds such as silver nitrate and silver sulfadiazine have been extensively used in many bactericidal applications, in topical ointments and creams containing silver to prevent infection of burns and open wounds and medical devices and implants prepared with silver-impregnated polymers. In addition, silver-containing consumer products such as colloidal silver gel and silver-embedded fabrics are now used in sporting equipment (Nomiya *et al* 2004, Silver and Gupta 1998, Becker 1999, Silver 2003)

Metal particles in nanometre size range exhibit physical properties that are different from both the ion and the bulk material. This makes them exhibit remarkable properties such as increased catalytic activity due to morphologies with highly active facets (Yacaman *et al* 2001, Somorjai 2004, Haruta 1997, Doraiswamy and Marks 1996, Iijima and Ichihashi 1986, Ajayan and Marks 1989). The development of new resistant strains of bacteria to current antibiotics (Kyriacou *et al* 2004) has become a serious problem in public health; therefore, there is a strong incentive to develop new bactericides (Sondi and Salopek-Sondi 2004). Bactericidal activity of silver nanoparticles has attracted a lot of interest (Li *et al*. 2005; Panac˘ek *et al*. 2006). The antibacterial activity of silver species has been well known since ancient times (Shrivastava *et al*. 2007) and it has been demonstrated that, in low concentrations, silver is non toxic to human cells (Zhang *et al*. 2003; Pal *et al*. 2007). The actual bactericide mechanism of silver nanoparticles is not well known. Some researchers support the idea that silver species release Ag^+ ions and they interact with the thiol groups in bacteria proteins, affecting the replication of DNA (Marini *et al*. 2007). It has also been reported that Ag^+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane (Holt and Bard 2005). Silver nanoparticles interactions with bacteria are dependent on the size and shape of the nanoparticles (Pana´c˘ek *et al*.2006; Morones *et al*.2005; Pal *et al*. 2007).

Chemical approaches are the most popular for the production of nanoparticles. However, some chemical methods can not avoid the use of toxic chemicals in the synthesis protocol. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals.

A number of unicellular and multicellular organisms produce inorganic materials either intra- or extracellularly. Some of the more well-known examples include magnetotactic bacteria (which synthesize magnetite nanoparticles or greigite nanocrystallites) (Blakemore 1982, Spring and Schleifer 1994), diatoms (which synthesize siliceous materials) (Simkiss and Wilbur 1989), and S-layer bacteria (which produce gypsum and calcium carbonate layers) (Mann 1993).

Biological methods of nanoparticles synthesis using microorganism (Klaus *et al* 1999, Konishi *et al* 2007, Nair and Pardeep 2002), enzyme (Willner *et al* 2006 Priyadarshini 2013), and plant or plant extract (Shankar 2004, Sathyavathi *et al* 2010 Ahmad *et al* 2014) have been suggested as possible eco-friendly alternatives to chemical and physical methods.

To enlarge the scope of biological agents in the synthesis of nanomaterials, we demonstrate herein that the fruit bodies of edible fungus Pleurotus florida when exposed to aqueous $AgNO_3$ solution, causes the reduction of the metal ions and formation of silver nanoparticles.

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MATERIAL AND METHODS:

Materials: *Pleurotus florida* was cultivated on wheat straw using standard cultivation protocol approved for Punjab conditions at Mushroom Research Complex, Punjab Agricultural University, Ludhiana. Analytical grade $AgNO_3$ was obtained from Hi-Media Laboratory chemicals. Bacterial cultures *Escherichia coli, Yersinia enteroclolitica, Aeromonas* spp., *Klebsiella pneumonia* and *Staphylococcus aureus* were obtained from Department of Microbiology, Punjab Agricultural University, Ludhiana and *Salmonella typhi* was procured from Department of Microbiology, Christian Medical College and Hospital, Ludhiana.

Synthesis of Silver Nanoparticles:

Fruit body (5 g) of *Pleurotus florida* was washed with distilled water thrice to remove any dirt or impurities, crushed in pestle and mortar, added into 500 Ml of Sterile distilled water and was kept on orbital shaker at 200 RPM overnight to collect clear. To the clear solution $AgNO_3$ was added to make a final concentration of 1 Mmol. This solution was divided into 7 aliquots. One was kept in dark and used as control. The rest were kept in diffused sunlight for 6, 12, 18, 24, 30 and 36 h, respectively for light catalyzed nanoparticle synthesis.

Characterization of Silver Nanoparticles:

UV-Vis Spectroscopy:

The samples were subjected to spectroscopy in the wavelength ranging from 200 to 900 nm using Elico SL 218 Double Beam UV-Vis Spectrophotometer. The absorbance was plotted against the wavelength to observe the surface plasmon resonance peaks.

Transmission Electron Microscopy:

TEM measurements were performed on a transmission electron microscope Hitachi H-7650, working at 80 kV. Samples were prepared by placing 20 µl of the sol a formvar-coated copper grid.

Dynamic Light Scattering Analysis:

The size of nanoparticles was confirmed using Beckman Coulter Delsa™ Nano HC Particle Analyzer.

Fourier Transform Infra-Red Spectroscopy:

Samples for FT-IR Analysis were prepared by acetone precipitation of proteins. The dry acetone powder was crushed with activated KBr and pressed into disc shaped pellet using hydraulic pellet maker. FT-IR spectra were recorded for unreacted mushroom extract and sol containing silver nano particles with a Nicolet 6700 FT-IR (Thermo Scientific, USA) using OMNIC Spectra Software. Spectra were recorded from 4,000 to 400 cm^{-1}. Before calibration, the FT-IR reflectance data were mean centred and baseline corrected.

Antibacterial Assay:

The silver nanoparticles synthesised from *P. florida* at different exposure times to light were tested for their antibacterial effects against pathogenic bacteria *Escherichia coli*, *Yersinia enteroclolitica*, *Aeromonas spp.*, *Klebsiella pneumonia*, *Salmonella typhi* and *Staphylococcus aureus*. The bacteria were grown on Nutrient Agar plates by spread plate technique. Wells of 0.5 mm diameter were prepared using a sterile cork-borer. These wells were loaded with 100 µl of nanoparticle sols. Silver nitrate (1mM), gentamicin, Tetracycline, Kanamycin and Ampicillin (100 µg/ml concentration) were used as control. The plates were incubated at 37°C for 24 hrs and the zones of clearance were measured for each sample.

RESULTS AND DISCUSSION

Charecterization of *Pleurotus florida* mediated silver nanoparticles

The absorption spectra of the silver nanoparticles are presented in Fig.1. All the samples showed SPR peaks in the range of 450-500 nm, characteristic for silver nanoparticles (Lee and El-Sayed 2006). Transmission Electron Micrographs (Fig. 3) showed the spherical shape of nanoparticles which appeared in clusters, embedded in a gelatinous, probably proteinaceous substance. This matrix also prevented the particles from sticking together in direct contact, hence stabilizing the sols. Similar observations have been made by Ahmed *et al* (2003) and Saiffudin *et al* (2009) in silver nanoparticles synthesized using *Fusarium oxyporum* and *Bacillus subtilis* respectively. ("Fig 3 about here")

DLS data (Fig. 4) showed that the particle size decreased with increase in time of exposure to light, but after 24 hours slight increase in size is observed again. This could be due to agglomeration of particles. At 6h exposure time, particle size distribution is broad and average diameter of particles is 1955 nm. This result is contradictory with the Transmission Electron Micrographs, showing particles mostly in the size range of 10-50 nm. This could be due to presence of crystals of bulk silver nitrate in the sol. In 12h sample, average particle diameter is 94.3 nm. The particle size distribution narrows down significantly in 12h sample and remains similar in samples hereafter. Particle diameter is 101 nm at 18h reaction time. Particle diameter comes out to be minimum (85.2 nm) in 24h sample and increases to 212 nm and 226 nm in 30 and 36h samples respectively. These results were complemented by Transmission Electron Micrographs of corresponding samples.

The *P. florida* unreacted protein sample and protein sample conjugated with nanoparticles showed peak at 1623 cm^{-1} and 1637 cm^{-1} for β sheet structure in Amide I region during FT-IR analysis (Fig. 5). Unreacted proteins show another band at 1686 cm^{-1} which may indicate β sheet or β turn structure. It also showed a band at 1317 cm^{-1} indicating α helix structure in amide III region. There is an increase in number of peaks as well as increase in frequencies for amide IV-OCN bending and Amide V- out of plane NH bending. It shows increase in H-bonding upon reaction with nanoparticles.

Antibacterial Assay:

Silver nanoparticles synthesized at 6h exposure time showed maximum antibacterial activity against *Yersinia enteroclolitica*, Aeromonas spp., *Klebsiella pneumonia, Salmonella* typhi and *Staphylococcus* aureus. This could be either (i) due to additive effect of unreacted $AgNO_3$ along with silver nanoparticles, as at 6h exposure time conversion into nano-silver was not complete, or (ii) presence of smaller nanoparticles due to initial stage of aggregation. This indicates that sols at initial stages of biosynthesis having a combination of bulk and nano-silver could be more effective bactericidal agents than either one of these in pure form. Inhibitory effect is less than antibiotic control Gentamicin and Tetracycline but more as compared to kanamycin, ampicillin and bulk $AgNO_3$. Kanamycin, however, had more inhibitory effect than nanoparticles against *Yersinia enterocolitica*. Fayaz *et al* (2010) showed that antibacterial activities of ampicillin, kanamycin, erythromycin, and chloramphenicol were increased in the presence of AgNPs. The highest enhancing effect was observed for ampicillin. No inhibitory effects were observed against E. coli while $AgNO^3$ solution showed significant effect against

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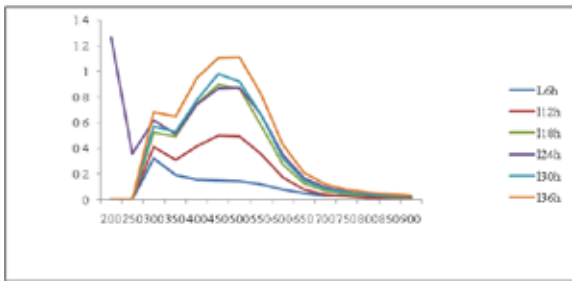
the same. Morones *et al* 2005 showed that silver particles interacting directly with *E. coli* had mean size of 16 nm with a standard deviation of 8 nm. The larger size of the bio-synthesized nanoparticles could be responsible for absence of activity against *E. coli*. Sondi and Salopek-Sondi (2004) and Kim *et al* (2007) reported that the antimicrobial activity of silver nanoparticles on gram-negative as well as gram-postive bacteria was dependent on the concentration of silver nanoparticles.

To conclude we can say that synthesis of silver nanoparticles using *Pleurotus florida* is cost effective and environment friendly method. The nanoparticles synthesized are stable due to protein capping and they also have significant bactericidal effects.

Table 1 Antibacterial activity of silver nanoparticles against various pathogens measured as diameter of inhibition zones.

Antimicrobial sample	Diameter of Zone of clearance (mm)					
	E. coli	Salmonella typhi	Staphylococcus aureus	Klebsiella pneumiae	Aeromonas spp.	Yersinia entero-colitica
Gentamicin	18	21	32	25	31	33
Kanamycin	0	11	-	21	-	-
Tetracycline	21	30	24	27	23	23
Ampicillin	27	-	-	15	-	-
Bulk $AgNO_3$	12	12	17	13	20	20
Nanoparticles at 6h	-	16	23	18	21	22
Nanoparticles at 12h	-	12	20	12	16	17
Nanoparticles at 18h	-	11	17	13	17	18
Nanoparticles at 24h	-	10	15	13	16	16
Nanoparticles at 30h	-	11	18	13	16	19
Nanoparticles at 36h	-	10	13	12	13	16

Fig 1. Absorption spectra of silver solun with solution as a function of time of exposure of *P. florida* extract and 1 mM $AgNO_3$ *solution* to visible light



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