Research Article

BIOSYNTHESIS, PARTIAL CHARACTERIZATION AND ANTIMICROBIAL ACTIVITIES OF SILVER NANOPARTICLES FROM PLEUROTUS SPECIES

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ABSTRACT
Integration of microorganisms to nanotechnology is one of the key issues in nanoscience research. There is growing need to develop environmentally benign metal nanoparticle synthesis process that do not use toxic chemicals in the synthesis protocols to avoid adverse effects in medical applications. The use of microorganisms in the synthesis of nanoparticles emerges as an eco-friendly approach. In the present study, biosynthesis of silver nanoparticles using edible mushrooms Viz., Pleurotus pulmonarius, Pleurotus djamor and Hypsizygus (pleurotus)ulumarius, partial characterization of silver nanoparticle and its antimicrobial study have been reported. It was found that the aqueous silver ions of 1-5mM concentration were reduced to silver metal nanoparticles by nitrate dependent reeducate and a shuttle quinone extracellular process when treated with fungal supernatant of Pleurotus pulmonarius, in 24hrs(2mM); Pleurotus djamor in 48hrs(5mM) and Hypsizygus ulmarius in 48hrs(3mM). Partial characterization of synthesized silver nanoparticles, investigated by UV-VIS Spectroscopy showed increased productivity at 386nm with sharp and intense surface plasmon. It is found that silver nanoparticles are bound to protein through carboxylate group of aminoacid residues. The synthesized silver nanoparticle showed high bactericidal activity against Gram-positive (Staphylococcus aureus) and Gram-negative (Pseudomonas aeruginosa) bacteria . The mechanism of the Ag NP bactericidal activity is discussed in terms of Ag NP interaction with the cell membranes of bacteria.

KEY WORDS: Silver nanoparticles, Pleurotus pulmonarius, Pleurotus djamor Hypsizygus(pleurotus)ulumarius, antimicrobial activity, extracellular synthesis.

INTRODUCTION
Nanotechnology is an emerging field in the area of interdisciplinary research, especially in biotechnology. The synthesis of silver nanomaterials/nanoparticles is extensively studied by using chemical and physical methods, but the development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. Biological synthesis process provides a wide range of environmentally acceptable methodology, low cost production and minimum period.

The microorganism probably play a role in providing a multitude of nucleation centers and establish conditions for obtaining high disperse nanoparticles system. They slow down or entirely prevent agitation by immobilizing the particle and providing a viscous medium (Sun et al., 2002)

Among various metal nanoparticles, biologically synthesized silver nanoparticles has many applications that includes catalysts in chemical reactions (Kumar et al., 2003), biolabelling, antimicrobial agent, electrical batteries (Peto et al., 2002), drug and gene delivery (Rao et al., 2001), DNA sequencing (Tripathi, 2003), staining pigments in glasses and ceramics and optical receptors (Klaus-Joerger, 2001, Krolikowska, 2003). It is known that, a large number of organisms, both unicellular and multi cellular are able to produce inorganic nanoparticles either intracellular or extracellular.

The use of fungi in the synthesis of nanoparticles is relatively a recent addition to the list of microorganisms. The use of fungi is potentially exciting since they secrete large amount of enzymes and their biomass are easy to handle. Using this unique property of fungi, it may be used to grow nanoparticles of silver as reported by Mukherjee et al., 2001b; Sastry et al., 2003; Lloyd, 2003; Bhainsa et al., 2006 and Vigneshwaran et al., 2006.

Mushrooms are known to have anti-inflammatory, cardiovascular, antitumor, antiviral, antibacterial, hepatoprotective and hypotensive activities in biological systems (Wasser et al.,1999; Rai et al., 2005; Bernardshaw, 2005). This indicates that, mushrooms could be valuable sources of antioxidant...
(Chen et al., 2006) and antitumor compounds (Cho et al., 2003; White et al., 2002). Studies on edible mushrooms have revealed valuable activities related to biological response modifications. Chemopreventive, chemotherapeutic, immunomodulatory, hypoglycemic and hypocholesteremic effects (Lee et al., 2006). Mushrooms characteristically contain many different bioactive compounds with diverse biological activity, the content and bioactivity of these compounds depend on how the mushroom is prepared and consumed (Chang 1996).

In the present investigation, biosynthesis, partial characterization of silver nanoparticles by using edible mushrooms viz., Pleurotus pulmonarius, Pleurotus djamor and Hypsizygus (pleurotus)ulmarius and their antimicrobial activities was studied.

MATERIALS AND METHODS

All the chemicals used were of analytical grade. The media components like glucose, malt extract powder, malt agar, Mueller Hinton agar, silver nitrate were obtained from Hi-Media chemicals, Mumbai (India).

Source of fungal mycelia and culture maintenance

The white rot fungal mycelia of Pleurotus pulmonarius (Plate1,2a,2b), Pleurotus djamor (Plate3,4a,4b) and Hypsizygus ulmarius (Plate5,6a,6b) were obtained from Directorate of Mushroom Research, Chambaghat, Solan, Himachal Pradesh. The mycelia were maintained at 4°C on malt agar slants. Fungal filtrate used for biosynthesis, experiments were grown aerobically in liquid media containing 5g/l malt extract powder and 10g/l glucose. The fungal strain was inoculated in the autoclaved media under sterilized and static conditions and was allowed to grow for 120 hrs at 25°C (150 rpm) with pH of 6.0 as reported by Rashmisani et al., 2009.

Biosynthesis of silver nanoparticle

The cell free filtrate was obtained by filtration of Pleurotus pulmonarius, Pleurotus djamor and
Hypsizygus ulmarius using Whatmann No.1 filter paper. For the synthesis of silver nanoparticles 20ml of the cell free filtrate was brought in contact with different milimolar concentration in 150 ml Erlen Meyer flask and agitated at 25°C in dark conditions under normal pH. Simultaneously control without silver ions was also run along with the experimental flasks (Nithya and Raghunathan, 2009).

Characterization of Silver Nanoparticles

**UV-VIS studies**
The reduction of silver ions was monitored by UV-VIS spectrum at 24hrs, 48hrs and 72hrs time interval by drawing 1cm³ of the sample. The absorbance was recorded at a resolution of 0.5nm at 350-800nm using UV-VIS spectrophotometer (Elico, UV-VIS SL 191).

**Bacterial susceptibility to nanosilver**
Susceptibility of the synthesised silver nanoparticles was done by well diffusion method as reported by Nithya and Raghunathan 2009, Nelson Duran et al., 2007 on Mueller Hinton agar plates with Gram positive- Staphylococcus aureus and gram negative- Pseudomonas aeruginosa organisms. The zone of inhibition was calculated for its antimicrobial studies.

**RESULTS**

**Formation of nanosilver**
Pleurotus pulmonarius depicts the color change from pale yellow to mild light brown at 2mM for 24hrs with further decreases in intensity of color at different mM concentrations of silver nitrate solutions. (Plates 7a and 7b). Pleurotus djamor shows the color change from pale yellow to light pinch brown at 5mM for 48hrs that further decreased in intensity of color with different mM concentrations of silver nitrate solutions. (Plates 8a and 8b)

Hypsizygus ulmarius when observed shows the color change from pale yellow to pale brown at 3mM for 48hrs with maximum intensity when compared to different mM concentrations of silver nitrate solutions with decrease in intensity of color. (Plates 9a and 9b).

**UV-VIS spectral analysis**
UV–visible spectra of different fungal supernatent treated with 1-5 different mM Concentration of silver nitrate solutions showed a characteristic surface Plasmon absorption band at 386 nm. Further incubation, lead to decrease or increase in intensity indicating complete or maximum reduction of silver ions.

Characteristic surface plasmon absorption band at 386 nm was observed at 2mM concentration for 24hrs (Graph 1) with optical density of 1.28 (Graph 2) for Pleurotus pulmonarius (Table 1); Pleurotus djamor showed at 5mM concentration for 48hrs (Graph 3) with optical density of 0.98 (Graph 4 and Table 2) and at 3mM concentration for 48hrs (Graph 5) with optical density of 1.222 (Graph 6) for Hypsizygus ulmarius (Table 3).
Graph 1: Depicts record of silver nanoparticles synthesised by *Pleurotus pulmonarius* at various time Intervals against optical density (Y-axis) and sample concentration (X-axis).

Graph 2: Transverse surface Plasmon absorbance band at 386nm of *Pleurotus pulmonarius* with absorbance at (Y-axis) and wavelength (X-axis)

Table 1: O.D values of *Pleurotus pulmonarius*in different mM concentration at different time intervals.

Graph 3: Depicts record of silver nanoparticles synthesized by *Pleurotus djamor* at various time intervals against optical density (Y-axis) and sample concentration (X-axis)
Graph 4: Transverse surface Plasmon absorbance band at 386nm of Pleurotus djamor with absorbance (Y-axis) and wavelength (X-axis)

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>24hrs</th>
<th>48hrs</th>
<th>72hrs</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.15</td>
<td>0.28</td>
<td>0.31</td>
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<tr>
<td>2</td>
<td>0.39</td>
<td><strong>0.45</strong></td>
<td>0.52</td>
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<tr>
<td>3</td>
<td>0.60</td>
<td>0.68</td>
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<td>4</td>
<td>0.79</td>
<td>0.81</td>
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<tr>
<td>5</td>
<td>0.89</td>
<td><strong>0.98</strong></td>
<td>0.78</td>
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Table 2: O.D values Pleurotus djamor in different mM concentration at different time intervals.

Graph 5: Depicts record of silver nanoparticles synthesised by Hypsizygus ulmarius at various time Intervals against optical density (Y-axis) and sample concentration (X-axis).

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>24hrs</th>
<th>48hrs</th>
<th>72hrs</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
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<td>5</td>
<td>0.46</td>
<td>0.42</td>
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Table 3: O.D values Hypsizygus ulmarius in different mM concentration at different time intervals.

Graph 6: Transverse surface Plasmon absorbance band at 386nm of Hypsizygus ulmarius with absorbance (Y-axis) and wavelength (X-axis)
Bacterial susceptibility to synthesized nanosilver
The antibacterial activity of synthesised silver nanoparticles of *Pleurotus pulmonarius*, *Pleurotus djamor* and *Hypsizygus ulmarius* on gram positive and gram negative organisms are recorded with clear zone of inhibition in centimeter, with ampicillin 10mcg/disc as control.

*Pleurotus pulmonarius* showed inhibition zone (Plates 10a and 10b) of 0.15 cm in *Staphylococcus aureus* at 0.5mg/ml and 0.17 cm in *Pseudomonas aeruginosa* at 0.4mg/ml along with control 10mcg/disc with inhibition diameter of 0.12 cm and 0.01 cm. (Graph 7, Table 4).

In *Pleurotus djamor* maximum inhibition zone (Plates 11a, 11b) was seen in *Staphylococcus aureus* at a concentration 0.4mg/ml along with control 10mcg/disc with inhibition diameter of 0.14 cm and 0.12 cm whereas *Pseudomonas aeruginosa* recorded at a concentration of 0.2mg/ml along with control 10mcg/disc with inhibition diameter of 0.19 cm and 0.02 cm (Graph 8, Table 5).

*Hypsizygus ulmarius* showed inhibition zone (Plates 12a, 12b) of 0.16 cm in *Staphylococcus aureus* at 0.3mg/ml and 0.16 cm in *Pseudomonas aeruginosa* at 0.05mg/ml along with control 10mcg/disc with inhibition diameter of 0.12 cm and 0.03 cm. (Graph 9, Table 6).
Table 4: Record of inhibition zone (cm) by synthesized silver nanoparticles of *Pleurotus pulmonarius* at different concentration with respect to *S.aureus* and *P.aeruginosa*

<table>
<thead>
<tr>
<th>Concentration of sample mg/ml</th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Pseudomonas aeruginosa</em></th>
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<tbody>
<tr>
<td>Control</td>
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<td>0.5</td>
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Table 4: Record of inhibition zone (cm) by synthesized silver nanoparticles of *Pleurotus pulmonarius* at different concentration with respect to *S.aureus* and *P.aeruginosa*

Culture plate showing the Antibacterial activity of synthesized silver nanoparticle by *Pleurotus djamor* against *Staphylococcus aureus* (11a) and *Pseudomonas aeruginosa* (11b)

Graph 8: Antibacterial activity of synthesized silver particles by *Pleurotus djamor* (mg/ml) representing zone of inhibition (cm) on *S. aureus* & *P. aeruginosa*
<table>
<thead>
<tr>
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<th><em>Pseudomonas aeruginosa</em></th>
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<tbody>
<tr>
<td>Control</td>
<td>0.12</td>
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Table 5: Record of inhibition zone (cm) of synthesized silver nanoparticles by *Pleurotus djamor* at different concentration with respect to *S.aureus* and *P.aeruginosa*

Culture plate showing the Antibacterial activity of synthesized silver nanoparticle by *Hypsizygus sulmarius* against *Staphylococcus aureus* (12a) and *Pseudomonas aeruginosa* (12b)

Graph 9: Antibacterial activity of synthesized silver particles by *Hypsizygus sulmarius* (mg/ml) representing zone of inhibition (cm) on *S. aureus* & *P. aeruginosa*
DISCUSSION

Nanotechnology is an existing area of scientific development and is a field of convergence among life sciences, material science and information technology. It is an emerging field of science capable of resolving issues and problems that are impossible to tackle in engineering and biological sciences.

Accumulation of metals by biological species may be metabolism-independent “adsorption” (biosorption) at the cell surface or metabolism-dependent internal “adsorption” to organelles, cytoplasmic ligands and cytoplasmic structures (Gadd, 1988). Extracellular production of silver nanoparticles by the filtered culture supernatants with aqueous silver nitrate solution, from 1-5mM for 24, 48 and 72hrs exist, produced the maximum synthesis of silver nanoparticles at 2mM concentration with in 24hrs for Pleurotus pulmonarius, 5mM concentration, 48hrs for Pleurotus djamor and at 3mM concentration, 48hrs for Hypsizygus ulmarius. The appearance of light brown colour clearly indicates the formation of silver nanoparticles in the reaction mixture along with the control. Sadowski et al., 2008; Nikhil et al., 2009; Maliszewska et al., 2009 and Kannanatranjan et al., 2010 reported that upon addition of silver ions into the filtered cell free filtrate in the dark, samples changes its color from almost colourless to brown with intensity increasing during the period of incubation. Kowshik et al., 2003 reported the conversion of 3mM silver nitrate solution to nanosilver by Fusarium oxysporum in an aqueous medium due to the change in color of the reaction mixture from pale yellow to dark brown. The characteristics brown colour of colloidal silver solution is due to the excitation of surface plasmon vibrations in the nanoparticle providing a convenient spectroscopic signature of their formation. Several hydroquinones with excellent redox properties were reported that could be act as electron shuttle in metal reductions as reported by Baker et al., 1998; Newman et al., 2000. Thus, it was evident that, electron shuttle or others reducing agents released by Pleurotus pulmonarius, Pleurotus djamor, Hypsizygus ulmarius are capable of reducing silver ions to silver nanoparticles. On the other hand, the reduction of silver ions did not occur in the absence of fungal cells. It indicates that the reducing agents that are released into the cultures of Pleurotus pulmonarius, Pleurotus djamor, Hypsizygus ulmarius are involved in the reduction process.

UV-VIS spectrum is one of the important and easy technique to verify the formation of metal nanoparticles provided surface plasmon resonance exists for the metal. UV–visible spectra of fungal supernatant treated with the silver nitrate solutions showed a maximum characteristic surface plasmon absorption band at 386 nm for Pleurotus pulmonarius at 2mM,24hrs with OD 128 when compared to Pleurotus djamor at 5mM, 48hrs with OD 1.98 and Hypsizygus ulmarius at 3mM 48hrs with OD 1.22 . Further incubation lead to decrease in intensity indicating complete reduction of silver ions.

According to Kowshik et al., 2003, the absorption at 280nm indicated the presence of tryptophan, tyrosine or phenylalanine residues in the protein, indicating the release of proteins into filtrate that suggests a possible mechanism for the reduction of metal ions present in the solution.

Observation of the strong but broad surface plasmon peak has been well known in the case of various metal nanoparticles over a wide size range of 2-100 nm by Kowshik et al., 2003. Shankar et al., 2003 suggested that the shoulder at 370-390nm corresponded to the Transverse plasmon vibration in silver nanoparticles, whereas the peak at 440nm due to excitation of longitudinal plasmon vibrations.

Various forms of silver ions and nanoparticles are widely studied in the biological systems and have reported to have strong inhibitory and antimicrobial effects against many fungal and bacterial pathogens causing economically important diseases in plants (Morons et al., 2005; pal et al., 2007; kim et al., 2008; 2009, kabir et al., 2011). Many patents are filed for nano silver for preservation and treatment of diseases in agricultural field (Anderson, 2009).

Silver is now and expected agrochemical replacement, which eliminates unwanted microorganisms in soils. Silver is an excellent plant growth stimulator (Morons et al., 2005; kim et al., 2008; 2009, kabir et al., 2011).

Silver nanoparticles were evaluated for use in increasing the antimicrobial activities of different antibiotic agents S. aureus and E.coli. The antibacterial activities of pencillin G, amoxicillin, erythromycin, clindamycin and vancomycin increased in the

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Table 6: Record of inhibition zone (cm) of synthesized silver nanoparticles by Hypsizygus ulmarius at different concentration with respect to S.aureus and P.aeruginosa


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presence of Ag-Np’s against the test strains. Shanmugam et al., 2006 reported that silver nanoparticles inhibit the growth of E. coli, S. aureus, salmonella species, pseudomonas species, bacillus species, and K. Pneumoniae, indicating broad spectrum of antimicrobial activity. Among Me-Np’s silver nanoparticles have been known to have inhibitory and bactericidal effect (cho et al., 2005).

In the present study, the synthesized silver nanoparticle solution of Hypsizygus ulmarius at concentration of 0.3mg/ml and 0.05mg/ml exhibited excellent antibacterial activity against the bacteria Staphylococcus aureus (0.16cm) and Pseudomonas aeruginosa (0.16cm) when compared to Pleurotus pulmonarius at a concentration of (0.05mg/ml), with 0.15cm inhibition zone for Staphylococcus aureus, 0.4mg/ml with 0.17cm for Pseudomonas aeruginosa and 0.4mg/ml, with 0.14cm inhibition zone for Staphylococcus aureus, 0.2mg/ml, 0.19cm for Pseudomonas aeruginosa.

The extent of inhibition of bacterial growth reported in this study was dependent on the concentration of nanoparticles in the muller-hinton medium. Interaction between nanoparticles and the cell wall of bacteria would be facilitated by relative abundance of negative charges on the Gram negative bacteria.

Reports on the inhibitory action of silver ions on microorganism’s shows that upon silver ion treatment DNA loses its replication ability (Feng et al., 2005) and expression of ribosomal subunit proteins as well as cellular proteins and enzymes essential to ATP and expression of ribosomal subunit proteins as well as DNA lose activity against two representative pathogenic bacteria, Staphylococcus aureus and Pseudomonas aeruginosa. These silver nanoparticles inhibit the growth of E. coli, S. aureus, salmonella species, pseudomonas species, bacillus species, and K. Pneumoniae, indicating broad spectrum of antimicrobial activity. Among Me-Np’s silver nanoparticles have been known to have inhibitory and bactericidal effect (cho et al., 2005).

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CONCLUSION
Microbes have been reported to reduce metal ions and stabilize nanoparticles with a wide size range (Chen et al., 2003; Vigneshwaran et al., 2007). In this study, three fungus Pleurotus pulmonarius, Pleurotus djamor, Hypsizygus ulmarius was used for the synthesis of stable silver nanoparticles which was quite fast, efficient, ecofriendly and formed within hours when silver ions came in contact with cell filtrate. The UV-VIS Spectra showed characteristic surface plasmon absorption band at 386nm. These silver nanoparticles showed excellent antibacterial activity against two representative pathogenic bacteria, Staphylococcus aureus and Pseudomonas aeruginosa. Since the fungus of Pleurotus pulmonarius, Pleurotus djamor, Hypsizygus ulmarius is commercially accepted edible mushroom, the byproduct after harvesting the mushroom is available in abundance for the large scale production of silver nanoparticles. The use of agricultural bio mass in nanotechnology will result in the growth of multidisciplinary research activities. Further development of eco-friendly process for the synthesis of metallic nanoparticles is an important step in the field of application of nanotechnology.

REFERENCES