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Synthesis of Silver Nanoparticles from *Pleurotus florida*, Characterization and Analysis of their Antimicrobial Activity

Tandeep Kaur[1], Shammi Kapoor[1*] and Anu Kalia[2]

[1]*Department of Microbiology, Punjab Agricultural University, Ludhiana 141004, Punjab, India*
[2]*Electron Microscopy and Nanoscience Laboratory, Department of Soil Science, Punjab Agricultural University, Ludhiana 141004, Punjab, India*
**Corresponding author*

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A B S T R A C T

In the present research, variable morphology of the silver nanoparticles (AgNPs) synthesized by using *Pleurotus florida* mycelia extract as a bioreductant at two different reaction conditions (shaking and static), has been reported. The formed AgNPs were characterized for the specific SPR (Surface Plasmon Resonance) peak position around 400 to 450 nm at different time intervals by UV-vis spectroscopy. Under shaking conditions silver nanoparticles took least time for synthesis. The particle/ aggregate size as deduced Transmission Electron Microscopy was in the range of 5-20 nm and 10-40 nm at shaking and static conditions respectively. The nano cyrstalline dimensions were further confirmed by X-ray diffraction (XRD) spectroscopy exhibiting the 2θ values corresponding to the face centered cubic crystal packaging of silver. The antibacterial potential of the synthesized silver nanoparticles showed effective inhibition of test pathogenic bacterial strains *viz*, *Staphylococcus* sp. and *Bacillus* sp.

Introduction

Edible mushrooms are used for nutritional and therapeutic purposes (Borchers *et al.*,2004, Chang 1996). They are perfect health foods as they are low in calories, fats, essential fatty acids and rich in vegetable proteins, vitamins and minerals (Murugkar and Subbulakshmi 2005). They are a rich source of natural antibiotics, where the cell wall glucans have immunomodulatory properties, and many secondary metabolites are known to kill bacteria, fungi and viruses (Gao, *et al.*, 2005; Lindequist *et al.*, 2005). Nanotechnology has recently become one of the most interesting research fields in technology and engineering for the purpose of manufacturing new materials at the nanoscale level (Albrecht *et al.*, 2006)having potential applications in various areas such as chemicals, textile industries, materials industry, medical diagnostic, drug and gene delivery and electronics, diagnosis, artificial implants, tissues engineering (Kumar *et al.*, 2011). It is well known that Ag^{+} ions and Ag-based compounds have strong antimicrobial effects (Furno *et al.*, 2004), and many investigators have shown nanoparticles to be effective antibacterial agents (Crabtree *et al.*, 2003). Now-a-days, metal nanoparticles have

been a subject of huge interest because of their unique physical and chemical properties. Metal/ metal oxide NPs have extremely high surface areas and unusual crystal morphologies that possess numerous edges or corner and other reactive surface sites. A wide variety of silver nanoparticle preparation techniques have been reported; notable examples include biological, chemical, electrochemical (Vorobyova *et al.,* 1999), γ-radiation (Choi *et al.,* 2005), photochemical (Li *et al.,* 2005), laser ablation (Tsuji *et al.,* 2003) etc. The physical and chemical methods involve high cost, require eco-toxic reagents and chemicals and therefore are non-economical. As the use of toxic reagents for preparation of the NPs may corrode or cause adverse effect in the medical applications; scientists are looking forward for some low cost, non-toxic and eco-friendly route(s) of synthesis. Interest for biological mediated synthesis using plants, fungi, microbes and yeast (Philip *et al.,* 2009; Tripathy *et al.,* 2009) are gaining impetus. Since mushrooms have higher nutritious value besides possessing anti-cancer, -hypertension, -diabetes and -high cholesterol properties (Ajith and Janardhanan 2007), these can be the model biological entities for generation of metal/ metal oxide NPs. The AgNPs have high specific area and high fraction of surface atoms, which will lead to excellent bactericidal effects (Mahendra *et al.,* 2009). In this work we present the synthesis of silver nanoparticles from *P. florida* followed by their characterization using different spectroscopy and microscopy tools. The task of this work was to investigate the anti-microbial activity of the generated AgNPs against various human pathogenic bacteria.

Materials and Methods

Culture procurement and maintenance

The culture of *Pleurotus florida* was collected from Culture Collection Bank, Mushroom Research Centre, PAU, Ludhiana. The culture was maintained on Potato Dextrose Agar slants at 25±2^{0}C by sub-culturing them every month. The mycelia agar bits of approximately 5-6 mm diameter were sliced and picked from mother culture slants and transferred to potato dextrose broth and kept at 25±2^{0}C for growth of fungal mycelia.

Chemical synthesis of silver nanoparticles

The Ag NPs were chemically synthesized by two different methods i.e. Hot and Cold process. In hot process, 0.001 M silver nitrate was boiled and tri-sodium citrate was added drop-wise to this solution along with vigorous stirring. The mixture was heated till development of pale brown color. Then the mixture was then cooled with stirring at room temperature. In cold process, 0.002 M sodium borohydride was chilled on ice bath and silver nitrate was added drop-wise to it. The reaction mixture was stirred vigorously on a magnetic stirrer. The solution turned to light yellow and later to bright yellow by addition of all the silver nitrate.

Biosynthesis of silver nanoparticles

The mycelia extract was mixed with $AgNO_3$ solution (0.001M). The mixture was incubated for 48 hours at different conditions (shaking and static) in the presence of sunlight for complete conversion of $AgNO_3$ to AgNPs. Positive control (mycelia extract without $AgNO_3$) and negative control (0.001M $AgNO_3$) were run simultaneously.

UV-vis spectroscopy of aqueous suspension

The samples were subjected to spectroscopy in the wavelength ranging from 300 to 600 nm using Double Beam Spectrophotometer (model Elico SL 159). The absorbance was plotted against the wavelength to observe the characteristic surface plasmon resonance peaks. UV-Vis spectra was generated at

different time intervals and was used to compare the incubation conditions.

Scanning electron microscopy (SEM)

The morphology of synthesized NPs was deduced on a Scanning electron microscope (model Hitachi S-3400N) working at 15 kV accelerating voltage. Samples were prepared by placing 10 µl of the sample on the stub followed by gold coating in a gold ion sputter coater (model Hitachi E-1010). Elemental composition and the percentage of atom and weight of metals present on the sample surface were analyzed by SEM-EDS (model Thermo Noran).

Transmission electron microscopy (TEM) using drop technique

The nanoparticle size and structure was determined by Transmission electron microscope (model Hitachi H-7650) operated at 80 kV in high contrast imaging mode. Samples were prepared by placing 10-20 µl of the sample on carbon/ formvar coated copper grid and were air dried before imaging in the microscope.

X-Diffraction Spectroscopy (XRD)

The purified nanoparticles were freeze dried at -50^{o} C under vacuum. The dried AgNPs were coated on XRD grid and diffraction pattern was analyzed using X-ray diffractometer (model XPERT-PRO) with anode material as Cu, K-α radiation at 1.54°A working at 45kV and current 40 mA. The samples were scanned at an angle between 2Θ= 20^{o}-70$^{o.}$

Determination of antimicrobial activity against pathogenic bacteria

The antimicrobial susceptibility of chemically and biologically synthesized NPs was evaluated against five pathogenic bacteria namely *Aeromonas hydrophila* MTCC 1739, *Bacillus cereus* MTCC 430, *Shigella flexneri* MTCC 1457, *Staphylococcus aureus* MTCC 96 and *Yersinia enterocolitica* MTCC 859 using disc diffusion method. The zones of inhibition were measured after 24 hours of incubation at 35^{0} C. Four antibiotics (Amikacin, Ampicillin, Cefotaxime and Gentamycin) were also tested for their activity against the test microorganism.

Results and Discussion

Biosynthesis of Silver Nanoparticles

Synthesis of AgNPs was observed when mycelial extract was incubated with aqueous solution of silver nitrate, a gradual change of color was observed after half an hour (Fig. 1). The control treatments comprised of mushroom extract in deionized water (positive control) and $AgNO_3$ salt in deionized water (negative control) remained colourless.

The silver nitrate treated mushroom extract turned brown in color. The color change could be due to the formation of silver nanoparticles of varying shape and size (Sudhakar *et al.,* 2014)and can be attributed to excitation of surface plasmon resonance (SPR) peaks of the nobel metal nanoparticles (Narasimha *et al.*,2011).

UV-vis spectroscopy

The absorption spectra of AgNPs at different incubation conditions is presented in Fig. 2.shows the surface plasmon band at 430-440 nm indicating the production of AgNPs. Characteristic peaks in the range of 200-500 nm gives the evidence for the formation of NPs (Kaviya *et al.,* 2011). AgNPs have generally been reported to show peaks in 400-500 nm wavelength range (Lee and El-Sayed 2006). The method of synthesis at shaking

conditions took less time as compared to static conditions.

Scanning electron microscopy (SEM)

The representative SEM images of the mycosynthesized AgNPs (Fig. 3) clearly showed the presence of nanoparticles in both aggregated and dispersed form. The size diameter of the nanoparticles has been observed to lie between 10 to 30 nm and the shapes were observed as spherical. Similar observation in the size range 20-50 nm was reported by Vanmathi *et al.,* (012). Similarly, Balashanmugam *et al.,* (2013) reported that SEM analysis of nanoparticles sythesized from a mushroom revealed the spherical nature of silver nanoparticles and size distibuted in the range of 40 nm.

Transmission electron microscopy (TEM)

The TEM image of the AgNPs indicated the formation of spherical nanoparticles with a few agglomeraions (Fig. 4). The average size of these nanoparticles at shaking and static conditions is 12.7 nm and 26.8 nm respectively.

The particle size histogram show that the particles range in the size from 5-20 nm and 10-40 nm at shaking and static conditions respectively.

X-Ray diffraction (XRD)

The crystalline nature of silver nanoparticles was confirmed by XRD spectroscopy (Fig. 5). Diffraction patterns at 2Ø values 38.023, 44.6113, 46.093, 68.483 and 67.523 indicated the reflections of metallic silver (Kalpana and Lee 2013). Along with the five peaks mentioned above some other unassigned peaks were also observed at 27.74, 32.11, 33.77, 41.352, 54.394 and 57.58. The high intensity of these peaks indicated strong X-ray scattering in crystalline phase (Shankar *et al.,* 2003).

Table.1 Actimicrobial activity of AgNPs synthesized from *Pleurotus florida* (mycelia) compared to chemically synthesized nanoparticles a against pathogenic microorganisms as diameter of inhibition zones

Synthesized AgNPs	*Aeromonas hydrophila*	*Bacillus cereus*	*Shigella flexneri*	*Staphylococcus aureus*	*Yersinia enterocolitica*
***Pleurotus* Mycelia AgNPs (static conditions)**	22	36	18	34	24
***Pleurotus* Mycelia AgNPs (shaking conditions)**	32	35	20	39	25
Mean	27	35.5	19	36.5	24.5
Chemically syth. AgNPs	48	38	18	42	33
C.D @ 5%	**8.11**	**6.92**	**4.47**	**3.99**	**7.06**
Amikacin	60	62	33	50	40
Ampicillin	30	59	28	45	65
Cefotaxime	40	40	20	50	55
Getamycin	44	50	25	40	48
Mean	45.33	48.66	24.6	44.33	38.5
C.D @ 5%	**8.11**	**6.92**	**6.47**	**NS**	**7.06**

Fig.1 Colour change as exhibited by incubation of silver nitrate solution before and after exposure to mycelia extract of *P. florida*

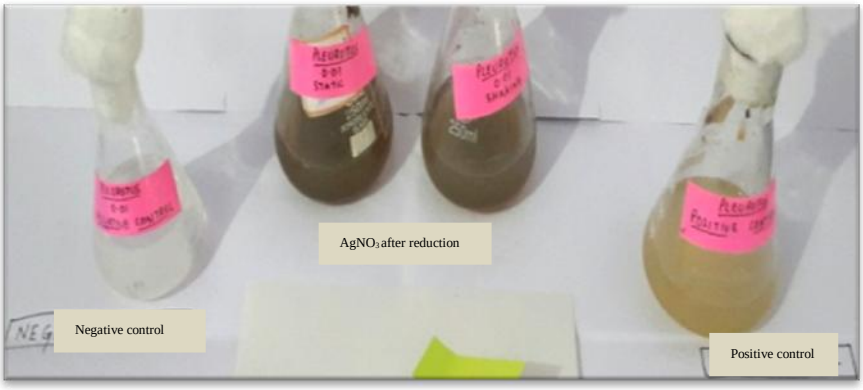


Fig.2 UV-visible spectrum at different incubation conditions (a) shaking (b) static

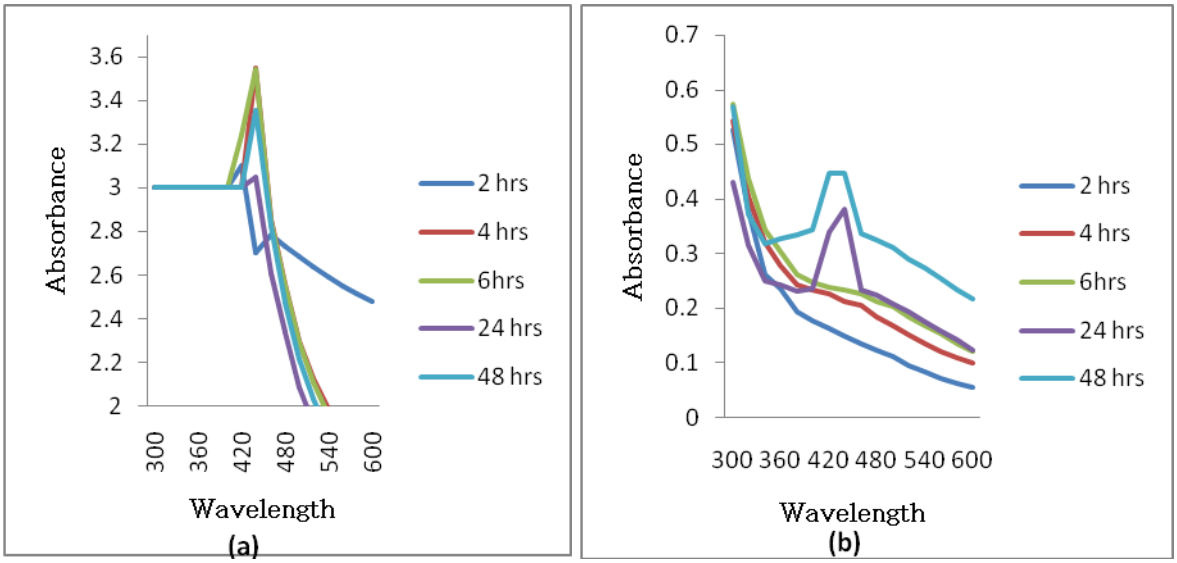
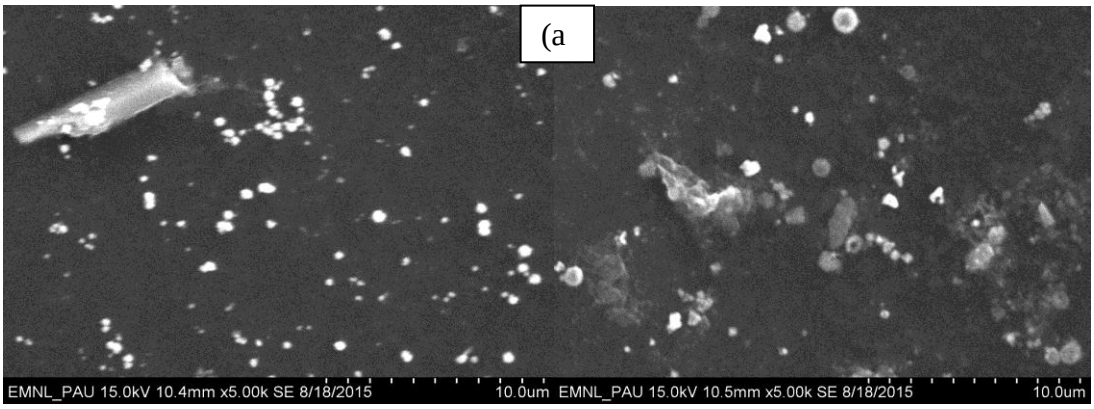


Fig.3 Scanning electron micrographs of *Pleurotus florida* synthesized silver nanoparticles at different incubation conditions (a) shaking (b) stati



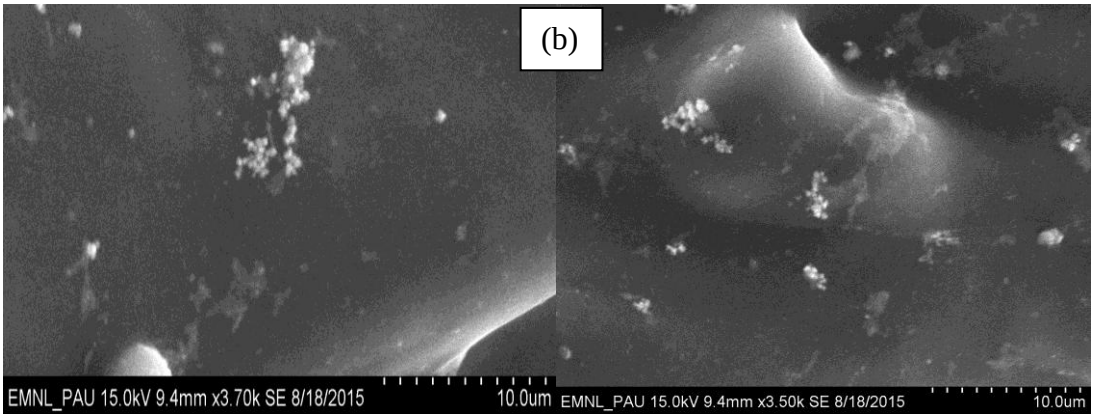


Fig.4 Transmission electron micrographs and particle size distribution of *Pleurotus florida* synthesized silver nanoparticles at different incubation conditions (a) static (b) shaking

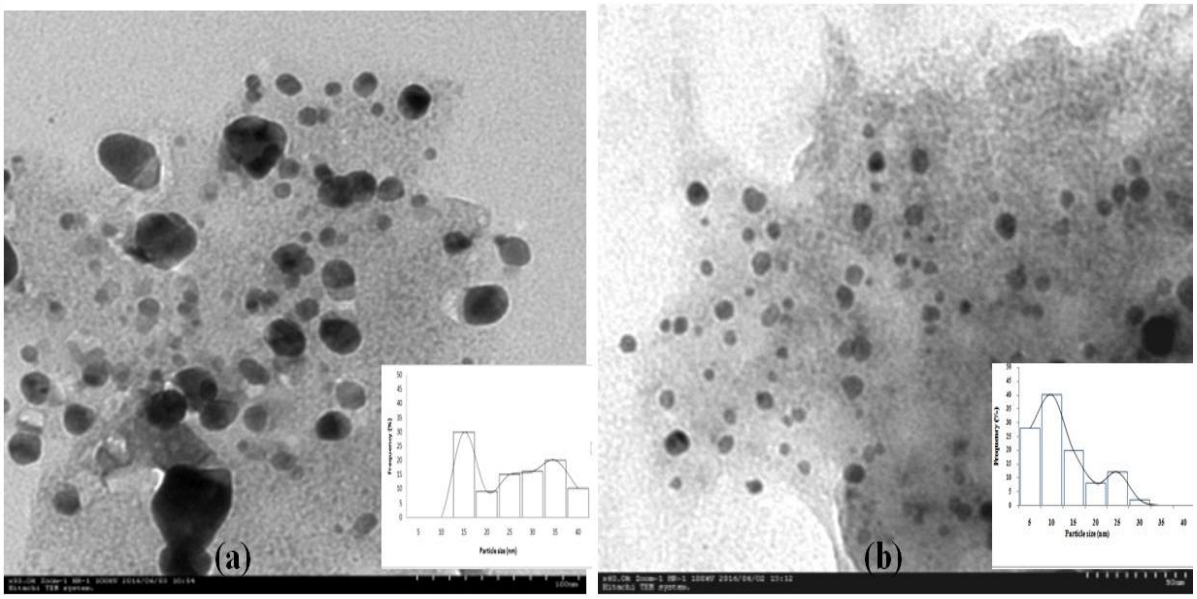


Fig.5 XRD spectra of *Pleurotus florida* synthesized Silver nanoparticles

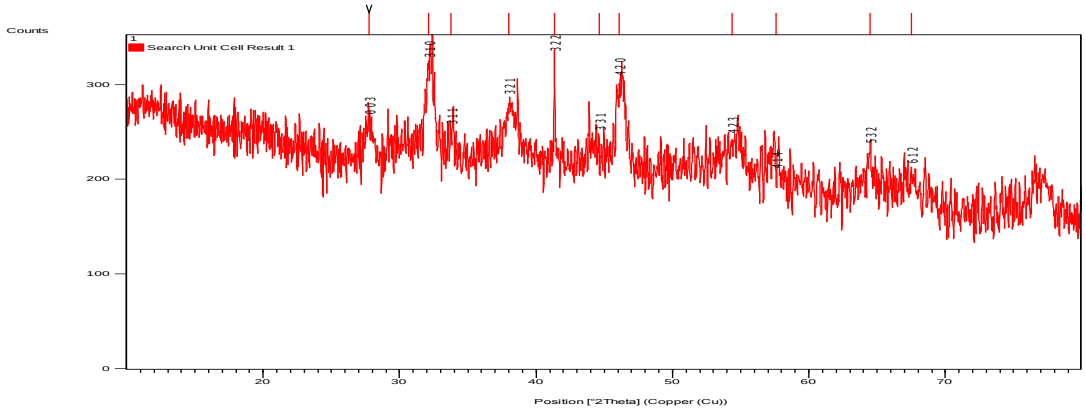
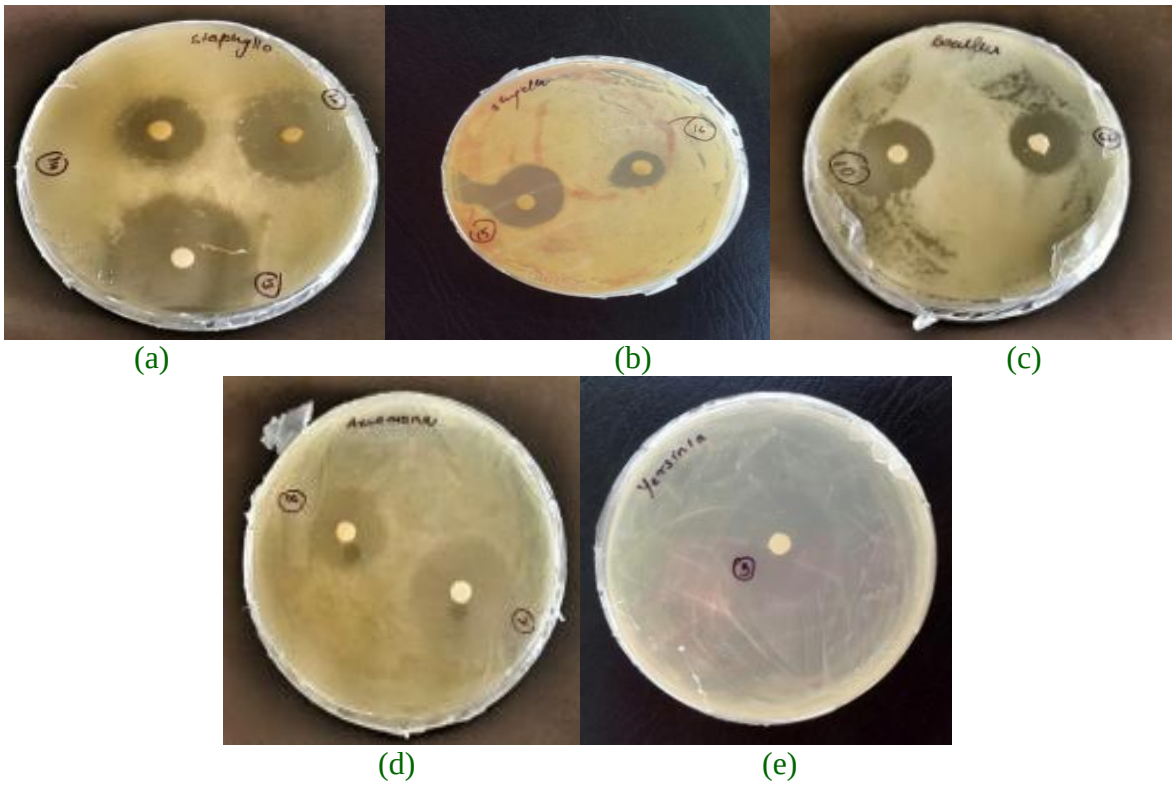


Fig.6 Antimicrobial activity of synthesized Ag NPs against (a) *Staphylococcus aureus* (b) *Shigella flexneri* (c) *Bacillus cereus*(d) *Aeromonas hydrophila* (e) *Yersinia enterocolitica*



(a) (b) (c)

(d) (e)

Antimicrobial activity of silver nanoparticles

The antibacterial effects of biologically synthesized silver nanoparticles have been investigated against five pathogenic bacteria. Fig. 6 provides the insights into the activity of microbially synthesized silver nanoparticles against various pathogenic bacteria. The highest zone of inhibition was shown against *Staphylococcus*, second mean inhibition zone was found against *Bacillus* and lowest activity was found against *Shigella* (Table 1).

The highest zones of inhibition were against gram positive microorganisms *Bacillus cereus* and *Staphylococcus aureus* than gram negative microorganisms. Similar results were given by Guzman *et al.,* (2009) that the Ag NPs showed good antibacterial action against gram positive organisms *Bacillus cereus* and *Staphylococcus aureus* when compared to *Pseudomonas aeruginosa*, *E.coli* etc.

Nanoparticles are considered as novel biocidal and antimicrobial agents. They possess unique physical, chemical and biological properties. AgNPs have high specific area than their volume, which lead to their excellent antimicrobial activity as compared with bulk silver metal (Mahendra *et al.,* 2009). Chemical synthesis methods lead to presence of some toxic chemicals that get absorbed on the surface and may lead to adverse effect in the medical applications. (Singh and Singh 2010).Therefore, researchers are showing much interest on biological mediated synthesis using plants, fungi, microbes and yeast (Philip *et al.,* 2009; Tripathy *et al.,* 2009). Extracts from bio-organisms may act both as reducing and capping agents in Ag NPs synthesis.. There

are various routes for the synthesis of NPs exhibiting homogeneity in their morphology and other properties. The microbial synthesis is cost effective, economically safe and environment friendly as compared to chemical and physical methods. AgNPs are the most promising nanomaterials having pronounced and documented antimicrobial activity. The present study is aimed to focus on the biological synthesis of AgNPs by using edible mushroom extract as bioreductant. The biosynthetic method developed in this study for producing silver nanoparticles has distinct advantage over chemical methods such as high bio-safety and being eco-friendly and non-toxic to the environment. There is a growing need to develop clean, nontoxic and environmentally friendly procedures for synthesis and assembly of nanoparticles, biosynthesis of silver nanoparticles using plants, bacteria (Kaliswaralal *et al.,* 2008), fungi (Jaidev and Narasimha 2010) and yeast (Kowshik *et al.,* 2003) are known to reduce silver ions into silver nanoparticles by both extra and intracellularly (Bhainsa and D'souza 2006).

Microbial synthesis of Ag NPs was performed using the cell free extract of *Pleurotus florida* along with the wet chemical synthesis of AgNPs and their antimicrobial potentials discerned at varying working concentrations. The extracts were treated with silver nitrate and placed under in different conditions for the appearance of color change. The synthesized nanoparticles were characterized by various microscopy and spectroscopy techniques. The UV-Vis spectroscopy of the synthesized sols exhibited characteristic absorption/ surface plasmon resonance peaks for the formation of AgNPs. Observation of this strong but broad surface plasmon peak has been well documented for various Me-NPs, with sizes ranging all the way from 2 to 100 nm (Sastry *et al.,* 1997; Sastry *et al.,* 1998). The Plasmon peak was observed between 420-460 nm. SEM analysis confirmed the formation of AgNPs and revealed their spherical nature. TEM photographs revealed that the nano sols consist of well dispersed particles with size ranging from 5-40 nm.

AgNPs have proved to be most effective because they have good antimicrobial activity against bacteria, viruses and other eukaryotic microorganisms (Gong *et al.,* 2007). The synthesized nanoparticles were exhibited greater variability in their antimicrobial potentials. It is well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, showing strong biocidal effects on as many as 12 species of bacteria including *E. coli* (Zhao and Stevens 1998). Recently, researchers have showed that hybrids of Ag nanoparticles with amphiphilic hyper-branched macromolecules exhibited effective antimicrobial surface coating agents (Aymonier *et al.,* 2002). The microbially synthesized AgNPs exhibited maximum antimicrobial activity against gram positive bacteria *Bacillus cereus* and *Staphylococcus aureus* as compared to gram negative bacteria *Shigella flexneri*, *Aeromonas hydrophila* and *Yersinia enterocolitica.* On the contrary, chemically synthesized AgNPs showed highest zone of inhibition against gram negative bacteria. The microbially synthesized AgNPs showed significant antimicrobial activity against all the four test pathogens but showed less inhibition against *Shigella flexneri.* Kaviya *et al.,* (2011) reported that the AgNPs exhibited good antibacterial activity against both gram negative and gram positive bacteria. But they showed higher antimicrobial activity against *E. coli* and *P. aeroginosa* (Gram negative) than *S. aureus* (Gram positive).The chemical controls showed less antimicrobial activity in comparison to synthesized NPs demonstrating that the metal ion toxicity has lesser antimicrobial potential than the

nanoparticulate metal/ metal oxide particles. The zones of inhibition formed in antimicrobial screening test indicated that AgNPs synthesized in this process has the efficient antimicrobial activity against pathogenic bacteria. The biologically synthesized nanoparticles could be of immense use in medical field for their antimicrobial function.

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