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Potential of *Pleurotus sajor caju* to synthesize Silver nanoparticles and evaluation of antibacterial activity and their role as antibiotic activity enhancer

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ABSTRACT

Pleurotus sajor caju, commonly known as oyster mushroom, has been used in food for a long time. Many useful properties of this fungus are still being studied. Presently the biosynthetic potential of silver nanoparticles using this fungus has been determined, characterized and their antibacterial and antibiotic activity enhancing properties reported. The synthesis of silver nanoparticles (AgNps) was judged by change in color of the reaction mixture and confirmed with UV-VIS spectroscopy. The characterization of synthesized silver nanoparticles for their size, shape and dispersity was done by Transmission Electron Microscopy (TEM) while the presence of different functional groups was characterized by Fourier Transform Infrared (FTIR) spectroscopy. The TEM study showed the formation of silver nanoparticles in the range of 4-22 nm and FTIR revealed the presence of proteins, amino acids, aromatic compounds, alcohols, aldehydes and carboxylic acids which may be responsible for the reduction and stability of the silver nanoparticles. Determination of antibacterial activities of the synthesized silver nanoparticles revealed remarkable antagonistic action against Methicillin resistance *Staphylococcus aureus* (*MRSA*), *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Also AgNPs were further evaluated for their role as antibiotic enhancers with some broad spectrum antibiotics (Methicillin, Penicillin, Amoxycillin and Ampicillin) which showed an increase in efficiency of these antibiotics when used in combination with AgNps.

Key Words: *Pleurotus sajor caju,* silver nanoparticles, antibacterial activity, antibiotic activity enhancer

INTRODUCTION

The applications of nanoparticles in various fields have been drastically increased during the last few years. These nanoparticles found applications in pharmaceutical, biomedical and other industries by virtue of their important characteristics. Different approaches have been used to synthesize metal nanoparticles but the biological method of synthesis has various advantages over the chemical and physical approaches. As chemical and physical methods involve the use of toxic solvents, high energy consumption and generation of hazardous by-products which constitute a high risk to the environment and human health (Thakkar *et al.,* 2010). Whereas, alternative to this, the biological approaches are ecofriendly, clean, require less energy consumption, low cost, give high yields and forms no toxic products. Among other noble metals, silver nanoparticles have proved to be the most effective as it has good antimicrobial efficacy against various microorganisms. The antibacterial and antiviral actions of silver, silver ions, and silver compounds have been studied earlier (Tokumaru *et al*., 1984). Various biological resources including plants and plant products, algae, fungi, yeast, bacteria, and viruses could all be employed for the synthesis of silver nanoparticles (Gardea-Torresdey *et al.,* 2003; Greene *et al.,* 1986; Vigneshwaran *et al.,* 2007; Lin *et al.,* 2005; Klaus *et al.,* 1999; Pokorski and Steinmetz, 2011). However, fungi appear to be more promising for large scale production of nanoparticles than the rest, as they are simpler to grow as well as secrete large amount of proteins which play a crucial role in extracellular synthesis of metal nanoparticles (Shedbalkar *et al.,* 2014). Mushrooms are fleshy fruiting bodies of the basidiomyceteous fungi, typically found above ground on soil, rotten wood or trees. Mushrooms have been used for centuries now due to their high nutritional and medicinal properties. Although, these are highly proteineous (75%) structures, very few studies have been reported on the use of mushroom for nanoparticle synthesis (Philip, 2009). The protein present in the mushrooms can act as reducing and protecting agents for nanoparticles. Interestingly, mushrooms contain different bioactive and aromatic compounds with diverse biological activities and studies have also suggested that a large number of active substances secreted by fungi play important roles as reducing and capping agents in the nanoparticle biosynthesis (Guangquan *et al.*, 2011). Therefore, with present study an attempt has been made to explore the biosynthetic potential of *Pleurotus sajor caju* for silver nanoparticles. Their characterization, potential as antibacterials and their role as antibiotic activity enhancers has also been studied.

MATERIALS AND METHODS

Materials used

Pleurotus sajor caju was procured from Mushroom Research Laboratory of Dr. Y.S Parmar University of Horticulture and Forestry Chambaghat, Solan (H.P), India. The chemicals and media used were of analytical grade.

Biosynthesis of silver nanoparticles

The synthesis of silver nanoparticles by fungal biomass was done as per methods of Hemath *et al.,* (2010). In this method *Pleurotus sajor caju* was grown in potato dextrose broth (PDB) at 28± 1°C for 14 days in shaking conditions (150 rpm). After the growth the mycelia was filtered or centrifuged (at 10000 rpm for 10 minutes) and all the traces of medium attached to mycelia (if any) were removed by washing in sterilized distilled water. The mycelia was then inoculated in fresh sterilized distilled water and incubated at 28± 1°C for 3 days. The cell filtrate obtained after filtering the mycelia by using Whatman filter paper no. 1 was challenged with 1mM silver nitrate ($AgNO_3$) by adding 0.5 ml of 0.1 M $AgNO_3$ in 49.5 ml of cell free filtrate for the synthesis of AgNPs. Cell free filtrate without silver nitrate was maintained as control.

The test and control were incubated in dark under shaking conditions (150 rpm) at 28± 1°C for 5 days. After 5 days, the samples were subjected to different analytical processes for detection and confirmation of silver nanoparticle synthesis. The biosynthesis of silver nanoparticles was detected visually by change in color of the reaction mixture from transparent/ light yellow to brown and confirmed by observing the peak of absorption spectra with the UV visible spectrophotometer (Hitachi-2900) in the range of 200800 nm at resolution of 0.5 nm. The observations were made periodically after every 24 hours for 5 days.

Characterization of silver nanoparticles

The characterization of size and shape of silver nanoparticles was done by analyzing with transmission electron microscopy (TEM), Hitachi (H-7500). The characterization for the type of chemical bonds and possible interactions between protein and silver nanoparticles was done by Fourier Transform Infrared (FTIR) spectroscopy, Model- Nicolet IR 200 (Thermo electron corp).

Evaluation of antibacterial and antibiotic activity enhancing potential of silver nanoparticles

The antibacterial activity of the biosynthesized silver nanoparticles was detected as per agar diffusion method of Bauer *et al.* (1966). The bacterial pathogens *Escherichia coli,* Methicillin resistant *Staphylococcus aureus* (*MRSA*), *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as test pathogens and inoculated on nutrient agar plate by spread plating method. Then wells on the plates were made by sterile borer, loaded with the 10 µl of silver nanoparticles under aseptic conditions and incubated for 24 hours at 37 ºC. The formation of zone of inhibition (if formed) was observed after 24 hours. Similarly, the antibiotic enhancing activity of biosynthesized silver nanoparticles in combination with commercial broad spectrum antibiotics (Penicillin, Methicillin, Ampicillin and Amoxycillin) was evaluated as per disk diffusion method of Bauer *et al.,* (1966). In this method, each of the antibiotic discs (10 µg) were impregnated with biosynthesized silver nanoparticles (10µl) and placed on nutrient agar plates inoculated with bacterial test pathogens (*Escherichia coli, MRSA, Pseudomonas aeruginosa, Staphylococcus aureus*). The plates were incubated at 37 ºC for 24 hrs and zones of inhibition (if formed) were observed, measured in millimeters (mm) and compared with control (i.e, disc impregnated with silver nitrate only and antibiotic discs with no silver nanoparticles). The increase in fold area was assessed as per Birla *et al.,* (2009) by calculating the mean surface area of the inhibition zone of each antibiotic (A) and antibiotic + silver nanoparticles (B). The fold increase area of bacteria was calculated by the equation $(B^2-A^2)/A^2$ where 'A' and 'B' were zones of inhibition for antibiotic and antibiotic + silver nanoparticles, respectively.

RESULTS AND DISCUSSION

The biosynthesis of silver nanoparticles by *Pleurotus sajor caju* was evaluated by observing the the change in color (from transparent/ yellow to dark brown) of the reaction mixture (containing cell free filtrate and silver nitrate) which indicated the formation of AgNPs due to reduction of silver ions to silver nanoparticles (i.e. Ag^+ to Ag^0). The control did not show any change in its initial color when incubated under the same conditions (**Figure 1**). This brown color was due to the excitation of the surface plasmon vibrations in the metal nanoparticles, as suggested by Ahmad *et al.* (2003).

Figure 1: The reaction mixture on 5th day of incubation, showing the biosynthesis of silver nanoparticles by *Pleurotus sajor caju* as change in color from yellow to dark brown (T); No change in color observed in control (C)**.**

Along with color change, the formation of AgNPs was also analysed and confirmed by UV/Vis spectrophotometry. The samples containing the synthesized silver nanoparticles, showed a peak in range of 380-680 nm which is the defined range of the silver nanoparticles, taken after every 24 hours for five days. The highest peak was indicated at 5th day of incubation (**Figure 2**).

The characterization of size and shape of AgNPs by transmission electron microscopy (TEM) revealed the shape of nanoparticles which were mostly spherical, distributed randomly and ranged between 4 to 22 nm (**Figure 3**).

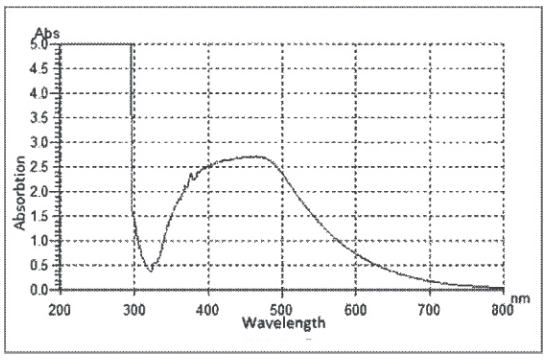

Fig. 2: Peak of silver nanoparticles synthesized by *Pleurotus sajor caju* on 5th day of incubation as obtained by UV/ Vis spectroscopy.

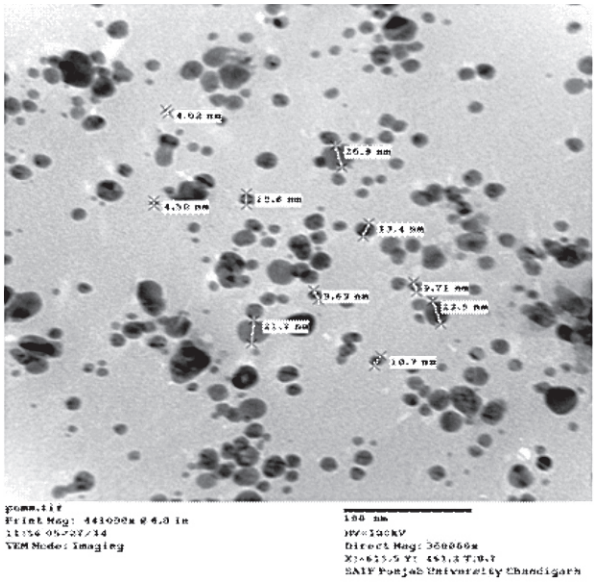

Fig. 3: TEM image of silver nanoparticles synthesized by *Pleurotus sajor caju.*

The FTIR spectra of *Pleurotus sajor caju* (**Figure 4**) revealed the band obtained at 3247cm^{-1} corresponding to OH stretch (alcohols, a broad, strong band), which arose due to carbonyl stretch. The bands at 2974, 2886 and 1655 cm^{-1} developed for CH (alkanes), O-H, carboxylic acid and C=C (alkenes) stretch, respectively and were commonly found in the proteins indicating the presence of proteins as ligand for AgNPs, which increased the stability of nanoparticles. The representative spectra of nanoparticles obtained manifests absorption peaks located at about 3852.29 cm^{-1}(N-H group of amines), 1454 cm^{-1}(aromatic C-C stretching) and 803 cm^{-1} (C- Cl). Therefore, the FTIR results made it clear that the synthesized nanoparticles were surrounded by proteins and amino acids which may be responsible for the stability of the silver nanoparticles

Table 1: Zone of inhibition (diameter in mm) obtained by silver nanoparticles produced by*Pleurotus sajor caju* against standard test bacterial pathogens.

Test Bacterial strains	*MRSA*	*S. aureus*	*P.aeruginosa*	*E. coli*
Control($AgNO_3$)	16mm	12mm	8mm	8mm
Test(AgNps)	18mm	26mm	32mm	24mm

and it can be assumed that the functional groups of alcohols, aldehydes and carboxylic acids present in the sample may be responsible for the reduction of silver nitrate to silver nanoparticles.

The antibacterial activity of the silver nanoparticles produced by the *Pleurotus sajor caju* was measured in mm as the diameter of the zone of inhibition (**Figure 5; Table 1**). The antibacterial activity was determined by comparing the zone of inhibition of test (AgNPs) with control ($AgNO_3$). The results revealed that the silver nanoparticles were quite effective in inhibiting the growth of almost all the test organisms. However, the highest activity on the basis of zone of inhibition, was reported against *Pseudomonas aeruginosa* (32 mm). The effect of silver nanoparticles on gram negative bacteria was shown to be enhanced over that of the gram positive. The reason behind this might be the cell wall composition of gram positive bacteria as described by Birla *et al.* (2009).

After evaluating the antibacterial activity of the mycosynthesized silver nanoparticles, their ability of enhancing activity of some commercial antibiotics was also evaluated. The combined effect of antibiotics, Penicillin(Pnc), Ampicillin (Amp), Amoxycillin (Amc) and Methicillin (Met) along with silver nanoparticles was seen against *MRSA, Staphyllococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* (**Figure 6**). Most of the bacteria have been reported resistant against these commercial antibiotics but in combination with

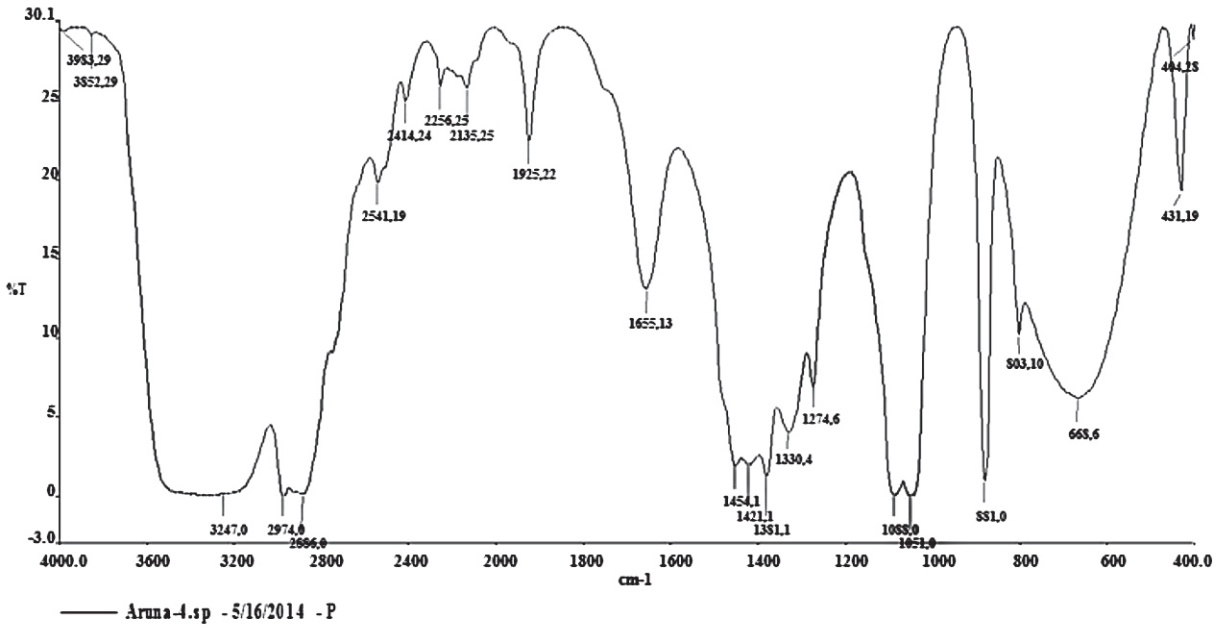

Fig. 4 FTIR spectra of silver nanoparticles synthesized by *Pleurotus sajor caju*.

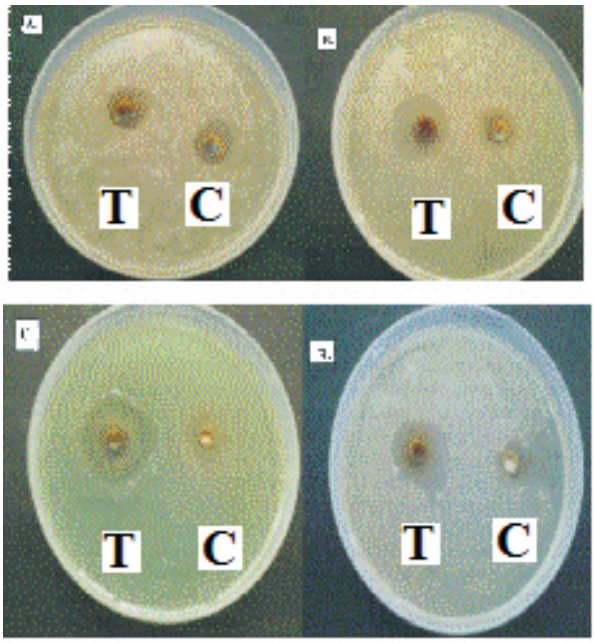

Fig. 5: Antibacterial activity of silver nanoparticles produced by *Pleurotus sajor caju* (T) and silver nitrate (C) against (A) *MRSA* (B) *Staphyllococcus aureus* (C) *Pseudomonas aeruginosa* D) *Escherichia coli.*

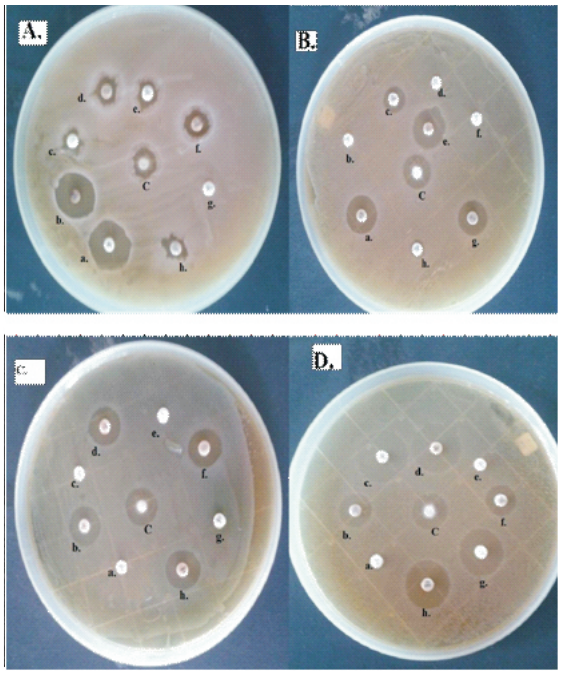

Fig. 6 Antibiotic enhancing activity of silver nanoparticles produced by *Pleurotus sajor caju* against:

[A] *MRSA* **a)** A mc **b)** A mc with AgNps **c)** Pnc **d)** Pnc with AgNps **e)** Amp **f)** A mp with AgNps **g)** Met **h)** Metwith AgNps.

[B] *S. aureus* **a)**A mc with AgNps **b)** Pnc **c)** Pnc with AgNps **d)** Met **e)** Met with AgNps **f)** A mp **g)** A mp with AgNps **h)** A mc

[C] *P. Aeruginosa* **a)** Met **b)** Met with AgNps **c)** Amp **d)** A mpwith AgNps **e)** Pnc **f)** Pnc with AgNps **g)** Amc **h)** A mc with AgNps.

[D] *E.coli* **a)** Pnc **b)** Pnc with AgNps **c)** A mp **d)** A mpwith AgNps **e)** Met **f)** Met with AgNps **g)** A mc **h)** A mc with AgNps

present silver nanoparticles their efficiencies were found to increase manifold. The increase in antibacterial activity of antibiotics in combination with silver nanoparticles was observed by fold increase in the zone of inhibition. In absence of zone of inhibition, the standard 6mm size of antibiotic disc was taken as zone as per Birla *et al*. (2009). The highest fold increase (15) was seen against *Pseudomonas aeroginosa* by methicillin when combined with AgNps (**Table 2**).

CONCLUSION

Various methods have been used for the synthesis of silver nanoparticles in recent years. However, the synthesis of nanoparticles by biological methods has advantages over others as it is easier to get a clear supernatant with biologically active molecules like enzymes, proteins, and other organic molecules from the separated biomass. Filamentous fungi like mushrooms in this regard have been explored very less despite having equal potential for biosynthesis of various metal nanoparticles. Besides, these higher fungi constitute a very favorable object of study, as they can accumulate high concentrations of reduced nanoparticles and this is especially true for cultivated edible mushrooms, as they are nontoxic and have a high biomass yield. Therefore, in present investigation an attempt was made for the synthesis of silver nanoparticles by using *Pleurotus sajor caju,* a well known edible mushroom. Still, efforts are required to screen out more mushroom species for their metal nanoparticle synthesizing potential and characterization for their antimicrobial properties.

Table 2 Antibiotic (Ab) enchancing activity of silver nanoparticles synthesized by *Pleurotus sajor caju* against the test organisms *MRSA, S. aureus, P. aeruginosa* and *E.coli.*

Antibiotics	*MRSA*			*S.aureus*			*P.aeruginosa*			*E.coli*		
	Ab	Ab+AgN	$[B]^{2-}$	Ab	Ab+AgN	$[B]^{2-}$	Ab	Ab+A	$[B]^2$-[A]	Ab	Ab+Ag	
	[A]	ps[B]	$[A]^2$	[A]	ps	$[A]^2$/	[A]	gNp[B]	^{2}/$[A]^2$	[A]	Np[B]	$[B]^2$-[A]
	(mm)	(mm)	/$[A]^2$	(mm)	[B](mm)	$[A]^2$	(mm)	(mm)		(mm)	(mm)	
Penicillin	8	12	1.25	-	14	4.44	-	22	12.4	-	18	8
Methicillin	-	18	8	-	12	3	-	24	15	-	16	6.11
Amoxycillin	20	30	1.25	7	20	7.1	-	22	12.4	20	30	1.25
Ampicillin	10	12	0.44	-	23	13.6	-	20	10.11	7	24	10.75

Where 'A' : Zone of inhibition by antibiotic only ; 'B' : Zone of inhibition in combination with Antibiotic and AgNps

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