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Synthesis and Characterization of Inorganic Silver Nanoparticles Using Calcocybe Indica Spawn and in Vitro Microbicidal Analysis and Pathogen city Inhibition Assay

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Abstract

Many reports have been published about the biosynthesis of metal nanoparticles using several biological materials, but the capacity of their natural reducing constituents to form silver nanoparticles has not yet been studied. In this study, the synthesis of silver nanoparticles using *Calocybe indica* spawn has been investigated. The formation of silver nanoparticles was monitored using color change, pH, UV – Vis spectroscopy where, the typical surface plasmon absorbance at 420nm was exhibited. The average size and purity of the nanosized particles were determined by SEM and EDAX respectively. The EDAX at the nanoparticle dispersion confirmed the presence of elemental silver signal; no maximum peaks of other impurities were detected. The SEM studies showed the formation of silver nanoparticles in the size range of 20 – 30nm, spherical shaped well distributed with aggregation in solution. The *in vitro* bactericidal activity showed maximum inhibition against *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, MRSA, *Staphylococcus aureus*, *Proteus mirabilis*, *Candida albicans* and *Cryptococcus neoformans*. The pathogenecity inhibition study showed very good effect than plating method highlighted the arrest in the motility and metallic sheen colonies (*E. coli*), capsule formation (*K. pneumoniae* and *C. neoformans*), Catalase and coagulase formation (MRSA and *S. aureus*), swarming (*P. mirabilis*) and germ tube formation (*C. albicans*). This study concluded that the spawn of *C. indica* have moderate microbicidal properties and better pathogenecity inhibition properties.

Keywords: *Calocybe indica*, silver nanoparticles, SEM, Microbicidal, Virulence

Introduction

In recent years, the field of nanotechnology is one of the most active and attractive areas of research in modern material science due to their unique electronic, optical, mechanical and chemical properties.[1,2] The metallic nanoparticles have found effective and have many other major applications in different fields like catalysis, electronics, therapeutics and information storage. Various methods with their appropriate mechanisms have been reported for the preparation of nanoparticles like salt reduction, microwave dielectric heating reduction, ultrasonic irradiation and electrochemical synthesis.[3,4]

In recent years, silver mediated microbial nanoparticles have been found to exhibit interesting antimicrobial,

antimutagenic, anticancer and antiviral properties.[31] It is well known that microorganisms like bacteria, yeast, algae and fungi play an important role in metal remediation where more scientists consider interest in constructing nanofactories using microbial resources recently.[5] The interaction between biological agents and metals have been well documented and the ability of the microorganisms to extract accumulated metals in dissolved and reduced form have different advantageous biotechnological process.[6,7]

Synthesis of nanoparticles employing microorganisms has attracted great interest due to their unusual optical,[8] chemical,[9] photoelectrochemical,[10] electronic[11] and biological.[12,13] It is well known that many microorganisms can synthesize inorganic metals either intracellularly or extracellularly.[14,15,16,17,18,19] The usage of fungi in the nanoparticle synthesis is a relatively recent addition in the nanofactory ability formulation field. Application of fungi to produce nanoparticles is potentially exciting because of their ability to secrete large amount of enzymes, easy to cultivate and less chance of contamination. Some of the microorganisms which have been widely used for the synthesis of silver nanoparticles include *Phoma,*[20] *Fusarium,*[5,21,22] *Pleurotus,*[23] *Aspergillus fumigatus,*[6,24] *Photopthora*.[25]

The biosynthetic method that are employing fungal filtrate as the major start-up solution has received some attention as simple and viable alternative to chemical procedures for synthesizing metal nanoparticles.[26] The metallic nanoparticles have made a remarkable comeback as potential antimicrobial, antimutagenic and anticancer agent.[27] This may be confirmed through one step protocol for the synthesis of microbial mediated metal nanoparticles that are analyzed broad antimicrobial and antiviral activity and less data in arising resistance. The main aim of this study is to synthesis *Calocybe indica* mediated silver nanoparticles, its characterization and exhibit the *in vitro* antimicrobial and pathogenecity inhibition properties. This study also includes the rapid formation of Ag fungal inorganic nanoparticles and understanding the surface plasmon resonance peak employing UV – Vis spectroscopy, determination of particle size and purity were carried out using Scanning Electron Microscope (SEM) respectively.

Materials and Methods

The spawns of *Calocybe indica* spawns obtained from the Mushroom Research Centre. Tamilnadu Agricultural University, Coimbatore, India and were species identified and certified. All the collected spawns were surface sterilized using 0.1% mercury chloride, 70% ethanol and washed thoroughly with distilled water for three times. The surface sterilized spawns (5g) were crushed and inoculated in sterile Potato Dextrose broth (Hi-media Laboratories, Mumbai). Broth incubated overnight was further filtered using Whatmann No 1 filter paper under aseptic condition.[13] The filtrate was stored at 4^{o}C and further used as reducing and stabilizing agent for 1mM $AgNo_3$ (sd fine chemicals, Mumbai).

For the synthesis of *C. indica* spawn mediated silver nanoparticles, the spawn filtrate (10ml) was added to 100ml of 1mM $AgNo_3$ aqueous solution and kept at room temperature. The experiment was done in triplicate for reproducibility. The reduction was observed by the periodical color change in the solution.[12] After ten minutes, the color of the solution changed from pale yellow to deep yellow. The bioreduced Ag nanoparticles solution was collected and monitored by periodic sampling of aliquots (5ml) of aqueous component and by measuring the UV visible spectra of the solution. UV- Vis spectroscopy measurement of *C. indica* spawn extract mediated reduction of silver to silver nanoparticles were carried out as a function of time of reaction at room temperature on

Systronics double beam PC based UV visible spectrometer 2202 with a resolution of 1nm at 420nm.[24,28,29]

The size and morphology of the silver nanoparticles was examined by SEM while the qualitative chemical composition of fungus mediated silver nanoparticle was analyzed by EDAX.[3] The dried samples of *C. indica* spawn mediated silver nanoparticles mixed with acetone, loaded onto the sample holder in SEM (JOEL model 6390) and dried under vacuum. The SEM analysis was done at an accelerating voltage of 20kV and elemental mapping was done by the Phoenix EDAX which was equipped along with SEM.

The synthesized *C. indica* mediated silver nanoparticles were further assessed for its antimicrobial potential (*in vitro* only) thereby the following two methods were followed

1. Antimicrobial activity by well cutting method
2. Pathogen city inhibition assay

Antimicrobial activity was performed by preparing Mueller Hinton agar (MHA) and sterility of the preparation was done by keeping the plates overnight in the room temperature. The bacterial isolates from the clinical samples including *Escherichia coli, Klebsiella pneumoniae,* MRSA, *Staphylococcus aureus, Proteus mirabilis;* fungal isolates like *Candida albicans* and *Cryptococcus neoformans* were seeded on the surface of the MHA plates. Well was cut and different concentration of 100µl of synthesized *C. indica* spawn mediated silver nanoparticles were loaded in the wells and incubated at 37^{o}C in uninverted position. The microbicidal activity of the test nanoparticles were assessed by measuring the zone of growth inhibition around the well.

The pathogen city inhibition test was done by assessing the motility and metallic sheen colonies (*E. coli*), capsule formation (*K. pneumoniae* and *C. neoformans*), Catalase and coagulase formation (MRSA and *S. aureus*), swarming (*P. mirabilis*) and germ tube formation (*C. albicans*). All these microbial characters were assessed before and after exposing the bacterial and fungal pathogens to the test nanoparticles, thereby the inhibition of the virulence may be determined and recorded as positive or negative.

Results and Discussion

Highly concentrated and stable silver hydrosol was synthesized and characterized in this study under the optimized conditions. We have observed that the fungus *C. indica*, when interacted with silver ion in solution leads to the reduction of higher molecular silver to nanoparticles. Reduction of silver ions was reflected in the changing color of the spawn filtrate from pale yellow to dark brown shade. The appearance of a dark brown color in the fungal biomass after reaction with Ag^{+} ions is a clear indicator of the reduction of the metal ions and formation of silver nanoparticles exhibit striking colors (pink to blue and light yellow to brown respectively) due to their excitation of surface plasmon vibrations in the particles.[3,23,30,31]

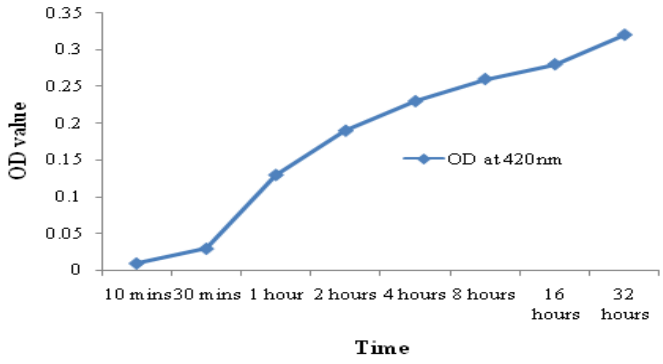
It was found that aqueous silver ions when exposed to various fungal strains are reduced in solution, there by leading to the formation of silver hydrosol. Table 1 shows the periodical color change occurred during *C. indica* mediated silver nanoparticle synthesis. The progress of the reaction between metal ions and spawn extract were monitored by UV-Vis spectra of silver nanoparticles in aqueous solution with different reaction times were easily followed by UV-Vis spectroscopy. The UV visible spectrum of the aqueous medium containing silver ions showed increase in optical density at 420nm corresponding to plasmon resonance of silver nanoparticle as shown in Figure 1. It showed an increased absorbance with increasing time of incubation at characteristic plasmon resonance absorbance band at 420nm indicative of relatively small mono disperse and spherical silver nanoparticles.[28,29,32,33]

Table 1: Periodical color change during the biosynthesis of *C. indica* silver nanoparticles

S. No	Time in Hours	Color change
1	0	-
2	1	+
3	2	++
4	4	++
5	8	+++
6	16	++++
7	32	++++

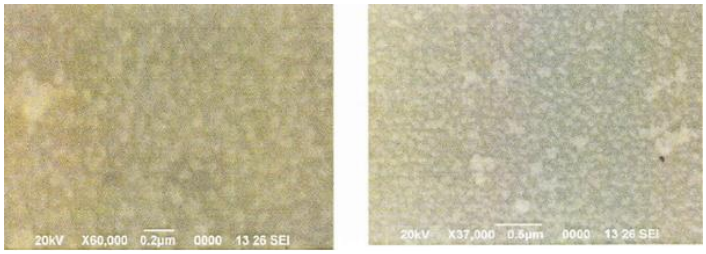
[- Deep yellow; ++ Yellowish brown; +++ Brown shade; ++++ Dark brown]

Figure 1: UV Vis spectra of *C. indica* silver nanoparticles at various time intervals



The micrographs of nanoparticles obtained in the filtrate showed that silver nanoparticles are spherical shaped, well distributed without aggregation in solution with an average size of about 20-30nm. Figure 2 shows SEM image of *C. indica* after exposure to 1mM aqueous $AgNO_3$ solution of 28 hours. SEM measurements of fungus mediated nanoparticle were carried out on JOEL model 6390 Scanning Electron Microscope with Phoenix EDAX attached of 20 kV.

Figure 2: SEM images of *C. indica* spawn mediated silver nanoparticles

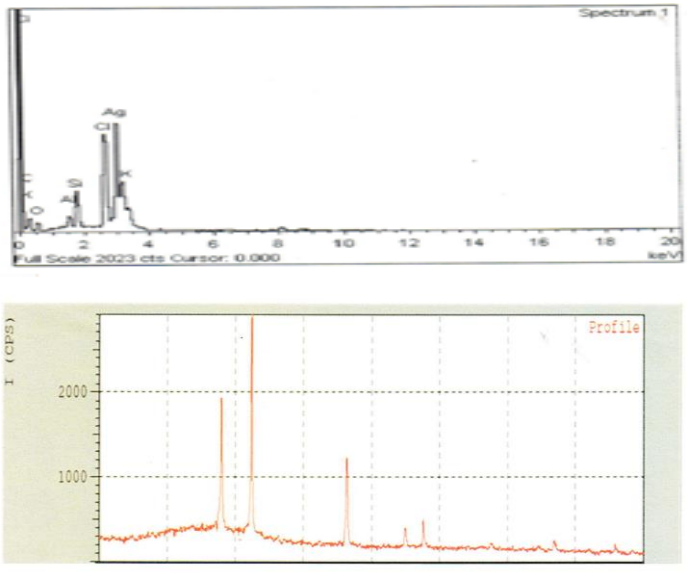


EDAX study showed strong signals from silver and weaker signals from C, Cl, O, K and Si. The weaker signals are likely due to the X-ray emission from proteins or enzymes present in the fungal biomass. Figure 3a shows EDAX spectrum recorded in the spot profile mode from one of the densely populated C. indica mediated silver nanoparticles (Ci-Ag-Nps) region on the surface of the clumps. SEM has provided further insight into the morphology and size details of the synthesized nanoparticles. The results suggest that protein might have played an important role in the stabilization of Ag nanoparticles.[3,23]

EDAX provides analytical data that cannot be obtained by other methods. It is a qualitative X-ray EDS spectrum corresponding to arbitrary nanoparticles (Figure 3b). The analysis confirms that the nanoparticles are pure silver.[2] The exact mechanism leading to the extra cellular formation of silver nanoparticles by challenging the fungus *C. indica* with the corresponding metal ions is not fully understood at the moment. Further studies are required to improve the biosynthesis conditions and the mechanism behind the production of nanoparticle synthesis.

Figure 3a: EDAX profile of Ci-Ag-Nps

Figure 3b: XRD pattern of Ci-Ag-Nps



The *in vitro* antimicrobial activity of the synthesized and characterized *C. indica* mediated silver nanoparticles were

well analyzed thereby the growth on the Mueller Hinton agar plate zones thereby the inhibition was analyzed. The maximum growth inhibition was observed against the bacterial pathogens like *E. coli*, *K. pneumoniae* and *Acinetobacter baumanii* even at 2% concentration followed by MRSA, *S. aureus*, *P. mirabilis* and the fungus C. albicans and *C. neoformans*. The lesser inhibition was observed against *P. vulgaris*, *Serratia marcescens*, *Salmonella typhi*, etc. The detailed bactericidal data was depicted in table 2.

Table 2: Antimicrobial activities of Ci-Ag-Nps

Test microbial pathogens	Zone of inhibition in mm verses various concentrations of Ci-Ag-Nps				Effective dose
	1%	2%	3%	4%	
Acinetobacter baumanii	20	25	26	26	2%
Bacillus cereus	7	7	9	11	-
Candida albicans	17	17	19	21	4%
Cryptococcus neoformans	17	18	18	21	4%
E. coli	20	22	25	28	2%
Klebsiella pneumoniae	21	27	29	32	2%
Micrococcus species	3	5	5	5	-
Proteus mirabilis	18	18	18	18	-
Proteus vulgaris	7	9	11	11	-
Pseudomonas aeruginosa	11	14	14	14	-
Salmonella typhi	11	11	11	14	-
Salmonella paratyphi A	9	9	11	13	-
Salmonella paratyphi B	9	9	11	13	-
Serratia marcescens	9	11	11	13	-
Staphylococcus aureus	18	19	19	20	4%
MRSA	19	22	22	22	4%

The pathogenecity inhibition in terms of study showed very good effect than plating method highlighted the arrest in the motility and metallic sheen colonies (*E. coli*), capsule formation (*K. pneumoniae* and *C. neoformans*), Catalase and coagulase formation (MRSA and *S. aureus*), swarming (*P. mirabilis* and *P. vulgaris*) and germ tube formation (*C. albicans*). The detailed results related to the pathogenicity inhibition were depicted in table 3.

Table 3: Description of pathogenicity inhibition using Ci-Ag-Nps

In nature, the silver has its own antimicrobial properties; when having synergy with effective medicinal herbs, the effect of the silver micro molecules is higher and sometime highest depends on the herbal constituents[34,35]. In this study also, it was effectively proved that the synergetic effect of silver ions with spawn of *C. indica* have wide antimicrobial properties and also confirmed the size of the nanomolecule synthesized.

Silver ions were reduced to silver nanoparticles using the aqueous extract of *C. indica*, indicating that *C. indica* can be used for the biosynthesis of silver nanoparticles effectively. The time and the concentration required for the conversion of the silver ions to silver nanoparticles was optimized using color change, UV-spectroscopy at 420nm[36,37]. The color changes are possible due to the silver ions reduced with some external and intrinsic effects of heat and produces complex. This complex was responsible for changing color. Generally the color change from pale to dark and sometimes threads indicated the synthesis of nanoparticles[34,38].

The observation and characterization of silver nanoparticles normally appear at a wavelength interval of 400 to 600nm. Further, the UV visible spectra of silver nanoparticles synthesized using the *C. indica* aqueous extract evince the blue shift of the absorption band with increasing silver nitrate concentration. This recorded information showed that the silver nanoparticles have formed in the extract, where the one plus has been reduced to zero. The encapsulated and insulated proteins and other secondary metabolites play very important role in both reducing and capping mechanism for nanoparticle formation[36,38].

The size dependent phenomena have been well documented in this study, thereby, the scanning electron imaging of the *C. indica* mediated silver nanoparticles showed mostly spherical particles of a size below 100nm. The blurred images were further clarified and final nanoparticles were oval and spherical in shape. Most of the nanoparticles were aggregated, and few identical particles were observed[39.40].

For determining the chemical composition and crystal structure of a material, XRD analysis was done; therefore, detecting the presence of silver nanoparticles in the test solution can be achieved by examining the diffraction peaks. In this study, the X-ray pattern of synthesized silver nanoparticles matches the bulk silver with the broad peaks that corresponding the planes[41]. In addition to the Bragg peak, additional and undifferentiated peaks were also recorded suggesting that the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles[42]. The broadening of the peaks is primarily due to small particle size. The X-ray diffraction results clearly show that the silver nanoparticles formed by the reduction of Ag^+ ions by the *C. indica* are crystalline in nature[43,44].

For many decades, silver salts have been used as antimicrobial agents where monodispersed small sized spherical nanoparticles provide a higher surface area that enhances the antibacterial potential of silver. In fact, the size-dependent interaction of silver nanoparticles with pathogenic microorganisms and viruses has been reported[45,46]. Thus, this study also having its own wide antimicrobial potentials using aqueous extract of *C. indica* and silver. The enhancing properties of the synthesis and characterization of *C. indica* mediated silver nanoparticles also showed wide microbicidal action including pathogenicity inhibiting nature. These are indicating the presence of biomolecules in *C. indica* and Ci mediated silver nanoparticles are possessing wide antibacterial potential.

Conclusion

Our present study shows the synergistic effect of *C. indica* mediated silver nanoparticles. We achieved spherical nanoparticles size in the range of 20-30nm determined from the SEM analysis and compound purity was confirmed using EDAX. We hope a case has been made for a serious study of fungal sources in the synthesis of nanoparticles as a possible, viable alternative to the more popular physiochemical methods in vogue. A rapid, simple, reliable, cost effective and eco-friendly method for silver nanoparticle synthesis has been developed where the degree of agglomeration of nanoparticle by SEM and particle purification by EDAX are well reported. The wide antimicrobial and pathogenicity inhibitory properties recorded in this study also step forward nanotechnological principles to achieve potent nano-molecules.

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