

Biosynthesis of Silver Nanoparticles Using an Endophytic Fungus, *Curvularialunata* and Its Antimicrobial Potential

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Abstract

The development of reliable green process for the synthesis of silver nanoparticles is an important branch of Nanobiotechnology. In the present investigation the use of the endophytic fungus *Curvularialunata* for the extracellular biosynthesis of silver nanoparticles (AgNPs) from silver nitrate solution is reported. It was observed that the aqueous silver (Ag^+) ions, when exposed to a filtrate of *C. lunata*, were reduced in solution, resulting in the formation of stable AgNPs. These AgNPs were characterized by means of several techniques. The nanoparticles show maximum absorbance at 422 nm on ultraviolet-visible spectra. The presence of protein was identified by Fourier Transform infrared spectroscopy. The reduction of Ag^+ ion to elemental silver was characterized by Energy - dispersive X-ray (EDX) Spectroscopy. Scanningelectron micrograph revealed the formation of polydispersed nanoparticles of 10-50 nm. The nanoparticles were also evaluated for their enhanced antimicrobial activities with various antibiotics against gram positive and gram negative bacteria. The antibacterial activities of Ampicillin, Rifampicin, Chloramphenicol, Erythromycin, and Kanamycin were increased in the presence of AgNPs against test strain. The highest fold increase of area was found for Erythromycin and Carbenicilin against *E. coli*, Ampicilin against *S. paratyphi*, Erythromycin against *B. subtilis*. The results showed that the combination of antibiotics with AgNPshas better antimicrobial effects.

Keywords

Endophytic Fungi, Silver Nanoparticles, SEM, FTIR, Antibacterial Activity

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1. Introduction

Antibiotic resistance by pathogenic bacteria and fungi has been continuously increasing over the past decade and there is a need for the development of new antimicrobial agents. In the present scenario, AgNPs have appeared as a promising antibacterial candidate in the medical field (SindhuPriya et al., 2013 [1]). Currently, nanobiotechnology represents an alternative for chemical and physical methods for the synthesis of NPs (Ahmed et al., 2003 [2], Anusuya and Sathiyabama 2014 [3]). Recognizing the importance of developing an eco-friendly synthesis of nanoparticles, number

of researches have turned to microorganisms that have shown an ability to reduce metal ions. Synthesis of NPs from microbes is a boon for advance research in nanotechnology. The use of fungus for the synthesis of NPs could be more advantageous, because of the tolerance, bioaccumulation (Slawson et al., 1992 [4]) and extracellular production. Further advantages includes its economic viability and ease in handling biomass, large scale production of enzymes. NPs such as Ag, Au, Pt and Pd are widely exploited due to its special physicochemical properties in biochemical applications. Several studies suggested that biomolecules like proteins, phenols and flavonoids play a vital role in the reduction of Ag^+ ions (Ahmad et al., 2011 [5]) which are

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present in the fungal cultures. Therefore the exploitation of the fungal culture filtrate in the synthesis of nanoparticles is fascinating. Colloidal silver has been known for a long time to possess antimicrobial properties and also it is non-toxic and environmentally safe (Sathishkumar *et al.*, 2012 [6]). Silver ions have more advantage against conventional antibiotics; (Antony *et al.*, 2011 [7]).

Endophytes are microorganisms that live to complete at least one stage of its life without causing apparent diseases are obviously a rich and reliable source of bioactive metabolites with large medical, agricultural and industrial potentials (Strobel and Daisy, 2013 [8]; Parthasarathy and Sathiyabama 2014,2015 [9-10]). However, very few endophytic fungi such as *Aspergillus clavatus*, *Epicoecum nigrum* have been reported to biosynthesize of AgNPs (Verma *et al.*, 2010 [11]; Qian *et al.*, 2013 [12]). Clearly, endophytes are relatively unexplored as a potential resource for the synthesis of AgNPs.

The present study involves with the extracellular synthesis and characterization of AgNPs using an endophytic fungus *C. lunata* and evaluating the antibacterial effect of synthesized silver nanoparticles against some human pathogens.

2. Materials and Methods

2.1. Microorganism and Production of Biomass

The fungus *C. lunata* (ITCC No.8428.11), isolated from leaves of *Catharanthus roseus*, was grown in 250 ml Erlenmeyer flasks containing 100 ml Potato dextrose broth (PDB) at 27°C for 72 hrs. After incubation, mycelial biomass were separated by filtration, washed with sterile distilled water to remove the traces of media components.

2.2. Synthesis of Silver Nanoparticles

Typically 15g of biomass (wet weight) was re-suspended in 100ml sterile double distilled water for 48 h at 27°C in an Erlenmeyer flask and incubated. After incubation, the cell filtrate was filtered by Whatman filter Paper No. 1. To the cell filtrate silver nitrate was added (1mM), and incubated at room temperature under dark condition for 24 h.

2.3. Characterization of Silver Nanoparticles

After 2 hours of incubation of the above mixture, the preliminary detection of AgNPs was carried out by visual observation of color change of the cell filtrate. The samples were later subjected to optical measurements, which were carried out by using a UV-visible spectrophotometer (Shimadzu, Japan) and scanning the spectra between 200 to 700 nm at the resolution of 1nm. The pellet was re-suspended in Milli-Q water and freeze dried. FTIR spectroscopic

studies were carried out to find possible bio-reducing agent present in the culture filtrate of *C. lunata*. The FTIR spectrum of the dried sample was recorded on a PerkinElmer 1600 instrument in the range of 4000 cm^{-1} and 400 cm^{-1} . To perform SEM analysis, thin films of the sample were prepared on a carbon coated copper grid. The morphology, size were measured at 100 Kev using Hitachi S-4500 SEM. The size of the distributed silver nanoparticles was also measured by Dynamic Light Scattering (DLS) technique using Malvern Zeta Sizer Nano series compact scattering spectrometer. The stability of the nanoparticle in suspension was determined by measuring the zeta potential (Zeta sizer Nano ZS, Malvern). EDX spectroscopy analysis were carried out for the confirmation of elemental silver in the sample. XRD patterns of silver nanoparticles were obtained by D max -2200, Rigaku, Japan.

2.4. Screening of Antimicrobial Activity

The antimicrobial effect of synthesized AgNPs was evaluated against some human pathogens such as gram negative (*E. coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*) and gram positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*) bacteria by disc diffusion method. Cultures were maintained at -80°C on glycerol stock. They were sub-cultured in Nutrient Broth for 24hrs at 30°C. Each strain was swabbed uniformly into the individual Muller-Hinton agar plates using sterile cotton swabs. Using sterile micropipette, 30 μL of crude culture filtrate, AgNPs were loaded on to sterile paper disc and it was allowed to dry. The sample loaded discs along with standard antibiotic disc were impregnated in the Muller-Hinton agar medium. The doses were selected based on the preliminary data obtained from a laboratory. After 24 hrs incubation at 37°C, the levels of zone of inhibition were measured.

3. Results

3.1. Synthesis of AgNPs

A detailed study on the extracellular biosynthesis of AgNPs by an endophytic fungus, *C. lunata* was carried out. The change in color (dark brown) of the filtrate of *C. lunata* was noted by visual observation, with an extinction of surface Plasmon resonance (SPR) observed at 422 nm in UV- vis spectrophotometer (Figure. 1). In an observation, the increase in reaction time increases the reduction process of AgNPs as noticed in the SPR peak at every 2hrs interval and stabilized after 24 hrs. The stability of the synthesized AgNPs was determined at room temperature for 20 days by UV-spectrophotometer. Synthesized AgNPs shows good stability at room temperature (28 \pm 2°C) without aggregation with an intense SPR peak at 422nm after 20 days.

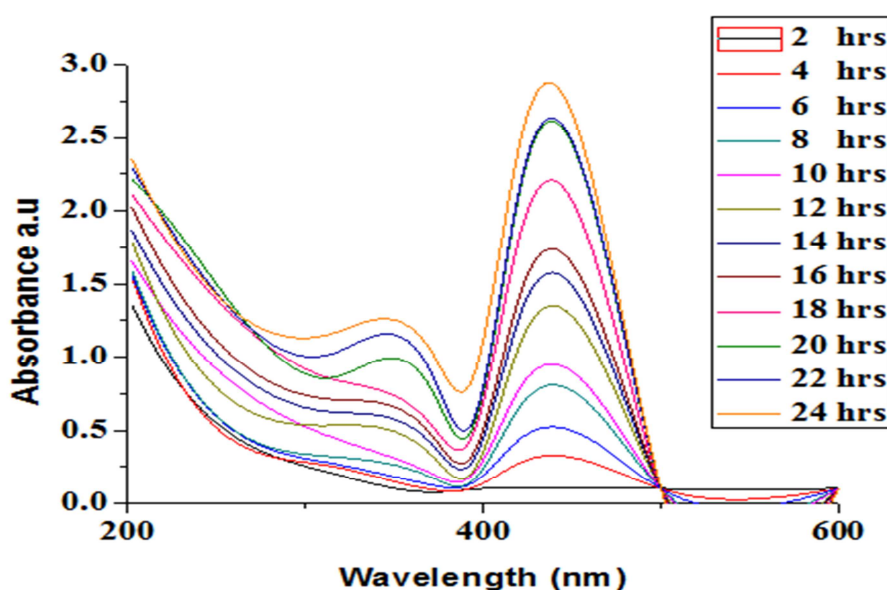


Figure 1. UV-vis spectra at different time intervals.

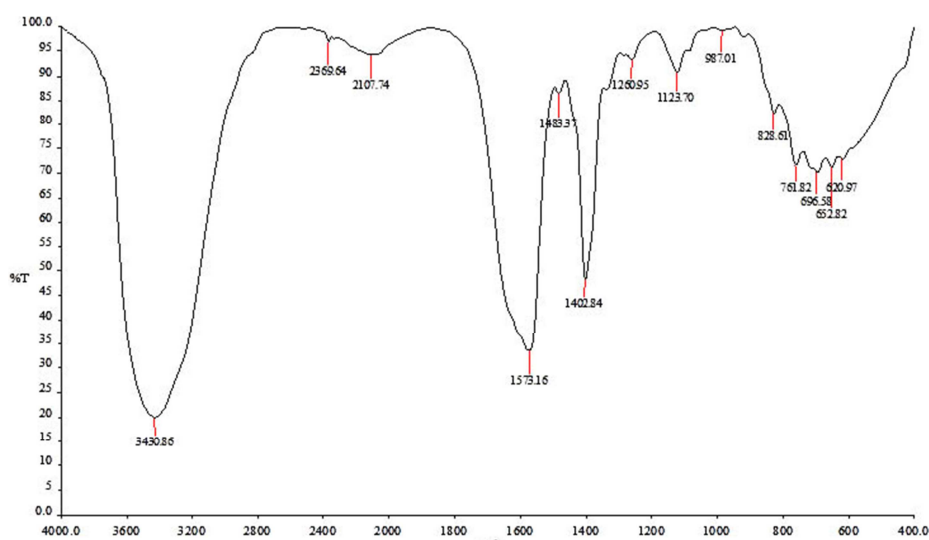


Figure 2. FTIR spectrum recorded from a drop-coated film of an aqueous solution incubated with *C. lunata* and reacted with Ag⁺ ions for 72 h. The amide I and II bands are identified in the figure.

3.2. Characterization of Silver Nanoparticles

FTIR results of synthesized AgNPs show an intense peak at 3430.86 and 1573.16 cm⁻¹ that correspond to the stretching vibrations of the amide I and amide II bands of the proteins. The peaks observed at 1483.37, 1402.84, 1260.95, 1123.70 cm⁻¹ can be assigned to the C-N stretching vibration of aromatic and aliphatic amino acid respectively (Figure. 2). These observations confirm the presence of protein in the sample which might have played an important role in the synthesis and stability of silver nanoparticles. SEM analysis, shows the AgNPs are spherical in shape and the sizes range from 10 to 50 nm in diameter with an average size of 26 nm (Figure. 3a, b). DLS evidenced the average size of the

synthesized nanoparticles of 64.3 nm with a narrow PDI value (0.516). The obtained single peak indicated that the quality of synthesized silver nanoparticles are good. (Figure 4a). The value of Zeta potential of the synthesized nanoparticles as -26.6 mV (Figure 4b) with a single peak, which indicates the stability of synthesized nanoparticles. The EDX spectrum showed a strong metal signal peak of Ag (Figure. 5), other peak of Cu was from the copper grid used for SEM analysis. XRD analysis showed four distinct diffraction peaks at 38.12, 44.30, 64.45 and 77.41 which indexed the planes 111, 200, 220 and 311 of cubic face-centered silver (Fig. 6). This was in agreement with the standard diffraction pattern of silver with cubic structure reported JCPDS File No. 04-0783.

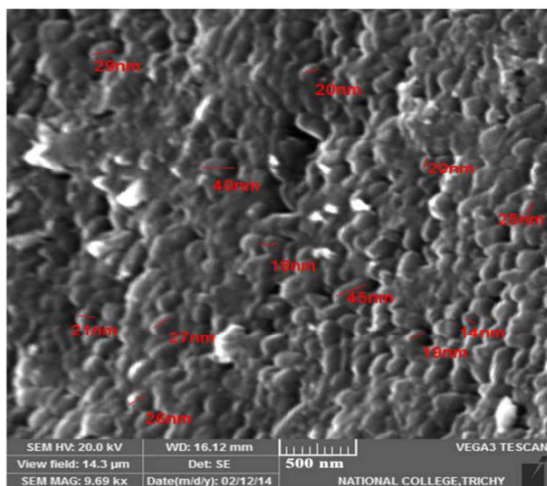


Figure 3a. Scanning electron micrograph of aggregated and protein-capped silver nanoparticles and spherical silver nanoparticles.

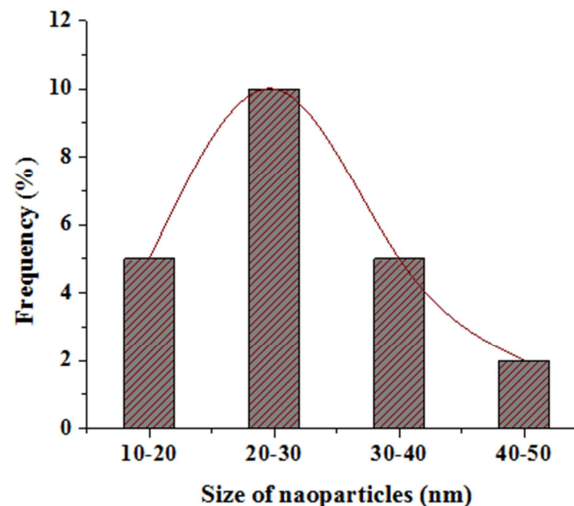


Figure 3b. Particle size histogram of AgNPs.

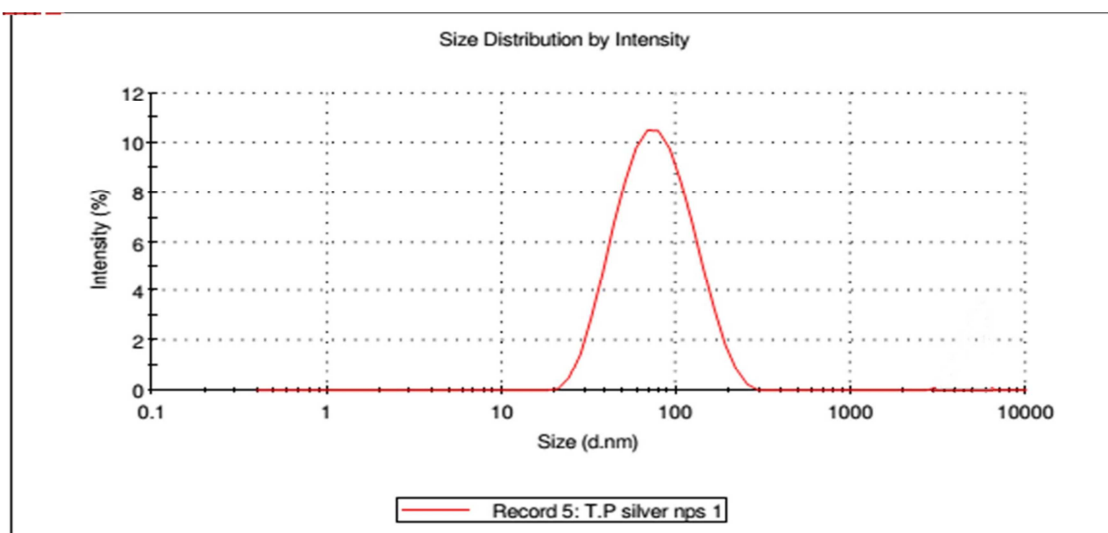


Figure 4a. Dynamic light scattering (DLS) of synthesized silver nanoparticles.

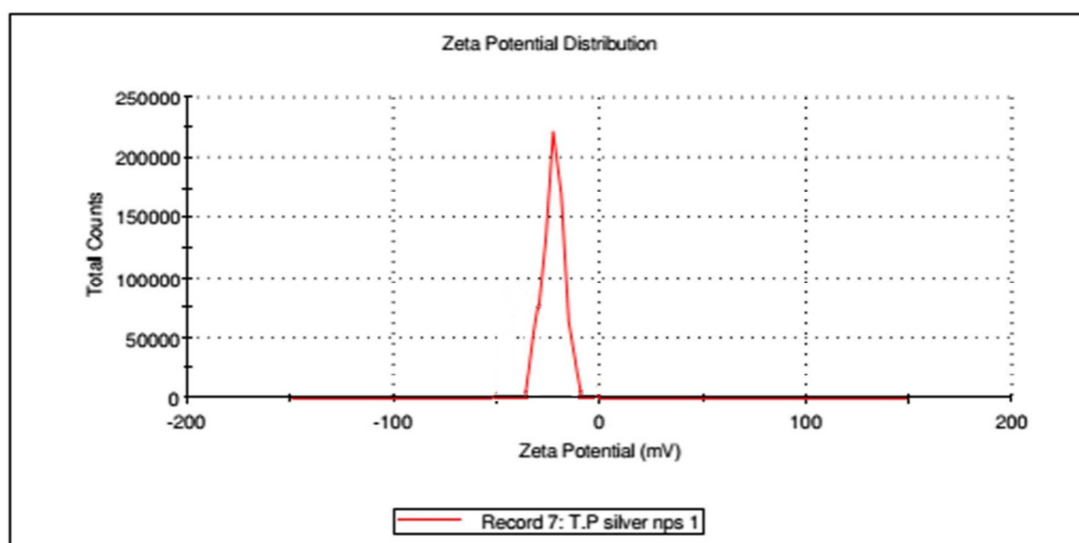


Figure 4b. The effective zeta-potential in aqueous solution were measured by particle characterizer and the mean values were averaged from 3 times assay data.

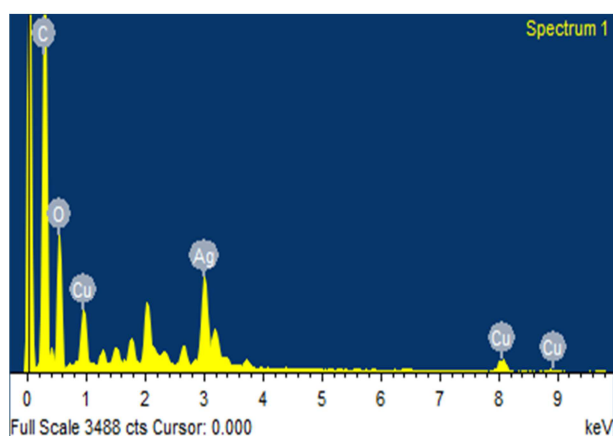


Figure 5. Energy-dispersive spectroscopy spectrum of silver nanoparticles. Various x-ray emission peaks are labeled.

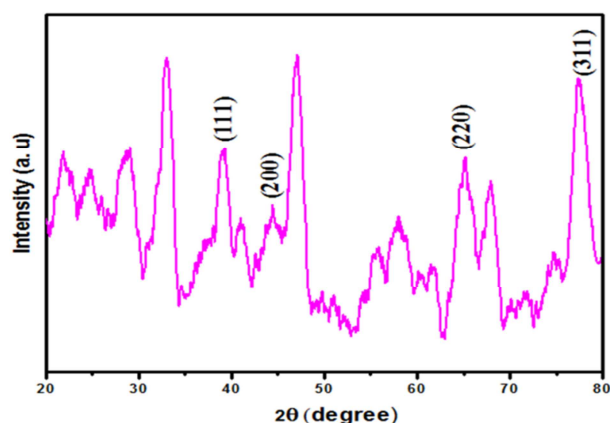


Figure 6. XRD Pattern of synthesized silver nanoparticles.

3.3. Antimicrobial Activity of AgNPs

Antimicrobial effects of synthesized AgNPs was tested against human pathogens like *E. coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Bacillus subtilis*, *Staphylococcus aureus* and *Bacillus cereus* (Table. 1). Synthesized AgNPs showed inhibitory activity against gram negative and gram positive bacterial pathogens.

Table 1. Zone of inhibition (mm) of different antibiotics against test strains (in absence of and in presence of AgNPs at concentration of 10 μ g/disk gram positive and gram negative bacteria).

Antibiotic	<i>E. coli</i>		
	Antibiotic (a)	Antibiotic with AgNPs (b)	Increase fold in area (b ² -a ² /a ²)
Penicillin	8	10.6	0.75
Ampicilin	5	6.66	0.77
Rifampicin	10.33	14.33	0.92
Erythromycin	10	14.33	1.05
Kanamycin	10.66	13	0.48
Chloramphenicol	11.33	14.66	0.67
Carbenicilin	15	22	1.15

Antibiotic	<i>Salmonella paratyphi</i>		
	Antibiotic (a)	Antibiotic with AgNPs (b)	Increase fold in area (b ² -a ² /a ²)
Penicillin	12.33	15	0.48
Ampicilin	8	11.33	1.00
Rifampicin	10	12	0.44
Erythromycin	10.33	13	0.58
Kanamycin	11	13.66	0.54
Chloramphenicol	14.33	17	0.40
Carbenicilin	11.33	15	0.75

Antibiotic	<i>Bacillus subtilis</i>		
	Antibiotic (a)	Antibiotic with AgNPs (b)	Increase fold in area (b ² -a ² /a ²)
Penicillin	6.33	8.33	0.73
Ampicilin	8	9.66	0.45
Rifampicin	10.66	12.66	0.41
Erythromycin	8.33	12	1.07
Kanamycin	10.66	11.66	0.19
Chloramphenicol	11.66	14	0.44
Carbenicilin	10.33	12	0.34

Antibiotic	<i>Staphylococcus aureus</i>		
	Antibiotic (a)	Antibiotic with AgNPs (b)	Increase fold in area (b ² -a ² /a ²)
Penicillin	7	9.33	0.77
Ampicilin	6.33	7.33	0.34
Rifampicin	9	10.66	0.40
Erythromycin	10.33	12.66	0.50
Kanamycin	10	11	0.21
Chloramphenicol	10.66	13	0.48
Carbenicilin	11.33	13	0.31

4. Discussion

The study of the biosynthesis of nanomaterials offers a valuable contribution to nanobiotechnology. In this present study an endophytic fungus was evaluated for synthesis of nanoparticles in short reaction time. When *C. lunata* culture filtrate was mixed with aqueous solution of silver nitrate, it starts to change the color from the yellowish to brown due to the reduction process of silver ions (Yang *et al.*, 2007 [13]; Wang & Su 2001 [14]). The synthesized AgNPs were confirmed based on the UV-vis spectra at 423 nm due to excitation of Surface Plasmon Resonance (Mulvaney *et al.*, 1996 [15]). This observation indicates that the reduction of the Ag⁺ ions takes place extracellularly. Absorbance band at 260nm is clearly visible and is attributed to electronic excitation in tryptophan and tyrosine residues in protein (Eftnick and Ghiron, 1981 [16]), which indicates the release of extracellular proteins in the colloidal solution.

The peaks observed in the FTIR spectrum shows the presence of proteins, which may act as a ligand for AgNPs. These evidence suggests that the release of extracellular protein molecules could possibly influence the reduction and stabilization of the silver nanoparticles. The presence of nitrate reductase was observed in the extracellular proteins secreted by the fungus *C. lunata* (data not shown). This might be responsible for the bioreduction (of Ag^+ to Ag^0 and the subsequent formation of silver nanoparticles), formation of stable nanoparticles (He *et al.*, 2011 [17]). Various studies have indicated that NADH, NADH-dependent nitrate reductase are involved in the biosynthesis of metal nanoparticles (Klittich & Leslie, 1988 [18]; Lloyd 2003[19]). Ahmed *et al.*, (2003) [20] have reported that certain NADH-dependant reductase was involved in the reduction of silver ions by *F. oxysporum*.

Size and shape of synthesized AgNPs was characterized through scanning electron microscope, it shows spherical particles with size less than 50nm, confirms that reduction of silver ions to AgNPs (Jain *et al.*, 2008 [21]). The data analysis of dynamic light scattering (DLS) supported the average size of the synthesized nanoparticles is 64.30 nm with 0.516 PDI value. The obtained single peak indicated that the quality of the synthesized nanoparticles is good. The values of the zeta potential of the nanoparticles were measured to be -26.6mV (Fig. 4a, b). The variation in size of the nanoparticles analyzed by SEM and DLS may be due to the different principle involved in these techniques (Manikandan and Sathiyabama, 2015 [22]). The reduction of Ag^+ ions to elemental silver by *C.lunata* is further confirmed by EDX analysis, which shows an optical absorption peak in the range of 3 to 4 keV (Fig 5.). This is typical for the absorption of metallic silver nanocrystallites (Magudapathy *et al.*, 2001 [23]). XRD patterns in agreement with a standard diffraction pattern of silver cubic structure (Qian *et al.*, 2013 [12]).

Synthesized AgNPs shows high inhibitory activity against gram negative and gram positive bacterial pathogens, but the mechanism of inhibitory action is not fully confirmed. Exact antimicrobial effect of AgNPs was still unclear. The results suggest that the inhibition may be due to either plasmolysis (Morones *et al.*, 2005 [24]), or silver ions may penetrate into the bacterial cell wall and cause damage to DNA (Feng *et al.*, 2000 [25]). This research provides helpful insight into the development of new antimicrobial agent.

5. Conclusion

The results of this work confirms the synthesis of silver nanoparticles. The synthesized AgNPs were characterized by UV-visible spectroscopy, FTIR, SEM, DLS and XRD analyses. The synthesis of AgNPs by the endophytic

fungus *C. lunata* may therefore serve as a simple, cheap and ecofriendly approach. The antimicrobial activity of AgNPs shows much potent inhibition against human pathogens. Hence AgNPs prepared by the cost effective reduction method described here have great promise as therapeutic agents.

Acknowledgements

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References

- [1] D. Sindhupriya, M. Amitava, N.Chandrasekaran. Synergistic effect of biogenic silver nanocolloid in combination with antibiotics: a potent therapeutic agent, International Journal of Pharmacy and Pharmaceutical Sciences.5, 0975-1491, 2013.
- [2] P. Ahmad, P. Mukherjee, S. Senapati, D. Mandal, M.I. Khan, M.Sastry. Extracellular biosynthesis of silver nanoparticles using the fungus *Fusariumoxysporum*. Colloids Surface B Biointerfaces. 28, 313–318, 2003.
- [3] S. Anusuya, M. Sathiyabama. Preparation of β -D-glucan nanoparticles and its antifungal activity, Int J BiolMacromol. 70, 440-3, 2014.
- [4] R.M. Slawson, J.T. Trevors, H. Lee. Silver accumulation and resistance in *Pseudomonas stutzeri*. Arch Microbiol. 158, 398-404, 1992.
- [5] N. Ahmad, S. Sharma, V.N. Singh, S.F. Shamsi, A. Fatima, B.R. Mehta. Biosynthesis of silver nanoparticles from *Desmodiumtriflorum*: A novel approach towards weed utilization. Biotechnol Res Int.1-8, 2011.
- [6] G. Sathishkumar, C. Gobinath, K. Karpagam V. Hemamalini K. Premkumar, S. Sivaramakrishnan. Phyto-synthesis of silver nano scale particles using *Morindacitrifolia* L. and its inhibitory activity against human pathogens. Coll Surf B 95: 235–240, 2012.
- [7] J. Antony, P. Sivalingam, D. Siva, S. Kamalakkannan, K. Anbarasu, R. Sukirtha, M. Krishnan, S. Achiraman,. Comparative evaluation of antibacterial activity of silver nanoparticles Synthesized using *Rhizophoraapiculata* and glucose. Colloids Surface B Biointerfaces.88, 134-140, 2011.
- [8] G. Strobel, B. Daisy. Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiology and Molecular Biology Reviews. 67(4), 491-502, 2003.
- [9] R. Parthasarathy, M. Sathiyabama. Gymnemagenin-producing endophytic fungus isolated from a medicinal plant *Gymnemasylvestre* R.Br. ApplBiochemBiotechnol. 172: 3, 141–3152, 2014.
- [10] R. Parthasarathy, M. Sathiyabama. Lovastatin-producing endophytic fungus isolated from a medicinal plant *Solanumxanthocarpum*. Natural Product Research. 14786419.2015-1016938, 2015.
- [11] V.C. Verma, R.N. Kharwar, A.C. Gange, Biosynthesis of antimicrobial silver nanoparticles by the endophytic fungus *Aspergillusclavatus*. Nanomedicine: Nanotechnology. Biology and Medicine. 5, 33–40, 2010.

- [12] Y. Qian, H. Yu, D. He, H. Yang, W. Wang, X. Wan, L. Wang. Biosynthesis of silver nanoparticles by the endophytic fungus *Epicoccumnigrum* and their activity against pathogenic fungi. *Bioprocess Biosyst. Eng.* 36, 1613-1619, 2013.
- [13] S. Yang, T. Chen. K. Li T. Tsai. Change in phenolic compound content, reductive capacity and ACE inhibitory activity in noni juiceduring traditional fermentation. *J. Food Drug Anal.* 15 (3), 290-298, 2007.
- [14] M.Y. Wang, C. Su. Cancer preventive effect of *Morindacitrifolia* (Noni). *Ann N Y Acad Sci.* 952, 161-8, 2001.
- [15] P. Mulvaney. Surface plasmon spectroscopy of nanosized metal nanosized metal particles. *Langmuir*, 12, 788-800, 1996.
- [16] M.R. Eftink, C.A. Ghiron, Fluorescence quenching studies with proteins. *Anal. Biochem.* 114, 199-227, 1981.
- [17] S. He, Z. Guo, Y. Zhang, S. Zhang, J. Wang, N. Gu, Biosynthesis of gold nanoparticles using the bacteria *Rhodopseudomonas capsulate*, *Materials Letter.* 61, 3984-3987, 2007.
- [18] C.J.R Klittich, J.F Leslie. Nitrate reduction mutants of *Fusariummoniliforme* (gibberella-fujikuroi) *Genetics.* 118,417-423. 1988.
- [19] J.R Lloyd. Microbial metal reduction. *FEMS Microbiol. Rev.* 27, 411, 2003.
- [20] A. Ahmad, P. Mukherjee, S. Senapati, D. Mandal, M.I. Khan, M. astry. Extracellular biosynthesis of silver nanoparticles using the fungus *Fusariumoxysporum*. *Colloids Surface B Biointerfaces.* 28, 313-318. 2003.
- [21] P.K. Jain, X.H. Hunag, I.H. El-Sayed, M.A. El-Sayed. Noble metals on the nanoscale: optical and phtothermal properties and some applications in imaging, sensing, biology and medicine. *Acc. Chem. Res.* 41, 1578-1586, 2008.
- [22] A. Manikandan, M. Sathiyabama. Green Synthesis of Copper-Chitosan Nanoparticles and Study of its Antibacterial Activity. *J NanomedNanotechnol.* 157-7439, 2015.
- [23] P. Magudapathy, P. Gangopadhyay, B.K. Panigrahi, K.G.M. Nair, S. Dhara. Ion beam sputtering and nanostructures of noble metals. *Physics B.* 299, 142-146, 2001.
- [24] J.R. Morones, Jose L. Elechiguerra A. Camacho, HoltK, J.B. Kouri J. Tapia Ramirez M. Jose Yacaman. The bactericidal effect of silver nanoparticles. *Nanotechnology* 16, 2346-2353, 2005.
- [25] Q.L. Feng, J. Wu, G.Q. Chen, F.Z. Cui, T.N. Kim, J.O. Kim, "A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*." *Journal of Biomedical Materials Research Part A.* Volume 52, issue 4, P, 662-668, 2000.