Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 2, 2013

Research Article

ENDOPHYTIC FUNGI MEDIATED EXTRACELLULAR SILVER NANOPARTICLES AS EFFECTIVE ANTIBACTERIAL AGENTS

SWETHA SUNKAR[1] AND C. VALLI NACHIYAR[2]

[1,2]**Department of Biotechnology, Sathyabama University, Chennai, India**

Received: 24 Nov 2012, Revised and Accepted: 05 Jan 2013

ABSTRACT

Background: Bionanotechnology has emerged in the recent past for developing facile, green and ecofriendly technology for synthesis of nanoparticles of variable size, shapes, chemical composition and controlled dispersity owing to their potential use for human benefits. Objective: The present study focuses on the biosynthesis of silver nanoparticles using one faction of microbes, the endophytic fungi as a "green" alternative to the chemical method. Methodology: The silver nanoparticles are synthesized extracellularly using the cell free filtrate facilitating the process of easy extraction and were evaluated for their antimicrobial and dye degrading abilities. The characterization of these biogenic silver nanoparticles was carried out by UV-VIS spectroscopy, Scanning electron microscopy, Transmission electron microscopy and FTIR analysis. Results: The formation of nanoparticles was initially observed by change in colour and was later confirmed by UV-VIS spectroscopy that showed an absorption peak at 420 nm characteristic of silver. The TEM and SEM in analysis revealed the size of the nanoparticles to be in the range of 13 - 35 nm with a smooth surface. The nanoparticles displayed significant antimicrobial activity against *E. coli, S. aureus, K. pneumoniae, S. typhi and P. aeruginosa.* The MIC was determined to be 1.08 µg/ml (GX2-AgNPs) and 0.72 µg/ml (ARA-AgNPs). A preliminary study was carried out to advocate the decolourising ability of these nanoparticles against azo dyes. Endophytes have made an entry in the catalogue of benign synthesisers of bionanoparticles generating silver nanoparticles as the new age antimicrobials.

INTRODUCTION

Biological entities and inorganic materials have been in constant touch with each other ever since inception of life on the earth. Due to this regular interaction, life could sustain on this planet with a well-organized deposit of minerals. Recently scientists become more and more interested in the interaction between inorganic molecules and biological species and were fascinated by the myraid uses identified from these interactions especially in medical field. Metals as nanoparticles with their small size and high surface to volume ratio provides a driving force for diffusion which is of paramount importance in the field of medicine to traverse through the biological barriers.

A wide variety of physical and chemical methods to synthesize nanoparticles are in practice but their inherent flaws that include contamination from precursor chemicals, use of toxic solvents and generation of hazardous by-products [1] makes their use inappropriate in biological systems. These disadvantages insisted the use of novel and well refined methods that opened doors to explore benign and green routes for synthesizing high-yielding, low cost, non-toxic and environment friendly nanoparticles. Nature has elegant and ingenious ways of creating the most efficient miniaturized functional materials.

Nanoparticles produced by a bio-enzymatic process are far superior, in several ways, to those particles produced by chemical methods despite the fact that the latter methods are able to produce large quantities of nanoparticles with a defined size and shape in a relatively short time. With an enzymatic process, the use of expensive chemicals is eliminated, and the more acceptable "green" route is not as energy intensive as the chemical method and is also environment friendly. Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities. It can be classified into intracellular and extracellular synthesis according to the location where nanoparticles are formed [2, 3]. The nanoparticles have been used in a variety of applications including drug carriers for targeted delivery, cancer treatment, gene therapy and DNA analysis, antibacterial agents, biosensors, enhancing reaction rates, separation science, and magnetic resonance imaging (MRI) [4].

The multi-resistant pathogens due to antigenic shifts and/or drifts are ineffectively managed with current medications. This resistance to medication by pathogens has become a serious problem in public health and therefore mandating the need to develop new bactericides and virucides. Silver nanoparticles (AgNPs), having a long history of general use as an antiseptic and disinfectant, are able to interact with disulfide bonds of the glycoprotein/protein contents of microorganisms such as viruses, bacteria [5, 6] and fungi [7]. Both silver nanoparticles and silver ions can change the three dimensional structure of proteins by interfering with S-S bonds and block the functional operations of the microorganism [8, 9].

Recently, scientists have made efforts to make use of microorganisms as possible eco-friendly nanofactories for the synthesis of silver nanoparticles. In an attempt to create miniaturized structures, the present was carried out to synthesise silver nanoparticles using endophytic fungi have been and study their antibacterial activity and dye degrading ability.

MATERIALS AND METHODS

Isolation of the endophytic fungi

Leaf samples of *Garcinia xanthochyumus* and *Aravae lanata* were cleaned under running tap water to remove debris and then air dried and processed within 5 hrs of collection. From each leaf sample, 4 segments of 1 cm length were separated and treated as replicates. Surface sterilization was carried out by submerging them in 75% ethanol for 2 min. The explants were further sterilized sequentially in 5.3% sodium hypochlorite (NaOCl) solution for 5 min and 75% ethanol for 0.5 min [10]. Samples were allowed to dry on paper towel in a laminar air flow chamber. Four segments per plant were placed horizontally on separate Petri dishes containing Potato Dextrose Agar (PDA). After incubation at 32°C for three days, the endophytic fungi was collected and placed onto PDA and incubated for 3 days and checked for culture purity. Eventually, pure cultures were transferred to PDA slant tubes and subcultured regularly.

Production of biomass and synthesis of Silver Nanoparticles

The fungi obtained were grown aerobically in liquid broth containing malt extract powder, glucose, yeast extract, peptone. The culture flasks were incubated on room temperature at 27°C. The biomass was harvested after 7 days of growth by sieving through a plastic sieve followed by extensive washing with sterile double-distilled water to remove any medium components from the biomass. Typically 20 g of biomass (wet weight) were brought into contact with 100 ml sterile double-distilled water for 72 hours at 27°C in an Erlenmeyer flask and agitated at 150 rpm. After incubation the cell filtrate was obtained by filtering using Whatman

filter paper No 1. 100 mL of cell filtrate is challenged 1 mM silver nitrate and incubated under dark conditions [11].

Characterization of Silver Nanoparticles

The formation of AgNPs was followed by visual observation of color that changes from pale white to brown and was further confirmed by the sharp peaks given by the AgNPs in the visible region from UV – vis spectrum of the reacting solution using Perkin-Elmer Lamda-45 spectrophotometer, in a 1cm path quartz cell at a resolution of 1 nm from 250 to 800 nm. The studies on morphology, size and the distribution of nanoparticles were performed by Transmission Electron Microscopic (TEM) analysis using a TEM, JEM- 1200EX, JEOL Ltd., Japan, Scanning Electron Microscope (SEM) using Hitachi S-4500 SEM. The probable biomolecules involved in the synthesis and stabilization of nanoparticles was recorded by FTIR spectrum using FTIR Nicolet Avatar 660 (Nicolet, USA) [1].

Antimicrobial activity of silver nanoparticles

The potential of silver nanoparticles as effective antimicrobial agents have been well appreciated. Hence the silver nanoparticles were checked for their antibacterial efficiancy using the agar well diffusion assay method [12]. The test organisms used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883. The respective test organisms were prepared by spreading 500 µL of revived culture on the nutrient agar plate. 6 wells were cut with the help of a sterilized stainless steel cork borer into which different concentrations of AgNP solution (10, 20, 30, 40, 50 and 100 µL) of was loaded and incubated at 37^0 C. The plates were examined for evidence of zones of inhibition, which appear as a clear area around the wells. The diameter of such zones of inhibition was measured for each organism and expressed in millimeter. The minimum inhibitory concentration was determined by the microdilution method using varying concentrations of the AgNPs.

Dye Degradation

The synthesied nanoparticles were tested for their ability to decolorize azodyes like Acid blue 113, Acid black 24 and Mordant black 17 at 1ppm and 10ppm. Using sterile micropipettes, Ag-NPs of varying concentrations (25µg, 50µg and 100µg) were added, while one was maintained as blank. The test tubes were incubated at 32^{0}C for 24hrs. After incubation, samples were withdrawn and analyzed spectrophotometrically using UV-Visible spectrophotometer.

RESULTS AND DISCUSSION

Nanomedicine is a burgeoning field of research with tremendous prospects for the improvement of the diagnosis and treatment of human diseases. The biosynthesis of nanoparticles by microbes is thought to be clean, nontoxic, and environmentally acceptable "green chemistry" procedures. The rate of formation and size of the nanoparticles could, to an extent, be manipulated by controlling parameters such as pH, temperature, substrate concentration, and exposure time to substrate. Fungi, the biofactories of nanoparticles, have taken the centre-stage in the biological metal particle generation due to their tolerance and bioaccumulation ability of metals [13]. Their efficacy in the production of enzymes on a large scale, the ease of handling biomass makes it a model system for nanoparticle synthesis.

The plants that were used to isolate the endophytic fungi were *Garcinia xanthochyumus* and *Aravae lanata*. One endophytic fungus was isolated from each plant and subcultured regularly. These were designated as GX2 and ARA. These fungi were grown aerobically in MGYP medium. The culture flasks were incubated on room temperature at 27°C (Fig 1).

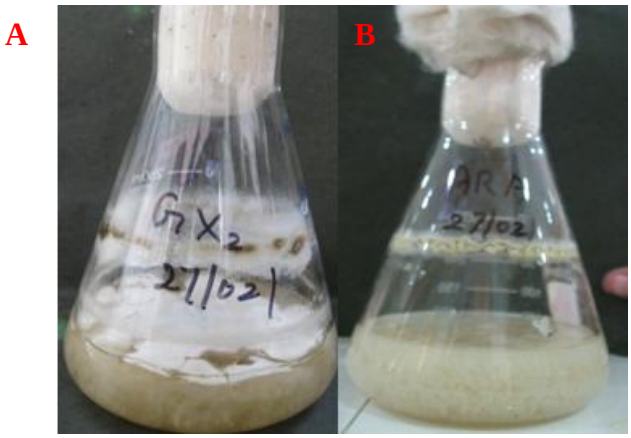

Fig. 1: Biomass of the isolate A) GX2 isolated from *Garcinia xanthochyumus* and B) ARA isolated from *Aravae lanata.*

Formation of silver nanoparticles

The endophytic fungi were grown in MGYP medium at 27°C until significant amount of biomass was produced. This separated and washed biomass was resuspended in MilliQ water and incubated for 3 days at 27°C. There has been an extracellular synthesis of silver nanoparticles by employing the cell free filtrate of the endophytic fungi. This mode of synthesis has an edge over the intracellular synthesis as the latter demands an additional step of releasing the nanoparticles from the biomass by certain chemical methods or ultrasound treatment. The filtrate which was initally in pale white in color turned deep brown (Fig 2) when challenged with $AgNO_3$ that indicated the formation of silver nanoparticles (AgNPs).

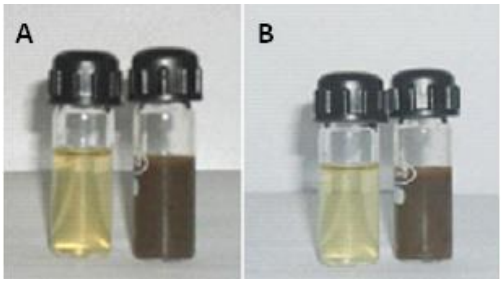

Fig. 2: Colour change observed from pale yellow to brown indicating the formation of silver nanopartilces A) GX2- AgNPS B) ARA-AgNPS

The characteristic brown color arises due to excitation of surface plasmon vibrations in the silver metal nanoparticles [14]. The color intensity of the cell filtrate with $AgNO_3$ was sustained even after 24 h incubation, which indicated that the particles were well dispersed in the solution, and there was no obvious aggregation.

Characterisation of silver nanoparticles

Primary conformation of the AgNPs was carried out by UV- Visible spectrophotometric analysis which showed a strong surface plasmon band at 400 nm and 423 nm by GX2-AgNPs and ARA-AgNPs respectively (Fig 3).

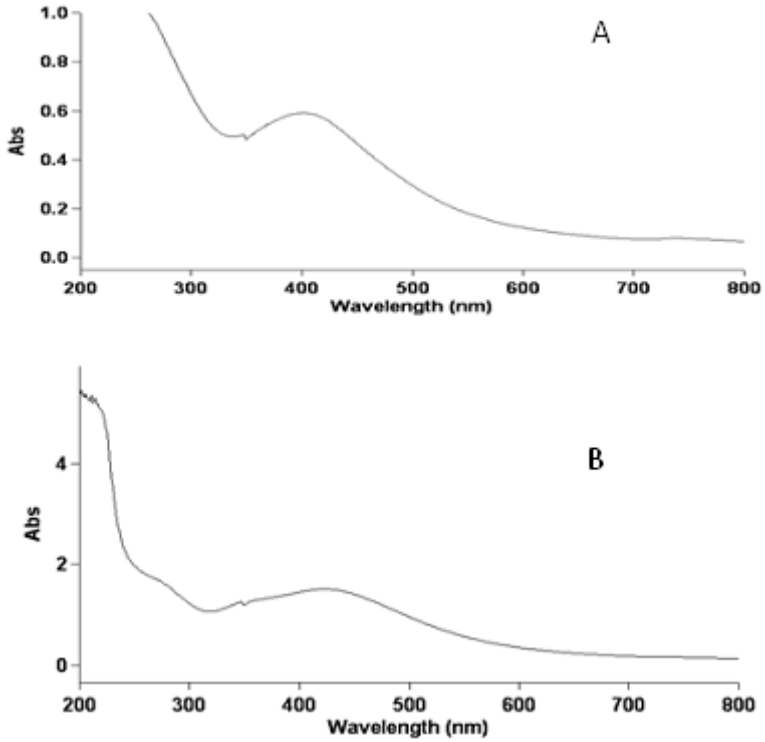

Fig. 3: UV- Visible spectra of the silver nanoparticles A) GX2-AgNPs B) ARA-AgNPs

Observation of this sharp clear peak, assigned to a surface plasmon, was well documented for various metal nanoparticles with sizes ranging from 2 to 100 nm [15, 16]. A long tailing on the large-wavelength side may be due to small amount of particle aggregation [14].

The size and shape of the nanoparticles that plays a significant role in their function is identified by SEM and TEM analysis (Fig.4). The SEM micrographs recorded showed comparatively spherical nanoparticles that were observed to be uniformly distributed. The TEM images from the drop-coated film of the silver nanoparticles showed a very clear picture in which the shape of the nanoparticles was found to be multivarient from round to square to sometimes polygonal and were found to be dispersed with minimum aggregation. Their size was in the range of 25 – 50 nm. Similar results were obtained by Kuber C Bhainsa [18] and Verma [19] where the size of the nanoparticles was in the range of 13-35 nm.

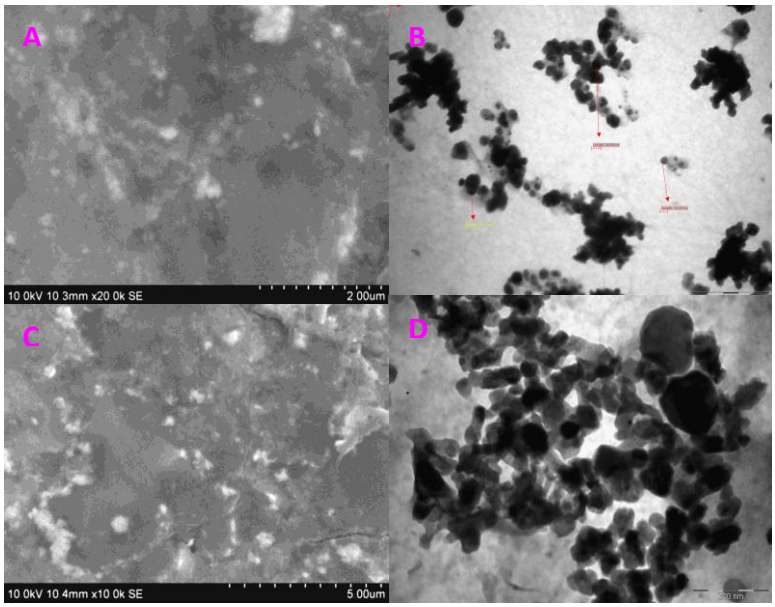

Fig. 4: A) SEM micrograph of GX2-AgNPS B) TEM image of GX2-AgNPS C) SEM micrograph of ARA-AgNPs D) TEM image of ARA-AgNPs

The mechanism leading to formation of silver nanoparticles is not definitely understood at the moment. It is stated that certain extracellular proteins released into the filtrate by the organism could play a role in the synthesis and stability of the silver nanoparticles. FTIR analysis of the silver nanoparticles provides information about the chemical bonds and molecular structures of a possible material that could play a role in the formation of nanoparticles. The FTIR measurements of the AgNPs formed by the endophytic isolates GX2 (Fig 5) and ARA (Fig 6) showed similar kinds of spectra with certain prominent peaks.

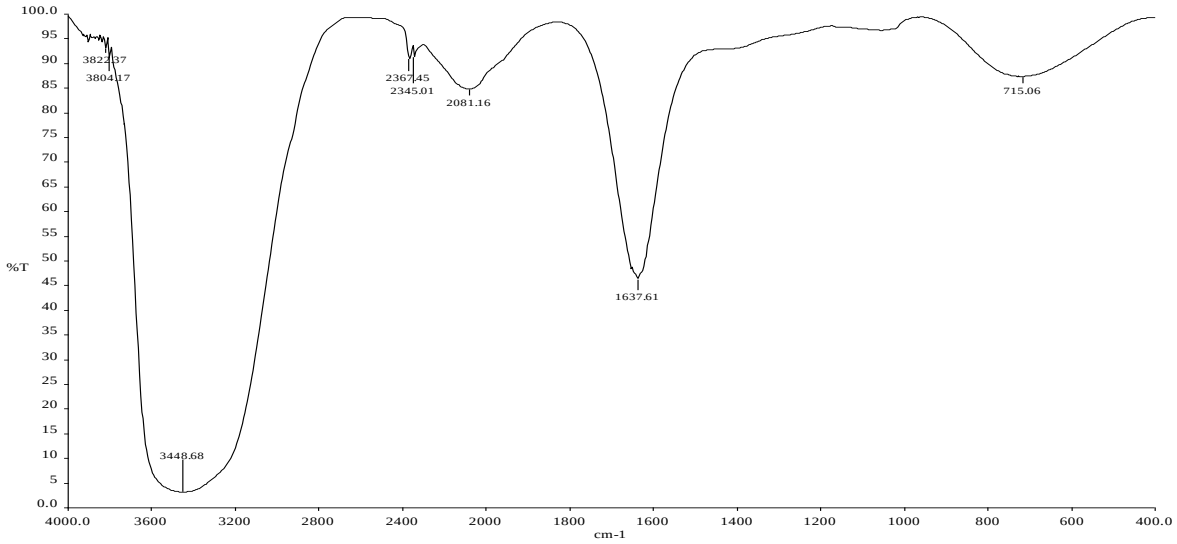

Fig. 5: FTIR spectra of the GX2-AgNPs

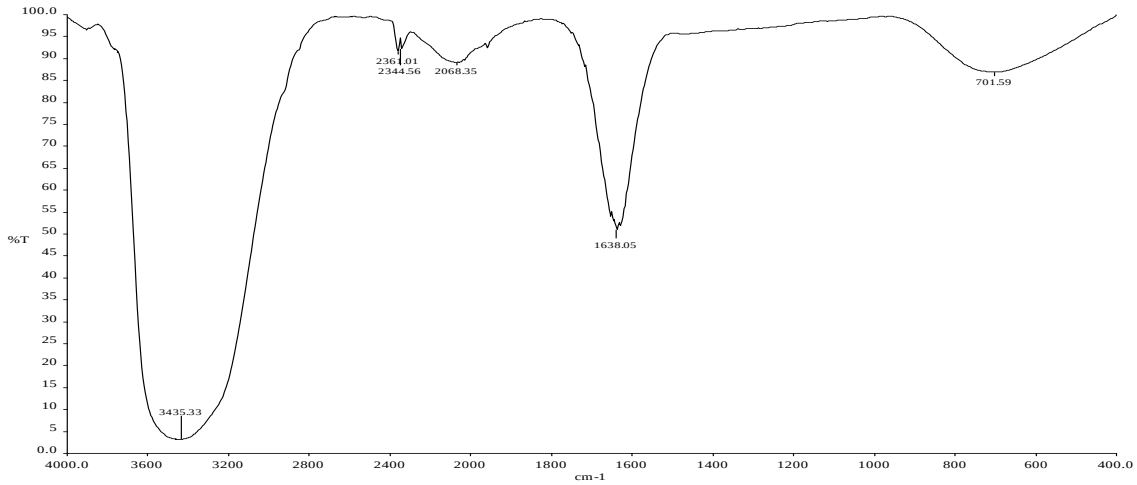

Fig. 6: FTIR spectra of the ARA-AgNPs

The broad peaks given by GX2-AgNPs and ARA-AgNPs at 3448 and 3435 cm^{-1} corresponds to the N-H stretching vibrations amines. The two peaks at 2367 cm^{-1} and 2344 cm^{-1} obtained in both the spectra corresponds to the stretching vibrations of carboxylic acids. A small peak at 2068 and 2081 cm-1 in both the AgNPs is indicative of transitional carbonyls. The bands at 1638 cm-1 correspond to the stretch molecule vibration while the peak at 715 and 701 cm-1 can be assigned to the aromatic C-H out of plane bending vibrations. This FTIR spectrum supports the presence of proteins in the synthesis of silver nanoparticles [20].

Table 1: Zones of inhibition shown by the biogenic silver nanoparticles at different concentrations.

S. No.	Organisms	Zones of Inhibition (mm)							
		AgNPs Conc (µl)							Ofloxacin
		Sample	10	20	30	40	50	100	
1	*Klebsiella pneumoniae*	GX2	--	--	8	8	11	12	13
		ARA	10	14	12	12	13	14	
2	*Salmonella typhi*	GX2	-	-	14	16	16	17	15
		ARA	--	10	10	10	12	14	
3	*Staphylococcus aureus*	GX2	--	--	8	10	10	11	15
		ARA	12	14	16	15	15	17	
4	*Pseudomonas aeruginosa*	GX2	--	--	8	12	14	16	12
		ARA	--	10	11	12	13	16	
5	*Escherichia coli*	GX2	--	--	8	12	10	12	13
		ARA		17	17	16	17	16	

Antibacterial activity and MIC determination

The resistance of bacteria to the available drugs forms a hassle to the treatment process of various diseases. Hence there are constant efforts to find novel antimicrobial agents from all available sources in combating this resistance [21]. Silver and its related compounds are in use as antimicrobial agents for long [22, 23]. Therefore a study on the antibacterial activity of the biogenic silver nanoparticles was carried out against the pathogens used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883 by agar well diffusion method against a standard antibiotic Ofloxacin and the MIC was determined (Table 1).

From table 1, it has been observed that the nanoparticles exhibited significant antibacterial activity reflected by the zones of inhibition in comparison with the standard antibiotic. The minimum inhibitory concentration for the silver nanoparticles was determined to be 1.08µg/1ml (GX2-AgNPs) and 0.72µg/1ml (ARA-AgNPs). The efficacy of silver nanoparticles can be attributed to the fact that their larger surface area enables them a better contact with the microorganisms. The toxicity of silver ions, though not very clearly understood, could be by their adhesion to the cell membrane and further penetration inside or by interaction with phosphorus containing compounds that may disturb the replication process or preferably by their attack on the respiratory chain [23].

Dye decolourisation studies

A preliminary study on the dye decoloursing ability of silver nanoparticles was carried out where these biogenic AgNPs were used to degrade three azo dyes namely Acid blue 113, Acid Black 24 and Mordant black 17. Dyes at 1 ppm and 10 ppm were exposed to different concentrations of AgNPs to evaluate their ability to decolourise the dyes which was given as % of decolourisation.

$$\% \text{ decolorisation} = (O.D_{control} - O.D_{test} / O.D_{control}) \times 100$$

After incubation of the dyes with AgNPs for 24 hrs, it has been observed that there is notable decrease in the colour intensity. The % decolourisation is given in Table 2.

Table 2: Percentage decolorisation of dyes at 1ppm and 10ppm using different concentrations of AgNPs

Dyes	Conc. of Dye (ppm)	Percentage decolourisation by the AgNPs at different concentrations (µl)					
		GX2-NPs			ARA-NPs		
		25	50	100	25	50	100
Acid black 24	1	8.44	29.22	31.02	7.39	12.73	28.75
	10	7.54	27.52	33.96	7.29	18.54	29.76
Mordant Black 17	1	2.33	17.81	21.45	3.16	9.35	13.02
	10	6.97	16.42	20.73	6.54	8.23	12.73
Acid Blue 113	1	5.57	10.30	16	5.96	17.36	25.73
	10	6.52	16.12	29.72	16.59	19.7	25.32

From the above table, it was observed that these AgNPs were able to bring about decolorisation of the dye to a certain extent. And it was also observed that as the concentration of nanoparticles increased, the decolorisation % also increased. The phenomenon behind decoloruisation is most commomly the adsorption as this experiment neither used a catalyst nor sunlight irradiation. It was reported earlier that the presence of additional factors like sunlight irradiation, catalyst or pH would enhance the rate of degradation of azodyes dyes [24]. Similarly rates of reduction of the dyes at 1ppm and 10ppm by the AgNPs were not showing much variation which indicated the fact that the higher concentrations of the dyes didnot facilitate the decolourisation process and probably demanded much higher concentrations of AgNPs.

CONCLUSION

This study reports the benign synthesis of silver nanoparticles using endophytic fungi as the nanofactories. Extracellular synthesis of nanoparticles using the cell free filtrate is advantageous as this doesn't require another step of separating the nanoparticles and hence makes the downstream processing easier. The silver nanoparticles synthesized were ranging in size from 24-55 nm and were found to be spherical and slightly clustered and displayed potential antibacterial activity. A prelimiary report on the ability of the silver nanoparticles to degrade azo dyes was also reported. With the recent progress and the ongoing efforts in improving particle synthesis efficiency and exploring their biomedical applications, it is hopeful that the implementation of these approaches with the optimisation of conditions on a large scale and their commercial applications in medicine and health care will take place in the coming years.

ACKNOWLEDGEMENTS

The authors would like to thank Sathyabama University for providing an opportunity to carry out this research work.

REFERENCES

1. Swetha Sunkar, Valli Nachiyar C. Microbial Synthesis and characterization of silver nanoparticles using the Endophytic bacterium *Bacillus cereus*: A novel source in the benign synthesisis. Global J Medical Research 2012; 12: 43-49.
2. Simkiss K, Wilbur KM. *Biomineralization*, Academic, New York, NY, USA, 1989.
3. Mann S. *Biomineralization: Principles and Concepts in Bioinorganic Materials Chemistry*, Oxford University Press, Oxford, UK, 2001.
4. Xiangqian Li, Huizhong Xu, Zhe-Sheng Chen, Guofang Chen. Biosynthesis of Nanoparticles by Microorganisms and Their Applications, Journal of Nanomaterials 2011; doi:10.1155/2011/270974
5. Furr JR, Russell AD, Turner TD, Andrews A. Antibacterial activity of Actisorb Plus, Actisorb and silver nitrate. J Hosp Infect 1994; 27:201-208.
6. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J Colloid Interface Sci 2004; 275:177-182.
7. Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M. Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 2009, 5:382-386.
8. Chung YC, Chen IH, Chen CJ. The surface modification of silver nanoparticles by phosphoryl disulfides for improved biocompatibility and intracellular uptake. Biomaterials 2008; 29:1807-1816.
9. Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett Appl Microbiol 1997; 25: 279-283.
10. Ravi Raja NS, Maria GL, Sridhar KR. Antimicrobial evaluation of endophytic fungi inhabiting plants of Western Ghats of India. Eng. Lifescience 2006; 6: 515-520.
11. Sadowski Z, Maliszewska I H, Grochowalska B, Polowczyk I, Koźlecki T. Synthesis of silver nanoparticles using microorganisms. Materials Science 2008; 26: 419-424.
12. Perez C, Paul M, Bazerque P. Antibiotic assay by agar well diffusion method. Acta Biol. Med.Exp 1990; 15:113-115.
13. Sastry M, Ahmad A, Khan MI, Kumar R. Biosynthesis of metal nanoparticles using fungi and actinomycete.Current Science 2003; 85:162-170.
14. Sadowski Z, Maliszewska IH, Grochowalska B, Polowczyk I, Oź lecki T. Synthesis of silver nanoparticles using microorganisms Mat Sci-Poland 2008; 26: 419-424.

15. Kowshik M, Ashtaputre SH, Kharazi SH. Extracellular synthesis of silver nanoparticles by a silver-tolerant yeast strain MKY3. Nanotechnology 2003;14: 95-100.
16. Henglein A. Physicochemical properties of small metal particles in solution: microelectrode" reactions, chemisorption, composite metal particles, and the atom-to-metal transition. J. Phys, Chem 1993;97: 5457-5464.
17. Minaeian S, Shahverdi AR, Nohi AS, Shahverdi HR. Extracellular biosynthesis of silver nanoparticles by some bacteria. J. Sci. I. A. U (JSIAU) 2008; 17: 1-4.
18. Vigneshwaran N, Ashtaputre NM, Varadarajan PV, Nachane RP, Paralikar KM, Balasubramanya RH. Materials Letters 2007; 61: 1413-1418.
19. Shankar SS, Rai A, Ankamwar B, Singh A, Ahmad A, Sastry M. Nature Materials 2004a; 3: 482-488.
20. Bhainsa KC, D'Souza SF. Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. Colloids and surfaces B Biointerfaces 2006; 47: 160-164.
21. Shaligram SN, Bule M, Bhambure R, Singhal SR, Singh KS, Szakacs G, Pandey A. Process Biochemistry 2009; 44: 939-943.
22. Wright GD. Adv Drug Deliv Rev 2005; 57: 1451-1470.
23. Kanchana A, Agarwal I, Sunkar S, Nellore J, Namasivayam K. Biogenic silver nanoparticles from *Spinacia oleracea* and *lactuca sativa* and their potential antimicrobial activity. Digest J Nanomat Biostruct 2011; 6: 1741-1750.
24. Gopalappa H, Yogendra K, Mahadevan K M. Solar Photocatalytic Degradation of Commercial Azo Dye Acid Orange 7 by Synthesized CaZnO2 Nanoparticle as an Effective Catalyst. Int J Res Chem Env 2012; 2: 39-43.