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Research Article

A COMPARATIVE ANALYSIS OF VARIOUS SPECIES OF *ASPERGILLUS* MEDIATED SILVER NANOPARTICLES SYNTHESIS AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT
Nanotechnology using biological principles provides wide range of novel antimicrobials resulting for the overcoming the antibiotic resistant bacterial strains. The usage of fungi for the development of silver based nanoparticles has added advantage that downstream processing and handling the biomass would be much simpler. Various species of *Aspergillus* included showing very good bactericidal effect which cannot be obtain by using conventional antibiotics. Further the results of color change, pH variations and uv spectra support the nanoparticle synthesis. The wide antimicrobial activity showed the need of rapid synthesis of nanoparticles would be suitable for the development of microbial nanotechnology biosynthesis process for the mass scale production.
Keywords: *Aspergillus* - silver nanoparticles – Antimicrobial activity

INTRODUCTION

There are remarkable anticipations in the study of nanoscale matter (matter having nanometer dimensions (1nm = 10^{-7}cm) with reverence to their elementary properties, organization to form superstructures and applications. Recognizing the importance of nanomaterials in key future pharmaceutical technologies, many countries have launched major initiatives into the development of a strong fundamental and applied knowledge base in the area of nanotechnology. It certainly does appear that "there are plenty of room at the bottom" in this fascinating area[1].

Synthesis of nanoparticles using biological entities has great interest due to their unusual optical, chemical, photoelectrochemical and electronic properties. Nature has devised various processes for the synthesis of nano and micro length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials[1]. More number of studies demonstrated the uses of microorganisms in the synthesis of nanoparticles are a relatively new and exciting area of research with considerable potential for the development[2,3,4]. On comparing with other microbial sources, the fungi play very important role in the synthesis of nanoparticles due to the secretion of large amount of enzymes and are simpler to deal in the laboratory[2,4].

The development of new resistant strains of bacteria to current antibiotics has become a serious problem in public health; therefore, there is a strong incentive to develop new bactericidals[5]. Thus the nanoparticles of silver have thus been studied as a medium for antibiotic delivery; synthesize composites for use as disinfecting filters and coating materials. However the bactericidal property of these nanoparticles depends on their stability in the growth medium, since this imparts greater retention time for bacterium-nanoparticle interaction. There lies a strong challenge in preparing nanoparticles of silver stable enough to significantly restrict the bacterial growth[6,7].

Nanomaterials are being actively developed for in vivo biomolecular profiling of infectious diseases biomarkers and targeted for drug delivery. These nanotechnological based techniques can be applied widely in the management of different infectious and non infectious diseases. Several nanotechnological approaches have been used to improve delivery of chemotherapeutic agents to infectious etiology with the goal of minimizing toxic effects on healthy tissues while maintaining antimicrobial efficacy. However it is well known that inorganic nanomaterials are good antimicrobial agents. Silver nanoparticles are the metal of choice as they hold the promise to kill microbes effectively.

Though, the biosynthesis of silver nanoparticles by free cell system and culture filtrate has not been explored yet. In this investigation, we report on the synthesis of silver nanoparticles by the reduction of aqueous silver ions by instantaneous reduction of aqueous silver with the culture broth of some fungal members. In the course of our screening process involving a number of fungal members were potential contender for synthesis of silver nanoparticles and also have extensive antibacterial effects.

MATERIALS AND METHODS

Synthesis of silver nanoparticles

Five fungal strains (*Aspergillus niger, A. fumigatus, A. flavus, A. terreus* and *A. versicolor*) isolated from various sources are included. The fungal isolates were cultivated in the petridishes using 2% malt extract with 0.5% yeast extract at room temperature until the complete biomass is observed. Then this biomass was grown aerobically in liquid medium containing (g/L): KH_2PO_4 – 7, K_2HPO_4 – 2, $MgSO_4$ – 0.1, $(NH_4)_2SO_4$ – 1.0, yeast extract – 0.6 and glucose – 10. This set up is incubated at 25°C with shaking (200rpm) for 72 hours.

After incubation, the biomass was filtered using Whatman filter paper No. 1 and then extensively washed with sterile distilled water to remove the medium components. Fresh and clean fungal biomass was taken into Erlenmeyer flasks containing de-ionized water and the flasks were agitated at the same conditions as described above, then the biomass was filtered again and the cell free filtrate was used for further experiments.

A solution of 0.01M $AgNO_3$ was prepared by dissolving 0.017g $AgNO_3$ in 100ml of de-ionized water. During this process, additives like ammonia (30%) were added drop wise, so that silver ions formed a stable soluble complex. The solution obtained was used as a precursor and inducer for the silver nanoparticles. A blend of reducing agents like glucose and hydrazine was used during the synthesis of the nanoparticles. Blending was essential to control the rate of reduction such that an optimum rate was achieved. A reduced rate of metals had been shown as clusters during size determination with reduced stability[6].

The 10ml of each of the fungal filtrate was taken in a sterile conical flasks and 100ml of $AgNO_3$ solution was added under aseptic condition and mix it thoroughly. Average size of the particles synthesized was 50nm with the size range of 30 to 80nm with irregular shape and sometimes clusters. Due to our special interest to achieve the smaller sized molecules, the mixture is centrifuged at 1200rpm for 15 minutes and the supernatant was taken for size determination.

Characterization of the nanoparticles

Colour change

When the silver binding fungal clones (As-Ag nanoparticles) were incubated in an aqueous solution of 0.01M silver nitrate for 24 – 48 hours at room temperature at various conditions from sunlight to dark conditions, the solution turned to reddish brown further to brown threads[1].

pH variation

The periodical changes in the pH are recorded. The increase in pH from acidic to alkaline condition was observed. This change in the pH range along with color change confirms that As-Ag nanoparticles have been synthesized[8].

UV Spectra

The biotransformation was routinely monitored by visual inspection of the biomass as well as measurement of the UV-vis spectra from the As-Ag nano liquid at various times and the absorbance was measured at the resolution of 540nm using a UV visible spectrophotometer[9].

Table 1: Colour change after exposed to $AgNo_3$ with various fungal members

Time	Colour change of various *Aspergillus* species exposed to $AgNo_3$				
	A. niger	*A. fumigatus*	*A. flavus*	*A. terreus*	*A. versicolor*
0	-	-	-	-	-
10 minutes	+	+	-	-	-
30 minutes	+	+	+	-	-
1 hour	++	++	+	-	-
2 hours	++	++	++	-	-
4 hours	+++	+++	+++	-	-
8 hours	+++	+++	+++	-	-
16 hours	++++	++++	+++	-	-
32 hours	++++	++++	++++	-	-
64 hours	+++++	+++++	++++	-	-
72 hours	++++++	++++++	+++++	-	-

[- No colour change; + Pale yellow; ++ Orange; +++ Red; ++++ Reddish brown; +++++ Tinge brown; ++++++ Brown threads]

Table 2: Percentage of growth inhibition of various fungal mediated silver (Ag) nanoparticles against bacterial pathogens compared to silver control

Test fungus	Concentration (µg)	Percentage of growth inhibition of various fungal mediated silver nanoparticles against bacterial pathogens					
		Amp Sensitive E. coli	*Amp. Resistant E. coli*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Staphylococcus aureus*	*Streptococcus pyogenes*
A. niger	5	80	50	20	10	20	30
	10	100	75	50	30	40	50
	25	100	100	70	60	40	70
A. fumigatus	5	80	50	20	-	20	30
	10	100	75	40	20	40	50
	25	100	100	50	40	50	60
A. flavus	5	40	30	10	-	-	10
	10	50	40	30	10	10	20
	25	70	60	50	20	30	40
A. terreus	5	30	20	-	-	10	10
	10	40	20	10	-	20	20
	25	40	30	20	10	20	30
A. versicolor	5	30	20	-	-	-	-
	10	40	20	10	-	10	10
	25	40	30	20	20	10	20
Ag control	5	30	20	-	-	20	20
	10	40	30	20	20	40	40
	25	60	50	40	40	60	70

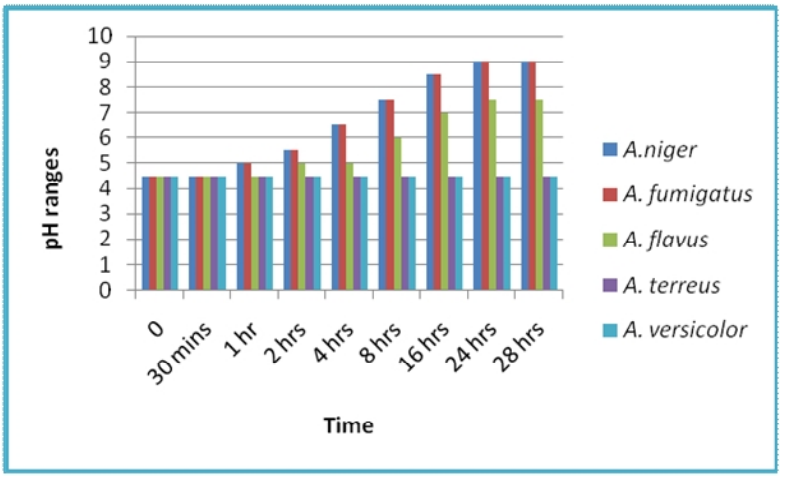

Figure 1: Variations of pH verses nanoparticle formation using *Aspergillus* sp.

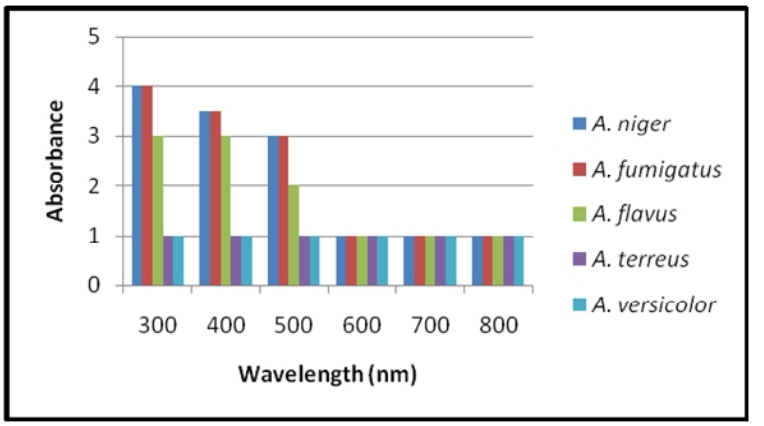

Figure 2: Temporal evolution of UV-Vis absorption spectra during the formation of silver nanoparticles using *Aspergillus* sp.

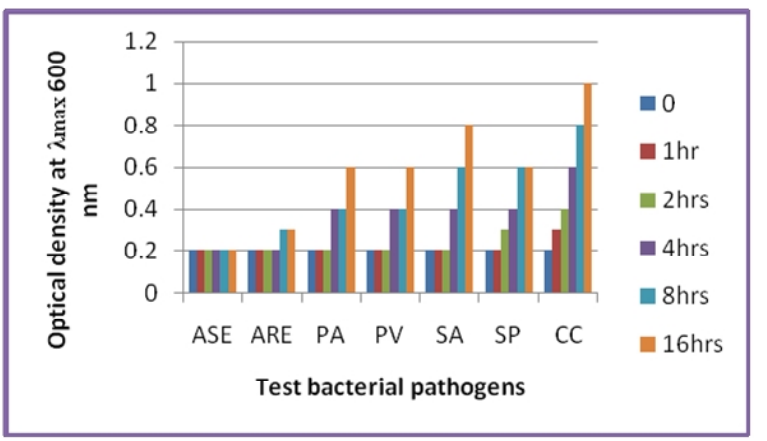

Figure 3: Optical density as a function of time for the estimation of bacterial growth in *A. niger* mediated silver nanoparticles solution studies
[ASE – Ampicillin sensitive E. coli; ARE – Ampicillin resistant *E. coli;* PA – *Pseudomonas aeruginosa;* PV – *Proteus vulgaris*; SA – *Staphylococcus aureus*; SP – *Streptococcus pyogenes*; CC – culture control]

Analysis of antibacterial activity

The effect of As-Ag nanoparticles on gram negative (*Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa*) and gram positive bacteria (*Staphylococcus aureus*, MRSA, *Streptococcus pyogenes*) was investigated by culturing the organisms in Luria Bertani (LB) agar plates (10^6 colony forming units of each strain per plate) supplemented with As-Ag nanoparticles at concentrations of 5, 10, 25 and 50µg ml^{-1}. Plates without nanoparticles were used as controls. Plates were incubated for 24 hours at 37°C and the number of colonies was counted. The counts on three plates corresponding to a particles sample were averaged[6].

To study growth of bacteria in the liquid broth, inoculations were given from fresh colonies on agar plates into 100ml LB culture medium. Growth was allowed till the optical density reached 0.1 at 600nm (Optical density of 0.1 corresponds to a concentration of 10^8 CFU ml^{-1} of medium). Subsequently 2 X 10^8 CFU form above were added to 100ml of liquid LB medium supplemented with 5, 10, 25 and 50µg ml^{-1} of As-Ag nanoparticles. Control broths were used without nanoparticles. Antibiotics (ampicillin 100µg ml^{-1}, kanamycin 25µg ml^{-1}, chloromphenicol 32µg ml^{-1}, trimethoprim 16µg ml^{-1}, amoxicillin 40µg ml^{-1}) were added to different media as appropriate. Growth rate was determined by measuring optical density at 600nm at regular intervals.

RESULTS AND DISCUSSION

Reduction kinetics

The reduction of As-Ag ions was visibly apparent from the colour change related with it. The Erlenmeyer flasks with the *Aspergillus niger, A. fumigatus* and *A. flavus* filtrates were a pale yellow color before the addition of Ag+ ions and this changed to a brownish color on completion of the reaction, but the Erlenmeyer flasks with the filtrates of *A. terreus* and *A. versicolor* were not changed. The table 1 shows the colour change after exposure to $AgNo_3$ solution for a period of 72 hours. Silver nanoparticles exhibit this striking colour due to excitation of surface Plasmon vibrations in the particles and thus provide a conventional means of visually determining their presence in the fungal biomass.

pH variations

The pH of the fungal silver nanoparticles varies from 4.5 to 9.0. Each and every fungal source has its own properties in the pH variations. In this investigation also, the pH of *A. niger* and *A. fumigatus* showed maximum upto 9.0 whereas *A. flavus* showed intermediate of 7.5 and there is no pH variations found in *A. terreus* and *A. versicolor* and the results in the variations of pH vs nanoparticle formation were interpreted in Figure 1.

Spectral changes

The UV visible spectra recorded from the aqueous $AgNo_3$ solution after 6, 24 and 48 hours of reaction with the biomass are shown in the Figure 2, while there is no evidence of absorption in the spectral window by *A. terreus* and *A. versicolor*. The absorption spectra during various stages of reduction of the precursors as they transformed into the silver nanoparticles and after different ageing times during its preservation under ambient conditions are easily understood by this investigation. The silver nitrate solution showed a high at about 300nm, which gradually underwent with appearance of hump at 400nm consistent with the formation of silver nanoparticles.

Effect of silver nanoparticles on bacterial growth

The LB agar plates incorporating increasing concentrations of silver nanoparticles were inoculated with 10^6 CFU from different bacterial strains. In case of non resistant *E. coli* 80% inhibition in growth is observed in plates supplemented with 5µg ml^{-1} of *A. fumigatus* mediated nanoparticles. The previous study showed the same of 60% inhibition rate using non microbial silver nanoparticles[7]. The extend of inhibition increased to 100% (complete inhibition) of bacterial growth in plates with 10µg ml^{-1} of the same nanoparticles. In the case of ampicillin resistant *E. coli*, 75% inhibition in growth was observed in plates supplemented with 10µg ml^{-1} of nanoparticles, whereas 25µg ml^{-1} of nanoparticles elicited complete inhibition of growth of bacteria.

The other *Aspergillus* species involved in the nanoparticle formation are also elicited some extends of bactericidal action against various pathogenic bacterial isolates (Table 2). Interestingly, the higher doses of *A. fumigatus* and *A. niger* mediated silver nanoparticles showed some inhibitory effect to other bacterial isolates including *Proteus, Pseudomonas, Staphylococcus, Streptococcus* etc. On comparing with gram negative bacterial isolates, the gram positive showed some extend of resistance to the nanoparticles. In broth inhibition assay, the *A. niger* mediated silver nanoparticles showed wide inhibition on *Pseudomonas, E. coli* and *Proteus*, whereas the OD value was found as equal and above the normal in the case of *S. aureus*. Thus the results of broth inhibition also support the same of plate inhibition assay. The inhibitory action of the various nanoparticles against bacterial pathogens in liquid broth as optical density determination is included in the Figure 3.

In additional experiments, strains of gram negative and gram positive bacteria were inoculated in liquid LB media supplemented with fungal mediated silver nanoparticles. Increasing concentration of nanoparticles progressively inhibited the growth of non resistant *E. coli* and other bacterial pathogens. The lag phase was found to be more prolonged than that described in the earlier reports[4,10]. This could be attributed to greater stability of the nanoparticles used in this study. The concentration of 25µg ml^{-1} of nanoparticles was found to be strongly inhibitory for bacteria, as it took about 8 hours to initiate any noticeable growth. Similar results were obtained with either resistant or sensitive strains of other gram negative bacteria included in this investigation.

In contrast these fungal based nanoparticles were found less effect on *Staphylococcus aureus*. No reduction is bacterial growth was observed till the concentration of 10 and 25µg ml^{-1} whereas 50µg ml^{-1} elicited some partial growth inhibition. The antibacterial effect of the nanoparticles was attributable to the mere presence of these particles in the growth medium. Bacterial cells were cultured for 60 minutes in the presence of nanoparticles followed by sedimentation and reculturing them in fresh medium without nanoparticles. Control also maintained correspondingly but without nanoparticles. A noteworthy retardation in growth of re-cultured gram negative bacteria and not of gram positive bacteria was observed in the fresh culture medium when they had been originally exposed to the nanoparticles. Control cells exhibited typical growth characteristics. These

observations were consistent with sustained interaction between nanoparticles and cellular components of gram negative bacteria compared to the control.

Biologically synthesized silver nanoparticles could have many applications, in areas such as non linear optics, spectrally coating for electrical batteries, as optical receptors, catalysis in chemical reactions, biolabelling and antibacterial capacity[3]. A number of issues need to be addressed from the nanotechnology and microbiology points of view before such biosynthetic procedures can compete with the traditional protocols. Genetic engineering techniques can potentially be used to improve the particle properties and to control their composition. The shift from bacteria to fungi as a means of developing natural nanofactories has the added advantage that downstream processing and handling of the biomass would be much simpler[2.3.4]. Further we can utilize the cheap source of material like waste biomass including industrial wastes gives an opportunity to cost effective preparation of various silver based nanoparticles.

Silver nanoparticles exhibit a broad size distribution and morphologies with highly reactive facets. The major mechanism through which silver nanoparticles manifested antibacterial properties is by anchoring to and penetrating the bacterial cell wall and modulating cellular signaling[5,11].

This study highlighted the importance of fungal induced nanoparticle synthesis, its characterization and antibacterial activities. Silver nanoparticles act primarily in three ways against gram negative bacteria:

1. The range of nanoparticles range of 1 – 10nm that attach to the cell membrane and disturb its functions and mainly arrest respiration.
2. Penetrate the cellular components and damage the DNA.
3. The silver ions have an additional contribution of bactericidal effect.

However, further studies must be conducted to verify if the bacteria develop resistance towards the nanoparticles and to examine cytotoxicity of nanoparticles towards human cells before proposing their therapeutic use.

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