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Assessment of antibacterial and anticancer capability of silver nanoparticles extracellularly biosynthesized using Aspergillus terreus

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Abstract

The present study explores biosynthesis of silver nanoparticles (AgNPs) employing extracellular extract of Aspergillus terreus ITCC 9932.15. Modulation of various variables that dictate the biosynthesis of AgNPs, suggested of optimal AgNPs synthesis using AgNO₃, 1 mM at pH 8 and temperature, 35 °C. The biosynthesis of AgNPs was observed to be time dependent with incremental particle synthesis till 24 h. Various studies were undertaken to authenticate formation and characterization of AgNPs for size, crystallinity and biomolecules involved. A sharp SPR peak observed at 420 nm in the UV–vis absorption spectra validated synthesis of nanoparticles. These particles exhibited spherical morphology with size ~25 nm and −16 mV of zeta potential. Further, the existence of proteins and other biomolecules onto the surface of AgNPs was confirmed with FTIR studies. The SAED pattern investigated by employing TEM authenticated the crystallinity of AgNPs. The AgNPs also exhibited potential antibacterial activity against Gram-negative and Gram-positive bacteria (E. coli and P. aeruginosa). In addition, remarkable anticancer activity was obtained in breast cancer cell line (MCF-7).

1. Introduction

Nanoparticles have found plethora of applications in different domains including diagnosis, energy, cosmetics, enzyme immobilization, biosensors and biodegradation. Amongst all nanoparticles, silver nanoparticles (AgNPs) have been thoroughly harnessed owing to its excellent antimicrobial properties. The antimicrobial effects could be attributed to interaction with the thiol groups of pivotal proteins and enzymes, introduction of oxidative stress and blockade of DNA replication [1]. In addition to antimicrobial efficacy, AgNPs have been shown to exhibit excellent anticancer effects that includes, lung cancer, breast cancer, skin cancer and hepatocellular carcinoma. Uptake of AgNPs by cancer cells could lead to ROS generation, diminished cell migration, enhanced oxidative stress that ultimately results in programmed cell death [2].

Chemical synthesis approach which involves reduction of silver salt using a reducing agent is the most customary approach of AgNPs synthesis. The green synthesis approach that utilizes extracts from plants or microbes is rapidly emerging as method of choice [3–7]. Interestingly, besides plant extracts researchers have also synthesized AgNPs using silk fibroin (from Bombyx mori) and UV radiation [8–13]. Application of fungal biomass for biosynthesis of AgNPs is preferred to be of great interest due to the inherent metabolic diversity involved. There are two approaches for biosynthesis of AgNPs using fungi i.e. extracellular and intracellular biosynthesis. Intracellular biosynthesis consist of addition of AgNO₃ to the fungal culture whereby it gets incorporated and reduced to AgNPs, accompanied by tedious extraction steps involving chemical exposure, filtration and centrifugation to dislodge biomass and free AgNPs [14, 15]. In contrast extracellular biosynthesis involves addition of filtrate obtained after cultivation of fungus in water to AgNO₃ [16–18]. The second approach is broadly implicated as it offers ease of recovery of AgNPs. Further, the fungi shows considerable tolerance to metals that facilitates enhanced secretion of extracellular proteins that in turn imparts high stability.
to the synthesized AgNPs [19, 20]. Furthermore, the fungal biomass appears to be more stable to high pressure and agitation in comparison to plant extracts, a positive requisite for large-scale synthesis. In addition, nanoparticles with desired characteristics could be synthesized by modulation of synthesis variables including pH, time, temperature and amount of fungal biomass [21]. In a recent study, AgNPs were synthesized from *Penicillium oxalicum* and tested against several pathogenic organisms [22]. A repertoire of fungi have been explored for synthesis of metallic nanoparticles that includes Ag, Au, Cu, Pd, Ti and Zn etc [23].

Amongst multifarious fungi, *Aspergillus* strains such as *A. fumigatus* and *A. flavus* have been reported for extracellular synthesis of AgNPs, while *A. niger* synthesized AgNPs has been investigated to decipher the antibacterial mechanism in *E. coli* [23–26]. Amidst numerous fungi, *A. fumigatus* has been reported to facilitate rapid synthesis i.e. within 10 min, where nanoparticles displayed high stability and monodispersity. The present study explores the biosynthesis of AgNPs employing extracellular extract of *A. terreus* ITCC 9932.15, accompanied by optimization of synthesis variables that consists of AgNO₃ concentration, pH, temperature and incubation time. The antibacterial potential of AgNPs was explored against *Pseudomonas aeruginosa* and *Escherichia coli*, followed by in vitro anticancer potential in human breast cancer cells (MCF 7).

2. Materials and methods

2.1. Materials

For the biosynthesis of AgNPs silver nitrate (AgNO₃) was procured from Merck Limited, India. Nutrient agar and Nutrient broth were purchased from Hi-Media Laboratories, India. Dulbecco’s Modified Eagle Medium high glucose (DMEM HG), Fetal Bovine Serum (FBS), 0.25% Trypsin-EDTA were purchased from Life Technologies, USA. Dulbecco’s phosphate buffered saline (PBS) without calcium and magnesium chloride, Sodium bicarbonate, Resazurin sodium salt was purchased from Sigma Aldrich, India. Kanamycin was purchased from Himedia, India. Filtration was done using Whatman No.1 filter paper and double distilled water (in-house prepared) was used for the preparation of extract and other solutions.

2.2. Fungus

*A. terreus* ITCC 9932.15 was evaluated for its ability to produce silver nanoparticles. The strain was acquired from the Microbial Catalysis and Process Engineering Laboratory, Department of Microbiology, Central University of Rajasthan, Rajasthan, India. Serial dilution technique was employed to isolate and purify the strain from soil samples collected from Ajmer (26° 27′ 0″ N, 74° 38′ 0″ E), Rajasthan, India. Identification of the purified culture was undertaken by Indian Type Culture Collection (ITCC), IARI, New Delhi, India. The strain was identified as *A. terreus* and sustained on PDA (potato dextrose agar) slants with periodical subculturing for further experiments.

2.3. Cultivation and preparation of *A. terreus* filtrate

Cultivation of *A. terreus* was done using MGYP (Malt extract, Glucose, Yeast extract, Peptone) media under shaking conditions (96 h, 30 °C, 150 rpm). Following incubation, the filtrate was centrifuged (10 000 rpm, 4 °C, 10 min; Hanil Combi 514R centrifuge) to obtain the mycelia. The supernatant was discarded and the pellet was weighted. The wet weight of the mycelial pellet was ~25 gm. Further, the pellet was suspended in pre-sterilized distilled water (50 ml) and incubated (120 rpm, 24 h, 30 °C). Following incubation, mycelium was obtained through filtration using Whatman filter paper. Further, the obtained filtrate was scanned using UV–vis spectrophotometer and stored (4 °C) for future studies.

2.4. Extracellular biosynthesis of AgNPs

*A. terreus* filtrate was employed for extracellular biological production of AgNPs. Briefly, 1 mM solution of silver nitrate (AgNO₃) was added to the fungal filtrate and incubated (35 °C, 150 rpm) under dark conditions. Additionally, AgNO₃ (1 mM) solution incubated under parallel settings was used as control.

2.5. UV–visible spectroscopic analysis of biosynthesized AgNPs

The extracellular biosynthesis of AgNPs was monitored by the observable variations occurred in the solution with time. Aliquots (1 ml) were withdrawn at regular intervals and scanned (300–600 nm wavelength) by UV–visible spectrophotometer (Evolution 201, Thermo Scientific, USA) for validation of biological synthesis of AgNPs.

2.6. Structural and functional characterization of AgNPs

Surface architecture and size of synthesized AgNPs was studied using TEM. Furthermore, the nanoparticles were subjected to zeta potential analysis employing Zetasizer and occurrence of silver was confirmed using EDX.
Biosynthesized AgNPs were evaluated for their antimicrobial activity against *E. coli* (Gram negative) and *P. aeruginosa* (Gram positive) by employing disk diffusion method. The disk diffusion was performed by inoculating the pure bacterial cultures in Luria-Bertini (LB) broth followed by incubation under shaking conditions (overnight, 37°C) till the optical density attains a value of 0.5 Mcfarland turbidity (1–2 × 10⁶ CFU ml⁻¹). Culture thus obtained was then evenly spreaded on agar plates. Whereas, Zeta potential and FTIR analysis was done by placing solution on the copper grid and further drying. Whereas, Zeta potential and FTIR analysis was done using whole suspension of AgNPs.

### 2.7. Derivation of various factors for AgNPs synthesis

Four key parameters affecting AgNPs synthesis viz concentration of AgNO₃, pH, temperature and time were optimized by varying one variable at a time. The range of parameters used for the optimization was: AgNO₃ concentration, 0.25–2 mM; pH, 4–12; temperature, 25°C–55°C and time, 0–24 h. Effect of the parameters was analyzed in terms of AgNPs synthesis by scanning the solution at regular intervals (300–600 nm).

### 2.8. Evaluation of antibacterial potential

Biosynthesized AgNPs were evaluated for their antimicrobial activity against *P. aeruginosa* (Gram positive) and *E. coli* (Gram negative) by employing disk diffusion method. The disk diffusion was performed by inoculating the pure bacterial cultures in Luria-Bertini (LB) broth followed by incubation under shaking conditions (overnight, 37°C) till the optical density attains a value of 0.5 Mcfarland turbidity (1–2 × 10⁶ CFU ml⁻¹). Culture thus obtained was then evenly spreaded on agar plates. Subsequently, sterile disks impregnated with 4–5 μl of volume with varying amounts of AgNPs (50, 100, 150, 200 μg ml⁻¹) were washed twice with double distilled water and resuspended in distilled water. TEM analysis was performed by placing solution on the copper grid and further drying. Whereas, Zeta potential and FTIR analysis was done using whole suspension of AgNPs.

### 2.9. Analysis of anticancer activity

#### 2.9.1. Cell culture

Human breast cancer cell line (MCF7), obtained from NCCS, Pune, India was used for the study. Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum (FBS) was used to maintain the cells. The cells were prepared using double distilled water and added to each well of 96 well plate and incubated (24 h) with subsequent Resazurin sodium salt (Sigma-Aldrich, USA) addition, followed by measuring either absorbance or fluorescence intensities. The deviation in the absorbance or fluorescence on the basis of cellular metabolic activity could be measured. Resazurin sodium salt (10 μl) was added at 0.1 mg ml⁻¹ concentration, following 24 h of AgNPs exposure and incubated (37°C, 5%CO₂ atmosphere) for 3 h. The absorbance was measured at both 570 and 600 nm using ELISA plate reader (Multiskan GO, Thermo Fisher Scientific).

### 3. Results and discussion

#### 3.1. Biosynthesis of AgNPs

The fungal strain employed in the study was identified as *A. terreus* ITCC 9932.15 by ITCC, IARI, New Delhi, India. The spores of *A. terreus* appear brown in colour with yellow pigmentation and irregular shape (figure 1(A)). The fungal filtrate displayed maximum absorbance at 290 nm, indicating the presence of proteins in the filtrate (1D). Further, it was confirmed by performing Bradford assay. The concentration of the protein was found to be 41.6 μg ml⁻¹. Following this, incubation of 1 mM AgNO₃ with the fungal extracellular filtrate resulted in biosynthesis of AgNPs. The synthesis of AgNPs was evident from the change in colour from brown to initial colourless, while, the control where only filtrate was taken remained colourless (figures 1(B) and (C)). This colour transformation could be due to the surface plasmon resonance (SPR) of the AgNPs. Further, the biosynthesis of AgNPs was confirmed by UV–vis spectrophotometer scans within range of 200–600 nm wavelengths (figure 1(D)). The AgNPs synthesis was observed to enhance with time i.e. 1 h to 24 h, with a prominent peak at 420 nm.

The series of biosynthesis studies were done in order to optimize the conditions for optimal AgNPs synthesis. The initial optimization studies were performed at room temperature and involved varied concentration if AgNO₃ solution (0.25 mM–2 mM). As evident from the data, AgNPs synthesis occurred at two
AgNO₃ concentrations i.e. 0.5 mM and 1 mM (figure 2(A)). Further, increase in the strength of AgNO₃ solution resulted in widening of the absorption spectra. This observation could be speculated to be due to either aggregation of AgNPs or simply increase in the size. Variation in the pH i.e. pH 4 to 12, of the AgNO₃ solution revealed of maximal AgNPs synthesis at pH 8, while lower nanoparticles synthesis took place at acidic and high alkaline pH (figure 2(B)). Extreme pH be it acidic or alkaline affects the structure of proteins present in the fungal extract or it may even lead to denaturation rendering unstable AgNPs [27, 28]. Our results were in concurrence to the studies where pH 9 was observed to be optimal for AgNPs synthesis [29]. It could be speculated that the pH may induce ionization by transfer of electron at alkaline pH of 8, with different metabolites present in the solution along side AgNO₃ salt; which resulted in formation of AgNPs. Furthermore, the studies done to investigate the influence of temperature suggested of optimal AgNPs synthesis at 35 °C (figure 2(C)). Remarkable inhibition in synthesis above 45 °C may probably be due to loss of protein activity owing to the denaturation [28]. Time duration of the biosynthesis reaction was also investigated that revealed enhancement of AgNPs formation up to 24 h (figure 2(D)). Post optimization studies, AgNPs were synthesized at 35 °C with 1 mM AgNO₃ at pH 8 accompanied with 24 h incubation. Further, no aggregation was seen upon incubation of AgNPs upto 15 days (figure 3), indicating stability of the suspension which is in agreement with the observation of Xue et al [30].

The size of the nanoparticles can be determined by the equation:

$$\gamma(R) = \gamma_0 + \left( \frac{A v_F}{R} \right)$$

where $A$ corresponds to scattering process (is 3/4 for silver) and $\gamma_0$ is the velocity of bulk scattering, and $v_F$ is the Fermi velocity as given by [31]. The range of size was found to be around 25 nm.

### 3.2. Characterization of AgNPs

High Resolution-Transmission Electron Microscope (HR-TEM) was employed to investigate the size and shape of the AgNPs; the particles thus synthesized were observed to be nearly spherical in shape along with even distribution throughout the sample with minor agglomerates. The average size of the AgNPs was found to be...
25.8 ± 0.59 nm (figures 4(A), (B), (D)). The selected area of electron diffraction (SAED) showed persistent ring patterns that could be designated to the presence of Ag 111, 200, 220, 311, and 222 planes of the face centered cubic structured (FCC); that confirmed the crystalline nature of AgNPs (figure 4(C)). Further, the elemental composition of AgNPs was investigated by EDX analysis (figure 5(A)). The EDX spectrum showed a strong peak between 3 and 3.2 keV, which could assigned to the binding energy of silver thus, inferring to the synthesis of AgNPs.

Figure 2. Optimization studies for AgNPs as assessed by UV-vis spectroscopy (A) AgNO₃ concentration, (B) Reaction pH, (C) Reaction temperature, (D) Reaction time.

Figure 3. Representative UV-vis spectra of (A) AgNPs after synthesis (B) AgNPs stability after 15 days.
AgNPs of crystalline nature. The spectrum revealed the presence of higher amounts of Ag (83.17%) and lower amounts of Cl (16.83%) weight % while the atomic % was of Ag and Cl was 61.9 and 38.1, respectively.

Zeta potential of AgNPs as evaluated on Zetasizer was observed as $-16 \text{ mV}$, which suggests of remarkable stoutness (figure 5(B)). The stability of the AgNPs could be corroborated to the negative values as it prevents aggregation \cite{32}. Another contributing factor could be the presence of capping molecules onto the surface of nanoparticles. FTIR studies of filtrate exhibited prominent peaks situated at 3414 cm$^{-1}$, 2990.35 cm$^{-1}$, 2825.45 cm$^{-1}$, 2883.65 cm$^{-1}$, 1639.49 cm$^{-1}$, and 1036.97 cm$^{-1}$ (figure 6). The peak at 3414 cm$^{-1}$ indicates the O–H bond stretching. The peaks 2990.35 cm$^{-1}$ and 2825.45 displays the presence of C–H stretching, indicating the presence of alkanes. Similarly other peaks at 1639.49 cm$^{-1}$, 1404.96 cm$^{-1}$, and 1036.97 cm$^{-1}$ denotes N–H, C=O and C=O stretching respectively. On the other hand AgNPs displayed peaks at 3377.80 cm$^{-1}$, 1644.02 cm$^{-1}$ and 1404.75 cm$^{-1}$ and 1045.25 cm$^{-1}$. The peaks were in concordance with the filtrate spectra where 3377.80 cm$^{-1}$, 1644.02 cm$^{-1}$ and 1404.75 cm$^{-1}$ and 1045.25 cm$^{-1}$ denotes O–H, C–N, S–O and C–N stretching respectively. Thus, the presence of these peaks establishes the participation of amide and imine bonds in capping of AgNPs. This could further be ascribed to the availability of proteins and enzymes in extracellularly secreted fungal extract. High molecular weight proteins have been speculated to be involved in the nanoparticle synthesis wherein the pivotal role is played by NADH-dependent reductase \cite{33}. The presence of this enzyme onto the surface of nanoparticles as well as into the extract was confirmed by fluorescence spectroscopy \cite{33}. Further, an absorption signature peak for the halo compounds was also observed at 671 cm$^{-1}$. Earlier, \textit{A. terreus} has been shown to produce metabolites with chlorine substitution including geodin and erdin \cite{34}. Looking at the FTIR data it can concluded that several

![Figure 4. Representative TEM images, (A), (B) Electron micrograph of AgNPs, (C) SAED of AgNPs shown in (A), (D) Graphical size distribution of AgNPs.](image-url)
biomolecules present in the extracellular fungal extract have potential role in biosynthesis and capping of nanoparticles.

3.3. Antibacterial potential of AgNPs

In the present study, disk diffusion assay was utilized to study the antibacterial potential of AgNPs towards Gram positive \((P. \text{ aeruginosa})\) and Gram negative bacteria \((E. \text{ coli})\) (figure 7). Effective antibacterial activity was observed when bacteria were exposed to high concentration \((200 \text{ µg ml}^{-1})\) of AgNPs. At AgNPs concentration of \(200 \text{ µg ml}^{-1}\) the zone of inhibition was 12 mm while at \(50 \text{ µg ml}^{-1}\) it was reduced to only 8 mm in case of \(P. \text{ aeruginosa}\). This trend was followed in case of \(E. \text{ coli}\) i.e. the zone of inhibition was 12 and 8 mm at 200 and 50 \text{ µg ml}^{-1}, respectively. The zone of inhibition of the positive control (Kanamycin) was found to be around 15 mm for both the bacteria. The antimicrobial potential of AgNPs could be attributed to plethora of mechanisms. It could be due to the degradation of bacterial DNA as suggested by Tamboli et al \([1]\). Cell membrane blebbing and escape of essential biomolecules due to alteration of membrane permeability followed by blockade of essential enzymes has also been reported to contribute to antimicrobial effects \([32, 35]\). Further, it was proposed that the silver nanoparticles impart antibacterial activity differently in gram-negative and gram-positive bacteria. The antibacterial effect was stronger in gram-negative bacteria than gram-positive. This was attributed to the cell wall thickness of gram positive bacteria \((30 \text{ nm})\) to gram-negative bacteria \((3–4 \text{ nm})\) \([36]\).

One of the most common factor that is responsible for antibacterial efficacy is the size of AgNPs. The smaller the size of the nanoparticles, the larger is its surface area. This enhances the contact area of small-sized AgNPs to the cell in relative to the large sized nanoparticles \([37]\). Also, it is evident from several results that the antibacterial activity depends on the concentration of AgNPs \([38, 39]\).

3.4. Anticancer potential of AgNPs

Human breast cancer cell line MCF-7 was engaged to evaluate the anticancer potential of AgNPs (figure 8). The cell viability decreased from 80%–35% with increase in the concentration of AgNPs from 2–128 \text{ µg ml}^{-1}. The
IC$_{50}$ value of the nanoparticles was calculated to be 25.24 ± 0.990 µg ml$^{-1}$. The size of AgNPs dictates the toxic potential, as it determines the uptake along with the potential interactions with the thiol enzymes [40]. Similarly, in another study it was demonstrated that the hemolysis, protein leakage and other cellular functions are affected by the AgNPs shape and size [41]. Thus, AgNPs display selective participation by disrupting the mitochondrial respiratory chain and formation of reactive oxygen species resulting in damage to the nucleic acids [42]. The disruption of vital cellular processes could also be cited as one of the reasons behind toxicity of nanoparticles [40]. In another study, dose dependent anticancer efficacy has been reported by AgNPs synthesized by Aspergillus fumigatus in vivid cancer cell lines [43, 44]. The AgNPs showed varying IC$_{50}$ values from 31.1–45.4 µg ml$^{-1}$ depending on the cell line studied [43]. The anticancer potential of AgNPs varies from one to another species, used for biosynthesis. The IC$_{50}$ value of 1.47, 2.46 and 3.12 µg ml$^{-1}$ has been reported for AgNPs biosynthesized using A. japonicus, A. niger and A. michelle respectively in MCF-7 cells [45].

4. Conclusion

This study evidenced the potential of fungal strain A. terreus ITCC 9932.15 for synthesis of AgNPs. Spherical shape particles with uniform size distribution of ~25 nm and −16 mV of zeta potential were synthesized by the extracellular fungal extract. In vitro assays suggested of potent antibacterial and anticancer efficacy of AgNPs. Thus, the AgNPs synthesized holds a great promise in effective anti-tumour drug delivery. This overcomes the
disadvantages of conventional therapies by crossing the biological barriers and targeted delivery of drugs. The plasmonic properties can be implemented in theranostic approaches, where the AgNPs can be utilised in diagnosis as well as treatment of cancer. However, clinical trials are one of the most important steps that is required to be followed for future direction of its applications. Despite of its valuable properties, AgNPs have toxicity issues that is necessary to be mitigated by meticulous pre-clinical study and elaborate datasets of its toxicities and pharmacological issues.

Figure 7. Antibacterial potential of AgNPs. Different concentrations of AgNPs were tested against two different bacteria. (A) E. coli and (B) P. aeruginosa (a-positive control, b-water, c-silver nitrate, d-extract and AgNPs concentrations; e-50 μg ml⁻¹, f-100 μg ml⁻¹, g-150 μg ml⁻¹ and h-200 μg ml⁻¹), (C) Graphical representation of the antibacterial data of the two bacteria.

Figure 8. Cell viability of MCF cells treated with different concentrations of AgNPs. Representative microscopic images of MCF-7 cells (A) Cells treated with AgNPs, (B) Control cells and (C) Graphical representation of the cell viability using resazurin sodium salt.
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