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Characterization Studies and Antimicrobial Efficacy of Silver Nanoparticles Synthesized Using *Aspergillus niger*: A Biological Driven Approach

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

For years, nanoparticles have been the subject of extensive research, continuously captivating researchers due to their minute size and distinctive surface properties. Among these, silver nanoparticles (AgNPs) stand out as particularly noteworthy, acclaimed for their remarkable biological, chemical, electrical, optical, and thermal characteristics. Moreover, the advent of biological synthesis methods has provided an environmentally friendly and cost-effective means of producing nanoparticles. In this study, the synthesis of silver nanoparticles (AgNPs) utilizing fungal isolates from marine soil samples was explored. A visible shift in color, from light to dark brown, signified the formation of silver nanoparticles through fungal mediation. Confirmation of the reduction reaction of silver ions in the fungal cell-free filtrates was achieved using UV-visible spectroscopy, which revealed a surface plasmon resonance peak at 420 nm. Scanning electron microscopy (SEM) affirmed the presence of spherical nanoparticles within the size range of 10-30 nm, while X-ray diffraction studies (XRD) validated the presence of metallic silver. The synthesized nanoparticles exhibited potent antimicrobial activity against both gram-positive and gram-negative clinical isolates, with minimum inhibitory concentration (MIC) values indicating their efficacy. This underscores the potential of these silver nanoparticles in combating major infections. Further assessment of this property was conducted through time-kill assays, revealing bacterial cell viability at various time intervals. The identification of fungi at the molecular level was accomplished through 16S rRNA sequencing.

Keywords: silver; nanoparticles; biological synthesis; antimicrobial activity; SEM.

1. INTRODUCTION

Nanoparticles, often referred to as minuscule particles with dimensions in the range of 1-100 nanometres, showcase properties and behaviours distinct from the larger forms of the same materials. Nanotechnology focusing on manipulating materials at nano scale, has emerged as a revolutionary force, reshaping industries spanning healthcare, defence, electronics, energy, food and manufacturing. Its precision in regulating matter at the atomic and molecular levels has unlocked unparalleled control over materials, driving the creation of innovative solutions and products [1,2].

Metal nanoparticles represent a versatile class of nanomaterials with broad-ranging applications across diverse sectors, including catalysis, electronics, biomedicine, and environmental remediation. Among these, silver nanoparticles stand out for their multifaceted role in nanotechnology. They continue to captivate researchers and industries alike, finding utility in an array of consumer products such as healthcare, textiles, cosmetics, and household appliances. Numerous physical, chemical and biological methods of nanoparticle synthesis are documented in literature and utilized in both laboratory and industrial settings [3].

The rise of unconventional synthesis methods is promising unparalleled synthesis capabilities characterized by enhanced control over morphologies, sizes, and size distribution. Physical methods, while straightforward, may be time-consuming and costly. Chemical methods are high-yielding and easily scalable. But biological or green synthesis methods serve as feasible substitutes to all other methods in terms of safety, sustainability and energy consumption [4]. These methods utilize natural sources such as plant extracts, microorganisms, or biomolecules, as reducing and stabilizing agents [5].

In recent years, there has been notable interest in employing a range of microorganisms including bacteria, viruses, fungi, actinobacteria, yeast, and microalgae for nanoparticle synthesis. These diverse microorganisms present multifaceted pathways for generating various types of nanoparticles, encompassing metals, metal oxides, and other vital nanomaterials, utilizing both intracellular and extracellular mechanisms. Bacteria and actinomycetes have been widely utilized for metal nanoparticle synthesis, whereas algae and yeast have emerged as a relatively recent focus for such synthesis [6].

Fungi are explicitly known to play a crucial role in ensuring sustainability by addressing challenges related to agriculture, bioremediation, food, drugs, and energy at global perspective. Various genera of fungi have been instrumental in

synthesizing a wide range of nanoparticles, capitalizing on their myriad advantages over other biological systems [7].

Flourishing under conducive conditions, fungi efficiently produce the enzymes and metabolites essential for nanoparticle synthesis. Extensive literature research unveils a rich diversity of fungal-derived nanoparticles, extending beyond traditional metals like gold, silver, and copper to encompass a broad spectrum of elements spanning the periodic table [8].

The intricate branching network of fungi serves as an optimal framework for the formation of nanoparticles with the prime locations for nucleation being the metal-binding functional groups present in the cell wall and the associated extracellular polymeric substances (EPS) [9].

In addition to all this, advancements in nanoparticle characterization have significantly enhanced our ability to understand and manipulate nanomaterials for various applications. By employing the various characterization methods, researchers can gain valuable insights into the properties and behaviour of nanoparticles, facilitating their development and optimization for various applications [10].

The high surface area and reactivity of nanoparticles can lead to several side effects. One concern is their non-specific antimicrobial action, which affects both pathogenic and beneficial microorganisms. This lack of specificity can disrupt the delicate balance of symbiotic microorganisms in various ecosystems, including the human body. Additionally, the potent reactivity of nanoparticles raises concerns about their potential toxicity, as they may interact with biological molecules and cellular structures in unforeseen ways, potentially causing harm [11].

Recent developments in nanoparticle characterization have expanded our capabilities to study and manipulate nanomaterials with unprecedented precision and efficiency. These breakthroughs lay the groundwork for the creation of innovative nanotechnologies applicable across diverse fields such as medicine, electronics, energy, and environmental science. In conjunction with exploring the potential of nanoparticles through characterization methods, it is imperative to investigate their toxicity and adverse effects [12,13,14].

In this study, the cell-free filtrate derived from isolated fungi was employed to reduce silver nitrate to silver nanoparticles. The synthesized nanoparticles underwent characterization through UV-visible spectroscopy, scanning electron microscopy (SEM), and X-ray diffraction (XRD). Furthermore, the antimicrobial properties of the nanoparticles were assessed against common human pathogenic microorganisms including *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. The minimum inhibitory concentration of the silver nanoparticles against these microorganisms was determined. Additionally, the bactericidal effect of the silver nanoparticles on the pathogens was evaluated through time-kill assay studies. The fungal isolate was identified through 16S rRNA studies.

2. MATERIALS AND METHODS

2.1 Collection of Samples

A total of 32 marine soil samples were collected from different geographical locations. The samples were taken from a depth of about 15 cm removing any superficial layers or contamination. The soil was then transferred into sterile polythene bags, labelled and stored for further use [15].

2.2 Isolation of Fungi from Samples

1g of the individual soil samples were suspended in 9 mL of sterile distilled water and serial dilution was carried out until the 10-7 dilution. This was followed by plating 1mL of each dilution on potato dextrose agar (PDA). The plates were then incubated at room temperature (28 ± 2 °C) for 7 days. Post the incubation period, individual colonies occurring in the plates were isolated employed for further screening [16].

2.3 Preparation of Cell-Free Filtrate

Each fungal isolate was grown separately in Potato dextrose broth for 7 days at 28℃. The fungal mat was washed several times with sterile double distilled water. Then, 10 ml of sterile double distilled water per gram of the wet weight of mat was taken in a conical flask and incubated in a shaker for 48 hours. After the incubation period, the contents of the conical flask were filtered using Whatmann Filter Paper No.1 to obtain the mycelial-free filtrate [17].

2.4 Screening the Isolates for Formation of Silver Nanoparticles

The filtrate combined with 1mM $AgNO_3$ was allowed to react in dark conditions at room temperature and observed for the formation of silver nanoparticles by analysing colour change up to 48 hours [18]. The samples showing suitable colour change alone were further subjected to characterization studies using UV-visible spectroscopy.

2.5 Characterization of the Synthesized Nanoparticle

2.5.1 Uv-visible spectroscopy

UV-visible spectroscopic analysis was performed to confirm the presence of nanoparticles in a solution at preliminary level [19]. The UV-visible spectra of the solution were recorded on UV-Vis Hitachi in a range between 200 and 700 nm. Based on the maximum absorption peak obtained peak and stability of the nanoparticles formed, one extract was utilized for further characterization studies.

2.5.2 Scanning Electron Microscopy (SEM)

Sample preparation was carried out by finely dispersing the supernatant comprising nanoparticles on to the substrate. The sample was then transferred to the stub and analysis was carried out [20].

2.5.3 X-Ray Diffraction analysis (XRD)

The freeze-dried AgNPs in pellet form were subjected to XRD analysis in a Phillips PW 1830 functioning at a radiation voltage of 40 kV, current of 20 mA and CuK_α radiation at a wavelength of 1.5406 Å [21].

2.6 Preliminary Identification of Fungi Selected for Nanoparticle Production

The colony morphology of the fungi capable of producing stable nanoparticles was identified using Lactophenol cotton blue staining (LPCB) method [22].

2.7 Antimicrobial Activity of Silver Nanoparticles

The antimicrobial activity of the synthesized AgNPs was tested using well-diffusion method against Gram positive *(Bacillus subtilis* ATCC 6633*, Staphylococcus aureus* ATCC 25923*) and Gram-negative (Escherichia coli* ATCC 25952*, Pseudomonas aeruginosa* ATCC 27853 *and Klebsiella pneumoniae* ATCC 700603*)* on Mueller Hinton agar plates. 1mM of freshly prepared silver nitrate solution, mycelial free extract and 1 mg/mL of standard antibiotic (Streptomycin) which served as positive control was added to the wells and incubated at 37℃ for 24 hours. Post incubation, the zone of inhibition (ZOI) was measured [23] (Table 2).

2.8 Minimum Inhibitory Concentration (MIC)

Based on the 96-well microtiter plate technique [24], two-fold serial dilutions were performed in Mueller-Hinton Broth using the synthesized AgNPs (known concentrations ranging between 0.19 µg/mL to 100 µg/mL) to check their minimum inhibitory concentration against the test microorganisms adjusted to (0.5 McFarland's standard) (Table 3).

2.9 Time-Kill Assay

In order to study the effect of the nanoparticles on the test microorganisms with respect to concentration and time factor, time-kill assay was performed according to Loo et al. [25]. Nanoparticle solution (1/2 MIC, 1 MIC, 2 MIC) was added to the test cultures followed by incubation under agitation of 150 rpm at 28℃. 25 µl from each tube was pipetted out on Nutrient agar plate at regular time-intervals in hours (0,2,4,6,8,10,12,14,16,18,20,24). The plates were then incubated for 24 hours and the colony count of each plate was noted and expressed as CFU/mL. Finally, a logarithm graph was plotted between the viable colony counts (CFU/ml) and time (in hours) (Fig. 5).

2.10 Molecular Identification of the Fungal Isolate

The ITS (Internal transcribed spacer) region of the fungal isolate was sequenced to identify the fungi from a pure sample. Mycelium from a ten-day old culture was used for DNA extraction which was carried out using QIAamp DNA minikit (QIAGEN, Hilden, Germany) following the manufacturer's directions. Further, the 16s-rRNA genes were amplified using Veriti 96-well thermal cycler (Applied Biosystems, USA) and then sequenced on ABI 3730XL sequencer (Applied Biosystems, USA) with ABI PRISM BigDye Terminator. Phylogenetic relatedness was

calculated with 23 closest BLAST matches and ML (Maximum likelihood) Tree was generated using FastTree v2.1 [26] (Fig. 6).

3. RESULTS AND DISCUSSION

Among the biological agents, fungi are the favoured choice for producing a diverse array of nanoparticles. This process, known as mycosynthesis, harnesses the unique capabilities of fungi for nanoparticle synthesis. Many researchers have utilized fungi for the synthesis of nanoparticles [7], [27]. From the 32 soil samples initially collected for this study (Table 1), 40 isolates of fungi were detected during the preliminary screening based on their colony morphology on Potato Dextrose Agar (PDA) [28].

The cell-free filtrate of these fungal colonies was screened for extracellular silver nanoparticle synthesis when treated with 1mM of silver nitrate solution [29]. In accordance with literature studies, positive results in terms of colour change depicting a gradual shift from light yellow towards brown colour were obtained in 12 of the fungal cell-free filtrate samples (Table 1) supporting the theory of reduction reaction. Such reactions are fuelled by the fungal enzymes when the metal precursor reacts with aqueous filtrate [30,27].

UV-visible spectra can be related to the phenomenon of surface plasmon resonance involving the interaction of conduction electrons of nanoparticles with the incident photons, thereby confirming the presence of silver nanoparticles [31]. Three of these samples passed the initial confirmation tests carried out with the help of UV-spectroscopic studies. But only one sample (DGV01) apart from showing a good colour change (Fig. 1) displayed a remarkable peak at 432 nm (Fig. 2) during UV-visible spectroscopic studies with reproducible results confirming extremely stable silver nanoparticles. This peak remained stable for a period of 25-30 days. All these factors served as the selection criteria to use these silver nanoparticles to carry out further research. The results were in accordance with several similar studies [32-34].

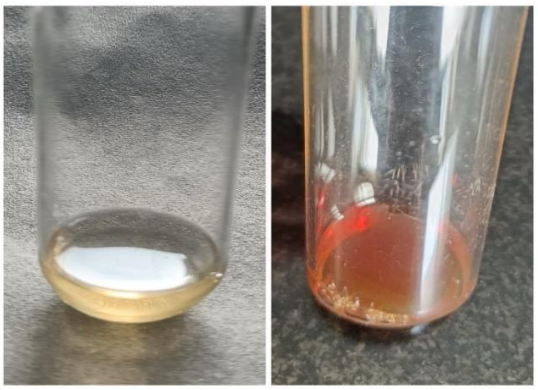


Fig. 1. Visible colour changes during the formation of AgNPs

The morphology and size of the synthesized nanoparticles were studied using Scanning Electron Microscopy (SEM). An illustrative SEM

Table 1. Screening of marine soil fungi for the biogenic synthesis of AgNPs

Source	Location	Number of samples collected	Number of isolates	Number of positive isolates
Marina Beach (Chennai)	13°03'02.2"N 80°16'57.6"E	24	15	02
Thiruvanmiyur Beach (Chennai)	12°58'16.0"N 80°15'58.2"E	15	09	01
Besant Nagar Beach (Chennai)	12°59'57.8"N 80°16'19.5"E	05	03	01
Kasimedu Beach (Chennai)	13°08'00.7"N 80°18'04.1"E	06	03	02
Pondicherry Beach	11°55'24.9"N 79°49'49.1"E	08	04	02
Karaikal Beach	10°55'06.5"N 79°50'59.5"E	05	02	01
Juhu Beach (Mumbai)	19°05'51.3"N 72°49'35.2"E	05	02	02
Nalaguraidhoo Beach- Maldives	3°29'03.3"N 72°48'13.4"E	04	02	01

micrograph (presented in the Fig. 3) of the synthesized AgNPs obtained after 72 hours of incubation was evidently distinguishable and indicated spherical scattered particles in the range of 11.93-37.71 nm. The results correlated with the studies of Mughal et al. [27].

The X-ray diffraction (XRD) pattern of AgNPs, synthesized from the extract of A. niger, is depicted in Fig. 4. The pattern exhibits peaks at 111, 200, 220, and 311, corresponding to 2 Theta values of 37.81, 45.79, 64.75, and 77.87, respectively. These values closely match the provided data for pure silver nanoparticles in the database of the JCPDS file no. 04-0783. This offers a glimpse into the crystalline structure, aiding in the comprehension of the physical characteristics of the produced nanoparticle. Similar observations were made by Singh et al. [35].

The selected fungal strain (DGV01) was initially identified through analysis of its cultural characteristics and microscopic examination using Lactophenol cotton blue (LPCB) stain. In the culture plate, the growth appeared white, transitioning to black due to the presence of darkly pigmented conidia. The colony's reverse exhibited a pale-yellow hue. Under the microscope, large dark brown conidial heads were visualized. Conidiophores displayed a dark coloration toward the globule, with conidial heads typically biseriate brown, sometimes with distinct metulae. Vesicles were observed to be globose, dark brown, and characterized by rough walls [36].

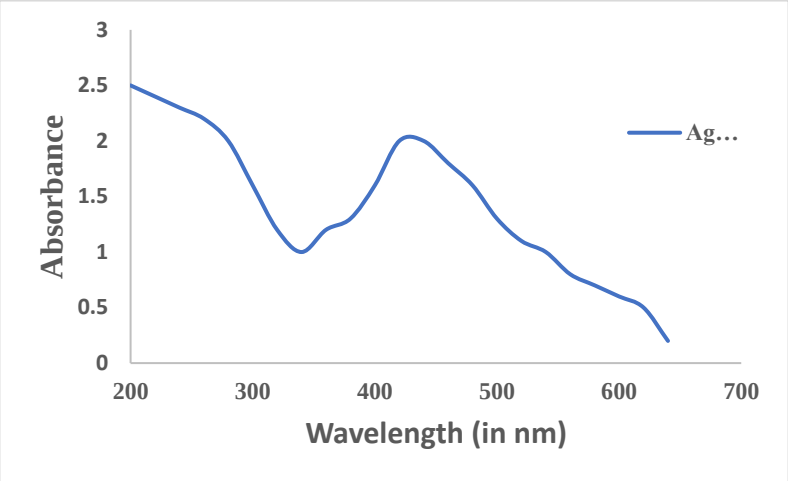


Fig. 2. UV-Visible spectrum of the biologically synthesized AgNPs

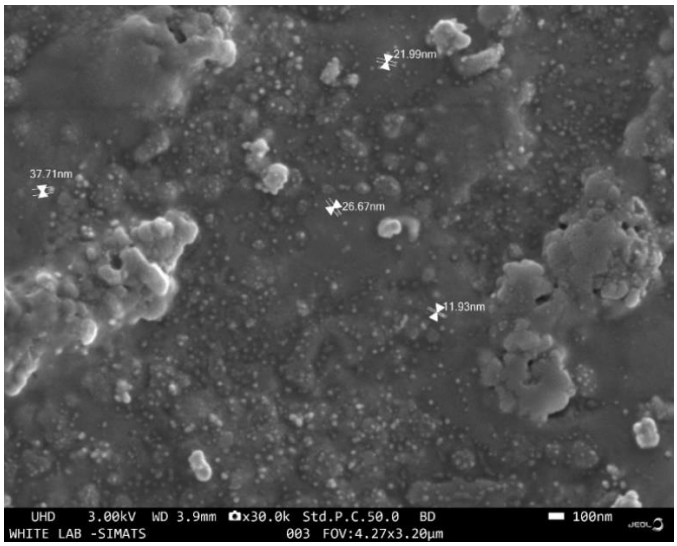


Fig. 3. SEM analysis of the biologically synthesized AgNPs

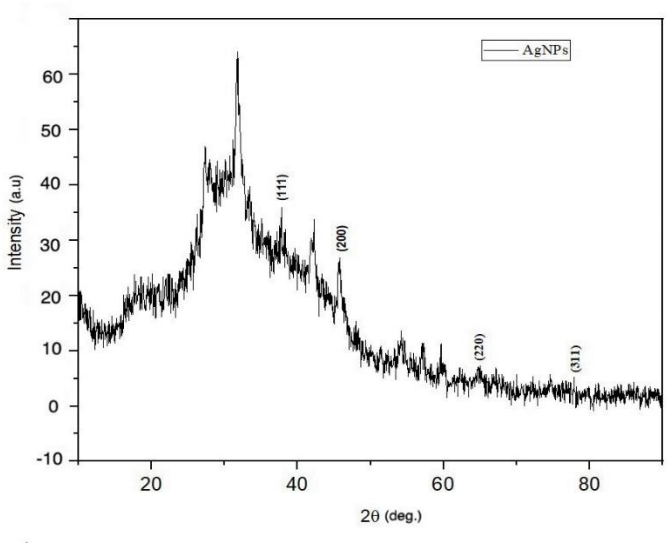


Fig. 4. XRD analysis of the biologically synthesized AgNPs

Table 2. Antimicrobial activity of the of the biologically synthesized AgNPs against the test strains

Organism	Mycelial free extract	$AgNO_3$ solution (1mM)	Ag Nps (1mg/mL)	Standard antibiotic (1mg/mL)
	Zone of inhibition (in mm)			
B. subtilis ATCC 6633	-	14	16	19
S. aureus ATCC 25923	-	14	16	26
E. coli ATCC 25952	-	13	27	30
P. aeruginosa ATCC 27853	-	16	20	24
K. pneumoniae ATCC 700603	-	10	12	16

Several studies have reported that silver nanoparticles when synthesized biologically and used in minimal concentrations, are non-toxic to humans and show good antimicrobial effect against a wide range of microorganisms. The antimicrobial ability of the mycofabricated silver nanoparticles to inhibit the test gram-positive and gram-negative bacteria is presented in Table 2. Based on the obtained results, it was noticed that *Escherichia coli* (27 mm) showed maximum sensitivity towards the synthesized Ag nanoparticles followed by *Pseudomonas aeruginosa* (20 mm), *Staphylococcus aureus* (16 mm), *Bacillus subtilis* (16mm) and *Klebsiella pneumoniae* (12mm). The results were in accordance to the findings reported by Funder et al. [37].

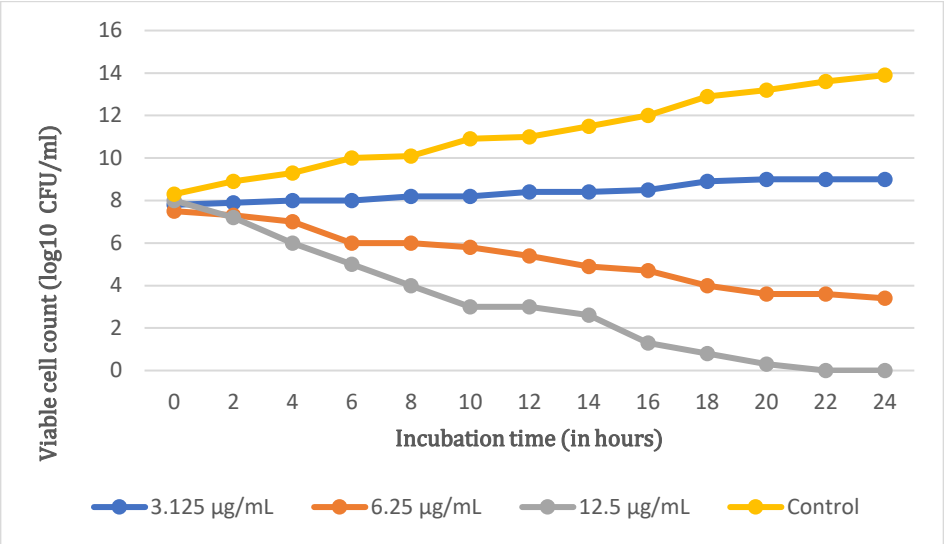
The results of minimum inhibitory concentration (MIC) are provided in Table 3. They signify the lowest concentration of the AgNPs (6.25, 6.25, 3.12, 3.12 and 12.5 µg/mL), required to inhibit the activity of *B. subtilis, S. aureus, E. coli, P. aeruginosa* and *K. pneumonia* respectively. Few studies have reported similar activity of AgNPs against clinical pathogens, however the MIC was found to be different from the ones we found in this present work [38,39,35] investigated the antibacterial activity of AgNPs against various gram-positive and gram-negative bacteria and the results were comparable to our study.

The swift multiplication of bacteria plays a significant role in their infectiousness. Nonetheless, controlling the bacteria's reproduction time could present an optimal strategy for averting viable infections. This potential was demonstrated in time-kill assay, where the biogenic AgNPs effectively restrained and eradicated bacteria in a dose and time-dependent manner. Time kill activity of the synthesized AgNPs against the pathogenic microbial test strains used during antimicrobial activity studies is shown in (Fig. 5a, 5b, 5c, 5d,

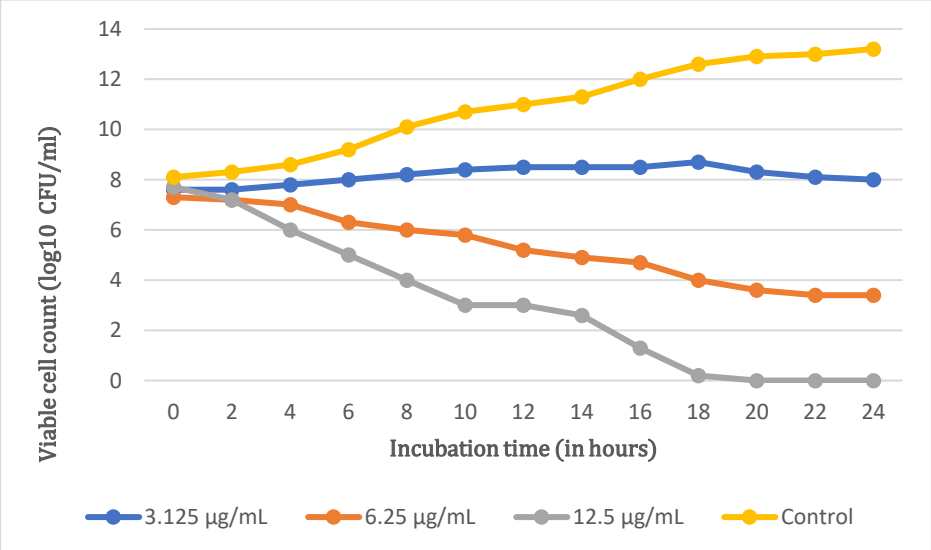
and 5e). The bactericidal endpoints were prominent at the respective MIC concentrations where the growth curve almost declined at the end of 18-24 hours. At concentrations higher than the MIC range, growth declined at a faster rate indicating the successful action of silver nanoparticles against the pathogens. A comparable discovery was made by Jenkins and Schuetz [24]. Similar experiment when performed by some other researchers using metal nanoparticles, parallel findings were noticed [40,41].

Table 3. Minimum Inhibitory concentration (MIC), of the biologically synthesized AgNPs against test microorganisms after 24 hours

Test organisms	MIC (in µg/mL)
B. subtilis ATCC 6633	6.25
S. aureus ATCC 25923	6.25
E. coli ATCC 25952	3.12
P. aeruginosa ATCC 27853	3.12
K. pneumoniae ATCC 700603	12.5



(a)



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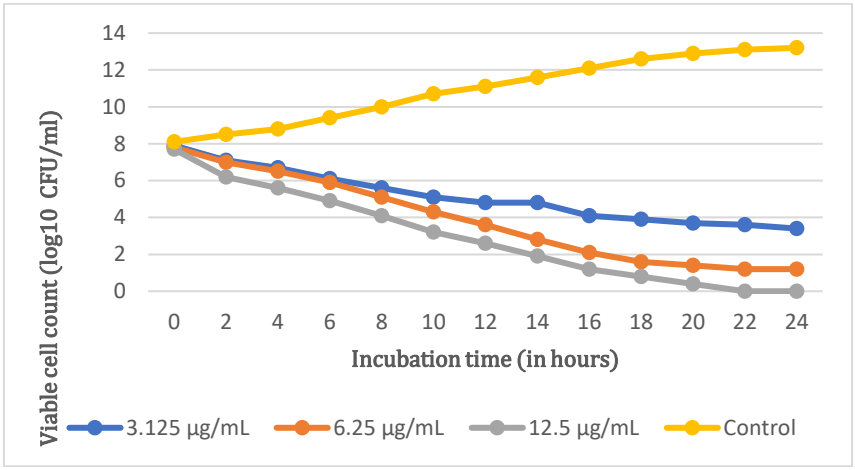

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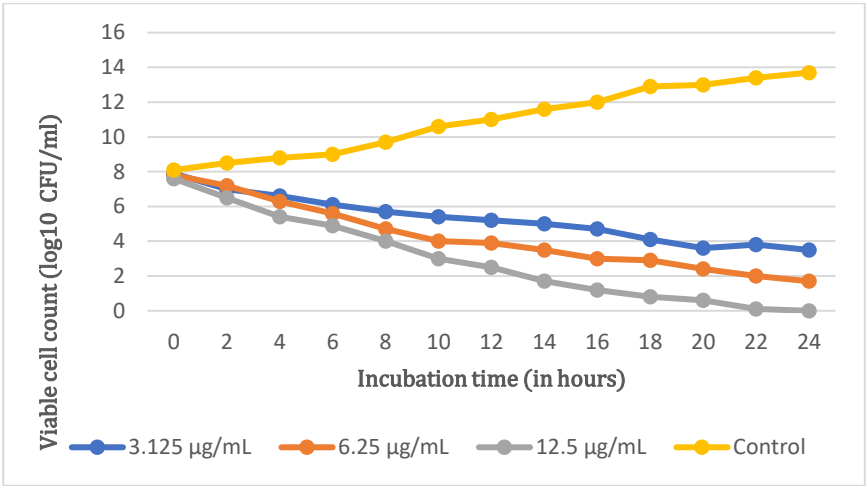

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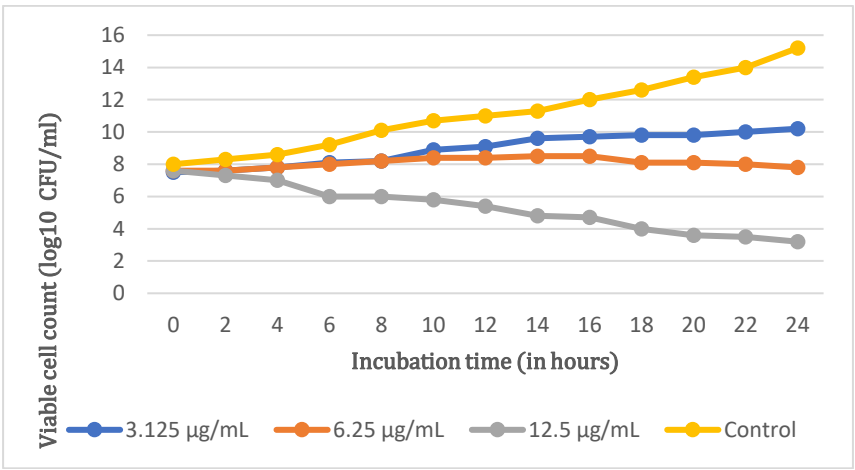

(e)

Fig. 5. Time-kill assay of the biologically synthesized AgNPs against the test strains (5a-*Bacillus subtilis* ATCC, 5b-*Staphylococcus aureus,* 5c-*Escherichia coli*, 5d- *Pseudomonas aeruginosa*, 5e-*Klebsiella pneumoniae*)

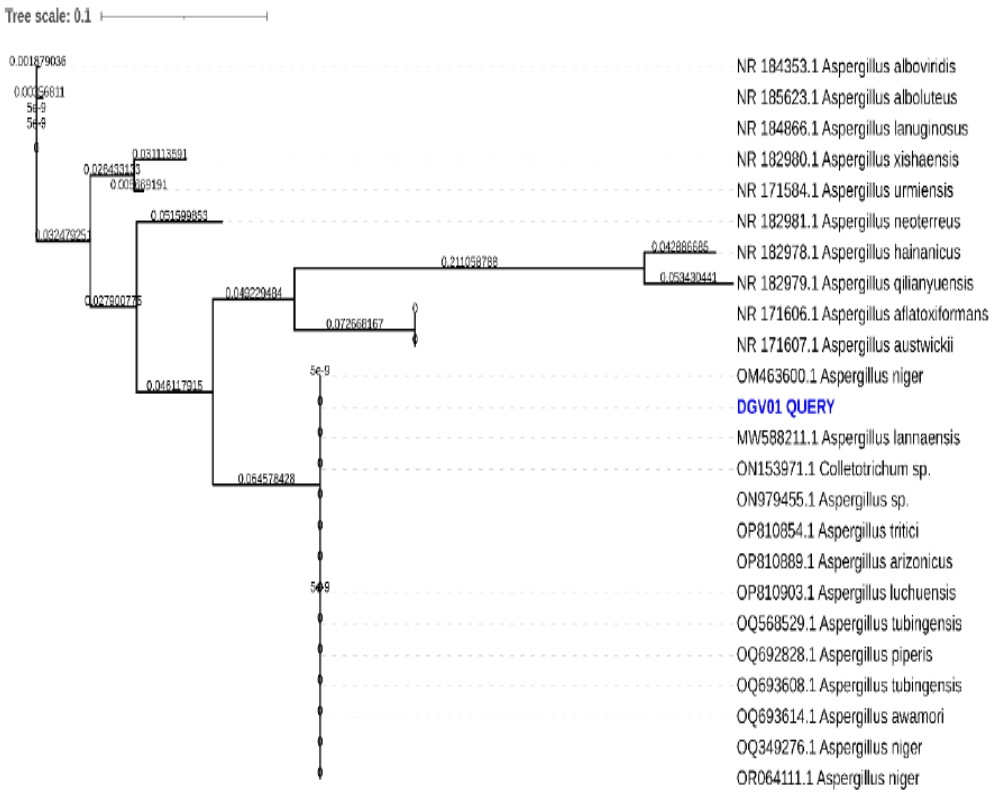

Fig. 6. Neighbourhood joining phylogenetic tree of the isolate *Aspergillus niger* DGV01 created using distance matrix analysis of 16S rRNA gene sequences

The isolated fungus (DGV01) exhibited good growth on Potato dextrose (PDA medium) with black colonies producing conidial spores. The fungus was identified by PCR amplification of 18S rDNA gene using ITS primers. The sequence was deposited in NCBI GenBank with Accession Number: OR484843. The nucleotide sequences of the 18 s rRNA gene region of *Aspergillus* isolates was aligned with other reference isolates by using the Blastn analyses, pairwise and multiple sequence alignment which revealed 100% identity with the sequences of *Aspergillus niger* strains (Fig. 6). This result is consistent with reports by Awad et al. [42].

4. CONCLUSION

This study sought to assess the utilization of fungus for the biological synthesis of silver nanoparticles. The culture supernatant of *Aspergillus niger* exhibited effectiveness in reduction of metal ions to silver nanoparticles. This was primarily confirmed with the presence of UV–visible spectral peak at the wavelength of 432 nm, stable for a period of 25-30 days. The Scanning Electron Microscopic image revealed the surface morphology and particle size of the nanoparticles which were spherical, well-dispersed in the range of 10-30 nm. X-ray Diffraction studies demonstrated the crystalline nature of the silver nanoparticles. The antimicrobial effectiveness assessed via the well-diffusion method showcased a maximum zone of inhibition against *E.coli* (27 mm) followed by *P. aeruginosa* (20 mm), *B. subtilis* (16 mm), and *S. aureus* (16 mm). Minimum susceptibility was displayed by *K. pneumoniae* (12 mm). The results signify the effectiveness of the synthesized AgNPs against gram positive and gram-negative pathogens indicating their broad spectrum and thereby presenting a promising avenue for antibiotic development. The potency of the nanoparticles was determined using MIC studies where it was seen that a concentration of 3.12 µg/mL was effective against *E.coli* and *P. aeruginosa*, 6.12 µg/mL against *B. subtilis and S. aureus* and 12.5 µg/mL against *K. pneumoniae*. Time-kill curve determined the effect of nanoparticles on bacterial viability over a specified period of time while indicating faster decline in growth at concentrations higher than the MIC values. Nevertheless, it is vital to critically analyse the molecular interactions between the synthesized nanoparticles and

fungal proteins to fully grasp the specific mechanism driving the process. Species level identification was confirmed by 16s rRNA sequencing. The fungus coded DGV01, showed 100% sequence similarity with *Aspergillus niger.* The results imply that AgNPs synthesized using *Aspergillus niger* harbour considerable promise as impressive antibacterial agents applicable in the realms of both medicine and biotechnology. They also suggest the role of biomolecules like proteins in functionalization, substantially boosting the antibacterial properties of nanoparticles, which can be further studied through investigations of underlying molecular mechanisms.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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