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FUNGUS MEDIATED SYNTHESIS OF SILVER NANOPARTICLES USING *ASPERGILLUS FLAVUS* AND ITS ANTIBACTERIAL ACTIVITY AGAINST SELECTIVE FOOD BORNE PATHOGENS

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Abstract:

In the present study, the aqueous extract of Aspergillus flavus was employed for the mycosynthesis of silver nanoparticles (AgNps). The fungal extract of A. flavus acts as a reducing agent for the synthesis of AgNps. The biologically synthesized AgNPs was well characterized using various techniques such as, UV–Visible spectrophotometer, TEM, EDX and XRD. The synthesized AgNPs was found to be spherical in shape with a size range between 10 to 40 nm, and presence of elemental silver was also confirmed by the EDX spectrum. The mycosynthesized AgNps showed great extent of antibacterial property against the selected food borne pathogenic strains. The results obtained revealed that the maximum antibacterial activity of AgNPs was found against B. subtilis followed by S. aureus. From the present study results, it is clear that the synthesized AgNPs possessing good antibacterial activity could be more effective in applications such as antibacterial food packaging materials.

Keywords: *Aspergillus flavus, AgNPs, Antibacterial activity, TEM*

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INTRODUCTION:

Nanotechnology has gained much importance in recent days and research on nanoparticles has also increased due to their size, shape, high surface area to volume ratio, optical, physical and chemical properties. Silver nanoparticles (AgNPs) are the widely used nanoparticles with a broad range of applications in the areas like biological product development, cancer therapy, water treatment, etc. [1]. According to Larue and co workers, silver is the most commercialized nano-material which is produced at the rate of five hundred tons per year as of 2015 and is estimated to further increase in future years [2]. Silver is mostly preferred because of their strong bactericidal, anti-fungal, anti-inflammatory activities [3, 4]. The high yield of nanoparticles and synthesis of uniformly sized nanoparticles are the two fundamental focus in the synthesis of silver nanoparticles [5].

In recent years, the emergence of multi drug resistant pathogens pose a significant problem which lead several researchers to develop new generation antimicrobial which posses good activity [6, 7]. Silver and silver based nano structures, especially the AgNPs was found to be one of the strong alternatives due to their potent broad spectrum of antimicrobial property against the pathogens [8, 9, 10, 11, 12]. Studies also show that the antimicrobial effects were greatly influenced by the size of the AgNPs [13].

The AgNPs can be synthesized by many routes via physical, chemical and biological methods [14, 15]. Most of the above methods involve the usage of hazardous chemicals which are found to be difficult in preparations as well as purifications [16]. As per environmental concern, biological methods gains advantage over other methods. In the biological methods, nanoparticles are using naturally synthesized with the help of reductants derived from plant extracts and microorganisms [17, 18]. The biological synthesis of nanoparticles also provides advantages over other methods as they are simple, cost-effective, eco-friendly, results stable materials and reproducible [19]. Recent investigations are concerned with use of fungal extract as reducing agent for the biosynthesis of silver nanoparticles [20]. In the present study, AgNps was biologically synthesized using extract of *Aspergillus flavus* and investigated for their antibacterial activity against food borne pathogenic bacterial strains.

MATERIALS AND METHODS:

Sample collection and strain selection

The oil contaminated soil samples were collected nearby Chennai and brought to the laboratory. The samples were serially diluted and fungi with different morphology were isolated using serial dilution followed by pour plate technique onto potato dextrose agar (Himedia Pvt Ltd) supplemented with chloramphenicol (50 mg/L). The fungi with different morphology were used for screened for the synthesis of AgNPs.

Preparation of fungal extract and screening of AgNPs synthesis

For the preparation of fungal extract, the fungal isolates were inoculated separately into 100 ml of potato dextrose broth under aseptic conditions and incubated for 4-5 days at 25˚C for biomass production. At the end of incubation, the biomass was washed well with sterile distilled water to remove the spores and other traces of media components. Ten gram of wet biomass was taken along with 100 ml of sterile distilled water and the preparation was kept in a rotary shaker incubator for 48 h at room temperature. Then, filtrate was collected using Whatman filter paper No. 1 and it was treated with silver nitrate to reach a final concentration of 1 mM. The preparation was incubated in dark condition and monitored for the formation of dark brown colour which was further analyzed using UV-Vis spectrophotometer to confirm synthesis of AgNPs [21, 22]. The fungal strain which mediates the synthesis of stable AgNPs was selected as a potential isolate and was used for further studies.

Characterization of silver nanoparticles

The formation of AgNPs was confirmed by recording the absorption spectra using UV-Visible spectroscopy at a wavelength of 300 - 700 nm (Elico UV-Visible double beam spectrophotometer). The size and surface morphology of the synthesized AgNPs were determined using Scanning electron microscopy (SEM) and Energy dispersive X-ray (EDX) on FEI Quanta FEG 200-High Resolution Scanning Electron Microscope. The crystalline nature of AGNPs formed was analyzed using X-ray diffraction (XRD) measurement using Single crystal X-Ray Diffractometer (XRD) instrument.

Identification of fungi

The potential isolate which mediates the formation of

stable AGNPs was identified using macroscopic, microscopic characters and molecular sequencing method. The microscopic identification of fungal isolates analyzed using lacto phenol cotton blue staining of the fungal specimen and observed under high power objective [23, 24]. Further, the potential isolate was identified using molecular sequencing method, briefly the genomic DNA of the potential isolate was extracted using CTAB method [25], then the ITS 1 and ITS 2 primers was used for amplification process (White et al., 1990). The amplified product was purified and sequenced using ABI 3730x1 Genetic Analyzer by Big Dye terminator method (Applied Biosystems, USA). The obtained sequence of the potential isolate was analyzed for sequence homology using BlastN using the NCBI website and also deposited in National Centre for Biotechnology Information (NCBI), the phylogenetic tree analysis was performed using maximum parsimony analysis method by MEGA 6 software [26].

Antibacterial activity of AgNPs

The antibacterial activity of the mycosynthesized silver nanoparticles against food brone pathogenic bacteria, *Escherichia coli* (MTCC 118), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 737) *and Enterobacter aerogenes* (MTCC 111) was assayed using standard well diffusion assay method. The test strains were cultured in Mueller Hinton broth and incubated at 37°C for 24 h. The overnight grown bacterial cultures were separately spread on the Mueller-Hinton agar (MHA) plates to get a lawn culture. Wells of 4 mm diameter were prepared using sterile well borer and loaded with 30 µl of fungal extract, $AgNO_3$, ampicillin (1mg/mL) and different concentration of AgNps. The plates were incubated at 37°C for 24 h and the antibacterial activity was determined by measuring the diameters of inhibition zones around the wells [27].

RESULTS AND DISCUSSION:

In the present study, fungi with different morphology were isolated from oil contaminated soils and were screened for the synthesis of AgNPs. Among the different fungal isolates screened for AgNPs, the strain PVGS 1 was found to mediate the synthesis of AgNPs under 18 h with stability of more than two months. The addition of fungal extract of *A. flavus* to the aqueous solution of silver nitrate results in change in the color of the solution to brown when incubated in dark condition (Fig. 1) after 24 h which is due to excitation of surface plasmon resonance (SPR) vibrations [4, 28].

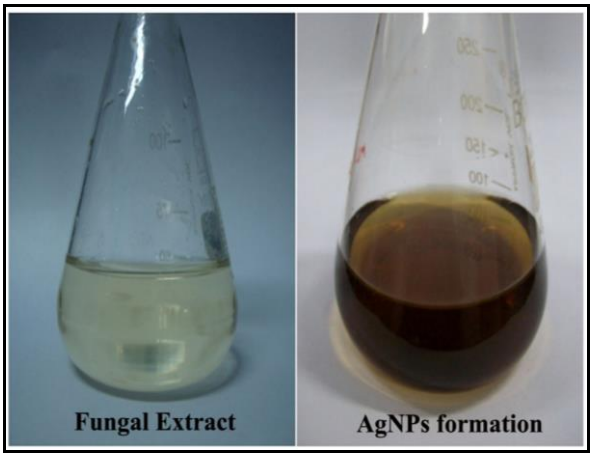

Fig. 1: Formation of AgNPs shown by brown color change after the addition of $AgNO_3$ to fungal extract.

The synthesis of silver nanoparticles in the aqueous solution was screened by recording the absorption spectra using UV-Visible spectroscopy. The maximum peak of fungus mediated AgNPs formation was recorded at 434 nm (Fig. 2) which is well accordance with earlier studies reported by various researchers [29, 30, 31](Mishra et al., 2011; Gupta and Bector, 2013, Nithya, 2016). Zaheer and Rafiuddin have suggested that the peak observed between 410 and 450 nm might be attributed to the SPR phenomenon which is characteristic of metal nanoparticles, especially AgNPs [32]. The mycosynthesis of silver nanoparticles is served to be a potential route for the synthesis of nanoparticles with good stability and less toxicity. Different fungi such as, *Fusarium oxysporum* [33], *Aspergillus niger* [20], *Fusarium acuminatum* [34], *Phoma glomerata* [35] were investigated for the synthesis of silver nanoparticles.

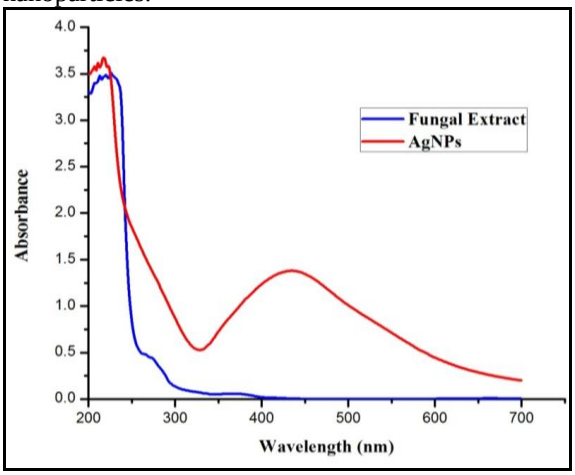

Fig. 2: UV–visible absorption spectrum AgNPs and Fungal extract

The synthesized AgNPs was further characterized using XRD, EDX and TEM analysis. The transmission electron Microscopy (TEM) results revealed the spherical shape of the synthesized silver nanoparticles (Fig. 3) with an average size of 10 to 40 nm (Fig. 3). The EDX spectrum also revealed the strong peaks corresponding to silver which confirmed the successful formation of AgNPs.

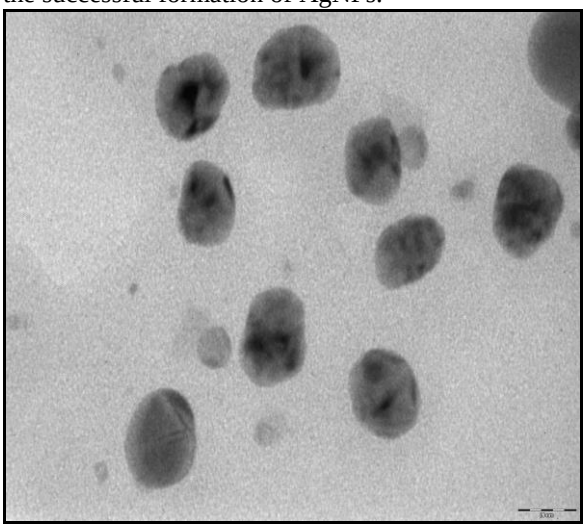

Fig. 3: TEM image of AgNPs with spherical in shape

The XRD spectrum of the synthesized AgNPs was analyzed and three distinct Bragg reflections were observed corresponding to (1 1 1), (2 0 0), and (2 2 0) orientations of the face-centered cubic silver. The obtained data was well matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783, confirming the crystalline nature of AgNPs. Similar studies were reported by various researchers who have also characterized fungus mediated AgNPs using XRD and EDX [36, 37, 38, 39].

The strain which mediated the synthesis of stable AgNPs was considered as the potential strain (PVGS1) and was identified based on microscopic and molecular characterization method. The genomic DNA of the potential strain PVGS1 was extracted and amplified for ITS region. The species level of identification was performed using the molecular sequencing of the amplified ITS region which resulted in 557 bp length of nucleotide sequence. The sequence was analyzed using Blast analysis tool, (NCBI) National Center for Biotechnology Information (MD, USA) and the potential isolate PVGS1 was identified as *Aspergillus flavus.* Further the nucleotide sequence was deposited in NCBI, GenBank database (MD, USA).

The application of silver and its components as an antimicrobial agents in the form of silver coins and silver vessels extend towards ancient times [36, 40]. Due to the potent antibacterial activity of the AgNPs, they have been recently used in various industries like healthcare, medicine, textiles, food storage, wound dressing, antiseptics, etc [41]. In the present study, we have analyzed the antibacterial potential of AgNPs synthesized from *A. flavus* against various food borne pathogens. The fungal extract and the AgNps were tested for antibacterial activities towards four different food borne pathogens (Table 1).

Table 1: Antibacterial activity of AgNPs against food borne pathogens

Bacterial pathogens	**Zone of Inhibition**					
	Fungal extract	$AgNO_3$ (1 mM)	AgNPs (10 µg)	AgNPs (20 µg)	AgNPs (30 µg)	Ampicillin (1 mg/ml)
Escherichia coli	-	-	7 mm	9 mm	10 mm	10 mm
Bacillus subtilis	-	-	9 mm	13 mm	16 mm	14 mm
Staphylococcus aureus	-	7 mm	9 mm	12 mm	12 mm	12 mm
Enterobacter aerogenes	-	-	7 mm	8 mm	9 mm	10 mm

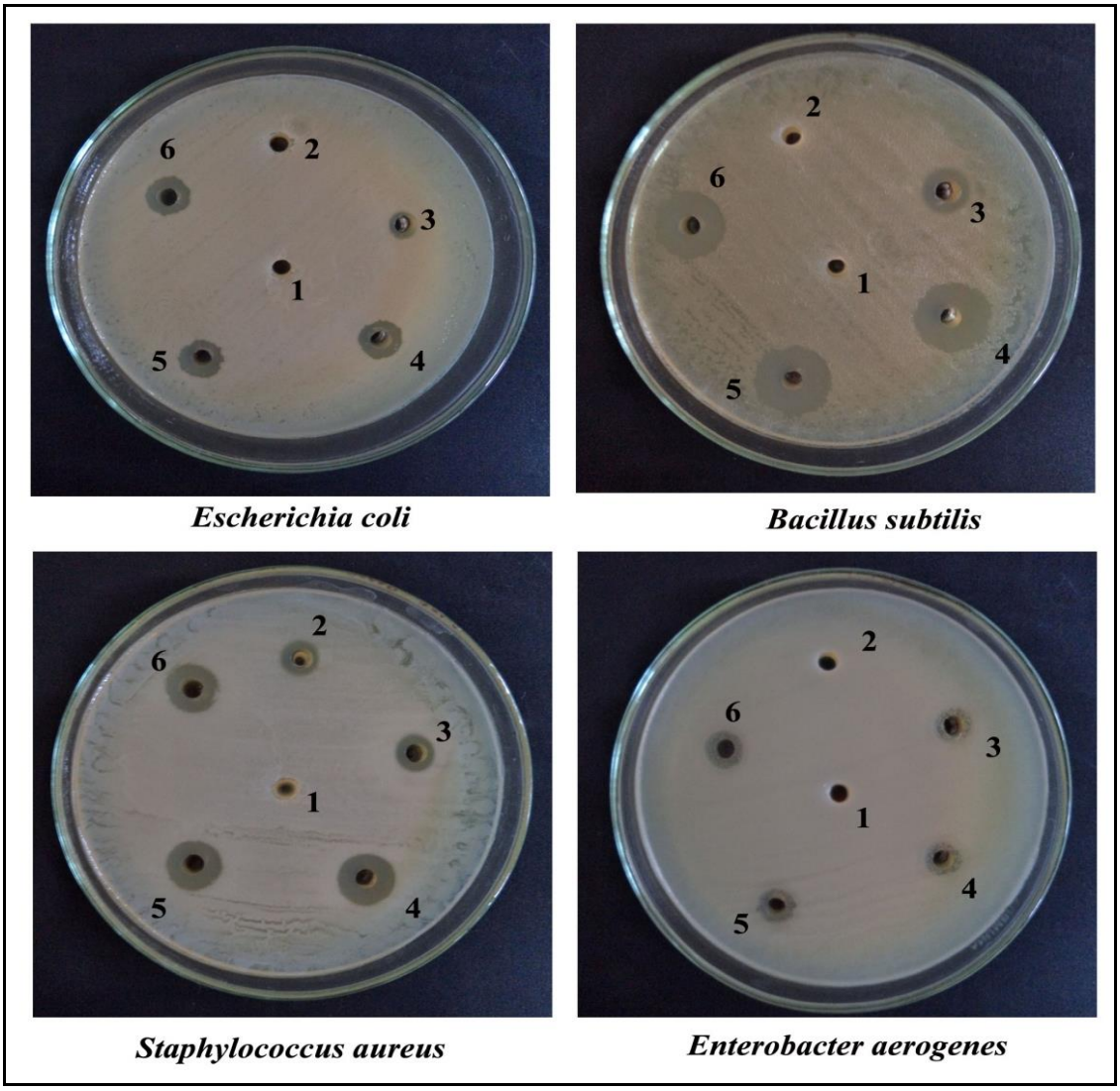

Fig. 4: Antibacterial activity of AgNPs against food borne pathogens
(1 – fungal extract; 2 – $AgNO_3$; 3 – AgNPs [10µg]; 4 – AgNPs [20µg]; 5 – AgNPs [30µg]; 6 – Ampicillin)

The results proved showed that the mycosynthesized AgNps exhibit good antibacterial activity against all the selected food borne pathogens. Maximum activity was found against *B. subtilis* (16 mm) followed by *S. aureus* showing zone of inhibition of 12 mm; the minimum activity was found against *E. aerogenes*. The biologically synthesized AgNPs showed better activity when the concentration was maintained at 30 µg/mL though the activity was also found at lesser concentration (Fig. 4). The fungal extract did not show any activity when tested against all the food borne pathogenic strains. The standard drug, ampicillin with 1mg/mL was also tested along with AgNPs as positive control.

There are many advantages of AgNPs to be used as effective antimicrobial agents. They are highly

effective against a broad range of microbes and parasites, even at a very low concentration with very little systemic toxicity toward humans [12]. According to earlier reports it is also found that AgNPs could be used and tested for several applications including prevention of bacterial colonization and elimination of microorganisms on various medical devices, antibacterial food packing, disinfection in wastewater treatment plants, and silicone rubber gaskets to protect and transport food and textile fabrics [10].

The exact mechanism of the antibacterial property of AgNPs against the pathogenic bacteria is yet to be completely understood. Very few researchers have investigated the antibacterial mechanism of AgNPs and they have reported that the electrostatic attraction between the positively charged metal ions with the negatively charged bacterial cells could be responsible the bactericidal effects [42]. Few reports also suggested that the AgNPs involves in inactivation of cellular proteins, DNA breakage and enzymes degradation in bacterial cells [43, 44, 45].

CONCLUSION:

In the present study, a novel approach for the mycosynthesis of AgNPs from aqueous extract of *Aspergillus flavus* was suggested. The mycosynthesized AgNPs were of spherical in shape with size ranging beween 10 to 40 nm. The synthesized AgNPs have shown excellent antibacterial activity towards all the tested food borne pathogens, with maximum activity found against *B. subtilis*. Thus it is concluded that the mycosynthesis of AgNPs using *A. flavus* extract serves as a simple, cost effective and eco-friendly method which could be easily scaled up for the increased yield of the nanoparticles.

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CONFLICT OF INTEREST

Authors claim that there is no conflict of interest.

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