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BIOGENIC SYNTHESIS OF SILVER NANOPARTICLES FROM *ASPERGILLUS ORYZAE* MTCC 3107 AGAINST PLANT PATHOGENIC FUNGI *SCLEROTINIA SCLEROTIORUM* MTCC 8785

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ABSTRACT

Phytopathogen including Sclerotinia sclerotiorum is a major problem for agricultural crops. Being safe, antifungal, and environment friendly, silver nanoparticles (AgNPs) are the first choices to combat phytopathogens. In view of this, the present study was designed to formulate AgNPs from Aspergillus oryzae MTCC No. 3107. Biosynthesis of AgNPs by A. oryzae was investigated using cell-free filtrates from fungi cultivated in potato dextrose broth (PDB) and amylase production media (APM). Fungal production media harbour inducers which upregulate secretion of specific enzymes. Amylase is known to catalyze the bio-reduction process. The cell- free filtrates containing extracellularly secreted fungal amylases when exposed to the metal salt solution (silver nitrate) at 1mM concentration, silver ions were reduced to zero oxidation state forming stable nano silver. The colour change was observed and the formation of AgNPs was further characterised by UV–vis spectrophotometry by scanning from 300-700 nm wavelength. Transmission electron microscope characterization revealed a size of 40nm. Further, the FTIR analysis identified the key functional groups involved in the stabilization and capping of AgNPs. Moreover, XRD analysis was done to identify the diffraction pattern in AgNPs. Antifungal effect of synthesized AgNPs on phytopathogen S. sclerotiorum MTCC 8785 was studied using variable concentrations of amylase mediated AgNPs. 100 percent inhibition was observed at 100 µg/ml concentration when compared to positive control.

Keywords: Extracellular enzymes, AgNPs, *Aspergillus oryzae*, Antifungal, *Sclerotinia sclerotiorum*

INTRODUCTION

The most commanding challenge for agriculture and food security these days is plant pathogenic fungi harming most of the commercial crops. *Sclerotinia* is one the most dreadful phytopathogens developing resistance to available fungicides. It spreads through hyphae in the target host and cause dreadful diseases like cottony rot, drop, and white mould **(Ranjan *et al.*, 2019)**. Eco-friendly new age nano-alternatives can be designed to alleviate the emergence of *Sclerotinia* mediated crop diseases and related resistance **(Tomah *et al.*, 2020)**.
With the advancements of urbanization and industrialization it is necessary to mitigate their harmful effects. Sustainability is promised with green practices which are easy, cost-effective, and nature friendly. Biogenic synthesis of nanoparticles is one of the sustainable processes with low energy and less waste generation **(Zhang *et al.*, 2020)**. Nanoparticles fall in the size range of 1-100 nm and are gaining much attention due to their diverse applications in industries, biomedical devices and antimicrobial action **(Khan *et al.*, 2020)**. The surface specific feature of AgNPs is its surface plasmon resonance (SPR) which results in their remarkable bio efficiency. Additionally, AgNPs display varied shapes, sizes, and morphology which control their physio-chemical and physiological properties within the system **(Elshafei *et al.*, 2021)**.
Nanoparticles synthesised from biological systems are termed as biogenic or green synthesis. The old conventional techniques to produce nanoparticles include thermal, chemical, and hydrothermal processes which involve toxic waste generation **(Huq *et al.*, 2022)**. AgNPs have been employed diversely due to their potency as antimicrobials **(Cheng *et al.*, 2018)**. AgNPs from biological routes like plants, microbes including bacteria, and fungi were reported by many researchers of concurrent times **(Bhatt *et al.*, 2018; Lahiri *et al.*, 2021)**. Biological routes can be scaled up for synthesis of stable nano silver **(Dawadi *et al.*, 2021)**.
Fungi is an excellent producer of extracellular enzymes which play a pivotal role in the bio reduction process of metal nanoparticle synthesis **(Raghav *et al.*, 2022; Guilger-Casagrande *et al.*, 2019)**. Various reports have been documented where fungal extracellular enzymes have been used for industrially relevant and agriculture friendly products **(El-Gendi *et al.*, 2021; Saxena *et al.*, 2017a; 2015)**. Recently, extracellular enzymes like microbial amylases have been employed for biological nanoparticle synthesis. Microbial enzymes exhibit free thiol and other functional groups which aid in the bio reduction process for metal nanoparticle synthesis **(Li *et al.*, 2022)**. *A. oryzae* belongs to non-pathogenic and generally recommended safe fungi by WHO and used for various industrial processes **(Barbesgaard *et al.*, 1992)**. It is a fast grower with high capacity of extracellular enzyme secretions which makes it ideal for experiments involving AgNP synthesis. There is an immediate need to explore untapped potential of its enzymes secreted extracellularly for generation of stable nanoparticles **(Obiedallah *et al.*, 2018; Elamawi *et al.*, 2018)**.
In the present study, we have utilized a green chemistry approach to synthesize AgNPs from *A. oryzae* MTCC 3107 and characterized using UV-vis spectrophotometry, Transmission Electron Microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and X ray diffraction (XRD) analysis. Furthermore, antifungal activity of AgNPs was also demonstrated against phytopathogenic fungi *S. sclerotiorum* MTCC 8785.

MATERIALS AND METHODS

Fungal strain and growth conditions

Both the fungal strains (*A. oryzae* MTCC 3107 and *S. sclerotiorum* MTCC 8785) required for investigations were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. These strains were routinely sub-cultured on potato dextrose agar (PDA) at 28ºC for 5-7 days and monitored as well as maintained on PDA slants.

Biogenic synthesis of AgNPs: Extracellular

A. oryzae MTCC 3107 grown on PDA was seeded in 100 ml APM containing 1% soluble starch as inducer and PDB at inoculum size of $1X10^5$ cells/ml. The conical flasks were incubated at 28ºC, 120 rpm for 5-7 days. Biomass grown after the incubation period was collected and processed with proper washing to remove media constituents. The biomass after washing was transferred to sterile 50 ml distilled water and incubated under shaking conditions (120 rpm) at 28ºC for 3 days. The cell-free filtrate (CFF) was harvested after removing biomass and further challenged with 1mm silver nitrate under dark conditions at room temperature. The CFF was continuously monitored for colour change. CFF without $AgNO_3$ was maintained as negative control **(Molla *et al.*, 2022)**.

Bio-stimulation: Extracellular amylase production using *A. oryzae* MTCC 3107

A. oryzae MTCC 3107 was grown on PDA containing 1% (w/v) starch in the

presence of antibacterial antibiotics. Iodine solution was poured onto the plates to observe the clear zone of hydrolysis surrounding the colony**.**

Characterization of AgNPs

Various analytical techniques were employed to characterize AgNPs

UV-Visible spectroscopy analysis

AgNPs synthesised using PDB and APM were further characterised by UV-vis spectrophotometer spectra scan (300-800 nm) at a resolution of 0.1 nm to record SPR peak values.

TEM analysis

To assess the shape and size of synthesized AgNPs using PDB and APM, TEM (Jeol, USA) analysis was done. AgNPs solution was loaded dropwise on the copper grids in TEM and images were captured followed by analysis for conferring to determine the shape and size of AgNPs **(Alves *et al*., 2022)**.

FTIR analysis

FTIR was carried out using freeze-dried AgNPs powder synthesized using APM in the range from 500–4000 cm^{-1}. To identify the major interacting chemical groups present during capping and stabilization of AgNPs, the peaks obtained were aligned with the standard **(Bhatt *et al*., 2018)**.

XRD analysis

AgNPs synthesised using APM were firstly dehydrated at 80°C and the diffraction pattern was acquired by Bruker AXS D8 Advance in Bragg–Brentano geometry and Johansson monochromator to produce pure Cu Kα1 radiation (1.5406 Å; 45 kV, 30 mA) **(Bhatt *et al*., 2018)**.

Purification of AgNPs

AgNPs from the solution were separated during centrifugation at 9000-10,000 for 5 min at 4ºC. The pellet obtained was washed with distilled water, dried and used for further experimental assays**.**

Antifungal assay

To evaluate the antifungal activity, the plant pathogen *S. sclerotiorum* was grown on PDA plates and was treated with AgNPs synthesis from CFF-APM at (25, 50, 100µg/ml concentration). Point inoculation was done on the PDA plates containing various concentrations of AgNPs and incubated at 28ºC for 7 days. The fungal radial growth was observed to assess the effect and the data were expressed as inhibition rate (%) **(Essghaier *et al*., 2022)**.

RESULTS AND DISCUSSIONS

Morphological and microscopic characterization

Aspergillus oryzae MTCC 3107 and *S. sclerotiorum* MTCC 8785 were sub-cultured as per standard operating procedures and their morphological characterization was performed routinely. *A. oryzae* MTCC 3107 was routinely sub-cultured and characterized morphologically **(Fig 1A)**. The colony of *A. oryzae* MTCC 3107 was fast growing, initially white and gradually developing yellowish-green to deep green tufts, in small zones or with concentric rings on the media surface. Microscopically the fungus exhibits conidiophores which are irregularly branched and bear flask shaped phialides. Condia are green and born on conidial tips clustered together (**Fig 1B)**.

Extracellular Synthesis of AgNPs

For synthesizing AgNPs from *A. oryzae* 3107, the fungal spores at inoculum size of $1x10^5$ spores/ml were seeded in 100 ml PDB and APM for 3-5 days at 28ºC under shaking culture (120 rpm) conditions. Fungal biomass was observed as a fully grown ball of cells which was further processed through filtration and washing with the distilled water. 10 gm weight of fungal biomass was then transferred to autoclaved distilled water (50 ml) and kept for three days under previously mentioned control conditions. Fungal biomass in distilled water secreted extracellular enzymes, specifically amylases in APM. Our data are in agreement with the previous reports where role of amylases from various sources have been confirmed in the synthesis of AgNPs **(Pandey *et al*., 2018; Mishra and Sardar, 2012)**. Additionally, nitrate reductase secreted by microbes reduces the Ag^+ to AgNPs. Our data are in agreement with Daniels (2015), who has also reported role of nitrate reductase in the reduction of Ag^+ to AgNPs **(Mughal and Hassan, 2022)**.

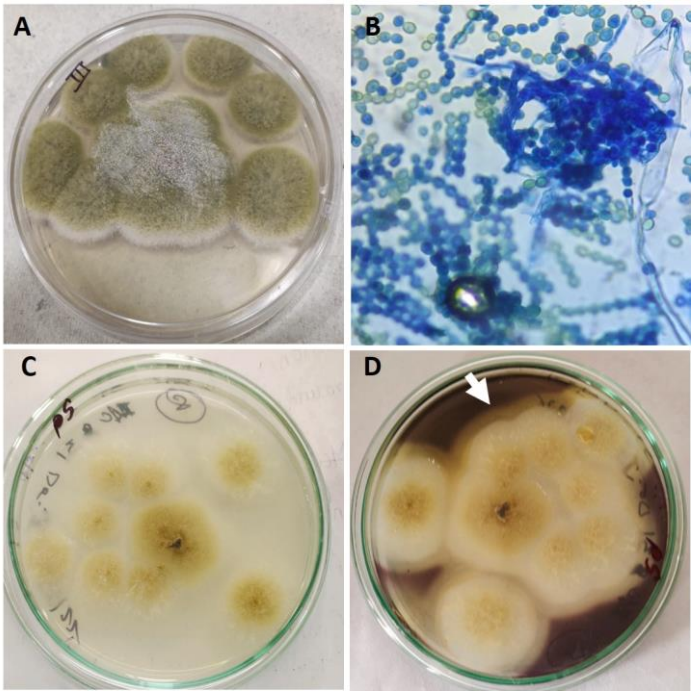

Figure 1 [A] Plate morphology of *A. oryzae* MTCC 3107 after 7 days of growth on PDA. [B] Microscopic features of the Fungal strain at 40 X**.** [C] Growth of fungal strain on Starch agar before adding KI-I_2 solution. [D] Hydrolysis of starch is depicted by a clear area [arrow marked].

Amylase plate assay

To assess the potential of *A. oryzae* MTCC 3107 for amylase production, the fungal strain was inoculated on a starch agar plate for starch hydrolysis. Initially, fungus was inoculated on the starch agar plate and the plates were incubated at 28ºC for 3-5 days. The amylase activity was checked by adding KI-iodine solution to observe halo zones. The clear zones due to breakdown of starch by the action of fungal amylases can be clearly observed **(Fig. 1C and D)**.

Characterization of AgNPs

UV-VIS Spectrophotometer

Cell-free filtrates exhibit colour change peculiar for AgNPs. The colour changes from yellow to dark brown with time. The characteristic golden-brown coloration for AgNPs was observed on the fifth day of incubation in CFF from APM **(Fig. 2A iii)** and further it is confirmed with UV-Vis spectroscopy **(Fig. 2B)**. The colour was light yellow in CFF-PDB, this may be due to low number of enzymes and proteins in the cell free filtrate **(Fig. 2A ii)**. Nano silver synthesised using these CFF-APM exhibits a strong band at 420 nm. The monodisperse nanoparticles always have sharp peaks in UV-Vis spectra when compared to polydisperse nanoparticles. Also, red or blue shifts in spectra can give an approximate idea about the size range of particles in nanoscale. The sharp, slightly narrow and specific band at 420 nm **(Fig. 2B)** for AgNPs from CFF-AgNPs indicates that amylases in CFF play a significant role in the synthesis and capping process of biogenic AgNPs. Proteins like amylases have free sulfhydryl group in cysteine which catalyze bio reduction, synthesis and capping process **(Mishra and Sardar, 2012)**. The possible reason for a change in color from yellow to brown is the phenomenon of surface plasmon resonance [SPR] where vibrations of photons at 260 nm are in resonance with the oscillations of electrons of AgNPs **(Ssekatawa *et al.*, 2021).** The electronic oscillation in AgNPs are in resonance with the photon at 430 nm which results in the characteristics brown color of AgNPs with peak at 430 nm **(Zhang *et al*., 2016).**

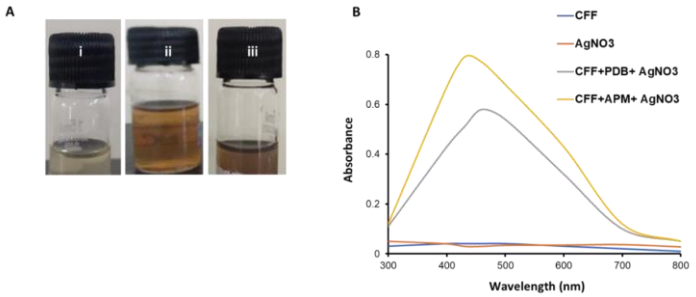

Figure 2 AgNPs synthesis. [A] Change in color in cell free filtrate harvested [i] without $AgNO_3$, [ii] using biomass grown in potato dextrose broth media, and [iii] using biomass grown in amylase production media. [B] UV–Vis spectra of AgNPs synthesized with CFF of *A. oryzae* MTCC 3107.

TEM analysis

Transmission electron microscopy studies revealed that AgNPs obtained from CFF of PDB **(Fig. 3A)** and APM **(Fig. 3B)** are 40 nm in size and spherical in shape. The difference in polydispersity was noted in both types of AgNPs due to variability in culture media and nature of enzymes secreted **(Johnston *et al*., 2018)**.

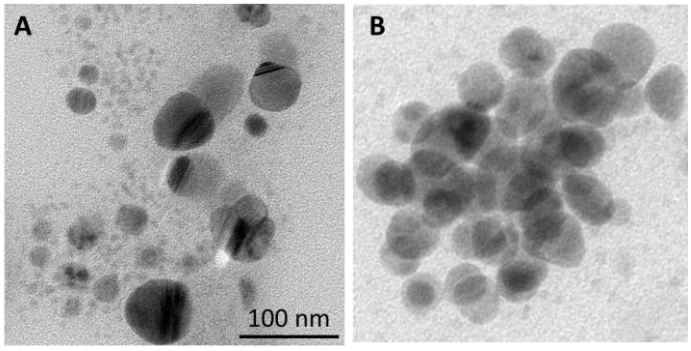

Figure 3 TEM micrograph of AgNPs synthesized in [A] PDB and APM [B] using CFF of *A. oryzae* MTCC 3107.

FTIR analysis

AgNPs synthesized using APM were more uniform as depicted using UV-Vis spectrophotometry and TEM analysis. Hence, further FTIR analysis was used for the characterization of the synthesized AgNPs in APM. The analysis revealed that the biological reduction and capping of Ag^+ ions to silver nanoparticles are due to the biomolecules mainly proteins of CFF. Peaks in **Fig. 4** are observed at 2923, 2853, 1741, 1634, 1506, 1455, 1158, 720, 600, and in the region of 450–400 cm^{-1}. The peaks at 2923 and 2853 cm^{-1} corresponds to C-H stretch. The signal at 1740 cm^{-1} is assigned to aldehyde group, whereas, the peaks at 1634 and 1506 cm^{-1} corresponds to primary and secondary amine groups respectively. Furthermore, peak at 1158 cm^{-1} represents the stretching vibrations of C-N bond and implies for functional aliphatic amines groups. The above data implies the role of certain proteins present in APM are involved in the capping and stabilization of AgNPs **(Saxena *et al*., 2016)**.

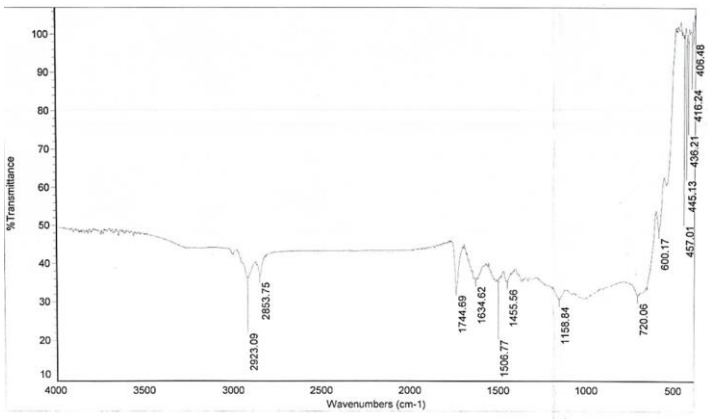

Figure 4 FTIR spectra of synthesized AgNPs

XRD analysis

The XRD pattern of AgNPs obtained using APM has been depicted **(Fig. 5)**. The diffraction pattern consisting of peaks at 28.257°, 30.280°, 33.139°, and 39.007° can be attributed to the face-centred cubic structure. The average crystal size obtained was 29 nm, which is smaller than those obtained from TEM. The size difference observed in TEM and XRD is mainly due to the limitation of use of Debye-Scherrer formula, which is only applicable to near-spherical shape particles **(Dhoondia and Chakraborty, 2012).** Our data are in agreement with the previous observation where AgNPs were synthesized and characterized using XRD **(Sallehudin *et al*., 2018; Bhatt *et al*., 2018)**.

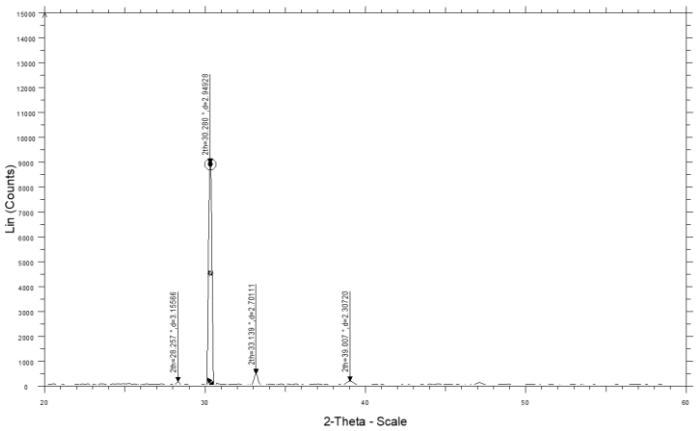

Figure 5. XRD patterns of AgNPs

The antifungal assay

Antifungal activity of AgNP was estimated by recording inhibition in radial growth of target pathogen *S. sclerotiorum* MTCC 8785. Plates revealed reduction in fungal growth in a concentration-dependent manner when exposed to 25, 50, and 100 µg/ml **(Fig. 6A-C)** of biogenic nano silver. Complete growth inhibition was observed with respect to control at 100 µg/ml concentration of AgNP **(Fig. 6C)**. Plates without antifungal or AgNPs have been used as negative control and showed full growth **(Fig. 6E)**, whereas complete inhibition in growth was observed in the presence of antifungal Fluconazole 100 µg/ml **(Fig. 6D) (Table 1)**. AgNPs inhibit fungal growth by blocking molecular and biochemical pathways as well as rupture of the cell wall **(Saxena *et al*., 2017b)**.

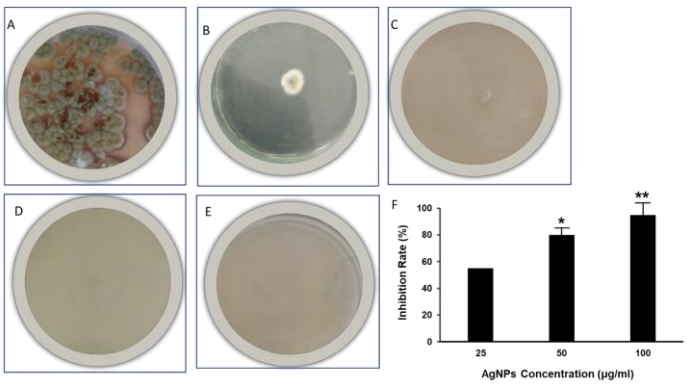

Figure 6 – Antifungal activity of AgNPs synthesised with enzyme stimulation from *A. oryzae* 3107. Inhibitory action of AgNPs at [B] 25 µg/ml, [C] 50 µg/ml, and [D] 100 µg/ml against plant pathogenic fungi *S. sclerotiorum* MTCC 8785. Fungal growth in the [E] presence of Fluconazole and [A] absence of AgNPs

CONCLUSION

The work highlights the significance of mycogenic AgNPs through green chemistry and can be employed as new nano weapons against pathogenic fungi *S. sclerotiorum* MTCC 8785. Here, we have taken *A. oryzae* MTCC 3107 as a source for extracellular enzyme amylases which are secreted upon cultivation of the same fungi in APM. Enzyme stimulation has resulted in improved synthesis of AgNPs from CFF and has shown an antifungal effect. Fungi is the diverse group of microbes thus can be exploited for such eco-friendly processes for metal nanoparticle synthesis with better characteristics. Attention is further needed to design research for process optimization of the variables involved during synthesis and capping.

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Conflict of Interest: Authors declare no conflict of interest.

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