Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454

Vol. 22 | No. 1 | pp. 253-259 | 2023

Original Research Paper

https://doi.org/10.46488/NEPT.2023.v22i01.024

Open Access Journal

Bioinspired Trichogenic Silver Nanoparticles and Their Antifungal Activity Against Plant Pathogenic Fungi *Sclerotinia sclerotiorum* MTCC 8785

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 10-06-2022
Revised: 04-08-2022
Accepted: 10-08-2022

Key Words:
Trichoderma
Enzyme stimulation
AgNPs
Antifungal
Sclerotinia

ABSTRACT

There is a pressing need for new nanomaterials for multipurpose functions. The biological synthesis of nanoparticles is environment-friendly, least toxic, and cost-effective. An experiment was designed to use extracellular amylases in the cell-free filtrate (CFF) for the biosynthesis of silver nanoparticles (AgNPs) from the *Trichoderma harzianum* MTCC 801 strain. Potato dextrose broth (PDB) as general-purpose growth media and amylase production media (APM) as enzyme-specific production media have been used for sub-merged fungal cultivation and nanoparticle synthesis. AgNPs synthesized in the CFF of PDB were compared with AgNPs synthesized from the CFF using APM. The cell-free filtrate obtained upon enzyme stimulation has contributed to the reduction and capping process of nanosilver. The synthesized AgNPs showed a spectral peak at 420 nm, a characteristic feature of AgNPs. The particles were monodispersed, 50 nm in size, and spherical in shape as well as have shown an antifungal effect (100% inhibition) against *Sclerotinia sclerotiorum* MTCC8785. This is the first report to synthesize trichogenic AgNPs using extracellular amylases against the phytopathogen *Sclerotinia* strain.

INTRODUCTION

The field of nanobiotechnology has gained massive attraction over the last few decades, curating the applications of nanomaterials into diverse fields of daily importance. Nanomaterials fall into the size range of 1-100 nm and exhibit remarkable surface, physical, chemical, and biological properties (Ahamed et al. 2010). There is a lot of attraction among researchers for biogenic AgNPs as it is a new-age nano alternative and is employed as a modern tool in agriculture, medicine, and industry. The biological route is the bottom-up approach with preferences over physical and chemical routes for nanoparticle synthesis. This approach offers advantages like less waste generation, low energy consumption, and simple and cost-effective steps (Vigneshwaran et al. 2007). All microbes and herbal extracts can be utilized for such type of blend as they served as a source for bio reductants and bio stabilizers (Siddiqi et al. 2018).

Fungi are easy to handle and culture in laboratories. Moreover, they are the excellent secretor of extracellular enzymes, metabolites, and other proteins. Due to these potent reasons, several fungi have prospected for the synthesis of metal nanoparticles (Duhan & Gahlawat 2014). Fungal enzymes and proteins are involved in the reducing and capping process of metal ions into a metal zero-oxidation state. Fungal proteins provide a better range to control the shape, size, and stability of nanoparticles (Saxena et al. 2017).

Enzymes can serve as a set of structurally assorted tools in bionanotechnology and play crucial roles in the self and dynamic assembly of nanostructures. The enzymes are available commercially, however, the pure form is very costly. Extraction of microbial enzymes offers cost-effective procedures and ease of handling. Microbes like bacteria and fungi have enzymes with specific amino acids, and chemical groups catalyzing biochemical and biological processes. The putative mechanism for nanoparticle synthesis involves the interaction of reducing thiol groups from cysteine with silver ions to form Ag-S bonds and complete bioreduction (Mishra & Sardar 2010, Thapa et al. 2017).

Phytopathogens are emerging threats to commercially important crops and harm the economy of developing countries the most. Over the last decade, *T. harzianum*, which is a mycoparasitic fungus has been used as a strong biocontrol agent against many plants' pathogenic fungi. *Trichoderma* strains release several hydrolytic enzymes upon the growth of fungal hyphae and degrade the cell wall of pathogenic fungi. The enzymes are amylases, Proteases, and chitinases which are also associated with the

mycoparasitic nature of Trichoderma strains. The promising role of *T. harzianum* as a biocontrol agent along with its simple handling opens avenues for its extensive use in the field of bionanotechnology to develop value-added products. Fungi that belong to the genus *Trichoderma* sp also secrete nitrate reductases, which are equipped for the synthesis and stability of nanoparticle cores (Guilger-Casagrande et al. 2019).

S. sclerotiorum is a very harmful plant pathogenic fungi that cause sclerotium disease on crop plants resulting in tremendous global economic losses. *S. sclerotiorum* exhibit a wide host range covering 278 genera in 75 families of plants. The most common disease is Sclerotinia stem rot (SSR) or white mold along with crown rot, drop, cottony rot, and blossom blight (Carpenter et al. 2021). Multicellular melanized sclerotia are the hardest structure known from these fungi that can live for many years in heterogeneous conditions. The pathogenesis initiates from the ascospores after the germination of these hardened black structures. The emergence of high-level resistance in sclerotinia strains against benzimidazole and carbendazim is alarming and has been difficult to control so it is imperative to find new alternatives against this pathogen to check for any sudden outbreak. Applications of fungicides could be possible solutions but may cause severe environmental hazards. Researchers have reported the effect of AgNPs from the Penicillium strain against *S. sclerotiorum* however there is a pressing need to explore more and develop a new breed of stable AgNPs with better bioefficacy (Saxena et al. 2017, Tomah et al. 2020).

MATERIALS AND METHODS

Fungal Growth Conditions

T. harzianum MTCC 801 and *S. sclerotiorum* MTCC 8785 were acquired from Microbial Type Culture Collection (MTCC), Chandigarh, India. Both the fungal strains were grown on potato dextrose agar (PDA) at 28ºC for 96 h and routinely maintained on PDA slants.

Extracellular Synthesis of Biogenic AgNPs

The mycelium of *T. harzianum* MTCC 801 was inoculated in PDB as well as APM (containing 1 % soluble starch as inducer) and kept at 28^{o}C for 120 h. After incubation, mycelia were collected by washing with autoclaved distilled water to get rid of media components and transferred to autoclaved distilled water followed by incubation for 48 hrs at 28°C. The cell-free filtrate procured after filtration of mycelia was exposed to 1 mM silver nitrate ($AgNO_3$) and incubated at room temperature in the dark for 3-5 days. A CFF without $AgNO_3$ was used as a negative control (Guilger-Casagrande et al. 2019).

Extracellular Amylase Production Using *T. harzianum* MTCC 801

T. harzianum MTCC 801 was cultured on PDA containing starch 1% (w/v) as a sole source of carbon. After incubation for three days, the plates were exposed to Iodine-KI solution and the clear zone of hydrolysis surrounding the colony was observed (Molla et al. 2022).

Characterization of AgNPs

Morphological Valuation

The color change of the incubated solution was recorded which further suggested the synthesis of AgNPs.

UV-Visible Spectroscopy Analysis

The synthesized AgNPs using PDB and amylase production media were scanned in scales between 300-800 nm at 0.1 nm resolution using a UV-Vis spectrophotometer.

Transmission Electron Microscope (TEM) Analysis

To assess the proportions of synthesized AgNPs using PDB and APM, TEM analysis was done. A small amount of AgNPs solution was loaded on copper grids in TEM and images were captured followed by the analysis of AgNPs (Alves & Murray 2022).

Purification of AgNPs

AgNPs from the solution were separated during centrifugation at 9000-10,000 for 5 min at 4^{o}C. Dark brown pellets obtained were splashed with distilled water, dried, and used for further experimental assays.

Antifungal Assay

To demonstrate the antifungal activity of synthesized AgNPs using PDB and amylase production media, PDA plates were prepared using different concentrations of AgNPs (25, 50, and 100 $\mu g.mL^{-1}$). Point inoculation of *S. sclerotiorum* MTCC 8785 was done onto each plate and incubated at 28^{o}C for 7 days. The growth of fungal mycelium over the plate was recorded and compared with the control. Plate prepared using antifungal fluconazole (100 $\mu g.mL^{-1}$) and inoculated with 10^5 spores of *S. sclerotiorum* MTCC 8785 was used as a positive control (Essghaier et al. 2022).

RESULTS AND DISCUSSION

Morphological and Microscopic Characterization of *T. harzianum* MTCC 801

In this present investigation, fungal strains were routinely sub-cultured and characterized morphologically (Fig 1A). Colonies of *T. harzianum* MTCC 801 were fast-growing, initially white and gradually developing yellowish-green

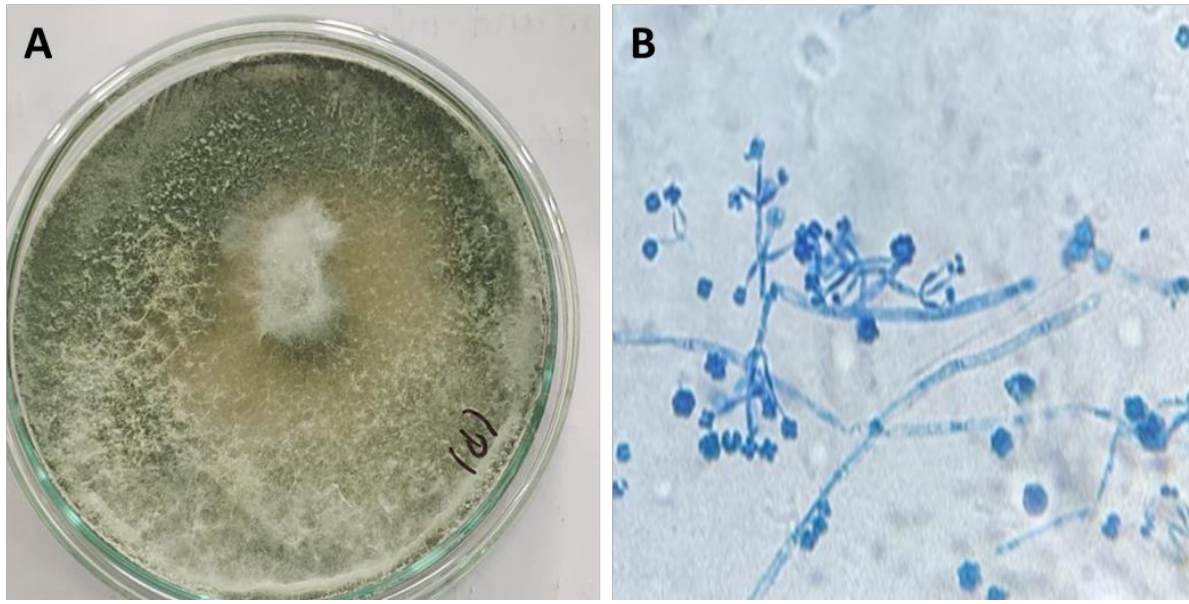

Fig. 1: Fungal characterization. (A) Plate morphology of *T. harzianum* MTCC 801 after 7 days of growth on PDA. (B) Microscopic features of the fungal strain at 40 X.

to deep green tufts, in small zones or with concentric rings on the media surface. Microscopically the fungi exhibit conidiophores which are irregularly branched and bear flask-shaped phialides. Conidia are green and born on conidial tips clustered together. (Fig. 1B).

Synthesis of AgNPs

At the first step for synthesis of Trichogenic AgNP, the fungal (1X105 spores.mL^{-1}) strain was inoculated and cultivated separately in 100 mL PDB and Amylase production media for 3-5 days at 28^{o}C, 120 rpm under submerged batch cultivation. The growth of fungi can be observed as a round ball of biomass which is characteristic of submerged cultivation (Fig. 2A and 2C). Through filtration,

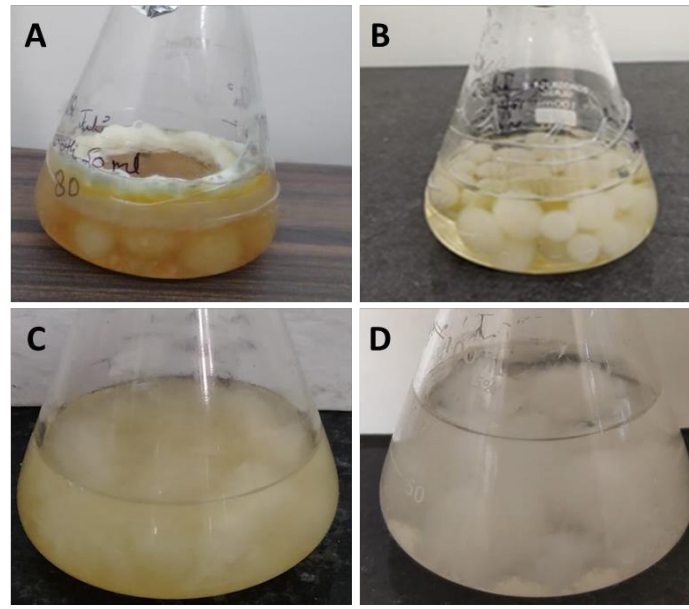

Fig. 2: (A) Subculture of *T. harzianum* MTCC 801 using PDB at 28^{o}C, (B) Fungal biomass in deionized distilled water for harvesting extracellular proteins, (C) Subculture of fungal strain using amylase production media (with starch as inducer) at 28^{o}C, (D) Fungal biomass in deionized distilled water for harvesting extracellular proteins (amylases).

media components were then removed, and the biomass (10 gm, fresh wet weight) after washing was transferred to 50 mL distilled water, which was kept under the same conditions for three days (Fig. 2B and 2D). In cell-free filtrate, fully, grown biomass secretes extracellular enzymes by reverse osmosis, which are agents for bioreduction and capping during the synthesis process. Amylase production media (APM) was used because amylase-mediated bioreduction is well documented (Mishra & Sardar 2010). Moreover, starch is used as an inducer in APM, which stimulates the strain to secrete more extracellular amylase under controlled conditions. In this paper, we have for the first time utilized extracellular amylases from the *Trichoderma* strain for nanoparticle synthesis. Moreover, microbes secrete nitrate reductase enzymes in the extracellular milieu which reduces the Ag^+ to AgNPs (Das et al. 2014). Further, Guilger-Casagrande et al. (2019) reported that *Trichoderma harzianum* utilizes nitrate reductase for the reduction of Ag^+ to AgNPs (Guilger-Casagrande et al. 2019).

Amylase Assay

Colonies of *T. harzianum* MTCC 801 were assessed for amylase production using starch hydrolysis assay. The fungus grown with inducer starch agar media is capable of growing by producing extracellular amylases. The amylolytic activity was monitored by the qualitative KI-iodine halo zone assay. *T. harzianum* MTCC 801 showed a clear zone of hydrolysis (Fig. 3).

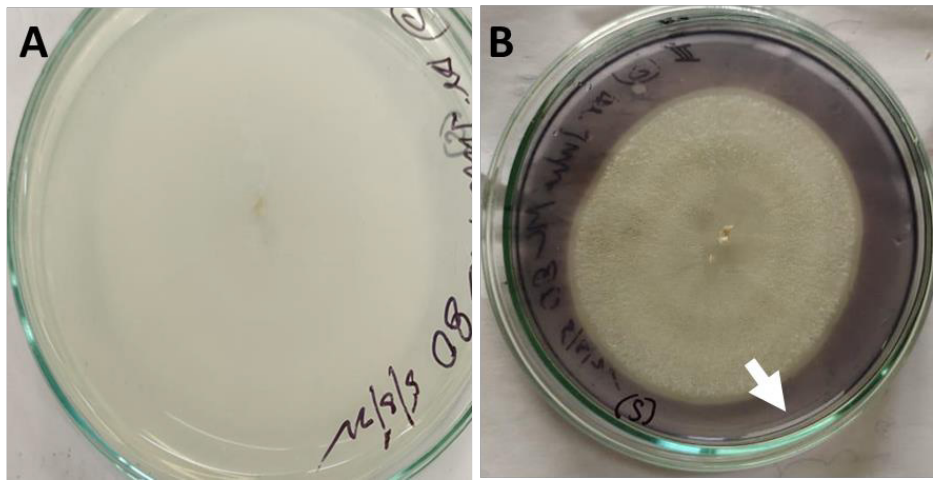

Fig. 3: Primary (Starch Hydrolysis Plate assay) screening of *T. harzianum* MTCC 801 for extracellular amylase production. (A) Growth of fungal strain on Starch agar before adding KI-I_2 solution. (B) Clear areas (marked with red arrow) show positive detection of fungal strain for starch hydrolysis as shown by an arrow.

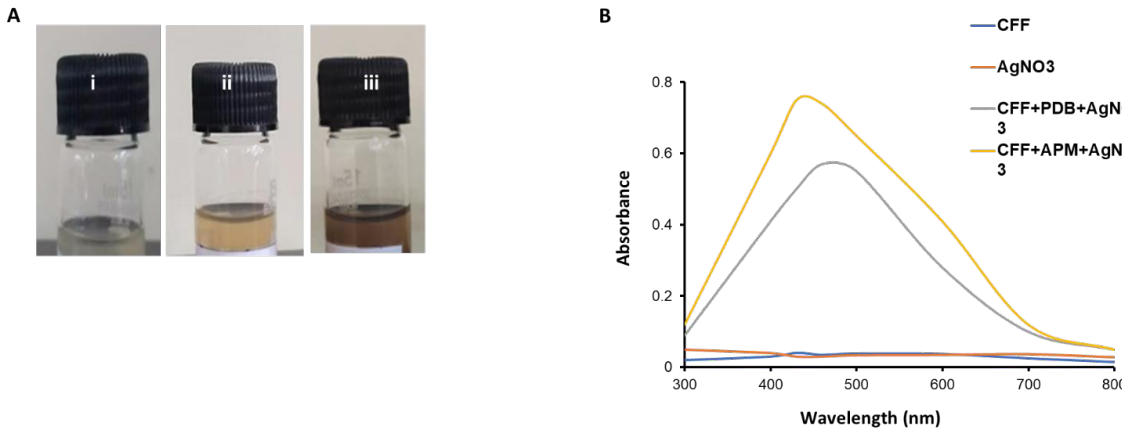

Fig. 4: AgNPs synthesis. (A) Change in color in cell-free filtrate harvested (i) without $AgNO_3$, (ii) using biomass grown in PDB, and (iii) using biomass grown in APM. (B) UV–Vis spectral peak of AgNPs.

Characterization of AgNPs

UV-VIS Spectrophotometer: Fungal biomass in CFF was removed by filtration and challenged by metal stress with $AgNO_3$ at 1 mM concentration under dark conditions. The color changed gradually to reddish-brown from yellow in CFF indicating the synthesis of AgNPs (Fig. 4A). The sharp color change was observed on the fifth day of exposure to metal ions with reductants under dark conditions at room temperature. However, the color change was dark brown in CFF-APM as compared to CFF-PDB which was further confirmed with a UV-Vis spectra scan (Fig. 4B). The color changes differently in both the cell filtrates because of the difference in enzymes present in CFF (Pandey et al. 2018). Also, the silver metal ion upon reduction in presence of these enzymes shows characteristic surface plasmon resonance (Fig. 4B). The synthesis was confirmed and compared initially by UV-Vis spectroscopy, indicating the peculiar peaks for nanosilver exhibit intense absorption at 422 nm. The oscillation waves of electrons of AgNPs are in resonance with the light waves, giving rise to a unique surface plasmon resonance (SPR) absorption band (400-440 nm), which is also the origin of the observed color (Zhang et al. 2016). The sharp peak in CFF-APM indicates monodispersed particles and a size scale in nanometers. The band in CFF-APM was intense when compared to CFF-PDB, clearly correlating that the intense color change in CFF-APM was due to amylases. Fungal amylase has -SH groups that bind, reduce and stabilize AgNPs (Khan et al. 2013). Our data is in agreement with Saxena et al. (2016), who synthesized AgNPs using cell-free filtrate of *S. sclerotiorum* MTCC 8785 grown in PDB media.

Table 1: Antifungal activity of AgNPs.

Treatment	Inhibition rate (%)
AgNPs [25 µg.mL^{-1}]	45
AgNPs [50 µg.mL^{-1}]	75
AgNPs [100 µg.mL^{-1}]	90
Flucanazole [100 µg.mL^{-1}]	100

TEM analysis: TEM analysis revealed that particles synthesized using PDB (Fig. 5A) and APM (Fig. 5B) are spherical at 50 nm in size. APM-mediated synthesized AgNPs were monodispersed in nature whereas PDB mediated are polydisperse. This could be because of the difference in enzymes present in CFF (Pandey et al. 2018).

Antifungal Assay

The antifungal activity was evaluated using radial growth inhibition assay in the presence and absence of different concentrations of AgNPs (25, 50, and 100 µg.mL^{-1}) on the growth of *S. sclerotiorum* MTCC 8785. Inhibition in the fungal growth was observed in a concentration-dependent manner with the maximum at 100 µg.mL^{-1} concentration of AgNPs (Fig. 6A-D; Table 1). Plates devoid of AgNPs showed fully grown fungus (Fig. 6E). Fluconazole (100 µg.mL^{-1}) was used as a positive, hence no growth was observed for fungus in the presence of Fluconazole (100 µg.mL^{-1}) (Fig. 6F). AgNPs are known to disintegrate the membrane-bound respiratory enzymes, hence retards the growth of fungi (Saxena et al. 2014).

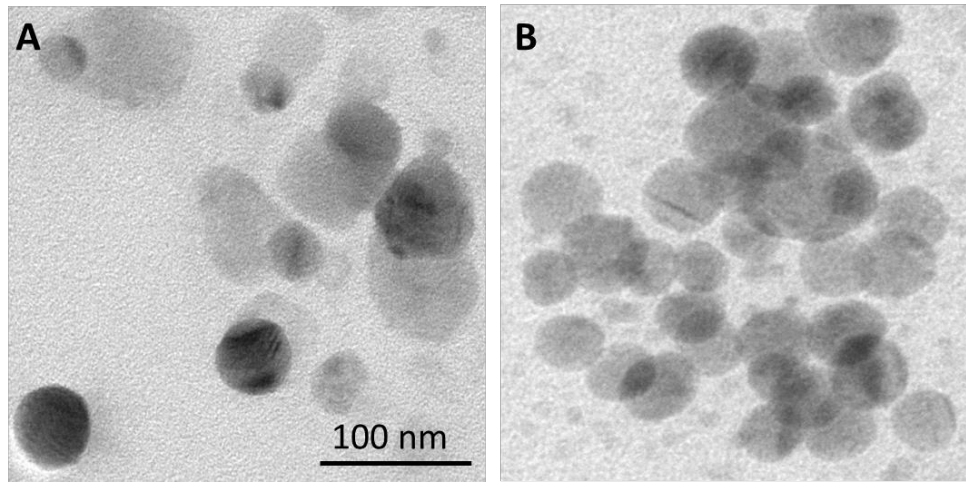

Fig. 5: TEM micrograph of AgNPs synthesized in (A) PDB and APM (B) using CFF of *T. harzianum* MTCC 801.

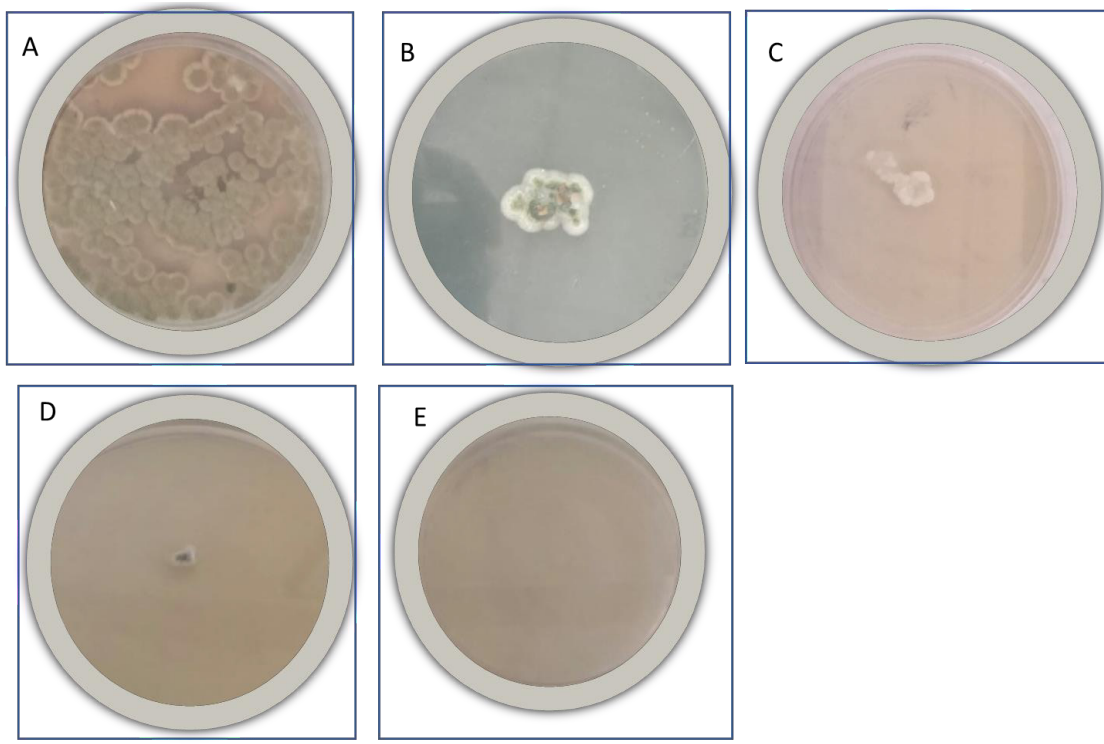

Fig. 6: Antifungal activity of AgNPs synthesized with enzyme stimulation from *T. harzianum* MTCC 801. Inhibition effect of AgNPs at (B) 25 µg.mL^{-1}, (C) 50 µg.mL^{-1}, and (D) 100 µg.mL^{-1}against phytopathogenic fungi *S. sclerotiorum* MTCC 8785. Fungal growth in the (E) presence of Fluconazole and (A) absence of AgNPs.

CONCLUSION

This work establishes a simple and bio-friendly procedure to synthesize AgNPs from cell-free filtrates of *T. harzianum* MTCC 801. We have used amylase-mediated AgNP synthesis which is beneficial for nanoparticle stability and bioefficacy. The secreted amylases in CFF-APM are directly utilized for synthesis instead of using amylases purified through cumbersome steps of downstream processing. The process offers a one-step method for producing stable nanoparticles. This bioinspired enzyme-mediated synthesis can be further explored for optimization models and industrial-scale production of AgNPs as new antifungal agents.

ACKNOWLEDGEMENT

We are indebted to Chandigarh University for supporting and providing the necessary infrastructure for the completion of this work.

REFERENCES

Ahamed, M., AlSalhi, M.S. and Siddiqui, M.K.J. 2010. Silver nanoparticle applications and human health. Clin. Chim. Acta, 411(23-24): 1841-1848.

Alves, M.F. and Murray, P.G. 2022. Biological synthesis of monodisperse uniform-size silver nanoparticles (AgNPs) by fungal cell-free extracts at elevated temperature and pH. J. Fungi, 8(5): 439.

Carpenter, K.A., Sisson, A.J., Kandel, Y.R., Ortiz, V., Chilvers, M.I., Smith, D.L. and Mueller, D.S. 2021. Effects of mowing, seeding rate, and foliar fungicide on soybean sclerotinia stem rot and yield. Plant Health Prog., 22(2): 129-135.

Das, V.L., Thomas, R., Varghese, R.T., Soniya, E.V., Mathew, J. and Radhakrishnan, E.K. 2014. Extracellular synthesis of silver nanoparticles by the Bacillus strain CS 11 isolated from industrialized area. Biotech., 4(2): 121-126.

Duhan, J.S. and Gahlawat, S.K. 2014. Biogenesis of nanoparticles: A review. Afr. J. Biotechnol., 13(28): 616.

Essghaier, B., Smiri, M., Sehimi, H., Ben Jalloul, A., Zid, M.F. and Sadfi Zouaoui, N. 2022. Antifungal potential of two synthetic vanadium (IV) oxalate compounds to control blue mold of oranges and apples under storage conditions. J. Food Process Preserv., 111: e16860.

Guilger-Casagrande, M., Germano-Costa, T., Pasquoto-Stigliani, T., Fraceto, L.F. and Lima, R.D. 2019. Biosynthesis of silver nanoparticles employing Trichoderma harzianum with enzymatic stimulation for the control of Sclerotinia sclerotiorum. Sci. Rep., 9(1): 1-9.

Khan, Z., Singh, T., Hussain, J.I., Obaid, A.Y., Al-Thabaiti, S.A. and El-Mossalamy, E.H. 2013. Starch-directed green synthesis, characterization and morphology of silver nanoparticles. Coll. Surf. B, 102:578-584.

Mishra, A. and Sardar, M. 2012. Alpha-amylase mediated synthesis of silver nanoparticles. Sci. Adv. Mater., 4(1): 143-146.

Molla, A.H., Islam, M.J., Mosharaf, M.K., Sultana, S., Talukder, M. and Miah, M. 2022. Extracellular enzymes production and non-

phytopathogenic potential fungi isolation for the purpose of solid waste management of safe food production. Waste Biomass Valoriz., 13(1): 369-381.

Pandey, A., Shankar, S. and Arora, N.K. 2018. Amylase-assisted green synthesis of silver nanocubes for antibacterial applications. Bioinspired. Biomim. Nanobiomater., 8(3): 161-170.

Saxena, J., Sharma, M.M., Gupta, S. and Singh, A. 2014. Emerging role of fungi in nanoparticle synthesis and their applications. World J. Pharm. Sci., 3(9): 1586-1613.

Saxena, J., Sharma, P.K., Sharma, M.M. and Singh, A. 2016. Process optimization for green synthesis of silver nanoparticles by Sclerotinia sclerotiorum MTCC 8785 and evaluation of its antibacterial properties. Springer Plus, 5(1): 1-10.

Saxena, J., Sharma, P. and Singh, A. 2017. Biomimetic synthesis of AgNPs from Penicillium chrysogenum strain FGCC/BLS1 by optimising physico-cultural conditions and assessment of their antimicrobial potential. IET Nanobiotechnol., 11(5): 576-583.

Siddiqi, K.S., Husen, A. and Rao, R.A. 2018. A review on biosynthesis of silver nanoparticles and their biocidal properties. J. Nanobiotechnol., 16(1): 1-28.

Thapa, R., Bhagat, C., Shrestha, P., Awal, S. and Dudhagara, P. 2017. Enzyme-mediated formulation of stable elliptical silver nanoparticles tested against clinical pathogens and MDR bacteria and development of antimicrobial surgical thread. Anna. Clin. Microbiol., 16(1): 1-10.

Tomah, A.A., Alamer, I.S.A., Li, B. and Zhang, J.Z. 2020. Mycosynthesis of silver nanoparticles using screened trichoderma isolates and their antifungal activity against Sclerotinia sclerotiorum. Nanomaterials, 10(10):1955.

Vigneshwaran, N., Ashtaputre, N.M., Varadarajan, P.V., Nachane, R.P., Paralikar, K.M. and Balasubramanya, R.H. 2007. Biological synthesis of silver nanoparticles using the fungus Aspergillus flavus. Mater. Lett., 61(6): 1413-1418.

Zhang, X.F., Liu, Z.G., Shen, W. and Gurunathan, S. 2016. Silver nanoparticles: Synthesis, characterization, properties, applications, and therapeutic approaches. Int. J. Mol. Sci., 17(9): 1534.