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MYCOSYNTHESIS OF SILVER NANOPARTICLES: A STUDY ON OPTIMIZATION PARAMETERS

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ABSTRACT

Nanotechnology's broad scope was harnessed in developing management strategies against plant diseases especially using metal-based nanoparticles. Nano-silver has huge potential as antimicrobial agents. Mycosynthesis of AgNPs offers enhanced antimicrobial properties by the synergistic metabolites and also an ecofriendly approach. Multiple parameters play a crucial role the yield of synthesized AgNPs. Hence, this research was aimed for optimization of two key parameters for the mycosynthesis of AgNPs. Parameter-A was reaction mixture ratio of *T. asperellum* culture filtrate (TCF) to that of silver nitrate (1mM $AgNO_3$) with levels of 1:1 (A_1), 1:10 (A_2), 1:100 (A_3). Parameter-B was pH of the reaction mixture with levels of pH 8 (B_1), pH 9 (B_2) and pH 10 (B_3). The mean yields of AgNPs recorded in treatments were 12 mg in A_1B_1, 21 mg in A_1B_2, 29.33 mg in A_1B_3, 17.66 mg in A_2B_1, 28.33 mg in A_2B_2, 40.33 mg in A_2B_3, 26 mg in A_3B_1, 37 mg in A_3B_2 and 51.33 mg in A_3B_3. Based on the results, the best treatments recommended for higher yield of mycogenic AgNPs were 1: 100 mixture ratio and pH of 10 (A_3B_3) followed by 1: 10 mixture ratio and pH of 10 (A_2B_3) and 1: 100 mixture ratio and pH of 9 (A_3B_2). U.V. Visible spectroscopy was done for the best treatment *i.e.*, A_3B_3 and the results showed the absorption peak at 385 nm. This peak represents the small and stable mycogenic AgNPs synthesized. The higher volumes of substrate solution consist of more quantity of $AgNO_3$ molecules available for the metabolites to reduce and form more AgNPs. Alkaline pH of 10 provided optimal conditions for the interactions of metabolites and silver ions and increased the metabolites reactivity thereby leading to formation of small and more stable AgNPs. Notable other parameters, kept constant, were reaction mixture temperature of 80 ± 1°C, 1mM $AgNO_3$ substrate concentration, 1:10 suspension ratio of biomass to distilled water for metabolite production and 800 rpm of continuous stirring of reaction mixture.

Key words : $AgNO_3$, Mycosynthesis, Optimization, pH, Silver nanoparticles, *Trichoderma*.

Introduction

Nanotechnology has recently gained a lot of scientific attention due to its wide applications in variety of fields such as agriculture, science, engineering, and health etc. The nano-scale of the particles offer remarkable scientific innovations which were previously not possible such as drug delivery, nano-based sensors, nano-fertilizers, etc. Pioneers used different physical and chemical methods to synthesize nanoparticles, which were costly as well having environmental concerns. A huge leap in nanotechnology was taken by the innovation of green synthesis. Green synthesis was primarily utilisation of biological molecules as the reducing agents for the production of nanoparticles. Green synthesis was a fascinating alternative to other methods with advantageous of high yields, relative ease of production, less energy utilisation and low cost, environment friendly by less output of toxic waste and also additional synergistic effects of the reducing biomolecules (Hussain *et al.*, 2016). Mycosynthesis of AgNPs primarily depends on the use of fungal cell free extracts. Mycosynthesis involves high proteins with residue molecules gives more

biocompatibility and stability in comparison to bacteria and plants (Oyebamiji *et al*., 2025). The well-established biocontrol fungal genus *Trichoderma* was a rich source of antimicrobial metabolites that can be used for nanoparticles synthesis (Herrera Pérez *et al*., 2024). *T. asperellum* has ability to produce different volatile and non-volatile organic compounds against plant pathogens (Birari *et al*., 2025). The metal nanoparticles especially nano-silver has many distinct properties like antimicrobial nature, potential in bio-sensors, drug delivery etc. (Sati *et al*., 2025). *Trichoderma* metabolites when combined with the silver possess high antimicrobial activity due to the synergistic effects of the secondary metabolites of the fungus as well as the nano-silver. Silver in the nanoform offer large surface area by which the disease management becomes easy. Nano-scale of the silver also prevents the metal toxicity. These properties were being exploited against plant pathogens and sustainable plant disease management. The mycosynthesis of silver nanoparticles was challenging due to varied synthesis conditions. Bulk and high yields of mycogenic AgNPs can ease in evaluating and designing novel applications in different fields. Multiple parameters influence on the final yield of the synthesized AgNPs. To harness the full potential of nanoparticles, the key parameters need to be optimized for their bulk synthesis. Therefore, the present research was conducted to optimize the two key parameters for obtaining high yields of mycogenic AgNPs.

Materials and Methods

Procurement of pure culture of *Trichoderma asperellum*

Trichoderma asperellum culture previously identified and preserved at Dept. of Plant Pathology, Post Graduate Institute, Dr. PDKV, Akola was procured and utilised in the mycosynthesis of silver nanoparticles. The culture was maintained by sub-culturing on PDA at 28°C.

Production of biomass of *T. asperellum*

Petri dishes with active growing and pure culture plates of seven days old culture of *T. asperellum* were selected for broth inoculation and biomass production. 2-3 mycelial discs of 5 mm diameter was added to potato dextrose broth (PDB) and incubated at 28°C in the orbital shaker with 100 rpm for 10 days. The mycelial mat grown in PDB was used as biomass for the production of secondary metabolites and culture filtrate (Fig. 1A).

Production of culture filtrate of *T. asperellum*

The 10 days old mycelial mat of *T. asperellum* was then harvested by discarding the broth. The mycelial mat was left in the conical flask after draining out the broth. The mycelial mat was washed twice with sterile distilled water and left for starvation for 24 hrs so as to activate the secondary metabolite production by the fungal mat (Fig. 1B). 10 g of biomass was weighed and transferred into a conical flask with 100 ml of sterile distilled water (1: 10 suspension ratio) and incubated at 28°C, for 10 days in the orbital shaker with 150 rpm to attain the maximum yield of aqueous metabolites. Filtration of the culture filtrates rich in secondary metabolites was done to remove the mycelial mat and spore debris using Whatman No-1 filter paper. The filtration step was done twice. The cultural filtrates were transferred to 50 ml centrifuge tubes. Centrifugation was done at 7000 rpm for 10 min at 4°C to remove the spore or cell masses. After centrifugation, the cells/spore mass debris was precipitated at the bottom of centrifuge tubes. The aqueous supernatant of metabolites was carefully transferred to fresh tubes leaving debris at bottom. The centrifugation step was repeated once to ensure complete removal of cells/spore masses. The pure aqueous metabolites were obtained and used further in mycosynthesis of silver nanoparticles.

Synthesis of silver nanoparticles

Mycosynthesis of silver nanoparticles was done according to Gowda and Sriram, 2023 with some modifications. The Silver nitrate ($AgNO_3$) (SRL, Pvt. Ltd.) was extrapure, 99.5% and molecular weight of 169.87. $AgNO_3$ was dissolved instantly upon mixing in distilled water. Preparations were made to obtained required molarity of the stock and working standard solutions of $AgNO_3$ (Table 1). The culture filtrate was used as a reducing agent for 1mM $AgNO_3$ substrate and form AgNPs.

Reaction mixture ratios

The ratios of 1:1 (A1), 1:10 (A2) and 1: 100 (A3) was applied for addition of *T. asperellum* cultural filtrate (TCF) to 1mM $AgNO_3$. In 1: 1 ratio, equal volumes viz., 50 ml of TCF and 50 ml of 1mM $AgNO_3$. In 1: 10 ratio, 10 ml of TCF and 90 ml of 1mM $AgNO_3$. In 1: 100 ratio, 1 ml of TCF and 100 ml of 1mM $AgNO_3$. The temperature was slowly raised to 80 ± 1°C meanwhile

Table 1 : Preparation of stock and working solutions of $AgNO_3$.

	$AgNO_3$	Distilled water	Concentration
Stock solution	169.87 g	1000 ml	1 Normal
	10 g	~59 ml	1 Normal (1N = 1000 mM)
Working solution	1 ml of 1N $AgNO_3$	999 ml	1 mM

drop by drop of cultural filtrate was added into the substrate solution under constant stirring at 800 rpm placed on the hot plate magnetic stirrer for a period of 20 min.

pH of the reaction mixture

The pH of the solution was adjusted to 8 (B1), 9 (B2), 10 (B3). 16 g of NaOH pellets were dissolved in distilled water and volume was made up to 1 litre and a final concentration of 0.4 M NaOH was prepared. Drops of 0.4 M, NaOH were added to the reaction mixture and increase in pH to desired level was simultaneous recorded with pH meter.

Colour change reaction

After setting of the pH, the reaction was allowed for 30 min for complete synthesis of AgNPs. The colour change reaction to brown was recorded indicating the synthesis and formation of mycogenic AgNPs. The reaction mixture was then allowed to cool down and then centrifugation was done at 7000 rpm for 15 min at 4°C. The supernatant was discarded and precipitate of AgNPs was obtained at the bottom of centrifugation tubes. The precipitate was washed with distilled water by centrifugation. The wet aggregate of AgNPs was collected using spatula and placed in the bottom lid of petri dishes and placed in hot air oven for drying at 60°C for 24 hrs. The dried aggregates of AgNPs were collected into mortar and crushed using pestle into fine grounded powder. The yields of AgNPs were recorded by weighing the fine powder on butter paper in precision analytical weighing balance and collected into 5 ml glass vials for storage and further use. The best treatment was subjected to U.V. visible spectroscopy using U.V. Visible double beam spectrophotometer.

Statistical analysis

The data was analysed using two factorial completely randomized design (2-factor CRD) with factor-1 (A) as reaction mixture ratios (TCF: $AgNO_3$) and factor-2 (B) as pH. and the statistical analysis using two-way analysis of variance (2-way ANOVA) and the significance of treatment means was compared at 1% level of significance ($p < 0.01$). The statistical analysis was conducted in online statistical analysis tool (OPSTAT-http://opstat.somee.com/opstat/) (Sheoran *et al.*, 1998).

Results and Discussion

The initial colours of the 1mM $AgNO_3$ was pure white or transparent and aqueous secondary metabolites was pale green colour. The synthesis of mycogenic AgNPs was started upon immediately in the reaction mixture immediately upon addition of aqueous metabolites into 1mM $AgNO_3$ solution and confirmed with change of the

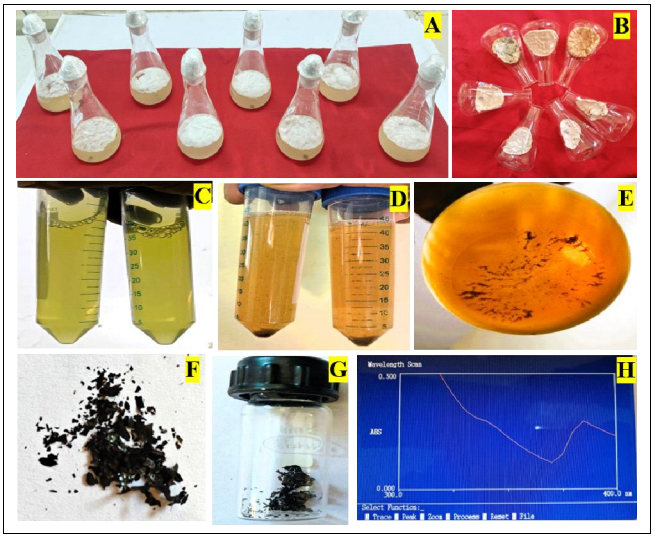

Fig. 1 : Mycosynthesis of silver nanoparticles; A- Mass culturing of *T. asperellum*, B- Mycelial mats used for metabolites secretion, C- Aqueous metabolites, D- Colour change reaction and formation of AgNPs, E- Aggregates of AgNPs formed, F- AgNPs powder, G- Storage of AgNPs in vials, H- U.V. Visible spectroscopy characterization.

colour of the solution from pale green to brown colour (Fig. 1C, 1D). Minute black aggregates of silver nanoparticles were visible in the reaction mixture indicating the start of the synthesis reaction. As the reaction progressed, the quantity of the black aggregates was increased (Fig. 1E). The reaction mixture was allowed to cool down and centrifugation was done leaving the AgNPs precipitate in the bottom. The synthesized silver nanoparticles were visible to naked eye as black shiny powder (Fig. 1F). The synthesized AgNPs powder was collected and stored in glass vials (Fig. 1G).

This colour change reactions were recorded by several previous researchers as light yellow to dark brown (Cui *et al.*, 2022); yellow to brown (Ogunleye *et al.*, 2022; Heikal *et al.*, 2024); light yellow to dark brown (Thepbandit *et al.*, 2024; Li *et al.*, 2025). The synthesis was confirmed by the formation of a black precipitate settled overnight (Singh *et al.*, 2024).

The data of mean yields of two parameters and their interactions was presented in Table 2 and Fig. 2.

A_1B_1: The reaction mixture ratio of 1:1 (equal volumes) of TCF: $AgNO_3$ and pH of 8 has successfully yielded the AgNPs. The colour change was recorded from light green to light brown colour. The mean yield of the mycogenic AgNPs was 12 mg.

A_1B_2: The reaction mixture ratio of 1:1 (equal volumes) of TCF: $AgNO_3$ and pH of 9 has successfully yielded the AgNPs. The colour change was recorded

Table 2 : Yield of AgNPs obtained under combination of A×B parameters.

Reaction mixture ratio (TCF: $AgNO_3$) (A)	pH (B)			Mean of A (TCF: $AgNO_3$)
	pH – 8 (B_1)	pH – 9 (B_2)	pH – 10 (B_3)	
1: 1 (A_1)	12	21	29.33	20.77
1: 10 (A_2)	17.66	28.33	40.33	28.77
1: 100 (A_3)	26	37	51.33	38.11
Mean of B (pH)	18.55	28.77	40.33	-
-	TCF: $AgNO_3$ (A)	pH (B)	Interaction of A×B	-
SE ± m	0.654	0.654	1.133	-
C.D. (1%)	1.959	1.959	3.393	-

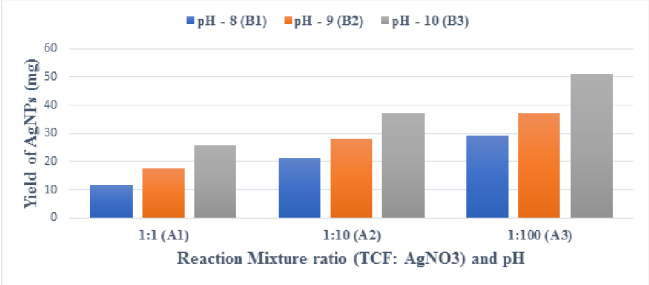

Fig. 2 : Yield of silver nanoparticles (AgNPs) by the interactions of mixture ratios and pH values.

from light green to light brown colour. The mean yield of the mycogenic AgNPs was 21 mg.

A_1B_3: The reaction mixture ratio of 1:1 (equal volumes) of TCF: $AgNO_3$ and pH of 10 has successfully yielded the AgNPs. The colour change was recorded from light green to light brown colour. The mean yield of the mycogenic AgNPs was 29.33 mg.

A_2B_1: The reaction mixture ratio of 1:10 of TCF: $AgNO_3$ and pH of 8 has successfully yielded the AgNPs. The colour change was recorded from light green to brown colour. The mean yield of the mycogenic AgNPs was 17.66 mg.

A_2B_2: The reaction mixture ratio of 1:10 of TCF: $AgNO_3$ and pH of 9 has successfully yielded the AgNPs. The colour change was recorded from light green to brown colour. The mean yield of the mycogenic AgNPs was 28.33 mg.

A_2B_3: The reaction mixture ratio of 1:10 of TCF: $AgNO_3$ and pH of 10 has successfully yielded the AgNPs. The colour change was recorded from light green to brown colour. The mean yield of the mycogenic AgNPs was 40.33 mg.

A_3B_1: The reaction mixture ratio of 1:100 of TCF: $AgNO_3$ and pH of 8 has successfully yielded the AgNPs. The colour change was recorded from light green to dark brown colour. The mean yield of the mycogenic AgNPs was 26 mg.

A_3B_2: The reaction mixture ratio of 1:100 of TCF: $AgNO_3$ and pH of 9 has successfully yielded the AgNPs. The colour change was recorded from light green to dark brown colour. The mean yield of the mycogenic AgNPs was 37 mg.

A_3B_3: The reaction mixture ratio of 1:100 of TCF: $AgNO_3$ and pH of 10 has successfully yielded the AgNPs. The colour change was recorded from light green to dark brown colour. The mean yield of the mycogenic AgNPs was 51.33 mg.

The means yields of parameter A and parameter B represented were on par with each other. The mean yields of A1 and B1 were 20.77 mg and 18.55 mg respectively. The mean yields of A2 and B2 were both 28.77 mg. The mean yields of A3 and B3 were 38.11 mg and 40.33 mg respectively. Therefore, based on the results, the best treatments giving higher yields of mycogenic AgNPs were 1: 100 mixture ratio and pH of 10 (A_3B_3) followed by 1: 10 mixture ratio and pH of 10 (A_2B_3) and 1: 100 mixture ratio and pH of 9 (A_3B_2).

Water, the universal solvent, was used to disperse the synthesized nanoparticles because of the properties like neutral pH and no specific organic compounds affecting the quality and properties of the nanoparticles. The aggregates were broken down for better uniform distribution in distilled water by ultrasonication using probe. Ultrasonication also provides enhanced stability. The best treatment A_3B_3 was subjected to U.V. Visible spectroscopy. The optical absorption spectral curve of the sample with dispersed AgNPs of A_3B_3 showed a single peak which indicated the particles shape as spherical shape and shorter wavelengths represented

small size of the mycogenic AgNPs by U.V. Visible double beam spectrophotometer. The surface plasmon resonance (SPR) absorbance was recorded and a peak was observed at 385 nm (Fig. 1H). This result indicates that the best treatment also had optimal properties like small size and spherical shape of AgNPs. Earlier researchers characterized the AgNPs using U.V. Visible spectroscopy and recorded SPR absorbance peaks at 415-420 nm (Chowdappa *et al.*, 2014), 400-410 nm (Gowda and Sriram, 2023), 400 nm (Singh *et al.*, 2024), 420 nm, 323 nm and 320 nm (Li *et al.*, 2025).

The critical observations made were higher pH (alkaline) values leads to the formation and synthesis of black coloured aggregates of silver nanoparticle. A pH is potential of the hydrogen as determined by the concentration of available H^+ ions and OH^- ions of the solution. The role of these ions makes it a critical factor for silver nanoparticle synthesis. When the pH of the mixture was adjusted to alkaline, the OH- ions concentration was more than H+ ions and which may form secondary and tertiary bonds with residual molecules forming a more stable metabolite-Ag complex. Therefore, increasing the stability and enhanced production of silver nanoparticles. The pH of the reaction mixture influences the size of the nanoparticles by the complex formation and also the rate of reduction. The higher volumes of substrate solution consist of more quantity of $AgNO_3$ molecules available for the metabolites to reduce and form more AgNPs. Alkaline pH of 10 provided optimal conditions for the interactions of metabolites and silver ions and increased the metabolites reactivity thereby leading to formation of small and more stable AgNPs. Alkaline pH of 9-11 were utilised and successfully in synthesis and formation of the silver nanoparticles (Islam *et al.*, 2024). While some studies utilised a neutral pH of 7 and synthesized silver nanoparticles (Abu-Elghait *et al.*, 2025). Contrarily an acidic pH of 5 was also recommended for the higher yields of silver nanoparticles (Cui *et al.*, 2022). Alteration of pH of reaction mixture effects the size of the silver nanoparticles (Alqadi *et al.*, 2014).

The overall inference of the results was that the reaction mixture of 1: 100 in combination with an alkaline pH value of 10 in resulted in higher yields of mycogenic AgNPs along with their optimal properties. The other critical parameters which were kept as constant and used throughout the experiments were 80 ± 1°C temperature of the reaction mixture, substrate concentration of 1mM $AgNO_3$, 1:10 suspension ratio of biomass to distilled water for metabolite production and 800 rpm of continuous stirring of reaction mixture.

Conclusion

The full potential of mycogenic AgNPs can be exploited only when sufficient quantity of the nanoparticles was synthesized. The synthesis depends on multiple parameters. Hence, two key parameters viz., reaction mixture ratio and pH were optimised for higher yields of AgNPs. 1:100 (TCF: $AgNO_3$) and pH of 10 (A_3B_3) was found as best treatment with a mean yield of 51.33 mg followed by 1:10 (TCF: $AgNO_3$) and pH of 10 (A_2B_3) with a mean yield of 40.33 mg and 1:100 (TCF: $AgNO_3$) and pH of 9 (A_3B_2) with a mean yield of 37 mg. Therefore, it was concluded that by using the best treatments in this study, higher yields of mycogenic AgNPs can be obtained.

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