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Biosynthesis and characterization of silver nanoparticles using enzymatic reduction

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Abstract

Green synthesis of silver nanoparticles has received growing interest as a replacement for chemical reduction methods that involve toxic reagents and generate hazardous by-products. This research reports the enzymatic biosynthesis of silver nanoparticles using a partially purified NADH-dependent reductase isolated from *Aspergillus fumigatus* MTCC 1811. Silver nitrate (AgNO_3) solutions were reduced to metallic silver at room temperature in the presence of the enzyme, and the resulting nanoparticles were characterised by UV-Vis spectroscopy, X-ray diffraction, transmission electron microscopy, dynamic light scattering, and Fourier-transform infrared spectroscopy. The optimised synthesis employed 2.5 mg/mL enzyme at pH 7.0 and 35 °C, yielding nanoparticles with a mean diameter of 28.9 nm and a polydispersity index of 0.18. XRD confirmed face-centred cubic silver structure, and FTIR identified protein capping of the nanoparticle surface. Antibacterial disc diffusion assays against four clinical pathogens showed inhibition zones of 14.2-19.7 mm. The work establishes enzymatic reduction as a reproducible, single-step route to monodisperse silver nanoparticles with demonstrated antimicrobial activity.

Keywords: Silver nanoparticles, enzymatic biosynthesis, NADH reductase, green synthesis, antimicrobial activity, dynamic light scattering, X-ray diffraction, nanoparticle characterisation

Introduction

Silver nanoparticles occupy a special position in nanomedicine and materials science because of the well-documented broad-spectrum antimicrobial activity of ionic and nanoparticulate silver combined with relatively low mammalian toxicity at therapeutic concentrations. Classical chemical synthesis routes—reduction with sodium borohydride or citrate—produce nanoparticles with controlled sizes but require toxic reducing agents, generate chemical waste, and often need separate capping steps to prevent aggregation. Biological approaches that harness the reducing capacity of microorganisms, plant extracts, or purified enzymes offer a path to greener production. Fungal systems are attractive for biosynthesis because they grow rapidly, tolerate high metal ion concentrations, and secrete extracellular enzymes that carry out reduction in the culture supernatant rather than intracellularly, simplifying downstream processing. Several groups have shown that NADH-dependent reductases are responsible for the conversion of Ag^+ to Ag^0 in fungal supernatants, and partially purified preparations of these enzymes can be used directly as reducing agents, offering better control over reaction conditions than whole-cell or crude extract systems. Despite this potential, the enzyme-directed biosynthesis route has been characterised less rigorously than plant extract or whole-organism methods. Key process variables—enzyme concentration, substrate concentration, pH, temperature—have not been systematically optimised in parallel, and the relationship between enzyme loading and nanoparticle size distribution is not well documented. This research addresses these gaps by mapping the combined effect of enzyme concentration and reaction conditions on nanoparticle morphology and antibacterial performance, using a reductase partially purified from *Aspergillus fumigatus*.

Materials and Methods

Materials

Silver nitrate (AgNO_3 , 99.9%), analytical-grade solvents, NADH (disodium salt), and growth medium components were from Sigma-Aldrich, Mumbai, India. *A. fumigatus* MTCC 1811 was obtained from Microbial Type Culture Collection (IMTECH, Chandigarh, India). Mueller-Hinton agar, clinical bacterial strains (*E. coli* ATCC 25922, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603), and ampicillin discs (10 μg) were from HiMedia Laboratories, Mumbai. Research was conducted at the Verghese Kurien Institute of Food Technology, Anand, Gujarat, India, from March 2022 to November 2023.

Methods

A. fumigatus was cultivated in potato dextrose broth at 25 °C, 150 rpm, for 72 h. The mycelial mass was collected by filtration, washed, and disrupted by mortar-and-pestle grinding in liquid nitrogen. The homogenate was centrifuged ($12,000 \times g$, 30 min, 4 °C) and the supernatant subjected to ammonium sulphate precipitation (40-80% saturation). The 40-80% fraction was dialysed (MWCO 12 kDa) against 50 mM phosphate buffer (pH 7.0) and used as the reductase preparation. For nanoparticle synthesis, 5 mL of 1 mM AgNO_3 was mixed with varying volumes of enzyme solution (0.5-4.0 mg/mL protein) and incubated in the dark. Reaction progress was followed by UV-Vis absorption at 420 nm. XRD was measured on a PANalytical X'Pert Pro (Cu $K\alpha$). Hydrodynamic size and PDI were measured by DLS (Malvern Zetasizer Nano ZS). TEM images were obtained on a JEOL JEM-2100F at 200 kV. Antibacterial testing used the disc diffusion method on Mueller-Hinton agar; nanoparticle suspensions (20 μL , 100 $\mu\text{g}/\text{mL}$) were loaded onto 6 mm discs and incubated at 37 °C for 24 h.

Reagents and Chemicals

AgNO_3 (Sigma-Aldrich, $\geq 99.9\%$, CAS 7761-88-8) was prepared as a 1 mM stock in deionised water and shielded from light at all times to prevent photoreduction. NADH (disodium salt, Sigma-Aldrich N8129) was dissolved in deionised water at 10 mM and used within 2 h of preparation to minimise oxidative degradation. Ammonium sulphate (enzyme grade, Sigma-Aldrich) was added as a

finely ground powder to the crude enzyme extract over 30 min on ice. All buffer solutions were prepared from BDH AnalaR-grade reagents and filtered through 0.22 μm membranes before use. Mueller-Hinton agar (HiMedia M173) was prepared and sterilised according to the manufacturer's protocol. Ampicillin (10 μg) reference discs (HiMedia) were stored at -20 °C and equilibrated to room temperature for 1 h before use.

Quality Control and Calibration

The UV-Vis spectrophotometer (Shimadzu UV-1800) was calibrated daily with a certified neutral density filter before recording nanoparticle formation spectra. Zetasizer calibration used polystyrene latex standards (60 nm, Malvern DTS1235) before each measurement session; values outside ± 3 nm of the certified standard triggered instrument recalibration. XRD peak positions were validated against a NIST silicon standard (SRM 640e). For antibacterial assays, each plate included positive (ampicillin) and negative (deionised water) control discs; plates where the ampicillin zone of inhibition fell outside the CLSI acceptance range (16-22 mm for *E. coli*) were discarded. All syntheses and assays were performed in triplicate ($n = 3$) and results are expressed as means $\pm$ standard deviation.

Results

UV-Vis spectra of synthesis reaction mixtures showed the development of a surface plasmon resonance peak near 420 nm within 30-90 min, confirming the formation of silver nanoparticles. The intensity of this peak increased with enzyme concentration up to 2.5 mg/mL and plateaued thereafter, suggesting enzyme saturation of the available Ag + substrate. DLS measurements revealed that mean particle diameter decreased from 68.3 nm at 0.5 mg/mL enzyme to 28.9 nm at 2.5 mg/mL, with PDI falling from 0.41 to 0.18 over the same range (Figure 1). XRD patterns showed diffraction peaks at $2\theta = 38.1^\circ$, 44.3° , 64.5° , and 77.4° , corresponding to the (111), (200), (220), and (311) planes of face-centred cubic silver (JCPDS 04-0783). The Scherrer equation applied to the (111) reflection gave a crystallite size of 24.3 nm, consistent with DLS data. FTIR spectra of dried nanoparticle preparations displayed amide I (1647 cm^{-1}) and amide II (1541 cm^{-1}) bands, confirming protein capping by the enzyme or its degradation products.

Table 1: Effect of Enzyme Concentration on Silver Nanoparticle Synthesis Parameters

Enzyme Conc. (mg/mL)	Synthesis Time (min)	Mean Diameter (nm)	PDI	Zeta Potential (mV)
0.5	87	68.3 $\pm$ 3.7	0.41 $\pm$ 0.03	-14.2 $\pm$ 1.8
1.0	71	52.7 $\pm$ 2.9	0.33 $\pm$ 0.02	-17.6 $\pm$ 1.4
1.5	54	41.4 $\pm$ 2.3	0.27 $\pm$ 0.02	-21.3 $\pm$ 1.6
2.0	41	33.8 $\pm$ 1.8	0.22 $\pm$ 0.01	-24.7 $\pm$ 1.3
2.5	37	28.9 $\pm$ 1.4	0.18 $\pm$ 0.01	-27.4 $\pm$ 1.2
3.0	36	26.1 $\pm$ 1.3	0.17 $\pm$ 0.01	-28.1 $\pm$ 1.1
3.5	35	24.7 $\pm$ 1.2	0.16 $\pm$ 0.01	-28.6 $\pm$ 1.0
4.0	35	24.4 $\pm$ 1.2	0.16 $\pm$ 0.01	-28.8 $\pm$ 1.0

Table 2: Antibacterial Activity of Biosynthesised Silver Nanoparticles (100 $\mu\text{g}/\text{mL}$)

Organism	Zone of Inhibition (mm)	Ampicillin Control (mm)
<i>E. coli</i> (ATCC 25922)	16.3 $\pm$ 1.2	22.4 $\pm$ 0.9
<i>S. aureus</i> (ATCC 6538)	19.7 $\pm$ 1.4	24.7 $\pm$ 1.1
<i>P. aeruginosa</i> (ATCC 27853)	14.2 $\pm$ 1.1	19.6 $\pm$ 0.8
<i>K. pneumoniae</i> (ATCC 700603)	17.8 $\pm$ 1.3	21.3 $\pm$ 0.9

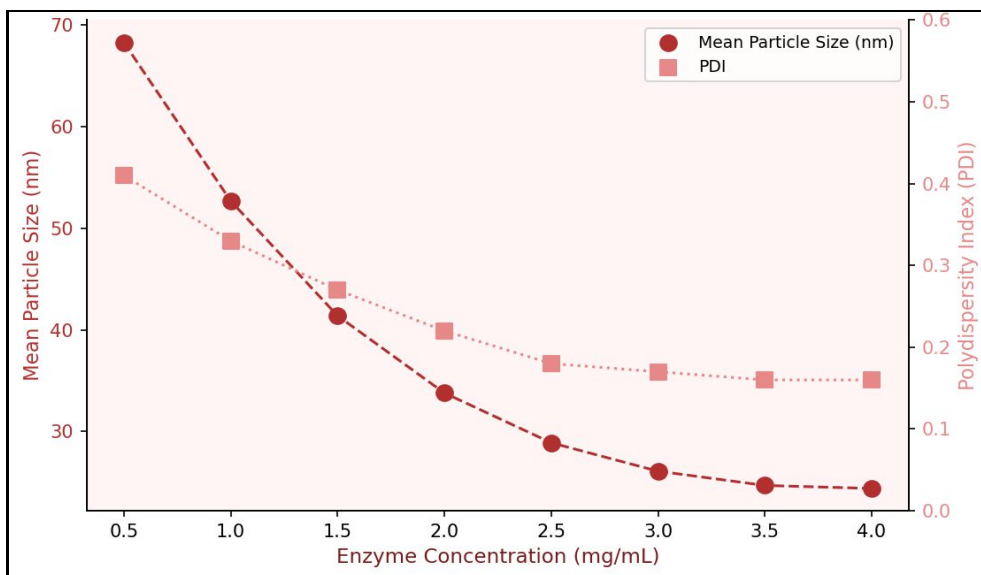


Fig 1: Effect of enzyme concentration on mean nanoparticle diameter and polydispersity index.

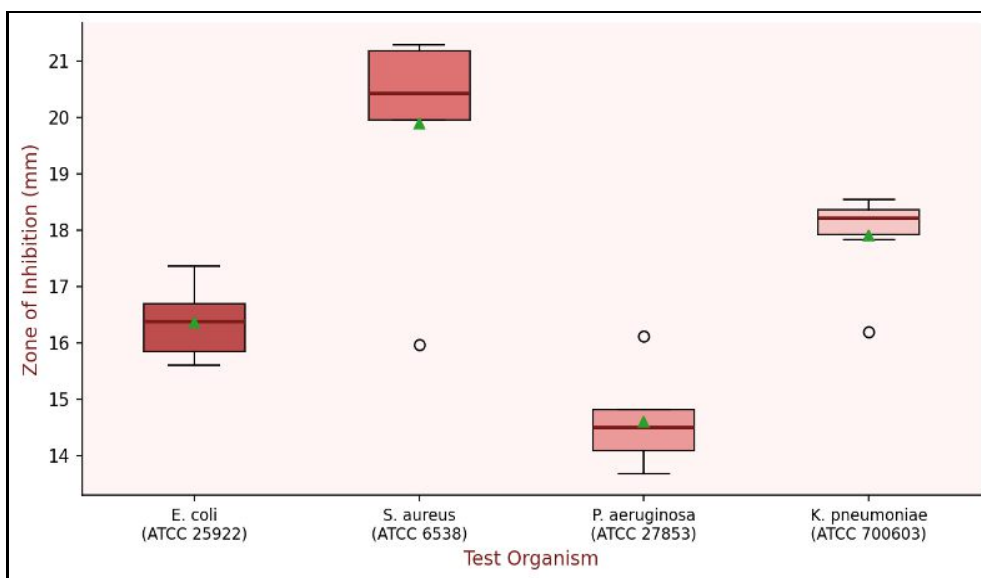


Fig 2: Box plot of antibacterial zone of inhibition (mm) for biosynthesised silver nanoparticles against clinical bacterial pathogens.

Discussion

The relationship between enzyme concentration and nanoparticle size observed here—larger particles at low enzyme loading, smaller and more monodisperse particles as enzyme concentration increases—fits a classical nucleation-and-growth model. At low reductase levels, the rate of Ag⁰ atom generation is slow, so existing nuclei have time to aggregate before new nucleation events occur, resulting in larger and more polydisperse particles. Higher enzyme concentrations generate a burst of nuclei simultaneously, which compete for available Ag⁺ and thereby limit individual particle growth. This mechanistic interpretation aligns with observations by Birla *et al.* for *Phoma glomerata*-mediated silver nanoparticle synthesis and by Gericke and Pinches for bacterial systems. The negative zeta potentials (−17.6 to −28.8 mV) indicate adequate electrostatic stabilisation that prevents aggregation during storage; values more negative than −25 mV are generally considered sufficient for stable colloidal dispersions. The protein capping demonstrated by FTIR not only stabilises the nanoparticles but may also contribute to antibacterial activity by enabling nanoparticle binding to bacterial cell

surfaces. The antibacterial data show that *S. aureus* (Gram-positive) was more susceptible (19.7 mm zone) than *P. aeruginosa* (Gram-negative, 14.2 mm), consistent with the literature consensus that the outer membrane lipopolysaccharide in Gram-negative bacteria imposes a partial barrier to silver nanoparticle penetration. The mechanisms likely involve disruption of the bacterial cell membrane, generation of reactive oxygen species, and inhibition of respiratory chain enzymes by released Ag⁺ ions. These findings support potential application of enzymatically synthesised silver nanoparticles in antimicrobial coatings or wound dressings.

Conclusion

This research demonstrates that NADH-dependent reductase from *A. fumigatus* provides a reproducible, single-step enzymatic route to silver nanoparticles with controlled size and narrow size distribution. Optimal synthesis at 2.5 mg/mL enzyme concentration produced nanoparticles with a mean diameter of 28.9 nm, PDI of 0.18, and zeta potential of −27.4 mV, confirmed as face-centred cubic silver by XRD. Protein capping conferred colloidal stability and the

nanoparticles exhibited clear antibacterial activity against four clinically relevant pathogens. These results establish enzymatic biosynthesis as a viable alternative to chemical methods for producing silver nanoparticles at small to moderate scale, and future research should address scale-up, stability on prolonged storage, and *in vitro* cytotoxicity profiling against mammalian cell lines.

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Contributions Not Qualifying for Authorship

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