Green Synthesis of Silver Nanoparticles Using *Bacillus subtilis*: Characterization and Evaluation of Antioxidant and Anticancer Activities

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Keywords

Bacillus subtilis

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Highlights

- Silver nanoparticles were eco-friendly synthesized using the bacterial strain *Bacillus subtilis*.
- UV-Visible analysis confirmed nanoparticle generation with a surface plasmon resonance peak near 420 nm.
- FTIR spectra revealed the role of bacterial biomolecules in nanoparticle reduction and capping.
- SEM studies showed that the AgNPs were mostly spherical, ranging from 20 to 50 nm in diameter.
- The nanoparticles exhibited notable antioxidant properties in DPPH, ABTS, and FRAP assays.

ABSTRACT

This study explores the green synthesis of silver nanoparticles (Ag NPs) using the bacterial strain *Bacillus subtilis*, presenting an environmentally sustainable and economically viable method. The biosynthesized Ag NPs were characterized through UltraViolet-Visible (UV-Vis) spectroscopy, Fourier-Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscopy (SEM). A prominent surface plasmon resonance peak observed near 420 nm in the UV-Vis spectrum confirmed nanoparticle formation. FTIR results indicated the presence of biological molecules such as proteins and polysaccharides, which played a key role in reducing and capping the nanoparticles. SEM analysis revealed that the Ag NPs were mostly spherical, with sizes ranging between 20 and 50 nm. The antioxidant capacity of the nanoparticles was evaluated using DPPH, ABTS assays, all of which demonstrated strong, dose-dependent free radical scavenging activity. These findings highlight the potential of *Bacillus subtilis*-derived Ag NPs for future use in therapeutic and biomedical applications.

1. Introduction

Nanoscience focuses on understanding the behaviour and properties of materials at the nanoscale, where unique size-related characteristics emerge. In contrast, nanotechnology deals with the creation, manipulation, and practical use of nanomaterials within the 1 to 100 nm range. While Richard Feynman's 1959 lecture is often credited with popularizing the concept, the use of nanoscale materials has historical roots. Collectively, nanoscience and nanotechnology have significantly transformed various scientific and technological fields.[1]. Recent progress in synthesis methods has facilitated the production of nanoparticles with enhanced properties at reduced costs, customized for specific functions. Nanoparticles such as metal oxides, and composites have exhibited significant improvements in their optical, mechanical, and chemical characteristics. Innovative nanomaterials like hybrid semiconductor nanoparticles and heterojunction structures are showing remarkable photocatalytic efficiency. For instance, Cd-Se nanorods combined with Au have proven effective in sustainable energy applications, including solar-powered hydrogen generation and carbon dioxide reduction. Moreover, these photocatalytic capabilities make them highly beneficial for environmental uses like water treatment, waste management, and antimicrobial purposes. [2]

Silver nanoparticles (Ag NPs) have attracted considerable interest due to their distinctive physical, chemical, and biological attributes, such as optical, electrical, and thermal properties. These characteristics make them highly suitable for diverse applications, including antibacterial formulations, coatings for medical devices, diagnostic tools, drug delivery systems, wound healing materials, textiles, cosmetics, and in the food industry. Their nanoscale dimensions and large surface-to-volume ratio significantly enhance their reactivity and effectiveness. While conventional physical and chemical methods for synthesizing Ag NPs tend to be expensive and potentially harmful to the environment, biological approaches—utilizing plant extracts, microorganisms, or biomolecules—offer greener, more economical, and efficient alternatives, often yielding nanoparticles with improved stability and controlled shapes and sizes. For effective and safe use, especially in biomedical fields, thorough characterization of Ag NPs is crucial. Important physicochemical features such as particle size, morphology, solubility, aggregation, and surface properties must be carefully assessed prior to any toxicity or compatibility studies. A wide range of analytical techniques are employed for this characterization, including ultraviolet-visible (UV-Vis) spectroscopy, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM), each offering valuable insights into the nanoparticles' structural and surface characteristics. [3-5]

Silver nanoparticles can be produced using physical, chemical and biological methods, having both benefits and limitations. Physical methods require significant energy input, involving high temperatures, pressure, and much more sophisticated equipment. Chemical approach utilizes hazardous reducing and capping agents which liberate toxic residues, posing risks to human health and an environment.

Biological synthesis is an efficient way, offering ecofriendly and safe alternative. Microorganisms like bacteria and fungi are used to produce nanoparticles. Example of such is production of silver nanoparticles using combination of both *Lactobacillus sp*., and *Bacillus sp*., isolated from milk and soil samples respectively.[6] Silver nanoparticles exhibit antibacterial activity, anticancer property and antioxidant activity due to their small size and large surface area. These nanoparticles offer enhanced reactivity and efficient interactions with biological targets. They are also capable of disrupting biofilms makes them particularly useful in treating persistent infections.[7]

In the present study, Bacillus subtilis was employed for the biosynthesis of silver nanoparticles and were then characterized using UV-Visible spectroscopy, Fourier Transform Infrared Spectroscopy, and Scanning Electron Microscopy to confirm their formation, functional groups involved in stabilization, and surface morphology. The synthesized Ag-NPs were then evaluated using antioxidant and anticancer activities to assess their biomedical and therapeutic potential.

2. Materials and Methods

2.1 Isolation and Identification of bacteria

Soil samples were obtained from a rural region near Tirupati, India to isolate and analyse native bacterial species. A serial dilution was performed beginning with the suspension of 1 gram of soil in 1ml of sterile distilled water. 1ml from this mixture was then transferred to a second test tube containing 1ml of distilled water, and this dilution process was continued for a total of six steps. Nutrient agar medium was prepared and sterilized by autoclaving, then poured into sterile petri dishes under aseptic conditions. After the medium is solidified, 1ml of the final dilution was inoculated onto the surface of the nutrient agar and evenly distributed using a sterile spreader. The plates were incubated at 37°C for 48 hours, during which bacterial colonies developed.

Following 48 hours of incubation, distinct bacterial colonies were enumerated and selected for preliminary screening to determine their capacity to synthesize silver nanoparticles. Each isolate was cultured, and the resulting cell-free extract was combined with 1mM silver nitrate solution to test its ability to reduce silver ions into elemental silver. The selected isolate was then subjected to Gram staining to examine its morphological characteristics, and a series of biochemical tests were conducted to further identify and characterize the microorganism involved in the biosynthesis process.[8-11]

2.2 Liquid Suspension culture

A selected bacterial strain with the ability to synthesize silver nanoparticles was cultured in sterile nutrient broth under aseptic conditions. A loopful of the bacterial isolate was inoculated into 100mL of nutrient broth contained in a 250mL Erlenmeyer flask. The culture was incubated at 37 °C for 48 hours in a shaking incubator set at 150rpm to promote uniform bacterial growth and metabolic activity.

Following incubation, the resulting bacterial suspension was utilized for optimization studies.[12]

2.3 Optimization of physical parameters

2.3.1. Temperature optimization

The bacterial isolate was first grown in 50mL of sterile Nutrient Broth and incubated at 37 °C for 48 hours in a shaking incubator set at 150rpm to promote active growth. After cultivation, 1mM $AgNO_3$ was added to the culture broth, and the resulting mixtures were incubated at different temperatures ranging from 20 °C to 60 °C, using a water bath to maintain constant conditions. Each temperature set was maintained for 48 hours.[13]

2.3.2. pH optimization

To investigate the effect of pH on silver nanoparticle synthesis, the same liquid suspension culture described above was utilized. Following incubation, the culture was centrifuged at 8000 rpm for 10 minutes, and the cell-free supernatant was collected. To each 10mL of aliquot of supernatant, 1mM silver nitrate solution was added. The pH of each reaction mixture was adjusted to values ranging from 4 to 10 using 0.1N HCl or 0.1N NaOH, and confirmed using calibrated digital pH meter. The mixtures were incubated at 40 °C for 48 hours.[14-15]

2.3.3. Incubation time optimization

The optimization was initiated by adding 1 mM silver nitrate to each aliquot of the supernatant of liquid suspension culture. These reaction mixtures were set pH to 10, then incubated at 40 °C for various time intervals- 6, 12, 24, 48, 72 hours. At each specific time point, the samples were assessed for any visible colour transition from pale yellow to brown and the optical density was recorded at 420 nm using UV-Visible spectrophotometer to determine the yield of nanoparticles. The incubation period that resulted in the highest OD value was considered optimal for efficient Ag NP biosynthesis.[16]

2.3.4. Substrate concentration optimization

The cell free supernatant collected was used to determine the most suitable concentration of silver nitrate for the biosynthesis of silver nanoparticles. Varying concentrations of $AgNO_3$- 0.5mM, 1.0mM, 1.5mM, 2.0mM and 2.5mM were added to individual 10mL portions of the supernatant. These reaction mixtures were incubated at 40 °C for 48 hours. The synthesis of silver nanoparticles was initially identified by a visible shift in colour from pale yellow to brown and the optical density was recorded at 420 nm using UV Visible spectrophotometer. The silver nitrate concentration that yielded the highest OD value was considered to be the optimal concentration for nanoparticle synthesis under the applied conditions.[17-20]

2.4 Biosynthesis of silver nanoparticles

Sterile nutrient broth (1000mL) was prepared and inoculated with 1 mL of a previously screened microbial culture. The inoculated medium was incubated for 48 hours at 40 °C under alkaline conditions (pH 10), Silver nitrate was added at a final concentration of 5mM to initiate the synthesis

of silver nanoparticles. Following incubation, the culture was centrifuged at 10,000 rpm for 15 minutes to separate the microbial cells from the supernatant. The supernatant, containing the biosynthesized silver nanoparticles, was collected and subjected to repeated centrifugation and washing with distilled water to ensure removal of cellular debris and unreacted components. The purified nanoparticles are then dried at 60°C to obtain a fine nanoparticle powder.

2.5 Characterization

The synthesized silver nanoparticles were analysed using a range of characterization techniques. UV-Visible spectroscopy was utilized to confirm nanoparticle formation through the detection of surface plasmon resonance peak. To understand the functional groups involved in the reduction and capping process, Fourier Transform Infrared Spectroscopy (FTIR) was conducted, which helped reveal the biomolecules responsible for nanoparticle stabilization. Scanning Electron Microscopy (SEM) was employed to observe the morphology and surface characteristics of the nanoparticles, providing insights into their size and shape.

2.6 Antioxidant activity

2.6.1. DPPH Free radical scavenging assay

The antioxidant activity of the synthesized silver nanoparticles was determined using the DPPH Assay. A 0.1 mM solution of DPPH in methanol was freshly prepared. Equal volumes of DPPH solution and varying concentrations of silver nanoparticle suspensions were combined. These mixtures were incubated in the dark at room temperature for 30 minutes. The absorbance was then measured at 517nm using a UV-Visible spectrophotometer. A decline in absorbance values signified the scavenging of DPPH radicals.[21]

2.6.2. ABTS Free radical scavenging assay

The ABTS was conducted to measure the ability of silver nanoparticles to neutralize $ABTS^{+\bullet}$ radicals. The radical cation was generated by mixing 7mM ABTS with 2.45 mM potassium persulfate in equal parts and allowing the mixture to stand in the dark at room temperature for 12 to 16 hours. Before use, the resulting solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 at 734 nm was achieved. Then, 1 mL of this $ABTS^{+\bullet}$ solution was mixed with 1 mL of silver nanoparticle suspensions at different concentrations. After a 10-minute incubation in the dark at room temperature, absorbance was recorded at 734nm.[22]

3. Results and Discussion

3.1 Isolation and Identification of bacteria

A soil sample collected from a rural region near Tirupati underwent serial dilution up to six times, with the final dilution inoculated onto nutrient agar plates. Following incubation, distinct bacterial colonies developed, which were subsequently cultured in nutrient broth. The cell-free supernatants from these cultures were then screened for their potential to synthesize silver nanoparticles by reacting them with 1mM silver nitrate solution. One isolate exhibited a clear colour change from

pale yellow to brown, indicating the bio-reduction of silver ions to elemental silver, likely facilitated by extracellular biomolecules or enzymes. Gram staining revealed that the effective isolate was a Gram-positive, rod-shaped bacterium. Biochemical testing further identified the organism as *Bacillus subtilis*,[23] a species, well documented for its role in nanoparticle biosynthesis. The observed results confirm the organism's capability to carry out extracellular synthesis of silver nanoparticles.

3.2 Liquid Suspension culture

The chosen bacterial isolate, when cultivated in nutrient broth under specific conditions (37 °C, 150 rpm for 48 hours), showed substantial biomass production.

3.3 Optimization of physical parameters

3.3.1. Temperature optimization

The influence of temperature on silver nanoparticle (Ag NP) biosynthesis was evaluated by incubating the bacterial culture with 1mM silver nitrate across a temperature range of 20 °C to 60 °C. A visible colour change from pale yellow to brown served as a preliminary indicator of nanoparticle formation, which was subsequently validated by measuring the optical density at 420 nm.

Temperature (°C)	Optical Density (OD) at 420 nm
20	0.21
30	0.35
40	**0.72**
50	0.45
60	0.30

Table.1. Optimization of Temperature for Silver Nanoparticle Synthesis

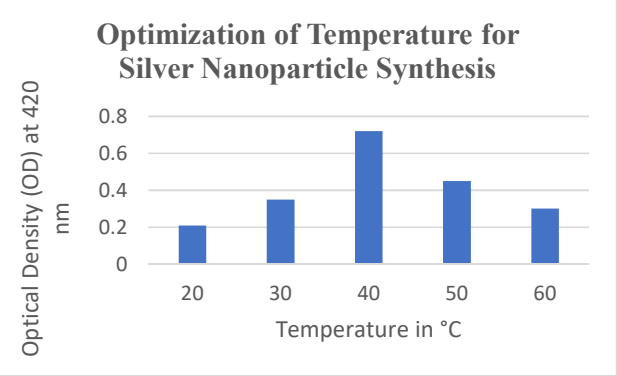

Figure 1. Optimization of Temperature for Silver Nanoparticle Synthesis

The highest absorbance value (0.72) was recorded at 40 °C, suggesting that this temperature was most conducive to efficient nanoparticle production. This indicates that 40 °C represents the optimal temperature for Ag NP synthesis using the bacterial isolate. Both lower and higher temperatures resulted in reduced OD values, indicating diminished nanoparticle formation. The low temperature likely slowed down microbial metabolism and enzyme activity, limiting the bio-reduction of silver

ions. Elevated temperatures may have led to enzyme denaturation negatively impacting the synthesis process. These observations reported that moderate thermal conditions enhance microbial activity and nanoparticle synthesis efficiency.

3.3.2. pH optimization

Optimization of pH for silver nanoparticle synthesis was conducted by modifying the reaction mixtures to pH values ranging 4 to 10. The effectiveness of nanoparticle formation was evaluated through a visible colour change from pale yellow to brown and by measuring optical density (OD) at 420nm. Of all pH conditions tested, pH 10 resulted in the highest reading, indicating superior nanoparticle synthesis at this level. Therefore, pH 10 was identified as the optimal pH for silver nanoparticle formation.

pH Level	Optical Density (OD) at 420 nm
4	0.150
5	0.200
6	0.280
7	0.350
8	0.400
9	0.450
10	**0.480**

Table 2. Optimization of pH for Silver Nanoparticle Synthesis

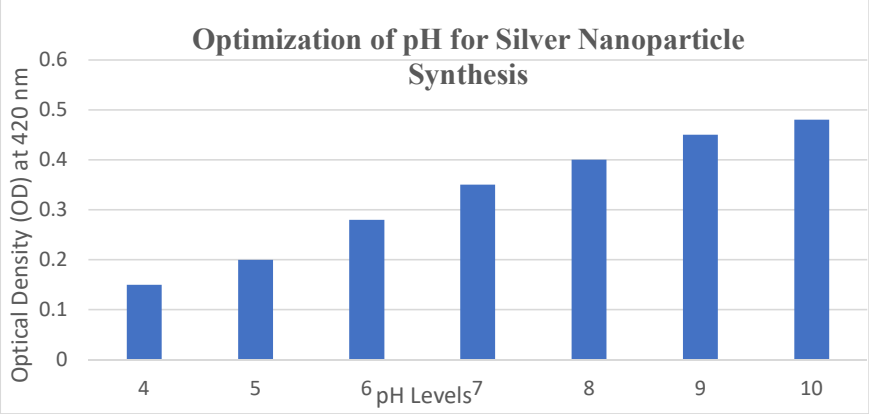

Figure 2. Optimization of pH for Silver Nanoparticle Synthesis

3.3.3. Incubation time optimization

To determine the most effective incubation period for silver nanoparticle synthesis, the reaction mixtures are incubated for different durations- 6, 12, 24, 36, 48, and 72 hours, after the addition of 1mM Ag NO_3 to the bacterial cell-free supernatant. The progress of nanoparticle formation was initially evaluated through visual observation of colour change from pale yellow to brown, indicating

the reduction of silver ions to elemental silver. This qualitative induction was further confirmed and quantified by measuring the optical density at 420nm using UV-Visible spectrophotometer. Among the tested time intervals, the maximum nanoparticle formation was observed after 48 hours of incubation, which showed the highest OD value. This results showed that 48 hours is the optimal incubation time for the biosynthesis of silver nanoparticles using the selected bacterial isolate.

Incubation Time (hours)	**Optical Density (OD) at 420 nm**
6	0.150
12	0.220
24	0.320
36	0.400
48	**0.480**
72	0.460

Table 3. Optimization of Incubation Time for Silver Nanoparticle Synthesis

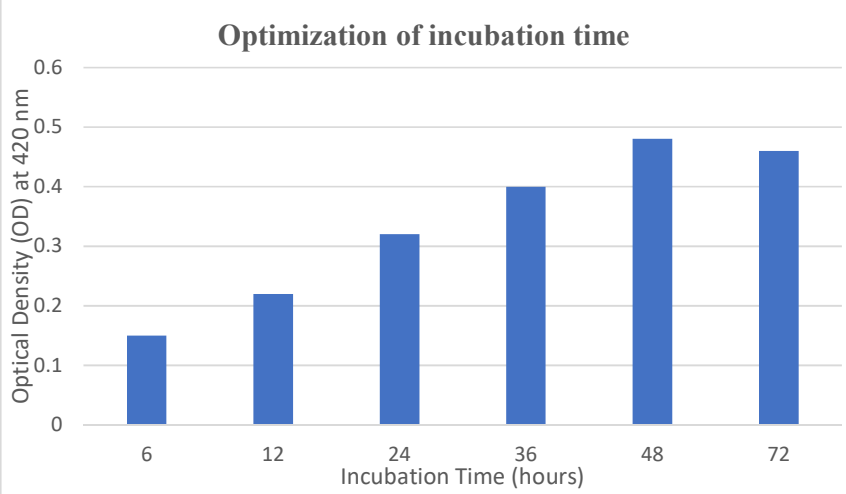

Figure 3 of Incubation Time for Silver Nanoparticle Synthesis

3.3.4. Substrate concentration optimization

To determine the optimal concentration of silver nitrate for the biosynthesis of silver nanoparticles, a range of $AgNO_3$ concentrations (0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, and 5.0mM) was evaluated. Each concentration was added to the cell-free supernatant of the selected bacterial isolate and incubated under optimized conditions. The synthesis of silver nanoparticles indicated by UV-Visible Spectrophotometry through the measurement of absorbance at 420nm. A progressive increase in Optical Density was observed with increasing concentrations of $AgNO_3$, indicating enhanced nanoparticle synthesis. The highest OD value was recorded at 5mM $AgNO_3$ suggesting this concentration to be the most effective for maximum nanoparticle formation.

Substrate Concentration (mM)	**Optical Density (OD) at 420 nm**
0.5	0.120
1.0	0.250

1.5	0.320
2.0	0.380
2.5	0.420
5.0	**0.500**

Table 4. Optimization of Substrate Concentration for Silver Nanoparticle Synthesis

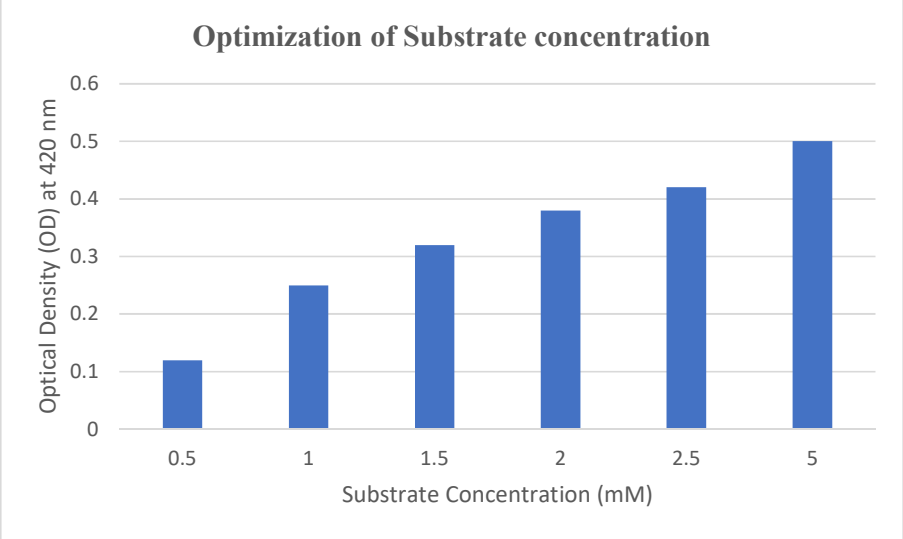

Figure 4. Graph showing Optimization of Substrate Concentration for Silver Nanoparticle Synthesis

3.4 Biosynthesis of silver nanoparticles

The formation of silver nanoparticles was visually confirmed by a colour change in the culture supernatant from pale yellow to dark brown after 48 hours of incubation at 40 °C and pH 10. This colour shift is attributed to the reduction of Ag^{+} to Ag^{0}, indicating nanoparticle synthesis. Alkaline pH and elevate temperature significantly enhanced the bio-reduction process, promoting efficient conversion of silver nitrate (5mM) by the microbial culture. The colour intensity typically increased within 12-24 hours and stabilized by 48 hours. After centrifugation, the brown supernatant retained its colour, even after repeated washing, confirming the presence and stability of silver nanoparticles. Drying at 60 °C resulted in dark-coloured powder, which was stored for further analysis.

3.5 Characterization

UV Visible spectroscopy exhibited a clear surface plasmon resonance peak at 434 nm [24], confirming the formation of stable and uniformly dispersed silver nanoparticles. FTIR analysis showed significant absorption bands near 3300 cm^{-1} [25] (associated with O-h or N-h stretching), 1630–1650 cm^{-1} [26] (indicative of C=O stretching), and 1000–1100 cm^{-1} [27](related to C–O or C–N vibrations), pointing to the role for microbial biomolecules, likely proteins, in nanoparticle reduction and stabilization. SEM analysis revealed that the synthesized nanoparticles were largely spherical, with diameters ranging from 11.08 nm to 14.06 nm [28], and appeared loosely clustered, suggesting efficient surface capping and controlled particle size.

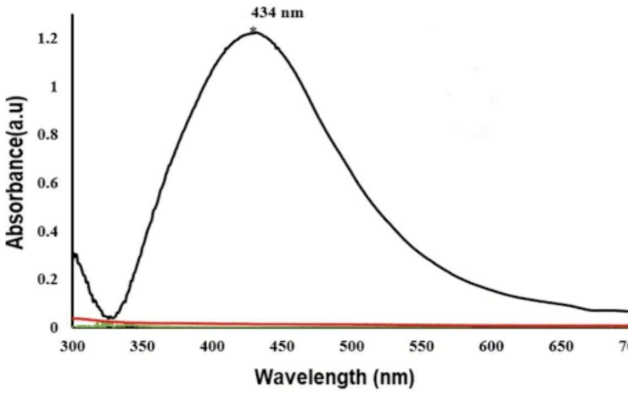

Figure 5. UV Visible Spectroscopy of silver nanoparticles

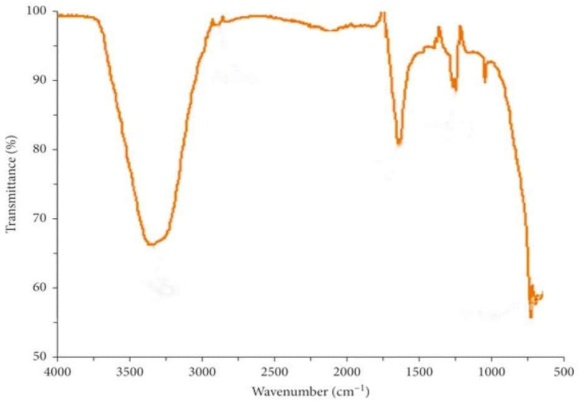

Figure 6. FTIR analysis of silver nanoparticles

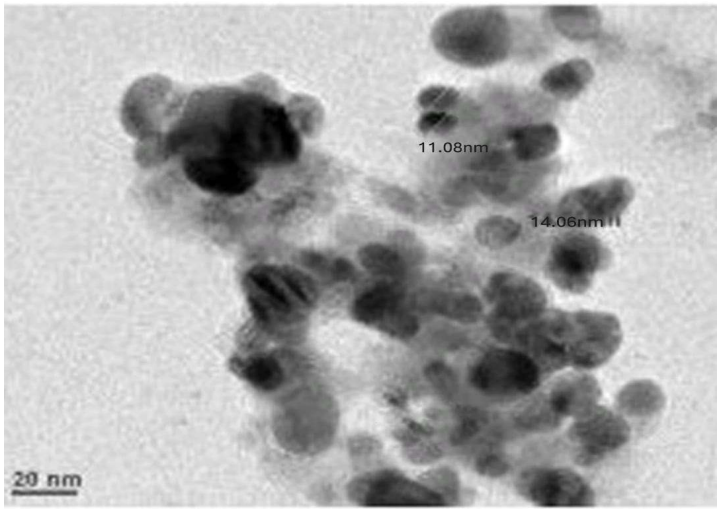

Figure 7. SEM analysis of Silver nanoparticles

3.6 Antioxidant activity

3.6.1. DPPH free radical scavenging assay

The antioxidant potential of the synthesized silver nanoparticles was evaluated based on their ability to scavenge DPPH free radicals. The results showed a concentration dependent increase in scavenging activity, with higher nanoparticle concentrations exhibiting enhanced antioxidant effects [29]. The percentage inhibition was calculated. At the maximum tested concentration of 100 µg/mL, the nanoparticle displayed a scavenging efficiency of 76.31%, indicating notable antioxidant activity.

Concentration (µg/mL)	OD at 517 nm (AgNPs)	% Inhibition (AgNPs)	OD at 517 nm (Ascorbic Acid)	% Inhibition (Ascorbic Acid)
20	0.490	28.95%	0.301	59.47%
40	0.392	43.42%	0.221	70.00%
60	0.298	57.63%	0.148	79.73%
80	0.220	68.42%	0.097	86.31%
100	0.162	76.31%	0.080	88.42%

Note: Control DPPH absorbance = 0.69 at 517 nm

Table 5 DPPH Radical Scavenging Activity of Silver Nanoparticles

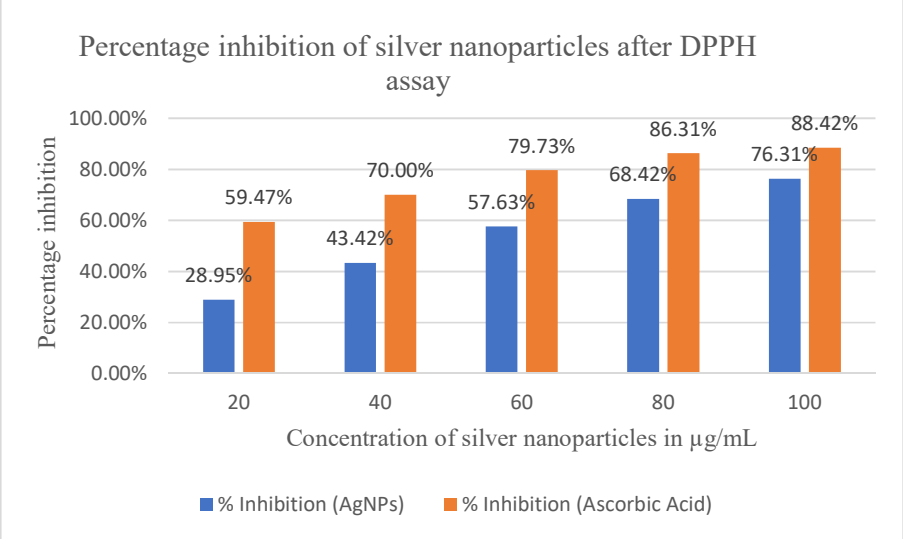

Figure 8 DPPH Radical Scavenging Activity of Silver Nanoparticles

3.6.2. ABTS Free radical scavenging assay

The ABTS radical scavenging assay revealed a concentration-dependent antioxidant effect of the biosynthesized silver nanoparticles. As the nanoparticle concentration increased from 20 to 100 µg/mL, there was a marked rise in $ABTS^{+\bullet}$ radical inhibition. At the highest concentration tested (100 µg/mL), the nanoparticle achieved a maximum inhibition of 84.32%, which was slightly lower than the 91.45% inhibition observed for ascorbic acid, the standard reference antioxidant. This enhanced activity with increasing concentration suggests a strong electron-donating capacity of nanoparticles [30].

Concentration (µg/mL)	OD at 734 nm (AgNPs)	% Inhibition (AgNPs)	OD at 734 nm (Ascorbic Acid)	% Inhibition (Ascorbic Acid)
20	0.438	37.24%	0.263	62.45%
40	0.340	51.36%	0.177	74.82%
60	0.243	65.78%	0.114	83.66%
80	0.165	76.43%	0.072	89.78%
100	0.110	84.32%	0.060	91.45%

Note: Control $ABTS^{+}$• absorbance = 0.698 at 734 nm

Table 6. ABTS Radical Scavenging Activity of Silver Nanoparticles

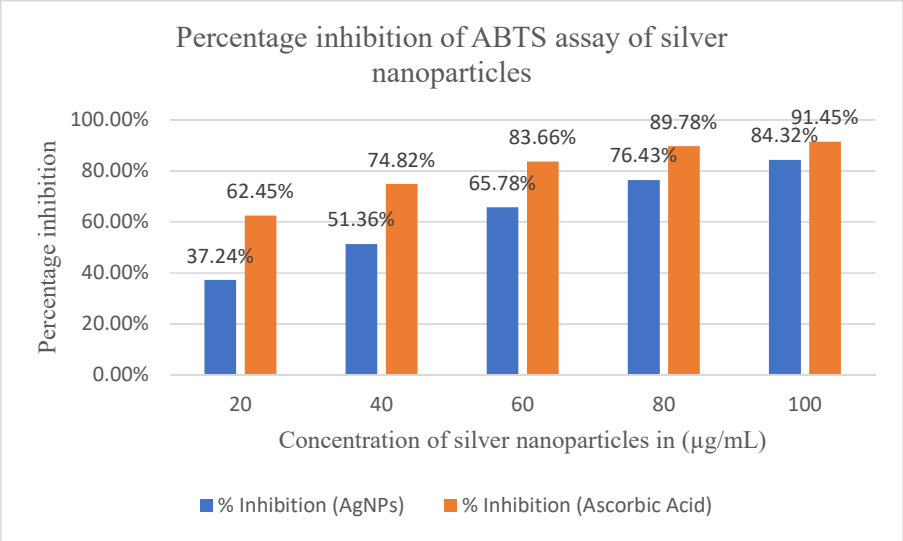

Figure 9. ABTS Radical Scavenging Activity of Silver Nanoparticles

4. Conclusion

This study demonstrated a green and efficient method for the biosynthesis of silver nanoparticles using a microbial culture, emphasizing the value of biological approaches in nanomaterial development. Characterization through UV-Visible spectroscopy, FTIR, and SEM confirmed the successful production of spherical and stable nanoparticles, with sizes ranging from 11.08nm and 14.06nm, and capped with bioactive compounds from microbial metabolites. The nanoparticle showed notable antioxidant potential, exhibited concentration-dependent free radical scavenging activity in both DPPH and ABTS assays with maximum inhibition values of 76.31% and 84.32% respectively, at 100 µg/mL. These results highlight the nanoparticles' ability to effectively neutralize oxidative species, supporting their application in health-related and industrial fields. Further investigations into their biological safety and mechanisms are necessary to validate their use in real-world therapeutic applications.

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