RESEARCH ARTICLE

Eco-Friendly Extracellular Biosynthesis of Silver Nanoparticles Using a Novel Bacillus Strain: Characterization and Potent Antibacterial Activity Against Multidrug-Resistant Pathogens

Mohammadali Nahofte[1], Fatemeh Tohidi[1,2]*, Abazar Pournajaf[2,3], Mehdi Rajabnia[2,3]

[1] Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran.

[2] Department of Medical Microbiology and Medical Biotechnology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran.

[3] Infectious Diseases and Tropical Medicine Research Center, Health ResearchInstitute, Babol University of Medical Sciences, Babol, Iran.

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ABSTRACT

Objective(s): Biosynthesized nanoparticles have attracted significant attention in biomedical research due to their biocompatibility, natural origin, and potent antibacterial properties. Silver nanoparticles (AgNPs), in particular, are valued for their cost-effectiveness, eco-friendliness, and strong antimicrobial activity. This study aimed to establish a sustainable and scalable extracellular green synthesis platform for AgNPs using Bacillus strains isolated from spring water.

Methods: Eighteen Bacillus isolates were screened for their ability to reduce silver ions. The most efficient strain-mediated synthesis was optimized under 3 mM $AgNO_3$, pH 10, 37 °C, for 72 h. The synthesized AgNPs were characterized using UV–visible spectroscopy, scanning electron microscopy (SEM), dynamic light scattering (DLS), X-ray diffraction (XRD), and Fourier-transform infrared (FTIR) analyses to assess morphology, size, crystallinity, surface charge, and biomolecular capping.

Results: UV–Vis spectroscopy revealed a distinct surface plasmon resonance peak at 430 nm. SEM and DLS analyses confirmed well-dispersed, nearly spherical nanoparticles with diameters of 30–50 nm and high colloidal stability (zeta potential −34/9 mV). XRD patterns indicated a crystalline face-centered cubic structure, while FTIR spectra suggested biomolecular capping via amide and hydroxyl functional groups. The AgNPs demonstrated 98 % antibacterial inhibition at 10 mg/mL and MIC values of 0.5–1 mg/mL against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, comparable to gentamicin.

Conclusions: This study presents a rapid, reproducible, and eco-friendly extracellular biosynthesis of AgNPs using a novel Bacillus strain, highlighting its potential as a sustainable nanoplatform for combating multidrug-resistant pathogens in biomedical applications.

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INTRODUCTION

Silver nanoparticles (AgNPs) with unique features are used in many fields, including molecular diagnostics, antibacterial applications, and cancer therapy[1]. These nanoparticles are composed of silver particles with an average size ranging from 10 to 100 nm, and they provide greater stability than other forms of silver solutions[2]. In addition to having a more significant environmental effect, AgNPs have a wider contact surface with the outside environment owing to their tiny size. Silver is a relatively inactive metal at larger sizes, but when it is reduced to nanoscale dimensions, it shows unique physicochemical and biological activity[3]. This enhances its antibacterial activity by more than 99%.

* Corresponding Author Email: *tohidi187@yahoo.com*

Nanoparticles have a dual property as compared to macroparticles, which is caused by their higher surface area. This results in increased reactivity and alignment with quantum effects in physics and chemistry. AgNPs' characteristics include rapidity, non-toxicity, harmlessness, absence of allergenicity, adaptability under diverse conditions (notably high stability), hydrophilicity, environmental friendliness, and heat resistance. Furthermore, studies have shown that AgNPs are more resistant and compatible with microbes[4]. Given these specific characteristics, AgNPs are well-suited for applications such as improving surgical tools, covering bone prostheses with AgNPs, and treating infections[5, 6]. Moreover, AgNPs have attracted the interest of many researchers due to their potential in healthcare, agriculture, food technology, and environmental applications. Particularly remarkable is their development as a topic in the field of biological applications, driven by their diversity of capabilities, including antibacterial, antiviral, anti-inflammatory, anti-angiogenic, and anti-permeability effects[7].

Considering the cost and toxicity of chemicals associated with chemical and physical methods for nanoparticle (NP) manufacturing, alternative approaches are considered more favorable. For example, biological synthesis techniques utilizing fungi, bacteria, and plants are preferred because they operate at very low temperatures and pressures. Compared to chemical or physical procedures, biological technologies are not only more cost-effective but also more time-efficient[8]. Bacteria are ideal candidates for producing biogenic nanoparticles due to their easy access to genetic material and ease of cultivation[9]. Bacteria are also capable of adapting to unfavorable environments. They synthesize nanoparticles via two mechanisms: extracellular pathways and intracellular processes. Notably, a broad spectrum of bacteria has been found capable of producing nanoparticles, including Escherichia coli, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Alteromonas, and Ochrobactrum species[8]. Recently, antibiotic-resistant bacteria have become increasingly prevalent. Nanoparticles have been proposed as a novel strategy to combat these harmful bacteria, owing to their large surface area relative to their size and ability to penetrate cell walls[10]. It is therefore necessary to develop green synthesis methods for AgNPs using a variety of microorganisms, as their applications and demand continue to increase.

Despite considerable progress in microbial synthesis of AgNPs, several challenges remain, including limited control over particle size, variable yields, and inconsistency in physicochemical properties caused by differences in microbial metabolism. Extracellular biosynthesis provides practical advantages for nanoparticle recovery, but optimization of reaction conditions remains challenging. In this study, we report the isolation of a Bacillus strain from natural spring water and the optimization of its extracellular synthesis conditions to produce AgNPs with uniform morphology and potent antimicrobial activity. These findings offer valuable insights for improving the reproducibility and efficiency of biogenic AgNP production.

MATERIALS AND METHODS

Isolation of bacterial strains

Water samples were collected from different locations of spring water in Ziarat village, Gorgan city. Sterile 50 mL Falcon tubes were used to collect 200 mL of water, which were then transferred to the microbiology laboratory of Babol University of Medical Sciences. 100 µL of each sample was spread on blood agar (BA) medium using a sterile loop and incubated at 37°C for 48 h. After incubation, bacterial colonies were used to prepare slides, and Gram staining was performed for preliminary identification.

Molecular identification

The isolated bacterial strain was molecularly identified via 16S rRNA gene sequencing. Genomic DNA was extracted using the High Pure DNA Isolation Kit (Roche, Switzerland). Proper 16S rRNA primers were selected and checked using NCBI BLAST before ordering from Cinnagen (Iran). PCR amplification was performed using forward primer F (5′-GAGTTTGATCCTGGCTCAG-3′) and reverse primer R (5′-AGAAAGGAGGTGATCC-3′) in a 50 µL reaction mixture containing 1 µL of genomic DNA, 0.2 U/µL Taq DNA polymerase, 20 pmol of each primer, 1× PCR buffer, and 200 µM of each dNTP. The PCR cycling program included initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products (~1500 bp) were visualized on 1% agarose gel, and sequencing was performed by Bioneer (South Korea). The resulting sequences were analyzed

Isolation of silver nitrate-reducing bacteria

To assess the potential for AgNP synthesis, a single bacterial colony was inoculated into 5 mL of Luria-Bertani (LB) broth and incubated at 37°C for 24 h. For scale-up, cultures were transferred to 100 mL LB broth in 250 mL Erlenmeyer flasks and incubated under the same conditions. Cultures were centrifuged at 4000 rpm for 10 min, and the supernatant was sterile-filtered through a 0.22 µm membrane using a 10 mL syringe. The filtered supernatant was stored in 100 mL Erlenmeyer flasks for further experiments.

Extracellular biosynthesis of AgNPs

Bacillus isolates were cultured on nutrient agar supplemented with 3 mM freshly prepared silver nitrate ($AgNO_3$). $AgNO_3$ stock solution (100 mM) was prepared in double-distilled water, sterilized through a 0.22 µm filter, and diluted immediately before use. The initial pH of the culture medium was adjusted to 9 using sterile 1 M NaOH and measured with a calibrated digital pH meter. After 24 h of incubation at 30°C, colonies were transferred to 500 mL LB broth and incubated at 30°C, 200 rpm for 24 h. Supernatants were separated by centrifugation (4000 rpm, 10 min) and filtered (0.22 µm). A control sample contained supernatant without $AgNO_3$.

Investigation of factors affecting synthesis of nanosilver

Concentration of silver nitrate solution

Final concentrations of 1, 2, 3, 4, and 5 mM were tested in bacterial supernatant(Fig. 1).

pH

The effect of pH (7–11) on nanoparticle synthesis was examined prior to $AgNO_3$ addition (Fig. 1)

Temperature and time

Temperatures of 25, 30, 35, 37, and 40 °C were tested, and AgNP formation was monitored over five days at specific intervals (Fig. 1)

Characterization of AgNPs

Nanoparticles were examined using visual color change, UV–visible spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and dynamic light scattering (DLS) .Absorption spectra (400–700 nm) were recorded during

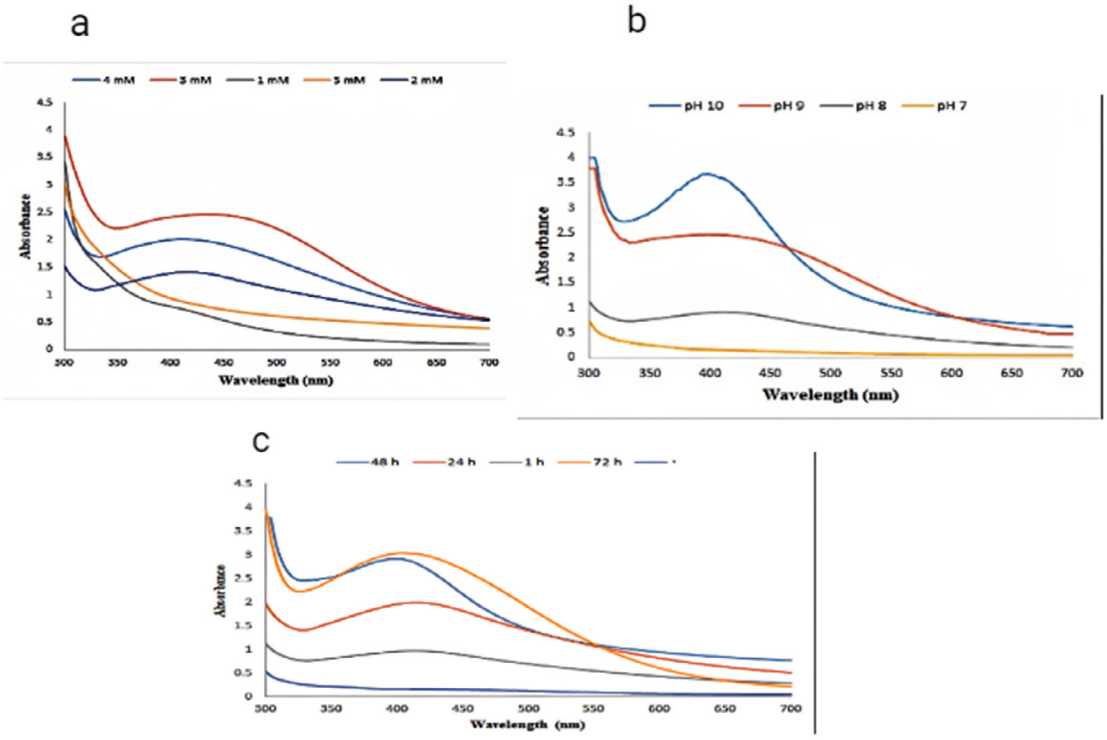

Fig. 1. UV–Vis spectra of AgNPs showing (a) $AgNO_3$ concentration, (b) pH, and (c) reaction time effects at 433 nm.

synthesis at different pH levels and time points using a UV-1800 spectrophotometer (Shimadzu, Japan)

Dynamic light scattering (DLS)

Zeta potential, polydispersity index (PDI), and particle size distribution were measured after diluting samples in normal saline to 200 µL. Analyses were performed at 25 °C using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Freeze-dried nanoparticles were used

Scanning electron microscopy (SEM)

Nanoparticle morphology was analyzed using SEM (AIS2100; Seron Technologies) at Bim Gostar facility (Tehran, Iran). Samples were cryopreserved at −20 °C for 24 h prior to imaging

Scanning electron microscopy (SEM, AIS2100; Seron Technologies) was used to characterize the morphology of cryopreserved nanoparticles at Bim Gostar facility (Tehran, Iran). For cryopreservation, nanoparticle solutions were frozen at -20°C for 24 hours under reduced pressure evaporation conditions.

X-ray diffraction (XRD) analyses

X-ray diffraction analysis was conducted using a Bruker D8 Advance diffractometer equipped with Cu Kα radiation (λ = 0.15406 nm) operating at 45 kV and 40 mA. A single droplet of nanoparticle solution was deposited onto a glass slide and air-dried at room temperature prior to XRD measurements.

Silver nanoparticles antibacterial activity

Minimum inhibitory concentration (MIC) testing was performed using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Three bacterial reference strains—*Escherichia coli* ATCC 25955, *Staphylococcus aureus* ATCC 35984, and *Pseudomonas aeruginosa* ATCC 10145—were obtained from the microbial culture collection of the Department of Microbiology, Babol University of Medical Sciences.A 96-well microplate was prepared by dispensing 100 µL of nutrient broth into each well. The highest concentration of silver nanoparticles (AgNPs) tested was 125 µg/mL. Serial twofold dilutions were prepared by adding AgNPs at 250 µg/mL to the first well and transferring 100 µL sequentially. One microliter of bacterial suspension adjusted to 0.5 McFarland was added to each well. The negative control contained only medium, and the positive control contained medium plus bacteria.

After 24 h incubation at 37 °C, bacterial growth was quantified by measuring optical density (OD) at 610 nm. All experiments were performed in triplicate. To validate the findings, an agar dilution assay was performed for selected strains using the same concentration range. Additional controls included wells containing cephradine (antibiotic control) and AgNP-free wells (nanoparticle control

Analyses of statistical data

Statistical analyses were performed using SPSS software, version 27.0 (IBM Corp., Armonk, NY, USA). Independent sample t-tests were used to compare differences between two groups, with $p < 0.05$ considered statistically significant.

RESULT

Isolation of silver-tolerance bacteria from water samples

Eighteen bacterial strains were isolated from spring water, four of which showed the highest tolerance to silver ions. These strains were tested in LB broth for extracellular reduction of Ag^+ to silver nanoparticles (AgNPs). The selected strain, presumptively identified as Bacillus licheniformis based on Gram-positive rods, catalase-positive, endospore-forming, and gelatin hydrolysis-positive traits, demonstrated strong extracellular AgNP synthesis.

The formation of AgNPs was confirmed by a distinct color change from light yellow to brown in the culture medium containing 3–4 mM $AgNO_3$ (Fig. 1). No change was observed in controls without bacteria or with $AgNO_3$ alone.

Molecular identification

16S rRNA gene sequencing revealed 99.51% similarity to Bacillus spp., confirming the isolate's identity (Fig. 2).

Optimal temperature for AgNP synthesis

The color intensity and UV–Vis absorbance at 420 nm increased with temperature, reaching a maximum at 37 °C. At 50 °C, absorbance decreased significantly, indicating that high temperatures inhibit synthesis (Fig. 3).

Characterization of AgNPs

UV–Vis spectroscopy

AgNPs showed a surface plasmon resonance peak at 400–450 nm, which was absent in the

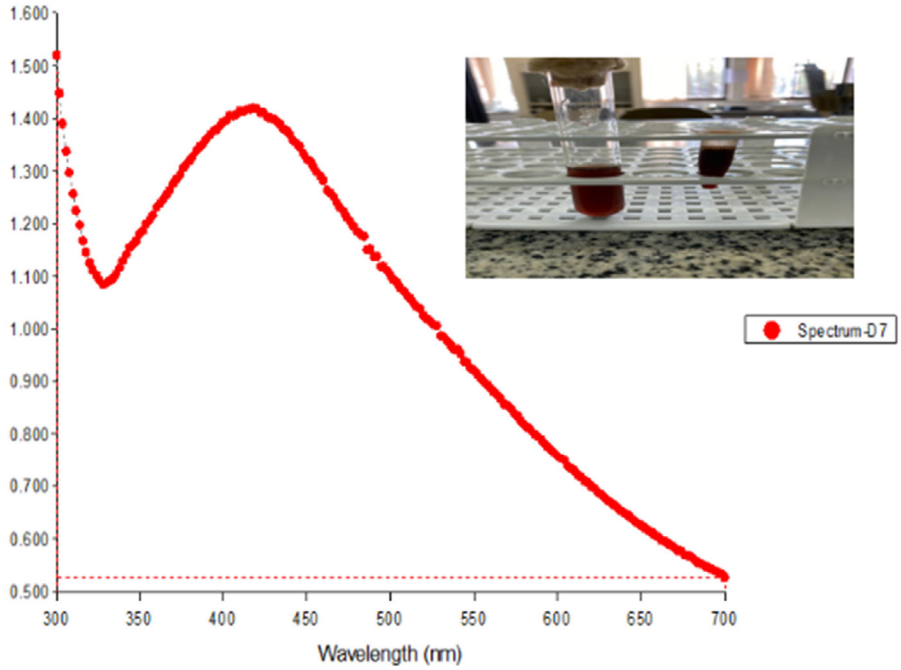

Fig. 2. UV–Vis spectrum of biosynthesized AgNPs showing the color change during the reaction.

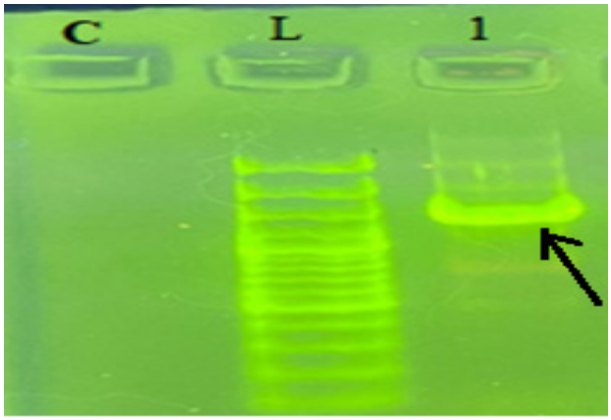

Fig. 3. Electrophoretic analysis of DNA on 1.5% agarose gel. Lane C: negative control; Lane L: 100 bp ladder; Lane 1: Bacillus producing AgNPs.

control samples (Fig. 4).

SEM analysis

Nanoparticles were well-dispersed, spherical, and measured 30–50 nm in size (Fig. 4).

FTIR analysis

Peaks at 3112 cm^{-1} (O–H/N–H), 1616 cm^{-1} (Amide I), 1539 cm^{-1} (Amide II), and 1393 cm^{-1} (carboxylate) indicated the involvement of proteins and biomolecules in the stabilization and reduction of AgNPs (Fig. 5).

XRD analysis

Four diffraction peaks at 38°, 44°, 64°, and 77° corresponded to the (111), (200), (220), and (311) planes, confirming face-centered cubic (fcc) crystalline silver and high purity (Fig. 6).

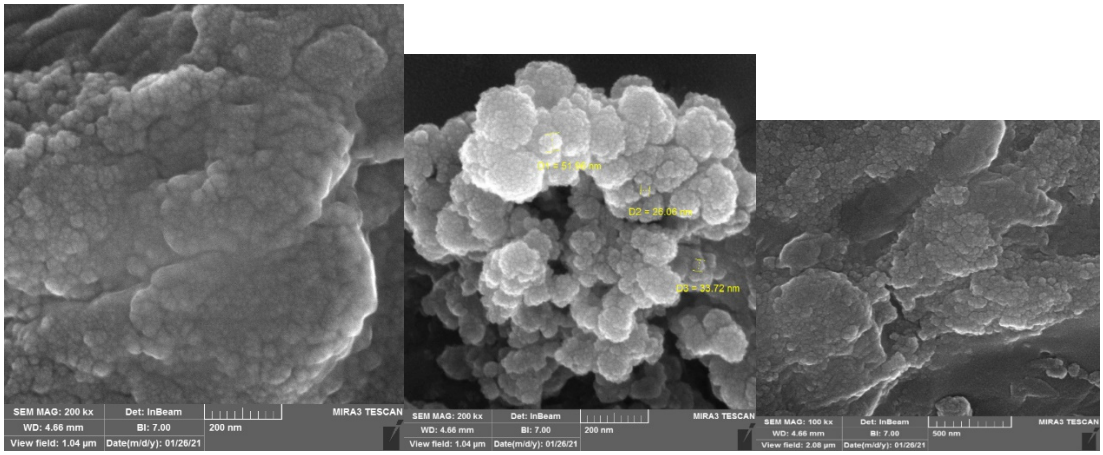

Fig. 4. SEM image of AgNPs synthesized by Bacillus, showing the nanoparticle morphology and estimated diameter.

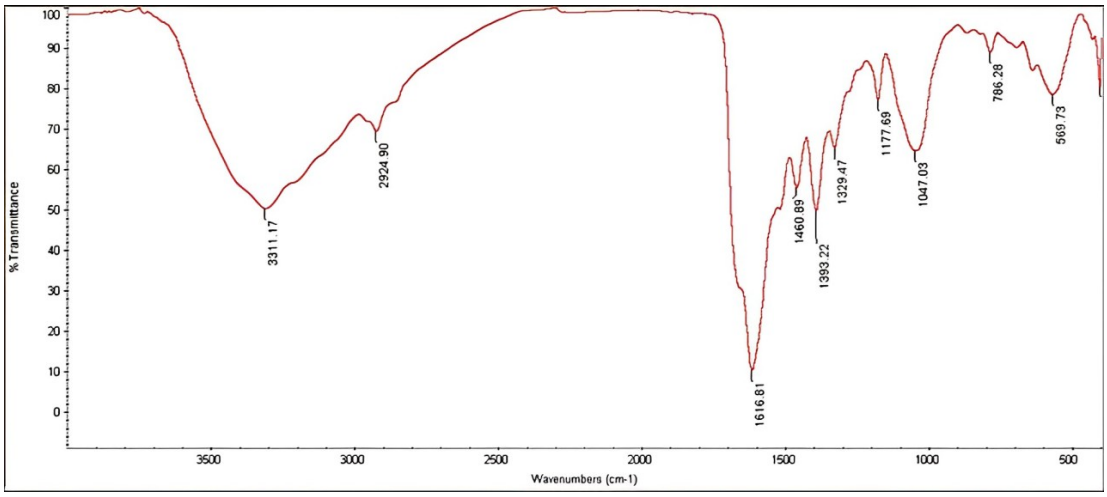

Fig. 5. FTIR spectrum of silver nanoparticles prepared from Baccilus culture supernatant

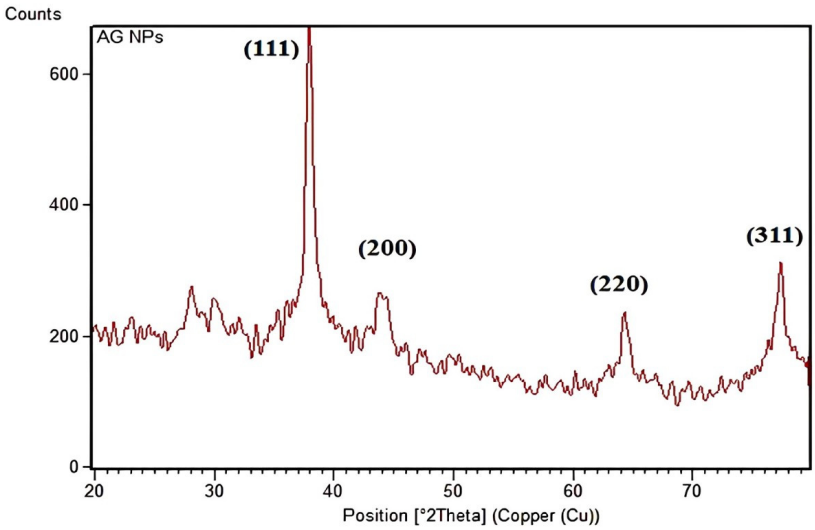

Fig. 6. XRD pattern of biosynthesized silver nanoparticles (AgNPs)

DLS & Zeta potential

The average hydrodynamic size of AgNPs was 98.2 ± 40.8 nm (mode 77.5 nm), and the zeta potential was –34.9 mV, indicating uniform particle distribution and good colloidal stability (Fig. 7).

AgNPs' antimicrobial activity

AgNPs exhibited strong antibacterial activity against E. coli, S. aureus, and P. aeruginosa, with inhibition zones comparable to gentamicin. No significant differences were observed in inhibition zones (E. coli: p = 0.251; S. aureus: p = 0.519; P. aeruginosa: p = 0.251). Even at low concentrations (10 mg/mL), AgNPs inhibited bacterial growth by up to 98% (Table 1, Fig. 8).

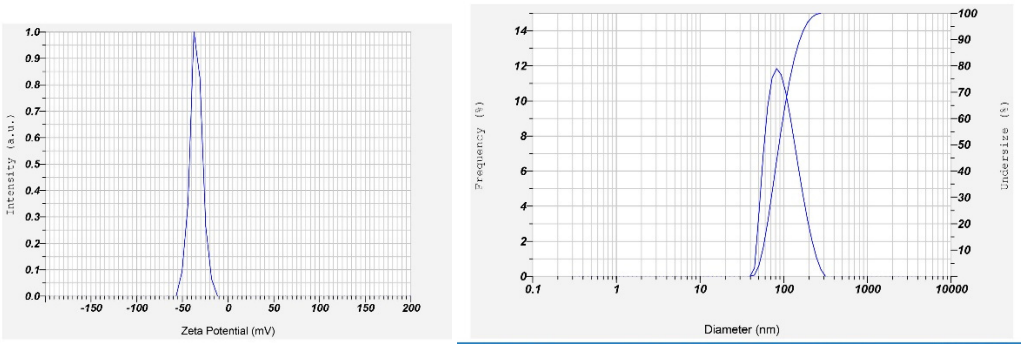

Fig. 7. Hydrodynamic size (DLS) and surface charge (Zeta potential) of the synthesized AgNPs.

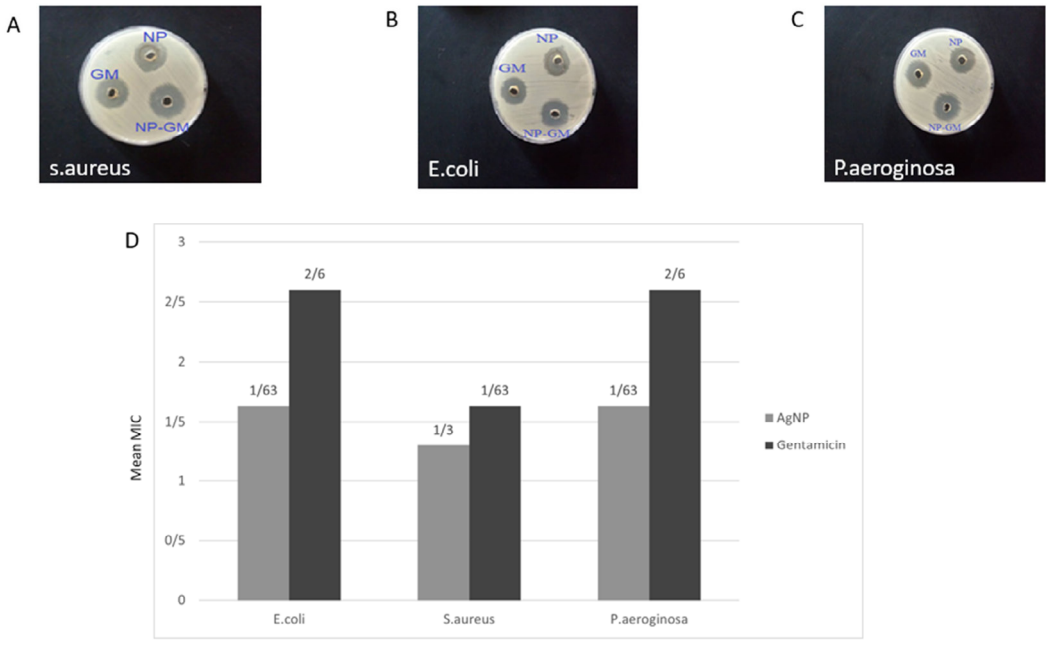

Fig. 8. A Comparison of antimicrobial activity (zone of inhibition and MIC) of AgNPs and gentamicin on S. aureus, E. coli, and P. aeruginosa.

Table 1. Inhibition halo diameters (mean ± SD) of antibiotics and AgNPs on three bacterial strains.

Microorganism	AgNps	Gentamicin	P-value
E.coli	1.63±0.56	2.60±1.13	0.251
S.aureus	1.30±0.56	1.63±0.56	0.519
P.aeruginosa	1.63±0.56	2.60±1.13	0.251

DISCUSSION

There are several methods to produce silver nanoparticles (AgNPs), but biological synthesis has proven to be one of the most effective approaches. The natural production of AgNPs has gained attention in recent years due to the growing interest in developing eco-friendly nanomaterials [11]. Numerous studies have demonstrated the broad-spectrum antibacterial activity of biosynthesized nanoparticles. However, a major challenge remains in engineering nanoparticles with precisely controlled physicochemical and biological properties [12].

The physicochemical properties of AgNPs strongly depend on the microbial species used in their synthesis. This underscores the importance of exploring novel microbial strains capable of producing nanoparticles with tailored characteristics. In this study, extracellular synthesis of AgNPs was investigated using a diverse range of soil-derived bacteria. Among them, only one Bacillus strain isolated from Ziarat converted silver ions into nanoparticles at 3–4 mM concentrations, showing the highest sharp absorption peak within two days after exposure to silver nitrate [10]. Genomic analysis revealed the presence of nitrate reductase, a key enzymatic catalyst for AgNP biosynthesis [10,13]. This enzyme likely plays a pivotal role in reducing Ag^+ ions and stabilizing nanoparticles through the production of NADH and NADH-dependent enzymes in the extracellular medium [13–15].

Besides microbial factors, pH and temperature significantly influence AgNP synthesis. High pH levels catalyze the conversion of monosaccharides into aldehyde chains, which are then oxidized to carboxyl groups in the presence of silver ions [14,16]. Acids generated during this process may induce particle aggregation or convert nanoparticles into microparticles [17]. In our experiments, the observed color change corresponded to an alkaline pH shift [9–10] during nanoparticle formation. This finding aligns with previous studies on Penicillium fellutanum-mediated AgNP synthesis, where increasing pH was associated with particle enlargement [17]. Gericke and Pinches (2006) also demonstrated a pH-dependent effect on nanoparticle size and monodispersity [18,19]. Research has shown that increasing pH from 3 to 9 can increase average particle size by more than 30 nm, significantly affecting size distribution and activating oxidoreductase enzymes, suggesting a dual mechanistic effect of pH on nanoparticle characteristics [20,21].

Reaction time and temperature are also crucial parameters. Experimental evidence shows that both factors significantly influence size distribution and morphology of AgNPs [22]. In this study, nanoparticles were successfully synthesized within 30 minutes at 37 °C, whereas B. persicus and B. licheniformis did not produce nanoparticles even after 48 hours [23]. Shorter exposure to silver ions generally yielded smaller particles with improved monodispersity, while longer exposure increased particle size [18,22].

The production of nanoparticles is influenced by various factors, including microbial species, pH, substrate concentration, growth phase, temperature, growth medium, and reaction time [24]. Despite extensive research, achieving consistent particle size and monodispersity remains challenging. FE-SEM analysis confirmed the formation of spherical nanoparticles with an average size of 35 nm, consistent with other studies reporting Bacillus licheniformis, B. amyloliquefaciens, Rhodobacter sphaeroides, and Streptomyces can synthesize spherical AgNPs [23,25,26]. Biologically produced nanoparticles typically range from 4 to 94 nm, with smaller particles (10–20 nm) exhibiting enhanced cytotoxicity and antibacterial activity [26]. In this study, AgNPs showed a suitable size distribution and no aggregation, indicating effective stabilization [27].

Fourier-transform infrared spectroscopy (FTIR) revealed absorbance peaks at 3300 cm^{-1} (–OH/N–H) and 2810 cm^{-1} ($–CH_3$/$–CH_2$), suggesting that amino acid residues and proteins play a role in binding silver ions, forming a protective layer that prevents aggregation and stabilizes the nanoparticles [28–31].

The antimicrobial activity of these biosynthesized AgNPs was evaluated against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa using disc diffusion and minimum inhibitory concentration (MIC) assays. All tested strains were highly susceptible, with AgNPs showing low MICs comparable to gentamicin, likely due to the release of Ag^+ ions. The diameter of inhibition zones increased with AgNP concentration, consistent with the known effects of nanoparticle size, morphology, and microbial strain sensitivity [32–34].

Combining AgNPs with antibiotics has been reported to significantly reduce bacterial resistance,

sometimes lowering MIC values by up to 2000-fold [35,36]. Extracellular synthesis using bacterial supernatant offers advantages over intracellular methods, including simplified purification, avoidance of hazardous reducing agents, and scalability for industrial production [37–42]. Nanoparticles produced extracellularly, with their small size and high surface-to-volume ratio, exhibit enhanced interactions with bacteria, presenting a promising approach against antibiotic-resistant strains.

To enhance the practical applicability of the synthesized silver nanoparticles (AgNPs), future research should focus on surface modification with polymers or amino/acidic compounds to improve stability, bioavailability, and therapeutic efficacy. Additionally, scaling up the extracellular synthesis for industrial production is essential. Optimization of culture conditions, reactor design, and downstream processing could enable large-scale, cost-effective, and eco-friendly production of AgNPs for applications in pharmaceuticals, medical devices, and water treatment, bridging the gap between laboratory research and industrial use [43,44]. While gentamicin served as the primary reference antibiotic in this study, expanding minimum inhibitory concentration (MIC) comparisons to a broader panel of antibiotics would provide a more comprehensive evaluation of the antimicrobial potency of AgNPs. Ultimately, polymer-coated AgNPs have the potential to enhance therapeutic outcomes in diverse contexts, including bacterial infections, skin infections, medical prosthesis-related infections, and contact lens-associated microbial complications. Despite these promising findings, the study is limited by the absence of cytotoxicity assessments and in vivo evaluations, highlighting the need for further investigations to fully assess the safety, biocompatibility, and real-world biological performance of the synthesized nanoparticles.

CONCLUSIONS

In this study, spherical silver nanoparticles (AgNPs) were successfully synthesized using an eco-friendly extracellular method with Bacillus subtilis supernatant. This approach is rapid (completed within hours), simple, cost-effective, and easily scalable. Critical factors, including silver nitrate concentration, light exposure, temperature, and pH, were found to significantly influence nanoparticle formation, size, and overall quality.

Importantly, the synthesized AgNPs exhibited potent antibacterial activity against human pathogens, demonstrating efficacy comparable to the standard antibiotic gentamicin. These findings indicate that biosynthesized AgNPs hold promise as alternative agents for combating antibiotic-resistant infections. Nonetheless, further studies are warranted to fully assess their clinical safety, biocompatibility, and therapeutic efficacy in vivo.

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AUTHOR CONTRIBUTION

All authors have read and approved the final version of the manuscript and have agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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