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ECO-FRIENDLY SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES MEDIATED BY THERMOPHILIC *BACILLUS* SP. B1

Gunay Abbasli
Baku State University, Baku State University, 23 Z. Khalilov St, AZ-1073, Baku, Azerbaijan

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The biological synthesis of silver nanoparticles is considered a green approach to nanoparticle fabrication that is both economical and environment-friendly and thus has been attracting considerable attention in recent years. In this study, the silver nanoparticles produced by the thermophilic bacterium *Bacillus* sp. B1 were characterized. According to the UV-Vis findings, there was a clear absorption peak at 421 nm, indicating the production of silver nanoparticles. The XRD findings showed that the silver nanoparticles were crystalline with diffraction peaks of metallic silver. Fourier transform infrared (FTIR) analysis identified the presence of organic molecules, including proteins and hydroxyl containing compounds, involved in the reduction and stabilization of the nanoparticles. The silver nanoparticles were analyzed using TEM. These were seen to concentrate in the bacterial cell, cell membrane, and were further isolated for analysis in the form of free particles. The size of the particles ranged from 11 to 13 nm with a predominance of oval-shaped particles. The intensity of color was found to be within the 5200–5400 range, indicating that the particles had metallic properties. In summary, the results of the research demonstrate that thermophilic bacteria can be used successfully for the biosynthesis of silver nanoparticles.

Keywords: Silver nanoparticles, green synthesis, thermophilic bacteria, UV-Vis spectroscopy, Transmission electron microscopy, X-ray diffraction, nanoparticle characterization

INTRODUCTION

Nanotechnology as a science plays a key role in the development of high-tech industries and is also widely used in biomedicine. There has been a growing amount of research on nanotechnology, particularly involving the green synthesis and characterization of nanoparticles [1, 2].

Control over the size and shape of nanoparticles allows for targeted modification of physicochemical properties such as optical, electrical, magnetic and biological ones. These properties make nanoparticles widely used in the development of new dosage forms, targeted drug delivery, tumor imaging and the creation of biocompatible coatings and functional materials [3]. The mechanism of silver nanoparticle antimicrobial action is based on interaction with the microbial cell membrane, the generation of reactive oxygen species, the inactivation of enzymatic systems and the disruption of nucleic acid replication. Silver nanoparticles are currently widely used in antiseptic coatings, medical implants, dressings, food packaging and wastewater treatment technologies [4, 5].

Despite the high efficiency of traditional physical and chemical methods for producing nanoparticles, their use is characterized by a number of limitations. These include the use of toxic reducing agents and stabilizers, the formation of byproducts, difficult purification and the high energy intensity of the processes. Furthermore, the presence of residual chemical reagents on the surface of nanoparticles limits their use in biomedical applications. In this regard, the biological synthesis of nanoparticles is particularly relevant.The biological synthesis process provides an environmentally friendly and cost-effective approach under

E-mail address: gunay.abbasli7505@gmail.com

relatively mild reaction conditions. Microorganisms such as bacteria, actinomycetes, and fungi are investigated in metal nanoparticles synthesis. Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities. Biomolecules such as proteins, enzymes, vitamins and polysaccharides perform dual functions acting as reducing agents and nanoparticle stabilizers preventing their aggregation and ensuring colloidal stability [6,7,8;9,10].

In recent years, particular attention has been paid to the search for new microbial strains capable of synthesizing silver nanoparticles. While many investigations have focused on the biogenic production of silver nanoparticles by mesophilic bacteria of the genus *Bacillus*, relatively little research has concentrated on the use of thermophiles in nanoparticle generation. Considering that thermophilic bacteria are able to exist and function at elevated temperatures their use in the synthesis of silver nanoparticles can contribute to increased process efficiency by accelerating reactions, reducing the risk of microbial contamination and increasing the stability of the resulting nanomaterials [11,12,13]. In addition, transmission microscopy makes it possible to study nanoparticles at the ultrastructural level, bioaccumulated in the cells of eukaryotic and prokaryotic organisms, including bacteria [14-19].

Thus, the biological synthesis of silver nanoparticles using thermophilic bacteria is a relevant area of research that combines the principles of environmental safety, economic efficiency and high functionality of the resulting nanomaterials. This research can help to further develop methods of biosynthesis of silver nanoparticles used in biomedicine and biotechnology.

MATERIALS AND METHODS

Test microorganism

A strain of the thermophilic bacterium *Bacillus* sp. B1 isolated from the Babazan thermal spring in the Salyan region of the Republic of Azerbaijan, was used as a test microorganism for the synthesis of silver nanoparticles. The isolation methods, genus identification, morphocultural characteristics, and some physiological and biochemical characteristics of the strain have been described in detail in our previous article [20].

Synthesis of silver nanoparticles

Silver nanoparticles were synthesized using biomass from the *Bacillus* sp. B1 strain, which had been previously separated from the culture fluid by centrifugation. Ten grams of wet biomass were added to 100 ml of a 1 mM silver nitrate solution, while 50 ml of cell-free supernatant were mixed with an equal volume of a 1 mM silver nitrate solution. The reaction mixtures were incubated in the dark at 55°C. The conditions for biomass production, the formation of the reaction mixture with the silver nitrate solution, and the color change from turbid white to brown during the incubation period have been described in detail in our previous article [11].

UV-Vis spectroscopy

UV-Vis spectrophotometric analysis of silver nanoparticles was carried out over the wavelength range of 300-900 nm using a Thermo Scientific GENESYS 30 visible spectrophotometer (Analytical Chemistry Laboratory, UFAZ), operating at a spectral resolution of 1 nm. The absorption spectra were recorded as a function of wavelength.

XRD analysis

The crystal structure of silver nanoparticles was studied using X-ray diffraction (XRD) (Bruker D2 Phaser). The spectrum was recorded by CuKα radiation with a wavelength of 1.5406 Å in the 2θ range of 5°-80° along with a LYNXEYE_2 one-dimensional (1D) detector. Crystallographic planes were identified by comparing the obtained diffraction patterns with the standard diffraction of silver (JCPDS 00-004-0783) and using reference patterns from the COD (Crystallography open database).The size of silver nanoparticles was calculated using Scherrer's equation.

TEM analysis

To determine the size and morphology of the synthesized silver nanoparticles, the samples were analyzed using a JEM-1400 transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80-120 kV in the Electron Microscopy Department of the Azerbaijan Medical University.

FTIR analysis

To determine the functional groups of biomolecules involved in the reduction and stabilization of silver nanoparticles, Fourier transform infrared spectroscopy (FTIR) was used (Bruker ALPHA, Germany). The spectra were recorded after the silver nanoparticle samples had been obtained and dried.

RESULTS

The formation of silver nanoparticles was first demonstrated using a UV-visible spectrophotometer, revealing a characteristic absorption spectrum at 421 nm, which is typical of silver nanoparticles. This phenomenon occurs due to surface plasmon resonance, which is explained by the oscillation of free electrons on the surface of the silver nanoparticles (Figure 1).The position and intensity of the absorption peak are influenced by several factors, including particle size, shape, distribution, and are often associated with the formation of relatively stable nanoparticles.

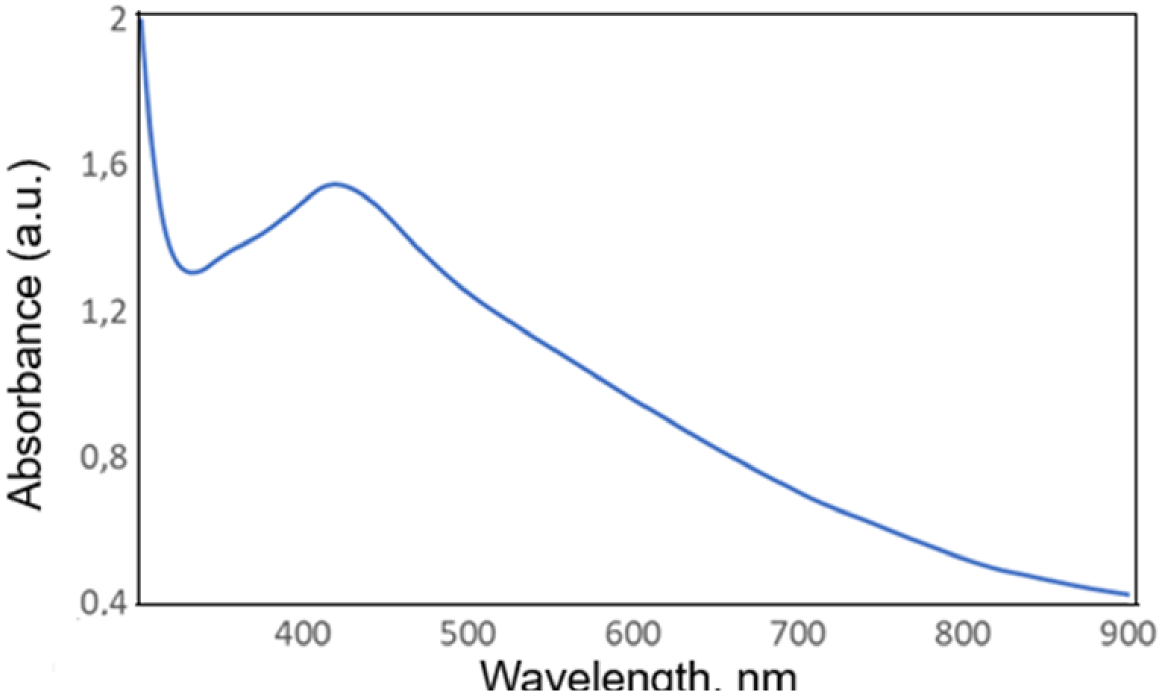

Figure 1. UV-Vis Spectrum of the produced silver nanoparticles by *Bacillus* sp. B1

Crystallinity in the synthesized silver nanoparticles was established through X-ray crystallographic analysis. The diffraction pattern is presented in Fig. 2. Sharp peaks appeared at 2θ values of 38.01°, 44.21°, and 64.29° respectively representing the diffractions at the Bragg planes of (111), (200), and (220). These planes can be indexed on the basis of the crystal structure of face-centered cubic structure of silver. The calculation of average crystalline size has been made by using Scherrer's equation - $D = \kappa \lambda / (\beta \cos \theta)$. Here D is the average crystallite size perpendicular to the reflecting planes, κ is the shape factor which is generally considered to have value 0.9. λ stands for the X-ray wavelength, β is the full width at half-maximum intensity while θ is the diffraction angle.The crystallite size of silver nanoparticles obtained by Scherrer's equation ranged between 5.9 to 11.0 nm with an average size of 8.1 nm.

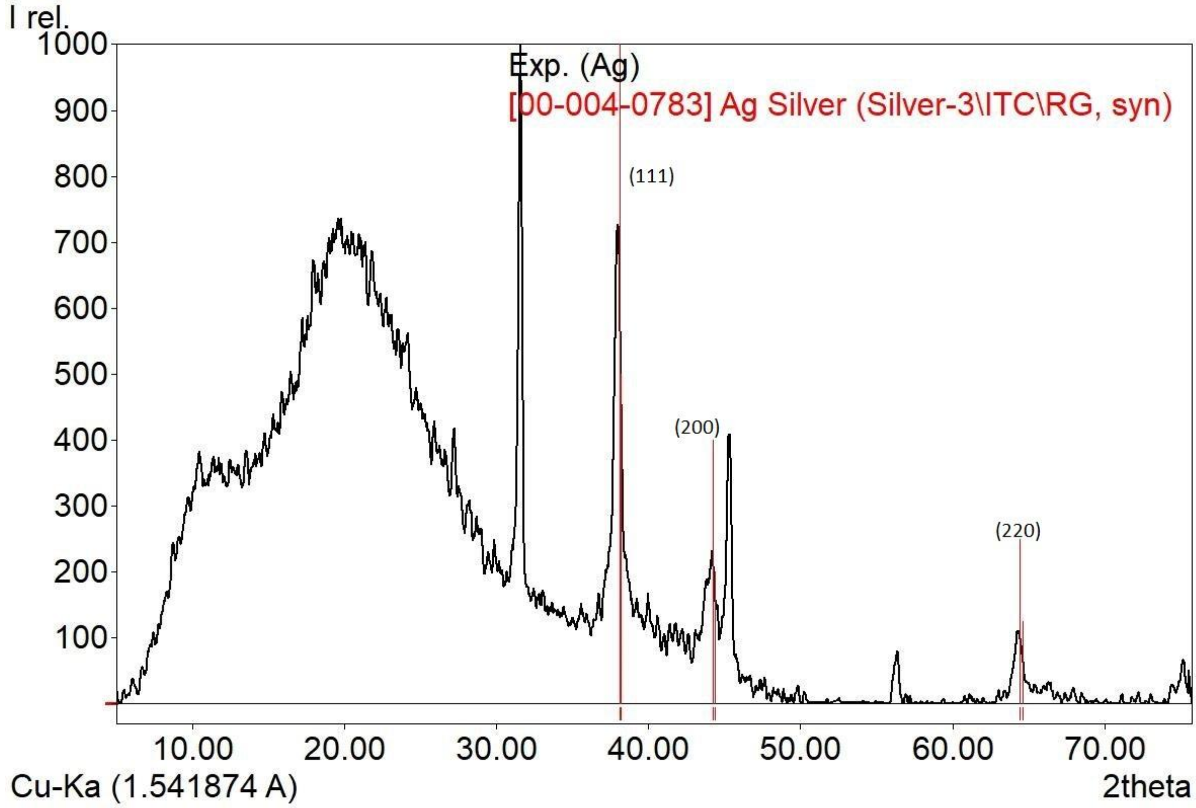

Figure 2. XRD analysis of silver nanoparticles synthesized by thermophilic *Bacillus* sp. B1

These data were also verified using transmission electron microscopy (TEM) aimed at identifying the localization of silver nanoparticles within bacterial cells, in the cell wall, and in the cell-free supernatant. First of all, it should be noted that after the experiments, bacterial samples were placed in a special fixative in accordance with the protocols adopted in TEM studies and after storage for at least one day, araldite-epon blocks were prepared. Samples were collected from these blocks, cut into semithin and ultrathin sections, examined under an electron microscope and electrograms were obtained. Based on the data obtained, Figure 3 was prepared. Electron micrographs and corresponding histograms were prepared based on the ultrastructural data of the *Bacillus* sp. B1 strain. Here, the general appearance of the aforementioned bacterium was determined at high magnifications of the electron microscope, i.e. at a magnification of more than 100,000 times, as well as the bioaccumulation of silver nanoparticles in the cell wall and cytoplasmic elements, both in free and aggregated form (Figure 3A). As a result of the analysis of this electron micrograph using the Intensity Profile program, it was found that the gray value of these particles on the histogram obtained using the red line drawn along these nanoparticles decreased from 6200 to 5400, and the size of the registered particle was 13.0 nm (Figure 3B). Analysis of the obtained data revealed that these parameters correspond to the size and gray value of the metal nanoparticles.

The silver nanoparticles synthesized in the cell-free supernatant of *Bacillus* sp. B1 after exposure to $AgNO_3$ were also studied using TEM. Figure 3C shows a high-magnification electron micrograph of silver nanoparticles in cell-free supernatant. When analyzing individual bioaccumulated particles using the program, the resulting histogram clearly shows that their color intensity decreases from 6100 to 5200 and their size is 12.0 nm (Figure 3D). The nanoparticles recovered from the reaction mixture of the cell-free culture supernatant and silver nitrate solution were also analyzed separately using TEM. Thus, the electron micrograph and corresponding histogram obtained from the precipitated particles after the experiments are presented in Figures 3E and 3F. It was established that the structures in the free state correspond to the shape and structure of the nanoparticles. In Figure 3E, these particles are visually noted at high magnification of the electron microscope and in the corresponding histogram in Figure 3E, the program determined that the intensity of the particles ranges from 6300-5200 and their size is 11 nm.

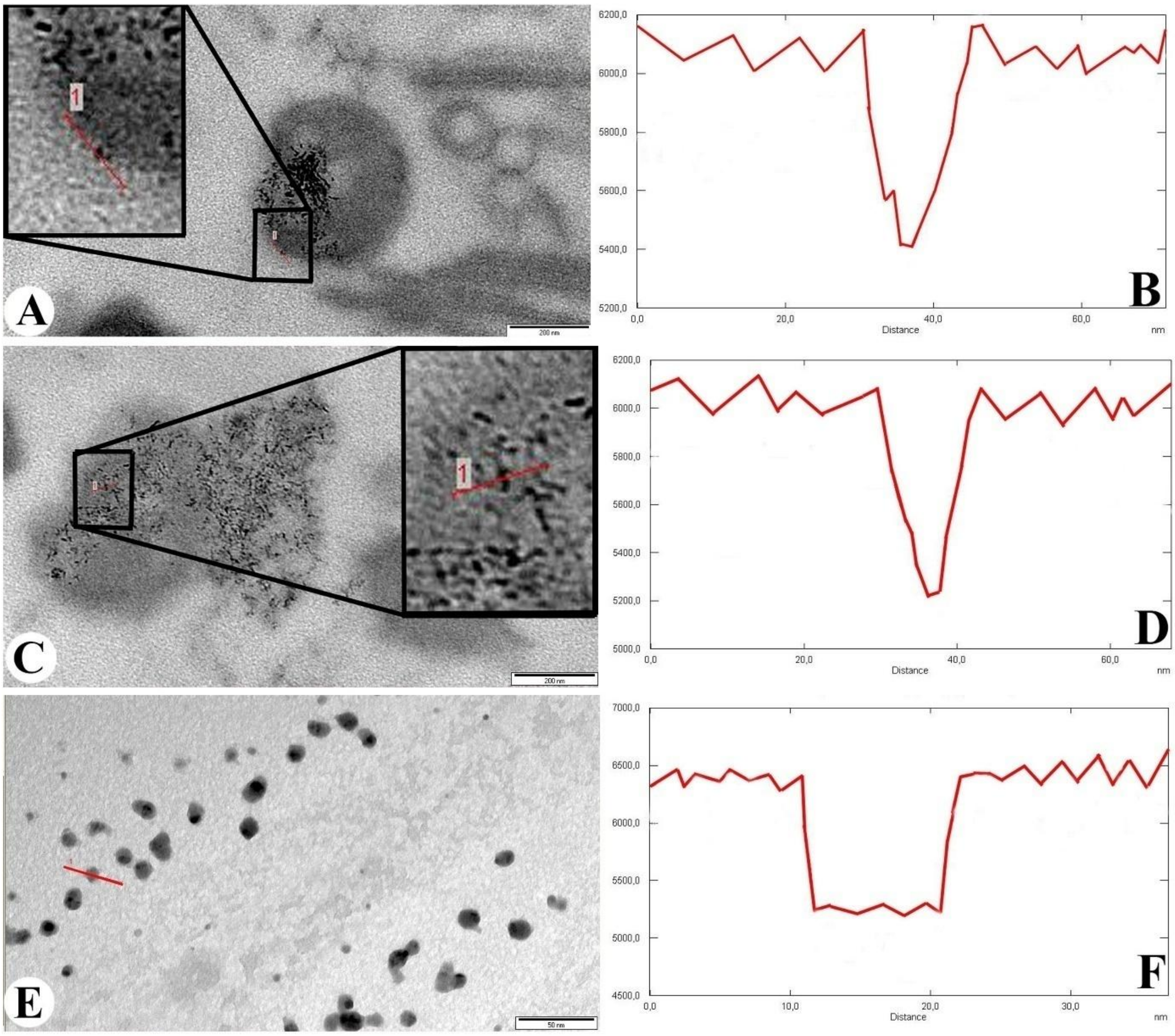

Figure 3.TEM characterization of nanoparticles obtained using *Bacillus* sp. B1. Ultrastructural images and corresponding histogram of silver nanoparticles inside and on the bacterial cell wall (A, B), in cell-free supernatant (C, D), and in free form (E, F).

Thus, as a result of the analysis and comparison of the electron micrographs and the histograms presented in Figure 3, it was established that when the *Bacillus* sp. B1 strain was exposed to $AgNO_3$, the gray value of silver nanoparticles recorded in their cell wall, mainly in their biomass and in free form, corresponded to 5200-5400 and their sizes were 11.0-13.0 nm.

The composition of the functional groups that participate in the reaction of reduction, resulting in the formation of silver nanoparticles, was established by means of FTIR spectroscopy. The data collected from the FTIR spectrometric study of the nanoparticles synthesized is given below in Fig. 4. The broad band at the position of 3285.78 cm^{-1} corresponds to O-H and N-H vibrational frequency, indicating the involvement of hydroxyl and amine group of biomolecules in the preparation of nanoparticles. The weak band found near the position of 2099.88 cm^{-1} can be attributed to vibrations of C≡C or C≡N group. The band at the position of 1636.44 cm^{-1} corresponds to amide I group of proteins, indicating the role of bacteria in nanoparticle preparation. The absorption bands formed at 566 – 433 cm^{-1} are generally associated with out-of-plane bending vibrations and skeletal vibration of organic molecules. These absorption bands may indicate the presence of functional groups attached to the surface of silver nanoparticles.

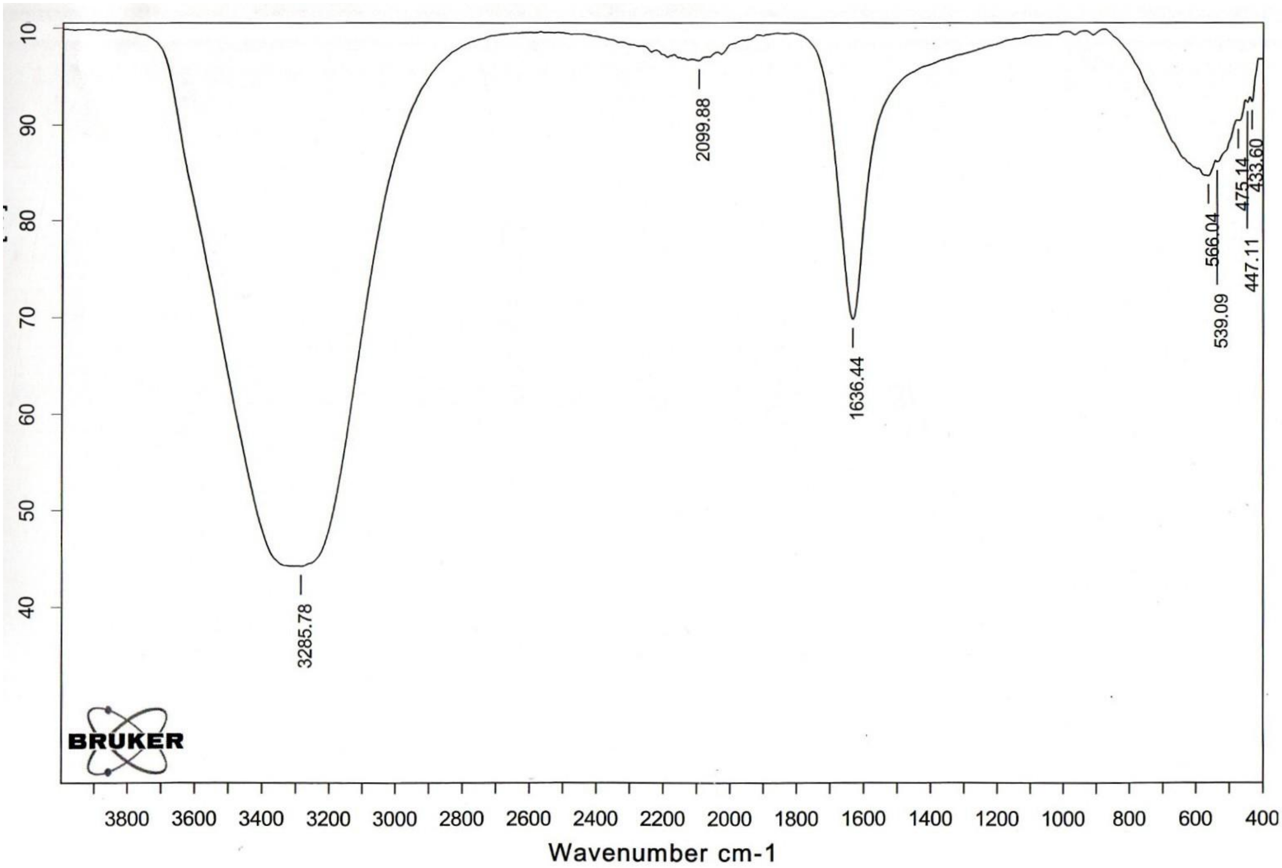

Figure 4. FTIR spectra of silver nanoparticles synthesized by thermophilic *Bacillus* sp. B1

DISCUSSION

Over the past few years, considerable effort has been put into the study of bacteria in order to produce metal nanoparticles as the synthesis of these nanoparticles using microbes has more advantages compared to conventional methods. Also, many bacterial systems are considered suitable for genetic and biotechnological manipulation.

The synthesis of silver nanoparticles using the *Bacillus* sp. B1 strain was studied and characterized in our previous work [11]. This study describes the detailed characterization of silver nanoparticles biosynthesized by this same strain.Spectrophotometric analysis revealed the presence of silver nanoparticles in the sample with absorption at a wavelength of 421 nm which is characteristic of silver nanoparticles. Appearance of such absorption peaks is regarded as the most important criteria for the formation of silver nanoparticles as it occurs due to the collective oscillations of free electrons when exposed to light at the surface of the nanoparticles. Moreover, the optical properties like peak width and absorption largely depend on the following factors: particle size, shape, surface charge, medium, refractive index of the surrounding medium, stability of particles, and surface-adsorbed components.Similar results were also obtained in studies by other researchers. As an example, silver nanoparticles synthesized by Bacillus licheniformis showed an absorption maximum at 440 nm with particles described as spherical and approximately 50 nm in size [21]. Another study found that, nanoparticles produced by Lactobacillus sp. and Bacillus sp. exhibited a peak around 420 nm with sizes ranging from 4.65 to 22.8 nm [22]. Therefore, the absorption range of 410-430 nm obtained in our study is consistent with existing scientific data and confirms the successful synthesis of silver nanoparticles.

TEM analysis revealed silver nanoparticles in the cell wall, cytoplasmic elements, and in the cell-free supernatant. Analysis of the silver nanoparticles using the intensity profile program revealed that the particle size was 11-13 nm, with a gray value of 5200-5400, corresponding to metallic nanoparticles. The small size of the nanoparticles allows them to migrate into the cellular structures of model organisms used in various experiments. Based on the literature data the sizes of silver nanoparticles in a number of studies were confirmed by experimental methods and amounted to 1–100 nm [23], 25.0–75.0 nm [24], in some cases 15.0–25.0 nm [25], in others 10.0 and 20.0–80.0 nm [26], in others 20.00–29.48 nm [27], 1.0–3.0 and 35.0 nm [28] and 78.5–100.0 nm [29]. It can be noted that the nanoparticles produced in the current research are considerably smaller compared to those produced in the other reports. The results indicate that the use of thermophilic Bacillus species for the production of smaller silver nanoparticles from silver nitrate is possible.

The crystalline nature of silver nanoparticles was confirmed via XRD analysis. The peaks obtained for 2θ = 38.01°, 44.21°, and 64.29° belong to the crystal faces of (111), (200), and (220) respectively, which is the face-centered cubic structure of silver metal. This is in agreement with previously reported works. The size of these crystallites calculated by applying the Scherrer's equation ranges between 5.9 to 11.0 nm on an average of 8.1 nm, which is in accordance with that found out via TEM analysis. The reason for the disparity could be that while XRD determines the size of crystalline domains, TEM determines the size of the particles in general, which can include organic capping and nanoparticle aggregation[30; 31].

Our study also revealed that the synthesized nanoparticles are primarily oval in shape with some being round and smooth-surfaced. These findings are consistent with scientific studies noting that silver nanoparticles are round and smooth-surfaced allowing them to easily pass through organelle membranes [29; 32;33].

The presence of organic compounds in the reduction and stabilization process was established through FTIR analysis. The peak at 3285.78 cm^{-1} represents vibrations of the O-H and N-H groups, and the one at 1636.44 cm^{-1} relates to the amide I group of proteins, suggesting the contribution of bacterial proteins in the nanoparticle synthesis. Bands at low frequencies in the range of 566-433 cm^{-1} might be the consequence of the interaction between silver nanoparticles and organic groups that stabilize them [34;35].

Overall, the results of the study show that the *Bacillus* sp. B1 strain is promising for the biological synthesis of silver nanoparticles and can be evaluated for industrial-scale use in the future.

CONCLUSION

This research suggests a biological and eco-friendly method for the synthesis of silver nanoparticles by thermophilic *Bacillus* sp. B1, isolated from the Babazanan hot spring in Azerbaijan.The finding was that the biomass produced by bacteria could effectively take part in the reduction and stabilization process of metal nanoparticles. From the results of the spectrophotometric analysis conducted in the UV-visible region, it was observed that there was a peak at an absorption wavelength of 421 nm for silver nanoparticles. The analysis of X-ray diffraction spectra confirmed the crystallinity of the synthesized silver nanoparticles. Results obtained by FTIR analysis indicated the involvement of organic compounds responsible for the reduction and stabilization of the nanoparticles. TEM images allowed the localization of these particles inside the cellular compartments along with their morphological characterization and size determination, ranging between 11 and 13 nm. These particles exhibited an oval morphology and had a relatively uniform distribution.

In general, the conducted study proved the feasibility of using thermophilic microorganisms for the synthesis of nanomaterials, which can be considered an environmentally safe technology due to its simplicity, low toxicity of the reagents used, and controllability of the characteristics of the final product. Future studies will focus on investigating the biological activity of the obtained nanoparticles, especially their antimicrobial activity, as well as the possibility of practical use of the obtained nanomaterials in various areas.

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