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Research Article

Silver Nanoparticles Synthesized through Green Methods Using *Escherichia coli* Top 10 (Ec-Ts) Growth Culture Medium Exhibit Antimicrobial Properties against Nongrowing Bacterial Strains

Esther Baltazar-Encarnación,[1,2] Carlos E. Escárcega-González,[1,2] Ximena G. Vasto-Anzaldo,[3] María Elena Cantú-Cárdenas,[1,2] and J. Rubén Morones-Ramírez[1,2]

[1]*Universidad Autónoma de Nuevo León (UANL), Facultad de Ciencias Químicas, Av. Universidad s/n, Cd. Universitaria, 66455 San Nicolás de los Garza, NL, Mexico*
[2]*Centro de Investigación en Biotecnología y Nanotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León, Parque de Investigación e Innovación Tecnológica, Km. 10 Autopista al Aeropuerto Internacional Mariano Escobedo, Apodaca, Nuevo Leon 66629, Mexico*
[3]*Universidad Autónoma de Nuevo León (UANL), Facultad de Ciencias Biológicas, Av. Universidad s/n, Cd. Universitaria, 66455 San Nicolás de los Garza, NL, Mexico*

Correspondence should be addressed to J. Rubén Morones-Ramírez; morones.ruben@gmail.com

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Finding novel antibiotics and antimicrobial materials has become of great importance to modern society due to the alarming increase in the development of multidrug resistance in various bacterial strains. This problem is even more complex when infections involve bacterial strains in stationary metabolic states, since most of the antibiotics found in the market do not have an effect on bacteria in dormant metabolic states. A promising field to aid in the solution of this problem is nanotechnology, since it offers a wide avenue for the development of potential therapeutics, specifically the use of silver metal nanoparticles. Silver nanoparticles have proven to be highly effective antimicrobial agents and excellent candidates to be engineered and designed into clever delivery systems, taking advantage of their rapid and potent toxicity on prokaryotic cells at low concentrations. Metal nanoparticles are most commonly synthetized through one or a series of redox chemical reactions using powerful but environmentally toxic-reducing agents. Therefore, in this work, we propose a biosynthesis method that allows the production of nanoparticles, with homogenous shapes and narrow size distributions, through an environmentally friendly technique that does not produce toxic residues. Here, silver nanoparticles were produced from silver salt ($AgNO_3$) using three different growth culture media residues from *E. coli* top 10. The three different culture media residues used included LB, LBN, and LBE; all of them displaying a different chemical and nutrient composition. Here, after characterization of the different silver nanoparticles produced with the different media, we demonstrated that the LB culture-conditioned media was the most suitable to produce them since they displayed the most narrow size distribution, with an average 10.6 nm in diameter, a relatively low standard deviation of 5.5 nm, and a narrow UV-vis spectrum absorption peak at 420 nm. The other methods presented larger nanoparticle sizes and broader size distributions. Furthermore, nanoparticles produced with LB Lennox were found to be, at very low concentrations, effective antimicrobial agent against *E. coli* top 10 at stationary phase. Therefore, these results seem to contribute knowledge linked to the production of antimicrobial nanoparticles (Ag-NPs) through green synthesis and represent a platform to treat infections caused by nongrowing bacteria.

1. Introduction/Background

Nanotechnology is a new branch of technology that studies different materials at a nanometric scale, generally materials with dimensions of less than 100 nanometers. It partakes in the design, production, and use of chemical structures with extremely small dimensions, which have been applied in diverse areas such as biology, materials science, medicine, engineering, and electronics. With regard to the medicine field, there is an increasing need to produce new antimicrobial agents that are able to compete against drug-resistant bacteria [1, 2].

One of the most promising nanomaterials for their use as antimicrobial agents are metallic nanoparticles, since they exhibit higher chemical activity due to their crystallographic structure and surface/volume ratio [1, 3]. Metallic nanoparticles have been produced with diverse elements like Au, Ag, Fe, Cu, Zn, Ti, Co, and Ni, being silver and gold nanoparticles the subject of research because of their ease of production, affinity to bind to biomolecules, and attractive physicochemical properties such as antimicrobial and electrical ones [4, 5]. In this context, Ag-NPs (silver nanoparticles) has been widely used as an antimicrobial agent, as well as in the elaboration of ointments and creams to prevent infections in burns and wounds [6]. In addition, one of the main problems in the medical field is the treatment of infections caused by bacterial strains in stationary metabolic phases. This is relevant since most of the available antibiotics do not have an effect on nongrowing bacteria.

There are numerous physical and chemical methods for the synthesis of nanoparticles, being chemical reduction, photochemical reduction, electrochemical reduction, vaporization, and thermal synthesis the most common [6, 7]. However, there has been a growing interest in the application of biological methods using microorganisms [8], plants, and their extracts [9] in what is known as "green synthesis." This type of synthesis allows the formation of nanostructures through techniques that are environmental friendly, cost-effective, and nontoxic [7, 10, 11]. These characteristics will allow the use of green synthetized nanoparticles in the pharmaceutical industry. In the case of synthesis of Ag-NPs using microorganisms, these nanoparticles has been synthesized by means of a novel *Nocardiopsis* sp. MBRC-1 [12], by *Brevibacterium frigoritolerans* DC2 strain [13], as well as by *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas jessinii* strains [14].

In our study, we describe the green synthesis of Ag-NPs using an *E. coli* top 10 (Ec-Ts) culture grown in 3 different media as a reducing agent. The results obtained by our group show that microbial synthesis of Ag-NPs is an appropriate method for the production of nanomaterials with antimicrobial properties against bacteria in nongrowing phases and that can be used in a safe manner in biomedical applications.

2. Methods

2.1. Strain Construction. The strain used in this study was *E. coli* top 10 (Ec-Ts), which was provided by the Biotechnology 3, Laboratory of the School of Chemical Sciences, UANL. The strain was transformed through the insertion of the Puc57-Kan plasmid, using kanamycin as a selection marker.

2.2. Biosynthesis of the Ag-NPs. The bacteria were grown in 3 different media: Luria-Bertani Lennox medium (LB), Luria-Bertani medium plus nitrate (LBN), and Luria-Bertani medium plus lactose (LBE) in 50 ml flasks, using kanamycin (Sigma-Aldrich St. Louis, MO, USA) as a selection marker in a concentration of 100 μg/ml. The experimental cultures were incubated at 37°C at 150 rpm for 48 hours until they reached an optical $OD_{600} = 0.6$. The media were filtered to obtain the supernatant that was used as a reducing agent. Subsequently, 5 ml of the supernatant of each media was transferred to 15 ml Falcon tubes with concentrations of 1 mM and 5 Mm of $AgNO_3$. This mixture was later incubated at 37°C at 150 rpm for 60 hours in darkness to allow the formation of the Ag-NPs in the exponential phase of the bacterial growth.

2.3. Sampling and Characterization of the Ag-NPs. A volume of 100 μl of each sample was taken at 0, 2, 4, and 6 hours. They were evaluated by measuring the turbidity at a 1 : 10 dilution in a GENESYS 20 spectrophotometer at 600 nm. Meanwhile, the absorbance spectra of the Ag-NPs were analyzed using a Varian Cary 50 spectrophotometer. The shape and size of the nanoparticles were confirmed through SEM micrograph and an energy-dispersive X-ray spectroscopy (Nova NanoSEM 200 FEI).

2.4. Antimicrobial Activity Analysis of the Ag-NPs. Aliquots of 100 μl of an *E. coli* top 10 (Ec-Ts) culture were taken, and serial dilutions in saline solution 0.85% were made until the dilution reached 10^6 CFU/ml. Then, 20 μl of each dilution were placed in a 96-well plaque and maintained in a purifier class II safety cabinet (Delta Series, Labconco) until the samples dried. The plaque was incubated at 37°C for 24 hours.

The concentration of Ag-NPs used in this analysis was determined through a sample of the supernatant of the nanoparticles which 5 mM of $AgNO_3$ were added as a source of Ag^+ ions. The detailed calculations followed to obtain the final nanoparticle concentrations can be found in the Supplementary Material (available here). Dilutions were made to obtain the concentrations (15, 30, and 60 μM) of Ag, and these were added to the LB media with *E. coli* top 10 (Ec-Ts) to evaluate their antimicrobial activity.

3. Results and Discussion

In this research, we cultivated *E. coli* in 3 different culture media: LB, LBN, and LBE. We decided to use this bacteria due to its rapid growth, its ability to grow in different media formulations, its widespread use as a model microorganism, and its ability to synthesize nanoparticles under several environmental conditions [15, 16]. Moreover, the supernatant of *E. coli* has been reported to present nitrate reductase enzymes which can carry out the reduction of silver ions during nanoparticle synthesis [17, 18].

When comparing the composition of each media (Figure 1), it is shown that the LB media differs from the LBN and LBE media because it does not contain KNO_3 or

LB Lennox media

Ingredient	Concentration (%)
Peptone	1
NaCl	0.5
Yeast extract	0.5

(a)

LBN media

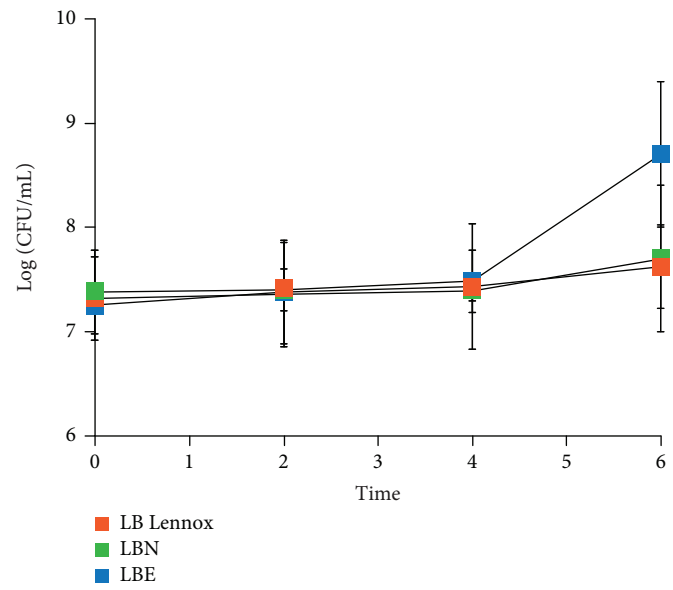
Ingredient	Concentration (%)
Peptone	1
NaCl	0.5
Yeast extract	0.5
KNO_3	0.1

(b)

LBE media

Ingredient	Concentration (%)
Peptone	1
NaCl	0.5
Yeast extract	0.5
KNO_3	0.1
Lactose	1

(c)



(d)

Figure 1: Composition of the media used for the production of the Ag-NPs. (a) LB media. (b) LBN media. (c) LBE media. (d) Bacterial behavior in the lag phase of growth in each media.

lactose. The behavior of *E. coli* top 10 (Ec-Ts) in the lag phase showed that the 3 media were adequate for bacterial growth, where the LBE media displayed optimal growth (Figure 1(d)). A SEM micrograph and an EDX analysis were performed for each sample, in order to confirm the presence of silver nanoparticles (Figures 2(a)–2(c)). This is possible due to the reducing environment provided by the components as enzymes released to the culture media [8].

An SEM and EDX analysis was performed, as shown in Figure 2. It can be observed that the Ag-NPs synthesized with LB, LBN, and LBE media (Figures 2(a)–2(c)) exhibit the presence of silver which confirms that Ag was reduced. It can be noted that Ag is more abundant when the synthesis was carried out in LB media (Figure 2(a)) than in LBN and LBE, so we infer that the reduction in this sample was more efficient. Moreover, the presence of carbon and oxygen suggests that organic compounds are responsible for the stabilization of the nanoparticles [19, 20].

A TEM micrograph was taken, and a total of 400 nanoparticles produced with the culture media were measured. When comparing the diameter of the Ag-NPs produced with the 3 culture media (Figures 3(a)–3(c)), it is shown that the LB media was the most efficient media for the production of Ag-NPs, since these NPs demonstrated a consistent and less disperse diameter of 10.6 nm between them, whereas many other studies have reported the production of Ag-NPs using bacterial cultures, obtaining nanoparticles with diameters that range from 15 to 97 nm [10, 13, 14, 21, 22].

We suggest that the lack of lactose and KNO_3 in the LB media allowed an effective reduction of Ag^+ ions that lead to a uniform diameter of the nanoparticles. This can be attributed to the time at which the supernatant was taken, since by the stationary phase it can be implied that the lactose and the KNO_3 will more than likely be consumed. As for the LB media, since there are no additional nutrients as in LBN and LBE, by the time that the supernatant is taken, the cells

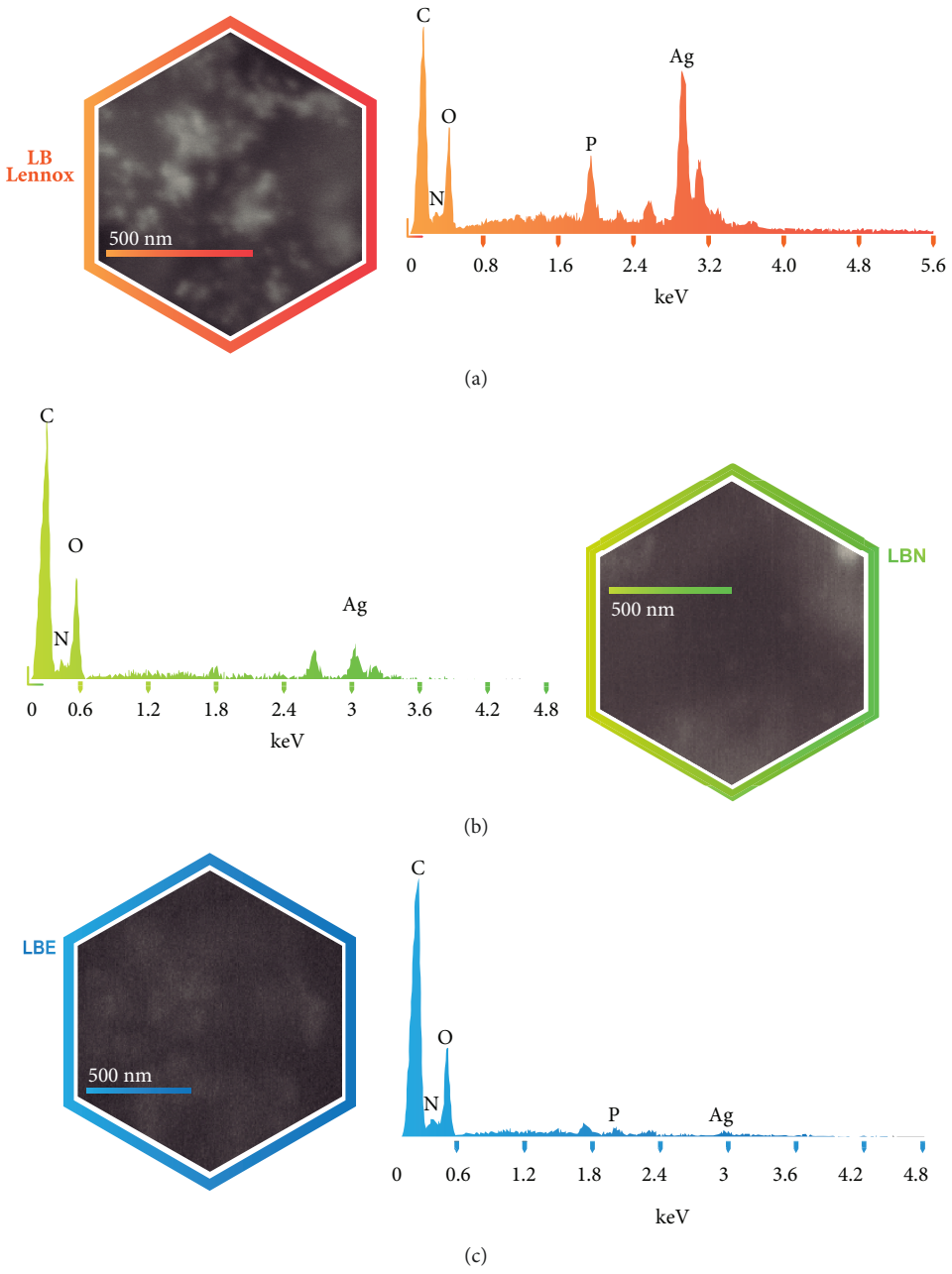


Figure 2: SEM and EDX micrograph of the Ag-NPs produced with the (a) LB media, (b) LBN media, and (c) LBE media.

are under stress conditions. During the stationary phase, this stress can cause the production and secretion of small and diffusible compounds, such as exopolysaccharides, enzymes, and proteins that are able to interact with insoluble metals and cause their reduction [23]. Specifically, in *E. coli*, it has been proposed that the extracellularly secreted enzyme nitrate reductase is responsible for the reduction of silver ions to produce metallic nanoparticles [10, 13].

The absorption peak of the Ag-NPs produced in the 3 culture media was compared at two concentrations (1 mM and 5 mM) of the precursor salt $AgNO_3$. In the Ag-NPs produced with the LB media (Figure 4(a)), the absorption peak was clear and prominent, as opposed to the absorption peaks displayed by the nanoparticles produced with LBN and LBE media (Figures 4(b) and 4(c)). Therefore, the Ag-NPs produced with the *E. coli* top 10 (Ec-Ts) culture in the LB media seemed to be more efficient for the production of nanoparticles with a uniform diameter than the other two media tested. The results could be explained by the composition of the media, since the more nutrients a medium has, the more it can cause metabolic changes in the bacteria which can lead to the emergence of substances that affect the reduction process of the Ag^+ ions to Ag-NPs, preventing a controlled reduction and homogenous nanoparticle size.

Finally, the antimicrobial activity of the Ag-NPs at 3 different concentrations was evaluated in *E. coli* strains in non-growing stationary phase. As the concentration of Ag-NPs increases, the number of bacterial cells decreases, which

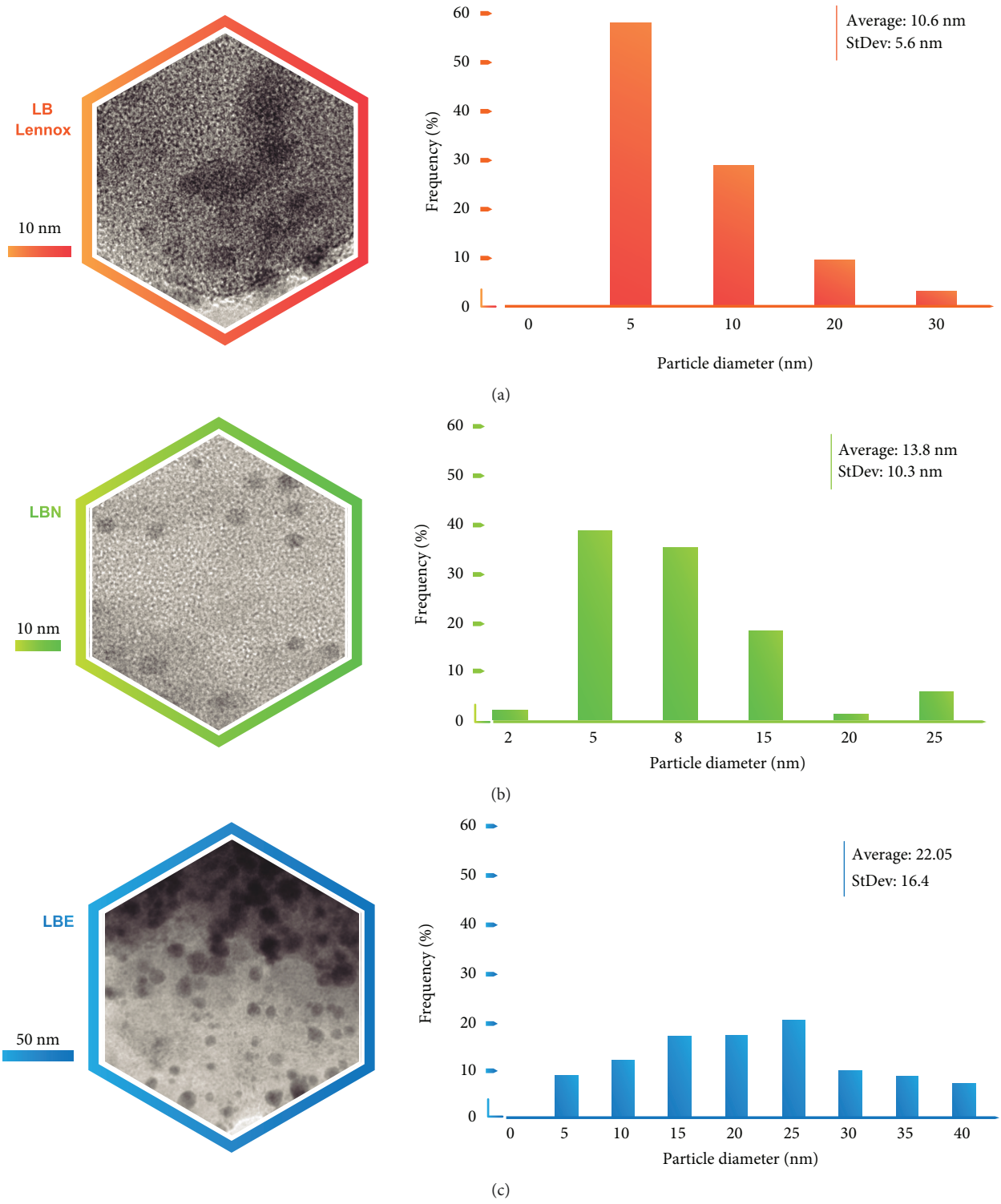


Figure 3: TEM micrograph and diameter distribution graphic of the Ag-NPs obtained with the (a) LB media, (b) LBN media, and (c) LBE media.

indicates that these nanoparticles possess antimicrobial properties against bacteria at nongrowing metabolic states (Figure 5). Similar studies have shown that Ag-NPs produced through green synthesis seem to exhibit antimicrobial properties [21, 22, 24–26]; however, these experiments are carried out in exponentially growing bacteria. To explain the toxic effect that the Ag-NPs have against bacteria, several mechanisms have been proposed, such as the following: (1) alterations in the cell membranes that cause disruption in its permeability and (2) interaction of the Ag-NPs with cell components that contain sulfur like DNA and proteins [27–29].

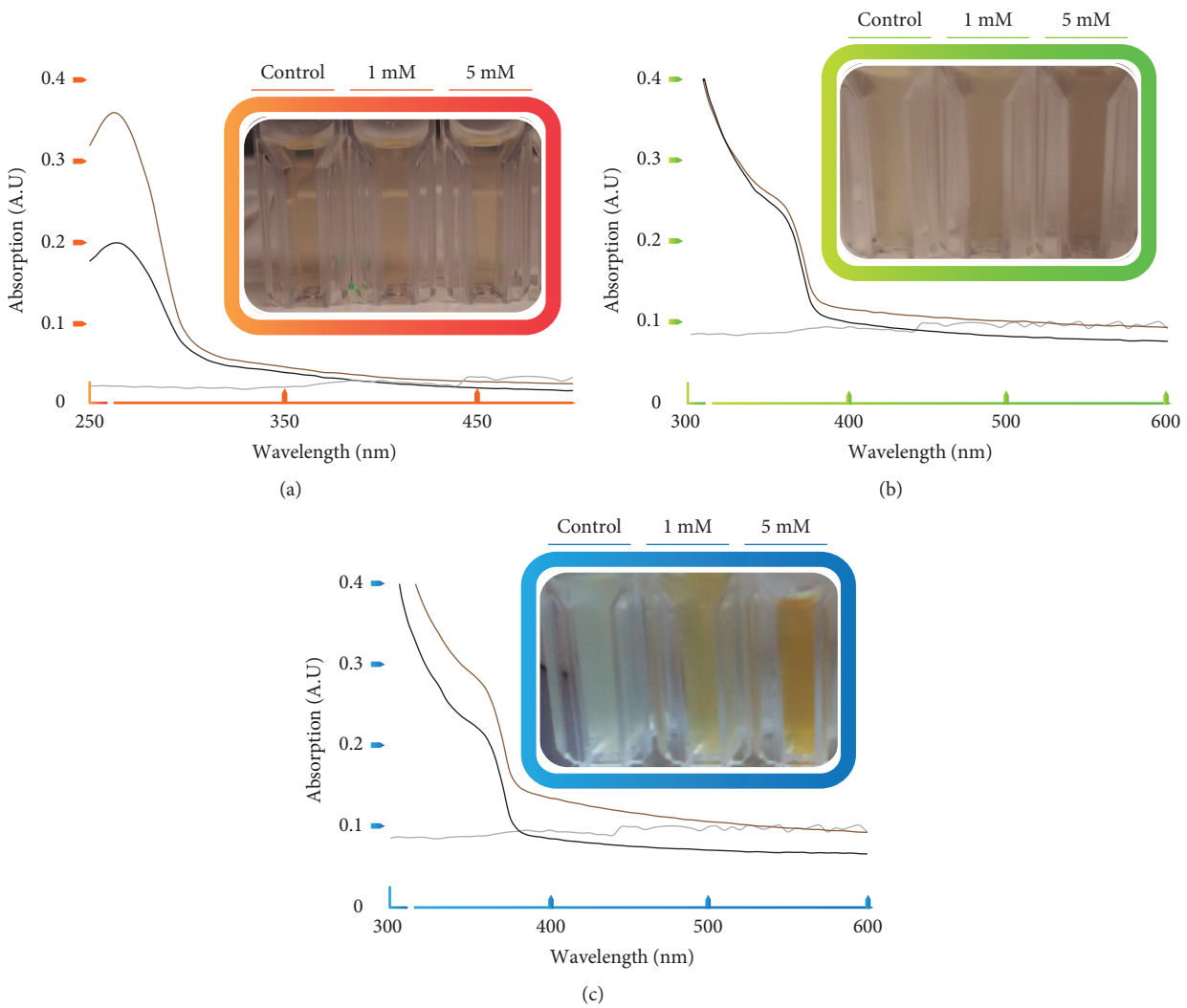


Figure 4: Absorption spectrum of the Ag-NPs produced with the addition of $AgNO_3$ at concentrations of 1 mM and 5 mM in the (a) LB media, (b) LBN media, and (c) LBE media.

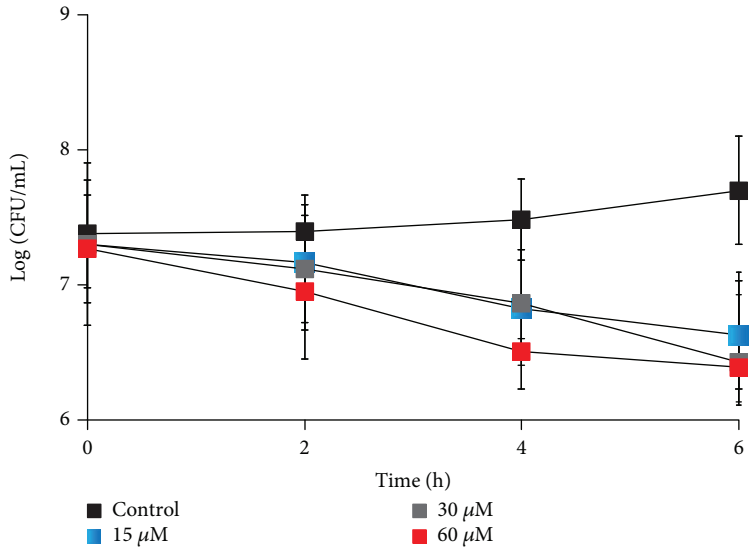


Figure 5: Antimicrobial activity of the Ag-NPs produced with the LB media against nongrowing *E. coli* top 10 bacterial strains.

4. Conclusions

The production of Ag-NPs using the supernatant of an *E. coli* top 10 (Ec-Ts) culture in 3 different media (LB, LBN, and LBE) was accomplished. These nanoparticles were characterized, presenting a uniform diameter and also displayed antimicrobial properties against the transformed bacteria *E. coli* top 10 (Ec-Ts). The results obtained contribute to the knowledge related to the production of Ag-NPs through green synthesis using bacterial cultures. In addition, the silver nanoparticles synthesized here exhibit antimicrobial properties against nongrowing bacteria, a crucial clinical problem in the treatment of infections. These materials propose a platform to develop antimicrobial agents against nonmetabolically active bacterial strains.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

EBE, CEEG, XGVA, MECC, and JRMR designed, performed, and analyzed all of the experimental data and wrote the manuscript. Esther Baltazar Encarnación and Carlos E. Escárcega-González contributed equally to this work.

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Supplementary Materials

Calculations of the estimation of the Ag-NPs concentrations used in the antimicrobial activity assay. *(Supplementary Materials)*

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