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Research Article

# Optimization of the biosynthesis of silver nanoparticles using bacterial extracts and their antimicrobial potential

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ABSTRACT

In the present study, silver nanoparticles (AgNPs) were biosynthesized using the supernatant and the intracellular extract of *Cupriavidus necator, Bacillus megaterium*, and *Bacillus subtilis*. The characterization of the AgNPs was carried out using UV–Vis spectroscopy, FTIR, DLS and TEM. Resazurin microtiter-plate assay was used to determine the antimicrobial action of AgNPs against *Escherichia coli.* UV–Visible spectra showed peaks between 414 and 460 nm. TEM analysis revealed that the synthesized AgNPs showed mostly spherical shapes. DLS results determined sizes from 20.8 to 118.4 nm. The highest antimicrobial activity was obtained with the AgNPs synthesized with supernatant rather than those using the intracellular extract. Therefore, it was determined that the bacterial species, temperature, pH, and type of extract (supernatant or intracellular) influence the biosynthesis. This synthesis thus offers a simple, environmentally friendly, and low-cost method for the production of AgNPs, which can be used as antibacterial agents.

## 1. Introduction

Nanoscience and nanotechnology are research areas that involve the creation and modification of structures, devices, and systems with novel properties and functions due to the atoms' arrangement in the scale of 1–100 nm [1]. Within the different types of nanoparticles (NPs), the most employed are the metallics, which include Ag, Au, Pt, Pd, Cu, Ni, Se, and Fe [2]. These metallics have been used widely because of their optic, magnetic, catalytic, and antimicrobial properties, which allow them to be used in fields like medicine, agriculture, environmental studies, electronics, and more [3,4]. Silver nanoparticles (AgNPs) are currently one of the most used metallics, largely due to their antimicrobial properties against several infectious and pathogenic microorganisms, including multi-resistant bacteria [5]. It has been shown that AgNPs inhibit the bacterial growth on bacteria like *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium,* and others [6,7]. This inhibition may occur because AgNPs can interact with enzymes, proteins, and biological surfactants present on microbial cells, affecting membrane permeability and certain processes like cellular respiration, replication, and transcription; furthermore, it has been demonstrated that AgNPs have antifungal and antioxidant properties [5,8]. AgNPs have this antimicrobial property because they act directly on the cell membrane of bacteria, which generates structural changes such as the formation of membrane pores, increasing the permeability of the membrane and, finally, bacterial death [9,10]. Furthermore, it has been reported that the small size of synthesized AgNPs increases their stability and biocompatibility, since their smallness facilitates internalization through cellular membranes, which is related to efficient antimicrobial activity [11]. Additionally, multiple studies indicate that the non-agglomeration of the nanoparticles plays a key role in their high efficiency in various nanoparticle applications, in this case antimicrobial activity [12]. Currently, synthesized AgNPs are used in food preservation, disinfection, water filtration systems, insecticides, cosmetics, and antimicrobial agent in the medical field, among other applications [5,13].

While AgNPs can be synthesized through physical or chemical processes, green synthesis is most advantageous because it minimizes the use of environmental hazardous substances, costs less than chemical

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synthesis, and is considered an easy protocol for reproduction; in addition to producing nanoparticles with a greater antibacterial effect and through more energy efficient processes [14–16]. Also, it has been determined that the synthesis using microorganisms is faster than using plant extracts [17].

Biological synthesis generally involves the reduction of metallic ions by biological organisms like plants [16–19], yeast, and fungal or bacterial extracts [20,21].Otherwise, the synthesis of AgNPs is a complex process that requires the optimization of different parameters such as pH, temperature, agitation, metal concentration, microorganisms, inoculum size, among others [22] Bacteria are the most used because of their large-scale production, fast cell growth rate, and easy handling. There are numerous examples of AgNPs synthesized from diverse microorganisms, including, *Paenibacillus anseongense* [21], *Bacillus pumilus* [23], *Terrabacter humi* [24], *Pseudomonas alloputida* [25], *Bacillus subtilis*, and *Bacillus megaterium* [26,27]. To our knowledge, there are no studies that use *C. necator* for the green synthesis of AgNPs using supernatants and intracellular extracts [28]. *Cupriavidus necator* is a facultative chemolithoautotrophic bacterium, it has the ability to use nitrate/nitrite as an alternative electron acceptor and has resistance mechanisms towards heavy metals [29–31]. Due to these characteristics, this bacterium was selected as a possible agent for the AgNPs synthesis. Furthermore, *B. subtilis* is a fast-growing Gram-positive bacterium that is easy to cultivate, that has genes encoding for a respiratory nitrate reductase [32,33]. Besides, *B. megaterium* is considered a powerful cell factory widely studied and used in many biotechnological applications such as small molecule biosynthesis, recombinant protein expression, biotransformations, among others [34]. Due to the widely used *Bacillus* spp. in similar research, and the novelty in the use of *C. necator* for the green synthesis of AgNPs, we decided to compare the AgNPs synthesis between these bacteria.

The synthesis and stability of these NPs is due to the biomolecules present in the microorganisms, such as enzymes, proteins, and biotensoactives [4]. Furthermore, it has been determined that bacterial supernatant and intracellular extract can synthesize stable AgNPs [23, 35,36]. Extracellular synthesis is a low-cost technique that has been reported to facilitate large-scale production and that requires simpler downstream processing, otherwise the synthesis through intracellular extracts requires a cellular lysis process (e.g. sonication) to release the molecules needed to carry out the synthesis [4]. To date, many studies focus on extracellular methods for AgNPs synthesis [23,37], while few studies have compared synthesis processes using bacterial supernatants and those using intracellular extracts [35,38]. The study and comparison of AgNPs green synthesis methods, using several bacterial strains and experimental conditions, are fundamental to demonstrate the advantages and drawbacks of the approaches, that can support the selection of adequate synthesis protocols in future studies.

In this study, we optimized the green synthesis of AgNPs mediated by two strains of *Bacillus* (*B. megaterium* and *B. subtilis*) and *Cupriavidus necator* using two approaches (extracellular synthesis and synthesis using intracellular extracts). In addition, for the extracellular synthesis we also evaluated the effect of pH (7, 8, 9, and 10) and temperature (30 and 60 °C) on the synthesis of AgNPs. Ultraviolet spectroscopy (UV–Vis), Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), and transmission electron microscopy (TEM) were used to characterize the synthesized AgNPs. These AgNPs were also studied for their antimicrobial activity against the non-pathogenic *E. coli* ATCC 25,922, which is a well-known strain usually used as quality control in many fields [39–41], to demonstrate their potential application in the medical field. This work determines the use of *C. necator* as an agent for the AgNPs synthesis.

## 2. Materials and methods

### 2.1. Microorganisms

Two Gram-positive bacteria (*Bacillus subtilis* DSM 10 and *Bacillus megaterium* DSM 32) and one Gram-negative (*Cupriavidus necator* DSM 545) bacteria used for the AgNPs green synthesis, were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). All bacterial strains were stored in 15 % glycerol containing nutrient broth medium at −70 °C. Prior to their use in green AgNPs synthesis, the cryopreserved bacteria were grown on lysogeny broth agar (LBA) at 37 °C for 24 h, and 140 rpm, on an 211DS incubator with orbital agitator (Labnet International Inc., Edison, NJ, USA).

### 2.2. Extracellular synthesis of silver nanoparticles

The microorganisms were cultivated on 75 mL of lysogeny broth medium (LB) and maintained at 37 °C on an 211DS incubator with an orbital agitator (Labnet International Inc., Edison, NJ, USA) at 120 rpm for 24 h. After the incubation period, the cultures were centrifuged at 10,000 rpm for 10 min, and the supernatants were used for the next experiments. To carry out the synthesis, 7.5 ml of the supernatant was added drop by drop with constant agitation (550 rpm) on 30 mL of 1 mM aqueous $AgNO_3$ solution. Subsequently, the mixture was maintained on constant agitation at 550 rpm for 27 h under darkness conditions. The temperature (30 °C and 60 °C) and pH (7, 8, 9, and 10) effects were studied on the AgNPs' formation. The pH was adjusted with NaOH and HCl. All the treatments were conducted in triplicate, using as control the microorganisms' supernatant without $AgNO_3$ under the same conditions described above. The AgNPs green synthesis was visualized by colorimetric changes in the solution, turning from yellow pale to brownish tones. Synthesis was also confirmed using the UV–vis spectrophotometer at 1 h intervals for 27 h.

### 2.3. Green synthesis of silver nanoparticles using intracellular extracts

The bacterial growth described in Section 2.2 was centrifuged at 5000 rpm, and then 2 g of biomass was resuspended on 10 mL Milli-Q® water and centrifuged for 10 min at 5000 rpm. This process was conducted three times to wash the biomass. Subsequently, the obtained suspension was subjected to cellular lysis by sonication for 12 min, with a 15 s pulse on and a 45 s pulse off, at 45 % amplitude, using the Qsonica Q700 sonicator (Cole-Parmer, Vernon Hills, EE. UU.). The lysed cultures were centrifuged for 30 min at 14,000 rpm. For the synthesis, 10 mL of the lysed culture were mixed with 10 mL of a 1 mM aqueous $AgNO_3$ solution. The mixture was maintained on constant agitation at 550 rpm at 60 °C for 24 h under darkness conditions. The treatment was conducted in triplicate. The AgNPs green synthesis was visualized by colorimetric changes in the solution. Synthesis was also confirmed using the UV–vis spectrophotometer after 24 h.

### 2.4. Quantification of proteins and DNA from the intracellular extract

The proteins and double-stranded DNA of the intracellular extracts (1:10 dilution) of *C. necator, B. subtilis*, and *B. megaterium* were quantified by a fluorimetric assay using the Qubit 4.0 Fluorometer (Life Technologies, Carlsbad, California, US). For each sample, 2 µl was examined using the Qubit dsDNA BR assay and protein assay kits, according to the manufacturer's instructions.

### 2.5. Characterization of silver nanoparticles

The techniques of ultraviolet visible spectroscopy (UV–Vis), Fourier transformed infrared spectroscopy (FTIR), transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential were used for the characterization of the AgNPs.

#### 2.5.1. UV–Vis characterization

To determine the formation of the AgNPs through time, the measuring of the surface plasmon resonance (SPR) band using UV–vis spectroscopy (Shimadzu, UV-1800) was conducted from 200 to 800 nm, operated at a resolution of 1 nm.

#### 2.5.2. FTIR characterization

The FTIR spectroscopy analysis (Nicolet 6700, Thermo Scientific) was conducted to determine the main functional groups of the supernatants, intracellular extracts, and biosynthesized AgNPs. The analysis was done in duplicate. The results were analyzed on the OMNIC 8.1 (OMNIC Series 8.1.10, Thermo Fischer Scientific) software.

#### 2.5.3. DLS and zeta potential

The particle size measurement, polydispersity and zeta potential of the synthesized AgNPs were determined using a nanoparticle analyzer (NanoPartica SZ-100V2, Horiba, Japan). The analyses were carried out at a 90° dispersion angle at a temperature of 25 °C. For the analysis, the AgNPs suspension was diluted 1:3 using Milli-Q water. Histograms of the size distributions of AgNPs were plotted on the OriginPro, version 2019b, Northampton, MA, USA.

#### 2.5.4. TEM characterization

The size and morphology of the synthesized nanoparticles were measured by TEM (JEOL, model JEM 2011), coupled with energy dispersive X-ray fluorescence (EDS) using the Inca X-sight Si (Oxford Instruments, model 7582-M). Window: ATW-2, resolution at 5.9 keV. The analyzes were carried out employing an accelerating voltage of 120 kV. For sample preparation before observing by TEM, 5 µl of AgNPs suspensions were drop-casting over carbon-coated copper grids, and dried for 16 h in a desiccator with silica.

### 2.6. Determination of minimum inhibitory concentration

The antibacterial activity of the biosynthesized AgNPs, against *E. coli* ATCC 25,922 was determined using a resazurin microtiter-plate assay (minimum inhibitory concentration, MIC) in a 96-well round bottom microtiter plate. A positive control with the commercial antibiotic kanamycin was done. A previous bacterial suspension of *E. coli* was prepared, and then centrifuged at 3500 rpm for 10 min. The resulting pellets were resuspended in a saline solution of 0.85 % m/v NaCl. Then, into the 96-well microtiter plates from column 12 to column 3, $5 \times 10^4$ UFC/well of *E. coli* was added, and the final volume was made up to 100 µL in each well, where the column 12 had the highest and column 3 the lowest concentration of AgNPs. Next, in each well 10 µL of a resazurin solution, with a concentration of 0.01 % (m/v), was added. Columns 1 and 2 served as the positive control (without AgNPs and kanamycin), and rows A and H served as the negative control (without bacterial suspension). For a period of 24 h at 37 °C, the plates were incubated. After that, an estimation, based on the change in color from blue to pink, was used to determine the presence of viable microorganisms in each well. The MIC value was taken at the lowest concentration of AgNPs that inhibited bacteria growth (the color remained blue), and all experiments were performed by triplicate, and then were reported the averages of the MIC values.

### 2.7. Statistical analysis

The statistical analyses of the results of the MIC assay were carried out using a one-way ANOVA, followed by post hoc analyses using Tukey's significant difference test. Differences at $P < 0.05$ were regarded as statistically significant. All results are reported as means ± standard deviation. These statistical analyses were performed using IBM SPSS (version 20.0; SPSS Inc, Chicago, IL, USA). Prior to performing the one-way ANOVA, the normality and the homogeneity of variances were tested by the Ryan-Joiner and Levene tests, respectively. The data were plotted on the OriginPro, version 2019b, Northampton, MA, USA.

## 3. Results and discussion

### 3.1. Green synthesis and characterization of silver nanoparticles

#### 3.1.1. Visual observation and UV–Vis spectroscopy

The green synthesis of AgNPs by reduction of silver nitrate using supernatants and intracellular extracts of *Cupriavidus necator, Bacillus megaterium*, and *Bacillus subtilis* was monitored based on the reaction mixture's change in color. The appearance of a yellowish-brown color is characteristic of the formation of AgNPs [23]. A clear difference in the color of the reaction was observed between the synthesized nanoparticles at 30 and 60 °C, with the reaction mixture appearing darker at 60 °C (Fig. 1). As shown in Fig. 1a (60 °C with *B. subtilis* supernatant), the color of the reaction slightly intensifies as the pH increases. With the *B. megaterium* supernatant at 60 °C (Fig. 1b), there was a noticeable difference between the color of the reaction at pH 10 and the reaction at other pH values: at pH 10, a dark brown color was observed. Regarding the *C. necator* supernatant at 60 °C (Fig. 1c), there was a darker color at pH 10 compared to the other treatments. The darker color of the reaction mixtures could be associated with a major AgNPs concentration [42]. Otherwise, at 30 °C and with *C. necator* supernatant (Fig. 1c), an intense coloration of the AgNPs solution was observed at pH 7 and pH 10.

Moreover, the AgNPs synthesized using the intracellular extract showed a yellow pale color in the reaction after 27 h (Fig. 2). It should be noted that a more intense coloration occurred on the treatment using the *C. necator* intracellular extract (Fig. 2c). The lower color intensity of the reaction for the AgNPs synthesized using the intracellular extract compared to those synthesized using the supernatant at 60 °C agrees with the findings of [43], who observed a slight color change in the reaction mixture in the intracellular synthesis of AgNPs compared to the brown color observed using the supernatant at pH 10 at 60 °C.

The UV–Vis spectra of the biosynthesized AgNPs are shown in Fig. 3.

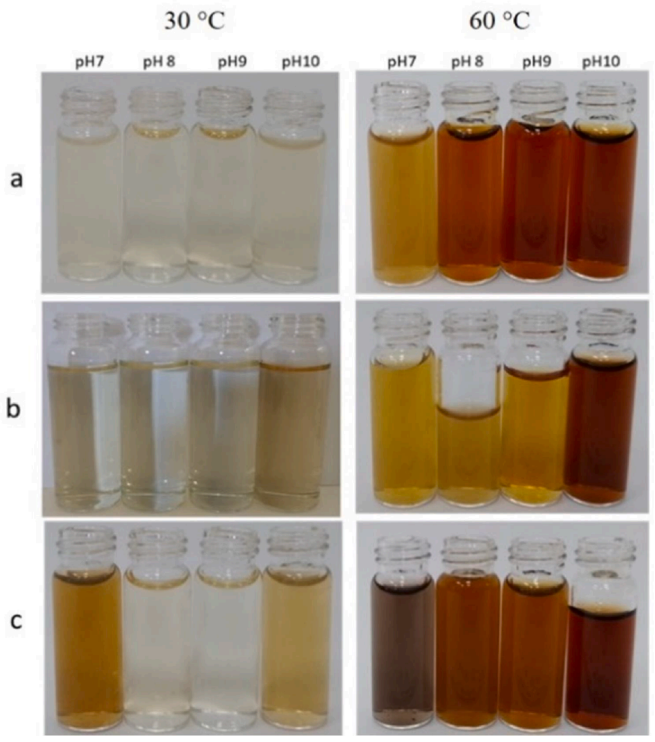


**Fig. 1.** Silver nanoparticles synthesized using the bacterial supernatants. (a) AgNPs synthesized using *B. subtilis*, (b) AgNPs synthesized using *B. megaterium*, and (c) AgNPs synthesized using *C. necator* at 30 °C and 60 °C.

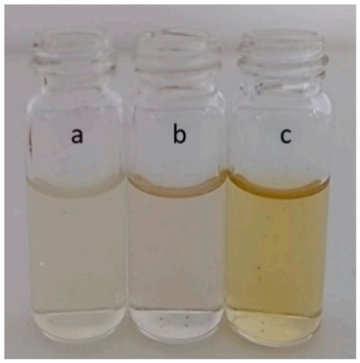


**Fig. 2.** Silver nanoparticles synthesized using the intracellular extract at 30 °C for 27 h. (a) AgNPs synthesized using *B. subtilis*, (b) AgNPs synthesized using *B. megaterium*, and (c) AgNPs synthesized using *C. necator*.

The absorbance was found to constantly increase in intensity as a function of time, in both the synthesis using the supernatant and the synthesis using the intracellular extract. Regarding the synthesis using the *C. necator* supernatant (Fig. 3a), the highest absorbance was found at pH 10 and 60 °C, followed by the treatment at pH 7 and 60 °C. On the other hand, the synthesis using the *B. megaterium* supernatant (Fig. 3b) showed a similar wavelength, about 425 nm, between the AgNPs synthesized at most pH levels at 60 °C; however, the highest absorbance was obtained at pH 10. The synthesis using the *B. subtilis* supernatant showed a wavelength close to 414 nm with treatments at pH 7, 8, and 9, and a wavelength of 407 nm at pH 10. The highest absorbance was obtained at pH 7; however, similar to the findings with the other bacterial supernatants, a wider band was observed at this pH, which could be related to the higher polydispersity of the AgNPs [44]. Regarding the AgNPs synthesized at 30 °C, the typical absorption band indicative of AgNPs formation was not detected for any of the evaluated bacteria, except for the synthesis using the *C. necator* supernatant at pH 10, which showed an absorbance of 0.5 and wavelength at 453 nm. The lack of absorbance at pH 7 and 30 °C agrees with the results of El-Bendary et al. (2021), who found that absorbance did not occur in the reduction of silver ions under the same pH and temperature conditions [45]. Therefore, according to the results of UV–Vis, the best AgNPs synthesis was obtained at pH 10 and 60 °C. Previous investigations have determined that the green synthesis on basic mediums produce AgNPs that are more stable and monodisperse and that show high growth rate and major absorbance [46], which agrees with the present findings. Also, it has been reported that temperatures above 60 °C disfavor the reduction process of silver ions because high temperature degrades the biomolecules responsible for the synthesis process and generates NP aggregates [45]. On the other hand, regarding AgNPs synthesis using intracellular extracts, the present study found that the highest absorbance occurred in the synthesis using *C. necator*, followed by *B. megaterium* and *B. subtilis*, respectively (Fig. 3d). Similarly, some authors have reported the use of intracellular extracts and supernatants from different bacteria to synthesize AgNPs [23,25].

### 3.1.2. FTIR spectroscopy

The FTIR characterization determined the presence of different

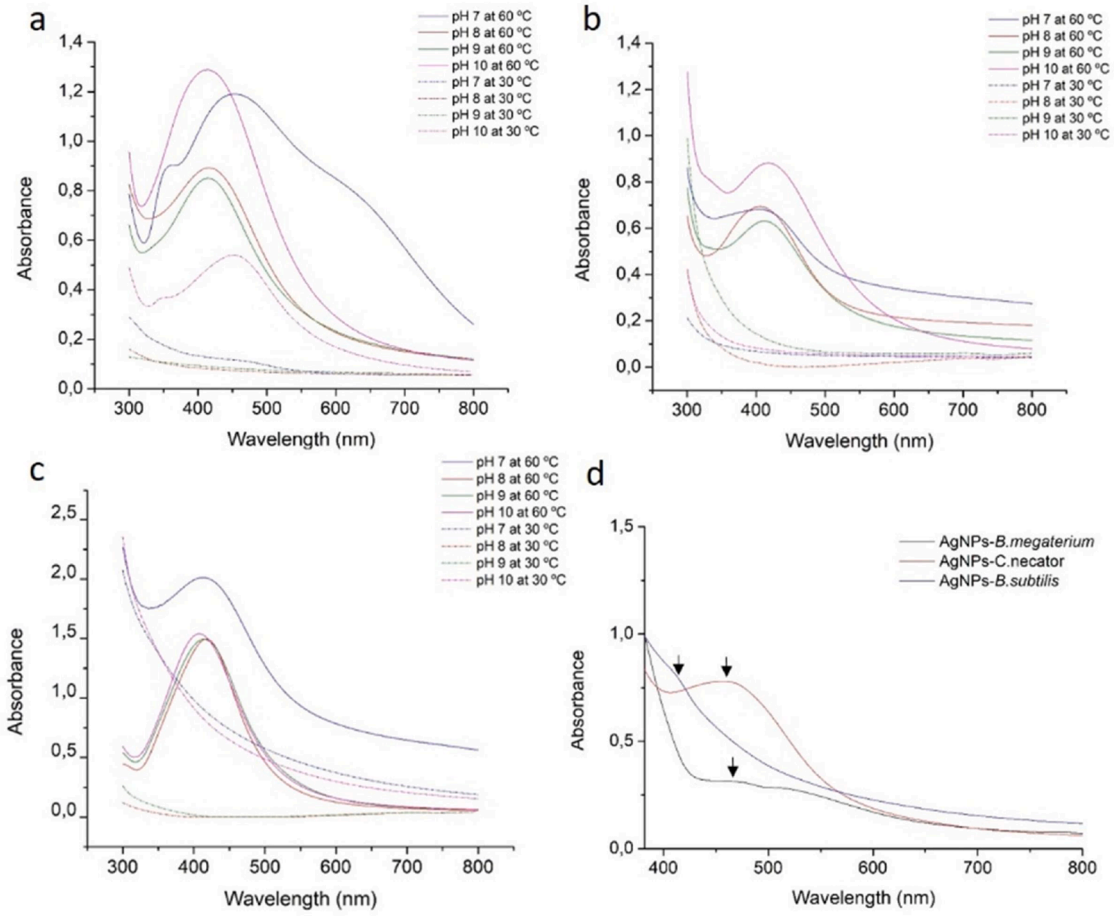


**Fig. 3.** UV–visible absorption spectra of AgNPs synthesized using the supernatant at 60 °C and 30 °C at different pH values for 27 h, and the intracellular extract at 30 °C for 27 h. (a) AgNPs synthesized using *C. necator* supernatant, (b) AgNPs synthesized using *B. megaterium* supernatant, (c) AgNPs synthesized using *B. subtilis* supernatant, and (d) AgNPs synthesized using the intracellular extracts.

functional groups associated with the green synthesis process and stabilization of the AgNPs. The FTIR spectra showed peaks on the 3500–3200 $cm^{-1}$, 1600–1500 $cm^{-1,}$ and 1300–1200 $cm^{-1}$ regions (Fig. 4a). The peaks in the 3500–3200 $cm^{-1}$ region could be associated with the -OH of the hydroxy functional groups [6], and the -NH of the amine functional groups [47]. Regarding the identified peaks in the 1600–1500 $cm^{-1}$ region, those could be related with the presence of C=O links from amides or esters [6]. On the other hand, the peaks between 1300 and 1200 $cm^{-1}$ and 1036 $cm^{-1}$ are related to aromatic and aliphatic amines [48]. It has been determined that the peak at 1078 $cm^{-1}$ could be a C—O stretch and the peak at 1038 $cm^{-1}$ a C—N stretch and aliphatic amines vibrations [49]. The presence of these functional groups in the synthesized AgNPs suggests the presence of proteins, enzymes, peptides, polysaccharides, and organics acids, which are involved in the reduction and stabilization of metallic ions in metallic nanoparticles [47,50]. Likewise, with the AgNPs synthesized using the intracellular extract (Fig. 4b), similar absorbance bands were observed as those obtained for AgNPs synthesized using the supernatants. Peaks were observed in the 3350–3290 $cm^{-1}$, 1650–1600 $cm^{-1}$, 1450–1350 $cm^{-1}$, and 1090–1050 $cm^{-1}$ regions. It should be noted that the stretching vibration of C—O increased to higher wavenumbers, shifting from 1078 to 1087 $cm^{-1}$ in the synthesis using the intracellular extract, suggesting the presence of higher protein content [51,52].

### 3.1.3. DLS and zeta potential

As shown in Table 1, the AgNPs synthesized using the intracellular extracts of *B. subtilis* and *B. megaterium* were found to be more stable, with zeta potential values of −34.1 mV and −33.9 mV, respectively, compared with *C. necator*, which yielded a zeta potential value of −21.4 mV. Fig. 5 shows the histograms of AgNPs synthesized. According to Dhiman et al. (2021), zeta potential values above −30 mV indicate high NPs stability [53]. On the other hand, in the AgNPs synthesized using the supernatant, all the zeta potential values were between −18.1 and −21.9 mV, which indicates a lower stability compared with the AgNPs synthesized using the intracellular extracts. These differences could be due to the different biomolecules present in the intracellular and supernatant extracts, such as carbohydrates, proteins and lipids [54]. In the case of the intracellular extract, the presence of DNA, c-type cytochromes, peptides, cellular enzymes such as nitrate reductase, and reducing cofactors could favor the synthesis and stabilization of AgNPs [55], preventing their aggregation and providing greater stability

**Table 1**
Dynamic light scattering (DLS) and zeta potential of AgNPs.

| | Treatment | Hydrodynamic diameter (nm) | Z-potential (mV) | Polydispersity |
|---|---|---|---|---|
| Intracellular extract | AgNPs-*C. necator* | 70.5 ± 2.1 | −21.4 ± 1.2 | 0.329 ± 0.025 |
| | AgNPs-*B. subtilis* | 118.4 ± 1.9 | −34.1 ± 1.9 | 0.170 ± 0.020 |
| | AgNPs-*B. megaterium* | 91.2 ± 2.4 | −33.9 ± 1.8 | 0.322 ± 0.012 |
| Supernatant | AgNPs-*C. necator* | 85.9 ± 5.9 | −19.0 ± 0.5 | 0.605 ± 0.050 |
| | AgNPs-*B. subtilis* | 20.8 ± 0.6 | −21.9 ± 0.7 | 0.907 ± 0.144 |
| | AgNPs-*B. megaterium* | 103.4 ± 7.6 | −18.1 ± 2.0 | 0.617 ± 0.086 |

*Values are presented as means ± standard deviation (SD).

compared to the AgNPs synthesized with the supernatant. Considering this information, it is possible to associate the different values of the zeta potential to the greater availability of stabilizing factors that can be found in the intracellular extract, which can be corroborated by means of the protein and DNA quantification results in Section 3.2. According to these results, it is important to take into account the stability over time of nanoparticles for the desired application, for example, nanoparticle formulations that need to be applied constantly, such as disinfectant sprays, may not require high stability over time compared to nanomedicines. Nanoparticles can also be modulated to generate better stability, using precision electrostatic/charge or precision polymer methods, as mentioned in the work by Sperling and Parak [56]. In addition, other factors such as antimicrobial activity should be taken into account in order to determine the potential use of AgNPs.

On the other hand, several authors have suggested that there are different proteins that can be attached to nanoparticles through amine or cysteine groups, which can contribute to their stabilization [57]. In addition, in the green synthesis mediated by bacteria, it has been determined that proteinous molecules with –OH, $–NH_2$, $–SH_2$, –CHO, or –COOH functional groups are responsible for the reduction of the silver nitrate and stabilization of AgNPs [58–61]. The secretion of extracellular enzymes, specifically nitrate reductase, has been suggested to be responsible for the reduction of Ag ions to AgNPs, which has been confirmed in studies that use extracellular extracts of *B. subtilis* and

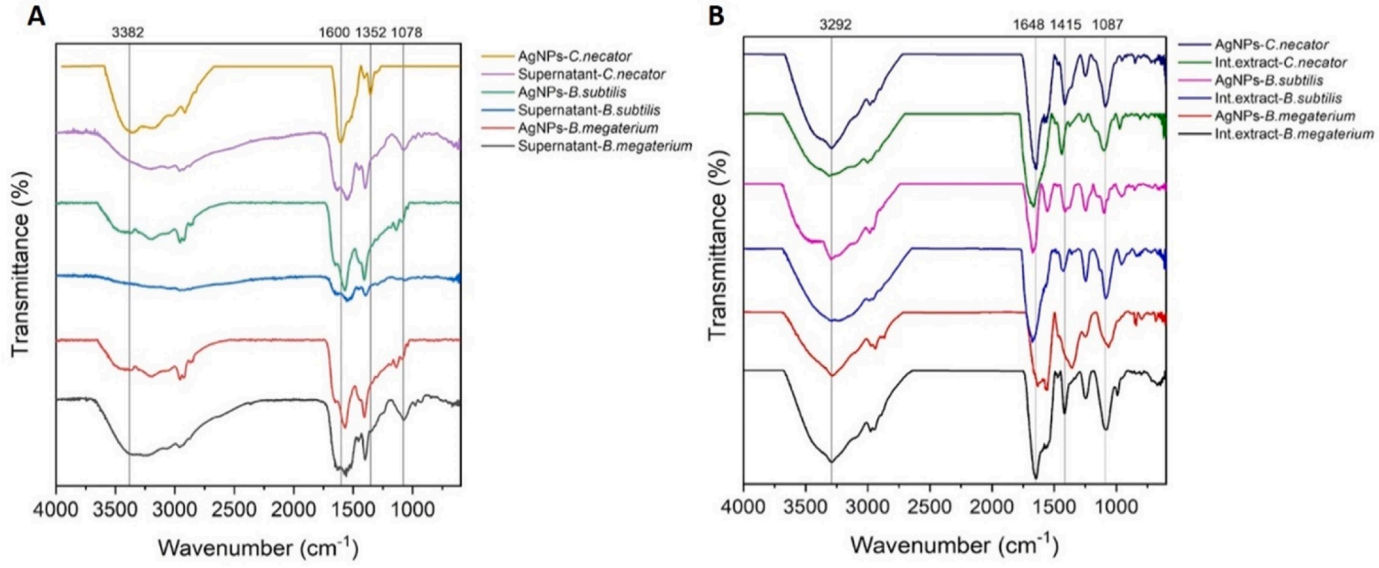


**Fig. 4.** FTIR spectra of bacterial supernatants, intracellular extracts and synthesized AgNPs. (a) FTIR of bacterial supernatants and AgNPs synthesized using the bacterial supernatants, at pH 10 and 60 °C, and (b) FTIR spectra of intracellular extracts and AgNPs synthesized using the intracellular extracts.

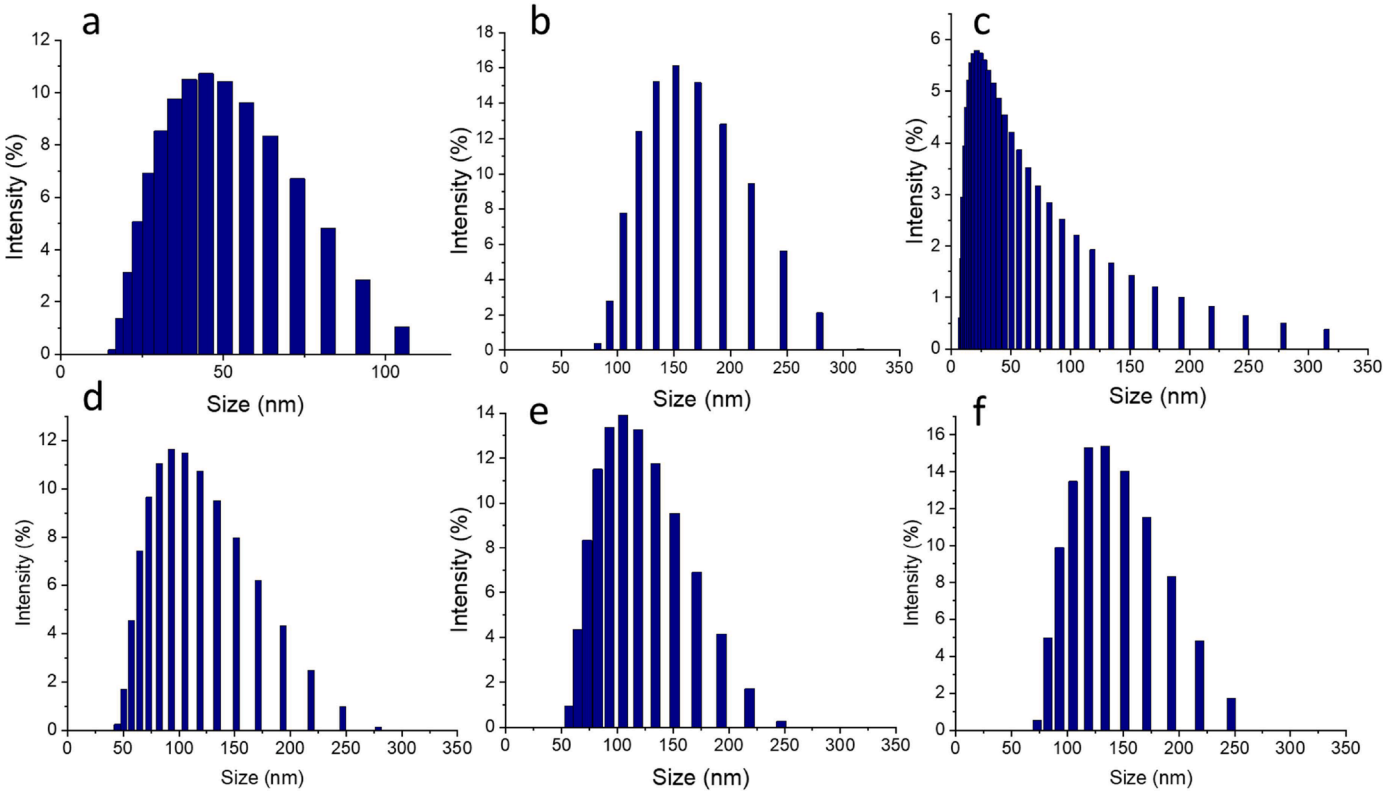


**Fig. 5.** Histogram of AgNPs synthesized using the supernatant at 60 °C pH 10, 60 °C, for 27 h (a–c), and the synthesized using the intracellular extract at 30 °C for 27 h (d-f). (a and d) AgNPs synthesized using *C. necator*, (b and e) AgNPs synthesized using *B. megaterium*, (c and f) AgNPs synthesized using *B. subtilis*.

*Cupriavidus* sp. [48,62]. Besides, other molecules such as oxidases, reducing cofactors, cyclic ring of monosaccharides, biosurfactants and exopolysaccharides are produced by bacteria [60,63–68].

Regarding the hydrodynamic diameter of the synthesized AgNPs using the intracellular extract, the AgNPs with a larger size were biosynthesized by *B. subtilis*, followed by *B. megaterium* and *C. necator*, with values of 118.4, 91.2, and 70.5 nm, respectively. On the other hand, according to the polydispersity data, the AgNPs synthesized using the intracellular extract from *B. subtilis* presented higher polydispersity. Concerning the AgNPs produced using the supernatant, the AgNPs formed by *B. subtilis* had an average size of 20.8 nm, which was significantly smaller than those formed by *C. necator* and *B. megaterium*, which had average sizes of 85.9 and 103.4 nm, respectively (Table 1). According to our results of zeta potential and particle size, using the supernatant of *B. subtilis* at pH 10, Zhao et al. (2019), obtained similar data for these parameters using *B. subtilis* [26]. However, different results were obtained with respect to polydispersity values. This could be associated with differences in the experimental conditions, such as temperature, agitation, light conditions, and pH, which influence the size, potential and polydispersity of AgNPs [45,53].

The variations in the particle size obtained between the different bacteria and type of extract, could be associated to the distinct biomolecules present in each extract, as explained previously. In addition, it has been reported that differences in the hydrodynamic diameter could be associated with some chemical characteristics of the nanoparticles, such as the rapid agglomeration of groups with low stabilization and the anisotropy of this type of AgNPs. Furthermore, part of the AgNPs could be very unstable due to their energy content and their reactivity, which explains the presence of different population sizes [69]. According to our results, it is important to highlight that the size of AgNPs has an impact on the antimicrobial activity, which has been reported by several studies, that found that smaller sizes of AgNPs seems to exhibit higher antimicrobial activity, since them can reach more easily the cytoplasm and nucleus of the microorganisms [70–72], however it is important to take into account that the antimicrobial activity can also be influenced by another factors, such as concentration, shape and charge of the nanoparticles [73,74].

### 3.1.4. Morphological characterization of AgNPs

TEM images (Figs. 6 and 7) revealed different shapes and sizes between the AgNPs synthesized with the different microorganisms and the type of extract (supernatant or intracellular extract), spherical being the most predominant shape. Similarly, Ameen et al. (2020), obtained AgNPs using *Cupriavidus sp*. with spherical morphology and sizes between 10 and 50 nm, while Stancu, 2021 performed a similar experiment using *B. megaterium* and obtained spherical AgNPs with a size between 1 and 85 nm [48,75]. It has been demonstrated that different microorganisms secrete different biomolecules in different proportions [76], such as carbohydrates, proteins, lipids, and polysaccharides [54, 57], which have been reported as molecules responsible for the green synthesis of AgNPs. In addition, associated with the EDS analyzes present in Fig. 6 (g, h and i) and Fig. 7 (g, h and i), silver was observed by a Ag peak in the EDS spectrum of all samples. Furthermore, the presence of elements such as copper (Cu), aluminum (Al), carbon (C) and oxygen (O) was also detected, however, it is important to emphasize that the other elements observed in the analyzes come from the chemical structure of the carbon tape and the copper grid used in the assembly of the samples.

## 3.2. Quantification of proteins and DNA from the intracellular extract

The protein and DNA concentration of the intracellular extract was determined by a fluorimetric assay (Table 2). The intracellular extract of *B. subtilis* had the highest concentration of protein and DNA, followed by *C. necator* and *B. megaterium*. Various studies have determined that these biomolecules are responsible for the reduction and stabilization of

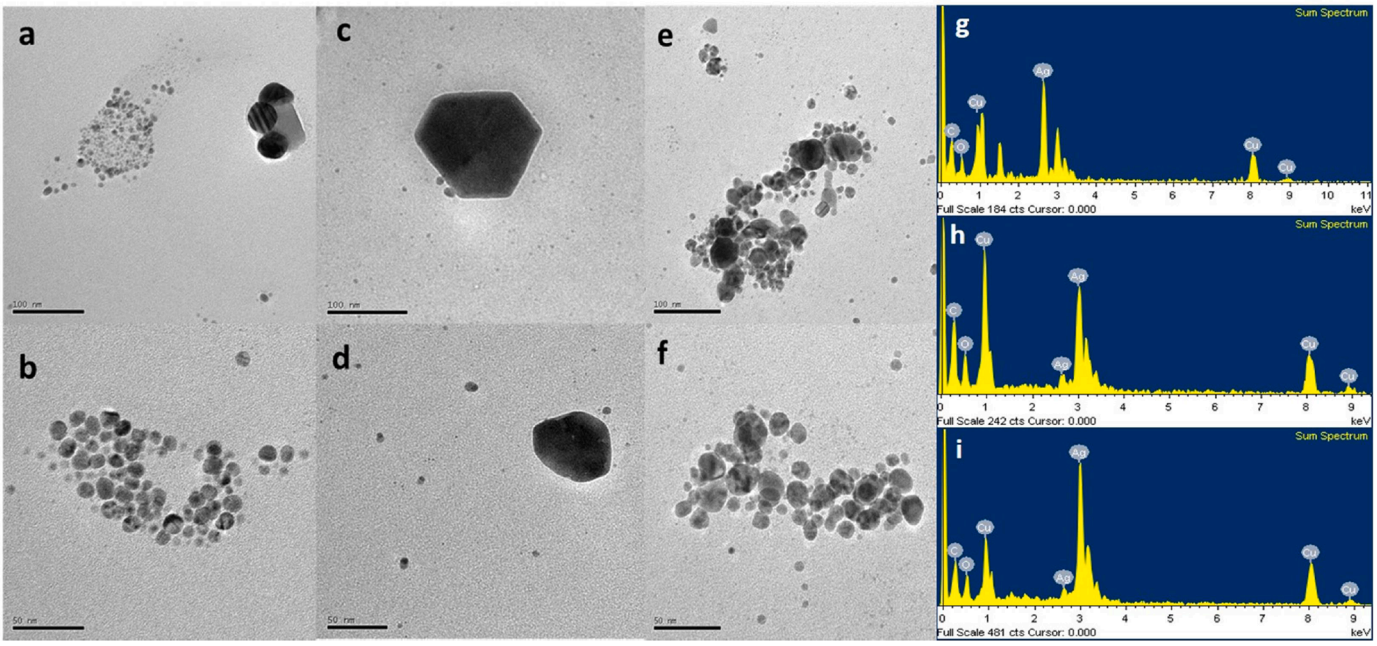


**Fig. 6.** Transmission electron micrograph and energy dispersive X-ray fluorescence of AgNPs synthesized using bacterial supernatant. (a, b and g) AgNPs from *C. necator* at pH 10, 60 °C, (c, d and h) AgNPs from *B. megaterium* at pH 10, 60 °C, and (e, f and i) AgNPs from *B. subtilis* at pH 10, 60 °C.

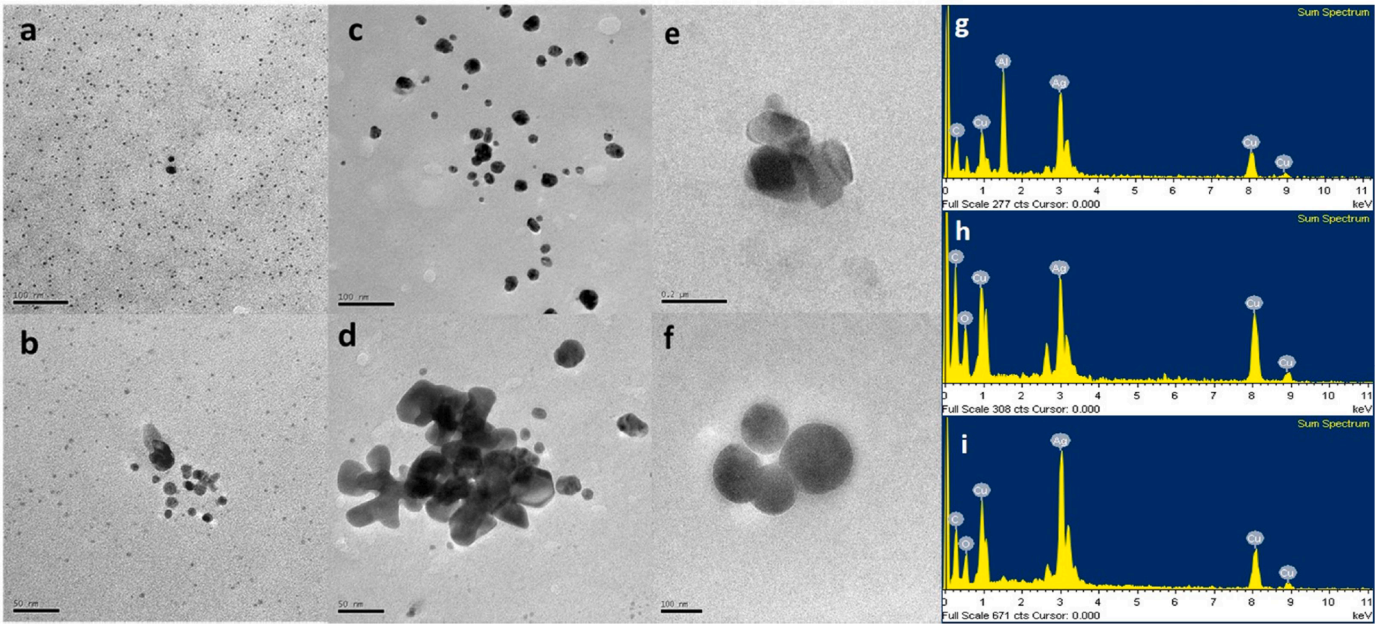


**Fig. 7.** Transmission electron micrograph and energy dispersive X-ray fluorescence of AgNPs synthesized using the intracellular extract (a, b and g) AgNPs from *C. necator* at pH 10, 60 °C, (c, d and h) AgNPs from *B. megaterium* at pH 10, 60 °C, and (e, f and i) AgNPs from *B. subtilis* at pH 10, 60 °C.

**Table 2**
Concentration of proteins and DNA from the intracellular extract of the microorganisms.

| Microorganism | Protein (mg/ml) | DNA (ng/ml) |
|---|---|---|
| *B. subtilis* | 0.127 | 10.6 |
| *B. megaterium* | 0.024 | 0.868 |
| *C. necator* | 0.0906 | 2.24 |

AgNPs [77,78]. Similarly, some studies have identified that certain bacterial enzymes (silver-binding proteins, metalloproteins, reductases) can perform the intracellular synthesis of AgNPs [43]. One of the most studied enzymes is the nitrate reductase, which has been reported to synthesize AgNPs [79]. This enzyme gains NADH electrons, which pass to NAD+ oxidized form and reduce the silver ions to AgNPs [3]. Furthermore, some biomolecules such as soybean and milk proteins have been used for the synthesis of AgNPs [78]. However, some studies have determined that the reduction of silver using soy protein occurs thanks to the tyrosine residue, while in milk protein the reduction occurs due to interactions between casein and whey protein. In addition, it has

been reported that DNA can be used as a capping molecule to control the morphology of metal nanomaterials such as gold and silver, among others [69]. Similarly, Chumpol & Siri, 2018 reported that bacterial genomic DNA obtained from a previous lysis and purification can synthesize AgNPs [77].

### 3.3. Antimicrobial activity of silver nanoparticles

According to the best results of green synthesis, the antibacterial activity was tested for the AgNPs synthesized using the bacterial supernatant at pH 10 and 60 °C, as well as for those synthesized using the intracellular extract. The AgNPs displayed antimicrobial activity toward the tested strain *E. coli* (Table 3). According to the results of the MIC (Table 3), the highest antimicrobial activity against *E. coli* was obtained using the AgNPs synthesized using the supernatant of *C. necator*. Among the treatments using intracellular extracts, the highest antimicrobial activity was obtained with the AgNPs synthesized with *B. megaterium*. These results prove the capacity of synthesized AgNPs to inhibit the growth of *E. coli*, confirming that these AgNPs have great potential as antimicrobial agents, a finding supported by other studies [36,80,81]. It has been reported that nanoparticles' antibacterial activity is determined by their size, concentration, shape, charge, and stabilizing agent and depends on the microbial strain. In similar investigations, it has been determined that the size of AgNPs influences the inhibition of microbial growth. Research has evidenced that small AgNPs have a greater surface area/volume ratio, which increases the interaction of NPs with the membrane surface of bacteria, which facilitates penetration through the membrane and causes its death [18,82,83].

In our research, it was clear that the AgNPs synthesized using the supernatant had lower MIC values compared to those synthesized using the intracellular extract. This difference in the antibacterial activity could be due to the different biomolecules that are present in the different extracts, such as carbohydrates, proteins, lipids, polysaccharides, enzymes, and DNA, which could produce AgNPs with different stabilizing molecules [54,84]. In the same way, it has been demonstrated that different microorganisms secrete different biomolecules in distinct proportions, which could affect the stabilization of AgNPs [76]. AgNPs with different stabilizing molecules influence antibacterial activity, becoming more or less antimicrobial by interacting differently with certain molecules of the microbial membrane, such as proteins [85–87].

On the other hand, the MIC values obtained in our research are lower than those found by other authors who have used plant extracts for the synthesis of AgNPs and have evaluated the antimicrobial activity against *E. coli*. For example, some research found MIC values between 7.8 and 20.0 μg/mL with AgNPs synthesized from plant extracts [76–78]. Otherwise, Rolim et al. [87], determined a MIC value of 15 ug/mL using AgNPs synthesized from green tea extract. While, Das et al. (2019), obtained a MIC value of 60 μg/ml using AgNPs synthesized from *Morus alba* leaf extract [88]. On the other hand, other investigations that have carried out AgNPs synthesis by microorganisms have found similar results to those obtained in our investigation. Akter and Huq (2020), obtained a MIC value against *E. coli* of 6.25 ug/mL using AgNPs synthesized from the extracellular extract of *Sphingobium* sp. MAH-11T [81], and other investigations have obtained MIC values between 3.12 - 12.5 μg/mL using AgNPs biosynthesized by bacteria [89,90]. Regarding the AgNPs synthesized by using plant extracts, some research has found MIC values between 7.8 and 20.0 μg/mL [91–93]. Besides, other biological extracts from fungus have been used for the synthesis of AgNPs, Mohanta et al. (2022) synthesized AgNPs from the extract of *Talaromyces funiculosus* and determined a MIC value of 43.94 μg/ml against *E. coli* [94].

On the other hand, some studies have compared the MIC value of the synthesized AgNPs with commercial antibiotics. For example, Vazquez (2017) have found major antimicrobial activity of commercial antibiotics (ampicillin, chloramphenicol, kanamycin, biapenem, aztreonam, amphotericin B, fluconazole and caspofungin) compared to commercial AgNPs, with MIC values of 0.05–2 μg/ml, and 10 μg/ml to inhibit the growth of *E. coli*, respectively [95]. Similarly, Wypij et al. (2022), found MIC values of AgNPs of 64 μg/ml and between 0.5 and 16 μg/ml for antibiotics [96]. The results of these research are in line with our findings, were the MIC values of the synthesized AgNPs were higher than the value of kanamycin (0.55 μg/ml), however it is important to highlight that this value was similar to the MIC value obtained with the synthesized AgNPs from the supernatant of *C. necator* (0.69 μg/ml). Our results revealed that the synthesized AgNPs exhibited potent activity against *E. coli* and can be used as antibacterial agents.

**Table 3**
Minimum inhibitory concentration of the synthesized AgNPs against *E. coli*.

| | Supernatant | Intracelular extract |
|---|---|---|
| Microorganism | MIC (μg/ml) | MIC (μg/ml) |
| *B. megaterium* | $2.750 \pm 0.487^{a}$ | $5.087 \pm 0.615^{a}$ |
| *B. subtilis* | $1.880 \pm 0.340^{b}$ | $5.500 \pm 0.001^{a}$ |
| *C. necator* | $0.687 \pm 0.151^{c}$ | $8.800 \pm 1.230^{b}$ |

*Values are presented as means ± standard deviation (SD). Values with different letters in the same column indicate significant differences ($P < 0.05$).

## 4. Conclusions

This study demonstrated that the optimal conditions to synthesize AgNPs were pH 10 and 60 °C using the bacterial supernatant of *C. necator*. In addition, a series of known techniques were used to characterize the AgNPs, including UV–Vis spectroscopy, indicating the highest absorbance in *C. necator*, followed by *B. megaterium* and *B. subtilis*. Regarding the intracellular extracts, *C. necator* presented the highest absorbance, followed by *B. subtilis* and *B. megaterium*. The FTIR analysis indicated the possible presence of functional groups of proteins, enzymes, peptides, and polysaccharides, among others, which is relevant because they are responsible for reducing and stabilizing the silver ions, thus carrying out the AgNPs' formation. Furthermore, the AgNPs synthesized with the supernatant showed greater antimicrobial activity against *E. coli* compared to those synthesized with the intracellular extract, whereas *C. necator* presented a lower MIC value when compared with *B. megaterium* and *B. subtilis*. Therefore, this green synthesis method appears to be an eco-friendly way to obtain stable AgNPs with antimicrobial properties. Besides, the extracellular synthesis is preferred over the intracellular synthesis because it is easier to recover the molecules responsible for the reduction and stabilization. In addition, according to several investigations, the advantage of the biological method over chemical synthesis of nanoparticles is that with the green synthesis of AgNPs a greater antimicrobial activity is obtained. On the other hand, to our knowledge, this investigation is the first to use *C. necator*, both its extracellular supernatant and intracellular extracts, for AgNPs synthesis. Likewise, it was possible to demonstrate that its use would be promising due to its greater antimicrobial activity compared to AgNPs synthesized with *Bacillus*. Finally, this investigation underscores the need for more analysis based on AgNPs green synthesis from intracellular extracts in order to elucidate the synthesis mechanisms with biomolecules such as DNA and intracellular proteins, an area which, to date, remains understudied.

## CRediT authorship contribution statement

**Iván Solís-Sandí:** Investigation, Writing – original draft, Visualization. **Sara Cordero-Fuentes:** Investigation, Writing – original draft, Visualization. **Reinaldo Pereira-Reyes:** Visualization, Data curation, Writing – review & editing. **José Roberto Vega-Baudrit:** Resources, Writing – review & editing. **Diego Batista-Menezes:** Investigation, Supervision, Writing – original draft, Visualization. **Gabriela Montes de Oca-Vásquez:** Investigation, Supervision, Data curation, Writing –

original draft, Visualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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