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Green Synthesis of Silver Nanoparticles Using a Biosurfactant from *Bacillus cereus* UCP 1615 as Stabilizing Agent and Its Application as an Antifungal Agent

Italo José Batista Durval [1,2], Hugo Morais Meira [2,3], Bruno Oliveira de Veras [4], Raquel Diniz Rufino [2,5], Attilio Converti [2,6,*] and Leonie Asfora Sarubbo [2,3,*]

1 Northeast Biotechnology Network (RENORBIO), Federal Rural University of Pernambuco (UFRPE), Dom Manuel de Medeiros Street, Dois Irmãos, Recife 52171-900, PE, Brazil; italo.durval@gmail.com
2 Advanced Institute of Technology and Innovation (IATI), Potyra Street, Number 31, Prado, Recife 50751-310, PE, Brazil; hugo.meira@iati.org.br (H.M.M.); raquel.rufino@unicap.br (R.D.R.)
3 Icam Tech School, Catholic University of Pernambuco (UNICAP), Príncipe Street, Number 526, Boa Vista, Recife 50050-900, PE, Brazil
4 Department of Biochemistry, Federal University of Pernambuco, Recife 50670-420, PE, Brazil; bruno.overas@ufpe.br
5 School of Health and Life Sciences, Catholic University of Pernambuco (UNICAP), Príncipe Street, Number 526, Boa Vista, Recife 50050-900, PE, Brazil
6 Department of Civil, Chemical and Environmental Engineering, Polytechnic School, University of Genoa (UNIGE), Via Opera Pia n. 15, 16145 Genova, Italy
* Correspondence: converti@unige.it (A.C.); leonie.sarubbo@unicap.br (L.A.S.)

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Abstract: Silver nanoparticles have great potential in a wide range of applications. Therefore, the purpose of this work was to synthesize, in a simple and green way, via the Tollens method, silver nanoparticles (AgNPs), using as a stabilizer the biosurfactant produced by *Bacillus cereus* UCP 1615 cultivated in a low-cost medium, with waste frying oil as a substrate. The obtained nanoparticles were identified and morphologically characterized using ultraviolet/visible (UV/vis) spectroscopy, scanning electron microscopy (SEM), and zeta potential. The maximum UV/vis absorption was observed at 400 nm for newly formed silver nanoparticles, while, for silver nanoparticles stored for 120 days, the peak was observed at 430 nm. SEM micrographs confirmed the formation of nanoparticles, with predominantly spherical structures. The average size of the formed nanoparticles was estimated to be 20 nm. The presence of the biosurfactant promoted stability, as a zeta potential of −23.4 mV was observed. The antimicrobial potential of AgNPs was evaluated at different concentrations against three pathogenic fungi (*Aspergillus niger*, *Penicillium fellutanum*, and *Cladosporium cladosporioides*). No less than 100% and 85% inhibitions of *P. fellutanum* and *A. niger* growth were observed, respectively, at the AgNP concentration of 16.50 μg/mL in potato dextrose agar medium. These results suggest the potential use of the biosurfactant as a stabilizer for silver nanoparticles and its application as an antimicrobial agent.

Keywords: biosurfactant; *Bacillus cereus*; nanoparticles; nanoscale materials; antimicrobial activity

1. Introduction

Nanotechnology has been growing due to the large number of effective applications in various sectors, ranging from traditional chemical techniques to medicinal and environmental technologies [1]. It is well accepted that a particle is nano if its diameter is from 1 to 100 nanometers (billionths of a meter), including in this range nanoparticles (NPs) considered especially practical and efficient which are traditionally synthesized by chemical means [2]. In particular, the size of silver nanoparticles (AgNPs) can be adjusted according to the desired application, as in the case of those prepared for drug delivery, which are larger than 100 nm to accommodate the amount of drug to be delivered [3].

Since AgNPs have nontoxic, inorganic, and antibacterial properties [4], they are used in several applications such as in drug delivery, ointments, nanomedicine, chemical detection, data storage, cell biology, agriculture, cosmetics, textiles, food industry, photocatalytic organic dye degradation activity, antioxidants, and antimicrobial agents [1]. The reason for the interest in using silver in the form of nanoparticles, especially in medical applications, is that the release of this metal is very fast and uncontrollable in the form of silver salts, but somewhat inefficient in the form of bulk metal [5]. In addition, AgNPs have new and distinct physicochemical properties compared to conventional counterparts such as very high intrinsic reactivity and surface-to-volume ratio [6].

AgNPs have an underexplored potential compared to relatively stable gold nanoparticles [7]. Studies have shown that the physical, optical, and catalytic properties of AgNPs are strongly influenced by their size, distribution, morphological shape (stem, triangle, round, octahedral, polyhedral, etc.), and surface properties, which can be modified by using various synthetic methods, as well as different reducing and stabilizing agents [3]. These NPs are composed of three layers: (1) the surface layer, which can be functionalized with a variety of small molecules, metal ions, surfactants, and polymers, (2) the shell layer, which is a material chemically different from the core in all respects, and (3) the nucleus, which is essentially the central part of the NP and generally refers to the NP itself [8]. AgNPs are traditionally prepared through physical and chemical methods; however, interest in the use of green synthesis methods is growing due to their ability to eliminate or minimize risks and to increase biocompatibility in environmental and biomedical applications [6,9]. Green synthesis is cost-effective and can be easily scaled up to large-scale applications, as it does not require high temperature, pressure, and energy or hazardous chemicals [10].

The concept of green synthesis is part of the atomic economy/efficiency principle of green chemistry, which adopts methodologies capable of reducing or avoiding the use or production of raw materials, products, byproducts, solvents, reagents, and so on that can significantly deteriorate human health and the environment [2]. Studies have reported the green synthesis of AgNPs using natural products such as plant extracts, vitamins, biodegradable polymers, biosurfactants, and enzymes as reducing agents and capping stabilizers [6].

Among the green synthesis approaches for AgNP preparation, there is the Tollens method, in which saccharides are used to reduce silver ions in the presence of ammonia [4]. Le et al. [5] proposed to associate with this method stabilizers, including surfactants, to compose the surface layer (outer core) of NPs. As a biological and sustainable alternative to chemical surfactants, biosurfactants can be used, which, in addition to their stabilizing activity, can act as reducing agents (dual functionality) [11].

Biosurfactants are surface-active compounds with different biochemical structures produced by microorganisms, which can be industrially applied in fermentation processes, especially using water-immiscible substrates as nutrient sources [6,12]. They have several advantages over chemical counterparts such as biocompatibility, low toxicity, diversity of applications, and functionality under extreme conditions [13]. Lipopeptides produced by bacteria of the genus *Bacillus* are examples of microbial biosurfactants obtained via fermentation [14], whose most important characteristics are the emulsification capacity and reduction in surface tension, along with antioxidant, antiadhesive, antibiofilm, antibacterial, antifungal, antitumor, and antiviral properties [15].

Considering the information given above, the aim of this work was to produce stable aqueous dispersions of AgNPs using a combination of the modified Tollens technique and the biosurfactant produced by *Bacillus cereus* UCP 1615 as a stabilizer. The produced nanoparticles were then evaluated for their fungicidal capacity against pathogenic fungi.

2. Materials and Methods

2.1. Microorganism

The bacterium *Bacillus cereus* UCP 1615 obtained from the culture bank of the Catholic University of Pernambuco (Recife, Brazil) was used as the biosurfactant-producing microorganism. This strain was previously isolated from environmental water samples contaminated with petroleum byproducts spilled from ships (port area) in the Atlantic Ocean in the state of Pernambuco, Brazil.

2.2. Production and Isolation of Biosurfactant

The lipopeptide biosurfactant was produced via fermentation as described by Durval et al. [16]. The bacterium was cultivated by adding 2% of cell suspension (0.7 optical density at 600 nm), corresponding to a 24 h inoculum of 10^7 CFU/mL, to an Erlenmeyer flask containing 500 mL of a mineral salt medium (0.087% K_2HPO_4, 0.65% trisaminomethane, 0.02% KCl, 0.06% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, and 0.005% yeast extract) supplemented with 2% waste frying soybean oil and 0.12% peptone, with pH adjusted to 7.0 ± 0.2. Cultivation was carried out for 48 h at 28 °C and 250 rpm.

The biosurfactant was extracted and isolated from cell-free broth, obtained by removing the cells via centrifugation (Heraeus Megafuge 16R Centrifuge, Thermo Scientific, Osterode am Harz, Germany) of the fermented broth at 5000× *g* for 30 min. A 6.0 M HCl solution was added to the supernatant to adjust the pH to 2.0, followed by the addition of a 2:1 (*v*/*v*) $CHCl_3/CH_3OH$ solution. After vigorous manual stirring for 15 min and phase separation, the organic phase was removed, and the operation was repeated twice more. The organic phases were combined, and the isolated biosurfactant was concentrated on a rotary evaporator (Q344B, Quimis, Diadema, Brazil).

2.3. Synthesis of Silver Nanoparticles

All reagents were analytical grade and were used without further purification. The technique used was adapted from Le at al. [5]. First, 1.7 g (1.0×10^{-2} mol) of silver nitrate ($AgNO_3$) was dissolved in 100 mL of deionized water. The $AgNO_3$ solution was then precipitated with 0.62 g (1.55×10^{-2} mol) of sodium hydroxide. The precipitate obtained, composed of Ag_2O, was filtered and dissolved in 100 mL of 0.4% (*w*/*w*) aqueous ammonia (NH_3) (2.3×10^{-2} mol) until a clear solution of silver–ammonium complex, $(Ag(NH_3)_2)^+$ (aqueous), was formed. Then, 2.5 g (8.9×10^{-3} mol) of the isolated biosurfactant (BS) was added dropwise to the complex, and the resulting solution was gently stirred for 2 h at room temperature until a homogeneous mixture was obtained. Finally, 2 g (1.11×10^{-2} mol) of glucose was added to the mixture at room temperature with gentle agitation.

The reduction of the silver complex solution (in quartz glass) was started with ultraviolet (UV) light irradiation. UV treatment was carried out for 8 h under vigorous stirring without heating. A UV lamp (λ = 365 nm, 35 W) was used as a light source to stimulate the reduction process. After 8 h of irradiation, a clear dispersion of AgNPs stabilized with biosurfactant was obtained. The synthesis of silver NPs was successfully conducted with a final silver concentration around 1%. The dispersion obtained, containing biosurfactant-complexed silver nanoparticles (BS-AgNPs), was stored at 4 °C for further experiments.

2.4. Properties and Characteristics of Silver Nanaoparticles

The absorption property of the BS-AgNP dispersion was analyzed using ultraviolet/visible (UV/vis) spectroscopy (Digital Spectrophotometer SP-22, Biospectro, Shanghai, China), at time zero and after 120 days of storage, in the wavelength range between 320 and 700 nm. On the other hand, the zeta potential and, consequently, the stability of the dispersed nanoparticles were determined using a Zeta-Meter 4.0 (Zeta-Meter Inc., Staunton, VA, USA).

Preliminary analysis of the morphology and size of the synthesized silver nanoparticles was performed using a scanning electron microscope (JSM-5600, JEOL, Tokyo, Japan)

using an accelerating voltage of 30 kV. Before analysis, a small aliquot of the nanoparticle suspension was deposited on the surface of a carbon ribbon, dried at room temperature, and subsequently coated by a thin layer of platinum to make the samples conductive.

2.5. Determination of Nanoparticles' Antifungal Activity

Three species of fungi (*Aspergillus niger* UFPEDA 5117, *Penicillium fellutanum* UFPEDA 6472, and *Cladosporium cladosporioides* UFPEDA 6703), which are well-known plant or human pathogens, were obtained from the Collection of Cultures of Microorganisms of the Department of Antibiotics of the Federal University of Pernambuco (UFPEDA), Recife, Brazil.

The in vitro assay of antifungal activity was performed in potato dextrose agar (PDA) fungal growth medium with added dispersion of synthesized silver nanoparticles. Here, 200 µL of the dispersion was added in order to obtain final concentrations of silver nanoparticles of about 1.65, 8.25, and 16.50 µg/L per Petri dish. After preparing the plates, agar plugs from each fungus were inoculated in the center of them and incubated at 30 °C until mycelial growth on the control dish reached the edge of the Petri dish, and the size of the inhibition halo was recorded [17].

The radial growth of fungal mycelium was measured and recorded after the incubation period of the fungus in culture media with different concentrations of BS-AgNPs. The following equation was used to calculate the inhibition rate (%) [18]:

$$\text{Inhibition rate } (\%) = \frac{R - r}{R}, \quad (1)$$

where R is the radial growth of fungal mycelium in the control plate, and r is the radial growth of fungal mycelium in the plate containing BS-AgNP.

2.6. Statistical Analysis

The data were analyzed statistically using the one-way procedure in the Statistica® version 7.0 software package (Statsoft Inc., Tulsa, OK, USA), followed by one-way linear analysis of variance (ANOVA). The results were expressed as the mean ± standard deviation (SD) determined from triplicate experiments.

3. Results and Discussion

3.1. Characterization of Silver Nanoparticles

The biosurfactant was successfully produced by *Bacillus cereus* UCP 1615 in mineral medium supplemented with residual frying oil, which makes the process more feasible, in addition to contributing to a reduction in input costs [16]. Because fermentation took place in 48 h at room temperature (28 °C), this method would be attractive for industrial use.

The synthesis of nanoparticles using the biosurfactant as a stabilizer was revealed, according to Tyagi et al. [18], by the change in the color of the solution (Figure 1), and confirmed in Figure 2 by the presence of a maximum UV/vis absorption peak characteristic of silver nanoparticles, between 400 and 450 nm, corresponding to localized surface plasmon resonance (LSPR) [18].

NPs made of coin metals have their unique optical properties arising from their specific behavior under light irradiation, i.e., the appearance of LSPR [19]. Garcia [20] highlighted that the main characteristic of LSPR in NPs is that the oscillation of excited electrons is limited by the volume of the NPs. Thus, the UV/visible spectral signature serves to assess the presence, uniformity, and size of AgNPs stabilized with the biosurfactant.

The plasmonic absorption peak, resulting from the LSPR characteristic of NPs, was observed in the newly synthesized (0 days) BS-AgNPs close to 400 nm, consistently with a previous study carried out on AgNPs stabilized with a lipopeptide from *Bacillus subtilis* grown in sunflower oil [6]. The diameter of the colloidal silver nanoparticles was found to be approximately 20 nm in the study with *B. subtilis*. Spherical silver NPs have the LSPR absorption light band normally close to 400 nm [21]; therefore, it is possible to

infer that BS-AgNPs could actually have this spherical shape and a size predominantly close to 20 nm. The peak observed after 120 days was close to 440 nm, which, in turn, corroborates the observations made for AgNP stabilized with a rhamnolipid produced by *Pseudomonas aeruginosa* that exhibited the characteristic peak at 428 nm and particle size around 160 nm [21].

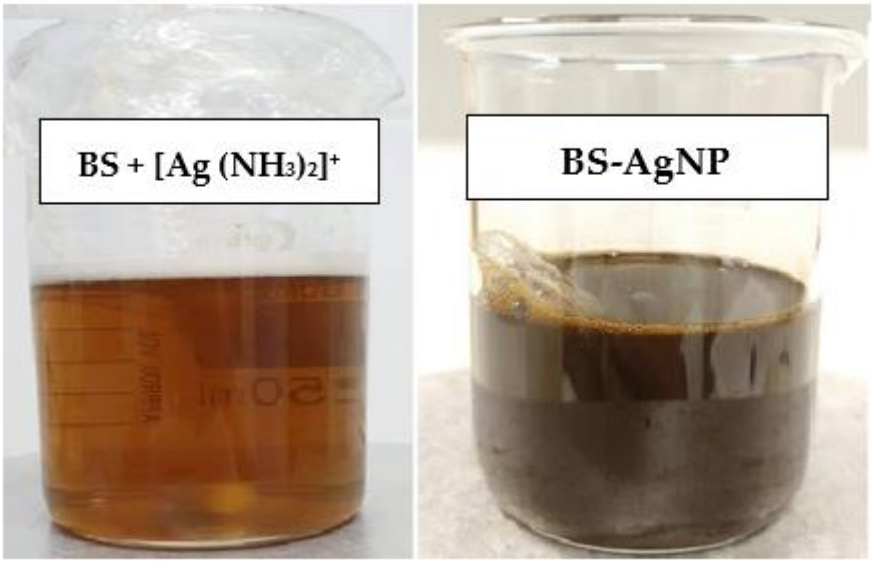

Figure 1. Change in the color of the solution after reduction of the silver complex, showing the formation of nanoparticles stabilized with the biosurfactant (BS-AgNPs).

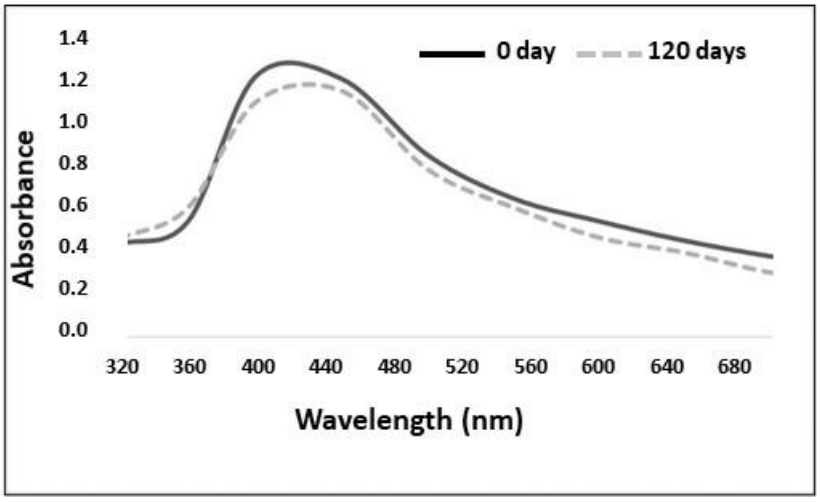

Figure 2. UV/visible spectrum of nanoparticles stabilized with the biosurfactant (BS-AgNPs) either at the start (0 days) or after 120 days of formation.

It can be noted that the peak was broad and had a strong tail, indicating a wide distribution of nanoparticle size, requiring studies to adapt the technique, such as the synthesis time and the amount of added metallic particles, to obtain a well-defined peak and, consequently, a solution with majority presence of particles of a single size [22].

The shift of the maximum absorption peak of BS-AgNPs from 400 to 440 nm after 120 days of storage shown in Figure 2 was indicative of an increase in the size of the nanoparticle diameter. Such a particle enlargement may be an example of the Ostwald ripening phenomenon, based on which small particles in solution lose stability and re-organize into larger particles so as to achieve greater thermodynamic stability, causing a reduction in the surface-to-volume ratio [23].

Scanning electron microscopy (SEM) was used to assess the morphology of nanoparticles (Figure 3), as well as to measure their actual diameter.

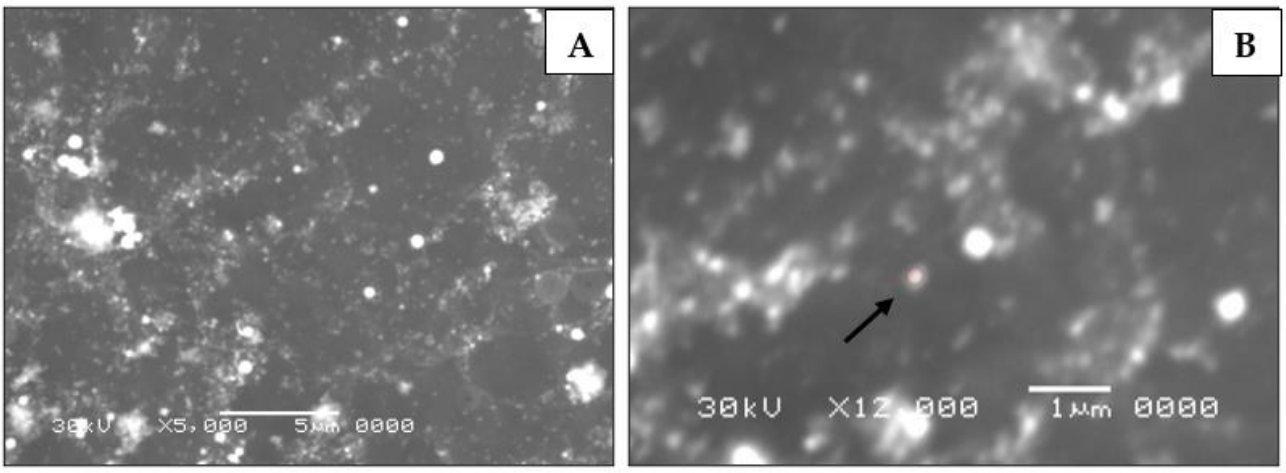

Figure 3. Scanning electron microscopy images of nanoparticles stabilized with the biosurfactant (BS-AgNP): (**A**) 5000× magnification; (**B**) 12,000× magnification.

Observing the BS-AgNP solution at a 5000× magnification (Figure 3A), points with NP agglomeration can be seen, which hindered the characterization of particle size; however, when the magnification was enlarged to 12,000×, non-aggregated and rounded AgNPs with a diameter of approximately 160 nm could be observed (indicative arrow in Figure 3B). This means that particles were not completely separated from each other, exhibited a high surface area, and formed clusters [24].

The SEM images obtained by Elakkiya et al. [21] for AgNP synthesized with a rhamnolipid biosurfactant revealed high-density structures with diameter in the range of 30–150 nm and a random distribution. The rounded shape corroborates the fact that silver NPs have the LSPR absorption light band normally close to 400 nm [19].

The colloidal stability of NPs was indirectly checked through the zeta potential. This parameter reached a value quite far from zero (−23.4 mV ± 1.4), which highlights a relatively strong electrostatic repulsion among particles, resulting in low chance of aggregation or precipitation.

In the work published by Nehal and Singh [24], a lipopeptide-rich cell-free extract, obtained by *Bacillus paramycoides* fermentation in a medium supplemented with olive oil, was added to a solution of $AgNO_3$ and sodium borohydride ($NaBH_4$). At the end of the reaction, stable nanosuspensions were generated with a zeta potential peak of −38.7 mV. Elakkiya et al. [21], using a similar methodology, but with a *P. aeruginosa* rhamnolipid, reported a value of −31.6 mV. Lastly, in another study, also carried out with a *P. aeruginosa* rhamnolipid, but where AgNPs were formed via reaction with an $AgNO_3$ solution at pH 12, NP stability was greatly enhanced by an increase in zeta potential (in absolute value) from −23.8 to −56.3 mV in the presence of the biosurfactant [25].

3.2. Antifungal Analysis

Many filamentous fungi that are plant and human pathogens can cause devastating damage to agriculture, as well as potentially fatal diseases in humans [26,27]. In agriculture, they cause a reduction in crop yield, loss in germination, development of discoloration and deformation due to plant diseases, and biochemical changes in the seed. In humans, damage to health is often associated with the action of these microorganisms either directly or through the ingestion of toxic chemical compounds and mycotoxins produced by them [28].

For this reason, the fungicidal potential of silver nanoparticles against fungi of genera known for their pathogenicity to humans and plants has been evaluated in the present

work. Table 1 shows the inhibition rate (%) of *Aspergillus niger*, *Cladosporium cladosporioides*, and *Penicillium fellutanum* growth promoted by the addition of BS-AgNPs at different concentrations to the growth medium.

Table 1. Inhibition rate (%) caused by biosurfactant-complexed silver nanoparticles (BS-AgNPs) at different concentrations on *Aspergillus niger*, *Cladosporium cladosporioides*, and *Penicillium fellutanum* growth in potato dextrose agar (PDA) medium. Data expressed as the mean ± SD of triplicate determinations.

BS-AgNP Concentration (μg/L)	Inhibition Rate (%)		
	A. niger	*C. cladosporioides*	*P. fellutanum*
1.65	74.20 ± 1.51	41.48 ± 4.19	97.78 ± 0.00
8.25	78.70 ± 0.68	66.67 ± 0.00	97.78 ± 0.00
16.50	85.78 ± 2.65	65.50 ± 1.65	100.00 ± 0.00

The inhibition of *P. fellutanum* growth stood out for being close to 100% at all BS-AgNP concentrations tested, with complete inhibition at 16.50 μg/L. Among the fungi selected for the study, the lowest growth inhibition rates, between 41.48% and 66.67%, were observed for *C. cladosporioides*, when compared to growth in a medium without BS-AgNP. The inhibition rate of *A. niger* growth achieved 85.78% at the highest concentration of BS-AgNP and 74.20% at the lowest one. In general, a higher concentration of nanoparticles in the growth medium led to a higher growth inhibition rate against all fungi. The inhibition halos caused by the addition of BS-AgNP at the highest concentration are illustrated in Figure 4.

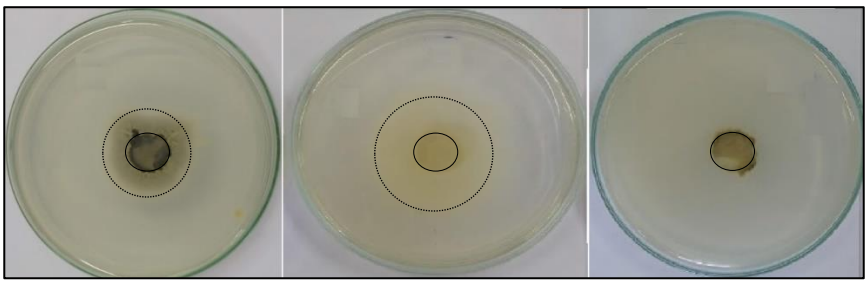

Figure 4. Halos of the growth of *Aspergillus niger*, *Cladosporium cladosporioides*, and *Penicillium fellutanum* (from left to right) in potato dextrose agar (PDA) medium supplemented with biosurfactant-complexed silver nanoparticles (BS-AgNP) at a concentration of 16.50 μg/L. The agar plugs are highlighted in the image by a solid line, while the halo of mycelium growth is represented by dashed lines.

A study on growth inhibition caused by silver nanoparticles synthesized by the green method against the phytopathogens *Alternaria solani*, *Corynespora cassiicola*, and *Fusarium* spp. revealed inhibition rates above 90% for all fungi in PDA medium at the highest concentration of nanoparticles (25 μg/mL) [18]. AgNPs biosynthesized through the mediation of the endolytic bacterium *Pseudomonas poae* showed strong antifungal activity against the pathogen *Fusarium graminearum*, with inhibition rates above 50% at concentrations between 10 and 20 μg/mL, which was attributed, at least partially, to inhibition of spore germination, growth of the germ tube, mycotoxin production, and cell membrane damage [29].

Another study on AgNPs ecologically synthesized using luteolin tetraphosphate as a reducing and stabilizing agent showed that a smaller diameter of NPs in the range of 9–21 nm led to a higher antimicrobial activity against plant pathogens [30].

The results described in the literature corroborate the data obtained in this work, thus indicating the potential of nanoparticles as antifungal agents, with the additional advantage

of having been prepared via a green method using the biosurfactant as a biocompatible stabilizing agent.

4. Conclusions

The present study was successful in the green synthesis, through the Tollens method, of silver nanoparticles (BS-AgNPs), using as a stabilizing agent the biosurfactant produced by *Bacillus cereus* UCP 1615, in a low-cost medium supplemented with waste frying oil. BS-AgNPs were shown to be stable for over 120 days, even if an increase in their size was observed over time. In this preliminary evaluation, the AgNPs appeared to be spherical and uniform, with such a small diameter being compatible with several biotechnological applications. The nanoparticles exhibited dose-dependent antifungal activity against pathogens that harm agriculture and human health. Therefore, it can be concluded that biosurfactants produced by green synthesis have potential for use as ecofriendly nanoparticle stabilizers.

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