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ORIGINAL RESEARCH ARTICLE

Biosynthesis of silver nanoparticles with *Chlorella* sp.

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ABSTRACT

This work shows the results of the biosynthesis of silver nanoparticles using the microalga *Chlorella* sp, using growth media with different concentrations of glycerol, between 5%–20%, and different light and temperature conditions. The synthesis of nanoparticles was studied using supernatants and pellets from autotrophic, heterotrophic and mixotrophic cultures of the microalga. The presence of nanoparticles was verified by ultraviolet-visible spectroscopy and the samples showing the highest concentration of nanoparticles were characterized by scanning electron microscopy. The mixotrophic growth conditions favored the excretion of exopolymers that enhanced the reduction of silver and thus the formation of nanoparticles. The nanoparticles obtained presented predominantly ellipsoidal shape with dimensions of 108 nm × 156 nm and 87 nm × 123 nm for the reductions carried out with the supernatants of the mixotrophic cultures with 5% and 10% glycerol, respectively.

Keywords: Green Chemistry; Silver Nanoparticles; Biosynthesis; Microalgae

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1. Introduction

The emergence of microorganisms resistant to common disinfectants has prompted an extensive search for new products that are effective in killing or inhibiting bacterial growth, and at the same time are harmless to humans. Silver nanoparticles (Np Ag) are a possible nanotechnology response to this challenge, as their high surface area per unit volume increases the already known antibacterial properties of silver[1].

Np Ag can be synthesized by various methods, such as chemical reduction reaction, photochemical reaction, thermal decomposition, radiation-assisted methods, electrothermal processes, sonochemical and microwave-assisted synthesis[2]. These methods produce silver nanoparticles efficiently, however, often these methods involve the use of toxic and hazardous chemicals, which present several harmful effects to the environment and human health[3], also the final product requires several stages of purification, in which reducing agents are used that are often adsorbed on the surface of the nanoparticles, it is also necessary to use stabilizers to avoid agglomeration of silver nanoparticles[4].

Some plant extracts can act as reducing leveling agents in the synthesis of silver nanoparticles. The reduction of Ag^+ ions by combinations of biomolecules found in these extracts such as enzymes/proteins, amino acids, polysaccharides and vitamins[5,6], are environmentally benign, although chemically complex. An extensive body of literature reports the successful synthesis of Np Ag using compounds from bioorganic living things; for example, the extract of unicellular green algae *Chlorella vulgaris* was used to synthesize crystalline Ag nanosheets at

room temperature[7]. The proteins in the extract provide dual functions of Ag^+ reduction and a morphology control in Np Ag synthesis. Where carboxyl groups on aspartic and glutamine residues and hydroxyl groups on tyrosine residues of proteins emerged as architects of Ag^+ ion reduction[7], which is confirmed by Ag reduction by the single bifunctional tripeptide Asp-Asp-Tyr-OMe. This synthesis process generated small Ag nanoplates with low polydispersity with good yield (55%)[8]. Various microorganisms have been used to grow silver nanoparticles intracellularly or extracellularly[9,13]. For example, Ag^+ containing nanocrystals of different compositions were synthesized by the bacterium *Pseudomonas stutzeri* (AG259)[9]. In the fungus *Fusarium oxysporum*, the reduction of Ag^+ ions is attributed to an enzymatic process, involving NADH reductase which is due to NADH reductase dependence[12]. The white-rot fungus, *Phanerochaete chrysosporium*, also reduced Ag^+ ion to form nanoparticles; suggesting that a protein from its metabolism caused the reduction. A protein was suggested to cause the reduction[13]; noting the possible involvement of proteins in Np Ag synthesis in filamentous cyanobacteria, *Plectonema boryanum* (UTEX 485)[14]. On the other hand, Ag^+ reduction by culture supernatants of *Klebsiella pneumoniae*, *Escherichia coli (E. coli)*, *Enterobacter cloacae* and *Enterobaceteriacae* produced silver nanoparticle formations[15].

In this work, one of the methodologies to synthesize silver nanoparticles, considered as part of the green synthesis group, was used, since the microalga *Chlorella* sp. was used as an agent for the formation of exopolymers[16,17], which promotes the formation of these nanoparticles. These exopolymers are products of the metabolism of *Chlorella* sp. when using glycerin as a substrate or when cultured under stress conditions, and being composed of monomeric units of sugars (with a large amount of hydroxyl groups)[18], it has the ability to easily attract silver ions from the silver nitrate solution, thus forming Np Ag.

2. Methodology

2.1 Stock culture of the microalgae *Chlorella* sp.

The strain of *Chlorella* sp. was tested by the Microalgae Biotechnology Laboratory of the Universidad del Atlántico. The culture started with 500 mL of a seed culture of the microalgae, which was diluted with sterile water to a volume of 2,000 mL, adding 4.7 mL of a 2.14 M solution in total nitrogen of Nutrifoliar® previously sterilized, to obtain a final nitrogen concentration of 5 mM. The cultures were grown in duplicate and provided with aeration, feeding and illumination (2,710 lm, Philips TL 65 W 25 fluorescent tubes, white light) and incubated for a period of 20 days, with light/dark cycles of 12 hours, respectively, using artificial light and room temperature of 24 °C. Cultures were gauged to 2,000 mL every 15 days using sterile water and 5.8 mL of Nutrifoliar® at 2.14 M total nitrogen.

2.2 Biosynthesis of silver nanoparticles

For the study of the synthesis of reducing agents produced by microalgae, under different culture conditions and their effect on Np Ag production, using a factorial design with two independent variables: illumination and temperature conditions, with three levels and glycerol concentration, as an organic carbon source, with 5 levels (0, 5, 10, 10, 15 and 20%) (**Table 1**).

Table 1. Factors and experimental levels

Experimental factor	Levels
Lighting and temperature conditions	Ambient temperature with continuous illumination
	Ambient temperature in continuous darkness
	−18 °C in continuous darkness
Glycerol concentration (% vol/vol)	0
	5
	10
	15
	20

The cultures were incubated for three days and then the cells were separated by centrifugation (10 min at 8,000 rpm). The synthesis of Np Ag was performed using reducing agent, the supernatant and precipitate solutions separately, in order to determine whether the microalgae actively participate in the synthesis of Np Ag or only the excreted metabolic products are responsible for the synthesis.

The synthesis procedure was as follows: 2 mL of precipitate or supernatant, for each of the exper-

imental combinations, were transferred to 10 mL culture tubes and 2 mL of silver nitrate ($AgNO_3$ J.T. Baker) with a concentration of 2 mM were added, and then subjected to vortex mixing for 2 min. The homogenized medium was again incubated under continuous artificial light for 3 days at room temperature. The experimental response variable was the UV-Visible spectrum of the samples, which allows the identification of the possible formation of silver nanoparticles. All assays were performed in duplicate. The UV-Vis spectra were obtained in a Genesys 60 s spectrophotometer (Thermofisher) using quartz cells with a step length of 1 cm and a spectrometric range from 290 to 900 nm.

2.3 Scanning Electron Microscopy (SEM)

The equipment used for this measurement is a SEM model VEGA3 TESCAN. Before the measurement, it was necessary to remove the organic matter, through successive washes of the samples using a volatile solution of 95% ethanol, as follows: the samples were centrifuged for 10 min at 8,000 rpm, followed by discarding the supernatant, adding 5 mL of 95% ethanol and resuspending the Np Ag in ultrasound for 10 min. Subsequently, for the second step, the Np Ag were centrifuged at 10,000 rpm, for 10 min. The supernatant was discarded and resuspended with 5 mL of 2-propanol in ultrasound for 10 min. This procedure was repeated 12 more times to ensure the removal of organic matter. The images obtained were processed with Image software to determine the shapes and sizes of the Np Ag.

3. Results and discussion

3.1 UV-Vis spectra of blank solutions

The UV-Vis spectra obtained from the aqueous solutions of silver nitrate and different concentrations of glycerol (5%, 10%, 15% and 20%) show flat absorption profiles in most of the photometric range analyzed, with slight absorption of light at wavelengths below 400 nm (**Figure 1**). This behavior is characteristic of non-reduced silver solutions[19]. These solutions were used as targets in the photometric measurements of the subsequent tests performed.

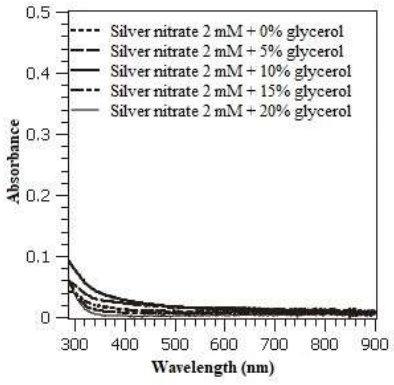

Figure 1. UV-Vis spectra of silver nitrate solutions with different concentrations of glycerol.

3.2 Effect of culture conditions on the production of silver nanoparticles

Figure 2 shows the UV-Vis absorption spectra of the silver nitrate and glycerol solutions subjected to each of the experimental treatments, using the cell-excretion metabolite-rich medium (supernatant, **Figures 2A**, **2B** and **2C**) and some representative treatments using *Chlorella* sp. cells (precipitate, **Figure 2D**). It is observed that most of the treatments in which silver reduction was performed using the supernatant of *Chlorella* sp. cultures grown at room temperature show the characteristic absorption peak of the plasmon effect in the wavelength range comprised between 400 nm and 500 nm (**Figures 2A** and **2B**), as well as the characteristic shift to a reddish-brown color[20,21]. In contrast, samples that underwent reduction with the culture supernatant obtained in the absence of light and low temperatures, as well as those in which precipitates were used, show no absorption peak between 400 nm and 500 nm. This suggests that the experimental treatments in which the reduction was done using as reducing agent the supernatant of the cultures of the microalgae grown at room temperature and under light or dark conditions there was formation of silver nanoparticles, while in the treatments in which supernatants of cultures obtained at low temperatures or the precipitate of any of the fermentations (cells) were used there was no reduction of this element.

In the case of the reduction carried out with the supernatants of the culture performed in the presence of light at room temperature, it is observed that the presence of glycerol in the culture medium

stresses the microalgae (probably osmotic stress) in such a way that it produces excretion metabolites that favor the reduction in the presence of light, at least up to glycerol concentrations of 10%. Above this glycerol concentration, a decrease in the intensity of the absorption peak corresponding to the plasmon effect, and thus of the Np Ag concentration, is observed, probably because at high glycerol concentrations, the microalga grows at a slower rate and is likely to produce fewer reducing metabolites[22,25]. This decrease in the intensity of the absorption peak associated with the plasmon effect at high concentrations also indicates that glycerol is not the reducing agent for silver, but rather the extracellular metabolites produced during microalgal growth. When comparing silver reduction with supernatants from microalgae cultures grown in the presence and absence of light at room temperature, a lower peak intensity (less than 50% in cases where there is evidence of Np Ag formation) associated with the plasmon effect is observed in the reductions carried out using supernatants from cultures grown in the dark, when compared to the curves corresponding to the same glycerol concentrations and cultures grown in the presence of light. This points to the fact that more reducing metabolites are formed during the microalgae growth process under mixotrophic conditions than under autotrophic (0% glycerol concentration) or heterotrophic (in the absence of light) conditions[23]. On the other hand, the results shown in **Figure 2C** indicate that in the absence of growth the silver-reducing metabolites are not produced. The results point out that the presence of glycerol during growth is determinant for the production of reducing metabolites and the formation of Np Ag and even these metabolites can be produced during strictly heterotrophic growth using glycerol as carbon source. However, the production process is favored under mixotrophic conditions, probably because higher growth rates are present under these conditions.

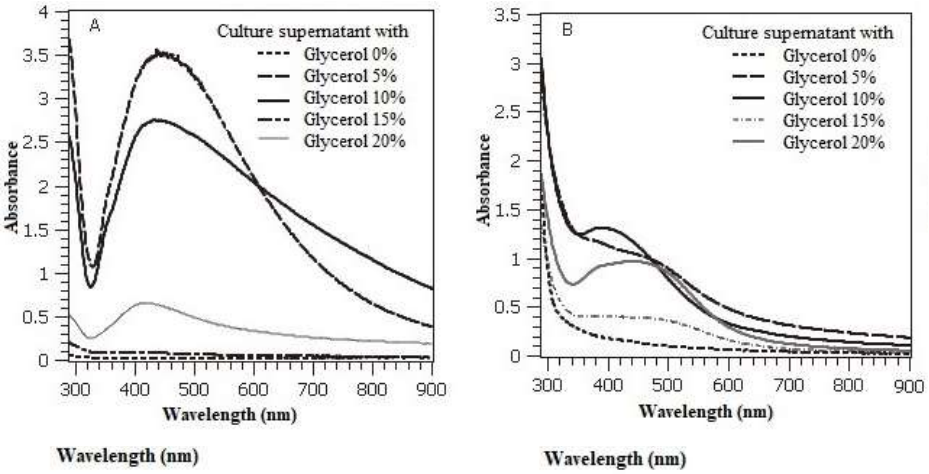

Figure 2. UV-Vis spectra of silver nitrate solutions with different concentrations of glycerol. (**A**) Supernatants of cultures grown in the presence of light at room temperature; (**B**) supernatants of cultures grown in the absence of light at room temperature; (**C**) supernatants of cultures grown in the absence of light at -18 °C; (**D**) precipitates obtained with 15% glycerol cultures and different culture conditions.

The results shown in **Figures 2A** and **2B** suggest that at concentrations of 20% glycerol, the microorganism also produces extracellular metabolites, which can reduce the nanoparticles that produce silver. However, the effect is less marked than for the 5% and 10% glycerol concentrations, particularly in assays with growth under mixotrophic conditions: for the 20% glycerol concentration, the peak of the surface plasmon effect has 0.4 absorbance units in the spectrum in the absence of light and 0.65 in the presence of light, for a difference of 0.25 absorbance units, whereas in the case of the spectra of the supernatants at 5% and 10% glycerol concentrations, these differences amount to 2.5 and 1.2 absorbance units, respectively. It has been reported that the mixotrophic growth of *Chlorella* sp. generates greater production of exopolymers than its growth under heterotrophic and autotrophic conditions[17]. These natural polymers are made up of both reducing and non-reducing sugar mono-

mers[16,17]. Sugars have been reported as reducing agents of silver and Np Ag production[26,29], so we suggest that it is the exopolymers produced by *Chlorella* sp. and found in the supernatant that are responsible for the production of Np Ag under the conditions studied in this work. The use of supernatants from bacterial[15,30,31], fungal[32,33], and microalgae cultures for the production of Np Ag has been reported.

3.3 Characterization of silver nanoparticles

The assays that showed peaks of higher intensity in the wavelength range between 400 nm and 500 nm, correspond to the reductions carried out with the supernatants of the cultures performed at room temperature and in the presence of light and initial glycerol concentrations of 5% and 10%. The nanoparticles obtained with these assays were characterized by scanning electron microscopy, as shown in **Figures 3** and **4**. In the case of the Np Ag obtained with the culture supernatant at room temperature, in the presence of light and 5% glycerol, they showed a predominantly ellipsoidal shape with typical dimensions of 108 nm × 156 nm and a standard deviation of 27 nm for both dimensions (**Figure 3**). On the other hand, Np Ag reduced with the culture supernatant carried out at room temperature, illumination and 10% glycerol showed ellipsoidal and spherical shapes, with average dimensions of 87 nm × 123 nm ± 15 nm × 27 nm. In both cases, the Np Ag were found to be aggregated on organic matter, despite the extensive washes performed on the samples, possibly the same exopolymers aiding in their reduction. In a similar study, but without overexpression of exopolymers[33], due to the presence of glycerol, predominantly spherical and smaller silver nanoparticles were obtained, suggesting that the use of glycerol may serve as an equalizing factor for the shapes and sizes of the nanoparticles produced.

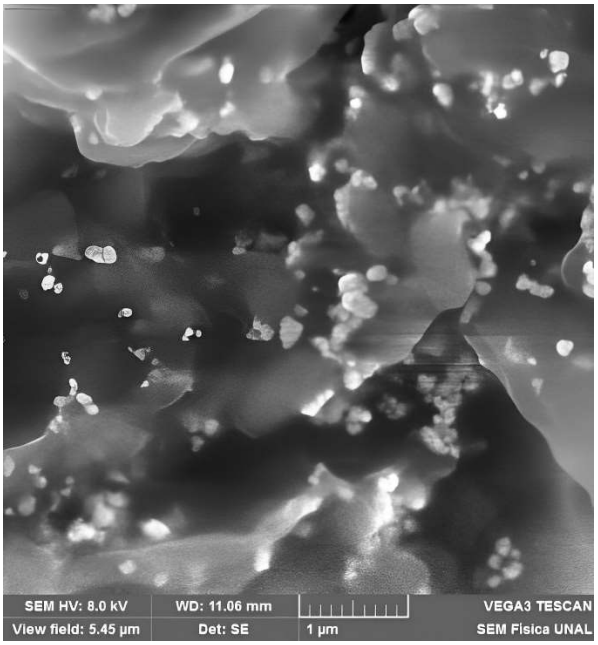

Figure 3. SEM image of the silver nanoparticles obtained from the supernatant of the *Chlorella* sp. Culture at room temperature and 5% glycerol.

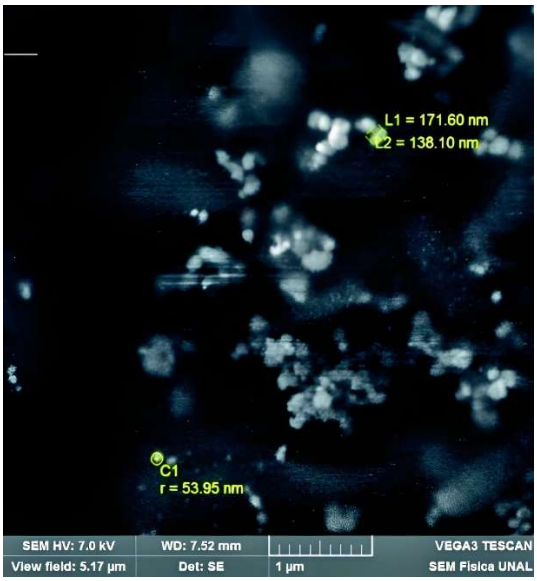

Figure 4. SEM image of the silver nanoparticles obtained with the supernatant of the *Chlorella* sp. Culture at room temperature and 10% glycerol.

4. Conclusions

Silver nanoparticles were successfully synthesized using the metabolites excreted during the mixotrophic culture of *Chlorella* sp. The use of glycerol as a source of organic carbon in the mixotrophic culture up to concentrations of 10% increases the height of the peak associated with the plasmon effect, which suggests an increase also in the concentration of silver nanoparticles when compared to the results obtained with the reduction using the supernatant of the *Chlorella* sp. culture obtained under autotrophic conditions. The culture conditions that promoted to a greater extent the production of reducing metabolites were the culture at room temperature and under mixotrophic conditions with initial glycerol concentration of 5% and

10%. The nanoparticles thus obtained presented predominantly ellipsoidal shape with dimensions of 108 nm × 156 nm, in the case of the Np Ag obtained with the supernatant of the culture at room temperature, in the presence of light and 5% glycerol; while the Np Ag reduced with the supernatant of the culture carried out at room temperature, illumination and 10% glycerol showed average dimensions of 87 nm × 123 nm.

Conflict of interest

The authors declare that they have no conflict of interest.

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