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ANTIMICROBIAL EFFECT OF BIOLOGICAL AND CHEMICAL SILVER NANOPARTICLES IN ENVIRONMENTAL SAMPLES

Efecto antimicrobiano de las nanopartículas de plata biológicas y químicas en muestras ambientales

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Abstract

The resistance of bacterial strains to antimicrobial agents and biofilm-associated infections causes considerable economic losses and worldwide deaths. If this problem continues it is estimated that in 2050, about 10 million human deaths could occur per year and the costs would reach 1 trillion USD globally. Most of the studies evaluating the antimicrobial effect of an antimicrobial agent focus on pure bacterial cultures, even when it is known that microorganisms live in communities interacting with each other, causing a less efficient antimicrobial effect on target compounds. Because of previous data, it is necessary the search for alternative and effective methods that, at the same time, do not generate bacterial resistance; silver nanoparticles (AgNPs) can be an excellent alternative; moreover, the evaluation of these antimicrobial agents on microbial communities from environmental samples are needed. In this paper, we synthesized spherical AgNPs by biological and chemical methods with an average diameter of 10.32 and 9.53 nm respectively; we evaluated the antimicrobial effect of both in microbial populations that came from three different environmental samples (computer keyboard, tap water, and pharyngeal exudate). Results showed that both AgNPs are excellent antimicrobial agents obtaining for both inhibition percentages higher than 90%.

Keywords: *antimicrobial effect, biological nanoparticles, chemical nanoparticles, environmental samples, silver nanoparticles*.

Resumen

La resistencia de las cepas bacterianas a los agentes antimicrobianos y las infecciones asociadas a biopelículas provoca pérdidas económicas considerables y muertes en todo el mundo. De continuar este problema, se estima que en el año 2050 podrían ocurrir alrededor de 10 millones de muertes humanas y los costos alcanzarían 1 billón de dólares a nivel mundial. La mayoría de los estudios de evaluación del efecto antimicrobiano se han enfocado en el estudio de cultivos puros, aun cuando se sabe que los microorganismos viven en comunidades que interactúan entre sí, lo anterior ocasiona que el efecto antimicrobiano de los compuestos objetivo sea menos eficiente. Debido a esto, es necesaria la búsqueda de métodos alternativos que sean efectivos y no generen resistencia bacteriana; las nanopartículas de plata (AgNPs) pueden ser una excelente alternativa, así también es muy importante la evaluación de estos agentes antimicrobianos en comunidades microbianas provenientes de muestras ambientales. En este estudio se reporta la síntesis de AgNPs esféricas por métodos biológicos y químicos con un diámetro promedio de 10,32 y 9,53 nm respectivamente; se evalúa el efecto antimicrobiano de ambos tipos de nanopartículas en la población microbiana proveniente de tres muestras ambientales diferentes (teclado de computadora, agua del grifo y un exudado faríngeo). Los resultados mostraron que ambos tipos de AgNPs son excelentes agentes antimicrobianos obteniendo en ambos casos porcentajes de inhibición mayores al 90%.

Palabras clave: *efecto antimicrobiano, nanopartículas biológicas, nanopartículas químicas, muestras ambientales, nanopartículas de plata*

1. INTRODUCTION

Bacterial resistance is a problem observed around the world since the appearance of antibiotics because microorganisms have genetic mechanisms that allow them to adapt to environmental pressures; resistance to antimicrobial agents is a natural evolution process; however, some factors increase the expression and dissemination of resistance genes such as the inadequate use of antibiotics, incorrect and interrupted supply in the human population and their indiscriminate use in non-health sectors such as agriculture, aquaculture and livestock [1]. Stated factors have caused the emergence of resistant bacterial strains, a situation that has generated a serious public health concern worldwide causing considerable economic losses, morbidity, and mortality [2].

Most studies report that antimicrobial agents are tested in pure bacterial cultures; however, in nature, bacteria are present in consortia formed by different species of bacteria which interact with each other; it has also been observed that these bacterial groups have greater resistance to antimicrobial agents; some bacterial species are even capable of forming biofilms, which are characterized by colonies of agglomerated microorganisms that are adhered to a substrate through the secretion of binding-molecules as adhesion proteins in addition to secreting peptidoglycans that create a covering that protects the microorganisms, making them more difficult to eliminate. Most of the studies explore environmental samples to isolate the bacteria and then evaluate antimicrobials on each one [3,4].

Currently, new alternatives to the use of antimicrobial agents have been assessed, such as the use of nanocomposites and metal nanoparticles, with a greater increase in silver nanoparticles (AgNPs) due to their well-known antimicrobial effect [5]. The specific toxicity mechanisms of AgNPs on microorganisms are not fully elucidated; however, they can act as follows; 1) they interact and destabilize the cell wall and membrane by disrupting cell permeability and respiration, 2) nanoparticles inside the cell alter the functions of proteins and DNA, 3) producing oxidative stress by the ROS production [6,7].

Therefore, it is more difficult for them to generate bacterial resistance due to the nanoparticles' non-specific action mechanisms [8], so they could be used against multi-resistant bacteria in the biofilm. The antimicrobial effect of AgNPs will depend on several factors such as size (greater toxicity for smaller AgNPs), shape, surface (smaller size, greater surface area more exposed atoms) surface charge, solubility, exposure time, and concentration [9,10]; thus, the antimicrobial effect of AgNPs could be different depending on synthesis parameters.

Calvo et al. [11], reported the antimicrobial effect of the biological AgNPs synthesized by extracellular filtrate of fungus ***Paecilomyces variotii*** on pure bacterial cultures demonstrating that the antimicrobial effect varied according to the type of microorganism, even between strains of the same species. The inhibition percentages obtained for all the analyzed strains were greater than 89%; the most sensitive strain was ***Proteus vulgaris*** with a minimum inhibitory concentration (MIC) of 99.8%. The most resistant was ***Staphylococcus aureus*** ATCC 25923 with 89.9% [11]. Based on the previous information (11), the objective of the present study was to evaluate the antimicrobial effect of both AgNPs synthesized chemically and biologically on the microbial populations present in three environmental samples and to determine if there is different inhibition behavior between both types of nanoparticles.

2. MATERIALS AND METHODS

2.1. Materials

The fungus *Paecilomyces variotii* was obtained from the microbial collection of the Environmental Biotechnology Laboratory at CICATA-QRO. The fungus was obtained and isolated from a wet spent catalyst (TiO) which contained $SO4^{2-}$, S and SO. Three different types of samples of microbial origin were studied, which were taken using a sterile swab covered with a culture medium, the first sample was taken from a computer keyboard, the second sample was taken from a biofilm found in tap water, and the third sample was taken from a 58 years old patient with a diagnosis of cholangiocarcinoma (bile duct cancer), liver and diabetic abscess at the ISSSTE hospital in San Luis Potosi, México.

Nutrient broth, nutritive agar, and D- glucose were purchased from Bioxon (México). Cadmium nitrate and sodium sulfide were obtained from Fermont (México), and Potato dextrose agar and Starch were purchased from Becton Dickinson and Company (Belgium). Malte dextrose broth was obtained from Dibico (México). $AgNO_3$ was purchased from Sigma Aldrich (Germany).

2.2. Methods

2.2.1. Biological Synthesis

The biological synthesis of AgNPs was carried out using the extracellular filtrate (EF) of the fungus ***Paecilomyces variotii.*** The fungus was cultured in potato dextrose agar (PDA) for 72 h at 30°C, after that, a solution of $3.3x10^5$ spores/mL was inoculated in 120 mL flasks with 30 mL of malt dextrose broth at 30°C, 140 rpm for 5 days. Afterward, the mycelium was separated by vacuum filtration, and 2 gr of mycelium was weighed and incubated with 30 mL of distilled water at 30°C, 140 rpm for 72 h; later, the EF was recovered by vacuum filtration. The EF was incubated with 1 mL of 30 mM $AgNO_3$ at 45°C, 180 rpm for 48 h under dark conditions. The inorganic negative control (NC) contained 30 mL of 1 mM $AgNO_3$ and the enzymatic activity control (EAC) consisted of 30 mL of the extracellular filtrate and 1 mL of sterile distilled water.

2.2.2. Chemical synthesis

The AgNPs were prepared by the methodology reported by Aguilar et al. [10], where 200 mL of starch at 0.17% was heated at 80°C until gelatinization, and then 3.33 mL of $AgNO_3$ 0.1 M and 5 mL of D-glucose were added; finally, the pH was adjusted at 10 by adding NaOH at 12%; the AgNPs synthesis was evidenced by the color change from yellow to brown. The NC consisted of 30 mL of 0.1 M $AgNO_3$.

2.2.3. Nanoparticles characterization

The AgNPs synthesized by both methods were analyzed by UV-Vis measurement at 200 to 800 nm in a spectrophotometer (Genesys 10S, Thermo Scientific, Spain), and data were plotted with the Origin Pro v8 program. The shape, size, and dispersion of the NPs were observed in an electronic transmission microscope (JEOL J2000FX, USA) with a resolution of 200000x. The ImageJ program was used to analyze the NPs images.

2.2.4. Assessment of antimicrobial activity

Three different types of environmental samples were under study (computer keyboard, tap water, and pharyngeal exudate), each sample was taken using a sterile cotton swab impregnated with nutrient broth, incubating in 10 mL of nutrient broth at 30°C for 24 h.

The MIC (Minimum Inhibitory Concentration) was determined, this value is defined as the lowest concentration of antimicrobial agent that inhibits microbial growth (12). The tests were performed with different nanoparticle concentrations in nutrient broth (5, 10, and 15 ppm) and inoculated with $1x10^6$ CFU/mL of each sample, this concentration was determined according to literature (3). All experimentation was done in triplicate using as control the sole inoculum of each sample in nutrient broth without nanoparticles. Finally, the tubes were incubated at 30°C for 24 h. Treatments, where the lowest AgNPs concentration had a negative effect on the microbial culture, were reported as the MIC.

The MBC (Minimum Bactericidal Concentration) is defined as the lowest concentration of antimicrobial

agent needed to kill 99.9% of the final inoculum after incubation for 24 h (12). For this assay, after 24 h of incubation, serial dilutions of the samples in which no visible bacterial growth were observed were prepared and inoculated (100 µL) in nutritive agar and incubated at 30°C for 24 h, afterwards, colonies were counted to determine the MBC and the microbial inhibition percent. The assays were performed in triplicate and the negative control consisted of nutrient broth without AgNPs.

2.2.4. Statistical analyses

The statistical analyses were carried out with an analysis of variance (ANOVA) and Tukey test ($P < 0.05$) using the Minitab software.

3. RESULTS

3.1. Synthesis and characterization of AgNPs

The synthesis of both types of AgNPs was evidenced by the change in the color from colorless to dark brown for biological AgNPs and brown for the chemical AgNPs, this color is indicative of the AgNPs formation due to the excitation of the vibrations of the surface plasmon resonance of silver nanoparticles that produce a characteristic absorption band around 400 to 450 nm [13]. The absorption spectrum for biological AgNPs showed a band around 420 nm (Figure 1 A) while the chemical AgNPs showed a band around 400 nm (Figure 1 B), this blue shift may be due to the different sizes of the nanoparticles, smaller NPs are blueshifted [13], which is consistent with the size difference determined by TEM.

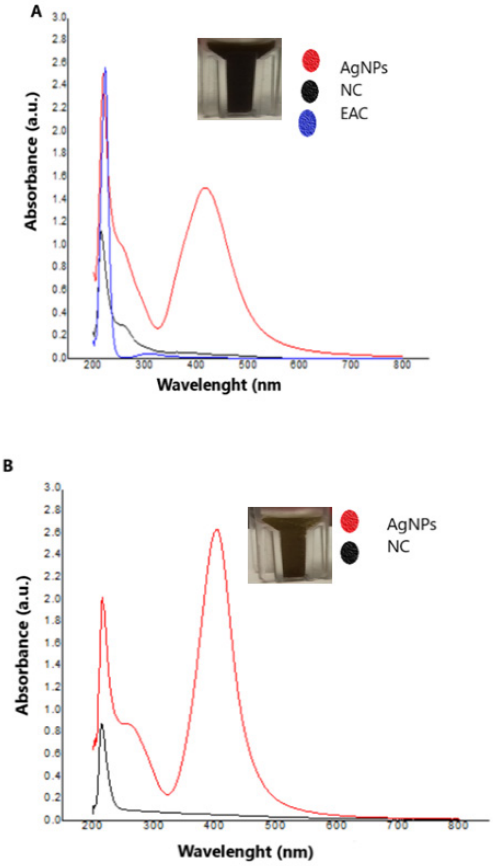

Figure 1. Optical absorption spectra of AgNPs. A) AgNPs synthesized by biological methods, red line (AgNPs), black line (negative control, NC), and the blue line (enzymatic activity control, EAC). B) AgNPs synthesized by chemical method, red line (AgNPs), black line (negative control, NC).

The analysis by transmission electron microscopy (TEM) confirmed the synthesis of spherical AgNPs by both chemical and biological methods. The micrographs were analyzed in the ImageJ program, it was determined that the average diameter for biological AgNPs was 10.32 nm (Figure 2 A) and the average diameter for chemical AgNPs was 9.53 nm (Figure 2 B).

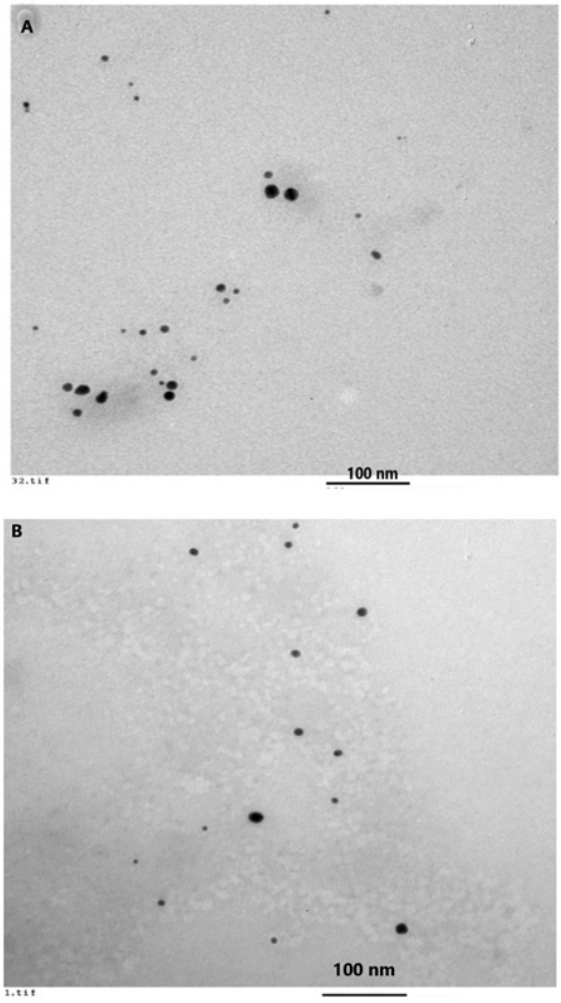

Figure 2. TEM micrographs show spherically shaped of AgNPs. A) Biological AgNPs with an average size of 10.32 nm B) Chemical AgNPs with an average size of 9.53 nm.

3.2. Antimicrobial effect of AgNPs

3.2.1. Determination of the minimum inhibitory concentration

To determine the bacteriostatic effect (bacterial growth is only transiently inhibited) an inoculum of $1x10^6$ CFU/mL of each sample was exposed to 5, 10, and 15 ppm of AgNPs solutions diluted in nutrient broth for 24 h as it was mentioned in the materials and methods section. Table 1 shows the results, the most resistant inoculum was the one obtained from the pharyngeal exudate with a MIC of 10 ppm for chemical AgNPs and 15 ppm for biological AgNPs. The chemical AgNPs allowed lower MIC values for all treatments.

Table 1. The minimum inhibitory concentration of AgNPs

Sample	MIC Chemical AgNPs (ppm)	MIC Biological AgNPs (ppm)
Computer keyboard	<5	10
Tap water	<5	10
Pharyngeal exudate	10	15

3.2.2. Determination of the minimum bactericidal concentration

The bactericidal effect (lethal action on the bacteria), was only observed with the concentration of 15 ppm with the biological AgNPs on the sample of pharyngeal exudate (Table 2), because it is necessary to kill 99.9% of the bacterial population, which was only achieved for this sample.

For the other samples, a few bacterial growth was observed, however, it did not kill 99.9% of the population, the results obtained are shown in the following table. The percentages obtained for the undetermined samples are shown in the following section as microbial inhibition percentages.

Table 2. The minimum bactericidal concentration of AgNPs

Sample	Concentration (ppm)	Chemical AgNPs	Biological AgNPs
Computer keyboard	5	+	+
	10	+	+
	15	+	+
Tap water	5	+	+
	10	+	+
	15	+	+
Pharyngeal exudate	5	+	+
	10	+	+
	15	+	-

****Positive* (+)**: Indicating growth; Negative (-): Indicating absence of growth.

3.2.3. Determination of the microbial inhibition percent

The inhibition percent obtained for all samples were above 90% and increased as the concentration increased for both types of NPs; the data are shown in figures 3 to 5. For the sample of a computer keyboard (Figure 3), the percentage of inhibition at 5 ppm was similar for both types of NPs, obtaining 90.6% for the biological and 90.8 % for the chemical ones, although there was not significant difference; but the highest inhibition effect was observed for biological AgNPs at concentrations of 10 and 15 ppm. For microorganisms from the tap water samples (Figure 4), the chemical NPs were more efficient as they achieved higher inhibition percentages than the biogenic NPs for all concentrations, the difference between treatments was significant. For the pharyngeal exudate sample (Figure 5), inhibition percentages were similar for both types of NPs at all evaluated concentrations.

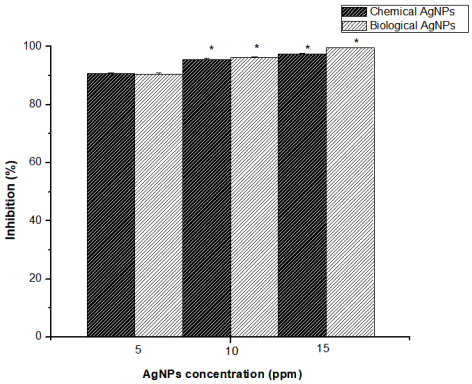

Figure 3. Inhibition percentages of microbial growth from a sample taken from a computer keyboard. (*) treatments with a significant difference, p-value <0.05.

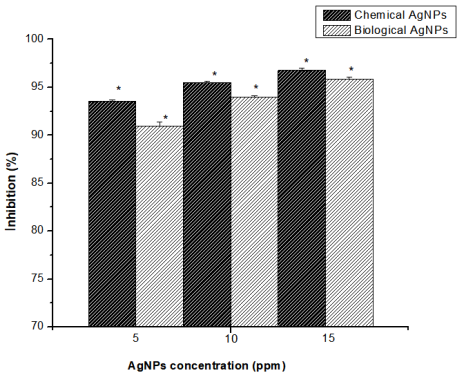

Figure 4. Inhibition percentages of microbial growth from a tap water sample. (*) treatments with a significant difference, p-value <0.05.

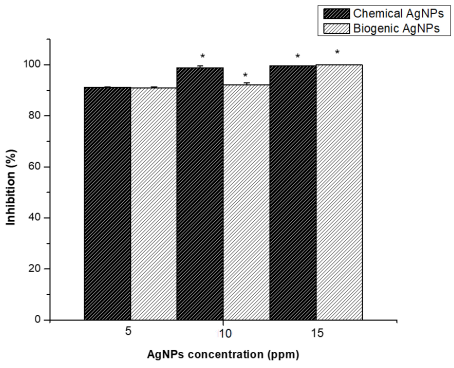

Figure 5. Inhibition percentages of microbial growth from pharyngeal exudate. (*) treatments with a significant difference, p-value <0.05.

4. DISCUSSION

The antimicrobial effect of AgNPs depends on several factors such as size, shape, surface charge, and concentration of silver ions, among others, that is the reason why it is difficult to compare their effectiveness even more with other studies or samples. Additionally, each study uses different bacterial strains which complicates the comparison. There are few studies on the evaluation of the AgNPs effect on microbial populations from environmental samples, most of them have been focused on evaluating the environmental impact of NPs or inhibiting the biofilm formation.

Colman et al. [14] evaluated AgNPs in the microbial population of stream water and sediment and they found that microbial respiration after exposure to 75 mg AgNPs was 39% lower than the control (untreated microorganisms) in the stream water microbes, and in the sediment microbes, there was no significant change in the measurement of biomass or respiration with respect to the control. On the other hand, Bhattacharuyya et al. [15] synthesized ZnONPs and evaluated their effect on the inhibition of ***Streptococcus pneumoniae*** biofilm formation and they found a MIC of 40 µg/ml on ***S. pneumoniae***.

As observed in the present study, the effect varies depending on the microbial population present in each sample. Results of this study showed a greater resistance for inoculum from pharyngeal exudate, which probably is due to the sample coming from an immunocompromised patient exposed constantly to antibiotics. Most of these types of samples have infectious agents and often present resistance to antibiotics that have intrinsic or acquired characteristics that could generate resistance to AgNPs, in comparison with those samples that came from the computer keyboard and tap water where mainly enterobacteria could be observed; these bacteria do not have a high selection pressure as bacteria in hospitals or patients. Although there are no available studies to compare the present results.

Leid et al [16], reported the MIC for N-heterocyclic silver carbene complexes (SCCs) encapsulated into L-tyrosine polyphosphate (LTP) nanoparticles on bacteria isolated from patient samples; *E. coli* was found and isolated from a sputum sample with a MIC of 22.7 ppm, *S. aureus* isolated from blood with a MIC of 22.7 ppm and *P. aeruginosa* isolated from sputum with a MIC of 2.9 ppm. On the other hand, Abdalhamed et al. [17], evaluated AuNPs on *Escherichia coli* and *Salmonella* species isolated from fecal ruminants' samples and they determined a MIC of 3.125 g/ml for *E. coli* and 5.5 g/ml for *Salmonella.*

The MIC determined in the past study [11] was lower for all strains except for *P. aeruginosa* where we obtained a MIC of 10 ppm; even though our samples were analyzed without isolating the microorganisms. In general, the antimicrobial effect of NPs is difficult to compare among reports because the effect depends on the characteristics of the NPs, which vary greatly, and on the microorganism under study. Nevertheless, silver nanoparticles have proven to be an effective antimicrobial (3).

Based on the results, we can propose that both types of nanoparticles are efficient antimicrobial agents since they reached inhibition percentages higher than 90%. The antimicrobial effect strongly depends on the origin/ type of the sample and the microbial population present in each sample.

5. CONCLUSION

The results showed inhibition percentages higher than 90% for both types of AgNPs which is a good indicator of their effectiveness especially if they were evaluated on environmental samples. Biological nanoparticles synthesized by ***P. variotti*** are outstanding candidates to inhibit microbial growth, so they would be an excellent alternative to the antimicrobials commonly used.

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