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DISINFECTION OF WATER CONTAMINATED BY PATHOGENIC BACTERIA USING GOLD MYCONANOPARTICLES

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ABSTRACT

The present work aimed at screening a batch of 10 fungal strains in order to identify those capable of biosynthesis of gold nanoparticles (AuNPs). Out of these, IBCLP15, IBCLP1212, IPT1011 and IPT1013 strains were found to be capable of biosynthesis of AuNPs. The obtained AuNPs were coded as $AuNPs_{IBCLP15}$, $AuNPs_{1212}$, $AuNPs_{IPT1011}$ and $AuNPs_{IPT1013}$ and characterized by size and morphology using dynamic light scattering (DLS), zeta potential(ζ), polydispersity index (PDI), and transmission electron microscopy (TEM). They were found to have an average size of 30–100 nm and a regular round shape. Only $AuNPs_{IPT1011}$ and $AuNPs_{IPT1013}$ exhibited stability over a month. The antimicrobial activity of $AuNPs_{IPT1011}$ in colloidal solution and immobilized in sodium alginate was evaluated against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. $AuNPs_{IPT1011}$ demonstrated antimicrobial activity for both bacteria at concentrations exceeding 50 $\mu g \cdot mL^{-1}$. In conclusion, the study demonstrated the effective antimicrobial action and low toxicity of mycogenic $AuNPs_{IPT1011}$, emphasizing their potential applications in freshwater treatment.

Keywords: Mycogenic gold nanoparticles. Alginate immobilization. *Escherichia coli*. Freshwater disinfection.

1 INTRODUCTION

World Health Organization (WHO) experts have found that 80% of all diseases and 50% of child deaths worldwide in the world are related to the unsatisfactory quality of drinking water and violations of hygiene and sanitation standards for water supply[1]. Water disinfection is a crucial step in the treatment of both drinking water and wastewater to ensure that undesired compounds and microbes in water are minimized for human health[2]. Siver nanoparticles and gold nanoparticles (AuNPs) have being described as a new class of antibacterial and anti-biofilm compounds by recent breakthroughs related to water treatment[3,4].

The antimicrobial activity of AuNPs consists of six important factors: size, concentration, dispersibility, dose, type of bacteria, and surface modification of AuNPs[5]. Previous studies by Shamaila and colleagues[6] demonstrated that chemically synthesized AuNPs exhibited antibacterial action against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus subtilis*, with the activity being size and dose dependent. Furthermore, different types of bacteria showed distinct responses to AuNPs.

Physical and chemical methods used to synthesize AuNPs involve or produce hazardous toxic chemicals[7]. Biological methods are eco-friendly and avoid the use of toxic chemicals thus, they are environmentally safe[8,9]. Microorganisms such as filamentous fungi (Ff) are commonly employed for AuNPs[10] and other metallic nanoparticles synthesis because of their ease of cultivation, rapid growth, and ability to thrive under stressful conditions of pH, temperature, and pressure[10,11].

However, there is still the necessity to screen Ff, exploring the synthesis mechanism, optimizing the synthesis conditions and evaluate their application. In this context, the present study aimed to: (1) identify different filamentous fungal strains capable of synthesizing AuNPs, (2) characterize the synthesized AuNPs, and (3) analyzing the nanoparticles' ability to disinfect water.

2 MATERIAL & METHODS

Microorganisms: Ten fungal strains were selected for this study. Eight strains were isolated from mangrove and tidal flat sediments at the Juréia-Itatins Ecological Station in São Paulo: IBCLP15, IBCLP1212, IBCLP1213, IBCLP1214, IBCLP1215, IBCLP1216, IBCLP1217, and IBCLP1218. Additionally, *Rhizopus arrhizus* IPT1011 and *Rhizopus arrhizus* IPT1013 strains were provided by the Culture Collection of the São Paulo State Technological Research Institute (IPT). Microorganisms' viability was assessed and carried out on Petri dishes containing Potato Dextrose Agar (PDA). All fungal strains were stored at 4 ºC with monthly culture renewal. For the assays, a 6 mm plug was collected from the peripheral area of a grown fungal colony, subcultured on another Petri dish containing PDA, and incubated at 30 ºC for 7 days. The Gram-negative bacteria *Escherichia coli* IPT245 and *Pseudomonas aeruginosa* IPT365 were provided by Instituto de Pesquisa Tecnológica do Estado de São Paulo (IPT, São Paulo, Brazil). The microorganisms were grown overnight in Tryptic Soy Broth (TSB). The final cell concentration was adjusted to $1.0x10^4$ $CFU \cdot mL^{-1}$.

AuNPs mycosynthesis and characterization: The mycogenic process of synthesis of AuNPs was adapted from Ottoni et al.[9]. For each strain, 5 plugs of 6 mm diameter were inoculated in 50 mL of Malt extract Glucose Yeast (MGYP) in a 250 mL Erlenmeyer flask. The cultures were incubated at 30 ºC, 150 rpm for 72 h. Subsequently, the biomass was filtered, washed in 500 mL of sterile deionized water, and weighed. Five grams of the biomass were added to a 250 mL Erlenmeyer flask containing 50 ml of sterile water. These were then kept at 30 ºC, 150 rpm for 72 h. After this period, the supernatant was collected by filtration using Whatman Grade 3 filter paper. Sequentially, the suspension was filtered through a 0.22 µm filter (Millipore), and for every 10 mL, 10 µL of a 1 mM solution of chloroauric acid ($HAuCl_4 \cdot 3H_2O$, Sigma Aldrich, Germany) was added, as the chemical precursor, followed by an incubation at 30 °C, 150 rpm for 72 h, in the dark. The AuNPs were characterized by UV-vis spectroscopy (wavelength range of 540-560 nm), colour change of the colloidal solution from translucent to purple, Dynamic Light Scattering (DLS) for the determination of the average hydrodynamic size, polydispersity index (PDI), and surface charge by Zeta Potential (ζ, Zetasizer Nano - ZS90, Malvern Instruments - United Kingdom). The size and shape of the AuNPs were determined by Transmission Electron Microscopy (TEM, JEOL, model JEM-2100) operated at 200 kV.

Alginate Gel Encapsulation: The microcapsules were obtained through ionotropic gelation with sodium alginate[12]. The biopolymer solution was prepared by solubilizing 4% (w/v) sodium alginate in 12 mL of biological AuNPs. Subsequently, the biopolymer solution (100 mL) was dripped into 100 ml of an aqueous solution of calcium chloride ($CaCl_2$ at 0.14 mol·L^{-1}). The system was kept under magnetic stirring (200 rpm) for 1 minute at 25 °C. After the gelation process, the microcapsules were vacuum-filtered and stored at 4 °C.

Antimicrobial Activity in Batch Mode with colloidal and immobilized AuNPs: The antibacterial activities of $AuNPs_{IPT1011}$ in colloidal solution against the bacterial strains IPT245 and IPT365 were evaluated using the minimum inhibitory concentration (MIC). In summary, 90 µL of $AuNPs_{IPT1011}$ at concentrations of 5, 10, 20, 50, 100, and 200 µg·mL^{-1} were added to each well of a 96-well plate containing 90 µL of bacterial suspension in TSB (final concentration 1x10^4 CFU·mL^{-1}) and incubated at 37 °C for 48 hours. A solution of 100 µg·mL^{-1} $AgNO_3$ was used as a positive control, and untreated bacterial suspension as the negative control. To indicate the presence or absence of pathogen growth, after 24 hours of the assay, 20 µL of 0.01% resazurin dye were added. The assay was conducted in triplicate, and the minimum inhibitory activity (MIC) was defined as the lowest NP concentration capable of inhibiting the growth of at least 90% of the pathogens. The minimum bactericidal concentration (MBC) was determined based on the absence of pathogen growth. For this purpose, 50 µL from two concentrations tested and prior to the MICs were collected from the plate and incubated on Petri dishes containing Trypticase Soy Agar (TSA), at 37 °C for 48 hours.

Parameters evaluated: bacterial concentration (1.0 x 10^2, 5.05 x 10^3, and 1.0 x 10^4 CFU·mL^{-1}), stirring speed (100, 150, and 200 rpm), and temperature (30, 35, and 40 ºC). Using 250 mL Erlenmeyer flasks, a volume of 50 mL of distilled water inoculated with IPT245 or IPT365 and 17,5 or 8.75 g of $AuNPs_{IPT1011}$ immobilized were added. The flasks were placed on a shaker platform for 2 hours. A solution of 100 mg·L^{-1} of $AgNO_3$ was used as a positive control, and the negative control consisted only of inoculum. Additionally, conditions with 8.75 g of alginate and with 8.75 g of the gold precursor ($HAuCl_4 \cdot 3H_2O$) were tested. The experiment was conducted in triplicate. Every 1 hour during the assay, a volume of 10 µL was withdrawn and transferred to Petri dishes containing TSA. After 18 hours of incubation at 37 ºC, CFU·mL^{-1} counts were performed.

3 RESULTS & DISCUSSION

Ten Ff were evaluated for their ability to synthesize AuNPs. Among them, IPT1013, IBCLP15, IPT1011, and IBCLP1212 showed a surface plasmon resonance (SPR) band corresponding to the gold (Au) valence layer. Only $AuNPs_{IPT1011}$ showed apparent stability, as there was no observed change in colour, turbidity or size for a period of 1 month. An intense dark purple colour was observed after the addition of $HAuCl_4 \cdot 3H_2O$ to the fungal supernatant and incubation for 24 h. The maximum absorption values of the extracellular supernatants for each strain after 72 h are summarized in Table 1.

Table 1 Physical-chemical characterization of the obtained AuNPs, including utraviolet-visible (UV-vis) spectroscopy, Dynamic Light Scattering (DLS), Zeta Potential (ζ), and polydispersity index (PDI).

Fungal strain	Colour	UV-vis spectrum (nm)	DLS (nm)	ζ (mV)	PDI
IPT1011	purple	533	29.3	-13.20	0.189
IPT1013	violet	556	73	-38.33	0.250
IBCLP15	purple	533	40.2	-24.97	0.273
IBCLP1212	purple	522	248.3	-11.23	0.669

The micrograph of $AuNPs_{IPT1011}$ exhibited a spherical shape, good dispersion, and an average size ranging between 40 nm.

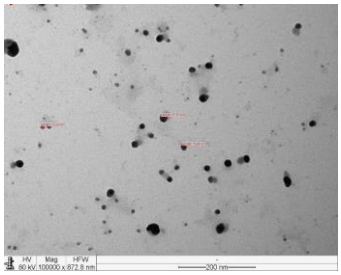

Figure 1 Transmission Electron Microscopy (TEM) of $AuNPs_{IPT1011}$ (Scale 200 nm, 100,000x).

The antibacterial activity of $AuNPs_{IPT1011}$ against Gram-negative IPT245 and IPT365 bacteria inhibited the growth of both pathogens with MICs ranging from 50 to 100 $\mu g \cdot mL^{-1}$ (Table 2). The $AgNO_3$ solution used as a positive control showed MICs between 1 and 10 $\mu g \cdot mL^{-1}$. The untreated pathogen suspension served as a negative control, demonstrating the growth of microorganisms and the change in colour of the resazurin dye from blue to purple.

Table 2 Antibacterial activity through MICs in $\mu g \cdot mL^{-1}$ of AuNPs against *Escherichia coli* IPT245 and *Pseudomonas aeruginosa* IPT365.

Sample	IPT245	IPT365
$AuNP_{IPT1011}$	≤ 50	≤ 75
$AgNO_3$	1	10

Our results reinforce those obtained by Sathyaraj' s and Ameen's groups[13,14], which described stronger antibacterial effect against the Gram-negative bacteria exposed to biogenic AuNPs. The enhanced antibacterial activity against gram-negative bacteria can be attributed to their thinner cell wall, turning them additionally susceptible. The thick layer of peptidoglycan in the cell wall of gram-positive bacteria impedes or reduce the uptake of AuNPs[14,15]. The AuNPs lead to disrupting bacterial cell membranes, inhibit bacterial enzyme activity, interfere with bacterial DNA, and induction of oxidative stress. These actions damage cellular components and leak the bacterial electrolytes, which leads to cell death of bacterial cells[16].

The immobilization of NPs prevents agglomeration and enhances their stability, allowing reuse. Additionally, alginate stabilizes the well-dispersed small AuNPs[17]. Our results revealed an effective antimicrobial action of mycological AuNPs at both concentrations (17.5 g and 8.75 g), with 100% cytotoxicity after 2 hours. On the other hand, the $HAuCl_4 \cdot 3H_2O$ precursor did not demonstrate toxicity after 48 hours of incubation, highlighting the importance of the unique properties of the produced NPs.

Subsequently, tests were performed with 8.75 g of the compounds at 40°C, 150 rpm, for 2 hours. Regarding having used a temperature of 40°C for the assays, $AuNP_{IPT1011}$ did not prove effective within the 2-hour period, similar to the precursor $HAuCl_4 \cdot 3H_2O$, a result akin to studies showing little or no effect of AuNPs when not coated with compounds exhibiting antimicrobial effects[18].

4 CONCLUSION

Mycogenic $AuNPs_{IPT1011}$ exhibited higher stability and structurally relevant characteristics, making them more suitable for studies related to antimicrobial activity. Antimicrobial assays using AuNPsIPT1011 in colloidal solution and encapsulated in sodium alginate against *E. coli* IPT245 and *P. aeruginosa* IPT365 demonstrated antimicrobial activity for both bacteria at concentrations exceeding 50 $\mu g \cdot mL^{-1}$ proving their potential for application in the treatment and disinfection of freshwater.

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