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Functional textiles impregnated with biogenic silver nanoparticles from *Bionectria ochroleuca* and its antimicrobial activity

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Abstract
Biogenic silver nanoparticles (AgNPs) were obtained throughout the fungal biosynthesis using extracellular filtrate of the epiphytic fungus *B. ochroleuca* and were incorporated in cotton and polyester fabrics by common impregnation procedure that was repeated once, twice or four times. Both fabrics were analyzed by scanning electron microscopy (SEM), and the effectiveness of impregnation was determined using inductively coupled plasma optical emission spectrometry (ICP OES). The AgNPs loaded fabrics showed potent antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* as well as on clinically relevant *Candida albicans, Candida glabrata,* and *Candida parapsilosis*, indicating that the AgNPs impregnation of cotton and polyester fabrics was efficient. AgNPs effectively inhibited the biofilm formation by *Pseudomonas aeruginosa* and was not toxic to *Galleria mellonella* larvae indicating a promising probability of biotechnological application.

Keywords Silver nanoparticles · Antimicrobial activity · Functional textiles · Biofilm

1 Introduction

Due to the unique optoelectronic, physicochemical and antimicrobial properties, the use of metallic nanoparticles (MNPs) in several sectors such as health, agriculture, environment and energy (Laloy et al. 2014; De et al. 2015; Patil and Kim 2017; Sarkar and Achary 2017) has received special attention in recent years. Physical and chemical methods are widely used for the synthesis of these nanomaterials; however, high energy and broad use of chemical reagents in the conventional methods have raised concerns about their impact on the environment (Qu et al. 2017). Several studies on the synthesis of MNPs by biological or bio-based means count on the use of extract plants (Davi et al. 2017; Hamedi et al. 2017) or microorganisms (Al-Bahrani et al. 2017; Hamedi et al. 2014), which are low cost and eco-friendly processes (Shivakumar et al. 2017).

The synthesis of MNPs using fungi is more advantageous than that by bacteria because fungal mycelia offer a larger surface area for interaction. In addition, fungi secrete higher amounts of protein than bacteria; therefore, the conversion of metal salts to MNPs is faster (Siddiqi and Husen 2016). Silver nanoparticles (AgNPs) are described as antimicrobial and antiviral agents (Amerasan et al. 2016; Singh et al. 2016) and the biological effect is enhanced by the large NP surface area, which facilitates the entrance into the microbial cell (Ballottin et al. 2016).

Recently, the use of AgNPs for microbial treatment in the textile industry has been the focus of many researchers. It has been described that the greater effectiveness of NPs, when compared to other forms of silver, arises from: (i) a greater ion release and catalytic activity due to the large surface area;

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(ii) the generation of reactive oxygen species in addition to that resulting from the released Ag^+; and, (iii) direct interactions with microorganisms wherein particles penetrate the cell membranes (Emam et al. 2013).

AgNPs are an interesting alternative against microbial infection and the antibacterial activity of textiles with higher antibiofilm activity against Gram-negative bacteria *P. aeruginosa* (Massironi et al. 2019) and *E. coli*, and on Gram-positive *Staphylococcus aureus* (El-Naggar et al. 2018) using synthetic AgNP was recently reported. However, few studies have been conducted using biogenic AgNP.

Previously, the synthesis of AgNPs by using the epiphytic fungus *Bionectria ochroleuca* was reported by our group (Rodrigues et al. 2013), such as its antibacterial activity on *P. aeruginosa*, *E. coli*, *S. aureus* and *Micrococcus luteus*. These AgNPs also showed pronounced antifungal activity against *Candida* sp., frequently occurring in hospital infections.

In this study, the effect of *B. ochroleuca* AgNPs was evaluated on biofilm formation by *P. aeruginosa*, and its toxicity on *Galleria mellonella* larvae model. Furthermore, the antimicrobial effect of fabrics impregnated with AgNPs was assessed on the yeasts clinically relevant *C. albicans, C. glabrata,* and *C. parapsilosis*.

2 Material and methods

2.1 Microorganisms

Fungus strain: Epiphytic fungus *B. ochroleuca* was previously isolated (Rodrigues et al. 2013) and deposited at the "Oswaldo Cruz Institute Collection (IOC, Rio de Janeiro, RJ, Brazil)", and at the "Collection of Microorganisms for Biocontrol of Plant, Pathogens and Weeds" from "Embrapa Recursos Genéticos e Biotecnologia (CENARGEN, Brasilia, DF, Brazil)", under the numbers IOC 4684 and CEN1065, respectively.

Bacterial strains: *Pseudomonas aeruginosa* from the Microbiology Department/ICB/USP collection, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Yeast strains: *Candida albicans* ATCC 36802, *C. glabrata* IOC 4565 and *C. parapsilosis* IOC 4564.

2.2 Silver nanoparticles biosynthesis and characterization

The AgNPs biosynthesis and characterization were performed as previously reported by our group using transmission electron microscopy (TEM), average diameter size determination, size distribution, and zeta potential by photon correlation spectroscopy NanoSizer (Malvern Instruments Corp., Worcestershire, UK) (Rodrigues et al. 2013).

2.3 Analysis of the effect of AgNPs on the biofilm formation by scanning electron microscopy

For the biofilm formation, the bacteria *P. aeruginosa* was cultivated on Müller-Hinton agar plates at 37 °C, and a suspension in PBS (pH 7.2) with an optical density of 1.0 at 600 nm was prepared. From this suspension, 2 mL were added to each well of a 12 well microplate containing sterile coverslips of 13 mm diameter in each well. Following that, the microplate was treated with 10 μL of $AgNO_3$ or diluted AgNPs, resulting in final concentrations of 0.01, 10, and 50 μM, and the plate was incubated at 28 °C at 150 rpm for 24 h. Controls were performed with bacterial cells without no treatment.

For the scanning electron microscopy (SEM), the bacterial cells were fixed with paraformaldehyde at 4% and glutaraldehyde at 2.5% in 0.1 M cacodylate buffer (pH 7.2) for 4 h (Karnovsky 1965). After fixation, the cells were washed three times with the same buffer for 15 min, dehydrated in a graded series of ethanol solution (30–100%), and then subjected to critical point drying with CO_2 in a CPD 030 equipment (Leica Microsystems, Heerbrugg, Switzerland). Samples were covered with a gold film and examined with a FEI QUANTA 250 SEM (Netherlands) and accelerating voltage of 10 kV. Images were obtained by secondary electron analysis (Heymann et al. 2006) performed considering modifications or ultrastructure arrangements on the cell morphology.

2.4 Impregnation of cotton and polyester fabrics with AgNPs by the padding method

Prior to the experiment, the cotton and the polyester fabrics were washed, sterilized in an autoclave, and dried at room temperature (R.T.). For impregnation, 10 cm^2 of each fabric was immersed into 10 mL of AgNPs dispersion until total wetting and subsequently compressed between two rolling pins, using a rolling machine (Marcato, Padova, Italy). The impregnation process was performed once, twice or four times, and the fabrics were totally dried at R.T. under light protection for 24 h. For each fabric, fragments of 1 cm^2 were cut and employed for the antimicrobial activity evaluation. Controls were performed with fabrics immersed in deionized water without AgNPs.

2.5 Concentration of silver in the AgNPs and on the impregnated cotton and polyester fabrics

The concentration of silver in the nanoparticles and on the impregnated fabrics was determined by inductively coupled plasma optical emission spectrometry (ICP OES 5100 VDV, Agilent Technologies, Tokyo, Japan), and compared to a standard calibration curve prepared with silver. To evaluate the incorporation of AgNPs on the fabrics, aliquots of 2 mL were taken from the dispersion before and after the fabric's

impregnation process. The standard curve of silver was prepared by diluting the standard solution of reference (Fluka, Sigma-Aldrich, Buchs, Switzerland) at 1 g/L to a range of 2.5 a 100 μg/L (r = 0.99997) in nitric acid purified at 5% (*v*/v), and before the analysis, the solutions were diluted 1:100. To transfer metallic silver from the nanoparticles into ions, 2.0 mL of AgNPs dispersion were digested by treatment with 2.0 mL of HNO_3 65% purified by sub-boiling distillation (Distillacid, Eningen, Germany). The sample was submitted to acid digestion in a dry bath at 60 °C for 2 h. Following that, it was quantitatively transferred to a volumetric flask of 25 mL using ultrapure water (Gehaka, São Paulo, Brazil) and filtered in a quantitative paper (Nalgon, Itupeva, Brazil) followed by silver quantification.

Measurements of silver were performed by ICP OES in the axial view, with a radiofrequency source (RF) of 27 MHz, using a simultaneous optic detector, a peristaltic pump, a double-step cyclonic nebulization chamber, a 1.8 mm quartz torch and a glass nebulizer *seaspray*. Analyses were carried out with liquid argon at 99.996% (White Martins, SP, Brazil) and the operational conditions were: plasma power: 1.4 kW; argon flow 12.0 L/min; auxiliary argon flow: 1.0 L/min; nebulization flow: 0.70 L/min. Stabilization and reading were performed in 15 s at 328.068 nm. Analyses were carried out in triplicate and blanks were conducted using the same procedure.

2.6 Analysis of cotton and polyester fabrics morphology by scanning electron microscopy

The morphology of the cotton and polyester fabrics with and without the AgNPs impregnation was analyzed by using SEM. Images were acquired in a JEOL JSM - T300 microscopy (Tokyo, Japan) at 20 kV with secondary electron detectors. Samples were prepared using aluminum support (stubs) covered with a double-sided tape recovered with gold by 100 s. Images were recorded by a Proscan high-speed slow-scan CCD camera and processed in the Analysis 3.0 system.

2.7 Antibacterial activity of fabrics impregnated with silver nanoparticles

To evaluate the antibacterial activity, fragments of 1 cm^2 of the cotton and of the polyester fabrics impregnated once with AgNPs were incubated with suspensions of a mid-logarithmic phase culture of *E. coli* ATCC 25922 or *S. aureus* ATCC 25923, in poor-broth nutrient medium [1% Bacto-tryptone and 0.5% (*w*/*v*) NaCl] in the concentration of 1×10^5 CFU/mL. Cultures were prepared in triplicate, and bacterial growth was assessed after 24 h at 30 °C and 150 rpm. The absorbance of the suspensions was determined at 595 nm. Gentamicin was used at 8 and 16 μg/mL (17.0–34.0 μM) as a positive control and untreated bacteria were used as negative control. The percentage of inhibition of the bacterial growth was determined by comparison with the controls containing untreated bacteria or bacterial culture free of fabrics containing AgNPs.

2.8 Antifungal activity of impregnated fabrics

To evaluate the antifungal activity of the cotton and polyester fabrics impregnated with AgNPs, fragments of 1 cm^2 were placed in glass tubes containing 800 μL of distilled sterile water and mixed for 10 min. in a rotary stirrer (Labnet 211DS, Edison, New Jersey, USA) at 220 rpm. Afterward, each sample received 2.2 mL of potato dextrose broth (PDB) and 10 μL of a suspension of *C. albicans*, *C. glabrata* and *C. parapsilosis* at 1.87×10^5 CFU/mL (according to a standard curve previously established in our laboratory). The mixture was incubated at 35 °C and 220 rpm for 24 h. After incubation, 10 μL of each suspension was serially diluted and the dilutions were incubated in a dish plate containing potato dextrose agar (PDA) at 32 °C. The assay was performed in triplicate for each sample, and the score of CFU/mL was calculated after incubation for 48 h. Glass tubes with fabrics without AgNPs impregnation were used as a control.

2.9 Effect of AgNPs on the *galleria mellonella* larvae model

AgNPs were diluted in PBS and 10 μL corresponding to 0.05, 0.25, 0.5 and 1 mM were inoculated in the hindmost larvae proleg using a gas-tight syringe (22 s gauge, Hamilton). Larvae non-inoculated or inoculated with PBS were used as a control. For each treatment were used ten larvae in two independent experiments. After the injection, the larvae were incubated at 28 or 37 °C and their survival was monitored up to 120 h. To analyze the larval survival, the Kaplan-Meier survival plot with Log-rank (Mantel-Cox) test and Bonferroni correction (GraphPad Prism 6) were used (Seed and Dennis 2008).

3 Results

3.1 Silver nanoparticles synthesis and physical characterization

The formation of AgNPs was monitored by surface plasmon resonance (SPR) band observed by UV-Vis at 440 nm. Nanoparticles were characterized as first reported by dynamic light scattering with the size in the range of 4–35 nm, with most of the particles falling in the range of 8–21 nm. As depicted in Fig. 1, by TEM analysis, NPs were spherical, uniform in size and well distributed without aggregates formation (Rodrigues et al. 2013).

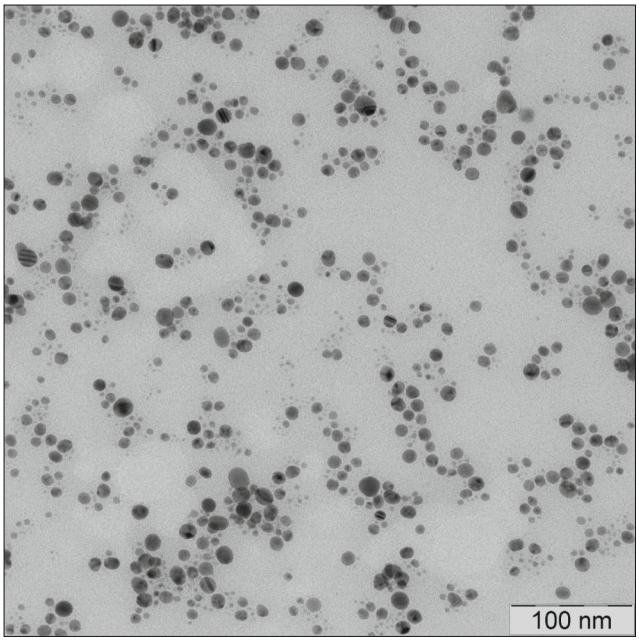

Fig. 1 Transmission electron microscopy (TEM) images of the AgNPs obtained from the *B. ochroleuca* culture

3.2 Effect of AgNPs on the biofilm formation by *P. aeruginosa*

In order to understand the effect of AgNPs on the growth of the pathogenic Gram-negative bacterium, the biofilm formation by *P. aeruginosa* was evaluated by SEM observation and it is shown in Fig. 2.

It is possible to note that the $AgNO_3$ did not influence the biofilm formation by *P. aeruginosa* and the presence of cells agglomerates, which is typical of biofilm organization, even when treated with $AgNO_3$ at 10 μM (Fig. 3). In this case, the bacterial growth was similar to that of the negative control without treatment.

In contrast, the biofilm formation by *P. aeruginosa* was reduced by the treatment with AgNPs from 0.01 to 50 μM. For the lower concentrations represented by Fig. 2, there was the presence of a few remaining cells but not the biofilm production such as aggregated cells or exopolysaccharide formation. These results showed that the biogenic AgNPs inhibited the activity of biofilms when compared to the control groups even that one treated with $AgNO_3$.

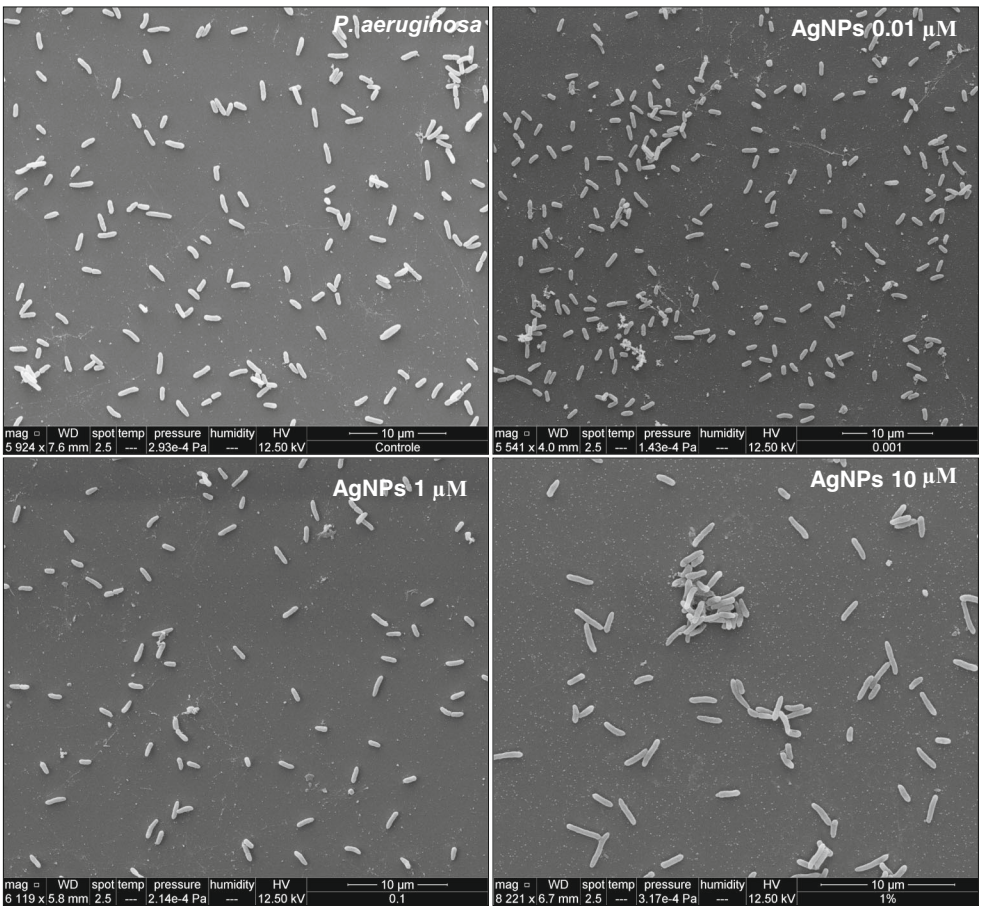

Fig. 2 Scanning electron microscopy (SEM) for *P. aeruginosa* biofilm exposed to 0.01, 1, and 10 μM of AgNPs obtained from *B. ochroleuca* culture. Bars are at 10 μm. Controls are only *P. aeruginosa* biofilm. Samples were covered with a gold film and examined with a FEI QUANTA 250 SEM an accelerating voltage of 10 kV. Images were obtained by secondary electron analysis

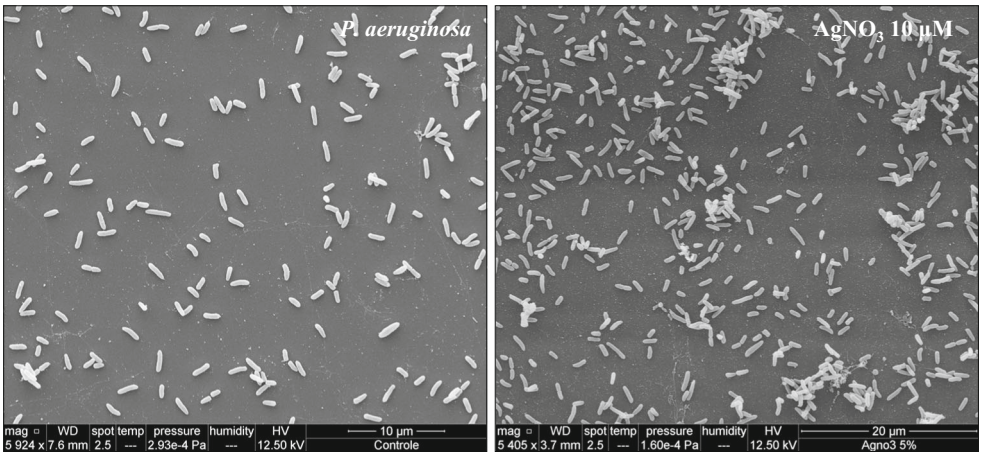

Fig. 3 Scanning electron microscopy of *P. aeruginosa* biofilm exposed to 10 µM of $AgNO_3$. Bars are at 10 and 20 µm. Samples were covered with a gold film and examined with a FEI QUANTA 250 SEM an accelerating voltage of 10 kV. Images were obtained by secondary electron analysis

3.3 Concentration of silver in the AgNPs and on the impregnated cotton and polyester fabrics

Elemental silver analysis by ICP OES showed that in the AgNPs, the silver concentration was 96.76 ± 2.11 mg corresponding to 89.70 ± 1.96% of the theoretical value (107.8682 mg). The efficacy of cotton and polyester fabrics impregnation with AgNPs is represented in Table 1 by percentage, and a substantial difference of impregnations capacity between cotton and polyester fabrics was observed by the process performed once, twice or four times.

For cotton fabric, the efficacy of impregnation with AgNPs increased with the number of impregnations and was higher for four times; for polyester, one impregnation was the most effective reaching 6.0%.

Figures 4 and 5 represent the SEM for cotton and polyester fabrics with and without impregnations process with AgNPs. In both fabrics, it is possible to observe the nanoparticles on the surfaces. The impregnation process in the polyester occurred in a low percentage, which is evident by the SEM analysis represented in Fig. 5. Furthermore, it is possible to see that the amount of AgNPs on the cotton fabric surface that passed by four impregnations, as expected, is higher than in the other impregnation procedures.

3.4 Antimicrobial activity of AgNP impregnated fabrics

The antibacterial effect of cotton and polyester fabrics impregnated once with AgNPs against *S. aureus* and *E. coli* was notably good in both species inhibiting 100% of the microorganisms' growth. Consequently, was not necessary to carry out this assay for the fabrics impregnated with AgNPs by twice or four times.

The antifungal activities of both, cotton and polyester fabrics impregnated with AgNPs by once, twice and four times were first evaluated on *C. albicans* since this species is frequently present in opportunistic infections caused by yeasts at hospitals. Impregnated fabrics were efficient in inhibiting *C. albicans* growth and more effective on the *C. parapsilosis* and *C. glabrata* species. The antifungal activity of the impregnated fabrics is represented in Table 1.

Considering the good effect on *C. albicans*, both fabrics impregnated by once were also evaluated on *C. glabrata* and *C. parapsilosis*. The fabrics inhibited the growth of *C. parapsilosis* and *C. glabrata*, more than in *C. albicans*. The polyester fabric, which was impregnated once, was more effective than the cotton fabric on *C. albicans*. On the other

Table 1 Percentage of AgNPs incorporated on the cotton and polyester fabrics by impregnation process performed by once, twice or four times and its antifungal activity against *C. albicans, C. parapsilosis, and C. glabrata*, in the percentage of inhibition (%)

	AgNPs	Pathogens		
Samples	Impregnations (%)	*C. albicans*	*C. parapsilosis*	*C. glabrata*
Controls	0	0	0	0
Cotton 1x	0.1	68.41	93.89	92.64
Cotton 2x	3.0	75.0	ND	ND
Cotton 4x	9.9	67.93	ND	ND
Polyester 1x	6.0	82.05	89.29	95.27
Polyester 2x	4.4	67.50	ND	ND
Polyester 4x	1.6	76.17	ND	ND

Control: cotton or polyester fabrics without AgNPs impregnation. ND = not determined.

two pathogens, the effect of both fabrics was similar inhibiting the growth of *C. glabrata* and *C. parapsilosis*, efficiently.

For both fabrics impregnated with AgNPs by twice or four times, the antifungal effect on *C. albicans* was not higher than that for once, and consequently, their effect was not evaluated on the other two yeasts species.

3.5 Toxicity of AgNPs in the *Galleria mellonella* larvae

The effect of the AgNPs was evaluated on the larvae in environmental temperature (28 °C) and in a clinical and physiologic temperature (37 °C) to verify if the effect could be temperature-dependent providing further understanding about its mechanism. Results showed that at 28 °C, 90% of larvae survived with 0.05, 0.5, and 1 mM of AgNPs, and 80% survived with 0.25 mM. For control larvae, no deaths were detected (Fig. 6).

At 37 °C, for NPs treatment until 0.5 mM and 120 h, 90% of larvae survived. Even for 1 mM, 80% of larvae survived until the end of the assay in 120 h with the early deaths being observed in 24 h. For 0.05 and 0.025 mM, no deaths were detected at the end of the experiment with 100% of survival.

For both temperatures, the overall toxicity of AgNPs towards *G. mellonella* was not statistically different among treatments (Log-rank test and Bonferroni correction; $P < 0.05$).

4 Discussion

Silver nanoparticles have been extensively used as antimicrobial agents in the health industry, food storage, and in several environmental applications and biomaterials approach (Abbasi et al. 2016).

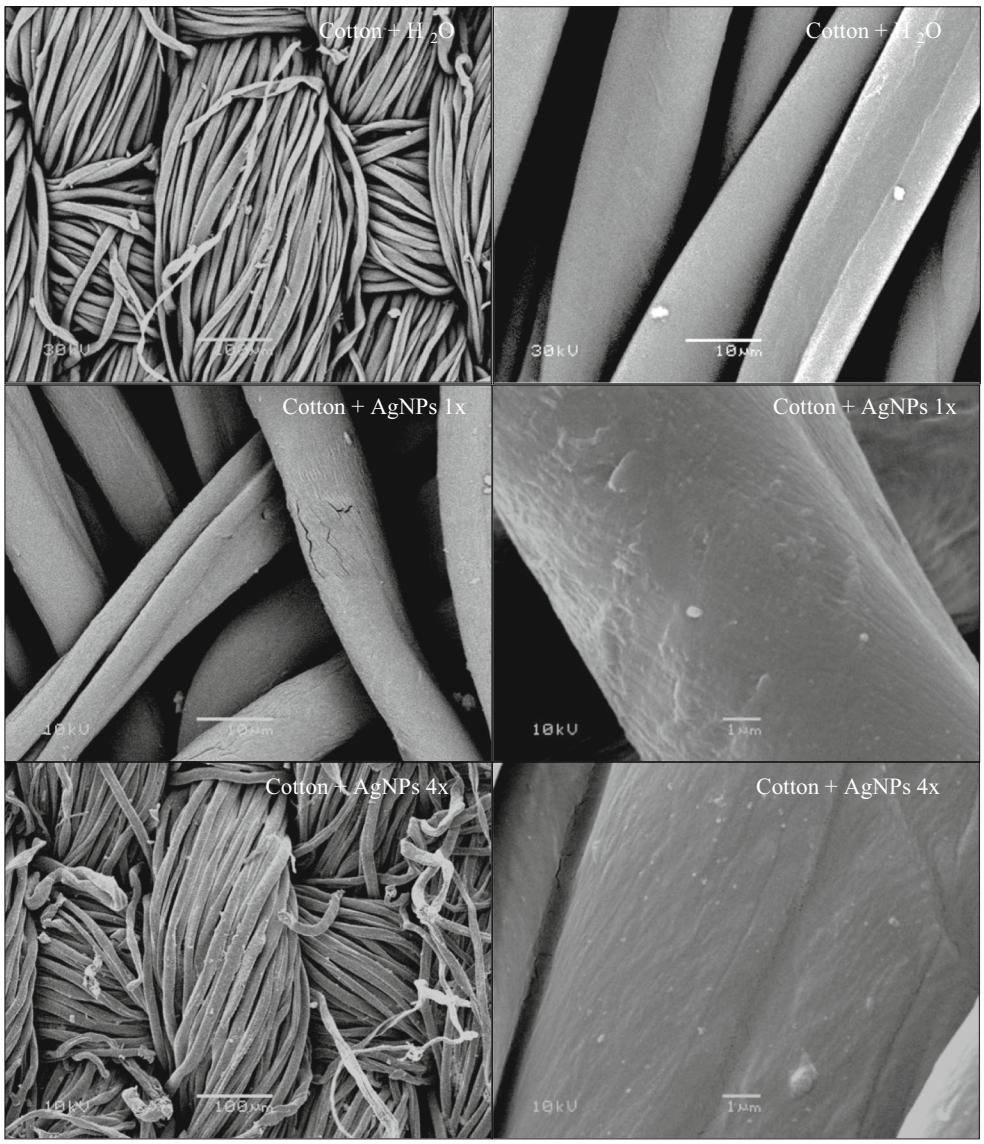

Fig. 4 Scanning electron microscopy (SEM) images of the cotton fabric without impregnations and impregnated with AgNPs by once or 4 times. Cotton + H_2O (100 and 10 μm); cotton + AgNPs 1x (10 and 1 μm); cotton + AgNPs 4x (100 and 1 μm)

P. aeruginosa is a Gram-negative and multidrug-resistant bacterium, which uses several strategies to survive, such as the biofilm lifestyle. Biofilms are complex, multicellular bacterial communities that can protect bacteria against antibiotics and the host immune system (Loutet and Valvano 2010), making more difficult the infections treatment's and increasing the demand for new antibiotics for biofilm (Habash et al. 2017).

The findings of the current research suggest that the $AgNO_3$ did not influence the biofilm formation caused by *P. aeruginosa.* On the other hand, AgNPs were very efficient in control the biofilm production even at lower concentrations.

The anti-biofilm ability of AgNPs against *P. aeruginosa* had already been reported. AgNPs synthetized with leaf extract of *Allophylus cobbe* decreased the *P. aeruginosa* biofilm formation by more than 90%, in treatment of 24 h (Gurunathan et al. 2014). In addition, it was reported that AgNPs potentiated the activity of the antibiotic tobramycin against *P. aeruginosa* biofilm production revealing that the association of AgNPs with antibiotic could be very effective in treating patients with chronic infections (Habash et al. 2017).

Electrochemically synthesized AgNPs were strongly active against planktonic and biofilm of *P. aeruginosa* strain DIN1 from cystic fibrosis patients. The anti-biofilm activity was relevant with the deconstruction of extracellular matrix and cell membrane damage. The viability of *P. aeruginosa* biofilm was reduced by at least 98% at concentrations of 8.5 μg/mL (Pompilio et al. 2018).

The results from the present study are showing the biogenic AgNPs efficiency against *P. aeruginosa* biofilm formation, suggesting that it could be an alternative for infections caused by Gram-negative pathogenic bacteria.

Previous studies showed antimicrobial activity of textile fabrics carrying synthetic AgNPs (Seino et al. 2015), even

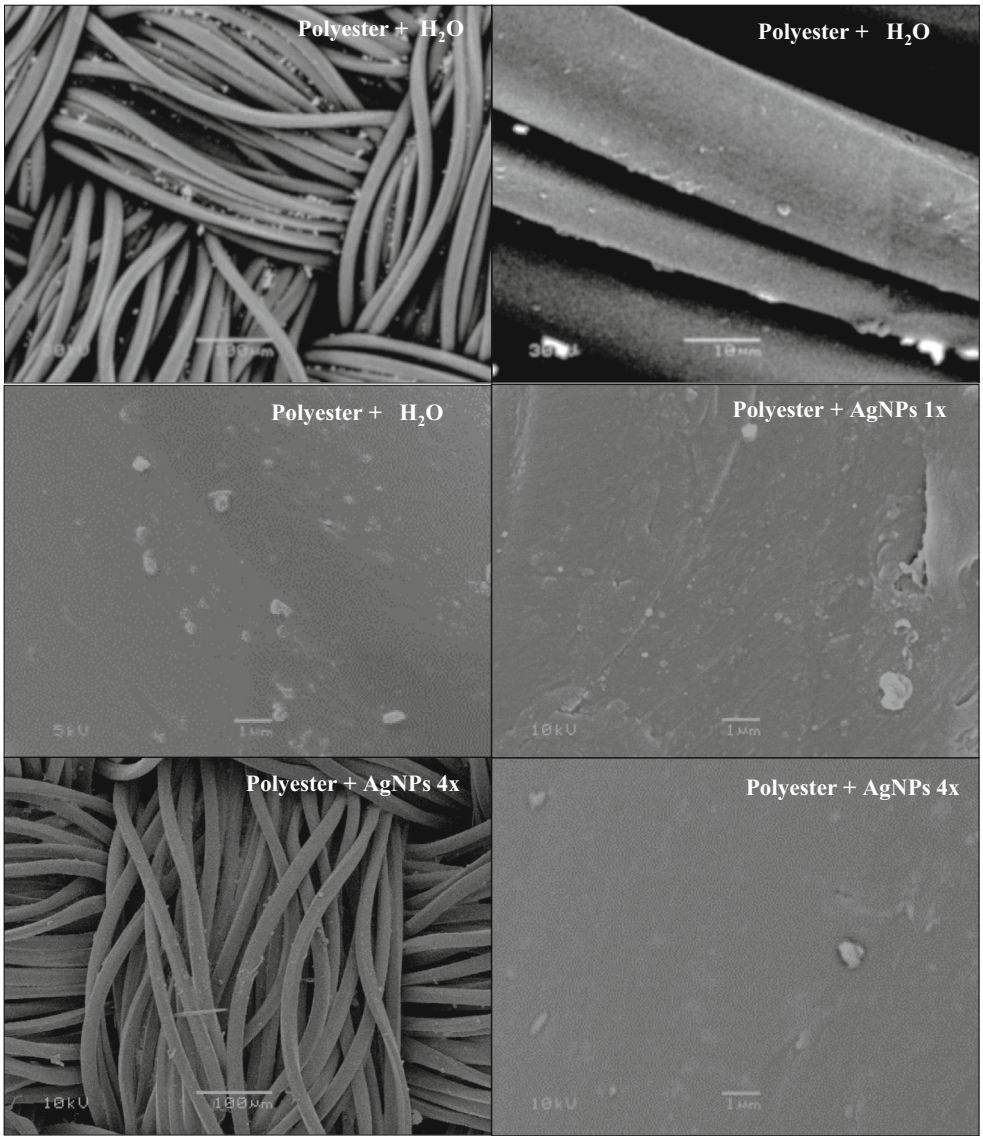

Fig. 5 Scanning electron microscopy (SEM) images of the polyester fabrics without impregnations and impregnated with AgNPs once or 4 times. Polyester + H_2O (100, 10 and 1 μm); polyester + AgNPs 1x (1 μm); polyester + AgNPs 4x (100 and 1 μm)

after 10 washing cycles (Mowafi et al. 2017), and of cotton fabric impregnated with low concentration of AgNPs produced by *A. terreus* (Balakumaran et al. 2016; Velhal et al. 2016), *Fusarium solani* (El-Rafie et al. 2012) or *Alternaria alternata* (Ibrahim and Hassan 2016). However, there are few reports using biological AgNPs and none applying nanoparticles obtained throughout the *B. ochroleuca* culture in textile or any other biomaterial. In this study, for the AgNPs impregnation process in the polyester fabric, no significant variation among the repetitions by once, twice or four times was observed. Most likely, after the first impregnation, there was a saturation of the polyester fabric. This effect was not observed for cotton, in which, the impregnation increased in accordance with the repetitions and reached 9.9% in the fourth time, probably due to the cotton capacity of adsorbing liquids, and consequently the nanoparticles present in it. The profiles of AgNPs incorporation in the cotton fabric are in accordance with those previously reported for AgNPs from *Fusarium solani* (Durán et al. 2007).

Cotton fabrics independent of the AgNPs concentrations presented good antifungal activity on *C. albicans* with a slightly higher inhibition when the fabric was impregnated by only once. This difference shows that the increase in the AgNPs impregnation from 0.1 to 9.9% was not relevant for improving the antifungal activity. Similarly, polyester fabrics also presented high inhibition of the *C. albicans* growth for the impregnations performed by once, twice or four times. In that case, the highest number of impregnations showed less pronounced inhibition.

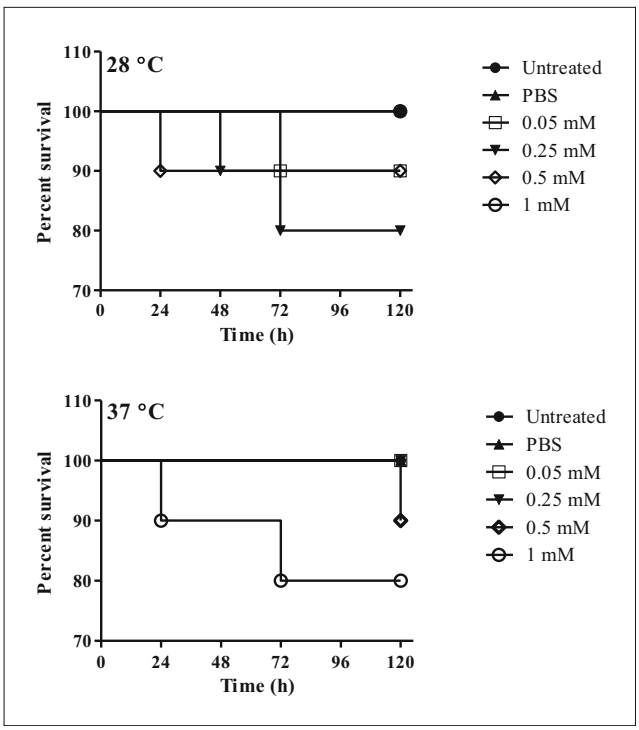

Fig. 6 Representation of *G. mellonella* larvae survival at 28 °C and 37 °C after injection with 10 μL of AgNPs of *B. ochroleuca* at 0.05, 0.25, 0.5 or 1 mM. Controls larvae were injected with PBS or even not injected (untreated). The survival was monitored daily during 120 h

The cotton fabric contains a higher percentage of AgNPs and inhibited efficiently the growth of the yeasts. Interestingly, the lower amount of AgNPs impregnated on polyester did not reduce its antimicrobial effect in relation to cotton fabric. This is an unexpected and remarkable result for a future scale-up process that could be performed in fewer steps and time, and with lower investment in the production of the final antifungal material. Overall, this approach might save costs while securing efficient antimicrobial activity against yeasts.

The caterpillar larvae or wax worm of *G. mellonella* is an animal model for toxicity assay (Seed and Dennis 2008) that has been used to evaluate the survival of larvae under treatments with bacteria, fungi and antimicrobial compounds, replacing the mammalian model in many cases due its innate immune system similar (Tsai et al. 2016).

The present study showed an effective and consistent approach to produce AgNPs by a biogenic method using the fungus *B. ochroleuca* for future applications as an antimicrobial material. The AgNPs presented anti-biofilm activity against *P. aeruginosa* and low toxicity in *G. mellonella* model. Besides that, the impregnation in cotton and polyester fabrics was very efficient and both the fabrics showed antimicrobial effect.

The results from this study are promising and encourage the application of biogenic AgNPs in different industrial areas such as in textile for hospitals. The applications of functionalized textiles or biomaterials containing AgNPs it is already a reality and will play a key role in the near future and different approaches can be designed to use these materials routinely in everyday life.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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