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Antibacterial Effect of Silver Nanoparticles Produced by Fungal Process on Textile Fabrics and Their Effluent Treatment

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Microorganisms play an important role in toxic metal remediation through reduction of metal ions. Studies demonstrated that silver ions may be reduced extracellularly using *Fusarium oxysporum* to generate stable gold or silver nanoparticles in water. These particles can be incorporated in several kinds of materials such as cloths. These cloths with silver nanoparticles are sterile and can be useful in hospitals to prevent or to minimize infection with pathogenic bacteria such as *Staphylococcus aureus*. In this work, the extracellular production of silver nanoparticles by *F. oxysporum* and its antimicrobial effect when incorporated in cotton fabrics against *S. aureus* were studied. In addition, all effluent was bioremediated using treatment with *C. violaceum*. The results showed that cotton fabrics incorporated with silver nanoparticles displayed a significant antibacterial activity against *S. aureus*. The effluent derived from the process was treated with *C. violaceum* and exhibited an efficient reduction in the silver nanoparticles concentration. In conclusion, it was demonstrated the application of biological synthesis to silver nanoparticles production and its incorporation in cloths, providing them sterile properties. Moreover, to avoid any damage to the environment the effluent containing silver nanoparticles can be treated with cyanogenic bacterial strains.

Keywords: Nanoparticles, Silver, Antimicrobial, Metal Ion, Microbiology, TEM.

1. INTRODUCTION

Recently, the development of resistant or even multi-resistant pathogens has become a major problem, for instance *Staphylococcus aureus* resistance to methicillin and *Candida albicans* resistance to fluconazole have to be mentioned.[1] On the other hand, the introduction of newly devised wound dressing has been a major breakthrough in the management of wounds or infections. In order to prevent or reduce infection a new generation of dressing incorporating antimicrobial agents like silver was developed.[2] It is well known that silver ions and silver-based compounds are highly toxic to microorganisms. Thus, silver ions have been used in many kinds of formulations,[3] and recently it was shown that hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules exhibit effective antimicrobial surface coating.[4] The wound dressing impregnated with colloidal silver (Contreet-H®) resulted in a strong decrease of pathogen-specific alterations in infected epithelium. The delivery of silver to infected keratinocytes in a moist healing environment improves the benefit/risk ratio as compared to wound dressing without silver.[1] Similar results with *E. coli* were obtained with silver nanoparticles.[3]

Nanometer sized silver particles synthesized by inert gas condensation or co-condensation techniques showed antibacterial activity against *E. coli*. The antibacterial efficiency of the nanoparticles was investigated by introducing the particles into a media containing *E. coli* and it was found that they exhibited antibacterial effect at low concentrations. In addition it was observed a relationship between the antibacterial properties and the total surface area of the nanoparticles. Smaller particles with a larger surface area were more efficient in the antibacterial activity tests.[5]

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In the last few decades there has been increased interest in reducing the availability of commercial textile containing antibacterial agents due to environmental pollution. Since silver is a good antibacterial agent and non-toxic and natural inorganic metal, it appears as an interesting material to be used in different kind of textile fibers. In this direction, polypropylene/silver nanocomposite fibers were prepared and the antibacterial tests showed that the fibers containing silver nanoparticles in core-part (inside the fiber) had no nearly significant antibacterial activity. However, the fibers having silver nanoparticles (30 nm size) in sheath-part showed excellent antibacterial effects.[6,7] Textile fabrics with antibacterial efficacy were easily achieved using nanosized colloidal silver particles (2–5 nm size), by padding process on cotton and polyesters. These fabrics showed laundering durability against *S. aureus* and *K. pneumoniae*.[8] Similar results were achieved with nanosized colloidal silver particles on polyester nonwovens. The growth of bacteria colonies was absolutely inhibited with only 10 ppm colloidal silver when the mean diameter of the silver particles was 2–5 nm. Consequently, a smaller particle size yielded better bacteriostasis on silver-padded nonwoven fabrics.[9]

Silver nanoparticles can be coated onto polyurethane foams in diverse forms. This material can be washed several times without any loss of nanoparticles. The performance of the material as an antibacterial water filter was studied and no bacterium (*E. coli*) was detected in the output water when the input water had a bacterial load of 10^5 to 10^6 CFU/ml.[10]

Many synthetic procedures for silver nanoparticles are available, but a narrow and controlled size preparation seems difficult to obtain because depend of the adjusted the concentration of reacting chemicals and controlled the reaction environment.[11] Colloidal metal particles can be obtained by chemical synthesis but these methods use toxic chemicals in the synthesis protocol, which raises great concern for environmental reasons.[12] Consequently, researchers have turned to biological synthesis because through this biological synthesis obtaining particles with good control on the size distribution than the other methods. The nanoparticles could also be stabilized directly in the process by proteins[13]. Although it is known that microorganisms such as bacteria, yeast, and fungi play an important role in the remediation of toxic metals through reduction of the metal ions, only recently this approach was considered interesting as nanofactories.[14] In this respect, the biosynthesis of inorganic nanomaterials using eukaryotic organisms such as fungi was achieved, with the intracellular production of silver nanoparticles by *Verticillium* strains.[12] Recently, it was found that aqueous silver ions may be reduced extracellularly using the fungus *F. oxysporum* to generate silver nanoparticles in water.[15] The mechanistic aspects were very recently described[13] and this process occurs probably by conjugation of reductase action and by electron shuttle quinones. Our aims in this research were to compare different impregnation processes published before with silver nanoparticle biosynthesized by the fungus *F. oxysporum*. Also important is our ecotoxicological concern, by recovering the silver nanoparticles generated in the process using a biotechnological approach involving *Chromobacterium violaceum*, which is able to metabolize or store metal ions in order to avoid any environment damage.[16] As recently described *C. violaceum* produces around 1–4 mM free cyanide[17] and it is able to metabolize several metals as cyanide complex. Among these metals are gold,[18,19] nickel,[17] and silver.[20]

2. MATERIALS AND METHODS

2.1. Silver Nanoparticles Preparation

The *F. oxysporum* strain used was 07 SD from ESALQ-USP Genetic and Molecular Biology Laboratory-Piracicaba, S.P., Brazil. The fungal inoculum was prepared in 2% malt extract and 0.5% yeast extract at 28 °C in Petri dishes. The liquid fungal growth was carried out in the presence of 0.5% yeast extract at 28 °C for 6 days. The biomass was filtrated and resuspended in sterile water. The biosynthesis of silver nanoparticles was carried out as following: approximately 10 g of *F. oxysporum* biomass was taken in a conical flask containing 100 ml of distilled water, kept for 72 h at 28 °C and then the aqueous solution components were separated by filtration. In this solution (fungal filtrate) $AgNO_3$ (10^{-3} M) was added and the system was kept for several hours at 28 °C. Periodically, aliquots of the reaction solution were removed and the absorption was measured in a UV-Vis spectrophotometer (Agilent 8453—diode array) at 440 nm. The silver nanoparticles were characterized by Transmission Electron Microscopy (TEM) and Elemental Spectroscopy Imaging (ESI). Bright field images and the elemental distribution within silver nanoparticles were obtained using a Carl Zeiss CEM-902 transmission electron microscope (80 KeV), equipped with a Castaing-Henry-Ottensmeyer energy filter spectrometer within the column. For the examination of the silver particle, one drop of the particle dispersion was deposited on carbon-coated parlodion films supported in 300 mesh copper grids (Ted Pella).

Elemental images were obtained for the relevant elements found in this sample, using monochromatic electrons corresponding to the silver K-edge, sulfur $L_{2,3}$-edge, and nitrogen L_3-edge. The energy-selecting slit was set at 367 ± 6 keV for Ag, 165 ± 6 eV for S, and 400 ± 6 eV for N. The images were recorded by a Proscan high-speed slow-scan CCD camera and processed in the AnalySis 3.0 system.

The size of silver nanoparticles was measured by X-Ray Diffraction (XRD, model XD3A from Shimadzu) with nickel-filtered Cu-Kα radiation (40 KV, 30 mA) at an angle of 2θ from 5° to 50°. The scan speed was 0.02°/min

and the time constant was 2 s. The size was calculated through of the Scherrer's equation:

$$D = (K\lambda)/(\beta_{cor}\cos\theta), \quad \text{with } \beta_{cor} = (\beta^2_{sample} - \beta^2_{ref})^{1/2}$$

where D is the average crystal size, K is the Scherrer coefficient (0.89), λ is the X-ray wavelength ($\lambda = 1{,}542$ Å), θ Bragg's angle ($2\theta = 25.1°$), β_{cor} the corrected of the full width at half-maximum (FWHM) in radians, and β_{sample} and β_{ref} are the FWHM of the reference and sample peaks, respectively.[21]

2.2. Silver Nanoparticles Loading on Cotton Fabrics

Cotton fabrics were washed, sterilized and dried before use. Experiments were performed on samples with maximum dimensions of 5 cm × 5 cm. The final filtrate (100 ml, 240 ppm) obtained above was treated by ultracentrifugation for 5 minutes and half of the filtrated (superior part) was eliminated to concentrate the silver nanoparticles. In order to impregnate cotton fabrics (5 cm × 5 cm), these were submersed in an Erlenmeyer (50 ml) and shaking at 600 rpm for 24 h and dried at 70 °C. The percentage of silver nanoparticles incorporated in the cotton fabrics was measured by X-ray fluorescence (XRF) (Shimatzu Mod EDX 7000), Source Rh, 50 kV and 15 kV.

2.3. Antibacterial Activity

The antibacterial behavior of the fabrics were evaluated against *Staphylococcus aureus* (ATCC 6538), a Gram-positive bacterium. The cotton fabrics were inoculated on agar plates inoculated with *S. aureus*. The inoculum was 1.3–1.6 10^5/ml. After 24 h, the plates were sterilized and the cotton fabrics were analyzed by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) at a voltage of 20 kV after previously coated with Au/Pd under vacuum.

In order to study the antimicrobial activity of the fabrics, squares of 1 cm of each fabric were prepared in aseptic manner. Each square was placed in a sterile vial and the fabrics subjected to pretreatment with 800 μl distilled water for 10 min. Tryptone soy broth (2.2 ml) was then added to each vial to make up to a total volume of 3 ml. An aliquot (10 μl) of *S. aureus* suspension was added to each vial (1.6×10^5/ml) containing the fabrics. Control broths with and without bacterial inoculation were also included. The vials were then incubated with agitation at 35 °C, 220 rpm. Aliquots of 10 μl broth were sampled at 24 h and serial dilution for the aliquots were prepared in broth. Duplicate aliquots (50 μl) of the serially diluted samples were spread on to plates. The plates were incubated at 35 °C and bacterial counts were performed. The bacteriostatic activity was evaluated after 24 h and calculated percent reduction of bacteria. Using the following equation: $R(\%) = [(A-B)/A] \times 100$. Where R = the reduction rate, A = the number of bacterial colonies from untreated fabrics, and B = the numbers of bacterial colonies from treated fabrics.

2.4. Microbial Treatment

The cotton fabrics previously loaded with silver nanoparticles were washed several times (as it could be used in a laundromat) and the effluent was treated as follows: a suspension of *Chromobacterium violaceum* CCT 3496 previously grown for 12 h at 30 °C in an orbital shaker at 120 rpm in 0.5% D-glucose; 0.5% peptone; 0.2% yeast extract; and 0.03% tryptophan.[22] The suspension were inoculated into 100 ml liquid collected from 5 consecutive water washes of the cotton fabrics loaded with silver nanoparticles. The concentration of *C. violaceum* inoculated were 10^2, 10^5 e 10^8 CFU/ml. Three inoculated flasks were incubated at 30 °C for 24 h in an orbital shaker at 120 rpm. The silver nanoparticles were measured in the effluent before and after *C. violaceum* treatment. The bacterial biomass was analyzed by SEM and EDS technique as described before.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization

The Erlenmeyer flasks with the fungal filtrate had a pale yellow color before the addition of Ag^+ ions which changed to a brownish color on completion of the reaction with Ag^+ ions for 28 h. The appearance of a brownish color in solution containing the biomass is a clear indication of the formation of silver nanoparticles in the reaction mixture.[13] Time-dependent increase in the intensity of the plasmon resonance (440 nm) was observed in the

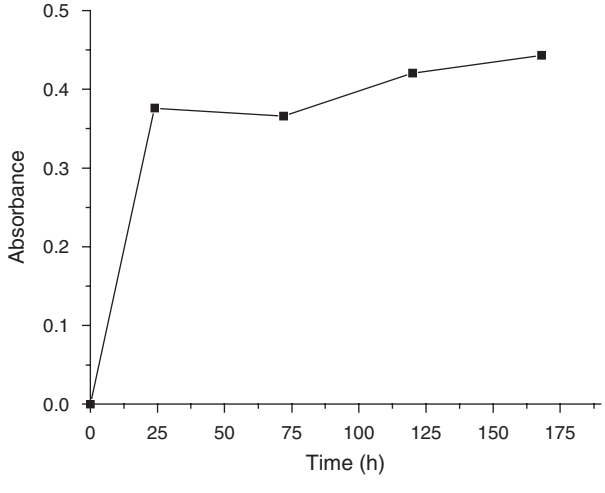

Fig. 1. Intensity absorbance of the plasmon resonance (440 nm) in function of time of reaction in an aqueous solution of 10^{-3} M $AgNO_3$ with the fungal filtrate.

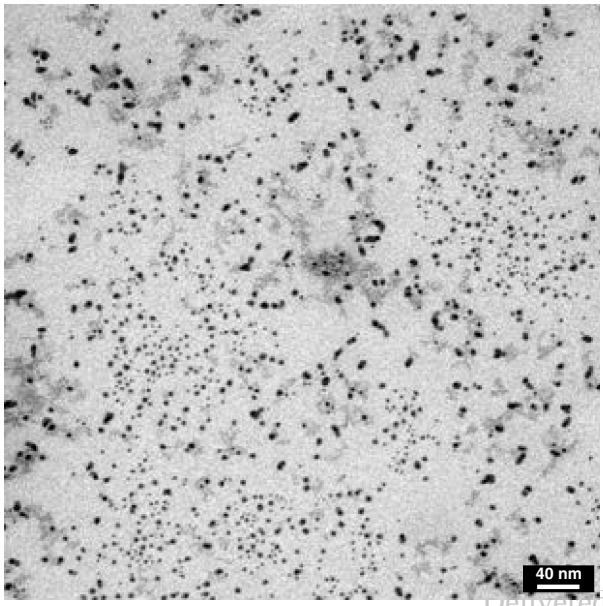

Fig. 2. TEM bright field image of the silver nanoparticles.

F. oxysporum reaction vessels (Fig. 1), confirming the silver nanoparticles formation.

The TEM micrograph (Fig. 2) showed spherical silver nanoparticles with size of 1.6 nm calculated by XRD through of the Scherrer's equation. These nanoparticles were analyzed by elemental spectroscopy imaging (ESI) (Fig. 3). The Figure 3(A) shows the bright field image of the silver nanoparticles and Figures 3(B), (C), and (D) show the ESI maps of this same region for Ag, N, and S atoms, respectively. As can be seen in the maps, particles were formed by silver and the presence of the N (Fig. 3(C)) and S (Fig. 3(D)) atoms around the silver nanoparticles are

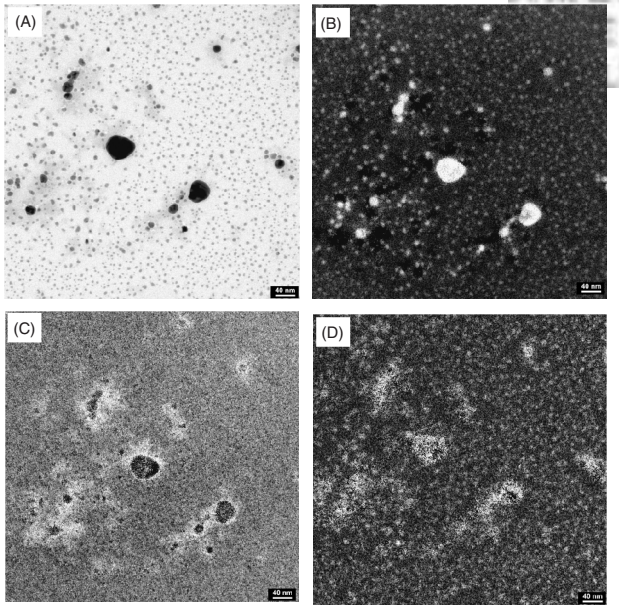

Fig. 3. (A) Bright field image of the silver nanoparticles, (B) ESI map for Ag atoms, (C) ESI map for N atoms, and (D) ESI map for S atoms.

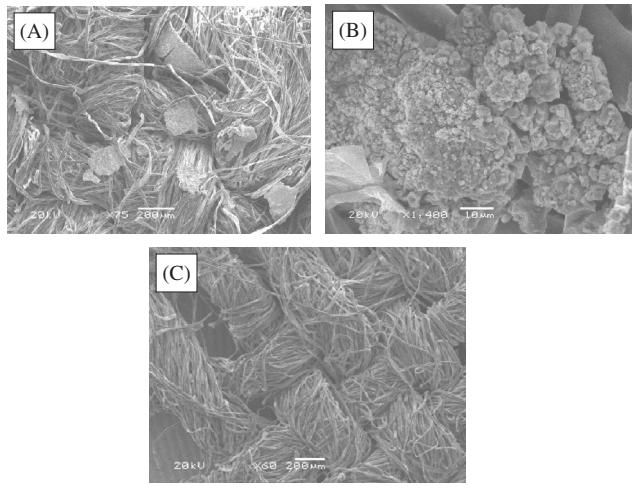

Fig. 4. SEM micrographs of the cotton fiber (A) without silver nanoparticles (control) ×75; (B) without silver nanoparticles (control) ×1400; (C) containing silver nanoparticles ×60.

indicated by the white regions. This result can be associated the particle stabilization by the fungal proteins.

3.2. Nanoparticles Incorporation in Cotton Fabrics and Their Antibacterial Effects

The cotton fabrics incorporated with silver nanoparticles were characterized by XRF. It was obtained 2% of incorporation. The bacteriostatic activity of the silver-impregnated fabrics against *S. aureus* was studied and this activity was indicated by a reduction of bacterial counts (1.6×10^5/ml to <10/ml, 99.9% reduction). The antibacterial activity of cotton fabrics with and without silver nanoparticles was evaluated and the fabrics was analyzed by SEM-EDS. In the fabrics without silver nanoparticles (control) a significant bacterial growth as shown in Figures 4(A) and (B) were observed. However, the cotton fabrics with silver nanoparticles presented antibacterial activity showing no bacterial growth in this one (Fig. 4(C)). This result demonstrated that silver nanoparticles can be used to turn sterile fabrics.

The incorporation of silver nanoparticles in the cotton fabrics also was verified by SEM-EDS (Fig. 5). In this figure of the cotton fibers containing silver nanoparticles was observed the presence of the silver peak and the absence of the contamination with bacteria (the inset Fig. 5). The silver nanoparticles dispersion will be reused for impregnation of other fabrics, for example, working in a closed circuit causing less damage to the environment.

3.3. Treatment of Effluent from Cotton Fabric Water Washes

Figure 6 shows the UV-Vis spectrum of the effluent containing silver nanoparticles before and after treatment with different inocula of the *C. violaceum*. In this spectrum was

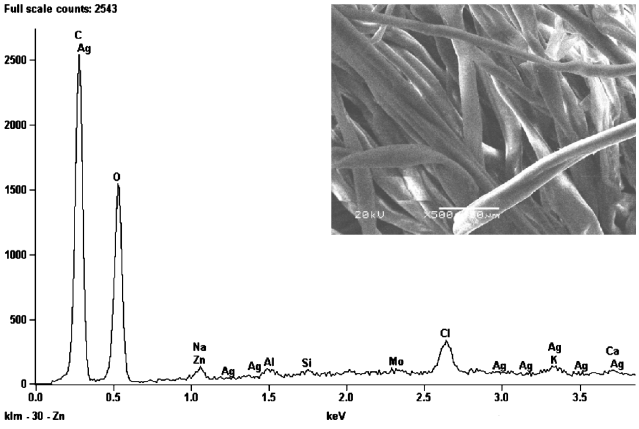

Fig. 5. EDS spectrum of the cotton fabrics containing silver nanoparticles. The inset shows the SEM micrograph of the cotton fibers ×500.

observed on intensity decrease of the plasmon resonance (440 nm) with increase of the bacterium concentration indicating a reduction in the silver nanoparticle concentration in the effluent. This reduction is due to the capture of these particles by *C. violaceum* as verified by SEM-EDS (Fig. 7). The Figure 7 shows the *C. violaceum* cells after bacterial treatment of the effluent with silver nanoparticles. Morphological changes of the cells from small rods to spherical forms were observed. This change was only observed in cells containing Ag atoms, as shown in the Figure 7 and Table I, demonstrating the influence of the silver in the morphology of these cells. A similar result was observed by Feng et al.[23] Apparently, the silver particles are concentrated at the surface and probably in the cytoplasm of the *C. violaceum*.

The metal capture by bacteria was already described in the literature. This process can be related with the presence of the cyanide or with atomic oxygen adsorption by the particles. Campbell et al.[18] verified that cyanide is formed as a secondary metabolite, and that for the leaching

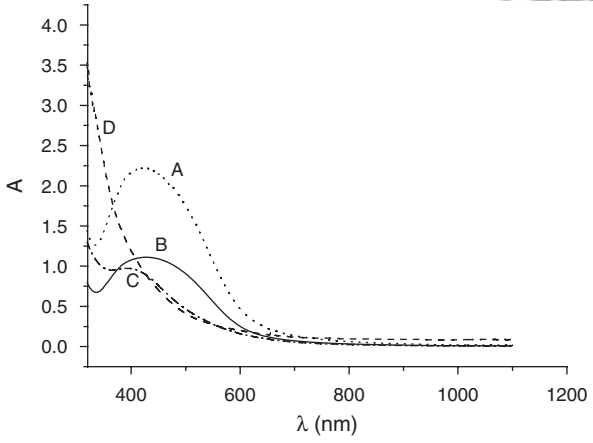

Fig. 6. UV-Vis spectrum of an effluent from cotton fabric water washes: (A) before treatment with *C. violaceum*, (B) after treatment with 10^2 CFU/ml of the *C. violaceum* culture, (C) after treatment with 10^5 CFU/ml of the *C. violaceum* culture, (D) after treatment with 10^8 CFU/ml of the *C. violaceum* culture.

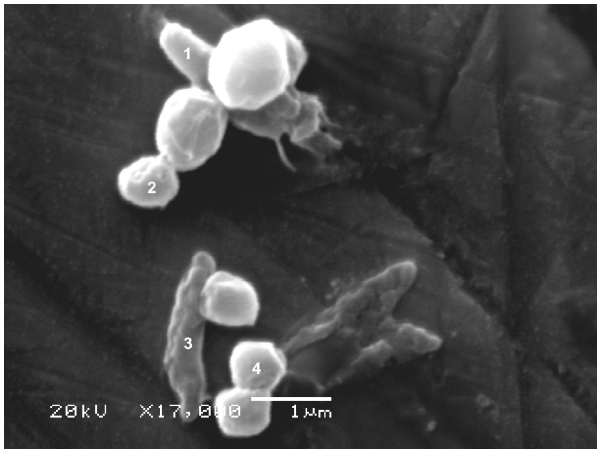

Fig. 7. SEM micrograph of the *C. violaceum* after effluent treatment ×17000.

Table I. Elements percentage by the EDS analysis of *C. violaceum* in presence of silver nanoparticles.

Point	C	O	S	Ag
1	12.45	0.48	0.20	0.00
2	12.48	0.28	0.14	0.46
3	14.65	1.22	0.09	0.06
4	8.49	0.49	0.11	0.26

process to occur, cyanide must be in close association with the silver surface leading to the silver oxidation and its posterior solubilization. Davies and Etris[24] showed that an adsorption of the atomic oxygen onto the surface of the silver exposed to the aqueous media occurs and that the particles readily react with pairs of sulfhydryl (-SH) groups on the surface of bacteria by replacing the hydrogen atoms (as water) thus resulting in the coupling of the sulfur atoms to form silver-S-S-bonds. Therefore, our data demonstrated that *C. violaceum* not only can colonize a silver surface, but can also solubilize the metal.

4. CONCLUSION

This study demonstrated the possibility of use biological synthesized silver nanoparticles and their incorporation in materials, providing them sterile properties. The cotton fabrics incorporated with these silver nanoparticles exhibited antibacterial activity against *S. aureus*. The effluents obtained from the cotton fabric wash process were efficiently treated by *C. violaceum.* This treatment was based on biosorption and showing to be very efficient for the elimination of silver nanoparticles remaining in the wash water, causing less damage to the environment.

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