academicJournals

Vol. 12(20), pp. 2917-2923, 15 May, 2013
DOI: 10.5897/AJB2013.12375
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antimicrobial activity of silver nanoparticles synthesized by the fungus *Curvularia inaequalis*

Helinando P. de Oliveira[1,2*], Leonardo S. Cavalcanti[1], Noelly B. Cavalcanti[1], Ismara de S. Nascimento[1], Sergio F. Pascholati[3], Luis F. P. Gusmão[4], Alan Greison C. Macêdo[1] and Mateus Matiuzzi da Costa[1]

[1]Instituto de Pesquisa em Substâncias Bioativas, Universidade Federal do Vale do São Francisco, 48920-310, Juazeiro, BA, Brazil.
[2]Instituto de Pesquisa em Ciência dos Materiais, Universidade Federal do Vale do São Francisco, 48920-310, Juazeiro, BA, Brazil.
[3]Escola Superior de Agricultura Luiz de Queiroz – USP, 13418-900, Piracicaba, SP, Brazil.
[4]Universidade Estadual de Feira de Santana, 44031-460, Feira de Santana, BA, Brazil.

Accepted 15 May, 2013

Silver nanoparticles have been widely reported in literature due to their vast industrial application in different areas. In this work, we explored a simple procedure for the biosynthesis of silver nanoparticles at room temperature from the action of *Curvularia inaequalis* as reduction agent. The degree of aggregation and size of biosynthesized particles were optimized from a factorial design involving combined variation of three different parameters of preparation. The resulting colloidal dispersion of silver nanoparticles presented strong antimicrobial activity against *Escherichia coli* and *Klebsiella pneumoniae* in an indication that *C. inaequalis* represents a new potential candidate for alternative biosynthesis of silver nanoparticles with antimicrobial activity.

Keywords: Antimicrobial activity, fungi, silver nanoparticles.

INTRODUCTION

Silver nanoparticles represent a class of materials with potential application involving catalysis, drug delivery and antibacterial activity (Homola et al., 1999; Yee et al., 1999; Ghosh et al., 2012; Birla et al., 2009; Kim et al., 2007; Mandal et al., 2012; Castro-Longoria et al., 2011; Antony et al., 2011). These structures are typically synthesized by electrochemical, chemical and biological methods (Bhaduri et al., 2013) with reduction of silver nitrate from the action of citrate, ammonia and sodium borohydrate (Bhaduri et al., 2013). Bio-inspired synthesis of metal nanoparticles (Cheng et al., 2011; Ahmad et al., 2012) can be considered as an emerging branch of green chemistry in which plant extracts, bacteria, fungi and algae have been applied in substitution of conventional chemistry reagents in order to promote the reduction of Ag^{+} to Ag^{0} (Birla et al., 2009; Chen et al., 2003; Joerger et al., 2000).

An additional advantage associated with the use of eco-friendly methods applied in the production of Ag NPs is related with typical pressure and temperature required during the synthesis, characterizing important advance for production of Ag NPs in large scale (Chen et al., 2003; Joerger et al., 2000; Basavaraja et al., 2008).

In the case of extracellular synthesis of metallic nanoparticles, it has been reported that filamentous fungi introduces important advantages over bacteria, if considering their large biomass (Kirthi et al., 2012), well-binding capacity (Mussrat et al., 2010) and superior

*Corresponding author: E-mail: helinando.oliveira@univasf.edu.br. Fax: 1-55-74-2102.7645.

Table 1. Combination of parameters applied in the chemometric study.

Sample	Parameter a (Reductase production)	Parameter b (Reaction time)	Parameter c (Silver nitrate)
I	24 h (-)	24 h (-)	5 mM (-)
C	24 h (-)	24 h (-)	100 mM (+)
B	24 h(-)	72 h (+)	5 mM (-)
A	72 h (+)	24 h (-)	5 mM (-)
BC	24 h (-)	72 h (+)	100 mM (+)
AC	72 h (+)	24 h (-)	100 mM (+)
AB	72 h (+)	72 h (+)	5 mM (-)
ABC	72 h (+)	72 h (+)	100 mM (+)

quality of resulting nanoparticles such as high stability (Castro-Longoria et al., 2011) and high-crystalline degree (Basavaraja et al., 2008). With this aim, different filtrate of fungi such as *Fusarium semitectum* (Basavaraja et al., 2008), *Neurospara crassa* (Castro-Longoriaet al., 2011), *Amylomyces rouxii* (Mussrat et al., 2010) and *Puccinia graminis* (Kirthi et al., 2012) have been applied in the nanoparticles production.

In this work, we developed a 3-factorial planning associated with the synthesis of Ag NPs induced by fungi *Curvularia inaequalis*. The optimal conditions for nanoparticles synthesis were explored in terms of reductase and silver nitrate concentration and reaction time.

MATERIALS AND METHODS

Preparation of reductase

Fungi *C. inaequalis* was obtained from PPBIO project/UEFS (Feira de Santana – Brazil). The growth of *C. inaequalis fungus* was established in potato dextrose agar (PDA) slants at 25°C during 48 h in a 250 mL Erlenmeyer flask containing 100 mL potato dextrose broth under constant agitation (120 rpm). The resulting incubated mycelial biomass (10 g) was filtered and washed with sterile distilled water in abundance. The resulting filtrate was dispersed in 100 mL of distilled water, with posterior incubation on orbital shaker at 25°C and agitation at 120 rpm. The suspension was filtered through a Whatman filter paper number 42.

We choose two different incubation times (24 and 72 h) in order to explore the influence of reductase on the size of silver nanoparticles which will be described as low and high concentration of reductase, respectively.

Synthesis of silver nanoparticles

The preparation of silver nanoparticles was established with the dispersion of silver nitrate (Aldrich, USA) at predefined concentration of reductase in aqueous solution. The reaction cell was maintained at room temperature and constant stirring (90 rpm) at fixed interval of time in a corresponding procedure established for different fungi (Gajbhiye et al., 2009). We explored two different concentrations of silver nanoparticles (5 and 100 mM) while the reaction time was established at two different levels (24 and 72 h).

Factorial planning applied to silver nanoparticles preparation

Factorial design (or factorial planning) is a powerful tool applied in the study of systems with multiple variables. The minimization in the number of experiments in comparison of one-factor-at-a-time systems is obtained with definition of two different levels (maximum and minimum) and the calculus of relative importance (obtained from contrasts of averages) (Olivier et al., 2007; Carvalho et al., 2010; de Oliveira et al., 2005).

Using this concept, the use of two different levels applied to three different preparation parameters viz. (reductase concentration – defined as parameter *a*, reaction time – parameter *b* and silver nitrate concentration – parameter *c*) can be explored in order to optimize the synthesis of AgNPs. Considering different possibilities of combination between different parameters (combined variation of minimum and maximum of each parameter), eight different samples can be obtained, as shown in the Table 1. To better understand how the preparation procedure affects the size and relative concentration of synthesized particles, we implemented a factorial design in which the response (absorbance in the UV-Vis and zeta-potential) is analyzed in terms of these three different parameters and their variation.

The absorbance in the UV-vis region represents an important parameter in the measurement of efficiency of synthesis since silver nanoparticles exhibit strong absorption of light due to the surface plasmon resonance (SPR peak) (Basavaraja et al., 2008). On the other side, the magnitude of zeta potential act as an indication of potential stability of colloidal dispersion. Large negative or positive values of zeta potential indicate elevated level of stability with no tendency of flocculation. By the use of both measurement techniques, the relative importance of main effects (a, b, c) or interaction effects (ab, ac and bc) can be calculated. As an example, the relative importance of parameter A measured from absorbance assumes a positive value if maximum in the specific parameter and contributes with elevation in the absorbance of resulting samples, as shown in the Equation 1.

$$\text{Importance of factor A} = [\,¼(f_a + f_{ab} + f_{ac} + f_{abc}) - ¼(f_i + f_b + f_c + f_{bc})\,] \quad (1)$$

Where, f_{ab} represents the response (absorbance or zeta potential) of sample ab, as previously defined in the Table 1.

Experimental setup

The absorbance of samples was measured using a spectrophotometer Hach DR5000, while size of particles and zeta-potential were measured using a Zeta Sizer Malvern (Nano ZS90). The SEM images were obtained using a Scanning Electron Microscope Hitachi TM1000. The action of synthesized silver nanoparticles against microogranisms was evaluated using the following procedure: the microdilution of nanoparticles in broth method was established according to CLSI (2006) with inoculation of 1 X 10^4 CFU ml^{-1} (cell formation unity), using concentrations of ½

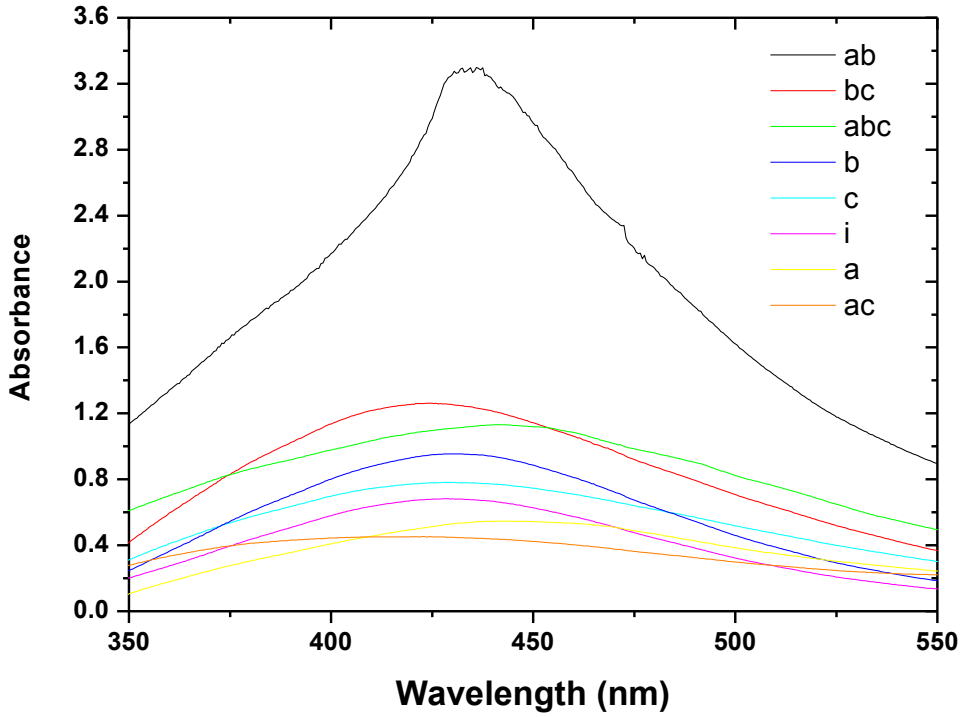

Figure 1. UV-vis spectrum of Ag NPS colloidal dispersion in aqueous solution.

Figure 2. Photograph of reductase before (left) and after (right) complete reaction with silver nitrate.

to 1/512 of resulting silver nanoparticles (measured in relation to 5 / 100 mM of silver nitrate) and incubated at 37°C for 24 h. Minimum inhibitory concentration (MIC) was determined using 2, 3, 5 triphenyltetrazole (TTC). Minimum bactericide concentration (MBC) measurements were performed with successive inoculation dilutions. As a result, negligible broth turbidity was observed. Then, samples were Mueller-Hinton Agar inoculated and incubated at 37°C for 24 h. The standard reference strains included in this study were: *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 1388, *Bacillus cereus* 11778, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, besides clinical samples of *Staphylococcus* spp. with methicilin resistance (n=1) and no biofilm producer *Staphylococcus* spp., verified by biochemical and genotypic tests (n=2).

RESULTS AND DISCUSSION

The spectrum of absorbance in the UV-vis region of silver nanoparticles (Wu et al., 2000), as shown in Figure 1, indicates that SPR peak is maximum to the sample ab (maximal reductase concentration, minimal concentration of silver nitrate and maximal reaction time) (Table 1) while the weaker peak was obtained in the sample ac (maximal reductase and silver nitrate concentration) since in this case, the coagulation of large aggregates of silver microparticles is verified at solution; a strong impediment for synthesis of colloidal systems with reasonable stability (Wu et al., 2000). The process of nanoparticles formation can be visually identified, as shown in Figure 2; a photograph of two flasks containing solution before and after the synthesis of silver nanoparticles, since this process is accomplished by change in colour of solution from transparent to yellowish (Yahyaei et al., 2013; Alani et al., 2012). Based on the value obtained for SPR peak of each sample (Figure 1), we can analyze the relative importance of isolated and combined parameters on the absorbance of colloidal solution and consequently on the efficiency of synthesis. It is important to report that to our

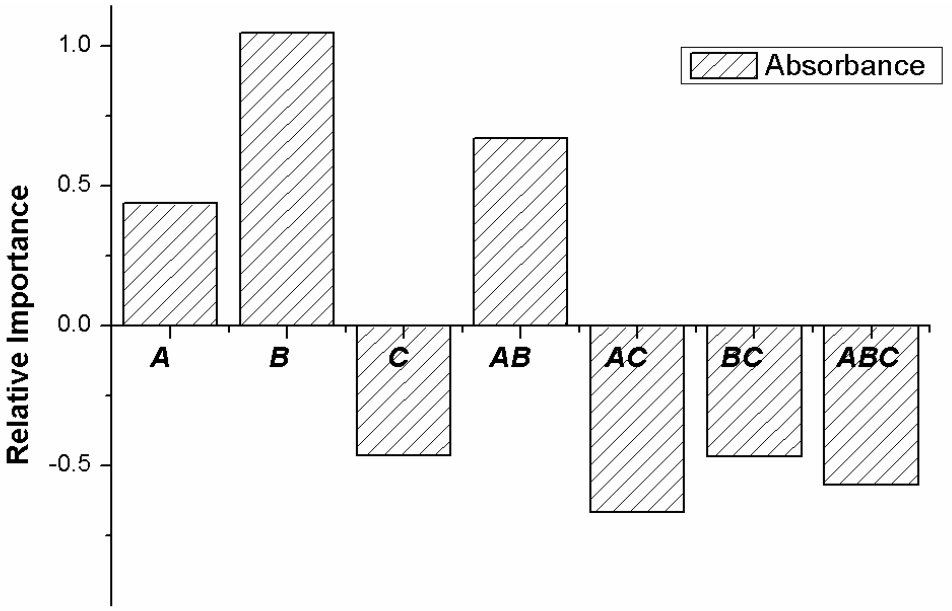

Figure 3. Relative importance of preparation parameters on the SPR absorbance peak

knowledge, it is the first time in which a factorial planning is applied in the study of bio-inspired synthesis of silver nanoparticles.

The results of relative importance of SPR peak, as shown in the Figure 3, indicate that parameter B (reaction time- relative to the interaction of silver nitrate with filtrate) represents the most important parameter in the optimization of SPR peak, followed by interaction of parameter AB and parameter A, respectively. On the other side, the parameter C (concentration of silver nitrate) introduces negative influence on SPR peak. Negative contribution for SPR peak is verified for parameters C, AC, ABC and BC, in a strong indication that elevation in the silver nitrate concentration induces a disordered growth of giant aggregates, as visually detected with precipitation of particles. Based on this information, it is possible to verify that minimal concentration of silver nitrate (5 mM) represents the adequate condition for regular growth of silver nanoparticles at increasing time. The relative importance of parameters measured from zeta-potential (Figure 4) indicates that if isolated, parameters B and C tend to reduce the zeta-potential and consequently the stability of colloidal dispersion. In spite of this result, the interaction of parameters A and C tends to increase the zeta-potential value, providing stability for colloidal dispersion during few days. It is an indication that high concentration of silver nitrate is compensated by high concentration of reductase, providing stable dispersion of nanoparticles in solution. The most negative relative importance parameter is the concentration of silver nitrate (variable C), in agreement with previous result of absorbance, in an indication that disordered growth of particles with low zeta potential is accomplished by progressive formation of precipitates.

Based on previous results, we explored the kinetics of silver growth using the most promising system from which is possible to define the most adequate condition for biosynthesis of silver nanoparticles. As an alternative for standard procedure of measurement of kinetics based on absorbance of light (Yahayaei et al., 2013; Alani et al., 2012), we explored the kinetics in terms of size of synthesized particles in order to correlate the antimicrobial activity with dimension of synthesized particles.

The kinetics of nanoparticles formation was analyzed from measurement of size of particles dispersed in water as a function of reaction time. The results in the Figure 5 indicate that at low concentration of reductase, the formation of nanoparticles is established after few hours and saturation in the size of particles is verified. If high concentration of reductase (as established in the Table 1) is considered after 100 h of reaction, the sample was composed by a distribution of large aggregates and particles with diameter in order of hundreds of nanometers, as shown in the Figure 6. The EDX of the sample indicates that silver represents 100% of synthesized particles, as expected.

The result indicates that low concentration of reductase (24 h of incubation) in a reaction with 5 mM of silver nitrate during 60 h is the most adequate condition for production of silver nanoparticles. Using these optimized parameters, we analyzed the antimicrobial activity of silver nanoparticles. Although the antimicrobial sensitivity of micro-organisms has been reported in a dose- dependent manner (Ghosh et al., 2012; Homola et al., 1999), our results prove that silver nanoparticles were effective until

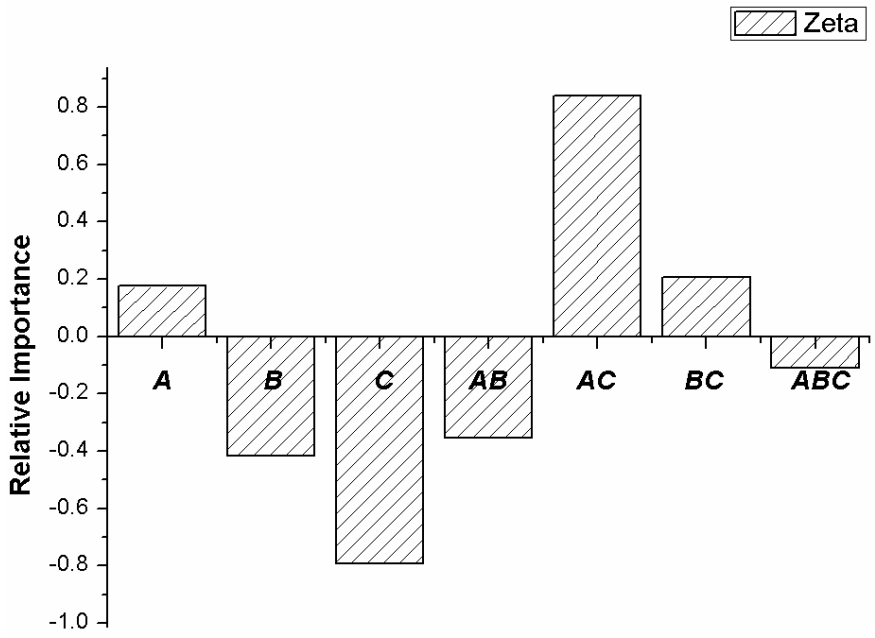

Figure 4. Relative importance of preparation parameters on zeta-potential of particles.

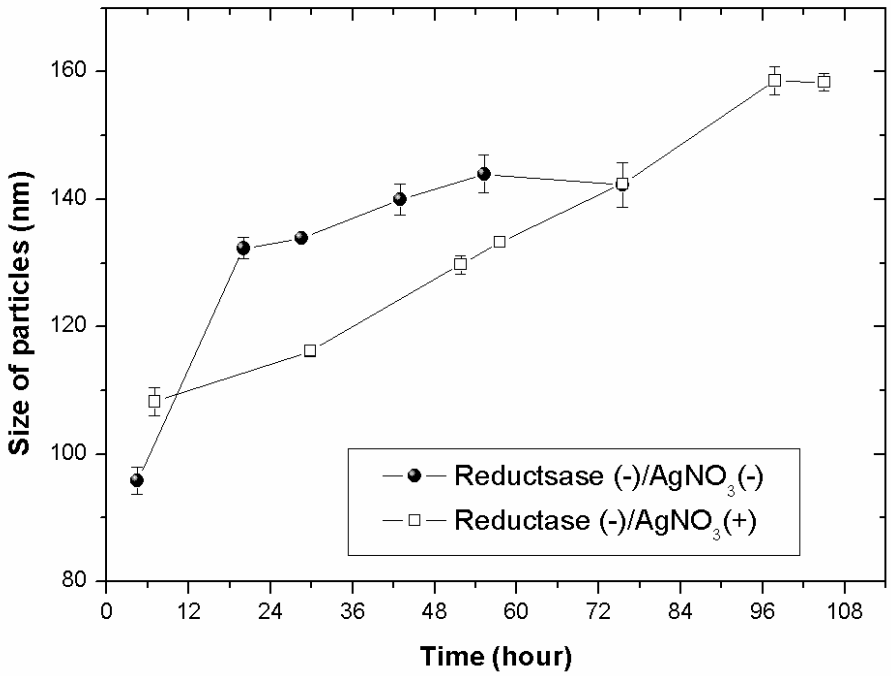

Figure 5. Kinetics of particles growth as a function of concentration of reductase.

1/256 dillution in *E. coli* and *K. pneumoniae*. Exception was verified in *E. coli* ATCC 25922; in Gram negative bacteria, the resistance to silver by efflux mechanisms coded by *sil* gene are reported in *Enterobacteriaceae* family and *Pseudomonas aeruginosa* (Sutterllin et al., 2012). The sensitivity of Gram negative bacteria was higher than for the tested Gram positive micro-organisms. The higher sensitivity of Gram negative bacteria is associated to the cell wall structure that permits a greater interaction with silver compounds (Antony et al., 2011;

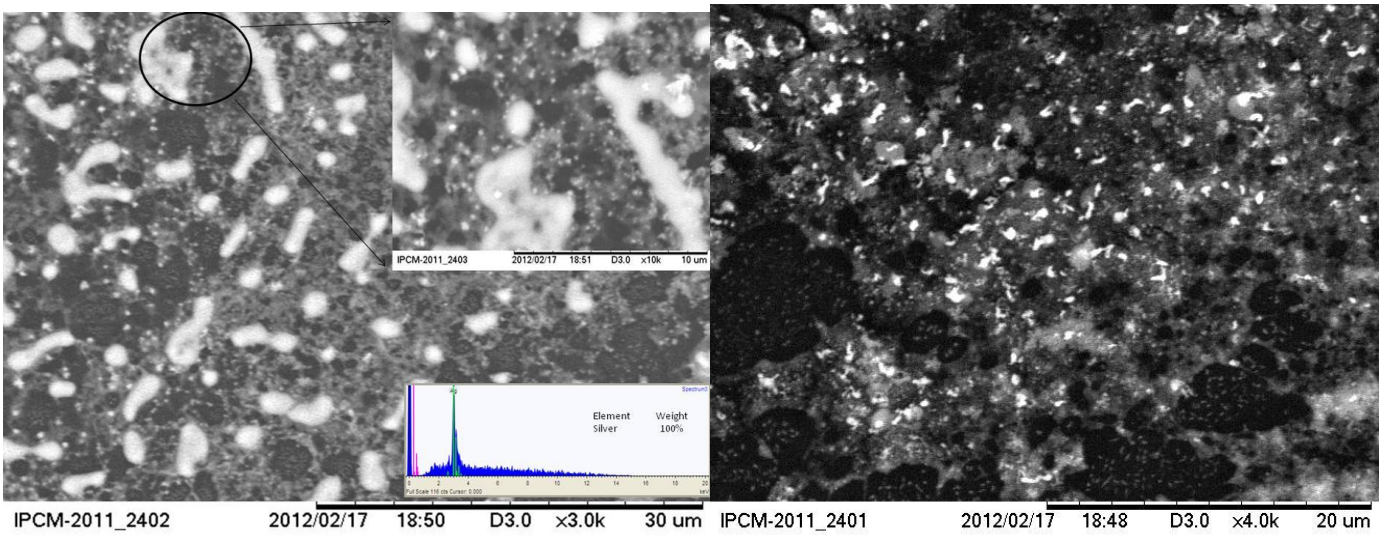

Figure 6. SEM of silver particles after 100 h of synthesis (high concentration of reductase-left side and low concentration of reductase – right side).

Table 2. Antimicrobial activity of silver nanoparticles against Gram negative and Gram positive bacteria.

Isolated microorganism	Reductase (-)		Reductase (+)	
	MIC	MBC	MIC	MBC
	Serial dilutions relative to 5 mM of $AgNO_3$			
Escherichia coli ATCC 25922	1/16	1/8	1/16	1/8
E.coli ATCC 35218	1/256	1/256	1/256	1/256
Klebsiella pneumoniae ATCC 1388	1/128	1/64	1/256	1/128
Bacillus cereus 11778	1/16	1/16	1/16	1/16
Staphylococcus aureus ATCC 6538	1/16	1/8	1/16	1/16
S.epidermidis ATCC 12228	1/32	1/16	1/16	1/16
S. aureus MRSA	1/64	1/64	1/64	1/16
Staphylococcus spp. 118	1/16	1/16	1/16	1/16
Staphylococcus spp. 131	1/16	1/16	1/32	1/16

Mohanty et al., 2011; Sutterlin et al., 2013). According to Ghosh et al. (2012), the silver nanoparticles produced by *Discocera bulbifera* were more effective against *E. coli* and *P. aeruginosa* if compared with *Salmonella typhi Bacillus subtilis* and *S. aureus*.

According to Mohanty et al. (2011), silver nanoparticles were more effective than $AgNO_3$ against medical interest pathogen such as *S. aureus*. Negligible difference was observed when biofilm producer and not producer *Staphylococcus* spp. isolates were compared. However, silver nanoparticles are associated to negative regulation of biofilm formation (Mohanty et al., 2011). Silver nanoparticles have shown antimicrobial activity against MRSA (Brandt et al., 2011; Ricco et al., 2012) as described in the present study. In Gram positive bacteria, the effect of silver nanoparticles may be associated to peptidoglycan fragmentation (Mirzajani et al., 2011). The antimicrobial activity of silver nanoparticles is summarized in Table 2.

Conclusion

The result of 3-factorial planning indicates that elevation in the silver nitrate concentration minimizes the stability of colloidal dispersion and provokes the aggregation and subsequent formation of giant precipitates. On the other side, increase in the reductase concentration contributes with reduction in the kinetics of silver nanoparticles formation. This information were fundamental for the optimization of parameters applied in the synthesis of silver nanoparticles. The silver nanoparticles produced by

C. inaequalis presented higher antimicrobial activity against *E. coli* and *K. pneumoniae* strains.

ACKNOWLEDGMENT

This work was partially supported by the Brazilian agencies FINEP, CAPES, FAPESB, FACEPE and CNPq.

REFERENCES

Ahmad N, Sharma S, Rai R (2012). Rapid Green synthesis of silver and gold nanoparticles using peels of *Punica granatum*. Adv. Mater. Lett. 3(5):376-380.

Alani F, Moo-Young M, Anderson W (2012). Biosynthesis of silver nanoparticles by a new strain of *Streptomyces* sp. compared with *Aspergillusfumigatus*. World J. Microb. Biotech. 28(3):1081-1086.

Antony JJ, Sivalingam P, Siva D, Kamalakkannan S, Anabarasu K, Sukirtha R, Krishnan M, Achiraman S (2011). Comparative evaluation of antibacterial activity of silver nanoparticles synthesized using *Rhisophora apiculata* and glucose. Coll. Surf. B 88:134-140.

Basavaraja S, Balaji SD, Lagashetty A, Rajasab AH, Venkataraman A (2008). Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*. Mat. Res. Bull. 43(5):1164-1170.

Bhaduri GA, Little R, Khomane RB, Lokhande SU, Kulkarni BD, Mendis BG, Siller L (2013) Green synthesis of silver nanoparticles using sunlight. J. Photochem. Photobiol. A 258:1-9.

Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009). Fabrication of silver nanoparticles by Phoma glomerata and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Let. Appl. Microbiol. 48(2):173-179.

Brandt O, Mildner M, Egger AE, Groessel M, Rix U, Posch M, Keppler BK, Strupp C, Mueller B, Stingl G (2011). Nanoscalic silver possesses broad-spectrum antimicrobial activities and exhibits fewer toxicological side effects than siver sulfadiazine. Nanomed.: Nanotechnol. Biol. Med. In press.

Carvalho RHR, Conceição MM, Souza AG, Souza EMBD (2010). Application of factorial planning and response surface methodology in the production of biodiesel from cottonseed oil *(Gossipium hisutum L.)*. Braz. J. Petroleum Gas 4(3):103-109.

Castro-Longoria E, Vilchis-Nestor AR, Avalos-Borja M (2011). Biosynthesis of silver, gold and bimetallic nanoparticles using the filamentous fungus *Neurospara crassa* Col. Surf. A 83:42-48.

Chen JC, Lin ZH, Ma XX (2003). Evidence of the production of silver nanoparticles via pretreatment of Phoma sp.3.2883 with silver nitrate. Lett. Appl. Microbiol. 37-2:105-108.

Cheng Y, Yin L, Lin S, Wiesner M, Bernhardt E, Liu J (2011) Toxicity reduction of polymer-stabilized silver nanoparticles by sunlight. J. Phys. Chem. C. 115: 4425-4432.

de Oliveira HP, Tenorio AC, de Lima EG, de Melo CP (2005). Dielectric characterization of colloidal solutions of retinoic acid embedded in microspheres of polyvinyl alcohol. Coll. Surf. A 257-258: 3-7.

Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M (2009). Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 5:382-386.

Ghosh S, Patil S, Ahire M, Kitture R, Kale S, Pardesi K, Cameotra SS, Bellare J, Dhavale DD, Jabgunde A, Chopade BA (2012). Synthesis of silver nanoparticles using Dioscorea bulbifera tuber extract and evaluation of its synergistic potential in combination with antimicrobial agents. Int. J. Nanomed. 7:483-496.

Homola J, Yee SS, Gauglitz G (1999). Surface plasmon resonance sensors: review. Sens. Act. B-Chem. 54 (1-2):3-15.

Joerger R, Klaus T, Granqvist CG (2000). Biologically produced silver-carbon composite materials for optically functional thin-film coatings. Adv. Mat. 12(6):407.

Kim J S, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang CY, Kim YK, Lee YS, Jeong DH, Cho MH (2007). Antimicrobial effects of silver nanoparticles. Nanomed. Nanotechnol. Biol. Med. 3(1):95-101.

Kirthi AV, Rahuman AA, Jayaseelan C, Karthik L, Marimuthu S, Santhoshkumar T, Venkatesan J, Kim SK, Kumar G, Kumar SRS, Rao KVB (2012) Novel approach to synthesis silver nanoaparticles using plant pathogenic fungi, *Puccinia graminis* . Mat. Lett. 81:69-72.

Mandal A, Meda V, Zhang WJ, Farhan KM, Gnanamani A (2012). Synthesis, characterization and comparison of antimicrobial activity of PEG/TritonX-100 capped silver nanoparticles on collagen scaffold. Coll. Surf. B 90:191-196.

Mirzajani F, Ghassempour A, Aliahmadi A, Esmaeili MA (2011). Antibacterial effect of silver nanoparticles on Staphylococcus aureus. Res. Microbiol. 162:542-549.

Mohanty S, Mishra S, Jena P, Jacob B, Sarkar B, Sonawane A (2011). An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. Nanomed.: Nanotechnol. Biol. Med. In press.

Mussrat J, Dwivedi S, Singh BR, Al-Khedhairy A, Azam A, Naqvi A (2010). Production of antimicrobial silver nanoparticles in water extracts of the fungus *Amylomyces rouxi* strain KSU-09. Bioresour. Tech. 101: 8772-8776.

Olivier S, Silva VL, Motta M (2007). Use of factorial planning in developing a methodology for galvanic zinc residue extraction. Quim. Nova 30(7):1750-1753.

Ricco JB, Assadian A, Schneider F, Assadian O (2012). *In vitro* evaluation of the antimicrobial efficacy of a new silver-triclosan vs a silver collagen-coated polyester vascular graft against methicillin-resistant *Staphylococcus aureus*. J. Vasc. Surg. 55-3:823-829.

Sutterlin S, Tano E, Bergsten A, Tallberg A-B, Melhus A (2012). Effects of Silver-based wound dressings on the bacterial flora in chronic leg ulcers and its susceptibility in vitro to silver. Acta Derm. Venereol. 92:34-39.

Wu P-W, Dunn B, Doan V, Schwartz BJ (2000). Controlling the spontaneous precipitation of silver nanoparticles in sol-gel materials. J. Sol. Gel Sci. Technol. 19:249-252.

Yahyaei B, Azizah S (2013). Rapid photogeneration of silver nanoparticles in ethanolic solution: A kinetic study. Spectrochim. Acta Part A 101:343-348.

Yee SS, Homola J, Gauglitz G (1999). Special issue: Surface plasmon resonance (SPR) optical sensors, current technology and applications - Preface. Sens. Act. B-Chem. 54(1-2):1-1.