

Research article

Bacteria mediated silver nanoparticles: comparison as potent antibiofilm agents

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Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):13-23

DOI: <http://dx.doi.org/10.4038/sljid.v9i1.8227>

Abstract

Introduction: Biosynthesized silver nanoparticles (AgNPs) have good antimicrobial properties comparable to chemically synthesized silver nanoparticles. Further, they have good potential as antibiofilm agents.

Methods: AgNPs were synthesized from *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Acinetobacter baumannii* (confirmed clinical isolate) and physically characterized by several techniques. The antibiofilm activity of the AgNPs against biofilms of *P. aeruginosa*, *S. aureus* and *C. albicans* was studied using crystal violet assay. Biofilms were formed in 96-well polystyrene plates and treated with biosynthesized AgNPs for 24 and 48 h.

Results: AgNPs synthesized by all bacteria except *S. aureus* mediated AgNPs displayed 50% biofilm inhibition at AgNP concentrations between 1.98 - 0.225 mg/ml. *S. aureus* mediated AgNPs showed 50% biofilm inhibition only against *S. aureus* biofilm. Scanning Electron microscopic images indicated that biosynthesized AgNPs were able to decrease surface coverage of biofilms and to reduce the extracellular matrix causing morphological changes in biofilms noticeably.

Conclusion: This study reports the antibiofilm activity of bacteria mediated AgNPs. This is the first report on antibiofilm activity of AgNPs synthesized by *Acinetobacter baumannii* and also as a comparison of antibiofilm activity of several bacteria mediated AgNPs. According to the

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Received 18 August 2018 and accepted 6 October 2018



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results, low dosages of green AgNPs can be applied in treating drug-resistant microbial infections in a cost effective manner. In conclusion, the bacterial synthesized AgNPs have antibiofilm activity and good stability suggesting its usefulness as economic and environmental friendly antibiofilm agents.

Keywords: Biofilms, Biosynthesis, Silver nanoparticles, Extracellular polysaccharides, Pseudo hyphae

Introduction

Bacterial biofilms pose a major challenge in medicine and are an important contributory factor towards the resistance of bacteria to antimicrobial treatment. Biofilms are a consortium of bacteria, fungi or other microorganisms which are embedded in a polysaccharide matrix where they can multiply, communicate with each other and persist, thus leading to biofilm infections specially associated with medical devices such as indwelling catheters^{1, 2}, contact lenses³ and prosthetic heart valves.⁴ Biofilms are responsible for disease outbreaks. Medically important biofilm forming pathogens include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Candida albicans*.

It is a well-established fact that biofilm organisms show greater resistance to antibiotics. Further, the biofilm formation is affected by the level of mutations and quorum-sensing mechanisms which results in antibiotic resistance leading to more virulent strains.⁵ Biofilms are more difficult to be penetrated through the polysaccharide matrix by even high concentrations of antibiotics. These biofilms have defensive mechanisms against antibiotics and antifungals under adverse conditions. Therefore development of novel antibiofilm agents is important in combating biofilm related infections.

Nanoparticles are emerging as novel antimicrobial agents and are widely used in various applications. Metal nanoparticles such as silver (AgNPs)^{6,7}, titanium dioxide⁷ have been reported to be effective against some biofilm forming bacteria indicating their potential for future development as effective biofilmicidal agents. AgNPs show a broad spectrum of bactericidal effects.⁸ Martinez-Gutierrez⁹ reported the inhibition of biofilm formation by AgNPs as a strategy to contain biofilm formation in medical devices. Due to the cost of production and toxicity of chemically synthesized AgNPs, biosynthesis of AgNPs involving bacteria is being considered as an attractive alternative. Bacterial enzymes act as reducing agents of Ag⁺ ions.¹⁰ Highly stable AgNPs can be produced from bacteria such as *P. aeruginosa*¹¹ and *Acinetobacter calcoaceticus*.¹²

In this study we have investigated the potential antibiofilm ability of bacterial biosynthesized silver nanoparticles *in vitro*.

Materials and methods

Microbial cultures

The bacterial strains *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), *A. baumannii* (confirmed clinical isolate) and *Candida albicans* (ATCC 10231) were obtained from the culture collection (Bacteriology laboratory, Department of Microbiology) and cultivated in Nutrient Broth Medium.

Ag nanoparticle biosynthesis

P. aeruginosa (ATCC 27853), *E. coli* (ATCC 25922), *A. baumannii* (confirmed clinical isolate) and *S. aureus* (ATCC 25923) were cultured in Nutrient Broth Medium (Himedia, India) and incubated for 72 h at 37 °C while shaking at 200 rpm. After the incubation period, the cultures were centrifuged at 6000 rpm for 20 min and the supernatants were collected.¹⁰ Aqueous silver nitrate solution (1 M, Park, UK) was added into culture supernatants to prepare a final concentration of 0.4 g/l and the mixture was kept at 60 °C to induce AgNP formation.

Biosynthesis of AgNPs was confirmed by UV–Visible Spectrophotometry. The AgNPs were diluted ten times and UV Visible absorption was measured using a UV-Visible spectrophotometer (PerkinElmer Lambda 35, USA). Transmission Electron Microscopy (TEM) was used to determine the particle size distribution of AgNPs (JEOL JEM 2100, Japan).

Preparation of microbial biofilms

Preparation of standard cell suspension (1.0 McFarland standard)

Colonies of 24 h old cultures of *C. albicans*, *S. aureus* and *P. aeruginosa* were inoculated in Brain Heart Infusion broth (Himedia, India) separately and incubated at 37 °C for 24 h. After 24 h of incubation, cell pellets were harvested by centrifugation at 3000 rpm for 10 min. Cell pellets were washed twice with PBS. An optical density (OD) of 0.1 (corresponding to 1×10^8 cells/ml) was obtained at 595 nm (Multiskan EX, Thermo Scientific, USA) as the standard cell suspensions for each organism.

Formation of 48 h old biofilms

Two hundred microliters of standard cell suspensions were added into each well and incubated for 24 h at 37 °C. After 24 h of incubation, 100 µl of the medium was removed and the well was replenished with 100 µl of fresh BHI medium followed by a further incubation at 24 h at 37 °C.

Assessment of biofilm formation

To interpret the biofilm production ability of the selected pathogens, a standard criteria described by Stepanovic et al.¹³ was applied as follows. The optical density of the cut off was calculated based on the OD of the negative control.

Optical density cut-off value (OD_c) = average OD of negative control (BHI only) + 3× standard deviation (SD) of negative control.

Average OD value	Biofilm production
≤ OD _c / OD _c < ~ ≤ 2x OD _c	Non/weak
2x OD _c < ~ ≤ 4x OD _c	Moderate
> 4x OD _c	Strong

Determination of Inhibitory concentrations for 48 hour mature biofilms

The 48 h old mature biofilms were washed twice with sterile PBS. Then 100 µl of serially diluted nanoparticle solutions were added into the corresponding wells in the microtiter plate. Negative control wells consisted of non-treated biofilms or sterile BHI. AgNO₃ (0.5% w/v) treated biofilms were used as positive controls. The plates were incubated at 37 °C for 24 h. The wells were washed twice with 200 µl of sterile PBS gently to remove free floating planktonic bacteria and biofilm viability was determined using Crystal violet assay. The biofilms were fixed with 200 µl of sodium acetate (2% w/v) and stained with 200 µl of crystal violet dye (0.1% w/v). Excess stain was rinsed off by thorough washing with sterile distilled water and plates were allowed to dry. After drying, 200 µl of 30% acetic acid was added into each well. The wells were incubated for 30 min. Acetic acid was aspirated and transferred to a new plate. The absorbance was measured at 595 nm using an ELISA plate reader and the percentage of biofilm inhibition calculated using the following equation. The experiment was carried out in triplicate, the data averaged, and standard deviation calculated.

$$\text{Percentage of biofilm inhibition} = \frac{(\text{OD}_{595} \text{ of untreated biofilm} - \text{OD}_{595} \text{ of treated biofilm})}{\text{OD}_{595} \text{ of untreated biofilm}} \times 100$$

Scanning Electron Microscopy (SEM)

The biofilms were cultured on 1 cm diameter sterile coverslips pretreated with conc. H₂SO₄ and absolute ethanol in 24 well culture plates (Corning, Germany) for 48 h and medium was replenished at 24 h of incubation. AgNPs were treated after 48 hours and further incubated for 24 h at 37 °C. Formed biofilms were fixed with 2.5% glutaraldehyde and washed with sterile PBS. Dehydration of samples was followed using an alcohol series (30%, 50%, 60%, 70%, 80%, 90%, 95% and absolute ethanol. After overnight drying in a desiccator, samples were coated with gold before imaging using a ZEISS EVO 18 Scanning Electron Microscope (Germany).

Results

The biosynthesis of AgNPs from all four bacteria were confirmed by the presence of narrow absorbance peaks observed approximately near 420 nm wavelength (Fig. 1). *S. aureus* mediated AgNPs gave a broad peak ranging from 420-450 nm.

According to TEM particle size analysis, all AgNPs were spherical in shape. The average sizes of the NPs were 11.14 ± 6.59 nm (S-AgNPs), 11.71 ± 2.73 nm (P-AgNPs), 12.87 ± 2.95 nm (E-AgNPs) and 12.22 ± 2.45 nm (A-AgNPs). Comparatively smaller AgNPs were produced by *S. aureus* and *P. aeruginosa*. Transmission electron microscopy identified all four AgNP species with average particle size < 20 nm in diameter suggesting the possibility of strong antimicrobial activity due to the small particle size.

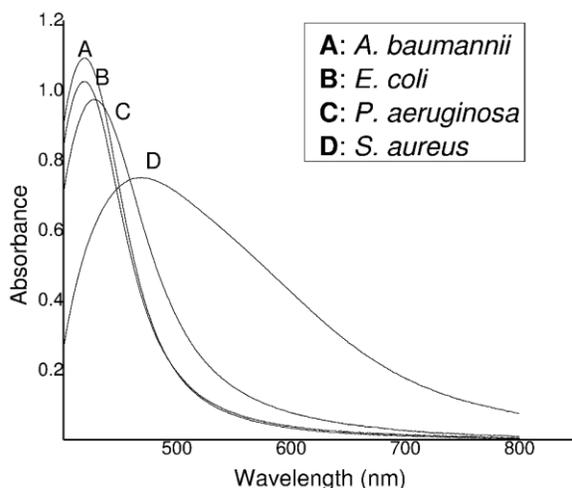


Fig. 1 UV-Visible absorption spectra of biosynthesized AgNPs

Biofilm formation

Biofilm formation of tested bacterial strains was determined using the crystal violet assay. Fig. 2 represents the biofilm formation assay at 24 h and 48 h. All three organisms were found to form strong biofilms according to the criteria specified by Stepanović et al.¹³ *P. aeruginosa* and *S. aureus* produced the strongest biofilms followed by *C. albicans* at both time points.

Bacterial biofilm formation was further confirmed using SEM imaging. Scanning electron microscopy

At the optimized conditions, the AgNP concentrations synthesized by *A. baumannii* (A-AgNPs)(0.665 mg/ml), *E. coli* (E-AgNPs)(0.99 mg/ml), *P. aeruginosa* (P-AgNPs)(0.45 mg/ml) and *S. aureus* (S-AgNPs) (0.435 mg/ml) were calculated by obtaining the dry weight.

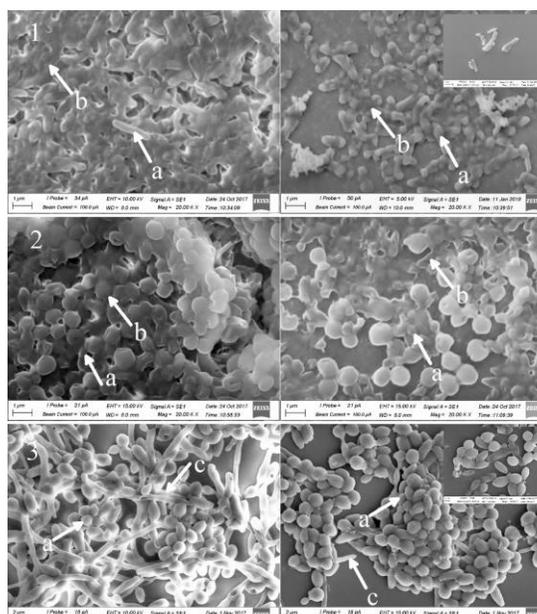


Fig. 2 SEM images of Biofilms of 1) *P. aeruginosa*, 2) *S. aureus* and 3) *C. albicans* **Left:** Before and **Right:** after treatment with AgNPs produced by *S. aureus* (a) cells, (b) extracellular matrix, (c) pseudo hyphae

of *P. aeruginosa* and *S. aureus* biofilms demonstrated a network of thick extracellular matrix surrounding abundant bacterial cells which suggests biofilm formation. The biofilm architecture included presence of interconnecting thread like structures, possibly formed due to water channels between the biofilm embedded cells. *C. albicans* biofilms demonstrated budding oval yeast cells with extensive production of pseudo hyphae and presence of budding scars. (Fig. 2)

Biofilm inhibition by AgNPs

Serial dilutions of bacterial synthesized AgNPs were prepared and used to treat 24 h old bacterial biofilms. Biofilm treatment was carried out for 24 h and 48 h. Highest inhibition of bacterial biofilms were observed at the highest tested AgNP concentrations of 1.33 mg/ml (A-AgNPs), 1.98 mg/ml (E-AgNPs), 0.9 mg/ml (P-AgNPs) and 0.87 mg/ml (S-AgNPs) at 24 h (Fig. 3).

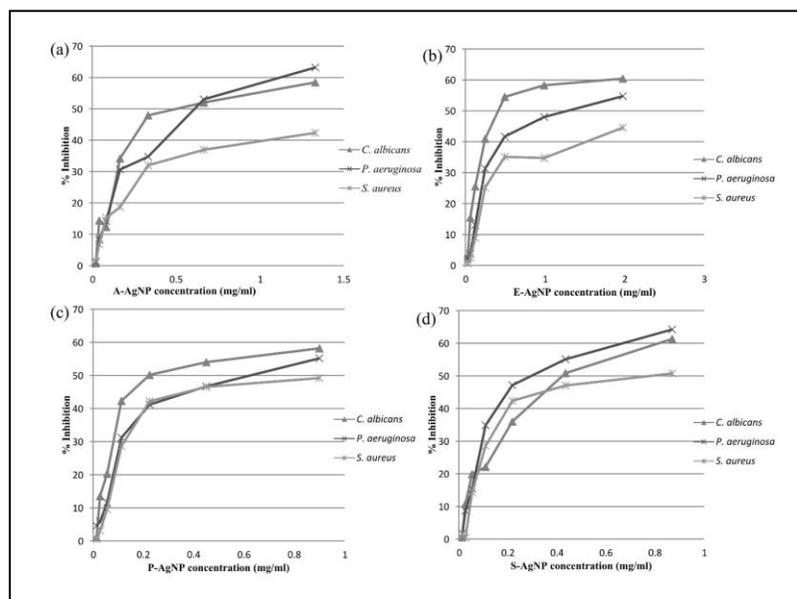


Fig. 3 Percentage inhibition of biofilms after 24 h treatment with the AgNPs produced by (a) *A. baumannii*, (b) *E. coli*, (c) *P. aeruginosa* and (d) *S. aureus*

Doubling dilutions of AgNPs were used to treat the 48 h old biofilms to determine 50% biofilm inhibitory concentration against the three bacterial biofilms tested. Biofilm inhibition was observed to be dose dependent. All four AgNPs showed more than 50% of biofilm inhibition against biofilms formed by *P. aeruginosa* and *C. albicans* while *S. aureus* biofilms showed 50% inhibition at this concentration only when treated with S-AgNPs (Table 1). With regard to *S. aureus* biofilm inhibition, A-AgNPs at 1.33 mg/ml was able to inhibit 42% of the biofilms, while highest AgNP concentration tested for E-AgNPs (1.98 mg/ml) were able to inhibit 45% of the *S. aureus* biofilms (Fig. 3). Further, 49% biofilm inhibition was seen when treated with 0.9 mg/ml, P-AgNPs. Fifty percent biofilm inhibition against *S. aureus* biofilms was achieved by treating the biofilms with 0.87 mg/ml AgNPs biosynthesized from the same organism.

Table 1: Concentrations of green AgNPs showing more than 50% inhibition of biofilms after 24 and 48 h treatments

Biosynthesized AgNPs	>50% Inhibitory concentrations of AgNPs (mg/ml)					
	<i>C. albicans</i> biofilms		<i>P. aeruginosa</i> biofilms		<i>S. aureus</i> biofilms	
	24 h treated	48 h treated	24 h treated	48 h treated	24 h treated	48 h treated
A-AgNPS	0.665	0.333	0.665	1.33	-	0.665
E-AgNPS	0.495	0.495	1.98	0.495	-	-
P-AgNPS	0.225	0.225	0.9	0.225	-	0.45
S-AgNPS	0.435	0.218	0.435	0.435	0.87	0.87

The highest biofilm inhibition at 24 hours when treated with A-AgNPs was observed against *P. aeruginosa* biofilm (63%) while treatment with E-AgNPs (60%) and S-AgNPs (61%) showed higher inhibition against *C. albicans* biofilms. *C. albicans* (58%) had stronger inhibition when treated with P-AgNPs while S-AgNPs were able to inhibit *P. aeruginosa* (64%), *C. albicans* (61%) biofilms. The biofilm inhibitory concentrations resulting in >50% biofilm inhibition by the four AgNPs after 24 h and 48 h of treatment against biofilms of *C. albicans*, *P. aeruginosa* and *S. aureus*. A-AgNP, E-AgNP, P-AgNP and S-AgNP are AgNPs biosynthesized using *A. baumannii*, *E. coli*, *P. aeruginosa* and *S. aureus* respectively. (Dashes indicate that the AgNPs did not give >50% biofilm inhibition at the tested concentration).

Electron microscopic imaging revealed that treatment of 48 h old biofilms with AgNPs for 24 h resulted in visible cell destruction leading to reduction in cell number, reduced extracellular matrix and damaged cells in all bacterial biofilms. Based on the crystal violet assay, S-AgNPs was the most effective against all microorganisms tested (Fig. 3). Fig. 2 shows the SEM images of S-AgNPs against *P. aeruginosa*, *S. aureus* and *C. albicans* biofilms after 24 h of treatment. AgNP treated cells (24 h) had altered cell morphology and disruption of cell membranes were also visible. Presence of silver nanoparticles was confirmed by the EDAX spectrum. Blebbing of the cell surface was seen in some cells which indicate the apoptosis process in these cells. Treated biofilms had less extracellular matrix when in contact with AgNPs for 24 h. After AgNP treatment of *C. albicans* biofilm, *Candida* cells showed rough outer cell wall with blebbing and markedly reduced presence of pseudo hyphae in AgNP treated specimens, while microcolonies were still present. The AgNPs were seen attached to the surface of the yeast cells.

Discussion

Bacterial biofilm formation is a challenge in treatment of infections due their intrinsic antimicrobial resistance. Thus, control of biofilm formation is critical in management of biofilm mediated infections. Silver nanoparticles have been reported to have multiple properties leading to antimicrobial activity and biofilm inhibition.

The nanoparticle size is a critical factor in determination of antimicrobial and antibiofilm properties⁸. According to Morones et al.⁸, AgNPs less than 10 nm of size can directly contact with bacterial cells and enhance antimicrobial activity. The nanoparticles reported in this study

had an average particle size of <20 nm and had a spherical morphology. The smaller size of the nanoparticles can increase the antimicrobial potency due to the higher surface to volume ratio which facilitates greater interaction with the cell surface. Nanoparticles with small size have superior penetrating ability.⁸

The appearance, size, shape and particle distribution depend on surface plasmon resonance energy of AgNPs.¹⁴ Further characterization of biosynthesized AgNPs using FT-IR spectroscopy indicated NH stretch vibrations present in the amide linkages of primary and secondary amines of proteins, presence of nitrocompounds from proteins or enzymes, C-H symmetrical stretch vibration of alkanes and -C-N- stretching vibrations confirming that the attached proteins in the biosynthesized AgNPs support stabilization of AgNPs.¹⁵

In the present study, silver nanoparticles synthesised by all four bacterial species demonstrated over 50% biofilm inhibition at a AgNP concentration between 1.98 mg/ml to 0.225 mg/ml (Fig. 3). The antibiofilm effectiveness of the AgNPs in the present study shows good promise when used at lower concentrations of approximately 1 mg/ml as seen in this study. Several studies have determined the effective biofilm inhibitory concentration of AgNPs. Martinez-Gutierrez et al.⁹ demonstrated strong biofilm inhibition against *Pseudomonas* and *Acinetobacter* biofilms while weaker biofilm inhibitory activity was observed against MRSA and *Candida albicans* biofilms using high AgNP concentrations ranging from 500 – 1000 mg/ml. In contrast, a study by Barapatre et al.¹⁴ using biosynthesized AgNPs (100 nm) had a minimum bactericidal concentration (MBC) of AgNPs between 16 µg/ml to 64 µg/ml against *S. aureus*, *E. coli* and *P. aeruginosa*. These differences may be due to the multiple factors contributing to the antimicrobial properties of the nanoparticles including the source of the AgNP, culture and biosynthesis parameters used, nanoparticle size and shape, surface charge, concentration, test organism and biofilm forming ability.

Biofilm formation is dependant on a number of factors and cell signalling through quorum sensing is one of the critical factors involved. AgNPs are involved in quorum quenching thereby diminishing the cell signalling as demonstrated by Chaudhari et al.¹⁶ against *S. aureus* biofilms. Further, AgNPs may arrest the synthesis of exopolysaccharides (EPS) which is an essential pre-requirement for biofilm formation.¹⁷ A study by Chandra et al.², demonstrated the three phases (early, intermediate and maturation) of biofilm formation of *C. albicans* using SEM imaging at different time points. The early phase of biofilm formation lacked detectable matrix, in the intermediate phase, cell density increased and formation of microcolonies was observed after 11 h while the mature biofilms were seen to have abundant matrix. The exopolysaccharides play an important role in protecting the biofilm from desiccation and antimicrobial agents.¹⁸ Further, EPS helps in cell adhesion and facilitates cell to cell communication. EPS also has a role in cell organization and contributes to structural integrity of the biofilm. Therefore targeting the EPS can be a useful approach in biofilm control. In the present study and in studies by others, the treatment of AgNPs to bacterial biofilms induced several structural changes as observed in the SEM images. Reduction in the biofilm associated EPS was observed for all the four test organisms comparable to other reported studies indicating the arrest of biofilm EPS secretion. The attachment of AgNPs to the surface of microbial cells

was observed in this study using SEM and has also been reported by several groups.^{17,19} Oza et al.²⁰ reported that the antibacterial effect of AgNPs may be due to penetration of released Ag ions in bacterial cell wall. AgNPs can disrupt the cell wall and rapidly detach the biofilms. Further, AgNPs can cause the formation of reactive oxygen species (ROS) which inhibits the antioxidant defense system and causes mechanical damage to the cell membrane.²¹ The overall antibiofilm activity of AgNPs may therefore occur by several mechanisms.

In the present study, AgNPs biosynthesized from different bacterial species were able to inhibit the bacterial and *Candida* biofilms. An interesting observation was, that against the *S. aureus* biofilms, the most effective AgNP was the NPs synthesized by *S. aureus* itself. Among the Gram negative bacterial biofilms tested, S-AgNPs were also found to be strongly effective against *C. albicans* and *P. aeruginosa* biofilms compared to other AgNPs synthesized. Especially in *P. aeruginosa* biofilms treated with AgNPs, the morphology change was visible with increased roughness, as reported previously for *E. coli*¹⁷, suggesting cell membrane damage by AgNPs. Comparable to previously published studies using planktonic cells, *S. aureus* biofilms were more resistant to AgNPs compared to Gram negative bacterial biofilms^{11,22}. The structure of the cell wall can influence the adhesion and penetration of the nanomaterial into the cell. Electron microscopic studies suggest that the higher cell wall thickness of Gram positive bacteria compared to the Gram negative bacterial cell wall may act as a barrier to the penetration of the nanoparticles into the cell.²³ Thus Gram positive bacterial cells possess an intrinsic resistance to the antimicrobial activity of nanoparticles compared to Gram negative bacteria as seen in this study and studies published by others.^{11,22,24}

Recently the focus on natural “green” methods of nanoparticle synthesis has been on the increase considering the low cost and environment friendly approach of biosynthesis compared to the chemical and physical methods of nanoparticle production. In this study, the focus was to compare different AgNPs biosynthesized by four bacterial species and to determine their antibiofilm potential. Using well defined culture and biosynthesis conditions in all experiments, it was possible to obtain AgNPs with reproducible properties which was confirmed by UV-visible spectroscopy, TEM, SEM and other methods. The exact mechanism of biofilm inhibition by green AgNPs is not fully understood.

Conclusion

The AgNPs synthesized by bacteria had significant antibiofilm activity after 24 h exposure against Gram positive, Gram negative and *Candida* biofilms at low concentrations displaying morphological changes and reduced biomass. The duration of AgNP exposure was important to obtain higher inhibitory effect. The results suggest the potential of green AgNPs as antibiofilm agents in therapeutics.

Abbreviations

AgNPs: Silver nanoparticles

EPS: Extracellular polysaccharides

MBC: Minimum bactericidal concentration

A-AgNPs: AgNPs by *A. baumannii*

E-AgNPs: AgNPs by *E. coli*

P-AgNPs: AgNPs by *P. aeruginosa*

S-AgNPs: AgNPs by *S. aureus*

Acknowledgement

The authors wish to thank the University of Sri Jayewardenepura, Sri Lanka for providing financial support (ASP/01/RE/MED/2016/42) and laboratory facilities.

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