# Green synthesis and antibacterial-antibiofilm properties of biogenic silver nanoparticles

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**Abstract**

The biosynthesis of metallic nanoparticles is gaining prominence as an alternative to traditional physicochemical methods, offering several advantages such as simplicity, non-toxicity, lower energy requirements and short reaction times leading to environmentally sustainable processes. The aims of this work were: to study the extracellular biosynthesis of silver nanoparticles (AgNPs) by *Pseudomonas extremaustralis* 2E-UNGS, to characterise the shape, monodispersity and size of AgNPs, to explore their antimicrobial and antibiofilm activities, and to evaluate the role of nitrate reductase activity in the biosynthesis process. The novelty of this work relies on the development of a green and sustainable method for the synthesis of stable AgNPs with optimal properties for potential applications in antimicrobial materials, especially when incorporated into polymeric matrices or used as agrochemical substitutes. Optimal conditions for the biosynthesis of spherical AgNPs were determined to be pH 7, 38 °C, 4 h of darkness and 120 rpm using stationary phase culture supernatants of *P. extremaustralis* 2E-UNGS. The involvement of extracellular nitrate reductase in AgNP biosynthesis was confirmed by enzymatic assays and supported by bioinformatics analysis, which identified the presence of the *napA2* gene linked to the *nirBD* cluster. Antimicrobial assays demonstrated the inhibitory effect of AgNPs against both Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* PA01 in both planktonic and biofilm states. In addition, the potential application of AgNPs in innovative antibacterial polymers was explored by incorporating them into polyurethane matrices either alone (PU-AgNP) or in combination with crystal violet as a photosensitizer (PU-AgNP-CV). Subsequent inoculation with a clinical isolate of *Pseudomonas aeruginosa* resulted in significant reductions in viable bacterial counts on both PU-AgNP-CV and PU-AgNP. Biogenic AgNPs showed antibacterial and antibiofilm properties for new antimicrobial material development.

**Keywords**

Biogenic Ag-nanoparticles; antimicrobial surfaces; green nanoparticle biosynthesis; antibiofilm activity.

## 1. Introduction

In the past few years, research in science and technology has been focusing on the manufacturing of atomic structures and materials at nanometer scales ($10^{-9}$ m), commonly known as "nanotechnology". There has been a growing scientific interest in this type of technology due to its potential impact in many different fields (energy, medicine, pharmaceutical and electronic industries, aerospace, textiles, etc.). Nanoparticles (NPs) exhibit unique chemical, physical and biological properties that differ from those of traditional materials (bulk materials) with the same chemical composition (Kumari et al., 2021; Venkatesh, 2018). The large surface area-to-volume ratio allows NPs to interact easier with other particles (Khan, 2019). These advantages make NPs very attractive for different applications were the surface/volume ratio display a crucial role, as antimicrobial activity and catalysis among others.

An important area of research in nanoscience is related to the synthesis of metallic nanoparticles. These particles show great diversity and have many applications. The most common methods for preparing nanoparticles are based on chemical and/or physical methods, which usually apply aggressive reagents for the environment and humans, along with complicated and expensive synthesis steps (by use of surfactants, solvents, elevated temperatures and pressures, undesired by-products, etc.), limiting the massive production. Therefore, there is a growing need to develop more economical, clean, non-toxic and environmentally friendly synthesis methods.

Some microorganisms, including bacteria, are well adapted to environments with high concentrations of metals. They can grow as a consequence of detoxification mechanisms, resulting in some cases in the formation of metallic NPs. The use of microorganisms for extracellular or intracellular NPs biosynthesis is emerging as an alternative to chemical methods and showing advantages over them: simplicity, non-toxicity, cleanness, fewer extreme temperatures and pressures applied with shorter reaction times (He et al., 2022).
It is estimated that silver nanoparticles (AgNPs) are the most widespread commercially; mainly due to their range of industrial applications. They are used in electronics, clothing, paints, cosmetics, biomedical practices, in the medical-pharmaceutical and food industries (Abou El-Nour et al., 2010; Calderón-Jiménez et al., 2017). AgNPs are becoming one of the fastest growing product categories in the nanotechnology industry. In addition, in the last decades, the prevalence of antimicrobial resistance has increased globally leading to an urgent need for new antimicrobial approaches. The strong antimicrobial activity of AgNPs is the central characteristic for the development of new medicinal products and in fact, a wide range is currently commercially available. Among them, antiviral properties of AgNPs promote their applicability for SARS-CoV-2 treatment, based on similarities observed with different virus families via in vivo studies (Bamal et al., 2021).
The incorporation of metallic NPs in polymeric matrices for textile (Syafiuddin et al., 2020) and paint industries (Bellotti et al., 2015) for antimicrobial purposes is a relatively new technology (Barberia-Roque et al., 2019; Fouda et al., 2019). Recent studies (Abou El-Nour et al., 2010) show an antimicrobial activity dependence on both the size (Dong et al., 2019; Raza et al., 2016) and shape of the NPs (Cheon et al., 2019). Smallest-sized spherical AgNPs demonstrated a better antibacterial activity against both *Escherichia coli* and *Pseudomonas aeruginosa* as compared to the triangular and larger spherical shaped AgNPs (Raza et al., 2016). Several researchers have reported the possibility of producing silver nanoparticles with proved antimicrobial activity using culture supernatants from *Pseudomonas aeruginosa* KUPSB12 (Jain et al., 2021), *Bacillus subtilis* (Loo et al., 2018), *Escherichia coli* (Pal et al., 2007) and *Pseudomonas antarctica* (Shivaji et al., 2011).
A more recent application of AgNPs is their use as acaricidal, insecticidal and fungicidal agents. This action against arthropods and fungi opens up its potential use, especially immobilised in biodegradable and inert matrices such as chitosan or diatomite (diatomaceous earth). This innovative alternative to phytosanitary products for the pest control in crops could avoid causing risks in the maintenance of the balance in soil microbial activities (Asadishad et al., 2018; Bapat et al., 2022; Benelli, 2018).
*Pseudomonas extremaustralis* 2E-UNGS (previously classified as *P. veronii* 2E) is an autochthonous microorganism isolated from the highly contaminated Reconquista River, Buenos Aires, Argentina (Vullo et al., 2008). Apart from being a non-pathogenic and innocuous bacterium, *P. extremaustralis* 2E-UNGS is able to self-aggregate forming flocs, develop biofilms on different inert supports (glass, polyurethane foam, Teflon, etc.), biosorb metals (cadmium, copper, lead, zinc) (Busnelli et al., 2021; Vullo et al., 2008) and biotransform chromium (VI) in lab-scale bioreactors (Alessandrello and Vullo, 2018), and secrete biosurfactants (Daniel et al., 2016) and exopolymeric substances (Ferreira et al., 2020). Regarding these properties, Cu(II), Cd(II) and online self-powered Cr(VI) biosensing devices were designed (Busnelli et al., 2021; Lazzarini et al., 2020). In addition, both extracellular and intracellular biosynthesis of Cu nanoparticles was evidenced in *P. extremaustralis* 2E-UNGS cultures (Busnelli et al., 2021). The wide spectrum of responses against external stimuli proved the ability to exploit its survival strategies as promissory tools for the development of innovative and sustainable environmental biotechnologies.
The aims of this work were: to study the extracellular biosynthesis of silver nanoparticles (AgNPs) by *Pseudomonas extremaustralis* 2E-UNGS, to characterise the shape,

monodispersity and size of AgNPs, to explore their antimicrobial and antibiofilm activities, and to evaluate the role of nitrate reductase activity in the biosynthesis process. The novelty of this work relies on the development of a green and sustainable method for the synthesis of stable AgNPs with optimal properties for potential applications in antimicrobial materials, especially when incorporated into polymeric matrices or used as agrochemical substitutes.

## 2. Materials and Methods

### *2.1 Microorganisms*

*Pseudomonas extremaustralis* 2E-UNGS is an autochthonous bacterium isolated from sediments associated to the Reconquista River basin-Buenos Aires Metropolitan Area and was the source of AgNP production. This isolate was identified by preliminary 500 bp 16S rRNA gene sequencing (MIDI Labs, USA) as *P. veronii* 2E (99.9% alignment with NCBI GenBank) in 2005. At the beginning of 2022 a complete sequencing of its genome was carried out. To move towards a more exhaustive identification through DNA hybridization isolate vs. DNA type strains, the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff et al., 2022; Meier-Kolthoff and Göker, 2019) platform was applied, resulting from the pairwise comparison of an alignment between 91.6% and 91.4% with the registered strains of the species *Pseudomonas extremaustralis*. According to the criteria established by the dDDH4 parameter used for this calculation, the strain was finally reclassified as *Pseudomonas extremaustralis* 2E-UNGS and registered in the NCBI GenBank with Accession Number CP091043.1.

*Pseudomonas aeruginosa* PA01, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* DH5α, *Mycobacterium smegmatis* $MC^2$ 155, *Micrococcus luteus* S66, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 14579, were the reference strains to determine antimicrobial activity by diffusion agar assay. *Pseudomonas aeruginosa* 1149, used for assessing antibacterial and antibiofilm activity, is a mucoid strain isolated from a cystic fibrosis patient in Intensive Care at King's College Hospital, London.

### *2.2 Silver nanoparticles biosynthesis*

To study the effect of culture medium composition on the AgNP production, *P. extremaustralis* 2E-UNGS was grown in nutrient medium PYG (g $L^{-1}$: 2.5 casein peptone, 1.25 yeast extract, 0.5 glucose) and in its 1:2 dilution, and the minimal medium M9 (g $L^{-1}$: 7.3 $K_2HPO_4$, 3.0 $KH_2PO_4$, 0.5 glucose, 6.6 $NH_4Cl$, 3.3 NaCl, supplemented with 0.1 yeast extract). After 24 h incubation (32 °C, 120 rpm) achieving a late exponential-early stationary phase biomass (0.683 g dry weight $L^{-1}$), cells were separated by centrifugation (7000 *g*, 15 min) and the supernatants were filtered through cellulose nitrate membrane (0.45 μm pore diameter). The cell-free solution was mixed with $AgNO_3$ (1 mM final) and incubated for 24 h at 120 rpm in darkness. The resulting yellow to brown solution was an indication of silver nanoparticles formation, confirmed by the absorbance spectrum (λ = 280-680 nm) in a PerkinElmer Lambda-25 spectrophotometer.

To check the effect of temperature on AgNP formation, PYG free-cell culture supernatants obtained at 20 °C or 32 °C were incubated in darkness for 24 h and 120 rpm at 20 °C, 32 °C or 38 °C in presence of 1 mM $AgNO_3$. The effect of pH was evaluated by 38 °C-incubation of *P. extremaustralis* 2E-UNGS culture supernatants grown in PYG at 32 °C, adjusting pH before biosynthesis with 0.1 M NaOH or 0.1 M HCl to 4, 6 and 9. The precursor concentration was also studied using 1 mM, 5 mM or 10 mM $AgNO_3$. Finally, the time effect was monitored up to 48 h under the optimal temperature, pH and precursor concentration conditions, previously determined.

Ultrapure water (18 MΩ cm, Millipore) was used to rinse the glass material and for the preparation of culture media and solutions. Non-inoculated sterile media (PYG and M9) were used as negative controls in each case and each assayed condition to confirm the absence of any potential non biological AgNP synthesis. All the experiments were performed in dark conditions.

### *2.3 AgNP purification and characterisation*

#### *2.3.1 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) analysis*

To purify the biogenic AgNPs, a suspension of 450 mL was centrifuged by 20 min (10000 rpm, 14000 *g*) resuspending the obtained pellet in 1 mM sodium citrate. The centrifugation step was repeated three times to ensure removal of impurities.
Sample drops for scanning electron microscopy observation (FE-SEM Zeiss SUPRA 40; CMA, FCEN-UBA) were seeded on silicon and dry at room temperature. The average particle size was measured after image captures, using ImageJ® as processing software.
For the elemental analysis, SEM images were captured with a Philips SEM-505-EDAX and a qualitative determination was performed (Instituto Nacional de Tecnología Industrial, INTI).

#### *2.3.2 Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR)*

To explore the functional groups belonging to biomolecules that could be associated to AgNPs, an ATR-FTIR spectrum was performed and analysed at 25 °C in the 4000-400 $cm^{-1}$ spectral range. To this aim, dried citrate-free samples were put on the diamond holder of an ATR-FTIR spectrometer (Thermo Nicolet iS10 Ultra Fast MX, Thermo Scientific, MA, USA, Instituto de Nanosistemas-UNSAM). Spectra were registered by co-adding 64 scans with 4 $cm^{-1}$ spectral resolution, using OMNIC software (version 8.3, Thermo Scientific, MA, USA).

### *2.4 Antimicrobial activity on reference strains*

#### *2.4.1 Agar diffusion assay*

Petri dishes (10 cm diameter) were prepared with 30 mL of Mueller Hinton (MH) Agar. After gelling, 5 mm diameter holes were made using a cork borer and dried 1 h at 32 °C. *P. aeruginosa* PA01, *S. aureus*, *E. coli*, *M. smegmatis*, *M. luteus*, *B. subtilis* and *B. cereus* cultures were grown overnight in MH broth (32 °C, 120 rpm) and then uniformly swabbed onto individual MH agar plates. Each hole was filled with 75 µL of the purified biogenic AgNP suspension and incubated 16-24 h at 32 °C. Antimicrobial activity of AgNPs was assessed by the visualisation of inhibition halos. A 1 mM sodium citrate solution was used as negative control.

#### *2.4.2 Antimicrobial assay against P. aeruginosa PA01 in liquid medium*

To evaluate the antimicrobial effects of silver nanoparticles on *P. aeruginosa* PA01 growth, 0 µg $mL^{-1}$, 5 µg $mL^{-1}$, 15 µg $mL^{-1}$ and 35 µg $mL^{-1}$ as final AgNP concentration was added to MH broth. Cultures were grown in a batch system, at 37 °C with constant shaking at 200 rpm containing an initial bacterial biomass (estimated by measuring Optical Density at 600 nm- $OD_{600nm}$) of approximately 0.01. Biomass as $OD_{600nm}$ was monitored up to 48 h. The concentration of the purified AgNPs was estimated by inductive coupling plasma mass spectrometry (ICP-MS, Agilent 7500cx, UNSAM). The experiment was carried out in triplicate and a sterile medium supplemented with nanoparticles was used as control.

### *2.5 Ag-NP antibacterial action on P. aeruginosa 1149*

*2.5.1 Minimal Inhibitory Concentration (MIC)*

The MIC of purified AgNPs in solution was determined against *P. aeruginosa* 1149 planktonic cells. A 96-well microtiter plate with MH broth was inoculated with $10^6$ Colony Forming Units (CFU) $mL^{-1}$ of *P. aeruginosa* 1149 and exposed to different AgNP concentrations (0-50 µg $mL^{-1}$). After incubating 48 h at 37 °C, the MIC endpoint was read as the lowest AgNP concentration with no visible growth, confirmed by Optical Density at 590 nm - $OD_{590nm}$- data obtained using a microtiter reader. Uninoculated wells were used as control.

*2.5.2 Antibiofilm efficacy*

The biofilm formation was studied according to O'Toole and Kolter (1998) with modifications. Different concentrations of AgNPs (0-50 µg $mL^{-1}$) were added to the 96-well microtiter plate containing MH broth and inoculated with a final concentration of $10^6$ CFU $mL^{-1}$ of *P. aeruginosa* 1149. After 48 h at 37 °C, the medium was removed, and the wells were rinsed three times with phosphate buffered saline (PBS). Biofilms attached onto the bottom of the wells were fixed with methanol and air-dried. Then, 180 µL of 0.1% (w/v) crystal violet (CV) was added to each well and incubated for 15 minutes. The CV solution was removed and the wells were rinsed with PBS until no violet colour was observed in the washing solution. As CV retained amounts are proportional to the attached biomass levels, after its solubilisation in 180 µL 30% (v/v) acetic acid, the remaining dye concentration was determined by measuring absorbance at 590 nm using a microtiter reader and contrasted with the corresponding calibration curve.

*2.5.3 Swell-encapsulation of AgNPs into polyurethane*

The method of Macdonald et al. (2016) was used for this purpose with minor modifications. Medical grade polyurethane (Branford, CT, USA) was cut into 1 × 1 cm squares. AgNPs were incorporated by swell-encapsulating into the polymer by immersing it into acetone/AgNPs solutions -75:25- with a final AgNPs concentration of 0.2 mg $mL^{-1}$. After 72 h in darkness, the swollen samples were dried 24 h at room temperature, rinsed with ultrapure water three times to remove residual nanoparticles from the surface and left to dry generating PU-AgNPs. As control, polyurethane was treated only with acetone/water 75:25 (PU).
To monitor encapsulation, the decrease in AgNP concentration in the applied solutions during these swelling experiments was recorded spectrophotometrically (Shimadzu UV-2600 spectrometer).

*2.5.4 Photosensitizers and AgNPs swell- encapsulations*

As mentioned above, the methodology applied by Macdonald et al. (2016) was used again with minor modifications: PU-AgNPs and PU samples (1 x 1 x 0,8 mm) were coated with crystal violet by immersing them in 1 mM CV for 72 h in the dark. After incubation, the samples (PU-AgNPs-CV and PU-CV) were left to dry at room temperature for 24 h and then rinsed with ultrapure water until non-violet colour remaining on water was observed. The samples were left to dry at room temperature under darkness before beginning with the experiments.

*2.5.5 Antibacterial surface properties*

To study the antibacterial activity of PU-AgNPs against *P. aeruginosa* 1149, the method described by Macdonald et al. (2016) was implemented. MH broth was inoculated with one

bacterial colony of *P. aeruginosa* 1149 and grown under aerobic conditions at 37 °C for 18 h and 200 rpm. Then, the bacterial pellet was recovered by centrifugation (21 °C, 2867.2 *g*, 5 min) and washed with PBS, centrifuged again under the same conditions, and finally re-suspended in 10 mL of PBS. The obtained suspension was diluted 1000-fold to achieve an inoculum of ~$10^6$ CFU $mL^{-1}$. Duplicates of each polymer (PU-AgNPs, PU, PU-AgNPs-CV and PU-CV) were inoculated with 25 µL bacterial suspension and incubated 4 h at 23 °C under a white light source at a distance of 30 cm (General Electric 28 W Watt Miser™ compact fluorescent lamp) emitting an average light intensity of 6.6 klux (~10 W $m^{-2}$) or under darkness. After incubation, the inoculated samples were submerged in 450 µL of PBS and vortexed for 20 s to recover the surviving bacteria. Viable counts were obtained by plating dilutions of the bacterial suspension on Mac Conkey agar at 37 °C, 24 h. Three biological replicates of the experiment were performed and statistical significance was analysed using the two-tailed t-test, verified with Mann-Whitney U test.

### *2.6 Biosynthesis mechanism: nitrate reductase activity*

Extracellular nitrate reductase activity from *P. extremaustralis* 2E-UNGS was analysed from cell-free PYG supernatants. Enzyme detection was measured according to Oza et al. (2012) method with a few variations. A 3 mL-sample was mixed with 250 µL of 0.4 M $KNO_3$ and incubated for 24 h at 32 °C. To ensure that the enzymatic activity was involved in AgNP production, three protein denaturation treatments were performed: heat (100 °C for 15 min), acid (HCl, pH 3) and detergent (0.1% sodium dodecyl sulphate, SDS) exposures. PYG broth and ultrapure water were used as negative controls, while $NaNO_2$ solution (0.06 mg $L^{-1}$) was used as a positive control. The reaction product was detected by addition of 1 mL 1% sulphanilamide (in 0.5 mL HCl (c)) and 1 mL of 0.01% N-(1-naphthyl) ethylenediamine dihydrochloride. After a 30 min-incubation at 30 °C and darkness for colour development, absorbance was measured in the 400-700 nm range with a maximum at 540 nm.
In parallel, treated supernatants with denaturing agents were also tested for AgNP production using the optimal pH, temperature, time and $AgNO_3$ concentration conditions previously obtained.

### *2.7 Identification of the nitrate reductase genes in P. extremaustralis 2E-UNGS genome*

The complete genome sequence of *P. extremaustralis* 2E-UNGS provided the possibility of studying the genes related to nitrate reductase activity and locating them applying bioinformatics tools such as Rapid Annotation using Subsystems Technology Server (RAST) (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014), the *Pseudomonas* Genome Database (Winsor et al., 2016), NIH/NCBI Basic Local Alignment Search Tool (BLAST, 2023) and Proksee-Genome Analysis (Grant at al., 2023).

## 3. Results

### *3.1 The effects of synthesis parameters on AgNP formation by P. extremaustralis 2E-UNGS*

*Pseudomonas extremaustralis* 2E-UNGS is an autochthonous and non-pathogenic bacterium isolated from the metal-contaminated Reconquista River. Although *P. extremaustralis* 2E-UNGS is able to survive in the presence of Cu(II) (Busnelli et al., 2021), Cd(II) (Ferreira et al., 2020; Ferreira et al., 2018; Ferreira et al., 2017), Zn(II) (Busnelli et al., 2021), and Cr(VI) (Alessandrello and Vullo, 2018), as described in previous studies (Vullo et al., 2008), this strain showed no tolerance to Ag(I) (data not shown). For that reason, AgNPs were obtained by exposing *P. extremaustralis* 2E-UNGS stationary-phase culture supernatants to $AgNO_3$ under different conditions. The effects of growth media on nanoparticle production were tested using the nutrient medium PYG, its 1:2 dilution and the minimal medium M9. Using

PYG culture supernatants, the reaction mixture changed from pale-yellow to caramel-brown indicating AgNP production, while with M9 no change in colour was observed. The location of plasmon absorption bands of metal nanoparticles depends on the shape and size of metal structures. Absorption spectra were obtained and a characteristic sharp peak at ~435 nm, associated with the surface plasmon resonance band indicated the formation of nearly spherical in shape nanoparticles (Torras and Roig, 2020). Fig. 1 A shows the typical maximal absorbance peak at 435 nm when PYG was used as culture broth. The corresponding spectra registered with the PYG 1:2 dilution and M9 supernatants, led to a lower intensity or null peak, respectively. Therefore, optimal nanoparticle production was achieved with PYG culture supernatants. As a control, the typical AgNP peak was not observed in the UV-visible spectra of PYG and M9 sterile media treated with $AgNO_3$. These results confirmed that the AgNPs were biologically produced, exclusively dependent on the bacterial growth products, discarding any possibility of a chemical synthesis mediated by carbohydrates, aminoacids or other components of PYG.

To evaluate the effect of experimental conditions on biosynthesis, different temperatures both on the bacterial growth (20 °C and 32 °C) and on the reaction mixture (20 °C, 32 °C and 38 °C) were studied (Fig. 1 B). AgNPs synthesised at 38 °C using supernatants from PYG cultures obtained at 32 °C showed a single intensive band at 435 nm in their absorption spectrum. Regarding culture supernatants obtained at 32 °C and biosynthesis reaction temperatures of 32 °C or 20 °C, both spectra presented a peak around 435-430 nm, corresponding also to spherical AgNPs and a less intense band around 600 nm, indicating either aggregation or the coexistence of two NP populations as described in literature (Abou El-Nour et al., 2010; Torras and Roig, 2020). AgNP formation was not observed with the control sterile culture media when incubated at 20 °C, 32 °C or 38 °C.

In order to study the effect of pH, cell-free supernatants obtained in PYG at 32 °C were used to synthesise nanoparticles. Fig. 1 C denotes the increase in absorbance intensity when reaction pH was 6. In the case of pH 4 and 9, the final absorbance values were much lower, indicating that no nucleation occurred. The influence of $AgNO_3$ concentration from 1 mM to 10 mM was evaluated in the reaction mixture at 38 °C and pH 6.0 (culture in PYG, 32 °C). In this study, 1 mM of precursor concentration revealed the maximal absorbance intensity at 435 nm, while an increase of $AgNO_3$ concentration did not improve these results (Fig. 1 D). The production of nanoparticles at various time intervals (0-5 h) was also investigated. An increase in the peak intensity near to 435 nm was detected along the reaction time, indicating the Ag(I) reduction with nanoparticle formation (Fig. 1 E). Optimal results were obtained using stationary phase *P. extremaustralis* 2E-UNGS culture supernatants obtained with PYG at 32 °C, under the following reaction conditions: 1 mM $AgNO_3$, pH 6, 38 °C and 3 h. Under these experimental conditions and applying a conversion factor related to the extinction coefficient at 435 nm (He et al., 2022), a maximum concentration of 53 mg AgNPs $L^{-1}$ was obtained, biotransforming approximately 50% of the total Ag(I) present in the solution. Therefore, these conditions were established for further studies.

### *3.2 Chemical characterisation of AgNPs*

Silver nanoparticles were characterised by SEM to determine morphology and size. For this purpose, a concentrated 80 mg AgNPs $L^{-1}$ suspension was obtained after the purification step. Fig. 2 A shows particles with quasi-spherical shape without significant aggregation. Frequency distribution determined from the histogram showed a particle size of 73 ± 33 nm, with a polydisperse of 43% (Fig. 2 B).

In addition, SEM-EDS qualitative analysis was applied to determine elemental composition of AgNPs. Fig. 3 A shows the results obtained by this methodology confirming an abundance of Ag in the assayed samples. To complement the chemical characterisation ATR-FTIR

spectrum was carried out to check the presence of associated biomolecules. Fig. 3 B represents the AgNPs ATR-FTIR spectrum overlapped with the soluble extracellular polymeric substances (EPS) secreted by *P. extremaustralis* 2E-UNGS. Peak assignment was performed according to Ferreira (2016), evidencing a clear polysaccharide-protein-lipopolysaccharide (LPS)-extracellular DNA (eDNA) structure present. The ATR-FTIR profiles obtained for AgNPs show typical signals for carboxyl, amide, phosphate and hydroxyl groups (Fig. 3 B). The spectrum revealed an intense band around 3200-3400 $cm^{-1}$, related to hydroxyl groups present in sugars, uronic acids, proteins, eDNA and LPS, and also of the N-H bond of proteins. The absorption at 2900 $cm^{-1}$ corresponds to symmetric stretching (νC-H s) of the $–CH_2$ group distinctive of polymers while the shoulder at 1650-1700 $cm^{-1}$ can be assigned to the asymmetric stretching of C=O groups (νC=O as) in -COOH suggesting the presence of carboxyl groups. Bands of amide I at ~1655 $cm^{-1}$ (νC=O and νC-N) and amide II ~1550 $cm^{-1}$ (νC-N and δN-H) were observed, corresponding to vibrational modes related to the peptide bond. The band that appears in the range of 1430-1470 $cm^{-1}$ could be assigned to C-H bending (δC-H) belonging to the peptide chain. The soft vibrational mode between 1390-1430 $cm^{-1}$ corresponds to symmetric stretching of the carboxylic ion groups (ν$COO^-$ s) and the peak at ~1250 $cm^{-1}$ could be related to the stretching of the phosphate groups present in nucleic acids and LPSs (νP=O). The absorbance at 1155-1180 $cm^{-1}$ corresponds to the stretching of the C-O-C group and the peak at 1100 $cm^{-1}$ to the C-O-C and –OH stretching, typical functional groups of polysaccharides. In the fingerprint area (<1000 $cm^{-1}$), characteristic soft peaks of C-O-P present in compounds with phosphate groups and C-O-C groups present in polysaccharides were observed.

To conclude, AgNPs are biosynthesised and potentially stabilised by their association with typical exopolymeric substances secreted by *P. extremaustralis* 2E-UNGS, evidenced by comparing band profiles of both ATR-FTIR spectra.

### *3.3 Antimicrobial activity on reference strains*

In this study, the antibacterial activity of AgNPs was detected by agar diffusion method against Gram-negative bacteria (*P. aeruginosa* PA01, *Escherichia coli*), Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus*) and *Mycobacterium smegmatis*. All the microorganisms exhibited inhibition halos indicating antimicrobial activity of AgNPs (Fig. 4 A).

The antibacterial activity in liquid media was determined against *P. aeruginosa* PA01 with different concentrations of AgNPs. A gradual decrease in the exponential phase slope (specific growth rate, μ) together with a marked extension in lag phase (4 and 8 h with 5 μg AgNPs $mL^{-1}$ and 15 μg AgNPs $mL^{-1}$, respectively) was observed with increasing AgNP concentration, indicating antibacterial activity. Complete inhibition of bacterial growth was apparent at 35 μg AgNPs $mL^{-1}$ (Fig. 4 B).

### *3.4 AgNP antibacterial action on P. aeruginosa 1149*

#### *3.4.1 Minimal Inhibitory Concentration (MIC).*

The MIC of purified AgNPs in solution was determined against *P. aeruginosa* 1149 planktonic cells. After 48 h incubation under aerobic conditions at 37 °C, a significant decrease in bacterial development was detected with 3.13 μg AgNPs $mL^{-1}$ ($p < 0.01$) while no turbidity was observed with concentrations higher than 6.25 μg AgNPs $mL^{-1}$ ($p < 0.001$), indicating bacterial growth inhibition (Fig. 5 A).

#### *3.4.2 Antibiofilm efficacy*

Antibiofilm activity by purified AgNPs was evaluated against *P. aeruginosa* 1149. A clear inhibition of biofilm formation was observed with a minimum of 6.25 µg AgNPs $mL^{-1}$ ($p < 0.001$, t-test) as determined by CV staining and dye concentration determination. Although not statistically significant, there was a clear reduction in biofilm mass at 1.56 and 3.13 µg AgNPs $mL^{-1}$. Results presented in Fig. 5 B are consistent with the growth inhibition detected above in MIC experiments.

The development of a mature biofilm is associated with the production of exopolymeric substances (EPS), within which the bacterial community is embedded and protected. Bacteria growing as a biofilm often show increased resistance to antimicrobial treatment compared to planktonic growth (Kalishwaralal et al., 2010). In this case, AgNPs were active against both planktonic bacteria and biofilm at 6.25 µg $mL^{-1}$ and above.

*3.4.3 Antibacterial surface properties*

Polyurethane polymer squares were exposed to solutions containing CV and AgNPs for 72 h resulting in change of colour, correlating to an uptake of either the dye or nanoparticles. Fig. 5 C, images a and c show the polyurethane squares before and after being coated in the AgNPs solution, respectively. Fig. 5 C, images b and d, show polyurethane-incorporating CV dye and AgNPs-CV, respectively.

The antibacterial activity of the modified polyurethane samples (PU-AgNP and PU-AgNP-CV) was tested against *P. aeruginosa* 1149 cultures, a mucoid strain isolated from a patient with cystic fibrosis in the Intensive Care Unit. After 4 h-white light exposure (~6 klux), PU-AgNP and PU-AgNP-CV demonstrated efficient bactericidal activity against *P. aeruginosa* 1149, resulting in a significant CFU $mL^{-1}$ reduction of 0.9 log ($p < 0.01$) and 5.5 log ($p < 0.001$) respectively, while compared with PU-CV. However, in the dark, no decrease in the number of viable bacteria was observed. Furthermore, no significant differences in bacterial counts among PU and PU-CV were detected under both light and dark conditions (Fig. 5 C).

***3.5 Biosynthesis mechanism: nitrate reductase activity***

Nitrate reductase activity was detected in *P. extremaustralis* 2E-UNGS culture supernatant grown in PYG broth and none or low activity was registered after denaturing treatments. Positive and negative controls confirmed that the reaction could be clearly associated with the presence of nitrate reductase. To study if the enzyme was involved in the AgNP production, the treated supernatants were tested under the optimal biosynthesis conditions found (1 mM $AgNO_3$, pH 6, 38 °C and 3 h). As shown in Fig. 6 A, the sample exposed to different denaturing agents decreased AgNP biosynthesis and specifically no NPs were obtained under acid denaturation, indicating that nitrate reductase activity is potentially involved in this mechanism.

Additionally, the *narK1K2GHJI* gene cluster was detected by a genomic study performed using the already mentioned bioinformatics tools at position 880 kbp with significant alignment results with the closest reference species *Pseudomonas veronii* G2 and is represented in Fig. 6 B. The nitrate reductase encoded in this cluster is well described as a cytoplasmic activity and no extracellular activity was reported up to now. In addition, a lack of the complete denitrification genes was detected by the absence of *nirS/nirK*, involved in nitrite reduction to NO, experimentally proved by the detection of only $NO_2^-$ after growing *P. extremaustralis* 2E-UNGS in $NO_3^-$ supplemented nutrient broth. A periplasmic nitrate reductase encoded by *napA2* gene was also detected -position 6155 kbp- in *P. extremaustralis* 2E-UNGS genome coupled to *nirBD* cluster, which is involved in nitrate-nitrite-ammonium assimilatory reduction pathway (Fig. 6 B). This cluster also denoted significant alignment with the corresponding genes in *P. veronii* G2 (Fig. 6 B).

## 4. Discussion

Many physicochemical methods for obtaining nanoparticles were reported involving the use of toxic agents (reducers, organic solvents and stabilisers) leading to a limited application (Gupta and Xie, 2018). The green synthesis, mediated by microorganisms, results in an advantage because of the simplicity accompanied by a restrictive use of potentially dangerous and expensive chemicals as well as the fully biodegradability of the bacterial products and the extracellular production (Moradi et al., 2021). Considering an intracellular production, an additional step would be necessary for the global process that increases complexity (Das et al., 2014), which is not the case in this study. Biogenic AgNPs were successfully obtained by a simple and rapid process using culture supernatants of *P. extremaustralis* 2E-UNGS. Monodispersion, shape and size of biosynthesised nanoparticles depend on physicochemical factors such as temperature, pH, precursor concentration and exposure time, growth medium composition and microorganism (Khan et al., 2019). Optimal conditions were set up leading to stable, non-aggregated, spherical and adequate shape AgNPs. These characteristics could be potentially associated to the presence of a coating mainly composed by bacterial biostructures such as soluble EPS, strongly supported by the ATR-FTIR analysis performed. Regarding the efficiency of the process, a 50% of total Ag(I) in solution was biotransformed to AgNPs, opening the possibility of metal recovery and recycling by this procedure.

In recent decades, significant research efforts have been focused on exploring the biomedical applications of metallic nanoparticles, particularly silver nanoparticles (AgNPs), owing to their unique antimicrobial properties (Torras and Roig, 2020). The antimicrobial efficacy of AgNPs is closely related to their shape and size, as highlighted by Dong et al. (2019) and Tang and Zheng (2018). Although the exact mechanism underlying their antimicrobial action is not yet fully understood, it was postulated that their small size enables easy penetration through microbial cell walls, leading to the generation of reactive oxygen species (ROS) and free radicals, increasing the oxidative stress in cells and ultimately resulting in cell lysis (Prasher et al., 2018). Regarding susceptibility, AgNPs are in fact more effective against Gram-negative bacteria since the thick cell wall of Gram-positive bacteria may reduce their penetration (Bamal et al., 2021). Given the escalating global threat of antibiotic resistance, there is an urgent need for the development of alternative antibacterial materials to prevent infections usually acquired from contaminated surfaces or other supplies in healthcare settings (Hwang et al., 2016; Owusu et al., 2019). In addition, surfaces treated with light-activated antimicrobial agents as crystal violet, methylene blue and toluidine blue O dyes exhibit photo-activated antimicrobial activity. This innovative development is considered promising because it can be easily applied to polymers which are widely used as hospital surfaces and in medical devices including tracheal or urinary catheters, and tubes for intravenous drips (Hwang et al., 2016). By producing reactive singlet oxygen ($^1O_2$) and ROS (superoxide, hydrogen peroxide and hydroxyl radicals), these agents cause adverse effects in bacteria such as loss of membrane integrity, inactivation of enzymes and DNA damage when exposed to a light source. The antimicrobial activity is dependent on the concentration of the agent, the exposure time, and the intensity of light, and they are typically more effective against Gram-positive bacteria compared to Gram-negative bacteria. This photobactericidal activity promoting bacterial sensitisation by ROS generation using light-active surfaces formulated with dye-modified polymeric materials has been widely reported (Macdonald et al., 2016 and references therein). As described, by combining diverse medical grade polymers embedded in dyes as CV, methylene blue or toluidine blue O with Au-nanoparticles, using the simple well-encapsulation-shrink method enhanced the antibacterial effects useful in healthcare environments such as hospital surfaces and/or other medical devices such as catheters.

In this study, the use of light-activated antimicrobial polymers along with the incorporation of AgNPs was examined as a potential innovative self-disinfecting surface. The inclusion of the

biogenic AgNPs on polyurethane (PU) coated with CV (PU-CV) samples were prepared by a two stage swell-encapsulation process (PU-AgNPs-CV). New developed light-active materials showed an effective antibiofilm and antibacterial activity against Gram-positive or Gram-negative reference strains including *P. aeruginosa* PA01 and even more against *P. aeruginosa* 1149, pathogen belonging to clinical samples from an intensive care unit of King's College Hospital, London.

In addition, another application of AgNPs consists in the development of a new generation of low environmental impact phytosanitary products. Acaricidal, insecticidal and fungicidal effects are currently under study complemented with ecotoxicological assessments in order to implement sustainable agricultural practices in future (Asadishad et al., 2018; Bapat et al., 2022; Benelli, 2018). In fact, AgNPs biosynthesised by *P. extremaustralis* 2E-UNGS culture supernatants are being evaluated as potential agrochemicals in periurban horticulture crops.

According to the literature, there is a strong hypothesis for silver nanoparticle biosynthesis suggesting that the enzyme nitrate reductase plays a key role in the process, assisted by the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) (Hietzschold et al., 2019; Rose et al., 2023). Primarily the nitrate reductase activity was associated to a cytoplasmic membrane-bound protein or outer membrane vesicles (OMVs) in *P. aeruginosa* PA01 (Arai, 2011; Deatherage and Cookson, 2012; Van Alst et al., 2009; Winsor et al., 2016). OMVs were described as spherical nanostructures (10-400 nm) and are secreted by bacteria specially during late exponential or stationary phases for the transport of proteins -mostly enzymes-, toxins, lipopolysaccharides, DNA and RNA, autoinducers contributing to the transmission and exchange of information between cells (Zhao et al., 2022). Regarding the soluble EPS release in *P. extremaustralis* 2E-UNGS containing carbohydrates 48% (m/m), protein 37% (m/m), total phosphorus 14% (m/m) and uronic acids in low concentration (ca. 1%), with an exopolisaccharide: LPS ratio ca. 1:1 (55% vs. 45%) (Ferreira et al., 2017), the presence of OMVs in this extract should be considered as a subject for further studies. Although a periplasmic nitrate reductase activity was also found in *P. aeruginosa* PA01, encoded within the *napEFDABC* gene cluster, as a compensatory supporting respiratory system in anaerobic growth (Van Alst et al., 2009), while aligning sequences of *P. extremaustralis* 2E-UNGS genome with *P. aeruginosa* PA01 *napEFDABC* gene cluster, non-significant identity results were obtained. However, the presence of a different periplasmic nitrate reductase *napA2* gene was identified and located close to the *nirBD* cluster aligned to *P. veronii* G2 gene. This *napA2* together with the *nirBD* cluster are responsible for the assimilatory nitrate reduction pathway. As a summary, a deeper exploration on periplasmic nitrate reductase expressed by *napA2* gene contained in OMVs or other potential secretion mechanism is the next step for the understanding of the extracellular detected activity. Adjusting environmental factors would contribute to the regulation of gene expression and hence to modulate the production of the extracellular nitrate reductase linked to the AgNPs biosynthesis efficiency.

## 5. Conclusion

The green biosynthesis of AgNPs provided a new nanomaterial of 73 ± 33 nm and quasi-spherical without significant aggregation, leading to appropriate size and shape for biotechnological purposes. Being a simple obtaining process mediated by culture supernatants of a non-pathogenic microorganism -*Pseudomonas extremaustralis* 2E-UNGS - indicated that the mechanism could be associated to an extracellular nitrate reductase activity. This was confirmed by the enzymatic reaction performed and supported by the presence of the corresponding gene cluster. Biogenic AgNPs exhibited antibacterial and antibiofilm activities against a range of bacterial species including known human pathogens. The inhibition properties were proved by manipulating AgNPs both in solution and incorporated in

polymeric matrices, demonstrating their potential application for the development of new and innovative antimicrobial materials.

**Acknowledgments**

This work was financially supported by the Universidad Nacional de General Sarmiento, Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica grant (ANPCyT, PICT 2014-0964).

**Author Contributions**

MLF work was focused on experimental AgNPs biosynthesis and characterisation and antimicrobial/antibiofilm studies. ICLB, MAD and GLS were involved in AgNPs at large-scales and characterisation. EO collaborated with the development of the antimicrobial materials under IP supervision. RJC collaborated with AgNPs manipulation and SEM. EA supervised the experiments to test the AgNPs antimicrobial/antibiofilm activity. DLV conceived the study and designed the experimental setup. All authors were involved with manuscript ideas and editing of relevant sections.

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**Figure captions**

Fig. 1. AgNPs production by supernatants from *P. extremaustralis* 2E-UNGS cultures in different media (PYG (1:2), PYG, M9). Inserted image shows photo of AgNPs produced by cell-free PYG supernatant (A). AgNPs production by *P. extremaustralis* 2E-UNGS at different temperatures (B), pH (C), $AgNO_3$ concentrations (D) and time (E).

Fig. 2. Scanning electron microscopy (SEM) of AgNPs synthesised by PYG supernatant from *P. extremaustralis* 2E-UNGS cultures (A). Nanoparticle size distribution of AgNPs; measurements were performed using ImageJ® software (B).

Fig. 3. SEM-EDS elemental analysis of AgNPs (A). Comparison of ATR-FTIR spectra belonging to AgNPs (blue) and soluble EPS from *P. extremaustralis* 2E-UNGS (grey). Band assignments correspond to typical stretching (ν) or bending (δ) symmetrical (s) or asymmetrical (as) vibrational modes (B).

Fig. 4. Antimicrobial activity detection by agar diffusion assay. Halos of inhibition produced by AgNPs on: (a) *Bacillus cereus*; (b) *Mycobacterium smegmatis*, (c) *Micrococcus luteus*, (d) *Pseudomonas aeruginosa* PA01, (e) *Bacillus subtilis*, (f) *Escherichia coli* and (g) *Staphylococcus aureus* (A). Antibacterial activity of AgNPs (◆ 0 µg, **x** 5 µg, ∗15 µg and • 35 µg $mL^{-1}$) in liquid media against *P. aeruginosa* PA01 (B).

Fig. 5. Antibacterial activity against *P. aeruginosa* 1149 (Minimal Inhibitory Concentration - MIC) of purified AgNPs obtained from culture supernatant of *P. extremaustralis* 2E-UNGS (* indicates significance, $p < 0.01$; * * indicates significance, $p < 0.001$) (A). Antibiofilm activity against *P. aeruginosa* 1149 of purified AgNPs obtained from culture supernatant of *P. extremaustralis* 2E-UNGS (**indicates significance, $p < 0.001$, t-test). Inserted image illustrates the CV-stained biofilm in the positive control well (*P. aeruginosa* 1149), while no violet colour is observed in the negative control (cell-free medium) well and in the highest AgNP concentration tested well (B). Viable counts of *P. aeruginosa* 1149 on modified PU squares after incubation at 23 °C with light (6.6 klux) or in darkness for 4 h.* indicates bacterial numbers reduced below the detection limit of 100 CFU $mL^{-1}$; * * indicates statistical significance ($p < 0.01$) compared to PU-CV; * * * indicates significance ($p < 0.001$) compared to PU-CV. Inserted image shows photo of each polyurethane polymer samples from antibacterial testing prepared by immersion in: (a) acetone: water (PU); (b) CV (PU-CV); (c) acetone: AgNPs (PU-AgNPs); (d) acetone:AgNPs:CV (PU-AgNPs-CV) (C).

Fig. 6. AgNPs production by *P. extremaustralis* 2E-UNGS supernatants after different protein denaturation treatment: heat, acid and detergent (SDS) exposures (A). *P. extremaustralis* 2E-UNGS *narK1K2GHJI* and *napA2* gene clusters, indicating the % of sequence identity while compared to the reference strain *Pseudomonas veronii* G2 (B).