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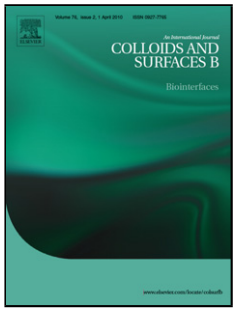

Biogenic Nanoparticles: Synthesis, Stability and Biocompatibility Mediated by Proteins of *Pseudomonas aeruginosa*

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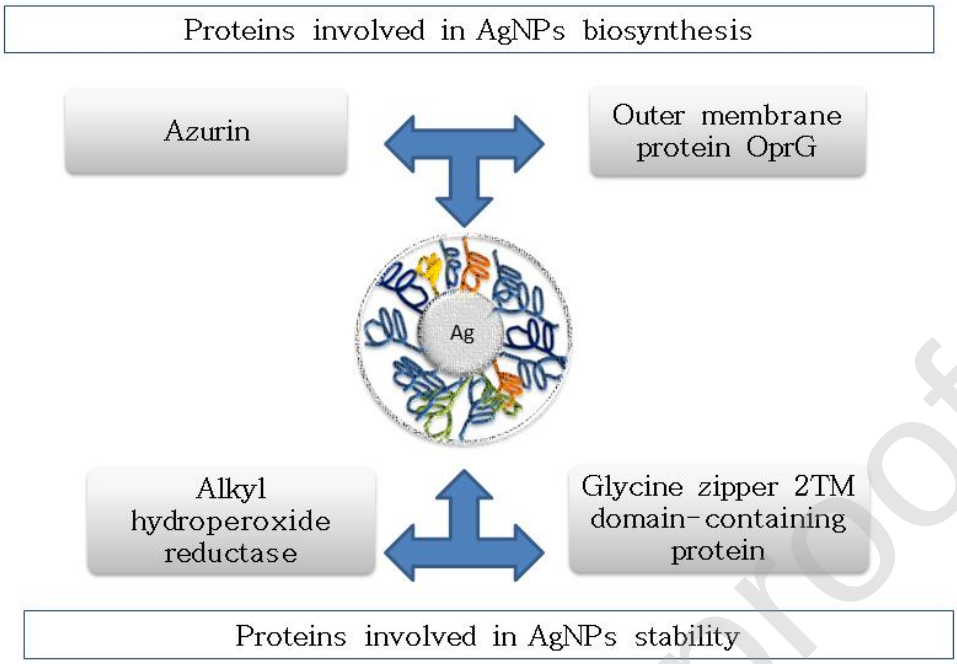
Graphical abstract



Highlights

- Silver nanoparticles (AgNPs) show higher biocompatibility compared to the chemically-synthesized ones.
- The TEM images showed that AgNPs were surrounded by an irregular coverage.
- The IR spectrum showed that this coverage was composed of carbohydrates and/or proteins.
- Different proteins were identified in the *capping* associated to biosynthesized AgNPs.

ABSTRACT

The development of environmental friendly new procedures for the synthesis of metallic nanoparticles is one of the main objectives of nanotechnology. Plants, algae, fungi and bacteria for the production of nanomaterials are viable alternatives due to their low cost, the absence of toxic waste production and their highly energy efficiency. It is also known that biosynthesized silver nanoparticles (AgNPs) show higher biocompatibility compared to the chemically-synthesized ones. In previous results, biosynthesized AgNPs were obtained from the supernatant of *Pseudomonas aeruginosa*, and they showed a bigger antimicrobial activity against different bacterial species compared to the chemically-synthesized ones. The aim of this work was to analyze the *capping* of biosynthesized AgNPs using techniques such as transmission electron microscopy (TEM), infrared spectroscopy (IR), and protein identification through mass spectrometry (MS) in order to identify the compounds responsible for their formation, stability and biocompatibility. The TEM images showed that AgNPs were surrounded by an irregular coverage. The IR spectrum showed that this coverage was composed of carbohydrates and/or proteins. Different proteins were identified in the *capping* associated to biosynthesized AgNPs. Some proteins seem to be important for their formation (Alkyl hydroperoxide reductase and Azurin) and stabilization (Outer membrane protein OprG and Glycine zipper 2TM domain-containing protein). The proteins identified with the capability to interact with some biomolecules can be responsible for the biocompatibility and may be responsible for the bigger antimicrobial activity than AgNPs have previously shown. These results are pioneers in the identification of proteins in the *capping* of biosynthesized AgNPs.

KEYWORDS: bacteria, silver nanoparticles, *capping*, proteins.

1. INTRODUCTION

Great advances in nanoscience had exposed the excellent physical and chemical properties of matter at nanometric size and its differences with the macro/micrometric state. The green synthesis of nanoparticles (NPs) consists in the use of plants, algae, fungi or bacteria for the production of low-cost, energy-efficient and non-toxic nanomaterials [1,2]. The biosynthesis of NPs has become an important object of study for nanotechnology, due to the great number of advantages that it offers. Chemical methods have some weaknesses that include the use of toxic solvents, the generation of dangerous by-products and the high consumption of energy [3]. When a particle decreases in size, a greater proportion of the atoms are present on its surface compared to the interior. These particular characteristics of nanoparticles improve the interaction with other molecules. The surface/volume ratio of the nanoparticles is big, which gives them an excellent capacity to adsorb substances. This property makes them good carriers of other molecules, such as chemical compounds, drugs, probes and proteins attached to the surface [3]. Biosynthesized NPs are more acceptable for medical applications because they offer biocompatibility superior to NPs produced by chemical methods [4]. This biocompatibility would be mediated by the biomolecules that act as natural stabilizers of NPs, preventing not only the aggregation over time but also giving them a particular additional stabilization [5]. The biomolecules that form part of the coverage would mediate the interaction with other biological molecules (proteins, sugars or even whole cells) and could improve the interaction with the pathogens involved[5]. This particular property could be responsible for the best antimicrobial activity of the NPs with respect to the NPs synthesized by other methods [1, 6-10] . However, there are no studies devoted to the identification of compounds such as proteins present in the *capping* of the

biosynthesized NPs.

In previous studies, our group reported a simple and green method for the biological synthesis of AgNPs using a cell-free supernatant of *Pseudomonas aeruginosa*. These biosynthesized AgNPs were stable, uniform, spherical and showed a great antimicrobial activity at picomolar levels against representative human Gram-positive and Gram-negative pathogens including multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii* and *Escherichia coli* [11].

The main objective of this work was to analyze the coverage of biosynthesized silver nanoparticles (AgNPs) to determine which components would be involved in the synthesis of the NPs and would be responsible for their stability and for the antimicrobial activity they previously showed.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Luria Bertani medium (MP Biomedicals, France), Mueller Hinton medium (Britania, Argentina). Silver nitrate ($AgNO_3$), potassium bromide (KBr), Phosphate Buffer Solution (PBS): sodium chloride (NaCl), potassium chloride (KCl), Sodium phosphate di-basic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4); Ethylenediaminetetraacetic acid (EDTA); Ethanol and Formaldehyde from Cicarelli (Argentina). Bradford Reagent (Bio-Rad). Bis-acrylamide and acrylamide, Bovine serum albumin (BSA), Tris, Glycine, Sodium dodecyl sulfate (SDS), Ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) from Sigma-Aldrich. Hydrochloric acid, Acetic acid, Sodium thiosulfate, Sodium acetate, and Sodium carbonate from Tetrahedron (Argentina).

2.2. Biosynthesis of AgNPs

In previous studies, a satisfactory methodology was developed to obtain stable nanoparticles with uniform size and shaping [11]. A culture of *Pseudomonas aeruginosa* ATCC 27853 was carried out in liquid LB medium at 37°C for 24 h. The culture was centrifuged at 10000 *xg* for the elimination of the biomass and the obtained supernatant was filtered. 10 mM silver nitrate solution (70:30 ratio) was added to one aliquot of the obtained supernatant. The resulting solution was incubated at 37 °C for 24 h with agitation and darkness. After the incubation time, the UV-vis absorption spectrum of the obtained solution was carried out, which indicated the formation of AgNPs by the appearance of surface plasmon resonance (SPR) at 412 nm.

2.3 Characterization by TEM

The morphological analysis of the AgNPs was carried out using a microscope at JEM-JEOL 1120 EXII operated at 80 kV. 10 µL of the biosynthesized samples were taken and placed on a grid for carbon-coated carbon microscopy. The samples were dried at room temperature and in darkness [12].

2.4 Infrared spectroscopy (IR)

Samples were prepared following the potassium bromide disk method (solid phase method) and then lyophilized. 50 mg of potassium bromide were added to 2 mg of the solid sample, and they were pulverized in an agate mortar, being careful with moisture absorption [13]. The mixture was compressed in a suitable mold for the formation of the IR discs. Not-uniform discs obtained were discarded for the analysis. The samples were

analyzed in an FT-IR team (Avatar 360).

2.5 Characterization of AgNPs corona

In addition to the results obtained by using TEM and IR, AgNPs corona composition was analyzed by identifying the proteins presents.

2.5.1 Sample Preparation

After incubation with Ag^+ ions, cell-free supernatant of *P. aeruginosa* used for the AgNPs biosynthesis was centrifuged for the AgNPs precipitation and the supernatant was removed. AgNPs were re-suspended in mQH_2O. Additionally, the proteins of the cell-free supernatant without the incubation with Ag^+ were also analyzed.

2.5.2 Electrophoresis

The total protein concentration of the supernatants was determined by the Bradford method, using BSA as reference (0-10 $\mu g\ mL^{-1}$) [14]. Proteins were concentrated by precipitation with cold acetone and were re-suspended in Urea-Thiourea-CHAPS buffer. 15 µg of proteins were placed into the wells. Finally, the electrophoresis under denaturing conditions (SDS-PAGE) in a gel prepared at 12% was performed at constant 180v for 2h [15]. The gel was stained by using a silver staining MS-compatible procedure. The visualized bands were excised from the gel and conserved in mQH_2O for the protein identification analysis.

2.5.3 Protein identification analysis

Samples were reduced with 20 mM DTT for 45 min at 56 °C and were alkylated with 20 mM Iodoacetamide for 45 min. Proteins were in-gel digested using sequencing grade Trypsin. The extraction of the peptides was carried out with acetonitrile. Samples

were lyophilized by Speed Vac and re-suspended with 10 µL of 0.1% Formic Acid.

Peptides were separated by nanoHPLC (EASY-nLC 1000, Thermo Scientific) and were analyzed by tandem mass spectrometry (spectrometer Q-Exactive, Thermo Scientific) with Orbitrap technology (nanoHPLC-ESI-MS/MS).

The bioinformatics analysis of the data obtained was performed with the Proteome Discoverer v1.4 software (Thermo Scientific), using the specific database for *P. aeruginosa*.

2.6. Statistical analysis

The assays were performed in triplicate. Data were expressed as means ± SD.

3. RESULTS

3.1 Synthesis and Characterization of AgNPs by TEM

The TEM technique was used to visualize the shape and size of the AgNPs obtained by *P. aeruginosa* supernatant. The AgNPs showed a homogeneous distribution in size and shape. They were spheroidal and the size was 25 nm ±8 nm. The TEM images revealed that AgNPs were surrounded by a corona, which was probably made up of components of the supernatant of the culture of *P. aeruginosa* (Figure 1).

3.2. Characterization of AgNPs by IR

Bacteria supernatant and AgNPs were both analyzed by IR. In the supernatant sample, peaks at 3,417 cm^{-1} were observed and they could correspond to the presence of amide groups (voltage vibrations of the N-H bond). Other peaks were also observed, such

as 1,663 cm^{-1} (vibrations of the link voltage C=0), 1,401 cm^{-1} (bending vibration of C-OH) and 1,074 cm^{-1} (vibration of the C-O-C bond in polysaccharide groups). The appearance of these characteristic peaks suggests the presence of carbohydrates and proteins in bacteria supernatant (Figure 2A).

In the AgNPs spectrum, some differences were noticed with respect to the supernatant sample. Some decreases and increases were observed in the peaks described previously (Figure 2B). The decrease of the peak at 3,417 cm^{-1} could be considered as the main factor that acts as a reducer in the synthesis reaction. Likewise, the increase of the peak at 1,401 cm^{-1} could be part of the stabilizing agent in AgNPs.

3.3 Characterization of AgNPs corona

In addition to the results obtained by using TEM and IR, AgNPs corona composition was analyzed by identifying the proteins present in AgNPs.

After the SDS-PAGE, eight bands were visualized in the gel: two bands were observed in the lane corresponding to the cell-free supernatant without the incubation with Ag^{+} ions, while six bands were observed in the lane corresponding to biosynthesized AgNPs (Figure 3). In addition, it could be seen that the concentration of some of the proteins was higher in the AgNPs sample (NP03 - 08). The increase observed in the AgNPs bands could be due to a greater affinity of proteins of *P. aeruginosa* supernatant by the AgNPs surface.

Table 1 show the proteins presents in the surface of AgNPs biosynthesized. From MS analysis of the excised bands, 20 proteins were identified. It is important to highlight

that most of the proteins were identified in AgNPs sample, while only one protein was identified from the crude supernatant sample. Unfortunately, proteins from bands Nº 2 and 5 could not be identified.

The proteins identified in bands corresponding to AgNPs sample were classified according to their function registered in the Uniprot database. Among proteins, we found outer membrane structural proteins, such as Outer membrane protein OprG and Glycine zipper 2TM domain-containing protein; proteins of interactions with biomolecules, namely Phospholipid-binding protein MlaC, PhoP/Q and low Mg^{2+} inducible outer membrane protein H1 (interaction with lipopolysaccharide), Uncharacterized protein PA1579 (lipid binding), Azurin (interaction with transition metal ions), cold-shock protein (interaction with nucleic acids), Bacteriohemerythrin (metal binding); proteins characteristics of response to oxidative and environmental stress, such as Alkyl hydroperoxide reductase subunit C and RNA-binding protein Hfq; and important proteins involved in metabolic reactions, e.g. Inorganic pyrophosphatase, Lipid A deacylase PagL, Pterin-4-alpha-carbinolamine dehydratase, Glycine cleavage system H protein 1 and YgdI/YgdR family lipoprotein.

4. DISCUSSION

The most important challenge in the synthesis processes is to obtain stable NPs with uniform size and shape. The chemical synthesis of NPs generally occurs under extreme conditions of pH or temperature, and the chemicals substances used can have a negative impact on health and the environment [16]. The use of microorganisms in the synthesis of

NPs is a relatively new promising research area, with a potential for considerable expansion. Biosynthesis has as main advantages, a lower use of energy and little environmental impact, compared to conventional methods [16-18]. Imtiyaz Hussain [19] summarizes the benefits of the biosynthesis like a low cost and high energy-efficiency process with absence of toxic waste or use of organic solvents. Currently, the precise mechanism of NP synthesis by biological agents is under study. This is because each type of agent reacts differently to the metals that lead to the formation of NPs [19].

There are many studies that support that biogenic NPs have a better antimicrobial activity compared to those of chemical origin. Sintubin *et al*. [20] demonstrated that the biosynthesized NPs had an antimicrobial activity 20 times bigger than those chemically synthesized. Botes and Cloete [21] propose that this is due to the fact that biological molecules not only give stability to NPs but also significantly increase the interaction with microorganisms, fostering their antimicrobial activity.

The biomolecules involved in the biosynthesis process give the AgNPs a differential stability due to the *capping* function it performs. One of the possibilities offered by biosynthesis is the use of fungi members. Some authors showed that the synthesis of NPs from fungi gives rise to AgNPs with excellent antibacterial activity. However, the main disadvantage is that the methodology involves numerous steps and longer time in which, in some cases, obtaining NPs can take up to 5 days of incubation [22-24]. The use of bacteria (in this case, the *P. aeruginosa* supernatant) as a route of production of AgNPs is more advantageous than other microorganisms, since the time of synthesis takes usually only a few hours [25-27].

Ahmed *et al*. [28] proposed the possible *capping* mechanism of the nanoparticles using FTIR studies in fungi. To determine the interaction between the biopolymer (Levan)

and NP, this group compared the FTIR spectra of Levan and stabilized AgNPs with this biopolymer. The IR peaks at 1650 cm^{-1} and 3400 cm^{-1}, which are characteristic of the carbonyl and hydroxyl groups respectively, were present in both the Levan AgNPs spectrum and the Levan stabilized, indicating the presence of this biopolymer around the particles. These results are in accordance with the IR analysis obtained in this work, where the carbonyl and hydroxyl groups are present in AgNPs and in the supernatant of *P. aeruginosa*. This indicates the participation of components of the supernatant of the bacteria in the formation of nanoparticles.

According to the literature, numerous studies have shown that the NPs stability can be achieved by the interaction of proteins on its surface, forming a complex called corona protein [29-32]. The initial corona that forms on the surface of the nanoparticle, the "hard corona" (HC), consists of proteins with high association rates. By contrast, the proteins that constitute the soft corona (SC) quickly exchanged the surrounding environment in short timescales [33]. The HC is considered to play an important role in the interaction of the nanoparticles with their surrounding cells since it is more stable and closely associated with the surface of the nanomaterial.

In thermodynamic terms, Walkey *et al*. explains the interaction of the nanoparticle with the protein corona. They affirm that the HC adsorbs onto the surface of NPs in a thermodynamically favorable manner with a large net binding energy of adsorption (ΔGads). This binding energy determines the stability of the protein–nanomaterial complex, as a consequence, proteins that adsorb with a large ΔGads have a low probability of desorption and tend to stay associated with the nanomaterial [34]. The proteins that adsorb with a small ΔGads, easily desorb and return to solution, as in the case of SC [34].

In other hand, a work published by Jain *et al*. [35], bands corresponding to two

proteins of 32 and 35 kDa were observed by SDS-PAGE. However, these proteins were not identified and the authors concluded that the proteins would have the function of reducing agents and *capping*. In the same line of research, Elumalai *et al.* [36] assumed that AgNPs synthesized using the coconut water were surrounded by some proteins and metabolites such as terpenoids and flavonoids, which are *capping* ligands of the nanoparticles.

To the best of our knowledge, there are no works devoted to the identification of the proteins which can be part of the corona surrounding the NPs synthetized by biological methods. In this work, the information obtained from the electrophoresis analysis was completed with the data acquired from the nanoHPLC-ESI-MS/MS studies followed by bioinformatics analysis to identify the proteins from the *P. aeruginosa* supernatant. Different proteins were identified in the corona associated to AgNPs biosynthesized. The presence of a bigger number of bands in the AgNPs sample could indicate that the proteins were concentrated around the nanoparticles and were precipitated after centrifugation. Therefore, we are not able to see these proteins in the crude supernatant sample, indicating that these proteins were present in very low concentration in the extracellular medium and the sensitivity of the silver staining method is not enough to reveal the bands of these proteins. Among the identified proteins, some proteins seem to be important for the formation, stabilization and interaction with some of the bacterial species tested in previous studies [11]. Some authors claim that the amount of proteins plays an important role in controlling the size and shape of the nanoparticles. Sotnikov *et al*. [37] asseverate that the interaction of macromolecules with nanoparticles depends on several factors, including the chemical nature of the adsorbed material, the composition of the medium, the pH, among others. The extracellular proteins also act as a *capping* agent stabilizing the particles [38].

Alkyl hydroperoxide reductase possesses a reductase function and it is one of the

proteins which could be responsible for the reduction of Ag^{+} ions into Ag^{0} in the AgNPs. Azurin is a protein with interactions to transition metal ions like silver and, at the same time, this protein had demonstrated to be capable of transfering electrons, both being responsible for the formation of AgNPs. The interaction between azurin and silver nanomaterials was studied by some authors, such as Martinolich *et al.* [39], who demonstrated that NP interactions impacted on protein structure, function, and modified the NP reactivity with implications for targeting, uptake, and cytotoxicity. Similarly, Freitas *et al.* [40] studied the alteration of the 10-40 nm Ag engineered nanomaterial (ENM) surface by adsorbed Azurin. The results showed an adventitious protein-ENM redox reaction that altered both metal ENM and protein reactivity [40]. Xie *et al*. [41] used an algal extract solution to produce single-crystalline gold nanoplates and demonstrated the presence of proteins involved in the synthesis of the gold nanoplates. A protein of 28 kDa called gold shape-directing protein (GSP) was isolated and purified by RP-HPLC and was found to possess both reduction and shape-directing functionalities. In a review published in 2016, the authors highlighted the importance of microbial cellular and extracellular oxidoreductase enzymes in the reduction processes for the formation of AgNPs. Additionally, these authors concluded that those reducing agents or other constituents present in the cells act as stabilizing and *capping* agents [42].

Some of the proteins that can be involved in the stability of the NPs are the identified structural proteins like Outer membrane protein OprG and Glycine zipper 2TM domain-containing protein. Likewise, the proteins identified with capability to interact with some biomolecules can be responsible for the biocompatibility and may be responsible for the bigger antimicrobial activity that biosynthesized AgNPs have previously shown compared to the chemically-synthesized AgNPs [11]. Among these proteins, we can

mention the following proteins: cold-shock protein, uncharacterized protein PA1579, Phospholipid-binding protein MlaC and PhoP/Q and low Mg^{2+} inducible outer membrane protein H1.

5. CONCLUSION

These results pioneer the identification of proteins in the *capping* of biosynthesized AgNPs. Some proteins seem to be important for their formation (Alkyl hydroperoxide reductase and Azurin) and stabilization (Outer membrane protein OprG and Glycine zipper 2TM domain-containing protein). The proteins identified with the capability to interact with some biomolecules can be responsible for the biocompatibility and may be responsible for the bigger antimicrobial activity of AgNPs biosynthesized extracellularly by *P. aeruginosa.*

This is of great interest not only because it allows understand the biogenic synthesis of the AgNPs obtained from the supernatant of the bacteria, but also highlights because it provides relevant information in the identification of those proteins that make up the corona around the AgNPs. It is very important to know the nature of the protein corona in biogenic NPs since it represents the "way in which the cells see the particle" [43].

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CAPTIONS

FIGURE 1. TEM image of biosynthesized AgNPs: A) scale 50 nm 500,000X B) scale 200 nm 150,000X.

FIGURE 1. Quinteros *et al.*

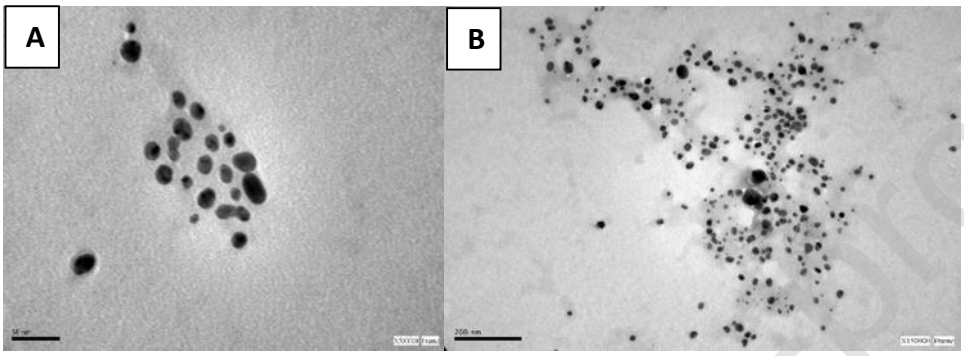

FIGURE 2. (A) IR spectrum of *P. aeruginosa* supernatant and (B**)** IR spectrum of AgNPs.

FIGURE 2. Quinteros *et al.*

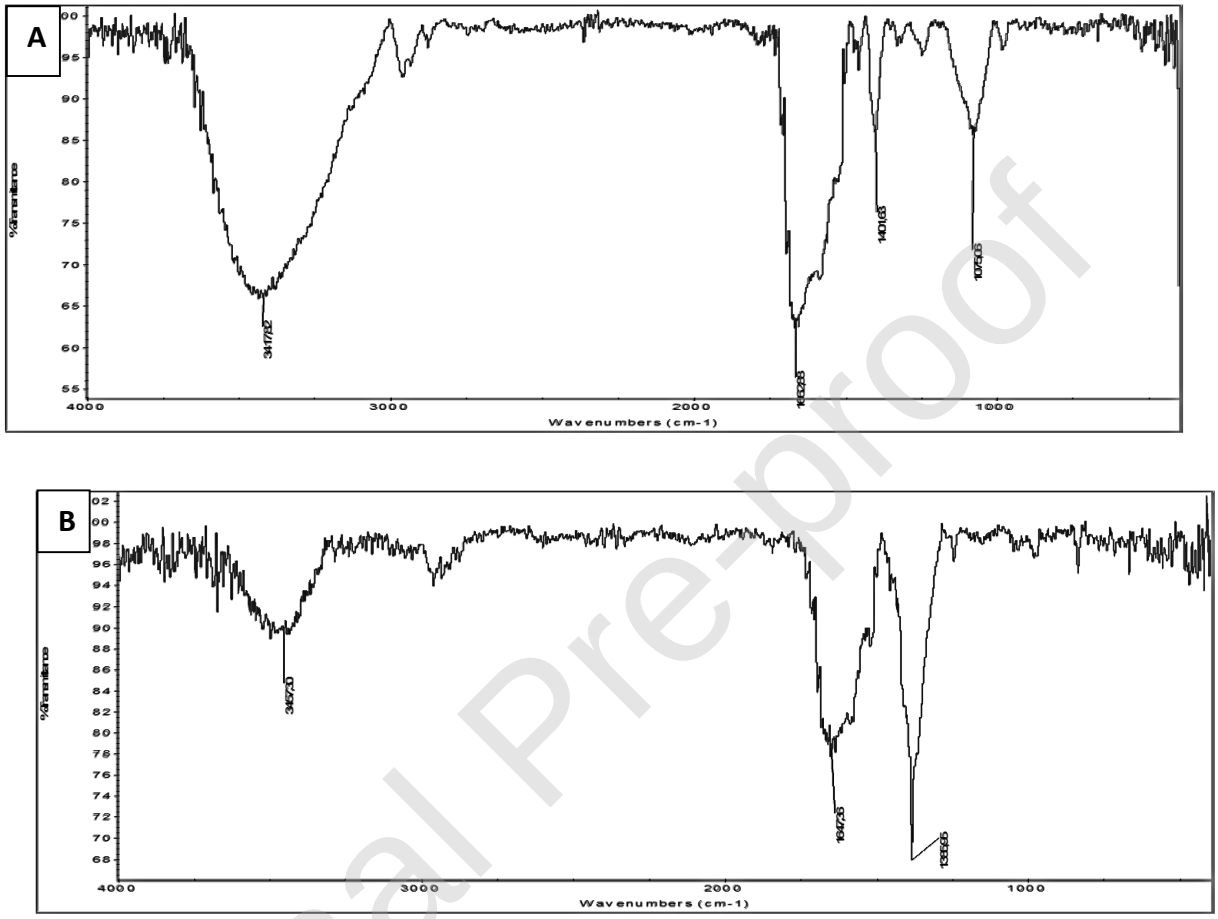

FIGURE 3. SDS-PAGE gel obtained from *P. aeruginosa* supernatant (lane 1) and AgNPs sample (lane 2).

FIGURE 3. Quinteros *et al.*

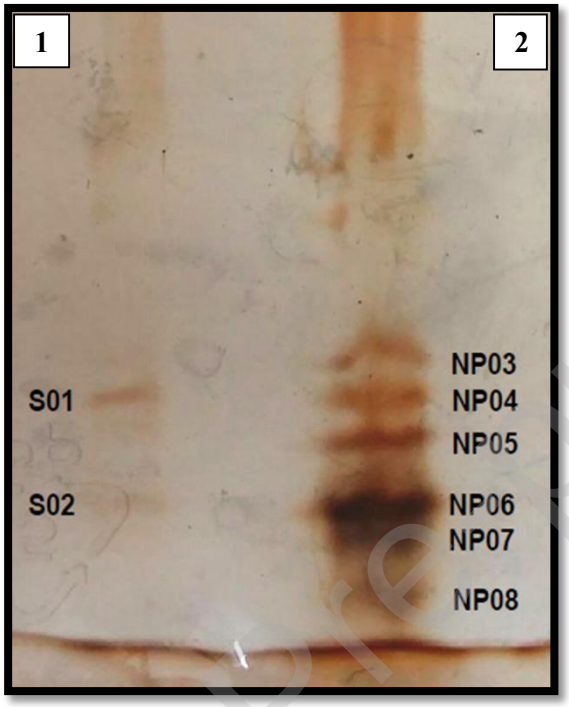

Table 1. Proteins identified from excised bands of the gel applying nanoHPLC coupled to MS/MS analysis.

Table 1. Quinteros *et al.*

Band number	Accession	Description	Coverage (%)	Peptides	MW [kDa]	calculated pI	Score Sequest HT
S01	Q9I690	UPF0312 protein PA0423	4,71	1	20,8	6,55	2,04
S02		**Unidentified**					
NP03	Q9HWW1	Outer membrane protein OprG	12,93	2	25,2	5,12	4,45
	Q9HVW4	Phospholipid-binding protein MlaC	11,63	2	23,7	9,14	5,42
NP04	G3XD11	PhoP/Q and low Mg2+ inducible outer membrane protein H1	27,00	5	21,6	9,03	21,68
	Q9I690	UPF0312 protein PA0423	25,65	4	20,8	6,55	14,31
	Q9HWZ6	Inorganic pyrophosphatase	6,29	1	19,4	5,25	4,57
	Q9I6Z3	Alkyl hydroperoxide reductase subunit C	5,35	1	20,5	6,33	2,16
	Q9I3D8	Uncharacterized protein PA1579	10,89	2	22,1	7,94	4,1
NP05		**Unidentified**					
NP06	P00282	Azurin	12,16	2	16	6,92	8,82

	Q9HVD1	Lipid A deacylase PagL	8,67	1	18,4	6,3	3,37
	Q9I4S1	Glycine zipper 2TM domain-containing protein	20,78	2	15,6	9,61	8,04
NP07	Q9I6G2	UPF0339 protein PA0329	10,00	1	11,8	9,28	2,51
	P43335	Pterin-4-alpha-carbinolamine dehydratase	13,56	1	13,3	6,4	2,51
	Q9I136	Glycine cleavage system H protein 1	7,09	1	13,8	4,3	2,23
	Q9HTL9	RidA family protein	39,68	4	13,6	5,15	16,73
	Q9HY15	Uncharacterized protein	11,76	2	15	8,65	4,18
NP08	Q9I4H8	Probable cold-shock protein	44,93	3	7,7	7,42	13,58
	Q9I352	Bacteriohemerythrin	7,84	1	17,8	6,21	1,95
	Q9HUM0	RNA-binding protein Hfq	15,85	1	9,1	9,52	1,94
	Q9I129	YgdI/YgdR family lipoprotein	12,33	1	8,1	8,18	2,22