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Production and Characterization of Silver Nanoparticles in Cultures of the Cyanobacterium *A. platensis* (Spirulina)

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The increasing application of Silver nanoparticles in biologically-relevant areas (including production of textiles, cosmetics, and biomedical devices), where their presence provides a continuous release of silver ions to provide protection against bacteria and other unwanted microbial contaminants urges adoption of intrinsically biologically safe production processes. Various species of cyanobacteria and algae have been known to absorb and take up heavy metal ions. This capability is shown also by Arthrospira platensis (Spirulina), a cyanobacterium that enjoys the Generally Recognised as Safe (GRAS) status and has been declared by WHO one among the greatest superfood. The present study aims at investigating the coupling between the recognised beneficial effects of Spirulina biomass to the antimicrobial activity of Ag nanoparticles (SNPs).
In this work, Spirulina was grown in sequential cultures targeting biomass production and nanoparticle formation. The cultures were conditioned during their lifetime in order to assess the effect of pH and added polysaccharides on the size and on the stability of the obtained SNPs. The synthesized SNPs were characterized as to their size and stability (Nanosizer), composition (XRD) and structural aspect (Scanning Electron Microscope).

1. Introduction

The synthesis of nanoparticles (NPs) with chemical or physical methods has drawbacks, being costly and producing materials superficially contaminated by adsorbed toxic chemicals, therefore potentially cheaper and intrinsically safer alternatives are investigated in the biologic synthesis domain. Biologic extracts can be a valid replacement to chemical reductants in the design of bio-based metal ion reduction protocols (Prabhu et al. 2012). Microorganisms secrete a large amount of enzymes capable of hydrolyzing metals and thus bring about the enzymatic reduction of metals ions (Rai et al. 2009). Biological reduction has a fast kinetics already at room temperature and pressure and requires less chemicals than their chemical counterparts, thus helping create a greener nanosynthesis approach (Ingle et al. 2008). Intracellular microbial nanoparticles are produced thanks to the electrostatic interaction between the negatively charged cell wall and the positively charged metal ions, thus operating a reduction. The enzymes present between the cell wall reduce the metal ions to small size nanoparticles that diffuse through the cell wall. Extracellular biosynthesis is mediated by secreted enzymes, nitrate-reductase, simplifying the NP recovery (Mukherjee et al. 2001, Gade et al. 2008). Many factors (e.g. temperature and pH) could influence the nanoparticle synthesis and their shape and size. Gericke and Pinches (2006) obtained different shape morphologies (triangle, hexagons, spheres, and rods) by modulating the pH of reaction mixture to 3, 5, 7 and 9. Riddin et al. (2006) also showed that less amount of nanoparticles are synthesized at 65 °C than at 35 °C. The ability of marine organism to complex metal ions and synthesize nanoparticles was reviewed by Asmathunisha (2013). Target of this study is the biosynthesis of Ag nanoparticles by the cyanobacterium *A platensis* (Spirulina), which was shown to be very interesting for the synthesis of inorganic nanoparticles thanks to its fast growth rate and its safety (Govindaraju, 2008). The intrinsical safety of *A. platensis*, which is a GRAS microorganism and has been declared by WHO one among the greatest superfood being in itself a wealth of completeness and a treasure of desirable properties addressing nutrition, and prevention of and therapy against diseases; furthermore *A. platensis* is able to

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produce Extracellular Polysaccharides (EPS); The standard applications of microbial EPS are as food coatings, emulsifying and gelling agents, flocculants, hydrating agents but it shows a potential antioxidant action (Sed et al. 2017). Applications of Ag nanoparticles are very wide: they have been used as antibacterial agents in biomedical and pharmaceutical industry, such as disinfection of medical instruments, for storing food, textile coatings and environmental applications such as air filters or water treatment. The present work aims to investigate the action of pH and EPS on SNPs synthesis, size, structure and stability when *A. platensis* is used as the synthesis agent.

2. Materials and Methods

2.1 Growth

The cyanobacterium *Arthrospira platensis* (courtesy of CNR-ISE Sesto Fiorentino) on Zarrouk agar plate, was inoculated in the same liquid culture medium, in 6 cm diameter cylindrical glass tube and grown until it reached the mass concentration of 3.5 g/L. Then in was transferred in 5000 mL cylindrical PCA photobioreactor, with a diameter of 12 cm and grown in axenic conditions, feeding it with filtered and humidified air (flow rate $130*10^3 Nm^3/h$). 16 hours photoperiod of light, provided by cold white fluorescent lamps (400-700 nm, 865 K, 32 W, 80 mmol photons m^2 s^{-1}), was followed by a period of darkness equal to 8 hours. The temperature was maintained constant at 28 ±1 °C. According to Lambert-Beer law, microalgae concentration is directly proportional to absorbance. The cellular density was correlated with the absorbance measured at a wavelength of 690 nm, corresponding to the absorption peak of chlorophyll by means of a spectrophotometer (UV1800PC by Shanghai Mapada Instruments Co., Ltd).
At the stationary phase of growth, 200 mL of biomass was collected, centrifuged, the exhausted culture medium was stored, and the cellular pellet was washed twice with distilled water. Afterwards, the collected algae were weighed, transferred in a moisture-free glass flask and dried in a vacuum oven at 110 °C overnight. The mass of dried microalgae was calculated by subtracting the flask tare from the recorded gross weight (Figure 1).

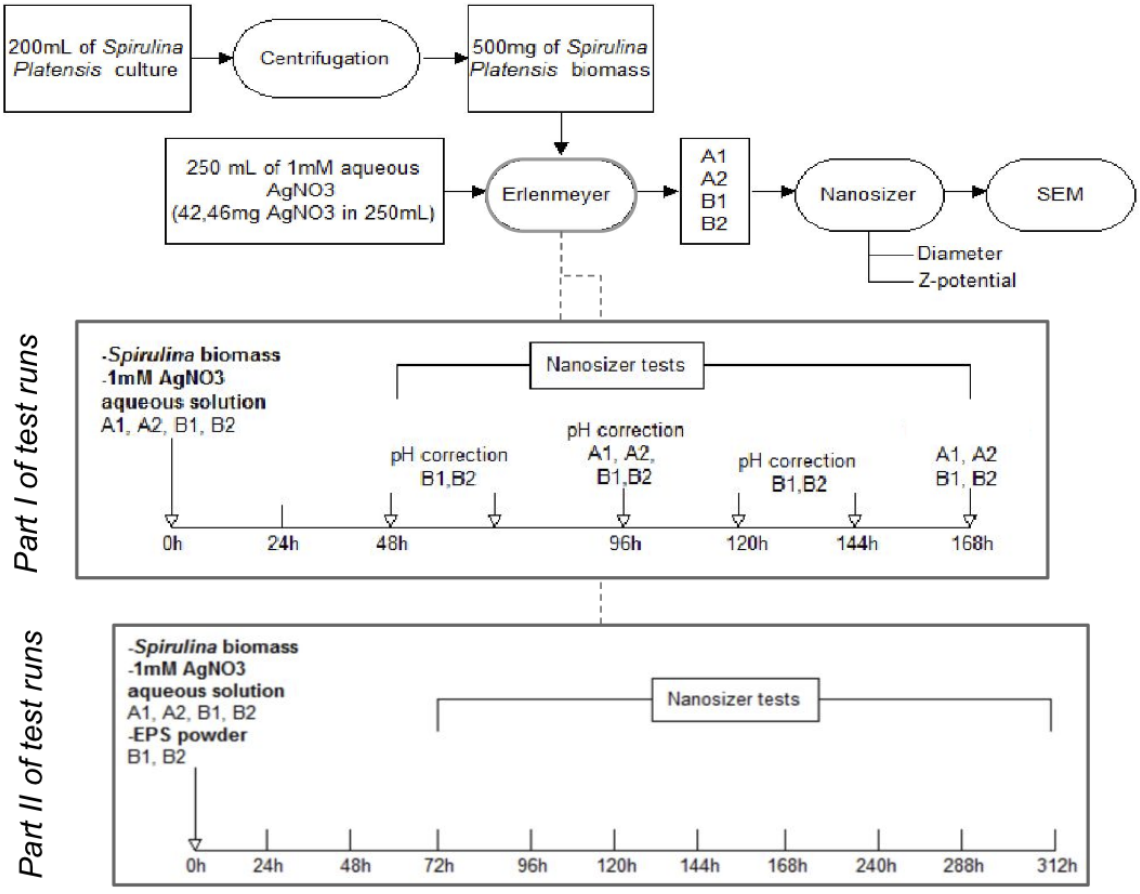

Figure 1: Overall sketch of performed experiments

2.2 Nanoparticles production

To obtain silver nanoparticles, 500 mg of *A. platensis* centrifuged biomass were taken and added to a 10^{-3} M aqueous solution of $AgNO_3$ at room temperature. No further conditioning was performed in Part I experiments, while EPS obtained from exhausted *A. platensis* media according to the protocol described in Sed et al. (2017) was added in Part II ones. The suspension was shaken in an orbital shaker. The culture was periodically sampled for characterisation and corrections according to the specific protocol. The tests were executed in two parts. In part I experiments, a correction of pH with HNO_3 after 96 and 168 hours (runs I-a) and at times 48, 72, 96, 120, 144, 168 hours (runs I-b) was performed; in part II experiments, pH was never adjusted along the culturing process but particles formation and growth conditions in the presence of *A. platensis* exopolysaccharides were investigated against the control (no added EPS).

2.3 Ag nanoparticle characterization

The size distribution of the Ag nanoparticles was measured by dynamic light scattering (DLS) (Brookhaven). This instrument was also used to measure the Z-potential of all the produced nanoparticles suspensions. Quantitative elemental analysis was performed by Energy Dispersive x-Ray Spectroscopy (by Bruker). Scanning electron microscopy (SEM) carried out by an Auriga Zeiss instrument was used to observe the overall morphology of the nanoparticles.

3. Results and discussion

During nanoparticles synthesis cultures by *A. platensis*, pH tends to naturally evolve toward alkalinity. The *A. platensis* cultures in this study are not aimed at thriving, but reducing Ag^+ ions to metallic silver. In the *production* medium, that only contains silver nitrate beyond the suspended cyanobacterium itself, pH tends to decay in a biomass-regulated manner (Figures 2A to 2D), always staying well above neutrality. When pH was adjusted down to 6, to attain the synthesis conditions used by Govindaraju et al. (2008), it spontaneously returned to a high value. This spontaneous regulation effect is apparently not due to buffering components contained in the medium, such as exopolysaccharides, which were not included in the formulation during phase I of the study. In all cases, the measured particles size grows beyond 10 nm but undergoes an oscillation (an increase up to about 30 nm, followed by a decrease) during the allotted culturing time (Figures 2A and 2C). In the cultures that underwent infrequent regulation the most frequently recorded size value during the culture lifetime is 10 nm (in those that underwent frequent pH regulation the most frequent size was slightly higher, 15 nm). The recorded zeta potential, which provides an indication of suspension stability, varies during the described pair of cultures in a similar manner, with an initial upward ramp touching, but never remaining inside the unstable range ($|z_{pot}| < 30$ mV), then rapidly falling to values characteristic of intrinsically stable suspensions (down to -60 mV). It can be questioned whether the largest size is actually due to individual nanoparticles, since a rapid breakdown is unlikely. The evolution of particles to (unstable) larger sizes may therefore be attributed more to agglomerated, rather than individually suspended particles.

During the second part of the experiments, the effect of added exopolysaccharides was investigated to assess whether it is capable of stabilising the nanoparticle suspension and production cultures were monitored for 300 h. The zeta potential time profile of EPS-conditioned cultures appears slightly stabler than that of unconditioned ones (Figure 2F). Both production cultures exhibit an initial jump of particle size (the first 150 h of culture), reaching values in excess of 100 nm and then falling down to the order of a few tens of nm (Figure 2E). Size appears to be slightly smaller for EPS-conditioned cultures than it is for unconditioned ones. While similar trends appear with and without added EPS both for size and stability, by looking at the gray lines indicating the (average - standard deviation; average + standard deviation) interval, it can be concluded that significance is scarce. As far as the zeta potential is concerned, the slight difference between the two distinct and regular time profiles lies between the (average - standard deviation; average + standard deviation) interval drawn, which means that it is scarcely significant. The same can be concluded for size.

The formed nanoparticles have been imaged by Scanning Electron Microscopy (Figure 3A) and the presence of silver nanoparticles surrounding the trichomes during production is confirmed by Energy Dispersive x-Ray Spectroscopy. Figure 3B shows trichomes covered by nanoparticles, and the actual position of silver within the rectangular detailed analytical frame is given by Figure 3C. Finally, the Elemental analysis of said detailed analytical frame, confirming silver presence, is shown in Figure 3D. Therefore the observed phenomena are indeed characteristic and significant, and include the formation of silver nanoparticles, the formation and the subsequent disaggregation of nanoparticle clumps, but the effect of adding EPS on either stability and size appear scarcely significant.

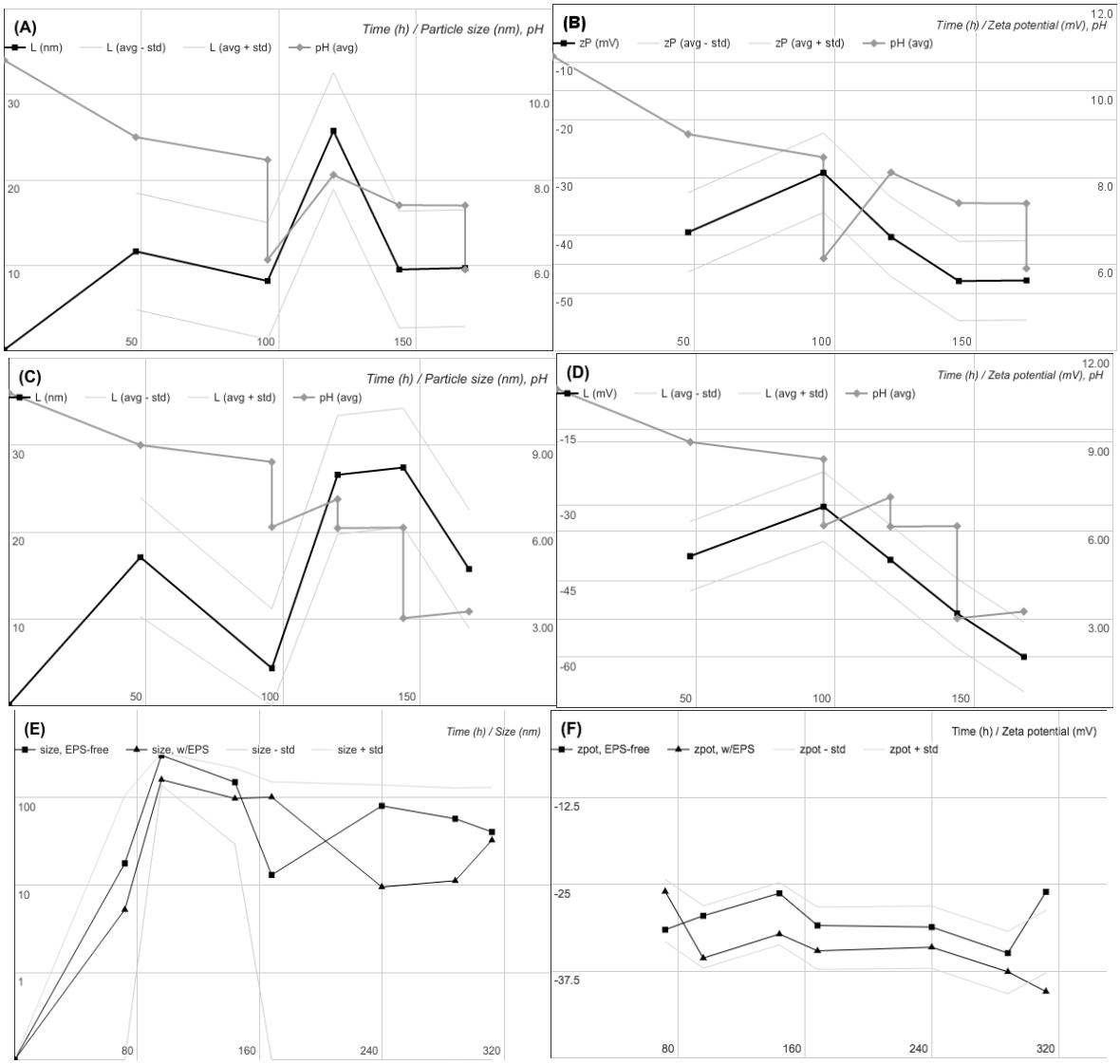

Figure 2: pH control and EPS role in time-dependent nanoparticle size (A-C-E) and stability (B-D-F). A-B-C-D: controlled-pH, EPS-free experiments: A-B at 96 h and 168 h, C-D at 48, 72, 96, 120, 144 and 168 h. E-F: uncontrolled pH experiments. Axis meaning and units are given as x-quantity (x-unit) / left-y-quantity (left-y-unit), right-y-quantity at the upper right of each chart. Upper and lower gray lines in each chart indicate confidence intervals at ±1 standard deviations calculated on pooled variance (variance pooling span: A-B-C-D: across replicates; E-F: across replicates and culturing modes).

EPS has been suggested to have a key role in silver ion reduction by Patel et al., 2015, although they demonstrate it for a *Scenedesmus* strain and, rather, speculate about the role of the C-phycocyanin protein for *A. platensis*-mediated formation of silver nanocrystals. The time profile of silver nanocrystals size during our Phase II experiments for runs carried out with a supply of exogenous EPS generally lies below that obtained without it. This fact is indeed compatible with the larger rate of nanocrystals production expected during the runs carried out with a supply of exogenous EPS because of the higher number of silver crystals formation sites competing for the same overall supply of silver ions.

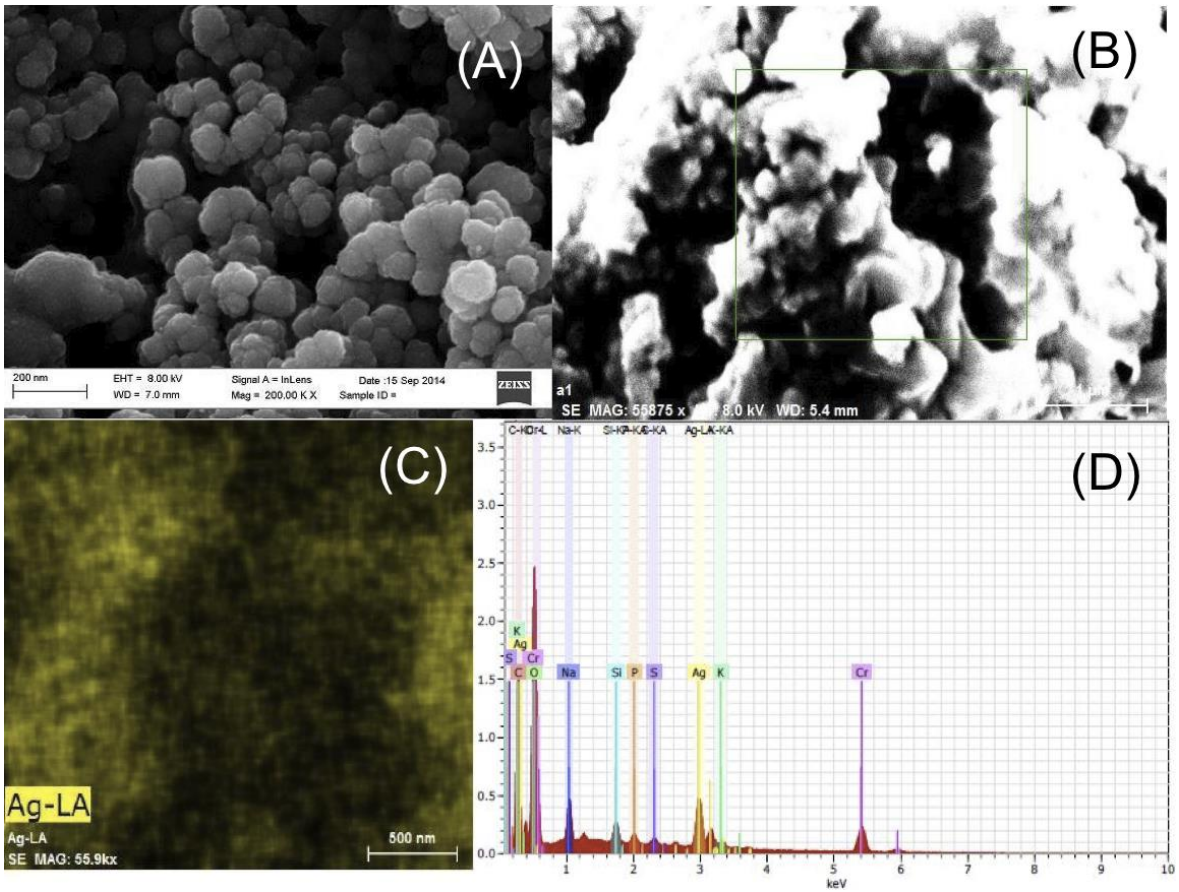

Figure 3: (A) SEM image of Ag nanocrystals obtained from EPS-free nanoparticle formation media; (B) XRD image of A. platensis *trichomes covered by Ag nanocrystals, and detail analysis area (green square); (C) Location of Ag in the analysis area of the (B) sub-image; (D) Elemental analysis in the analysis area of the (B) sub-image.*

4. Conclusions

The performed investigation showed that silver nanoparticles can be formed by placing *A. platensis* biomass in production cultures containing only silver nitrate. Nanoparticles are observed after 48 h. Observed size evolves over time. pH correction has a definite effect: cultures where pH is left uncorrected pH exhibit greater size than cultures where pH is corrected over time. Size was found to generally range between 4-42 nm. Added exopolysaccharides seemingly have a small, although statistically scarcely significant, effect. XRD analysis proved that silver nanocrystals are actually present and SEM observation showed very definite and regular crystals are formed.

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