Green Synthesis, Long-Term Stability and Toxicity of Colloidal Ag Nanoparticles

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Abstract: - The influence of algae life cycle and the solutions pH on the green synthesis of colloidal Ag nanoparticles (AgNPs) as well as effect of different storage conditions on AgNPs long-term stability, and toxicity was investigated. The extracts of *Parachlorella kessleri* algae were used for synthesis of silver nanoparticles. The results confirmed formation of polyhedron and/or near polyhedron AgNPs, (~5-60 nm in diameter). The synthesis rate, size, stability and an amount of AgNPs in solution can be influenced by the age of algae, and depend on the pH of solution. The best results were obtained using extract of tree weeks old algae. AgNPs formed in solutions of higher pH (8 and 10) are fine, with narrow size interval and stabile. Nanoparticles formed in solutions of low pH (2, 4 and 6) started to lose their stability on 10th day of experiment, and the particle size interval was wide. The long-term stability of AgNPs can be influenced by light and temperature conditions. The most significant stability loss was observed at day light and room temperature (21°C). After 200-days significant amount of agglomerated particles settled on the bottom of the Erlenmeyer flask. AgNPs stored at dark and room temperature showed better long-term stability, weak particles agglomeration was observed. AgNPs stored at dark and at temperature 5°C showed the best long-term stability. Such AgNPs remained spherical, fine (5-20 nm), with narrow size interval and stable (no agglomeration) even after more than six months. The AgNPs had strong toxic action against algae and microorganisms found in indoor air, the storage time did not influence the antibacterial effect of AgNPs.

Key-Words: - Silver nanoparticles; Green Synthesis; Stability; Toxicity; TEM.

1 Introduction

In recent years a significant amount of attention was given to silver nanoparticles due to their possible applications in different fields (sensors, bandages, water filtration, catalysis, commercial products and antimicrobial surface coatings) [1-3]. Discoveries in the past decades have demonstrated that the preparation of uniform nanoparticles with narrow particle size distribution is crucial for their utilization; the electromagnetic, optical, and catalytic properties of nanocrystals are strongly influenced by their shape and size [4-6].

Therefore, lots of physical (vapour deposition, molecular beam epitaxy etc.) and chemical methods focused on AgNPs formation have been developed [7-10]. Physical methods are efficient but they require expensive and complicated technologies. Chemical methods are more affordable but they are not environment friendly. Therefore, there is a need to support different activities in searching for new synthesis routes that allow better control of shape and size distribution during nanoparticle formation, and are environment friendly, and low costs.

Considering the promises shown in the green synthesis many different kinds of plant and fungi extracts, bacteria, and algae have been used as biological materials for the nanoparticles synthesis [11-18]. Reducing sugars, ketones/aldehydes, amine groups, water soluble heterocyclic compounds and proteins naturally present in such materials are proposed to play a key role not only for the reduction of silver ions but also for AgNPs stabilization. Furthermore, overall material and energy consumptions in biological methods are extremely lower, offering a low-cost green alternative [10]. Nowadays biochemical pathways responsible for the production of metal NPs using biological materials are well researched. However, there are a few data about biosynthesis of AgNPs by utilizing the algae, about the influence of algae's life cycle and pH of solution on colloidal AgNPs synthesis. Also the long-term stability of AgNPs

prepared by green synthesis is still not researched in detail, so lots of questions are not answered.

In the present study, the extract of *Parachlorella kessleri* algae for the synthesis of AgNPs was used. The aim of the study was to investigate:

- the impact of culture age (1, 2, 3 and 4 weeks old algae) and pH (2, 4, 6, 8 and 10) on AgNPs synthesis,
- the relationship between the solutions pH and the major characteristics of nanoparticles (size, size distribution and morphology),
- the impact of storage conditions (exposure to light or dark at different temperatures) on long-term stability of AgNPs,
- the influence of time on AgNPs toxicity.

Since knowledge in the field of AgNPs biosynthesis and long-term stability are not well known, the results reported in this study will shed light on entire AgNPs lifecycle. This preliminary study might result in new knowledge about the process which potentially occurs in natural ecosystems.

2 Materials and Methods

The algae *Parachlorella kessleri* were used for synthesis of colloidal Ag nanoparticles. The algae were cultivated in Petri dishes at the ambient temperature. Nutrient medium consisted of 2% agar and Millieu Bristol nutrient solution. After cultivation the algae were collected and treated by boiling in water bath for 15 min and centrifuging at 3000 rpm for 15 min. The liquid phases obtained after centrifugation was removed and transferred into four Erlenmeyer flasks containing 0.92 mM $AgNO_3$ solution (concentration of Ag 100 mg/l). A series of experiments were carried out to evaluate the effect of algae age, pH and storage conditions on AgNPs formation and stability, and to evaluate the influence of time on AgNPs toxicity:

1. the influence of algae age on AgNPs synthesis:
   - the algae were cultivated in Petri dishes for 1, 2, 3 and 4 weeks and subsequently treated as was mentioned above. The cells amount used for extracts preparation was determined by automatic cell counter and was the same for all extracts. Prepared solutions with different algal extracts were labelled as follows: one week old algae extract – *1wE*; two weeks – *2wE*; three weeks - *3wE*; four weeks - *4wE*. The solutions were left for 24 hours and the AgNPs formation was monitored by measuring the UV-vis spectra. Subsequently two solutions (with the worst and the best results) were chosen and left another 3 days at room temperature,
2. the influence of pH on green synthesis:
   - AgNPs were prepared using three week old algae by method mentioned above. The pH values (2, 4, 6, 8 and 10) of solutions were adjusted by 10% HNO_3 or 10% NaOH respectively. All experiments were carried out in triplicates. After 24 hours (1st day) the sample of each solution was taken and the absorbance was measured. The procedure was repeated on the 3rd, 7th, 10th, 14th, 21st and 28th day,
3. the long-term stability evaluation:
   - AgNPs were prepared using three week old algae (pH of solution was ~8) by method mentioned above. The AgNPs solutions were divided into three Erlenmeyer flasks and stored (200 days) as follows: at room temperature (21°C) in daylight; at room temperature in dark; and in dark at 5°C (in refrigerator),
4. the impact of storage conditions and time on toxicity of AgNPs:
   - the antibacterial activity of 4-day AgNPs and 300-day AgNPs against *Parachlorella kessleri* algae and fungus/molds presented in the indoor air was compared.

The AgNPs was monitored by measuring the UV-vis spectra of the solutions in 10-mm optical-path-length quartz semimicrocuvettes (UNICAM UV/vis Spectrometer UV4) and Atomic Absorption Spectroscopy (AAS). The size and morphology of the nanoparticles were studied by means of a Transmission Electron Microscope (JEOL model JEM-2000FX microscope operated at an accelerating voltage of 200 kV). The EVE™ automatic cell counter was used to obtain *Parachlorella kessleri* exact cell count.

3 Results and discussion

3.1 The influence of algae age on AgNPs synthesis

All organisms, from little one cell algae to moss, go during their life through a number of biological phases. Therefore, the composition and amount of trace elements, enzymes, bioflavonoids, lipoid acids, vitamins, amino acids, proteins, as well as plant substances and fatty acids changed with time. The contribution and ability of culture to create the AgNPs logically changed during their life. The extracts prepared from algae with different age (1, 2,

3 and 4 weeks of cultivation) was used for comparing their ability to form AgNPs.

Addition of the green algae extracts to $AgNO_3$ solutions led to changes of solutions colour, Fig.1. After 24 hours the colours changed in dependence of the extracts type from light brown (*1wE*) to dark brown (*4wE*) what confirmed the presence of AgNPs in all experimental solutions. The colour change is the result of the radiation absorption in the visible region of the electromagnetic spectrum (380 – 450 nm) due to the localised surface plasmon of AgNPs [19-21].

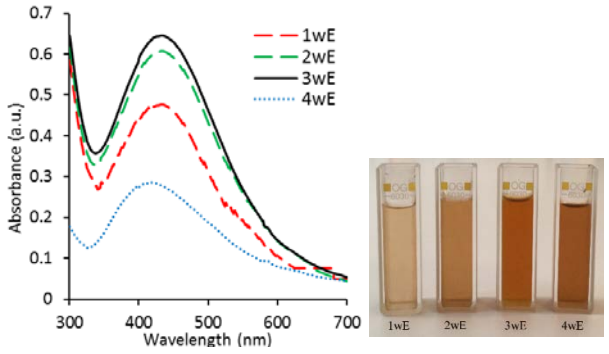


Fig.1. UV-vis absorption spectra and the solutions colour of the AgNPs (after 24 hours).

UV-vis spectra on Fig.1 show surface plasmon resonance (SPR) bands of AgNPs. Depending on the SPR bands shape it is possible to assume uniformity and size of the AgNPs shape [21]. Small spherical nanoparticles exhibit a single, symmetrical SPR band, whereas large and/or different shaped particles reveal two or three peaks [22].

It is clear that regardless of culture age is possible to prepare AgNPs. Based on the SPR bands shape and absorbance values, Fig.1, it is possible to say that the nanoparticles prepared by *1wE*, *2wE* and *3wE* extracts are symmetric with narrow interval of size distribution, but using of *2wE* and *3wE* extracts is most efficient. This two extracts gave symmetric and narrow SPR bands with the highest values of absorbance. The weaker SPR band symmetry of nanoparticles prepared by *4wE* extract indicates larger particles with wider size distribution and in comparison to above mentioned SPR bands the absorbance value is the lowest. According to Villanueva-Ibáñez et al. [28] the intensity of the UV–vis absorption also increases as the concentration of the synthesized NPs increases.

For further observation and for comparison of AgNPs behaviour during the time the best and the worse results (*3wE* and *4wE* solutions) were chosen. To increase the amount of NPs in solution they were left for another three days at room temperature exposed to light. The SPR bands of these AgNPs measured on the 1st, 2nd and 4th day of experiment are shown in Fig.2.

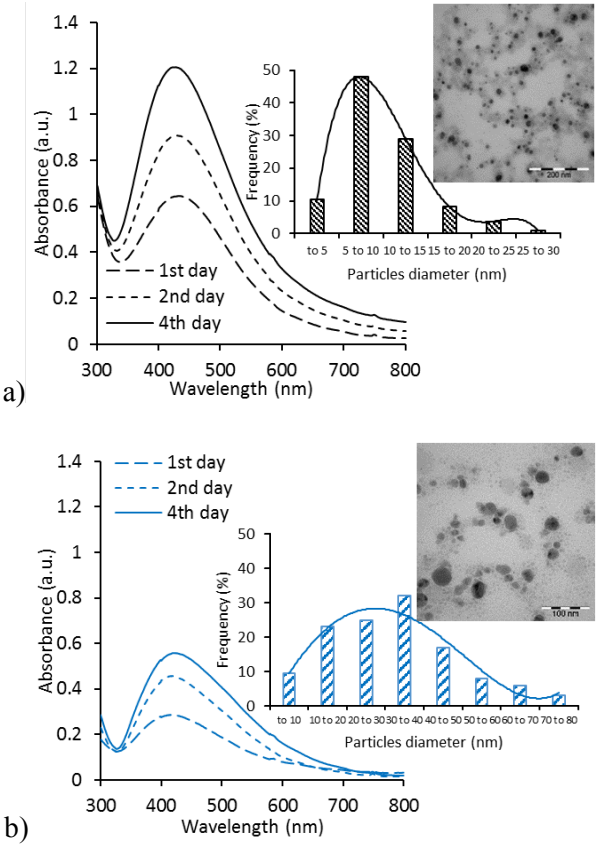


Fig.2. UV-vis absorption spectra of the AgNPs prepared by *3wE* (a) and *4wE* (b) extracts. The insets show the size distribution and TEM images of AgNPs on 4th day of experiment.

The UV-vis spectroscopy, Fig.2a, show symmetric SPR bands with a maximum wavelength at app. 428 nm (typical for Ag nanoparticles). The increasing of absorbance intensity with time indicates that Ag^+ ions are still presented in solution [23]. The TEM image of AgNPs formed on 4th day, Fig.2a, clearly confirmed the formation of fine, spherical AgNPs surrounded by a thin layer of organic material which is characteristic of AgNPs prepared in plant extracts. Particle size histogram revealed that more than 90% of AgNPs are down to 20 nm in diameter.

The SPR bands of AgNPs prepared by *4wE* extract, Fig.2b, show SRP bands with weaker symmetry, the shoulder become apparent and slight red shift of wavelength is evident. Nanoparticle size

histogram (on 4th day of experiment) shows wider interval of AgNPs size.

It is clear that the algae life cycle is important for AgNPs synthesis. Hence silver nanoparticles were successfully prepared using all types of algae *Parachlorella kessleri* extracts, the best results (the highest rate of nanoparticle production, nearer interval of size distribution and the highest concentration of AgNPs in solution) were obtained using three weeks old algae extract (*3wE*). But there are more possibilities to influence AgNPs synthesis. The major characteristics of nanoparticles (size, morphology, size distribution and stability) can be influenced by the solutions pH.

3.2 The influence of pH on green synthesis

SPR bands of experimental nanoparticles prepared in solutions with different pH (2, 4, 6, 8 a nd 10), measured on the 3rd, 7th, 10th, 14th, 21st and 28th day are in Fig.3. The UV-vis spectroscopy confirmed creation of AgNPs in all solutions except the solution of pH 2. On day 1 (for pH 2) light grey transparent solution and grey sediment of Ag (confirmed by AAS) on the bottom of the flask were observed. These findings indicated that no nanoparticles developed, however, the presence of the sediment pointed out either fast agglomeration of Ag particles or greater Ag particles formation. It might be due to subdued surface protection from algae.

We suppose that in AgNPs production the algae may play role of both stabilizer and reductant. Despite various studies on plant mediated synthesis of AgNPs have been conducted, there is no clear explanation regarding the chemistry involved in the multifunctional nature of extracts during the synthesis of stable NPs [24]. It is very likely that stabilization of AgNPs may be caused by natural polymers [25, 26]. The pH decreases up to 2 could cause polymer disruption resulting to loss of AgNPs stability.

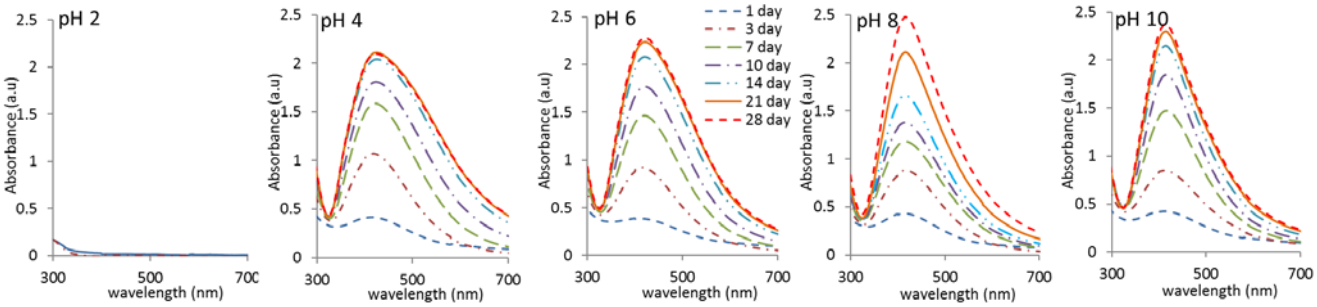


Fig.3. UV-vis absorption spectra of the AgNPs prepared at different pHs as a function of reaction time.

The SPR band of AgNPs formed in the solutions with pH 4 and 6 started to broaden on 10th day, Fig.3, and a slight red-shift of SPR band, Fig.4, was observed. According to literature [26-28] significant broadening and red-shift of SPR band indicate the presence of various shaped and/or sized nanoparticles. However, in our experiments no changes in shape only small changes in AgNPs size were observed. It is obvious that the broadening and red-shift, Fig.4, of SPR band were not so significant to cause such changes.

AgNPs formed in the solutions with higher pH (8 and 10) exhibited symmetrical and narrow SPR band throughout the experimental period, Fig.3. The red-shift of SPR band, Fig.4, was insignificant, what indicates formation of stable, uniform AgNPs with narrow size interval.

The information gathered by the UV-vis spectra was supplemented by TEM analysis. The particle sizes varied depending on pH of solutions and experimental time. The TEM image showed AgNPs in all solutions, but nanoparticles formed at lower pH grown with time and TEM images showed a number of aggregates. On the other hand, nanoparticles formed in the solutions with higher pH remained stable, even on 28th day no change in their size was observed.

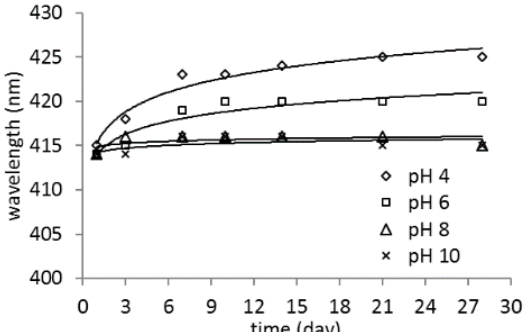
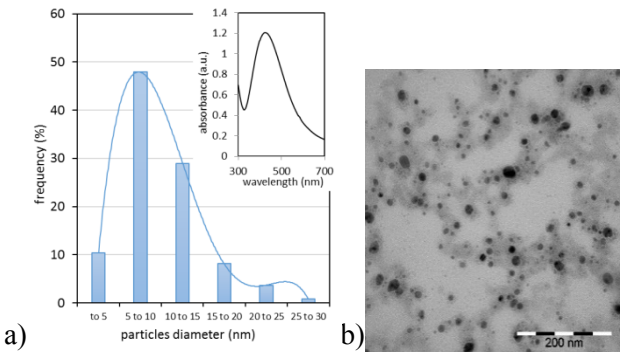


Fig.4. Wavelength changes as a function of time.

Based on above mentioned experiments we can assume that the extract of three weeks old algae and controlled pH of solution can guarantee uniform AgNPs with narrow interval of size distribution. Successful synthesis of nanoparticles should be followed by appropriate storage, to guarantee stabile and unagglomerated nanoparticles.

3.3 The long-term stability evaluation

For the analyse of different storage conditions influence on c olloidal AgNPs long-term stability (200 days) we have synthetized colloidal AgNPs where more than 90% of AgNPs were down to 20 nm in diameter, Fig.6. The SPR band was symmetrical, Fig.6a, what indicated spherical well dispersed particles confirmed by TEM, Fig.6b. The long-term stability of colloidal AgNPs was analysed at room temperature (21°C) in daylight/dark and at 5°C in dark.



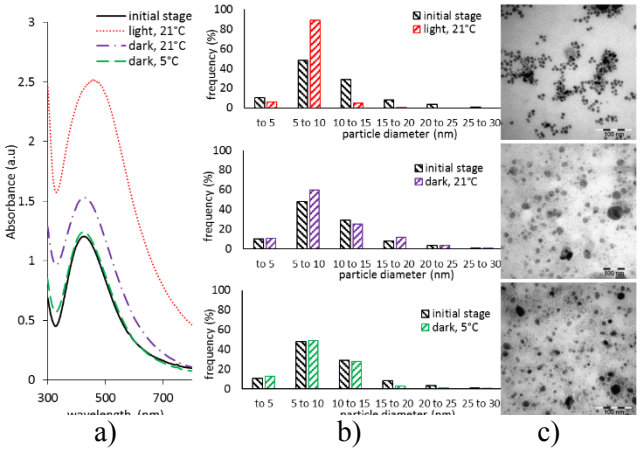
a) b)

Fig.6. The size distribution of AgNPs, the inset show UV-Vis absorption spectrum of the AgNPs (a). The TEM image of AgNPs (b).

The information gathered by a nalysing of the UV-Vis spectra for all experimental solutions are in Fig.7a. The storage of AgNPs at room temperature in daylight caused substantial changes in their long-term stability. After 200 days the SPR band lost symmetry, the SPR band´s peak noticeably shifted to higher wavelength (from 428 nm to 458 nm), λ_{max} (values of absorbance) increased from 1.206 to 2.514.

According to our results and as it was reported earlier [14, 29, 30], broading and/or symetry loss of SPR band indicates that various shaped and/or sized nanoparticles are present. Izak-Nau et al. [19] assumed that such changes in AgNPs' stability under this condition could be attributed to the elevated temperature of the dispersions exposed to daylight, which can increase the NPs collision rate and subsequently induce faster agglomeration. Additionally, daylight can cause photo-reduction of already dissolved Ag^+ that consequently may lead to the production of new NPs increasing the overall sample polydispersity.

Considering that the different shaped AgNPs were not confirmed by TEM images, Fig.7c. Such SPR bands deviation could be also caused by NPs dissolution, agglomeration and sinking to the bottom [31, 32]. Agglomeration was confirmed by the considerable amount of AgNPs sediment settled on the bottom of the Erlenmeyer flask. Only small, well dispersed particles (to 10 nm) remained in solution. The TEM image of AgNPs, Fig.7c and the size distribution on 200th day clearly indicated their presence, Fig.7b.



a) b) c)

Fig.7. The UV-Vis spectra (a), the size distribution (b) and TEM images (c) of AgNPs stored at different conditions after 200 days.

Fig.7b shows size distribution of AgNPs stored at room temperature in dark. Almost 90% of AgNPs was down to 20 nm and they size did not significantly change with experimental time. The SPR bands were symmetrical, Fig.7a. The max wavelength of peaks negligibly changed from 424 nm to 426 n m on 200th. The position of the peaks can be considered stable over time what indicated stabile, small and spherical AgNPs. However, increase of λ_{max} against time of experiment (from 1.206 to 1.533 on 200th day) and slight increase of SPR bands width against time may indicate some changes in solution. This behaviour could be possible if the large aggregated nanoparticles are formed in solution. The visual inspection of the stored solution confirmed slight sediment of AgNPs on the bottom of Erlenmeyer flask.

Combination of dark and low temperature has crucial influence on AgNPs stability, Fig.7b. This conditions can guarantee the best long-term stability

of AgNPs. UV-Vis did not change at all, Fig.7a, no agglomeration and no significant increase in particle frequency in dependent of time was observed, Fig.7b, c.

3.4 The impact of time on AgNPs toxicity

The antibacterial activity of 4-day and 300-day AgNPs stored at two different temperatures in dark against *Parachlorella kessleri* algae and fungus/molds presented in the indoor air was observed, Fig.8. We did not analyze the NPs stored at room temperature exposed to light because NPs (|after an almost half of year) lost their stability (agglomeration occurred and all AgNPs settled on the bottom of the Erlenmeyer flask).

The antibacterial activity was observed on agar plates, where the sterile Whatman paper disks (diameter 5 mm) were impregnated with a drop (10 μl) of colloidal AgNPs.

Petri dishes initially supplemented with *Parachlorella kessleri* algae show the clear circular inhibition zones, Fig. 8a.

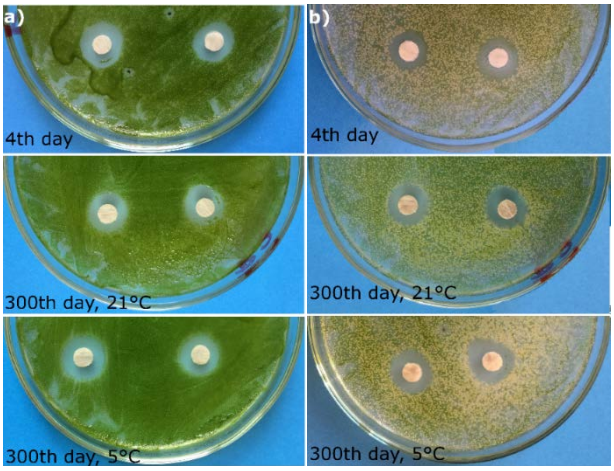


Fig.8. The toxicity of colloidal AgNPs on algae a) and fungus/moulds b)

The average diameters of inhibition circles did not differ significantly, for 4-day AgNPs it reached ~13 mm, for AgNPs stored at 21 and 5°C for 300 days it was 11 and 12 mm in diameter respectively. Even after exposure of agar plates to variety of bacteria, molds and fungi occurred in ambient environment the presence of AgNPs prevent contamination, Fig. 8b. The fungus/molds presented in the indoor air noticeably contaminated the agar plates but the inhibition zones stayed clear. It is obvious that the time (300 days) did not influence the antibacterial effect of AgNPs, small radius makes it easy for AgNPs to penetrate the cell membrane. Colloidal AgNPs even after a half of year are able to cause structural changes and damages of cellular membrane [33] that lead to cell death irrespective of age and storage conditions of colloidal AgNPs.

4 Conclusion

Silver nanoparticles were successfully prepared using extracts of algae *Parachlorella kessleri*. Experimental results are as follows:

- the algae life cycle is important for AgNPs formation, the rate of nanoparticle production and amount of AgNPs in solution can be influenced by the algae age.
- it is possible to control synthesis and the size of the nanoparticles by changing of pH, the best results were obtained using solution with pH 8.
- the long-term stability of Ag nanoparticles can be influenced by storage conditions, the strongest transformations occur in the sample stored at room temperature and exposed to daylight,
- dark positively influence the AgNPs stability, combination of the dark and temperature ~5°C has the crucial positive influence on AgNPs stability. This way stored nanoparticles are stable dispersed in solution and no phase separation or agglomeration is observed even after 6 months,
- the experimental results indicated that AgNPs had strong toxic action against *Parachlorella kessleri* the algae and against microorganisms presented commonly in air. The time did not influence the antibacterial effect of AgNPs.

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