DIFFERENTIAL RESPONSE OF *HAEMATOCOCCUS PLUVIALIS* TO SILVER NANOPARTICLES: ROLE OF INOCULUM TYPE IN ASTAXANTHIN ACCUMULATION

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Abstract

Silver nanoparticles (AgNPs) have been extensively investigated for their biostimulatory or toxic effects on microalgae. However, the influence of the inoculum's developmental stage on astaxanthin biosynthesis in the microalga *Haematococcus pluvialis* remains largely unexplored. This study aimed to assess the effects of AgNPs on biomass and astaxanthin production in *Haematococcus pluvialis* by comparing cultures initiated with two distinct inoculum types: green vegetative cells and aplanospores. Experimental cultures were exposed to silver nanoparticles of 40 and 60 nm, each applied at five concentrations (from 0.01 to 1.0 mg/L). Biomass and astaxanthin content were quantified to evaluate the metabolic responses induced by AgNPs exposure. Both inoculum types exhibited a similar response profile: exposure to 40 nm AgNPs did not induce astaxanthin synthesis at any tested concentration. When astaxanthin was determined in the aplanospore stage, the green-cell inoculum showed inhibited accumulation at all concentrations, with over a 20% reduction, while the aplanospore inoculum-maintained control levels. For 60 nm AgNPs, final astaxanthin content in *Haematococcus pluvialis* cultures with green motile cell inoculum increased by 8.7–13.3%, mainly at higher concentrations. With the aplanospore inoculum, red-stage astaxanthin increased by 50–80% at all concentrations. A slight reduction in biomass was observed in green-cell cultures treated with 40 nm AgNPs, while aplanospore-based cultures maintained more stable biomass growth. The results underline the critical role of the inoculum's developmental stage in modulating the metabolic response of *Haematococcus pluvialis* to AgNPs and provide a foundation for optimizing astaxanthin yield through nanoparticle-assisted cultivation strategies.

Keywords: *Haematococcus pluvialis*, silver nanoparticle, inoculum stage, biomass, astaxanthin

1. INTRODUCTION

Silver nanoparticles are increasingly used in microalgal biotechnology due to their capacity to modulate cellular metabolism [1]. Their action is mediated through two distinct mechanisms: (1) induction of physiological stress, leading to the synthesis of biotechnologically relevant compounds that support culture maintenance of the, or (2) hormetic stimulation at moderate concentrations, which enhances biosynthetic pathways and optimizes metabolites production [2,3]. The effect of silver nanoparticles on microalgae depends on several key factors, including applied concentration, nanoparticle size, and cultivation conditions. In general, moderate doses can stimulate metabolic processes, whereas higher concentrations exert an inhibitory effect [4,5]. Microalgal species-specific tolerance also plays a decisive role; some microalgae display resilience while others are more sensitive to AgNPs interaction [4]. Additionally, the physiological stage of the culture is an essential factor influencing how cells respond to treatment [6]. In the context of modern microalgal cultivation strategies aimed

at producing bioactive compounds, AgNPs are gaining importance as priming agents and in combination with other stimuli to direct specific biosynthetic processes, depending on the targeted microalgal species [7]. A notable example is *Haematococcus pluvialis*, one of the most valuable microalgae in biotechnology due to its extraordinary capacity to accumulate astaxanthin, a carotenoid with strong antioxidant activity and multiple applications in the pharmaceutical, food, and aquaculture industries [8]. This species exhibits two major developmental stages: the green stage, marked by active cell division and growth, and the red cyst stage, where astaxanthin accumulates in response to stress or nutrient limitation [9]. In this study, we investigated how the physiological stage of the inoculum influences the response of *Haematococcus pluvialis* to AgNP treatment (40 and 60 nm), focusing on astaxanthin accumulation and biomass production.

2. EXPERIMENTAL DESIGN

The green microalga *Haematococcus pluvialis* CNMN-AV-05 strain, maintained in the National Collection of Nonpathogenic Microorganisms at the Institute of Microbiology, Technical University of Moldova, was used as the study object. For inoculum preparation, green cells were cultivated for 7 days until reaching the exponential growth phase, while red aplanospores were formed after a complete 16-day cultivation cycle. Cultivation was carried out in a standardized mineral medium with the following composition (g/L): $NaNO_3$ – 0.30; KH_2PO_4 – 0.02; K_2HPO_4 – 0.02; NaCl – 0.02; $CaCl_2$ – 0.05; $MgSO_4 \cdot 7H_2O$ – 0.01; $ZnSO_4 \cdot 7H_2O$ – 0.0001; $MnSO_4 \cdot 5H_2O$ – 0.0015; $CuSO_4 \cdot 5H_2O$ – 0.00008; H_3BO_3 – 0.0003; $(NH_4)_6MoO_{24} \cdot 4H_2O$ – 0.0003; $FeCl_3 \cdot 6H_2O$ – 0.0175; $Co(NO_3)_2 \cdot 6H_2O$ – 0.0002; EDTA – 0.0075. The experiments were performed in 100 mL Erlenmeyer flasks containing 50 mL microalgal suspension, at 26°C, under continuous illumination of 1500 lx and periodic agitation during the first ten days.

Astaxanthin induction was achieved by increasing the light intensity to 3000 lx for 72 hours, which promoted the transition of cultures to the red aplanospore stage. Silver nanoparticles stabilized with citrate (sizes: 40 ± 0.2 nm and 60 ± 0.2 nm (SIGMA-ALDRICH CHEMIE GmbH, Germany), were added to the culture medium. Two experimental series were conducted, depending on the type of inoculum used and the timing of nanoparticle addition: (1) inoculum with green cells and (2) inoculum with aplanospores (red cysts). The cultures were maintained under astaxanthin-inducing light conditions until the end of the development cycle.

At the end of the cultivation cycle, the biomass was harvested and quantified spectrophotometrically using a calibration curve. The astaxanthin content was determined in the ethanolic extract obtained after acidic hydrolysis of the aplanospore biomass (0.1 N HCl, 90 °C, 10 minutes), followed by extraction in 96% ethanol for 120 minutes under continuous agitation. The absorbance of the extract was measured at 478 nm, and the astaxanthin amount was calculated based on a calibration curve constructed for synthetic astaxanthin (98.9% purity, SIGMA-ALDRICH CHEMIE GmbH, Germany).

All experimental determinations were performed in triplicate. Statistical analysis was conducted in Microsoft Excel (version 2108, Microsoft 365) by calculating the arithmetic mean and standard error. Differences between control and experimental samples were evaluated using the Student's t-test and were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Upon completion of the *Haematococcus pluvialis* cultivation cycle, astaxanthin synthesis was induced by increasing the light intensity. Culture initiates with red aplanospores inoculum required an extension of approximately three days compared to those initiated with green cell inoculum, due to the germination phase of the red cysts, under the applied cultivation conditions. Treatment with silver nanoparticles (AgNPs) resulted in distinct effects on biomass accumulation (**Figure 1**).

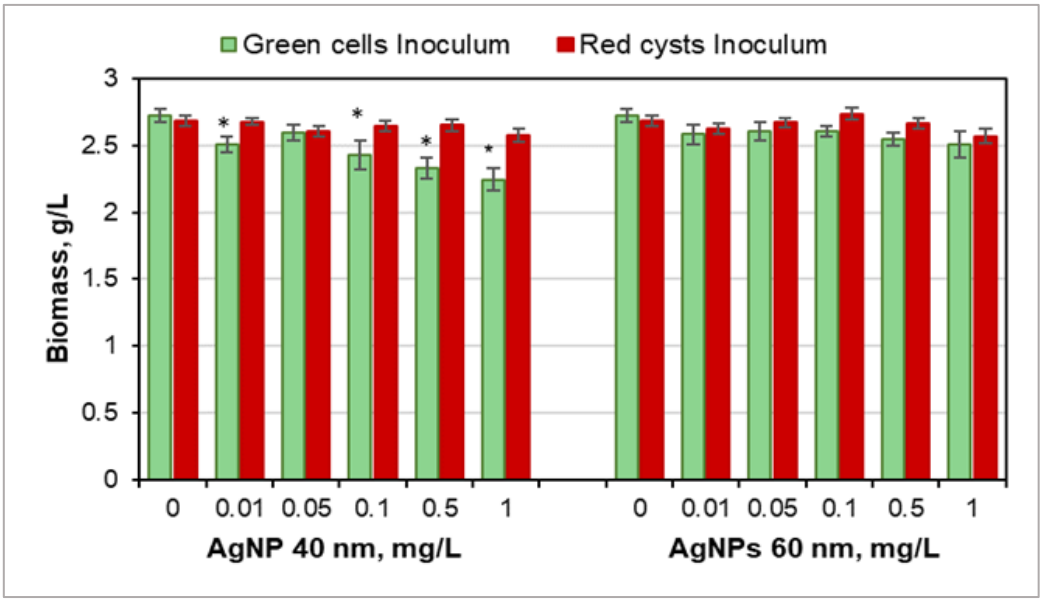

Figure 1 Biomass accumulation (g/L) in *Haematococcus pluvialis* cultures inoculated with green cells or red cysts after exposure to AgNPs (40 nm and 60 nm) at different concentrations. Data are expressed as mean ± SD (n = 3). *p < 0.05 vs. control

AgNPs with a size of 40 nm reduced the final biomass content in experiments where green cells were used as inoculum. This reduction was significant ($p < 0.05$), with biomass values being 10.71–17.46% lower compared to the control. The decrease was observed 0.1–1 mg/L. In contrast, for 60 nm AgNPs, the changes in biomass content were not significant, with values only 4.36–7.94% lower than the control at all tested concentrations. In experiments where red cysts (aplanospores) were used as inoculum, 40 nm AgNPs did not alter biomass content, with no significant effect observed at any of the tested concentrations. A similar trend was noted for 60 nm AgNPs, which also did not affect biomass production in *Haematococcus pluvialis* cultures.

Thus, a differentiated response of *Haematococcus pluvialis* cultures to AgNP treatment was observed, influenced not only by nanoparticle size and concentration, but also by the type of inoculum used. Upon exposure to 40 nm AgNPs, inoculation with aplanospores did not result in biomass reduction compared to the control. Cultures inoculated with green cells showed a clear and significant decrease. This observation is consistent with data from other studies, which demonstrated that the adverse effect of AgNPs on biomass accumulation is much more pronounced when the inoculum consists of green cells, with productivity decreasing by up to 85% at 4–8 mg/L [6]. This difference can be explained by the morphological and physiological characteristics of the cells: green vegetative cells have thinner cell walls and are more vulnerable to stress, whereas aplanospores possess multilayered cell walls and a higher tolerance to oxidative stress, partially due to their elevated astaxanthin content. Moreover, the application of small-sized AgNPs (10 and 20 nm) at 0.1 to 10 mg/L resulted in increased biomass when aplanospores were used as inoculum, and biomass harvested at the end of the cultivation cycle [10]. These findings confirm that resistance to AgNPs depends on the physiological stage of the inoculum and suggest that the use of aplanospores may represent an effective strategy to mitigate the negative impact of nanoparticles on final biomass.

Similar patterns have been reported for other nanoparticles. For instance, in the case of ZnONPs, cultures inoculated with green cells of *Haematococcus pluvialis* showed a significant biomass reduction after 72 hours of exposure to 10–200 µg/mL [11]. Likewise, for green-synthesized ZnONPs, concentrations of 50–400 µg/mL decreased biomass in green-cell inoculated cultures [12].

In the harvested biomass, the astaxanthin content was determined and used as an indicator of the cellular response to AgNP treatment, given its essential role in protection against oxidative stress. Variations in

astaxanthin levels, depending on nanoparticle size and concentration, reflected both the extent of cellular impairment and the adaptive capacity to the induced stress. An increase in astaxanthin accumulation indicated a defensive response, whereas a significant decrease signaled cellular damage (**Figure 2**). This correlation suggests that monitoring astaxanthin provides a reliable indicator of the impact of AgNPs on the physiological state and biosynthetic capacity of *Haematococcus pluvialis* cultures.

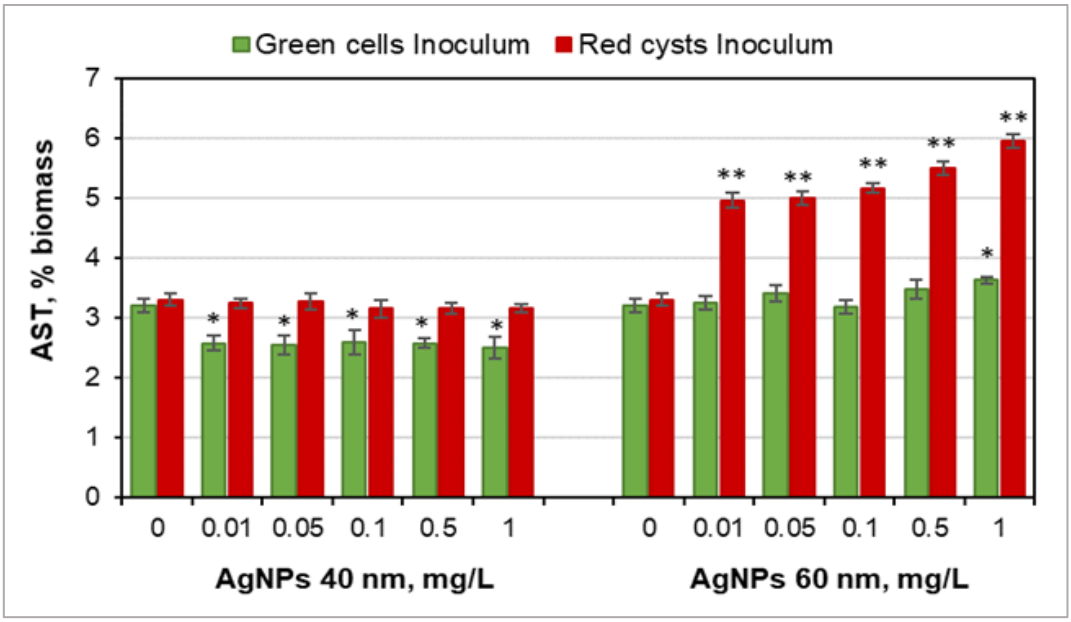

Figure 2 Astaxanthin content (% of biomass) at the end of the cultivation cycle in *Haematococcus pluvialis* cultures inoculated with green cells or red cysts after exposure to AgNPs (40 nm and 60 nm) at different concentrations. Data are expressed as mean ± SD (n = 3). *- $p < 0.05$, ** - $p < 0.01$ vs. control

Exposure *of Haematococcus pluvialis* to 40 nm AgNPs did not induce a significant increase in astaxanthin content, regardless of the inoculum type. In biomass derived from green cells, astaxanthin was significantly reduced ($p < 0.05$) by 18.96%–20.14% compared to the control. In contrast, in aplanospore-derived biomass, astaxanthin levels remained comparable to the control, with no significant changes observed.

In the case of 60 nm AgNPs, a stimulatory effect was evident. When green cells were used as inoculum, astaxanthin content increased by 8.70% at 0.5 mg/L and by 13.3% at 1 mg/L. In cultures initiated with red cysts, the effect was more pronounced: 0.01 and 0.05 mg/L resulted in increase of 50.11–51.25% ($p < 0.01$), while 0.1 and 0.5 mg/L resulted in increases of 56.0% ($p < 0.01$) and 66.21% ($p < 0.01$), respectively. At the highest tested concentration (10 mg/L), astaxanthin accumulation in aplanospore biomass reached nearly 80% above control levels.

These results demonstrate a stimulatory effect on astaxanthin biosynthesis, the intensity of which depends on the inoculum, with the germination period of cysts representing a favorable window for adaptation to the AgNPs exposure. At the same time, cultures inoculated with aplanospore maintained stable astaxanthin levels under conditions where no stimulation occurred, such as exposure to 40 nm AgNPs.

Findings from the literature support these observations. Several authors reported that exposure of green cells of *Haematococcus pluvialis* cells 1–8 mg/L AgNPs nearly completely inhibited astaxanthin accumulation within 72 hours, regardless of inoculum type. Conversely, aplanospores treatment under astaxanthin-inducing conditions (high light, nitrogen deprivation) showed a concentration-dependent reduction in pigment levels [6]. This inhibition likely results from combined stress, generated by both AgNPs and culture conditions. On the other hand, treatment of aplanospores with 10 and 20 nm AgNPs under two-phase protocol (growth in

optimized mineral medium under moderate light, followed by a 3-day induction phase) led to an increase in astaxanthin content by 17–66% [10].

Similar results have been reported with other nanoparticle. For example, exposure of green cells to green-synthesized ZnONPs (~30 nm) induced their transition to the aplanospore stage, with maximum astaxanthin accumulation of 19.98 mg/g dry biomass at 100 µg/mL, compared to 0.3 mg/g in the control [12]. In another study, ZnONPs (10–200 µg/mL) induced dose- and time-dependent decreases in astaxanthin in green cells, with the maximum reduction reaching 48% after 96 hours at 200 µg/mL [11].

Taken together, these observations emphasize that nanoparticle type, size, concentration, and the physiological stage of the inoculum, are decisive factors shaping the response of *Haematococcus pluvialis*. Depending on the interplay of these variables, nanoparticles may either stimulate carotenoid biosynthesis under favorable conditions or inhibit pigment accumulation. Therefore, optimizing nanoparticle parameters in correlation with the inoculum type represents a promising strategy to maximize astaxanthin production in *Haematococcus pluvialis* cultures.

4. CONCLUSION

The results obtained in this study demonstrate that the effect of AgNPs on biomass accumulation in *Haematococcus pluvialis* is closely related to nanoparticle size and concentration, and the type of inoculum used. Green cells were more sensitive to 40 nm AgNPs, exhibiting significant reductions in biomass, whereas aplanospores showed much greater resistance, maintaining culture stability even in the presence of nanoparticles. These findings highlight that the use of aplanospores as inoculum represents an effective strategy to reduce the negative impact of AgNPs on the culture. Not only did they maintain biomass, but they also accumulated higher levels of astaxanthin compared to green cells, emphasizing the critical role of the physiological stage of the inoculum in the response to nanoparticle-induced stress. 60 nm AgNPs stimulated astaxanthin biosynthesis, with the most pronounced effect observed in cultures inoculated with aplanospores, where increases reached up to 80%. In contrast, 40 nm AgNPs reduced astaxanthin content in samples inoculated with green cells, without affecting the values in aplanospore-inoculated cultures. These observations indicate that optimizing nanoparticle characteristics, together with the appropriate selection of inoculum type, may represent an effective strategy for maximizing astaxanthin biosynthesis in *Haematococcus pluvialis* cultures, opening new perspectives for the controlled application of nanoparticles in microalgal biotechnology.

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