THE EFFECT OF SILVER NANOPARTICLES ON THE MICROALGAE *HAEMATOCOCCUS PLUVIALIS*

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Abstract

The green alga *Haematococcus pluvialis* is highly regarded as a producer of astaxanthin. Due to its antioxidant properties, astaxanthin has become a popular nutritional ingredient. Astaxanthin has demonstrated potential pharmacological effects including antidiabetic, anti-inflammatory, and antioxidant activities and cardiovascular and neurological protective properties. Nanoparticles have become integral components of microalgal technologies, serving as stimulators for producing biologically active compounds. The stimulating effect of nanoparticles is based on the induction of oxidative stress. In this study, oxidative stress was induced by silver nanoparticles (AgNPs) and combined with stress from a nutrient-deficient cultivation medium. Citrate-stabilized AgNPs of 10 nm and 20 nm were used. They were applied to the mineral medium from the first day of the cultivation cycle in concentrations ranging from 0.01 mM to 10 mM. The microalgal culture in the red cyst phase was used as the inoculum. The results demonstrated a significant increase of 50-80% in the astaxanthin content in the biomass of the red cysts without altering the biomass content. Additionally, the lipid content in the biomass also increased significantly. The dose/effect relationship between the concentrations of 20 nm AgNPs and astaxanthin values was direct. For 10 nm AgNPs, low concentrations resulted in increased astaxanthin values. Combined stress, with the variable factor being the concentrations of silver nanoparticles, can be proposed as a strategy in the biotechnology of *Haematococcus pluvialis* for astaxanthin production.

Keywords: *Haematococcus pluvialis*, silver nanoparticles, biomass, astaxanthin, lipids

1. INTRODUCTION

The green microalga *Haematococcus pluvialis* is one of the most well-known producers of astaxanthin, a carotenoid with superior antioxidant properties. Astaxanthin has demonstrated anti-inflammatory, antitumor, antidiabetic, and immunomodulatory effects [1]. It is widely used in the pharmaceutical, nutraceutical, cosmetic, and food industries [2]. Technologies applied to *Haematococcus pluvialis* for astaxanthin production include adjustments to cultivation conditions such as pH, temperature, and light intensity and modifications to the mineral composition of the growth medium [3,4]. Nutrient deficiencies and excesses can significantly affect cell biomass productivity and astaxanthin accumulation [3,5]. Studies on the application of nanoparticles in microalgae cultivation technologies show varied results. Nanoparticles can undoubtedly be applied to biomolecule production [6]. For example, various types of nanoparticles have stimulated lipid accumulation in microalgae, demonstrating the potential for biofuel production, while others have influenced the accumulation of photosynthetic pigments and biomass production [7]. Some nanoparticles have demonstrated properties as stimulators of astaxanthin production by the microalga *Haematococcus pluvialis*, significantly increasing biomass content [8]. Silver nanoparticles, at low concentrations, have stimulated carotenoid synthesis in *Haematococcus pluvialis* biomass [9]. Several studies compare the action of nanoparticles on astaxanthin

synthesis based on their origin, highlighting this as an essential factor in nanoparticle characteristics [10,11]. Toxic effects on growth and carotenoid accumulation have been observed for various types of nanoparticles, with different forms of oxidative stress being identified [12,13]. In this study, we aimed to identify the effect of AgNPs with sizes of 10 nm and 20 nm, in combination with nutrient deficiency, on astaxanthin accumulation in the biomass of *Haematococcus pluvialis*.

2. EXPERIMENTAL DESIGN

The green microalga strain *Haematococcus pluvialis* CNMN-AV-05, deposited in the National Collection of Nonpathogenic Microorganisms at the Institute of Microbiology and Biotechnology, Technical University of Moldova, was used as the study object. In the experiments, the microalga was cultivated in a mineral medium with the following composition: $NaNO_3$ – 0,3 g/L; KH_2PO_4 – 0,02 g/L; K_2HPO_4 – 0,02 g/L; NaCl – 0,02 g/L; $CaCl_2$ – 0,05 g/L; $MgSO_4 \cdot 7H_2O$ - 0,01 g/L; $ZnSO_4 \cdot 7H_2O$ – 0,0001 g/L; $MnSO_4 \cdot 5H_2O$ – 0,0015 g/L; $CuSO_4 \cdot 5H_2O$ – 0,00008 g/L; H_3BO_3 - 0,0003 g/L; $(NH_4)_6MoO_{24} \cdot 4H_2O$ – 0,0003 g/L; $FeCl_3 \cdot 6H_2O$ – 0,0175 g/L; $Co(NO_3)_2 \cdot 6H_2O$ - 0,0002 g/L; EDTA - 0,0075 g/L, in 100 mL Erlenmeyer flasks containing 50 mL of microalgal culture, at a temperature of 26˚C, with constant illumination of 1500 lx and periodic agitation during the first ten days of cultivation. The induction of astaxanthin production was carried out by excessive illumination with an intensity of 3000 lx for 72 hours. Silver nanoparticles (AgNPs) stabilized with citrate, 10 ± 0.2 nm and 20 ± 0.2 nm particle size (TEM) (SIGMA-ALDRICH CHEMIE GmbH, Germany), in various selected concentrations, and were added to the mineral medium. The biomass content collected at the end of the growth phase of red aplanospores was determined spectrophotometrically, with a quantitative calculation based on the calibration curve. The astaxanthin content was determined in the ethanolic extract from pre-treated red aplanospore biomass. For this purpose, native *Haematoccus pluvialis* biomass was subjected to acid hydrolysis with 0.1N HCl at a temperature of 90°C for 10 minutes. The residual acid was removed by washing the biomass with distilled water. The extraction mixture consisted of 10 mg of pre-treated aplanospore biomass and 1 mL of 96% ethanol. Astaxanthin extraction was carried out under continuous agitation for 120 minutes. The absorbance of the ethanolic extract was recorded at 478 nm. The quantitative calculation was based on the calibration curve constructed for synthetic astaxanthin. The lipid content was determined spectrophotometrically with phospho-vanillin reagent. All measurements and determinations were performed in triplicate. Statistical analysis was performed using Microsoft Excel software (Microsoft 365 Excel version 2108). The data were statistically analysed by calculating the arithmetic mean and standard error. The t-Student test was used to determine statistically significant differences between the experimental and control values

3. RESULTS AND DISCUSSION

The mineral medium used for the cultivation of the microalga *Haematococcus pluvialis* CNMN-AV-05 is characterized by a low phosphate content (KH_2PO_4 – 0.02 g/L and K_2HPO_4 – 0.02 g/L), with a P/N ratio of 0.164. In the red aplanospore phase, astaxanthin constitutes 3% of the obtained biomass, with a total biomass production of 2.2-2.4 g/L and a cultivation cycle of 14 days. Under nutrient-deficient conditions, *Haematococcus pluvialis* cells enter a state of moderate stress, triggering protective mechanisms, including synthesizing carotenoids to neutralize free radicals. This represents a survival strategy under stress conditions, stimulating the production of biologically valuable compounds such as astaxanthin [14].

Under such conditions, when the microalgal culture is subjected to moderate stress, the addition of potentially toxic compounds, such as AgNPs, may influence either astaxanthin production or the productivity of the culture. The effect may depend on the nanoparticles' size and/or concentration. The presence of silver nanoparticles in the cultivation medium of the microalga induced changes in the biomass content during the red aplanospore phase. **Figure 1** shows the changes in the biomass content of *Haematococcus pluvialis*,

collected in the red phase, due to the action of silver nanoparticles at various concentrations in the cultivation medium.

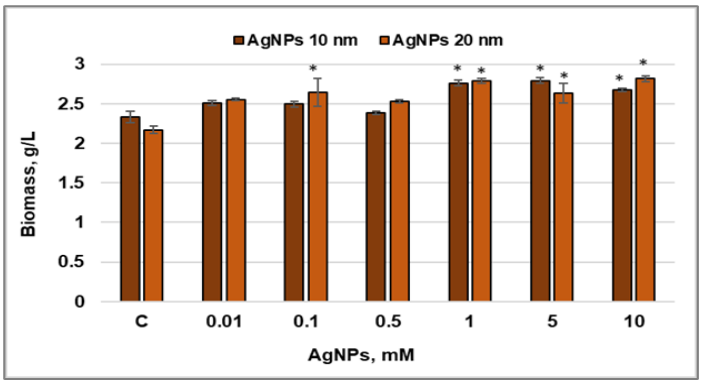

Figure 1 Biomass content (g/L) of *Haematococcus pluvialis*, red aplanospore phase, cultivated in the presence of AgNPs with sizes of 10 nm and 20 nm, C-control; *p < 0.05

The addition of 10 nm AgNPs to the mineral medium of *Haematococcus pluvialis* at concentrations of 1.0, 5.0, and 10 mM led to biomass increases of 18.6% (p > 0.01), 19.8% (p > 0.05), and 14.9% (p > 0.05), respectively. Lower concentrations, ranging from 0.01 to 0.5 mM, resulted in a 7% increase in biomass content. In the case of 20 nm AgNPs, applied at the same concentrations, an increase in biomass accumulation between 17.7% and 29.8% (p > 0.05) was observed compared to the control. Higher biomass content values are associated with higher nanoparticle concentrations. Thus, at the applied concentrations, citrate-stabilized silver nanoparticles did not exhibit toxic effects on the *Haematococcus pluvialis* culture, suggesting good tolerance of this microalga to silver nanoparticles in the growth medium.

Figure 2 illustrates the changes in astaxanthin and lipid content in the aplanospore biomass due to the exposure of *Haematococcus pluvialis* culture to AgNPs.

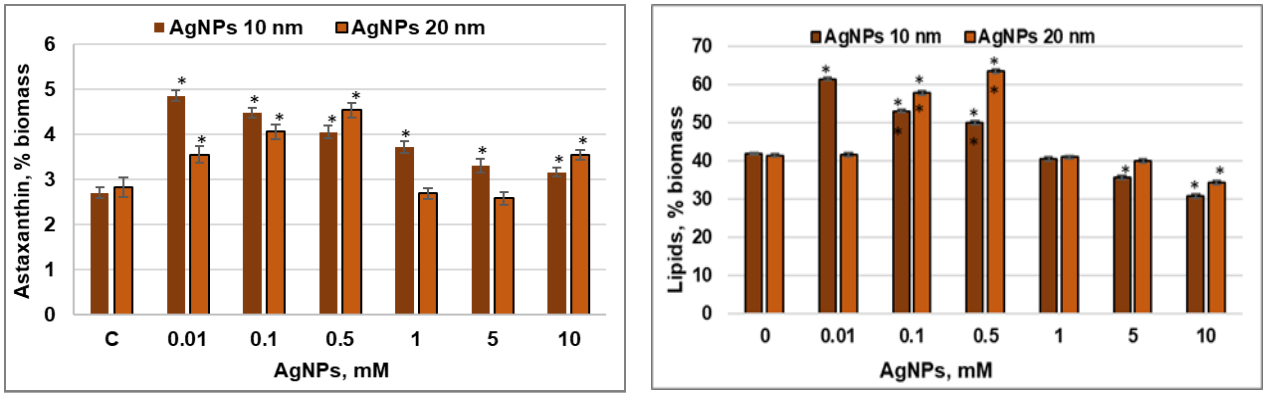

Figure 2 Astaxanthin content, % **(A)** and lipid content, % **(B)**, in the biomass of *Haematococcus pluvialis*, red aplanospore phase, cultivated in the presence of AgNPs with sizes of 10 nm and 20 nm, C-control; *p < 0.01

Figure 2A shows the changes in astaxanthin content in the aplanospore biomass as the result to the exposure of *Haematococcus pluvialis* to AgNPs. AgNPs of 10 nm, applied at concentrations between 0.01 mM and 0.5 mM, led to a significant increase in astaxanthin content in the biomass. The concentration of 0.01 mM AgNPs resulted in an 80% increase in astaxanthin content (p<0.01) compared to the control. Concentrations of 0.1 mM and 0.5 mM induced increases in astaxanthin values by 65.9% (p<0.01) and 50% (p<0.01), respectively. For 10 nm AgNP concentrations of 1.0-10 mM, an increase in astaxanthin of 37.8% (p<0.01) to 17% (p<0.01) was recorded. The stimulatory effect directly depends on the concentration of nanoparticles in the

Haematococcus pluvialis cultivation medium. The Pearson coefficient r = -0.9976, calculated for the relationship between 10 nm AgNP concentrations and astaxanthin content, shows a strong negative correlation. As the AgNP concentration increases, the astaxanthin content decreases proportionally. For 20 nm AgNPs, a concentration of 0.05 mM led to a 26.8% increase ($p<0.01$) in astaxanthin content in the microalgal biomass. Concentrations of 0.1 and 0.5 mM resulted in pigment content increases of 45% ($p<0.01$) and 62% ($p<0.01$), respectively, compared to the control. Therefore, increasing the concentration of AgNPs in the cultivation medium led to a proportional increase in astaxanthin content in the microalgal biomass, with a strong positive correlation (r = 0.9326). The concentrations of 20 nm AgNPs, 1.0 and 10 mM, considered high, also showed a tendency to increase astaxanthin levels, though the stimulatory effect was moderate. The values obtained at concentrations of 1.0 and 5.0 mM were similar to those of the control sample. An increase of 26.9% ($p<0.01$) in astaxanthin content was observed for the concentration of 10.0 mM AgNPs in the cultivation medium. Thus, *Haematococcus pluvialis* responds in two distinct ways to the presence of AgNPs in the culture medium, depending on the size and concentration of the nanoparticles. Low concentrations of AgNPs stimulate astaxanthin synthesis: for 10 nm AgNPs, the correlation between nanoparticle concentrations and astaxanthin content is inverse; for 20 nm AgNPs, the correlation is direct.

The size and concentration are the determining factors in the influence of nanoparticles on astaxanthin biosynthesis in *Haematococcus pluvialis.* Within the applied concentration limits, no inhibition effect on astaxanthin synthesis was established, suggesting a potential adaptation of the culture to the presence of AgNPs in the microalgal growth medium. As evidence of this statement, the results of changes in lipid content accumulated in the biomass of *H. pluvialis* (**Figure 2B)**. Silver nanoparticles (AgNPs) of 10 nm, applied in the *Haematococcus pluvialis* cultivation medium at low concentrations, stimulated lipid synthesis, and their content in the red aplanospore biomass increased considerably. AgNPs of 10 nm, at concentrations of 0.01 and 0.5 mM, led to a lipid content increase of 53.6% ($p<0.01$) and 24.7% ($p<0.01$), respectively. Nanoparticle concentrations of 1.0 and 5.0 mM generated a neutral response, while at 10 mM AgNP concentration, a 17.3% decrease in lipid content was observed ($p<0.05$). AgNPs of 20 nm, at concentrations of 0.01 and 0.5 mM, resulted in a lipid content increase of 19.6% ($p<0.01$) and 46.9% ($p<0.01$), respectively. Nanoparticle concentrations of 5.0 mM and 10.0 mM decreased lipid content by 14.6% and 16.8% ($p<0.05$) in the microalgal biomass. Lipid accumulation in the experimental variants with increased astaxanthin levels in the biomass is characterized by the same correlations between AgNP concentrations and astaxanthin values. The correlation between the concentrations of 10 nm AgNPs with a stimulatory effect and the lipid content is negative and strong (r=-0.9455). In the case of 20 nm AgNPs, this correlation is positive and very strong (r=0.9959). Lipid synthesis in the *Haematococcus pluvialis* biomass in the presence of AgNPs in the cultivation medium is not determined by astaxanthin accumulation. Increasing AgNP concentrations leads to a decrease in lipid content in the biomass. The correlation between the applied concentrations of 10 nm AgNPs and the lipid content in aplanospore biomass is negative and strong (r=-0.7956). For 20 nm AgNPs, this correlation is also negative and strong (r=-0.7490). It can be assumed that the stimulation of astaxanthin synthesis results from strong oxidative stress, considering lipid content values are decreasing.

The general trend of AgNPs influencing *Haematococcus pluvialis* culture in the growth medium is to reduce lipid synthesis, which generates a stressful environment for the microalga. Higher concentrations of AgNPs disrupt the metabolic processes of the microalga, negatively affecting its ability to accumulate lipids. At the same time, it can be stated that low concentrations of AgNPs have a safe stimulatory effect on the microalgal culture. There is limited evidence indicating a stimulatory effect on the growth of the microalga *Haematococcus pluvialis* due to nanoparticle exposure. Studies on the effects of nanoparticles, such as ZnONPs and TiO_2NPs, on the growth and astaxanthin synthesis by *Haematococcus pluvialis* have shown negative effects, manifested by the inhibition of growth and pigment synthesis [10,12]. Silver nanoparticles have also been studied from the perspective of their toxicity. AgNPs obtained by reducing a biological substrate were toxic to *Haematococcus pluvialis* culture. At concentrations up to 8 mg/L, AgNPs harmed cell morphology, culture productivity, and astaxanthin synthesis. Culture productivity in the green phase was reduced by up to 85% as AgNP

concentration increased to 8 mg/L, while the effects on the red aplanospore phase culture were less severe. Additionally, AgNPs reduced the rate of astaxanthin production [11]. A significant increase in astaxanthin production in response to nanoparticles in the cultivation medium was reported due to the application of magnesium aminoclay nanoparticles (MgAC) [8]. A concentration of 1.0 mg/L of MgAC nanoparticles induced a 13.7-fold increase in astaxanthin content in the *Haematococcus pluvialis* microalga cells. Lipid content in the cells increased 13.6 times. The Tris-Acetate-Phosphate mineral medium, specifically for photoautotrophy, with a high phosphate content (KH_2PO_4 – 0.34 g/L and K_2HPO_4 – 1.72 g/L), was used for microalga growth. The astaxanthin increase was calculated by comparison with the control, with the total astaxanthin production being 7.3 ± 0.6 mg/L. Astaxanthin content increased by 40% as a result of applying MgAC nanoparticles. A nutrient-poor mineral medium significantly stimulates astaxanthin production [14]. In this context, AgNPs were added to the *Haematococcus pluvialis* CNMN-AV-03 cultivation medium, which had a reduced mineral content. In the control, without nanoparticle supplementation, astaxanthin production was 60.7 mg/L. Adding a concentration of 0.01 mM of 10 nm AgNPs led to a 97.7% increase in astaxanthin production compared to the control. The combined stimulatory effect of the nanoparticles and the deficient mineral medium was evident. Higher lipid content values were recorded in variants with increased astaxanthin content due to the action of 10 nm and 20 nm AgNPs. A similar result was obtained in experiments using MgAC nanoparticles, which can be evidence of specific nanoparticles' stimulation of astaxanthin synthesis [8]. Nanoparticles are used as stimulators of lipid synthesis in microalgal biomass, and this process is based on the induction of oxidative stress. Citrate-stabilized AgNPs, with sizes of 10 and 20 nm, demonstrated a stimulatory effect on the microalga *Porphyridium cruentum*, manifested by a significant increase in lipid content in the biomass [15]. In the study of the action of AgNPs on the *Haematococcus pluvialis* microalga culture, lipid synthesis induction was a secondary process, with the primary effect being the stimulation of astaxanthin synthesis. The absence of toxicity can be attributed to the stabilization of the nanoparticles, which prevented their aggregation and, consequently, the subsequent damage to cell membranes and the associated consequences of this process

4. CONCLUSION

An important conclusion of this study is that 10 nm and 20 nm citrate-stabilized AgNPs act as stimulators of astaxanthin synthesis in the green alga strain *Haematococcus pluvialis* CNMN-AV-03 when cultivated in a nutrient-poor mineral medium. This microalgal response results from moderate cell stress, which did not lead to reductions in biomass content at the end of the cultivation cycle. The stimulatory effect of silver nanoparticles on astaxanthin synthesis by the *Haematococcus pluvialis* CNMN-AV-03 strain used in this study depends on the size and concentration of the nanoparticles in the cultivation medium. Low nanoparticle concentrations significantly increased astaxanthin content, and no evident toxic effects were observed within the applied concentration range.

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