Effect of Azide on Growth Parameters of *Haematococcus pluvialis* in Green and Red Stages

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Received: 2022-04-20 Revised and accepted: 2022-08-15

Abstract

Haematococcus pluvialis can accumulate large amounts of astaxanthin under stress conditions. Azide as an effective respiratory inhibitor can induce oxidative stress. In this study, the effect of pre-treatment (7 days) and treatment (14 days) of different concentrations of azide (0, 25, 50, and 100 µM) were investigated on growth and biochemical parameters in green and red cells. Azide treatment and pre-treatment caused a decrease in all measured parameters except for the carotenoid in the green stage. Carotenoid content did not show any changes in azide treatment but pre-treatment with low concentration induced carotenoid accumulation in the green stage. The dry weight, protein, and carbohydrate amount did not change in red cells treated with azide, but carotenoid content decreased in these cells. Pretreated with azide hurt the amount of protein and carbohydrates but increased the carotenoid content. Azide pretreatment had better performance in increasing the carotenoid amount in red cells. These results showed that *H. pluvialis* has good potential for phytoremediation of azide and carotenoid accumulation.

Keywords: Sodium azide, Carbohydrate, Carotenoid, Protein, Pretreatment

Introduction

The green microalga *Haematococcus pluvialis* (Volvocales) is widely distributed worldwide, from brackish water to rock surfaces (Czygan 1970; Lorenz 1999). This microalga is the richest astaxanthin source, which exhibits a strong antioxidant effect and a powerful scavenging ability against singlet oxygen (O'Connor and O'Brien 1998). This microalga has a complex life cycle with two distinct cell forms. Under suitable conditions, the cells are green, pear-shaped, and motile with two flagella.. *H. pluvialis* cells are transformed to red, spherical, and immobile cells under stress conditions. The red color of the cells is due to the accumulation of a significant amount of astaxanthin This stage is usually accompanied by the transformation of green vegetative cells into red cysts. At first, astaxanthin is accumulated in lipid droplets in the cytoplasm surrounding the nucleus, then a massive accumulation of carotenoids occurs, and astaxanthin-containing lipid

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droplets eventually fill the whole cytoplasm. Several factors play a role in obtaining the maximum algal biomass and astaxanthin content, including quality and quantity of light, culture medium, growth regulators, and temperature. These factors usually have a contrary effect on biomass production and astaxanthin content. It means stress conditions that increase astaxanthin content, on the other hand, decrease growth and biomass production. Sometimes exposure to extreme stress can cause cell death in the short term (Su et al., 2014).

There are several ways to manipulate biological systems to increase biomass or specific bioactive compounds. However, biotechnology method permanent changes may cause detrimental effects on the ecosystem and humans (Hunt et al., 2010). Therefore, increasing the production of bioactive compounds and biomass using alternative methods such as chemicals can be a safe method in this regard. Studies have indicated that biochemical stimulators significantly increase microalgae productivity (Cheng et al., 2012). It has been shown that reactive oxygen species (ROS) can involve in the carotenogenesis and induction of astaxanthin accumulation in *H. pluvialis* (Kobayashi et al., 1993).

Azide is an effective respiratory inhibitor and irreversibly binds to the fourth complex of the electron transport chain. Inhibition in the electron transport chain causes ROS produced and induction of oxidative stress (Apel and Hirt, 2004). Azide can also increase ROS and induce oxidative stress by inhibiting the activity of catalase and superoxide dismutase enzymes. It was reported that azide induces massive accumulation of triglycerides and production of lipids in different microalgae species (Chen et al., 2019; Yahya et al., 2018; Zalogin and Pick, 2014).In the present study, the effect of azide on increasing the amount of biomass and carotenoids in *H. pluvialis* under proper growth and stress conditions (without nitrate to induce carotenogenesis) over two periods (7 and 14 days) was investigated.

Material and methods

Haematococcus pluvialis (UTEX) used in this study was obtained from Arian Gostar. *Haematococcus pluvialis* has grown autotrophically in Bold's Basal medium (Tripathi et al. 1999). Pure cultures were incubated under 50 µmol photons $m^{-2}s^{-1}$ fluorescent light intensity with a 16/8 h light/dark cycle at 25 ± 1°C. An equal number of cells (3.5 x 104 cells ml^{-1}) were used to inoculate 150 ml of fresh culture medium in 250 ml Erlenmeyer flasks for all treatments. Two methods were used for the experiment; the cultures were exposed to different concentrations of sodium azide (0, 25, 50, and 100 µM for 14 days), and algal cells were affected by the same azide concentrations for seven days and then transferred to a new culture medium without azide. To induce astaxanthin accumulation, the green cells were transferred from the growth conditions to the stress conditions (fresh medium of BG11 without nitrogen) after centrifugation (Siegień and Bogatek,

2006).

Growth was measured by counting cell numbers using a haemacytometer. The dry weight of the algal biomass was estimated after drying at 60 °C in a hot-air oven until a constant weight was obtained. For pigment analysis, a specific amount of biomass was extracted with 96% methanol, and chlorophyll and carotenoids were quantified as per the procedure given by Şükran et al. (Şükran et al., 1998).

Soluble protein concentration was obtained according to Bradford and Fales method (1976) was used to assay soluble carbohydrate content (Fales, 1951).

All experiments were performed in three replicates. Data are presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance. Besides, Duncan's multiple comparisons test was used to estimate the significance of the differences ($P< 0.05$).

Results and Discussion

Algae treated and pretreated with azide showed fresh weight loss of green cells (Fig. 1a).

However, these treatments did not have a significant effect on the fresh weight of red cells except for cells treated with 100 µM azide which showed less fresh weight compared to control cells (Fig. 1b).

Azide treatment significantly decreased dry weight of algae cells (Fig. 2). This effect was dose-dependent which it means that with increasing azide concentration, a greater

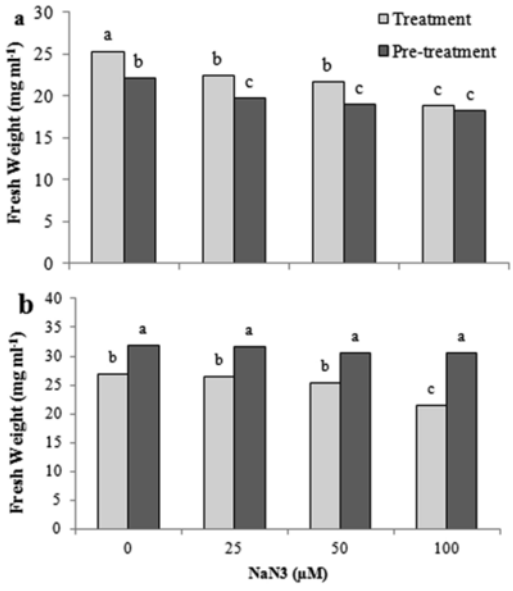

Fig. 1. Effect of azide treatment and pre-treatment on fresh weight in the green (a) and red (b) stage of *Haematococcus pluvialis*. Values are means of three-replication ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

decrease in dry weight was observed. The lowest dry weight was observed in culture treated with 100 µM azide with a 6-time decline. Pretreatment by azide also declined the cell dry weight of *H. pluvialis* in the green phase. However, it was not dose-dependent, and no difference was observed between different azide concentrations. Dry weight in the treated algae was about 60% control.

An incredible result was that azide treatment and pretreatment did not reduce the dry weight of microalgae in the red stage, just 100 µM azide treatment caused a 28% decrease in dry weight compared to control cells. Based on the decline in fresh weight and dry weight of the azide-treated algae in the green phase and no decline in the red phase, it can be concluded that the azide-treated cells grew better than the control cells in the stress phase (red stage). It was expected that this reduction would be observed in the red phase. Azide is a known metabolic inhibitor in plants and algae with many potential targets. Azide binds irreversibly to the heme cofactor in cytochrome C oxidase and inhibits catalase and superoxide dismutase, which scavenge ROS. Therefore, it enhances oxidative stress (Zalogin and Pick, 2014). The seedlings developed from treated seeds of Eruca sativa with sodium azide showed wide variation in plant growth (Al-Qurainy, 2009). In this study, azide was used to induce mutation in plants grown from treated seeds. Indeed, growth and biochemical parameters reduction can be due to oxidative stress

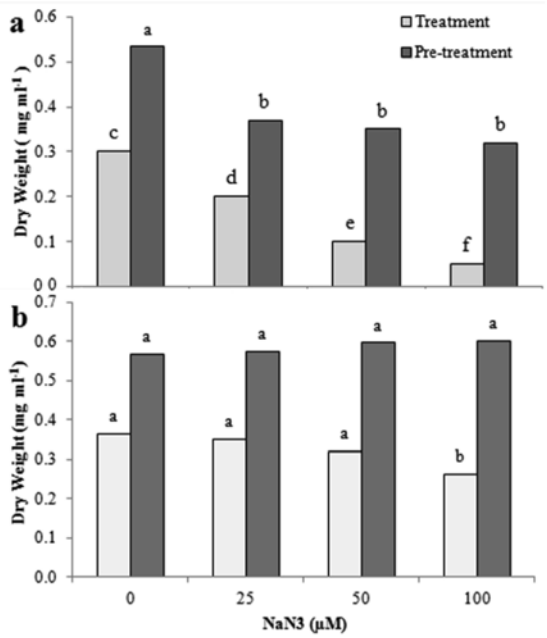

Fig. 2. Effect of azide treatment and pre-treatment on dry weight in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p< 0.05$ in one-way ANOVA and Duncan's tests

created by azide. It was reported that hydrogen peroxide production in *Anabaena nidulans* was increased many times by treatment with azide (Morales et al., 1992). The addition of azide had no significant effect on the cell biomass of *Anabaena* and *D. tertiolecta* (Chen et al., 2019; Nultsch et al., 1983). Contrary to our results, an experiment performed on different algae, reported that azide-treated cells had a higher rate of photosynthesis, more chlorophyll, and faster growth (Zalogin and Pick, 2014). Both treated and pre-treated algae with azide had lower amounts of chlorophyll a and b compared to control cells (Fig. 3). The lowest amount of chlorophyll a was observed in the treatment and pretreatment with 100 µM azide with a 50% decrease compared to the control.

The amount of chlorophyll b showed a 53 and 33% decrease in comparison to control in treatment and pretreatment by 100 µM azide, respectively. Similar to our results, a decrease in chlorophyll content decreased in *D. tertiolecta* treated with azide (Chen et al., 2019). Also, Al-Qurainy (2009) reported a significant decrease in chlorophyll content in Eruca sativa grown from seeds treated with azide.

Azide treatment had no significant effect on the carotenoid content of green cells (Fig. 4) . Pretreatment with an azide concentration of 25 µM resulted in a significant increase in carotenoid content, 28% increase compared to the pretreatment control and more than twofold compared to the treatment control.

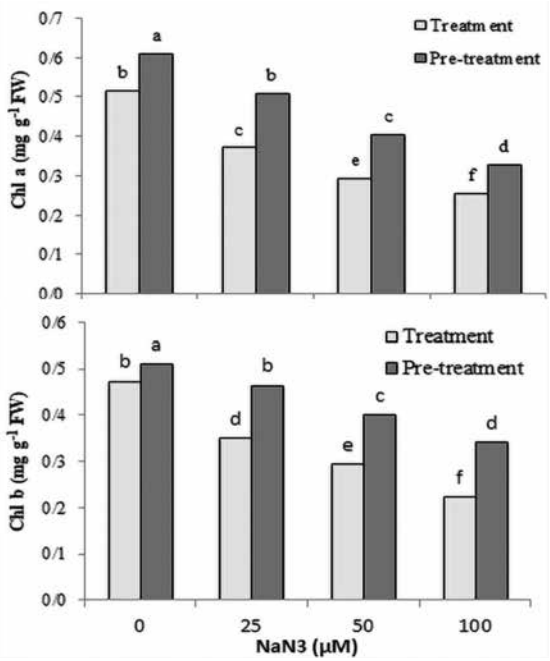

Fig. 3. Effect of azide treatment and pre-treatment on chlorophyll content in the green stage of *Haematococcus pluvialis*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

There was no significant difference between 50 and 100 µM concentrations of azide pretreatment, but these two concentrations significantly decreased the carotenoid level compared to the control. A decrease in the carotenoid content of Dunaliella tertiolecta and Eruca sativa was reported in cultures treated with azide (Al-Qurainy, 2009; Chen et al., 2019).

The results of the effect of treatment and pretreatment of azide on the carbohydrate content of green cells are shown in Figure 5. Although the number of carbohydrates significantly decreased at 100 µM azide, no significant difference was observed in carbohydrate content between the control, 25, and 50 µM azide treatments. In the pretreatment of sodium azide, the amount of carbohydrate in control and concentration of 25, 50, and 100 µM azide were 3.13, 2.35, 1.75, and 1.42 mg.g^{-1} FW, respectively. Carbohydrate content was reduced to half of the carbohydrate content in control by increasing azide concentration (by 100 µM azide).

Adding azide to the culture medium in the green phase for 14 days did not affect the carbohydrate content of cells in the red phase (Fig. 5). Azide pretreatment of cells in the green phase significantly reduced carbohydrate content in red cells. The effects of azide on the reduction of carbohydrate content were dose-dependent. The lowest amount of carbohydrate was observed in 100 µM azide with almost a 50% decrease compared to the control. Carbohydrate

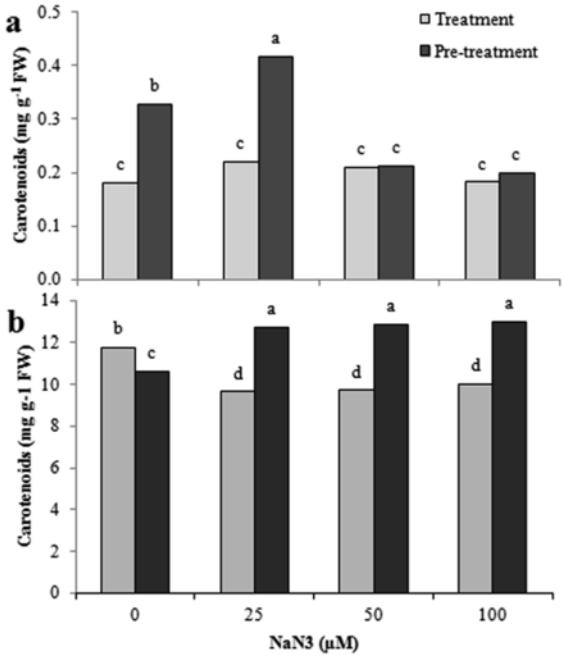

Fig. 4. Effect of azide treatment and pre-treatment on carotenoid content in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

reduction can be the result of the depletion of chlorophyll and photosynthesis rate. As a result, the inhibitory effect of azide on photosynthesis was proved in plants (Forti and Gerola, 1977). Besides, ATP synthesis decrease in the mitochondria due to the inhibitory action of azide is one reason for the decrease in the number of carbohydrates. The results showed that the amount of protein of green cells decreased in azide treatment and pretreatment (Fig. 6). The lowest protein content was observed in algae treated and pre-treated by 100 µM azide with 54 and 50% control respectively. The results of measuring the protein content of red cells *H. pluvialis* under sodium azide treatment showed that sodium azide treatment did not have a significant effect on the protein content of red cells. Sodium azide pre-treatment reduced the protein content of red cells significantly. The highest amount of protein was observed in pre-treatment red cells in the control group and the lowest amount of protein was observed in pre-treatment red cells at 100 µM concentration (50% reduction compared to control). As mentioned previously inhibitory effects of azide on nitrate reductase were confirmed (Zalogin and Pick 2014). This enzyme has a key role in nitrogen assimilation; therefore, reducing the amount of protein in the presence of azide is a predictable result.

Despite the negative effects of azide on the dry weight of cells in the green stage, the dry weight of the algae treated in the red stage was equivalent to the control sample. This shows that the treated cells show better performance than the control sample under red phase stress conditions. Azide with the concentrations used in this experiment showed negative effects on the biochemical parameters of the algae. Only the carotenoid content of green cells increased by 27% with 25 µM azide pretreatment and by 18% with

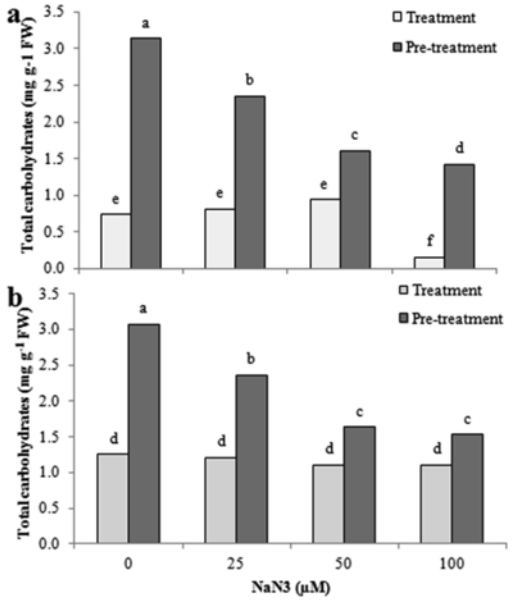

Fig. 5. Effect of azide treatment and pre-treatment on carbohydrate content in the green (a) and (b) red stage of *Haematococcus. pluvialis*. Values are means of

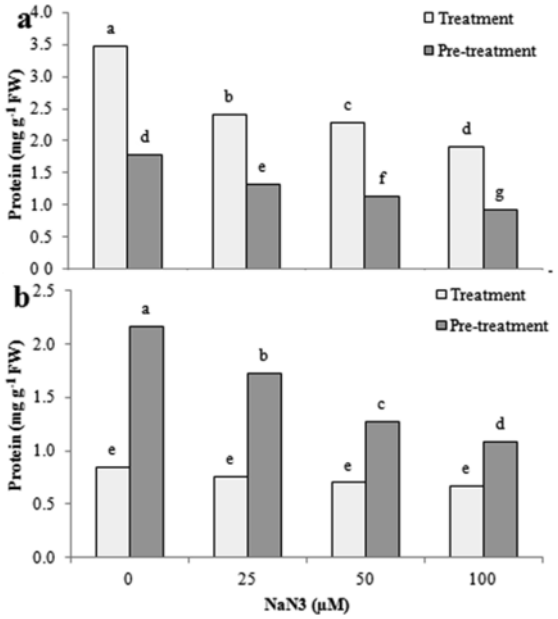

Fig. 6. Effect of azide treatment and pre-treatment on protein content in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

red pretreatment of all azide concentrations. Our results also suggested it may be that adding azide to induce carotenogenesis can be a good alternative for nitrogen deficiency conditions in this algae.

Acknowledgments

This work was carried out at Shahid Bahonar University of Kerman, Iran. There was no funding for this research.

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