

SHORT REPORT

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Enhancement of astaxanthin accumulation via energy reassignment by removing the flagella of *Haematococcus pluvialis*

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Abstract

Astaxanthin biosynthesis in *Haematococcus pluvialis* is driven by energy. However, the effect of the flagella-mediated energy-consuming movement process on astaxanthin accumulation has not been well studied. In this study, the profiles of astaxanthin and NADPH contents in combination with the photosynthetic parameters with or without flagella enabled by pH shock were characterized. The results demonstrated that there was no significant alteration in cell morphology, with the exception of the loss of flagella observed in the pH shock treatment group. In contrast, the astaxanthin content in the flagella removal groups was 62.9%, 62.8% and 91.1% higher than that of the control at 4, 8 and 12 h, respectively. Simultaneously, the increased Y(II) and decreased Y(NO) suggest that cells lacking the flagellar movement process may allocate more energy towards astaxanthin biosynthesis. This finding was verified by NADPH analysis, which revealed higher levels in flagella removal cells. These results provide preliminary insights into the underlying mechanism of astaxanthin accumulation enabled by energy reassignment in movement-lacking cells.

Keywords *Haematococcus pluvialis*, Astaxanthin, Flagella, Energy flow, pH-shock

Introduction

Natural astaxanthin, as a xanthophyll carotenoid, is one of the most powerful compounds with remarkable antioxidant activity and has been widely used in various fields, including medicine and food (Mahad et al., 2023). The chlorophyte microalgae *Haematococcus pluvialis* is

established as the richest source of natural astaxanthin due to its ability to accumulate a large amount of astaxanthin up to 4% of the total dry cell weight. Extreme environmental stress-induced conditions that induce the generation of reactive oxygen species have been extensively studied and well developed to promote the accumulation of natural astaxanthin, including high light, nitrogen starvation, high temperature, salt stress, and high carbon resources (Zhao et al. 2020).

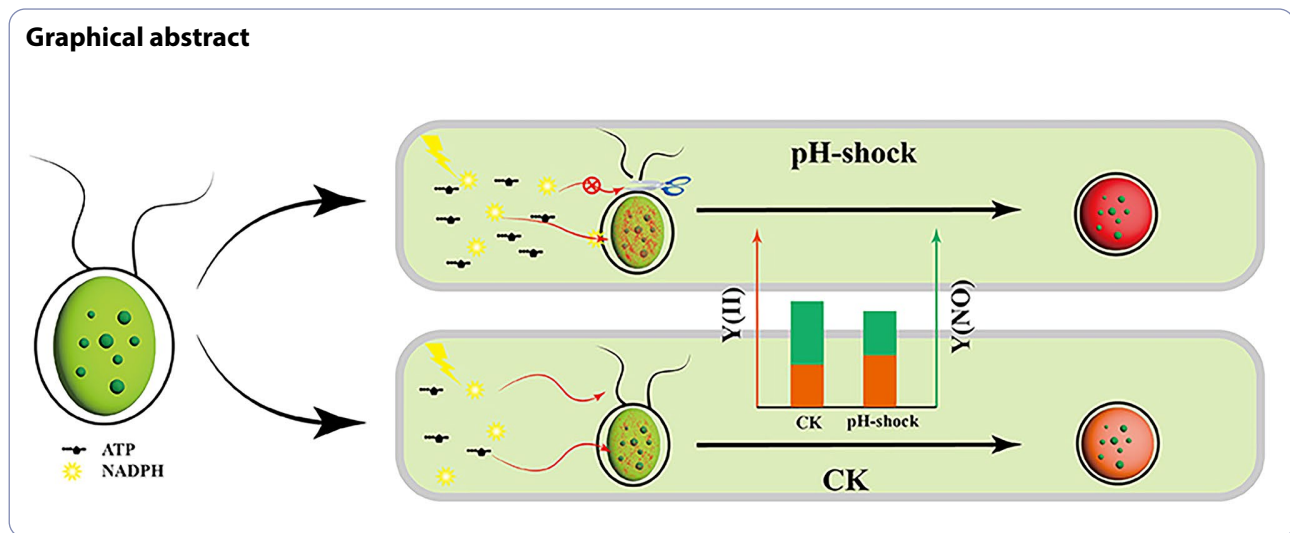
Reactive oxygen species (ROS) generated under environmental stress usually result in signal transduction and oxidative damage within the induction period. Multiple strategies have evolved to activate the antioxidant system, such as quenching ROS by biosynthesizing antioxidants such as astaxanthin and scavenging free radical substances to protect proteins and cells from

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damage (Li et al. 2021). Interestingly, the generation of an appropriate level of ROS facilitates the accumulation of carbohydrates by upregulating the activity of acetyl-CoA carboxylase (ACCase), and the enhanced carbon flux would be assigned to the production of astaxanthin (Li et al. 2023). Moreover, excessive carbon supply is another way to enhance carbon metabolism, thus facilitating astaxanthin production. It has been reported that 4.4 mM of acetate significantly enhanced the growth of *Haematococcus pluvialis*, but the cells were observed to contain a minimal amount of astaxanthin, resulting in a green color (Vidhyavathi et al. 2008). The addition of a higher concentration of acetate, up to 50 mM, resulted in the cells turning brown and the induction of carotenoid biosynthesis (Lv et al. 2022; Mansouri et al. 2022). Meanwhile, the expression of astaxanthin biosynthetic genes was determined to be upregulated in response to stress or high carbon supply, which provided an explanation for the observed biosynthesis of carotenoids (Vidhyavathi et al. 2008; Galarza et al. 2018; Mahadi et al. 2023).

Due to the high esterification level of astaxanthin molecules in *H. pluvialis*, both ATP and NADPH are required for the energy-intensive process of fatty acid biosynthesis, resulting in competition between the biosynthesis of astaxanthin and other physiological metabolic pathways (Harwood and Guschina 2009; Srinivasula et al. 2013; Xue et al. 2017; Zhao et al. 2020). Throughout the life cycle of *H. pluvialis*, a considerable quantity of carotenoids accumulated in conjunction with the cell type transformed from motile ones into red immotile spore cells by gradually losing the flagella (Li et al. 2019a, b; Feng et al. 2020; Zhang et al. 2023). These results prompted us to speculate that nonmotile cells without flagella may enhance astaxanthin biosynthesis by assigning more energy to the process than normal cells.

In analyzing cell motility, the majority of studies have concentrated on the well-established model organism *Chlamydomonas* (Sequeira et al. 2017; Boyd et al. 2018; Ahmad et al. 2022). With the protruding flagella from the cell surface, *Chlamydomonas* cells could swim towards or away from the light source enabled by the contraction and extension of microtubules, which highly relies on the efficient conversion of energy from ATP into mechanical work. Compared to extensive studies on *Chlamydomonas*, limited discussion has been reported on flagellar movement and its effect on astaxanthin accumulation in *H. pluvialis*. A previous study primarily tried to combine various conditions, including the addition of a high amount of carbon source and the removal of flagella to promote astaxanthin accumulation (Aflalo et al. 2007; Srinivasula et al. 2013; Han et al. 2020; Zhang et al., 2019). However, the underlying mechanism remains to be uncovered regarding the potential role of flagella removal in modulating astaxanthin biosynthesis.

In this study, rapid flagella removal in *Haematococcus* was carried out by pH shock and then used for further study after washing. The light energy absorbed by cells lacking flagella has not been negatively affected based on the results of photosynthetic activity measurements. The intracellular NADPH levels increased upon flagella removal, which would facilitate astaxanthin biosynthesis alongside the energy previously consumed by flagella-mediated movement. Thus, the present study provides new insight into the underlying mechanism of astaxanthin accumulation enabled by energy reassignment in motility-lacking cells in *H. pluvialis*. These findings will facilitate the improvement of astaxanthin accumulation, thereby enabling the development of more effective strategies to tackle future challenges related to natural products.

Materials and methods

Strain and culture conditions

The *Haematococcus pluvialis* (HA-3) strain used in this study was maintained at Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences (Zhao et al. 2020). The algae seeds were cultivated to logarithmic phase with two flagella as previously described (Liu et al. 2020). The cells were concentrated by centrifugation at 5000 rpm for 5 min and washed twice with 10 mM HEPES pH 7.4. Then the cells were transferred into a small beaker with stirring. The pH value of the medium was adjusted rapidly to 4.0 by the dropwise addition of 0.5 N acetic acid and incubated for 30 s, followed by neutralization to pH 7.0 through the dropwise addition of 0.5 M KOH (Luo et al. 2011; Han et al. 2020). The green vegetative cells were then collected by centrifugation at 3000 rpm for 5 min. Subsequently, the cell pellets were washed twice with sterile water and transferred to BG-11 medium with an initial cell density of 0.5×10^5 cells mL⁻¹ under continuous light of 120 μ mol photons m⁻²s⁻¹ at 25 °C. HA-3 cells were harvested every 4 h for morphological observation, pigment analysis and fluorescence parameter analysis.

Morphological observation and pigment determination

The cells harvested at the designated time points were used for the observation of morphological differences using a microscope with a camera (BX43F, COLYMPUS). For the determination of pigment contents, cells were harvested at 5000 rpm for 5 min. Then, the pellets were mixed with 1 mL of 5% KOH:30% CH₃OH and incubated at 75 °C for 5 min to degrade chlorophyll. After centrifugation at 10,000 rpm for 5 min, the pellets were subjected to a 5-minute water bath at 75 °C with the addition of 1 mL of dimethylsulfoxide (DMSO) and 0.05 mL of acetic acid until the pellets became colorless. Astaxanthin content was determined in a spectrophotometer at a wavelength of 490 nm and calculated as described in a previous study (Zhao et al. 2020). To determine chlorophyll content, cell pellets were ground using liquid nitrogen. Then, 2 mL of methanol was added to the samples, and the mixture was incubated at 4 °C for 24 h. The OD663 and OD645 values were measured using spectrophotometry (UV-1800PC, Shanghai Mapada Instrument., LTD) and then used for the calculation of chlorophyll contents according to the following formula (Ma et al. 2023), where V represents the volume of microalgae for pigment extraction.

$$\text{Chl a (mg/L)} = (12.7 \times \text{OD}_{663} - 2.69 \times \text{OD}_{645}) \times 2 / V;$$

$$\text{Chl b (mg/L)} = (22.9 \times \text{OD}_{645} - 4.86 \times \text{OD}_{663}) \times 2 / V;$$

$$\text{Chl (mg/L)} = \text{Chl a} + \text{Chl b}$$

Measurement of chlorophyll fluorescence parameters and NADPH contents

An IMAGING-PAM fluorometer (Walz, Germany) was used to measure chlorophyll fluorescence parameters according to the manufacturer's instructions. The maximum photochemical efficiency (Fv/Fm), actual photosynthetic efficiency of photosystem II (Y(II)), the effective quantum yield (Y(NO)) and the quantum yield of regulatory energy dissipation (Y(NPQ)) were determined. To quantify the NADPH content, cells were frozen in liquid nitrogen, ground at 4 °C, and then the NADPH content was measured by using an NADPH Assay Kit (S0179, Beyotime).

Statistical analysis

For the statistical analysis, each value was evaluated using three biological replicates. Values are reported as the mean \pm standard deviation. Data analysis was performed by one-way ANOVA using SPSS Statistics software. A significant difference was considered at the level of $p < 0.05$.

Results and discussion

Establishment of a pH shock method to remove flagella in *H. pluvialis*

The pH shock method has been employed to study flagellar motility in *Chlamydomonas* (Tian et al. 2009). To clarify the possible effect of flagellar motility on astaxanthin biosynthesis in *H. pluvialis*, we implemented and repeated the pH shock protocol as reported in a previous study (Luo et al. 2011). The flagella of *H. pluvialis* were indeed removed after pH shock compared to those in the control group (Fig. 1). Both the extracellular matrix and cell type were observed with no significant difference between the control and treatment groups, as transparent extracellular matrix and green oval cells were detected in both groups. However, the extracellular matrix of the treated cells narrowed, and the cells were detected with a light brown color at 4 h after pH shock (Fig. 1). Then, the cells underwent a change in color, becoming darker brown at 8 h, and immotile spore cells were observed in the experimental group, indicating the activation of astaxanthin biosynthesis (Zhao et al. 2020). Based on the established pH shock method here, we analyzed the potential impact of flagellar motility on astaxanthin biosynthesis in *H. pluvialis*.

Astaxanthin biosynthesis is enhanced by flagella removal

To assay the possible effect of flagella removal on pigment biosynthesis, we measured chlorophyll and astaxanthin contents at the set time points. As shown in Fig. 2A, chlorophyll contents were decreased in the treatment group compared to those in the control group. These results indicated that either chlorophyll biosynthesis is

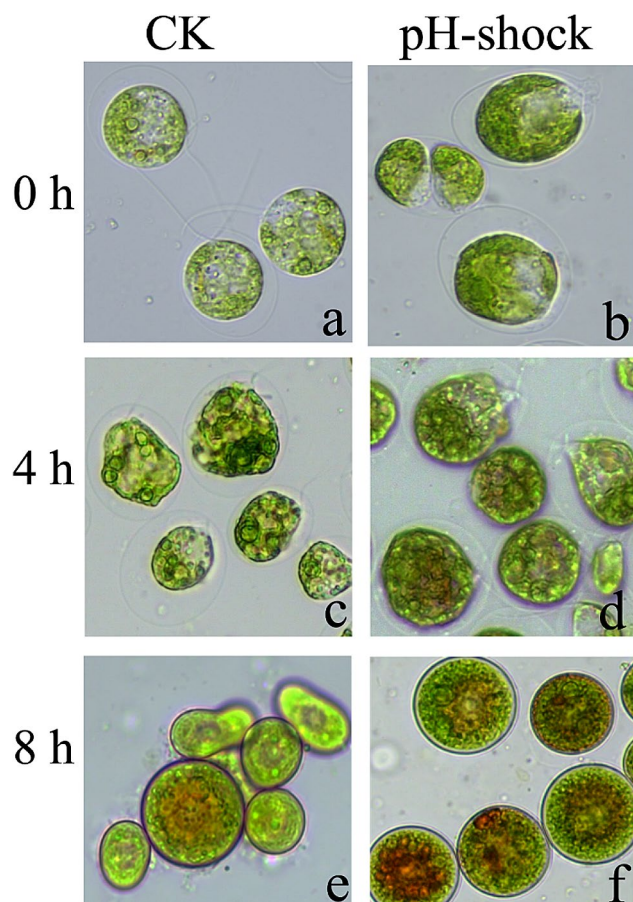


Fig. 1 The morphological changes of control and flagella removal cells at three distinct time points (0, 4, and 8 h). Representative photos from each experiment are presented here

inhibited or chlorophyll degradation is activated by pH shock. However, astaxanthin was significantly increased by 62.9%, 62.8% and 91.1% after flagella removal compared to the control samples at 4, 8 and 12 h, respectively (Fig. 2B). These results suggest that astaxanthin biosynthesis was activated and that the energy flux had been adjusted in favor of its biosynthesis by the treatment. As ATP is the energy resource for motility, flagella removal may benefit the energy requirements of the astaxanthin biosynthetic process (Ginger et al. 2008).

NADPH may be induced as the key factor for astaxanthin biosynthesis by flagella removal

NADPH is an essential cofactor for both antioxidant defense and reductive biosynthesis (Yang et al. 2023), and its availability may be a key factor in regulating carbon flux to astaxanthin biosynthesis. Thus, modulation of NADPH homeostasis might be an effective strategy to enhance natural products.

Since parts of the astaxanthin biosynthetic pathway are located in chloroplasts, NADPH could be generated through photosynthesis. To further investigate the impact

of flagella removal on NADPH production, we measured both chlorophyll fluorescence parameters and NADPH contents. As shown in Fig. 2C, the F_v/F_m and $Y(II)$ values in the experimental group increased by 9.2% and 28.3% compared to those in the control group at 8 h, respectively, indicating improvement in both maximum and actual photochemical efficiency. The pH-shocked treatment seems to have the potential to moderately impact cellular photosynthetic activity, leading to an increase in NADPH generation. Considering the elevated levels of astaxanthin observed in the treatment group and the fact that astaxanthin's primary structure is carbon-based, it can be speculated that more carbon flux and NADPH may have been assigned to the biosynthesis pathway of astaxanthin following flagella removal compared to the control group. Additionally, a higher $Y(NPQ)$ indicated that the cells in the pH-shock group possessed a superior ability to maintain photoprotective ability compared to those in the control (Approbato et al. 2023; Gebara et al. 2023). Similarly, the experimental group exhibited a lower $Y(NO)$ value, which suggested that flagella removal may favor the cells to protect themselves. Therefore, carbon flux and energy assignment could have been adjusted to favor astaxanthin biosynthesis by flagella removal, which is the main energy-consuming process under normal conditions.

To validate this hypothesis, we further determined the amount of available NADPH in cells. The intracellular NADPH of the flagella removal group was 15.6% and 26.1% higher than those in the control at 4 h and 8 h, respectively (Fig. 2D). Simultaneously, the higher NADPH could result in more ROS generation, which in turn prompted the super antioxidant biosynthesis to scavenge free radicals (Li et al. 2023; Yang et al. 2023). The yielding NADPH could be constantly used for astaxanthin accumulation in *H. pluvialis* over time, thus resulting in a decreased level after 12 h. As a result, the astaxanthin contents in both groups showed increased profiles. In particular, the astaxanthin content of the pH shock group was 91.1% higher than those in control (Fig. 2B). Correspondingly, the total amount of NADPH was 30.3% lower in the pH shock group than in the control group at 12 h, suggesting its participation in astaxanthin accumulation. Therefore, removal of flagella could favor astaxanthin biosynthesis by generating more ATP and NADPH.

Conclusion

To dissect the possible effect of cell motility and elucidate the mechanism of astaxanthin biosynthesis, we developed a flagella removal method for *H. pluvialis*. The removal of flagellar motility, which consumes both ATP and NADPH, allows for the reassignment of carbon and energy flux to the biosynthesis of natural products such

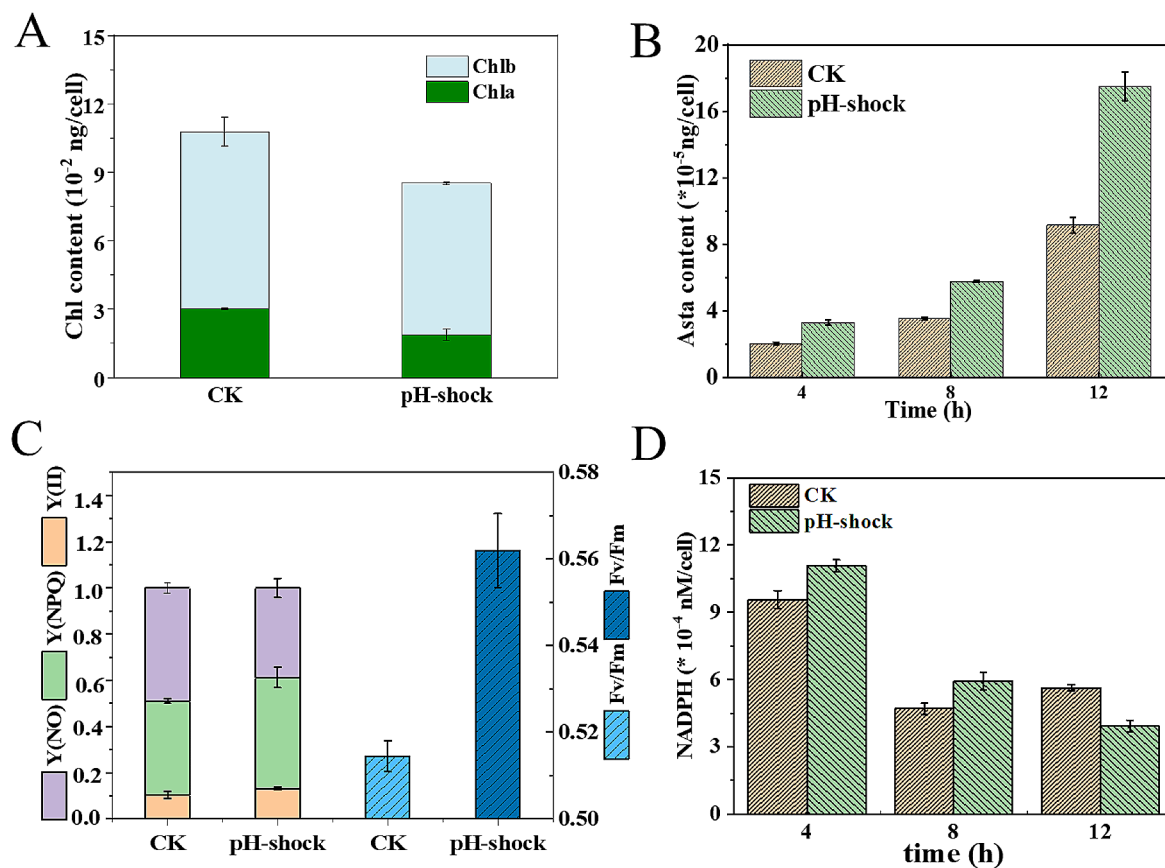


Fig. 2 The effect of flagellar removal on chlorophyll contents (A), astaxanthin contents (B), chlorophyll fluorescence parameters (C) and NADP⁺/NADPH content (D) after different hour-treatments in *H. pluvialis*. Chlorophyll contents measurements were carried out after eight hour-treatment. Chl, chlorophyll; Asta, astaxanthin

as astaxanthin. The study here provides preliminary clues to the underlying mechanism of astaxanthin accumulation enabled by flagella removal. These findings will favor the improvement of astaxanthin accumulation by biotechnological efforts to tackle future challenges.

Author contributions

Conceptualization: L.Z., L.Y., W. W., and Y.H. Investigation: Y.H., Z.G., Z.L., S.Y., M.C., F. C. Visualization: Y.H. and Z.G. Funding acquisition: Y.H. and L.Z. Writing—original draft and review & editing: Y.H., Z.G., W.W., L.Y. and L.Z. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its additional files. The authors are willing to provide any additional data and materials related to this research that may be requested for research purposes.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read this article and have approved its submission to *Bioresources and Bioprocessing*.

Competing interests

The authors declare that they have no competing interests.

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