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A new approach to promote astaxanthin accumulation via Na_2WO_4 in *Haematococcus pluvialis**

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Abstract The optimum concentration of Na_2WO_4 was explored in relation to the cell density and astaxanthin content in *Haematococcus pluvialis*. Then, the cellular morphology, nitrate reductase (NR) activity, soluble sugar and protein contents, and chlorophyll fluorescence were measured, and the transcriptional expression of carotenogenic genes was determined by quantitative real-time PCR. The results showed that 3.0 mmol/L of Na_2WO_4 was the optimum concentration to induce astaxanthin accumulation, with a maximum content of 49.41±0.13 pg/cell reached on the tenth day. The NR activity decreased significantly and continually after Na_2WO_4 treatment. The soluble sugar content increased gradually during the experimental period and was eventually significantly higher than that in the control. The soluble protein content increased rapidly, reached a maximum in day 0.5 and day 1 and then decreased. The effective photochemical efficiency of PSII (F_v'/F_m') and light saturation (E_k) first decreased and then tended to stabilize, and $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was correlated with photosynthesis. The transcriptional expression of *ipi*, *psy* and *bkt* was significantly increased compared with that in the control after application of Na_2WO_4, and the relative expression of *ipi* reached the highest level on the fifth day, with a 98.03±1.92-fold increase. Our results describe a new approach to promote the effective accumulation of astaxanthin in *H. pluvialis* by NR inhibitor Na_2WO_4.

Keyword: *Haematococcus pluvialis*; astaxanthin; Na_2WO_4; nitrate reductase; quantitative real-time PCR

1 INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione) is a keto-carotenoid with high antioxidant potency, which showed beneficial effects on human health and preventing diseases, such as diabetes, cardiovascular disease, cancer, and showed the effects of immuno-modulation and anti-aging activity (Yamashita, 2013). The U.S. Food and Drug Administration (FDA) and European Commission has approved the security of natural astaxanthin (Pashkow et al., 2008; Ambati et al., 2014), furthermore, it has been marketed as a new food resource for approximately 10 years without any adverse effects by the Ministry of Health, China. Astaxanthin has an estimated market of USD $2.57 billion by 2025 with irresistible business opportunity (Algae Industry Magazine, 2017).

The unicellular green alga *Haematococcus pluvialis* shows its highest astaxanthin content under different environmental stresses, including nitrogen deficiency, high light, or exposure to chemical regulators, such as ferrous sulfate and sodium acetate (Boussiba, 2000; Han et al., 2013). The astaxanthin content can be up to 4% of the dry weight (Lee and Ding, 1994; Ranga et al., 2009). Astaxanthin is stored in cytosolic lipid bodies in the form of monoesters or

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diesters (Ambati et al., 2013), which may enhance the stability, bioavailability and antioxidant properties of astaxanthin (Barbosa et al., 1999). The astaxanthin synthesis pathway in *H. pluvialis* is as follows. First, isopentenyl pyrophosphate (IPP) is synthesized from pyruvate and glyceraldehyde-3-phosphate by the non-mevalonate DOXP/MEP (Deoxyxylulose-5-phosphate) pathway (Disch et al., 1998). Then, isopentenyl diphosphate isomerase (Ipi), phytoene synthase (Psy) and so on catalyze the reaction from IPP to β-carotene. Finally, carotenoid ketolase (Bkt) and carotenoid hydroxylase catalyze the conversion of β-carotene to astaxanthin (Li, 2007).

Nitrate starvation is an effective method to increase the accumulation of astaxanthin, which can lead to extremely low levels of nitrate reductase (NR) activity and excess production of reactive oxygen species (ROS) in *H. pluvialis* (Dong et al., 2006; Vidhyavathi et al., 2008). For practical production, transferring large amounts of algae mass is difficult under nitrogen-deficient conditions; thus, a new approach to induce intracellular nitrate starvation in *H. pluvialis* is needed. Sodium tungstate (Na_2WO_4) is a NR inhibitor that significantly inhibits NR activity in the microalgae *Nannochloropsis oculata* and *Isochrysis galbana* (Shen et al., 2011; Fan, 2012). One possible mechanism for this action is that tungsten (W) can replace the molybdenum (Mo) in NR structure, which is the key element involved in the electron transfer process (Yu et al., 2010). Furthermore, Na_2WO_4 may induce metal stress in *H. pluvialis*. Because the effects of Na_2WO_4 in *H. pluvialis* have not been reported, we tested the effects and optimum concentration of Na_2WO_4 on astaxanthin accumulation in *H. pluvialis*. To elucidate the mechanism underlying astaxanthin accumulation in response to Na_2WO_4, we evaluated the cellular morphology, NR activity, soluble sugar and protein contents, and chlorophyll fluorescence of *H. pluvialis* at the optimum Na_2WO_4 concentration and examined the transcriptional expression of carotenogenic genes by quantitative real-time PCR.

2 MATERIAL AND METHOD

2.1 Algae culture and treatment

Haematococcus pluvialis was obtained from the Algae Collection Lab of Ningbo University and cultured in 250 mL Erlenmeyer flasks with 150 mL of NBU3# medium under a light intensity of 25 μmol/(m^2·s) on a 12 h:12 h light:dark cycle at 24°C. The NBU3# medium was composed of the following components (in mg/L): KNO_3, 100; K_2HPO_4, 10; $MnSO_4$, 0.25; $FeSO_4 \cdot 7H_2O$, 2.5; Na_2EDTA, 20; VB_{12}, 5×10^{-7}; and VB_1, 5×10^{-6} (Wang et al., 2014).

To explore the optimum concentration of Na_2WO_4, cells in the middle exponential stage were exposed to 1.0, 2.0, 3.0 and 4.0 mmol/L of Na_2WO_4 separately under continuous levels of high irradiance at 80 μmol/(m^2·s) (Lv et al., 2016). Algae were collected on days 5 and 10 to examine the astaxanthin content and cell density. Samples without Na_2WO_4 were used as the controls. The cell numbers were counted using a plankton counting chamber.

Haematococcus pluvialis cells in the exponential stage were exposed to an optimum concentration of Na_2WO_4 under continuous irradiance at 80 μmol/(m^2·s). The cells were harvested after treatment for 0 d, 0.5 d, 1 d, 3 d and 5 d, and the samples were used to examine the cellular morphology, NR activity, soluble sugar and protein contents, chlorophyll fluorescence, and carotenogenic gene expression.

2.2 Morphological observations and astaxanthin determination

A 400× optical microscope was used for the morphological observations and photography. The astaxanthin content was extracted and measured according to the methods of Boussiba et al. (1992). Algae cells were harvested and treated with a 5% KOH solution in 30% methanol to degrade the chlorophyll. Then, astaxanthin was extracted with dimethylsulfoxide (DMSO) three times until the precipitate became colorless. The absorbance of the extracts was determined at 490 nm with a spectrophotometer (Metash UV-6100A, China). The blank contained only DMSO.

2.3 Nitrate reductase activity and soluble sugar and protein contents determinations

The samples were frozen in liquid nitrogen and then ground completely into a fine powder. The powder was transferred and diluted to 2.0 mL. After centrifugation, the supernatant was used to determine the NR activity and the soluble sugar and protein contents. The NR activity was measured with a NR assay kit (Suzhou Comin Biotechnology, Suzhou, China), the soluble sugar was determined using the anthrone-sulfate method, and the soluble protein was determined using the Bradford method (Li, 2000).

Table 1 Quantitative real-time PCR genes and primers

Gene name	Primer sequence (5′→3′)	Products sizes (bp)	GenBank ID
GAPDH	Forward: TGCCACCACCTTGACCAT Reverse: CATCCAGACCGAGAAGAAGAC	198	SRX1136559
Isopentenyl pyrophosphate (*ipi*)	Forward: GCGAGCACGAAATGGACTAC Reverse: GCTGCATCATCTGCCGCA	181	AF082325
phytoene synthase (*psy*)	Forward: CGATACCAGACCTTCGACG Reverse: TGCCTTATAGACCACATCCAT	129	AF305430
beta-carotenoid ketolase (*bkt*)	Forward: AACAAGCGGAAGGCAGAC Reverse: GGAGCACCACAATCACACT	225	X86782.1
18*S* rRNA	Forward: CCGTCGTAGTCTCAACCAT Reverse: CCTTCCGTCAATTCCTTTA	149	KY364700.1

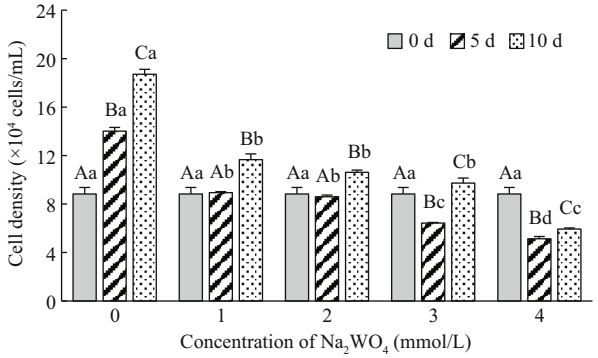

Fig.1 The effects of different concentrations of Na_2WO_4 on the cell density of *H. pluvialis*

Different uppercase letters among the different treatment times indicate significant differences (P<0.05). Different lowercase letters among the different Na_2WO_4 concentrations indicate significant differences (P<0.05). The same as that in the blow.

2.4 Chlorophyll fluorescence determination

Chlorophyll fluorescence was obtained using the Water-PAM (Walz, Germany). The samples were incubated in the dark for 15 min at 24°C. F_v'/F_m' was obtained from the induction curve under culture light intensity. The relative electron transport rate (rETR) was measured by applying a sequence of increasing actinic irradiance; each actinic light incubation lasted for 10 s (White et al., 2011). E_k was obtained from the curve-fitting model according to the method of Eilers and Peeters (1988).

2.5 RNA isolation and quantitative real-time PCR

The samples were frozen in liquid nitrogen and then ground into a fine powder. Total RNA was extracted using the Plant Total RNA kit (OMEGA, USA) according to the user manual. The cDNA used for the quantitative real-time PCR was synthesized from the total RNA using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech; China).

The gene-specific primers were designed using the Primer Premier 6 software (Table 1). Then, the PCR products were quantified with a real-time PCR system (Eppendorf, Germany) using SYBR green fluorescence. The PCR amplification profiles were 94°C for 30 s, followed by 40 cycles of 95°C for 5 s, 58°C for 15 s, and 72°C for 10 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the quantitative real-time PCR data (Livak and Schmittgen, 2001), and samples collected at 0 d were used as the control group.

2.6 Statistical methods

All experiments were conducted in triplicate, and the data were analyzed with one-way ANOVA (SPSS 22.0). Duncan's test was used to test differences among the groups from different trials, and p-values less than 0.05 were considered significantly different.

3 RESULT

3.1 Optimum Na_2WO_4 concentration

The cell densities of *H. pluvialis* after treatment with different Na_2WO_4 concentrations were shown in Fig.1. The cell density in the control group increased continually, with the maximum of 18.72×10^4 cells/mL obtained at 10 d. The cell density nearly stabilized after application of 1.0 and 2.0 mmol/L of Na_2WO_4, decreased significantly with the application of 3.0 and 4.0 mmol/L of Na_2WO_4 from days 0 to 5 (P<0.05), and increased again by day 10 in the 1.0, 2.0, 3.0 mmol/L, but there are no significantly different between them (P<0.05); treatment with 4.0 mmol/L of Na_2WO_4 resulted in the lowest cells density on the day 5 and 10. The astaxanthin content in the motile cells at day 0 was extremely low at 5.12 pg/cell (0.45 mg/L) but increased significantly after treatment with Na_2WO_4 (Fig.2). The value reached a peak at day 10 of 49.41 pg/cell (4.81 mg/L) after application of

3.0 mmol/L of Na_2WO_4; this astaxanthin concentration was 1.94-fold higher than the control. In summary, 3.0 mmol/L of Na_2WO_4 was the optimum concentration to induce the accumulation of astaxanthin in *H. pluvialis*.

3.2 Morphological observations

Changes in the cell morphology of *H. pluvialis* after exposure to Na_2WO_4 at the optimal concentration are shown in Fig.3. On the first day, most of these cells were round or oval motile cells with two flagella growing from the back of the protoplasts and extending through the periplasm to the cell wall (Fig.3a). After treatment with 3 mmol/L of Na_2WO_4 for 0.5 d, the algae cells progressively changed from motile cells to non-motile cells, accompanied by abscission of the flagella, a more uniform morphology, and disappearance of the periplasmic space (Fig.3b). At day 3, the algae color changed from green to red, which indicated that astaxanthin had accumulated in the cells. Meanwhile, the cell inclusions became more compact, and the cell wall tended to be thicker (Fig.3c). After 5 days of treatment, the chloroplasts were pushed to the edges of the cells, most of the area was filled with astaxanthin, and the cell wall was visible and thick (Fig.3d). We continued application of Na_2WO_4 to 10 d; microscopic observation showed that the algae cells became totally red and were obviously enlarged, which were typical characteristics of akinetes (Fig.3e).

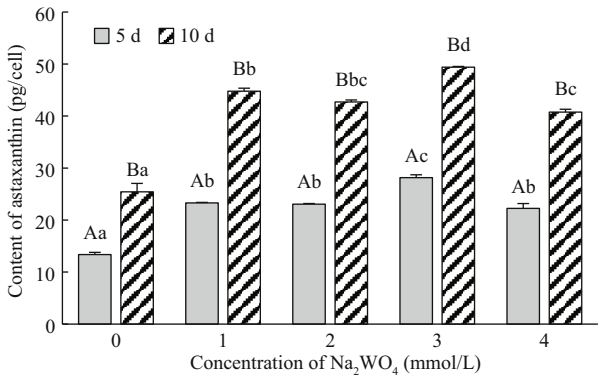

Fig.2 The effects of different concentrations of Na_2WO_4 on the astaxanthin content of *H. pluvialis*

3.3 Nitrate reductase activity and soluble sugar and protein contents

Haematococcus pluvialis cells in the exponential stage were exposed to 3 mmol/L of Na_2WO_4 under continuous irradiance at 80 μmol/(m^2·s). The NR activity was increased significantly in the control cells at day 0.5 and then tended to decline slowly. The NR activity in the treatment cells decreased significantly at day 0.5 and was much lower than the activity in the control at all experimental time points (P<0.05) (Fig.4), which indicated that Na_2WO_4 significantly inhibited NR activity.

The soluble sugar content is shown in Fig.5a. At 0.5 d, the soluble sugar content in both groups was significantly increased (P<0.05). The control group maintained a stable level through the subsequent timeframe, but the soluble sugar content in the treatment cells increased gradually and reached levels that were significantly higher than the level in the control cells. The maximum soluble sugar content was 2.23×10^{-5} μg/cell at day 5. The soluble protein content in the both groups increased rapidly and reached maximums of 7.12×10^{-6} μg/cell and 7.51×10^{-6} μg/cell at day 1, respectively (Fig.5b). Then, the soluble protein contents significantly decreased in both groups from 3 to 5 d (P<0.05), although the content declined more slowly in the treatment cells than in the control.

3.4 Photosynthesis and GAPDH gene expression

F_v'/F_m' reflects the actual photosynthetic efficiency of the algae and can be used to characterize the light-dependent photosynthesis reactions. Within the first 3 days, the F_v'/F_m' was lower in the treatment cells than that in the control, and the F_v'/F_m' in the treatment cells then remained stable from days 3–5 (Fig.6a). The results showed that Na_2WO_4 decreased the

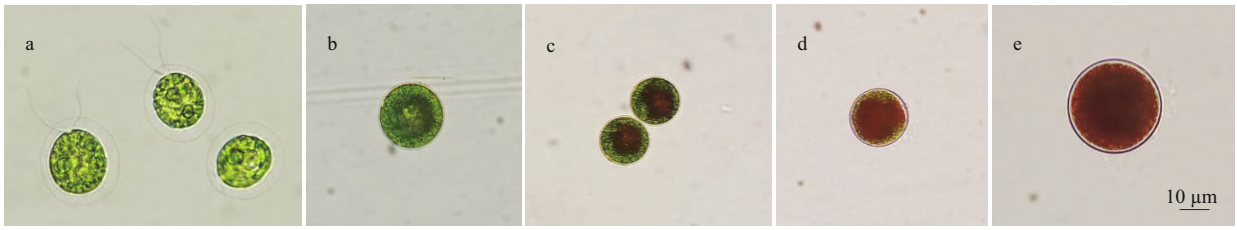

Fig.3 The morphology of *H. pluvialis* treated with 3.0 mmol/L of Na_2WO_4 (×400 microscope)

a. motile cells; b. non-motile cells; c. cells treated for 3 d; d. cells treated for 5 d; e. akinete.

photosynthetic efficiency, although the rapid accumulation of astaxanthin might protect the photosynthetic machinery.

E_k represents the minimum saturating irradiance and interrelates with the maximum photosynthetic rate of the algae (Ralph and Gademann, 2005). The initial E_k was 558.87 μmol/(m²·s), and the E_k of both groups declined after treatment with 3 mmol/L of Na_2WO_4 and continuous irradiance. At 0.5 d, the E_k of the treatment groups decreased rapidly to 36.19% of the initial value and then tended to stabilize (Fig.6b).

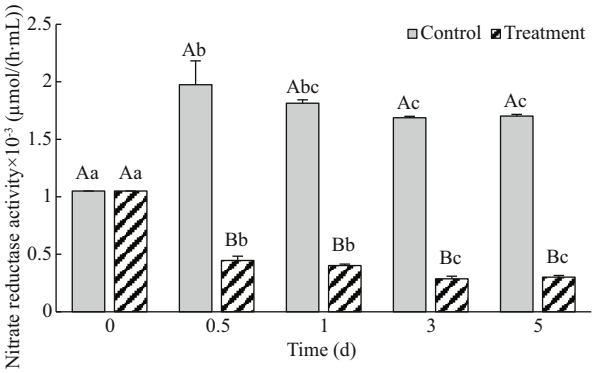

Fig.4 The effects of Na_2WO_4 on nitrate reductase (NR) activity of *H. pluvialis*

Treatment: 3.0 mmol/L of Na_2WO_4; control: without Na_2WO_4. The same as that in the blow.

The E_k of the treatment groups was always lower than that of the control, and the inducing irradiance (80 μmol/(m²·s)) was still within the E_k range. The results showed that Na_2WO_4 decreased the E_k of *H. pluvialis*.

GAPDH is a key enzyme in the Calvin cycle (dark reactions of photosynthesis). GAPDH gene expression was down-regulated in both groups (Fig.7). The treatment cells expression level was lower than the control at day 0.5 to 3 and then increased significantly at day 5 (P<0.05), which corresponded to the trend observed for F_v'/F_m'. These results revealed that GAPDH gene expression was affected by Na_2WO_4.

3.5 Transcription of carotenogenic genes

Ipi is the initial and key enzyme involved in carotenoid biosynthesis. This enzyme catalyzes the reversible isomerization reaction between IPP and dimethylpropyl pyrophosphate (DMAPP) (Wouters et al., 2003). As shown in Fig.8a, *ipi* expression was significantly lower in the control cells than in the treatment cells (P<0.05), and the peak transcriptional of 54.01-fold occurred on day 5 due to the effect of light (80 μmol/(m²·s)). In the treatment group, *ipi* expression increased continually and reached a peak on day 5 of 98.03-fold (P<0.05), which was 1.82 times

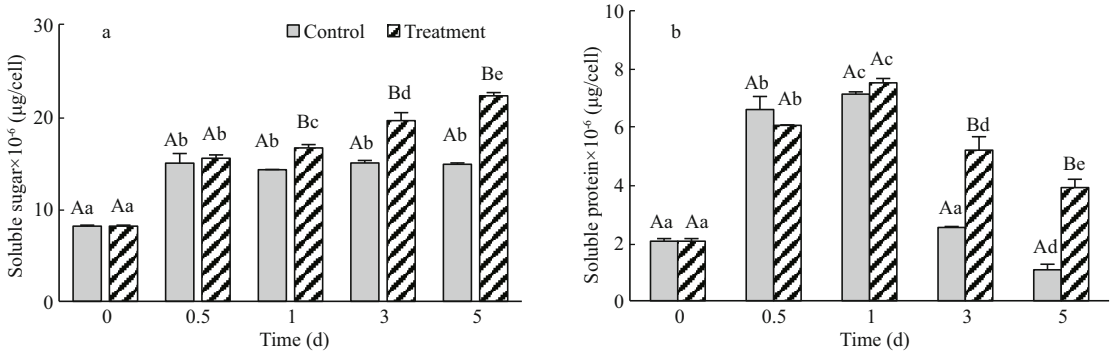

Fig.5 The effects of Na_2WO_4 on the soluble sugar (a) and protein (b) contents of *H. pluvialis*

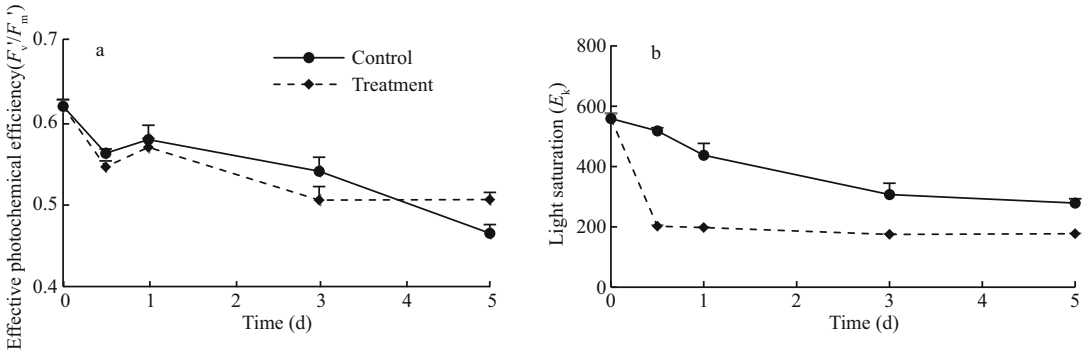

Fig.6 The effects of Na_2WO_4 on F_v'/F_m' (a) and E_k (b) of *H. pluvialis*

higher than the peak of the control. The results showed that high light levels promoted *ipi* expression and that the addition of Na_2WO_4 accelerated the effect remarkably.

Psy is a rate-limiting enzyme in carotenoid biosynthesis (Liang et al., 2006). After induction with high light and Na_2WO_4, the *psy* expression levels were down-regulated in both groups (Fig.8b), but the expression level was significantly higher in the treatment cells than in the control from days 0.5 to 5 (P<0.05). No significant change was observed in the treatment cells at days 0.5 to 3 (P>0.05), which was followed by a decrease in the expression level that was possibly due to feedback by astaxanthin. The results indicated that *psy* expression was down-regulated when the algae were exposed to high light conditions and that the addition of Na_2WO_4 increased the expression of this gene significantly.

Bkt participates in the final step of astaxanthin synthesis (Huang et al., 2006). As shown in Fig.8c, the *bkt* expression level was down-regulated in the control cells and was significantly lower than that in the treatment cells (P<0.05). In the treatment groups, the initial increase and maximum transcriptional expression of *bkt* occurred on day 3 with a 1.59-fold increase. The results indicated that the addition of Na_2WO_4 significantly promoted *bkt* expression, especially after 3 days of treatment.

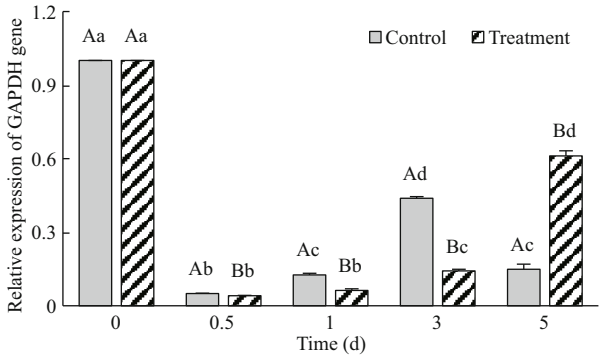

Fig.7 The effects of Na_2WO_4 on GAPDH gene expression in *H. pluvialis*

4 DISCUSSION

Na_2WO_4 is mainly used for dyes, electroplating, fabric, as a cellulose fire retardant, and as a catalyst but is also known for its anti-diabetic effects. Researchers have identified the pathways through which sodium tungstate improves pancreatic function and beta cell proliferation (Domínguez et al., 2003). Na_2WO_4 is a typic NR inhibitor for plant experiment, as well we in microalgae. For example, Shen et al. (2011) used it in *Nannochloropsis oculata* for fatty acid accumulation, and Fan (2012) reported that Na_2WO_4 could promote the lipid accumulation in *Isochrysis galbana*. In this study, Na_2WO_4 was used to induce astaxanthin accumulation in *H. pluvialis* through inhibition of NR activity. The cellular morphology changed obviously when algae were

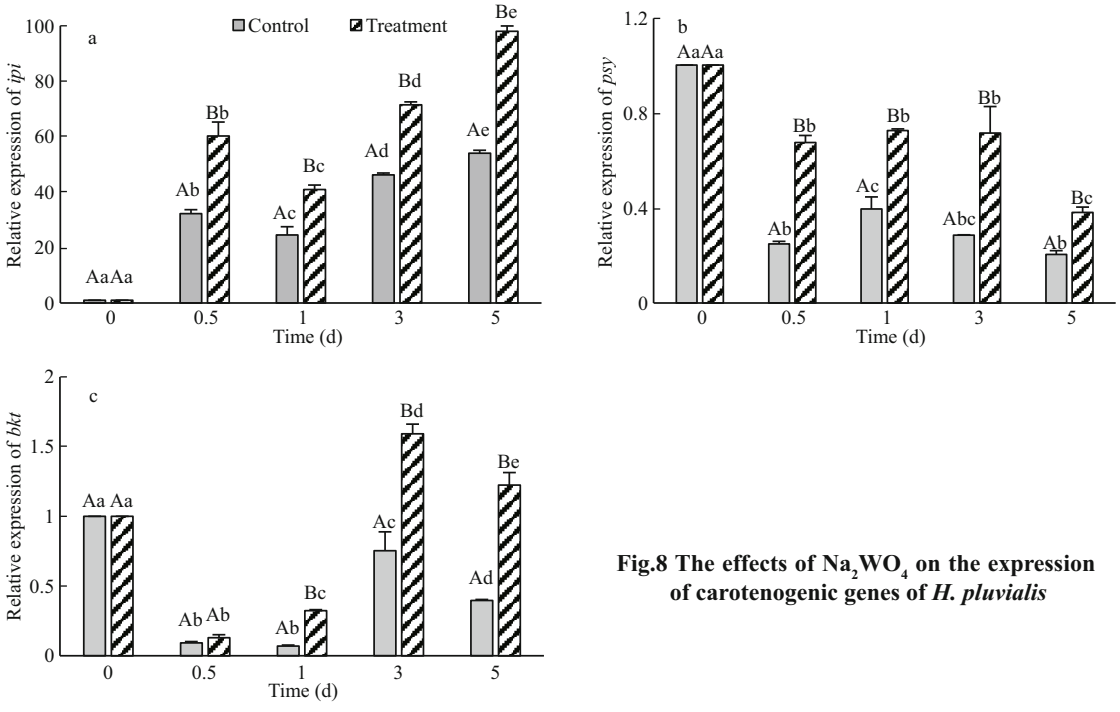

Fig.8 The effects of Na_2WO_4 on the expression of carotenogenic genes of *H. pluvialis*

exposed to Na_2WO_4. Astaxanthin accumulated rapidly after application of 3.0 mmol/L of Na_2WO_4, and the color of the cells completely turned to red at 10 d when the astaxanthin content reached 49.41 pg/cell. Boussiba and Vonshak (1991) reported that the astaxanthin content reached 65 pg/cell under 170 μmol/(m^2·s), and Kobayashi et al. (1997) obtained completely mature algae cells with an astaxanthin content of 49.7 pg/cell. By comparing our data with data from previous studies, we can confirm that Na_2WO_4 effectively promotes the accumulation of astaxanthin. But there is no doubt that Na_2WO_4 may have some toxic effect on algae cells, for the cells density decreased and some algae cells appeared bleach or rupture. On the other hand, Na_2WO_4 is easy to dissolve in water, while astaxanthin is usually extracted by organic solvent, then the product won't be affected.

NR is the initial and rate-limiting enzyme in nitrate assimilation, and its activity is closely related to algae growth and metabolism. Research has showed that Na_2WO_4 is a NR inhibitor in higher plants. Liang et al. (2008) reported that the NR activity of *Brassica campestris* was inhibited by 0.8 mmol/L of Na_2WO_4 and that higher concentrations of Na_2WO_4 showed toxic effects. Studies also showed that 1.0 mmol/L Na_2WO_4 had the best inhibitory effects on the NR activity of *Brassica napus* roots and that the rate of nitrate assimilation was significantly decreased by this treatment (Yang et al., 2012). NR is extremely sensitive to the nitrate concentration and light intensity (Zhao et al., 2004; Tian et al., 2009). The increase in NR activity in the control cells was probably caused by continuous light exposure. In the treated cells, Na_2WO_4 showed significant inhibitory effects on the NR activity of *H. pluvialis* from days 0.5 to 5. Under these conditions, we expect that the absorption of nitrate in the culture medium will be inhibited, thereby blocking amino acid and protein synthesis and inducing a form of nitrogen deficiency. At the same time, we confirmed that astaxanthin accumulation was significantly higher in the treated cells (3 mmol/L Na_2WO_4) than in the control cells at day 5, indicating that nitrogen deficiency could accelerate astaxanthin accumulation. This conclusion is consistent with the hypothesis that nitrogen deficiency has a greater effect on astaxanthin synthesis by exerting a stronger blocking effect on cell division (Fábregas et al., 1998).

Soluble sugar is a critical substance for maintaining cell osmotic pressure, increasing plant resistance to stress (Wang and Tang, 2014), and forming cell walls and other structures. Previous studies have reported that carbohydrates, such as sucrose, altrose and fructose-6-phosphate, are increased under various stress conditions in *H. pluvialis* (Su et al., 2014; Lv et al., 2016). In our study, the soluble sugar content of *H. pluvialis* was significantly increased in both groups at 0.5 d, which might increase the stress resistance of the cells rapidly. The soluble sugar content in the control cells exhibited a stable trend and in the treatment cells increased continuously, which demonstrated that the nitrogen deficiency caused by Na_2WO_4 promoted an increase in the soluble sugar content, which could improve the stress resistance of the cells. Similarly, other researchers concluded that the soluble sugar content increased with nitrogen deficiency in higher plant roots (Liang et al., 2008). We speculate that soluble sugar not only can be regarded as a resistance compound but can also be used to synthesize astaxanthin, the cell wall (Fig.1) and other secondary metabolites in *H. pluvialis*.

Soluble protein has functions such as regulating osmotic pressure and relieving metal stress and thus can also indicate cellular resistance to stress (Xu et al., 2008). Studies have shown that *H. pluvialis* produces a large amount of protease-resistant, heat-stable proteins under high light conditions and nitrogen starvation (Pelah et al., 2004). Our research showed that the soluble protein content in both groups increased significantly at 0–1 d, presumably to protect the cells. Then, the soluble protein content of the control cells decreased more rapidly than the content in the treatment cells, indicating that soluble proteins might play important roles in continual resistance to adverse environmental conditions.

Nitrogen is an important element in chlorophyll. Nitrogen deficiency affects the chlorophyll content and functions in *H. pluvialis* and suppresses photosynthesis. Previous studies noted that F_v'/F_m' and rETR were significantly decreased by a lack of nitrogen, which suppressed photosynthesis in *H. pluvialis* (Chen et al., 2012). In our study, Na_2WO_4 resulted in low NR activity and nitrogen deficiency, which led to a significant decrease in F_v'/F_m' and E_k. Then, F_v'/F_m' and E_k tended to stabilize following the rapid accumulation of astaxanthin, revealing the protective effects of astaxanthin on the photosynthetic system (Li et al., 2008). GAPDH participates in the reduction reaction of 3-phosphoglyceric and the conversion to glyceraldehyde-3-phosphate in the Calvin cycle, which relies on the electron carrier

NADPH that is generated by the light-dependent reactions (Raven et al., 2005). Kim et al. (2011) performed a transcriptomic analysis and showed that nitrogen deficiency inhibited the expression of chlorophyll biosynthesis genes and light-harvesting complex (LHC) related genes in *H. pluvialis*. In our study, GAPDH gene expression was down-regulated consistently with F_v'/F_m'. We speculated that the NADPH yield decreased with the decline of photosynthesis in the chloroplasts, which resulted in down-regulation of GAPDH gene transcription.

Many studies have examined the expression of carotenoid genes under different conditions by transcriptomics and quantitative real-time PCR techniques and have demonstrated that induction of carotenoid gene expression, including *ipi*, *psy* and *bkt*, plays an important role in regulating astaxanthin accumulation under various stress conditions (Gao et al., 2012; Gwak et al., 2014). Our results showed that transcriptional expression of *ipi* and *psy* was up-regulated at 0–48 h in response to high light stress with fast accumulation of astaxanthin (Li et al., 2010). Gao et al. (2013) showed that 24-epibrassinolide (EBR) enhanced the astaxanthin content and *ipi*, *psy* and *bkt* expression at 24–48 h. Although the stress conditions were different, we found that transcription of *ipi*, *psy* and *bkt* was up-regulated significantly after application of Na_2WO_4 compared with that in the control, and *ipi* showed maximal amplification. Our results indicated that Na_2WO_4 induced carotenoid gene expression and ultimately led to a high quantity of astaxanthin.

5 CONCLUSION

Our study provides the first evidence that Na_2WO_4 can effectively promote the accumulation of astaxanthin in *H. pluvialis* by inhibiting nitrate assimilation, which is a new approach to promote the accumulation of astaxanthin. We conjectured that WO_4^{2-} replaced or affected the molybdenum (Mo) in the NR structure, which inhibited NR activity and caused nitrogen deficiency. Then, chlorophyll, amino acid, and protein synthesis was blocked, and some metabolic pathways might be inhibited or disrupted, especially nitrogen assimilation and photosynthesis. When stress signals are transmitted to the algae nucleus, up-regulation of carotenogenic genes leads to high astaxanthin accumulation levels. Furthermore, the soluble sugars and proteins cooperated with astaxanthin to protect and relieve photosynthesis and other metabolic processes from stress.

6 DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the article.

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