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Influence of Sodium Orthovanadate on the Production of Astaxanthin from Green Algae *Haematococcus lacustris*

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Abstract Astaxanthin production is commonly induced under stress conditions such as nutrient deficiency (N or P), high light stress, and variations of temperature, high NaCl concentrations, and other factors. The objective of the present study is the analysis of the effect of oxidative stress by sodium orthovanadate (SOV), a nonspecific inhibitor of protein tyrosine phosphatases, on the cells growth and astaxanthin production of *H. lacustris*. In the presence of SOV (lower than 5.0 mM), maximum growth of *H. lacustris* obtained was 2.4×10^5 cells/mL in MBBM medium at 24°C under continuous illumination (40 $\mu E/m^2/s$) of white fluorescent light, with continuous aeration of CO_2 (0.2 vvm). Total carotenoids accumulated per cell biomass unit treated with 2.5 mM SOV has approximately shown 2.5 folds higher than the control after short period of SOV induction time as 2 days, despite that cells were grown under normal light. Meanwhile, maximal astaxanthin production from *H. lacustris* was 10.7 mg/g biomass in MBBM with 5 days of continuous illumination at 40 $\mu E/m^2/s$, which has been established as optimal light intensity for the control culture of *H. lacustris*. Treating algae *H. lacustris* with sodium orthovanadate showed promoting the accumulation of astaxanthin by advancing either the inhibition of dephosphorylation or synthesis of ATP. Its potential role of PTPases in microalgae *H. lacustris* is discussed. © KSBB

Keywords: Haematococcus lacustris*, astaxanthin, sodium orthovanadate, protein tyrosine phosphatase, photobioreactor*

INTRODUCTION

Astaxanthin extracted from green algae, especially from *Haematococcus* species, is desirable in the food supplements, cosmetics, and pharmaceutical industries due to its high antioxidant properties. *Haematococcus lacustris*, a green unicellular microalgae, has a high contents of astaxanthin and more than 80% of total accumulated carotenoids is astaxanthin [1]. Over the last several decades, many algal biotechnologists have studied the application of the photosynthetic machinery of algal cells toward the production of new bioactive compounds and application to environmental processes [2]. *H. lacustris* has been widely used in biosynthetic engineering of astaxanthin production, known as a class of diverse natural pigments. Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a high-value carotenoid pigment with potential applications in various fields including cosmetics, nutraceuticals, and food and feed industries. The potent antioxidant property of astaxanthin has been implicated in various biological activities demonstrated in both experimental animals and clinical studies. It has considerable potential and promising applications in human health and nutrition [3].

H. lacustris accumulates astaxanthin and its intermediates under various stress conditions such as high irradiance and high temperature [4], nutrient deficiency [5], oxidation from reactive oxygen species (ROS) [6-10], high salinity [11], and drought [12]. The availability of a carbon source, either acetate or CO_2 also affect the accumulation of astaxanthin [13,14]. Furthermore, astaxanthin, a secondary carotenoid in *H. lacustris*, has been proposed to have a photoprotective role [4,15]. Also, astaxanthin enables *H. lacustris* to resist and acclimate to high irradiance by dissipating the excessive light and shielding the photosynthetic apparatus [15]. The most pronounced change during the encystment process of *H. lacustris* is, of course, the massive accumulation of the red ketocarotenoid astaxanthin. In the green cells, chlorophylls followed by the carotenoid lutein and β-carotene dominate the total pigment content; thus cells appear green [16]. As encystment is induced, the amount of astaxanthin increases dramatically from a few picograms per cell to a few hundred grams at the end of the process [17]. At this stage, astaxan-

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thin esters (mono and di), constitute up to 98% of the total carotenoids profile and reach up to 4% of total cellular dry weight, is by far the highest value reported for any microorganism, including bacteria, fungi, and other microalgae [17]. For this reason, *H. lacustris* has sparked considerable interests as a potential route for the commercial production of this high value carotenoid [18]. The ability to accumulate astaxanthin in *H. lacustris* is an adaptation to habitats that exhibit strong radiation [19] in addition to the formation of cysts having rigid cell walls [20,21]. It is very important to understand the nature and relationship between the regulation and expression of β-carotene ketolases, because they play essential roles in expression and accumulation of astaxanthin in *H. lacustris* [22,23].

The culture of *H. lacustris*, both in laboratory conditions and for commercial purposes, has received much attention, even though research on the factors controlling growth and astaxanthin accumulation have yielded controversial results, postponing the management of the cause-effect association within reliable certitude. In part, this lack of conclusive results has discouraged research on the scale-up of astaxanthin production, unlike the culture of Dunaliella (for β-carotene production), which is successfully carried out in many countries. To a certain extent, this has been due to the complex life cycle of this microalgae, which exhibits different morphological cell types-macrozooids, microzooids, aplanospores, "palmella" stages, and cysts [24,25] and to a poor understanding of the optimal conditions for growth and astaxanthin accumulation of any of these morphological cell types. Moreover, the great variations in experimental designs, culture conditions, and strains utilized, make the comparison of the data almost impossible.

Protein tyrosine phosphatases (PTPs) have been implicated as key regulators of tyrosine phosphorylation-dependent cell signaling. Up-to-date, the attachment and removal of a phosphate group from a target protein involved in signaling pathways for cell proliferation and differentiation in mammalian cells and yeasts are well studied [26]. This process depends on the antagonistic interplay between protein kinases and serine/threonin/tyrosine phosphatases, in response to a variety of signals including hormones, mitogens, and oncogenes. While some protein kinases that regulate the phosphorylation state of protein have been described that can phosphorylate tyrosine residues of protein, nothing is known about the existence of the counteracting enzymes: protein tyrosine phosphatases (PTPs) in plant cells. Since the phosphorylation status or activity of ATPases is very important in photosynthesis of microalgae, we report the influences of orthovanadate, which is a transition element that is known to be a non-specific protein tyrosine phosphatases, alkaline phosphatases, and a number of ATPases [27,28] on growth of microalgae *H. lacustris*, as well as its involvement in astaxanthin biosynthesis. In the present study, carotenoid formation was studied under different sodium orhthovanadate (SOV) concentrations, in order to find the optimal concentrations for mass culture and maximum production of astaxanthin and other carotenoids of biotechnological interest.

MATERIALS AND METHODS

Strain and Culture Conditions

The unicellular green algae *H. lacustris*, UTEX 16 was purchased from the Culture Collection of Algae at the University of Texas at Austin and was cultivated photoautotrophically in the modified Bold's basal medium (MBBM) consisting of 246.5 mg/L of $NaNO_3$, 24.99 mg/L of $CaCl_2 \cdot 2H_2O$, 73.95 mg/L of $MgSO_4 \cdot 7H_2O$, 4.98 mg/L of $FeSO_4 \cdot 7H_2O$, 74.9 mg/L of K_2HPO_4, 175.57 mg/L of KH_2PO_4, 25.13 mg/L of NaCl, 49.68 mg/L of $C_{10}H_{16}N_2O_8$ (EDTA), 1.57 mg/L of $CuSO_4 \cdot 5H_2O$, 1.19 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 11.13 mg/L of H_3BO_3, 1.44 mg/L of $MnCl_2 \cdot 4H_2O$, 8.83 mg/L of $ZnSO_4 \cdot 7H_2O$, 0.49 mg/L of $Co(NO_3)_2 \cdot 6H_2O$, 6.06 mg/L of MoO_3, 30.86 mg/L of KOH, and 0.98 mg/L of H_2SO_4 in distilled water. A single colony of cells grown on the agar plate was inoculated into 100 mL medium, after adjusting the initial pH to 6.5 ± 0.5, in a 250 mL Erlenmeyer flask. The inoculated flasks were incubated at 25°C under continuous shaking (175 rpm) and irradiated at 40 μE/m^2/s with fluorescent lamps (Model FL 18D, OSRAM Korea, Ansan, Korea). The seed cultures were grown in 2.5 L bubble column photobioreactors containing 2 L MBBM. After 8 days of cultivation, the cells reached to late exponential phase and most of cells were in palmella stage with green color thick wall. The cells were transferred into smaller scale of 500 mL bubble column photobioreactors containing 400 mL of culture broth with 0.2 vvm aeration containing 5% CO_2 gas and 95% air under constant continuous light intensity of 40 μE/m^2/s at column surface. Compact fluorescent lamps (Model DULUX L®, OSRAM Korea, Ansan, Korea) were used for all the external illumination of photobioreactors. The temperature and pH were kept at 25°C and 6.5 ± 0.5 during cultivation time. The cell was inoculated at a density of 1.3×10^5 cell/mL from an exponentially grown cells in photobioreactor.

Photobioreactors (PBRs)

The 0.4 L bubble-column photobioreactors were designed and constructed. All photobioreactors were made of Pyrex glass tubes with 90 mm in diameter (OD) and 580 mm long. The bottoms were modified into cone-shape to reduce the cell sedimentation. In the center of each PBR, there was small inner tube (28 mm OD × 500 mm long) to host fluorescent lamp, a LED array or flashing light. The top of each photobioreactor was hermetically sealed with a silicon stopper that had three ports for sampling, gas outlet, and a nutrient inlet for fed-batch operation. The filtered air containing 5% CO_2 was injected into the conical bottom part of the photobioreactor at a constant flow rate of 0.2 vvm.

Microscopic Analysis

Morphological changes of the cell were observed with optical microscope (model CSB-HP3, Samwon Science, Korea) and were analyzed after photos were taken by a digital

camera (CoolPix 900, Nikon).

Analytical Methods

The cell concentration, the average cell size, the cell size distribution, and fresh cell weight (wet-based) were measured by a Coulter Counter (model Z2, Coulter Electronics, Hialeah, FL, USA) using AccuComp software (version 2.01, Coulter Electronics). Light intensity was measured with LI-COR quantum sensor (LI-190SA, LI-COR, Lincoln, NE, USA) equipped with a DataLogger (LI-1400, LI-COR). Following acetone extraction of chlorophyll and astaxanthin, the astaxanthin concentration was calculated by a calibration curve using synthetic astaxanthin (A9335, Sigma Chemical Co., St Louis, MO, USA) as a standard. For astaxanthin concentration less than 10 mg/mL, the following calibration was used: astaxanthin concentration (mg/L) = $0.0045 \times A_{475}$ [29]. The chlorophyll concentration was calculated using a previously reported equation [30]: chlorophyll a (mg/L) = $(12.7 \times A_{663})$ - $(2.69 \times A_{645})$ and chlorophyll b (mg/L) = $(22.9 \times A_{645})$ - $(4.64 \times A_{663})$. The nitrate concentration (NO_3^-) was analyzed by a spectrophotometer (model HP8453B, Hewlett Packard, Waldbronn, Germany) after treating the centrifuged sample with HCl according to the standard method [31].

Preparation of Soluble Proteins

Haematococcus lacustris cells were harvested by centrifugation at 3,000 rpm for 10 min and homogenized for 10 min in a mortar with liquid nitrogen using a lysis buffer consists of 20 mM Tris-HCl, 150 mM NaCl, 4.0 mM $MgCl_2$ (pH 7.5), 5.0 mM DTT, and plant protease inhibitor cocktail table (Roche Applied Science). The homogenized mixture was centrifuged at 13,000 × *g* for 30 min to separate cell debris. Total proteins in the supernatant were collected and the protein concentration of the extract was determined using 2-D Quant kit with BSA protein as a standard (Amersham Biosciences).

Protein Phosphatases Activity

Phosphatase activity was determined by monitoring the release of free phosphorus from *p*NPP used as a substrate [32]. Briefly, the reaction mixture contained 0.5 mM *p*NPP, 25 mM Tris-HCl, pH 7.2, and 2.0 mM DTT in 100 µL of total volume. Reactions were initiated by adding 0.1~1.0 µg protein into the reaction mixture. After incubating the reaction mixture at various range of temperatures, we terminated the reaction by adding 0.9 mL of sodium carbonate (1 M). Blank incubations were performed without the protein. The relative activity of the protein was monitored by absorbance at 405 nm (A_{405}) at various time points. The concentration of *p*NP formed was estimated using a molar extinction coefficient of 17,700 M^{-1}. One unit of phosphates activity was defined as the amount of enzyme that catalyzes the formation of 1 nmol of *p*NP per minute under the assay conditions. The results represented for enzyme activity of phosphatase are the means from at least three independent experiments. To confirm the PTPase assay, phospho tyrosine phosphatase assay kit (PTP-101, Sigma, USA) was used with $pTyr^{1018}$ (EGF receptor) as substrate. The assay was carried out according to the instruction of the supplier.

RESULTS AND DISCUSSION

Phosphatase Activity of *H. lacustris* under the Effect of Sodium Orthovanadate

Since the phosphorylation status or activity of phosphoprotein tyrosine phosphatases (PTPases) is very important in photosynthesis of microalgae, we report the influences of sodium orthovanadate, a nonspecific inhibitor of PTPases, on the cells growth and astaxanthin production of *H. lacustris*. Early studies demonstrated that most of β-carotene ketolases such as CrtW and CrtZ, containing highly conserved histidine motifs involved in the formation of ketocarotenoids, play essential roles in biosynthesis of high-value canthaxanthin and astaxanthin. However, still little is known about the potential key regulator controlling the astaxanthin biosynthesis in *H. lacustris* due to the lacking of genomic database. In addition, none of report describing the phosphorylation status of any specific proteins or ATPases of *H. lacustris* was defined. Therefore we strived to identify a potential regulator involving directly in astaxanthin biosynthesis in *H. lacustris*.

In the present study, we investigate the enzymatic assay for PTPase activity with protein samples prepared from non- and highlight-treated cells of *H. lacustris* by monitoring the release of free phosphorus from *p*NPP and phosphorylated tyrosine peptide used as a specific substrates for PTPases as described in "Materials and Methods". As shown in Fig. 1A, PTPases activity of non-highlight induction cells *in vitro* was estimated to be significantly higher than of its highlight-treated cells.

Furthermore, we have determined the PTPase activities using a protein tyrosine phosphatase assay kit (PTP-101, Sigma, USA) with a substrate $pTyr^{1018}$ (EGF receptor). As shown in Fig. 1B, the specific enzyme activities of proteins extracted from both non-induction and after induction by high light against $pTyr^{1018}$ substrate were estimated to be 0.23 nmol/µg/min and 0.16 nmol/µg/min, respectively. Therefore we proposed that accumulation of astaxanthin in *H. lacustris* might be related with the reversible tyrosine phosphorylation catalyzed by protein tyrosine kinase(s) (PTKs) and PTPase(s). Although in the present study we did not discuss any enzyme activity of PTKs and we have no understanding of their physiological roles in microalgae, our results suggested that PTPase(s) possibly mediated the signaling pathway of the production and accumulation of astaxanthin in photosynthetic micro algae *H. lacustris*.

Photobioreactor Bubble Columns

To investigate the effect of sodium orthovanadate on the cell growth and astaxanthin production in *H. lacustris*, the

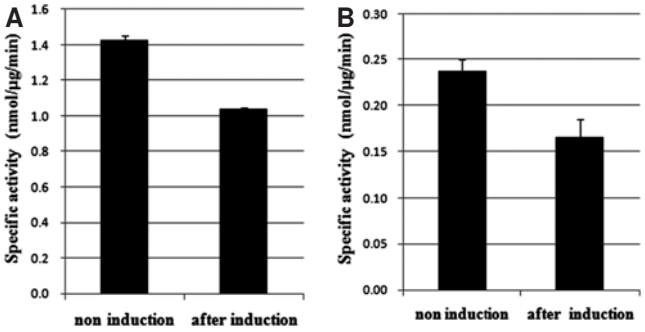

Fig. 1. Determination of PTPases activity. Total intracellular proteins from non-and highlight-treated cells were prepared and used for PTPases assay at room temperature in 25 mM Tris-HCl buffer (pH 7.2) containing of 1 mM DTT with 0.5 mM *p*NPP (A) or 1.0 mM $pTyr^{1018}$ (EGF receptor) (B) as substrates in 100 μL of total volume. The enzyme activity was estimated as described in the "Materials and Methods". The experiments were performed in triplicate and bars represent the standard deviation (S. D.).

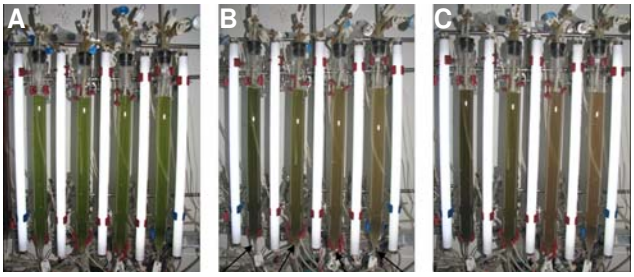

Fig. 2. Side view photograph of externally illuminated photobioreactor bubble columns. A, Day 0; B, 2-day SOV exposure; C, 4-day SOV exposure.

0.4 L bubble-column photobioreactors were used (Fig. 2). All photobioreactors were made of Pyrex glass tubes. The filtered air containing 5% CO_2 was injected into the conical bottom part of the photobioreactor at a constant flow rate of 0.2 vvm. After two days of adding sodium orthovanadate (SOV) to the culture, the color of the culture changed immediately from green to red with SOV concentration from low to high correspondently. After 4-day SOV exposure, the color of culture changed completely to red while the color of culture control still maintained green. This phenomenon has shown that astaxanthin with red color was produced significantly and accumulated in culture after induction with sodium orthovanadate.

Morphological Changes of the Cells after Treating with SOV

Morphological changes of the cells were observed with optical microscope and were analyzed after photos were taken by a digital camera (Fig. 3). After two-day SOV exposure,

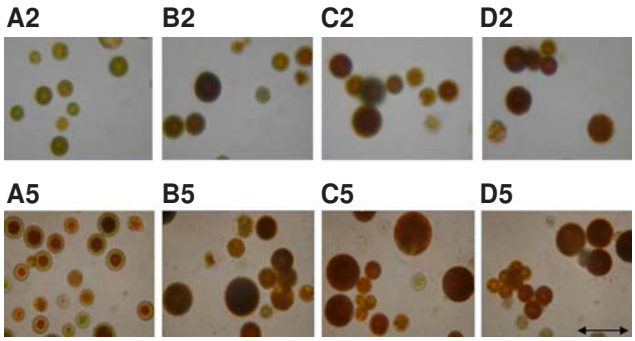

Fig. 3. Morphology of *Haematococcus lacustris*. A2, Control of day 2; B2, SOV 1.25 mM treated cell of day 2; C2, SOV 2.5 mM treated cell of day 2; D2, SOV 5.0 mM treated cell of day 2; A5, Control of day 5; B5, SOV 1.25 mM treated cell of day 5; C5, SOV 2.5 mM treated cell of day 5; D5, SOV 5.0 mM treated cell of day 5; ↔, 50 μm.

the morphology of *H. lacustris* changed from vegetative stage with thin wall and green color (panel A2) to heterocyst stage with thick wall and red color (panels B2, C2, and D2). Most of the cells treated with 2.5 mM and 5.0 mM SOV became bigger and accumulated astaxanthin in the cells. After four-day SOV exposure, although nitrogen source in the control culture was depleted (data not shown) and the control cells without SOV started to accumulate astaxanthin under nitrogen-depleted environment, only a portion of the control cells became red and smaller than SOV treated cells with full astaxanthin accumulation. The addition of SOV to cell culture increased cell size by 12% compared to untreated cells.

Cell Growth

H. lacustris was cultured in 2 L scale bubble column photobioreactor at 25°C under continuous light intensity of 40 $\mu E/m^2/s$. When the cells grew into exponential phase, they were transferred to four 400 mL bubble column photobioreactors with cell density of 1.3×10^5 cell/mL. Cells were continuously grown at 25°C under continuous light intensity of 40 $\mu E/m^2/s$ for one day more and then treated with sodium orthovanadate at different concentration as 1.25, 2.5 and 5.0 mM in turn. Growth rates of *H. lacustris* were decreased by the addition of SOV with respect to control culture without this compound. With supplementation of SOV to the culture, *H. lacustris* was induced to form heterocyst cells, which was closely associated with a concomitant increase in the astaxanthin content per cell. The addition of SOV to the medium had an immediate strong effect on the cellular accumulation of carotenoids. SOV also affected the amount of chlorophyll present. Total chlorophyll content decreased gradually once SOV in high concentration was added to medium (data not shown).

Over the range of concentrations tested, higher SOV concentrations caused a total growth inhibition. However, an increase in cell size was microscopically observed under high SOV concentration. Under the effect of SOV, PTPases

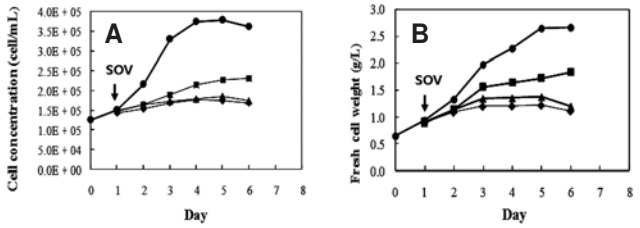

Fig. 4. Profile of cell density (A) and fresh cell weight (B) in culture of *Haematococcus lacustris*. The arrow represents the incubation of *H. lacustris* cells with SOV. ●, Control; ■, the culture with SOV 1.25 mM; ▲, SOV 2.5 mM; ◆, SOV 5.0 mM.

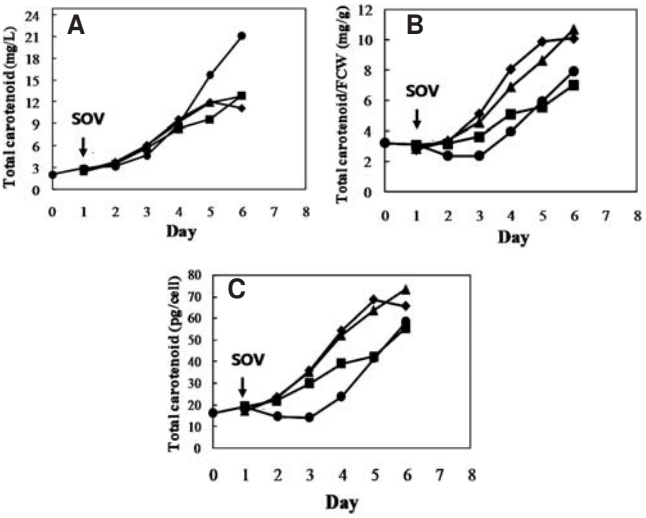

Fig. 5. Profile of total carotenoid production (A), total carotenoid per cell (B), and total carotenoid per fresh cell weight (FCW) (C) in culture of *Haematococcus lacustris*. The arrow represents the incubation of *H. lacustris* cells with SOV. ●, Control; ■, the culture with SOV 1.25 mM; ▲, SOV 2.5 mM; ◆, SOV 5.0 mM.

activity of *H. lacustris* was inhibited, so the cells treated with SOV could not grow well in comparison with the control cells. The control cells grew quickly and could reach a maximum cell density of 3.7×10^5 cell/mL at day 4 and fresh cell weight of 2.65 g/L at day 6 of cultivation as shown in Fig. 4. However, the cells treated with SOV grew slowly. We assumed that SOV inhibited PTPases activity of *H. lacustris* and somehow affect on the energy production ability and the growth of the cell. The cells treated with 1.25 mM SOV were only able to reach a cells density of 2.3×10^5 cell/mL and of fresh cell weight of 1.8 g/L at day 6 of cultivation time. At higher concentration of SOV as 2.5 and 5.0 mM, the cells grew very slowly and only reached to 1.86 and 1.75×10^5 cell/mL of cell density correspondently at day 5 of cultivation.

Carotenoid Accumulation

After treating with SOV, *H. lacustris* cells immediately

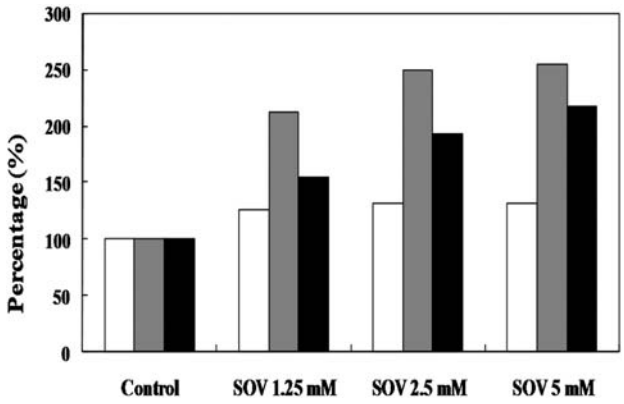

Fig. 6. The effect of sodium orthovanadate on carotenoid production of *H. lacustris* after two-day SOV exposure. □, total carotenoid production; ■, total carotenoid per cell; ■, total carotenoid per fresh cell weight.

accumulated astaxanthin and total carotenoid increased with the period of SOV exposure, while the control cells without SOV treatment started to accumulate carotenoid at the day 4 of cultivation only after nitrogen source in the culture was depleted. Although the cells treated with SOV grew slower than the control cells, total carotennoid in the SOV culture was produced higher than the control cells culture up to the day 4 of cultivation as shown in Fig. 5A. Maximal total carotenoid content per volume of culture was obtained in cultures with high SOV concentration (2.5 and 5.0 mM) at day 2 and 3 of SOV exposure (Fig. 5), but after the day 4 of SOV exposure, total carotenoid content per volume of these cultures was lower than the control with nitrogen deficiency. An increase in SOV concentration in the medium produced lower cell yield because the cell growth was inhibited. At day 2 of SOV exposure, total carotenoid concentration (mg/L) was almost 31% in cultures with 2.5 and 5.0 mM SOV higher than the control. At this period of exposure, SOV enhanced the accumulation of total carotenoid per cell, with values up to 2.5 folds higher than the control culture. However, the major accumulation occurred in culture with 2.5 and 5.0 mM SOV, which presented a strong growth inhibition accompanied by cell encystment.

The carotenoid production in *H. lacustris* was compared between sodium orthovanadate-treated and untreated cells (Fig. 6). In the comparison of total carotenoid production per biomass unit, the cells with SOV treatment can accumulated total catotenoids significantly higher than that of the control. Following the period of SOV exposure, the SOV treated cells maintained accumulating caroteinoids up to 2.5 times higher than the control approximately. A difference in the astxanthin accumulation was noted from the *H. lacustris* cells treated with SOV compared to untreated the control cells. There was a 31% increase in the total carotenoid production in the SOV-treated cells as compared to untreated the control. Total carotenoid concent per cells increased in SOV-stressed algae compared with the control. Maximum cellular carotenoid content was obtained at the higher SOV concentration assayed, in

culture with 5 mM SOV. In this culture, up to 2.5 times more carotenoids per cell were accumulated than in culture without this compound, but cell density was lower than the control because PTPase enzymes for energy production were totally inhibited in this culture. Total carotenoids per fresh cell weight can be produced 1.93 fold higher than the control and maximal astaxanthin production was 10.7 mg/g fresh cell weight with 5 days of continuous illumination at 40 $\mu E/m^2/s$. SOV significantly promoted the accumulation of carotenoid in *H. lacustris* implying that SOV is a factor which dramatically influenced on the carotenoid accumulation in *H. lacustris*.

The mortality of cells increased substantially with the increase of SOV concentration. In spite of this high mortality, an increase in red coloration of the surviving cells at higher SOV was evident in our work. An increase in total carotenoid content per cell and in astaxanthin content per dry weight occurred at increasing SOV concentrations. On the other hand, the age of the culture was crucial to trigger astaxanthin production in SOV stress induced cultures. Younger cultures (four to six days old) were very sensitive to SOV addition, while older cultures (eight to ten days old) were resistant and accumulated much more astaxanthin when SOV was added after a prolonged incubation time (eight days). This agrees with the results found by various authors who used different stress factors such as nitrogen-depletion, high irradiance, and high salinity to induce astaxanthin production in *H. lacustris*. For instance, Borowitzka *et al*. (1991) [33] and Orosa *et al*. (2001) [34] suggested that the shift from optimal growth conditions to inductive carotenogenic condition must occur when most of the cells in the culture are encysted and according to Orosa *et al*. (2001), the induction of astaxanthin production is triggered by stress factors that are cumulative of the physiological state of the cells and the culture conditions.

Assuming that total astaxanthin constitutes at least 80% of total carotenoid content in *H. lacustris* [1,35,36], the total carotenoid accumulation produced in the best condition in the present study, 1.1% fresh cell weight (w/w), would mean an astaxanthin accumulation of 0.88% fresh cell weight (w/w) after 5-day SOV exposure under the normal light condition. The mechanism, by which sodium orthovanadate is able to induce astaxanthin accumulation in *H. lacustris*, is still unknown. Sodium orthovanadate is also an inhibitor of PTPases in mammalian systems [37,38]. The protein tyrosine phosphatases are also existent in plants [39-43], but their role in plant signaling processes is still unknown. Although it is now clear that plants have PTPases, we have no understanding of their role in plant. Further identification of the target PTPases or its substrates will be important steps toward understanding the role of PTPases in the plant signaling network.

CONCLUSION

Thus far, several factors involved in astaxanthin production have been identified. In the present study we aimed to find out a novel key regulator involved in the regulation and/or accumulation of astaxanthin in micro algae *H. lacustris*. Since there is a simple evidence indicating that both enzymes phosphoprotein tyrosine phosphatases (PTPases) and protein tyrosine kinase(s) (PTKs) are implicated as key regulators in signaling pathway for cell proliferation and differentiation in mammalian cells and yeasts are well studied [26], we have investigated the influence of sodium orthovanadate (SOV) on the accumulation of astaxanthin in microalgae *H. lacustris*, although SOV function remains to be further determined. From our *in vitro* experiments we can conclude that SOV with optimum concentration 2.5 mM leads to an increase in the cell carotenogenesis accumulation in microalgae *H. lacustris*. In the presence of SOV for a definite short period of time as two days under normal light condition, in spite of the growth inhibition at higher SOV concentration, it is important to note the significant effect over the stimulation of astaxanthin synthesis and accumulation per cell that was more than 2.5 times higher than in culture of cells in the absence of SOV. Interestingly as shown in Figs. 1A and 1B, PTPases activity of non-highlight induction cells *in vitro* was estimated to be significantly higher than of its highlight-treated cells, when we assayed PTPase activities with both *p*NPP and $pTyr^{1018}$ EGF receptor. Therefore we propose that the accumulation of astaxanthin in *H. lacustris* might be related to the reversible tyrosine phosphorylation catalyzed by protein tyrosine kinase(s) (PTKs) and PTPase(s). Taken together, our results, strongly suggest that SOV might be a novel factor which can effectively stimulate astaxanthin accumulation in *H. lacustris*, in addition to the well-known environmental stress factors such as illumination, nitrogen-starvation, oxidation, and high salinity. This study reinforces the notion that PTPases involvement in the processing of astaxanthin production with a potential role in the photosynthetic microalgae *H. lacustris*. To the best of our knowledge, this is the first report describing the involvement of PTPase in microalgae. The exact mechanism underlying PTPase involvement during astaxanthin accumulation in *H. lacustris* is of interest and is currently being investigated in our laboratory.

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