Initial study of polymer-based nanoparticles effect on carotenogenesis of *Haematococcus lacustris*

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**Abstract.** The carotenogenesis in *Haematococcus lacustris*, accompanying by encystment starting from green motile cells to aplanospores, is suggested mediating by reactive oxygen species (ROS). Our previous study showed that isobutyl-cyanoacrylate nanoparticles (IBCA-NPs) induced the cell mortality, preceding by intracellular ROS accumulation, in most of Volvocales species but not *H. lacustris*. This study aimed to investigate the effect of IBCA-NPs exposure to *H. lacustris* on carotenogenesis. The cells were cultured on C medium then exposed to 100 mg \(\cdot\) L\(^{-1}\) of 180 nm of IBCA-NPs for the 14 days. The accumulation of astaxanthin was started after four days of IBCA-NPs exposure. The ratio of intermediate cells reached to 46\% after 14 days of IBCA-NPs exposure, while the ratio of intermediate cells of untreated culture was only 11\%. The results suggested that the addition of nanoparticles could enhance the production of astaxanthin. Further studies are required to investigate the correlation between nanoparticle exposure and ROS generation that leads to the biosynthesis of astaxanthin. The condition of cell growth and the addition of nanoparticles shall be optimized to increase the production of ketocarotenoid and to minimalize the negative effect of longer exposure time of IBCA-NP that could lead the cell mortality.

**Keywords:** Astaxanthin, carotenogenesis, *Haematococcus lacustris*, isobutyl-cyanoacrylate, nanoparticles

**1. Introduction**

*Haematococcus lacustris*, which synonym is *Haematococcus pluvialis* or *Sphaerella lacustris*, is a unicellular freshwater microalgae belong to Volvocales in Chlorophyceae [1]. *H. lacustris* is widely distributed in many habitats across diverse environmental and climate conditions [2-3]. This species can survive on an unfavorable abiotic condition such as extreme temperature and high salinity [2-3]. For industrial applications, *H. lacustris* is well-known as the natural sources of astaxanthin that can accumulate the pigment up to 3-5\% of the dry weight [3-4].

Astaxanthin (3,3' dihydroxy-ß-carotene-4,4'-dione) is a red-orange color lipid-soluble pigment belong to keto-carotenoid [5]. Astaxanthin has health-beneficial effects in the prevention and treatment of infectious and chronic diseases because of its free radical scavenging activity [6]. Astaxanthin has widely utilized in the food, feed, cosmetic, aquaculture, nutraceutical, and pharmaceutical industries [6]. For example, this pigment has been used as a color additive in salmon...
feeds and as a dietary-supplementing ingredient for human consumption [4]. Most of the astaxanthin is chemically synthesized, while the natural astaxanthin derived from H. lacustris covers less than 1% of commercial supply [7]. The development of astaxanthin production in H. lacustris is important due to the health concern for synthetic one, and the high demand for natural astaxanthin [4, 8].

The accumulation of astaxanthin in H. lacustris is affected by environmental conditions such as light, temperature, pH, salinity, and nutritional composition of growth medium [9]. Abiotic conditions also have influenced to change the cell ultrastructure, which is frequently associated with stress response of unfavorable condition in the environment [10-14]. Under the environmental stress, the vegetative green cells of H. lacustris start the encystment process to enter the resting phase described by the transformation of the motile cells followed by the increment of cell volume, the loss of flagella, and the formation of a thick cyst-like wall that may contain sporopollenin [8-9, 15]. During the encystment, astaxanthin is synthesized and deposited in the lipid droplets around the nucleus then radially extended until all the cyst cell acquires a red coloring [15-16]. This phenomenon suggests that encystment and astaxanthin accumulation occur simultaneously.

In our previous study, the acrylic nanoparticles, made from the polymer of isobutyl-cyanoacrylate (hereafter IBCA-NP), induced the cell mortality in Chlamydomonas reinhardtii and other Volvocales species (class: Chlorophyceae) but not in H. lacustris [17]. Unexpectedly, we observed the transformation of H. lacustris from a green vegetative motile cell to a thick cell-walled non-motile cell by the exposure of acrylic nanoparticles [17]. This compelling phenomenon suggested that the cell transformation was a response to cell stress that was most probably generated by IBCA-NPs exposure.

This study is the preliminary research to investigate the possible utilization of IBCA-NP as the stress inducer to enhance the production of astaxanthin in H. lacustris. We observed the encystment ratio of H. lacustris, which is associated with carotenogenesis in vegetative-phase and resting-phase cells. To prove the hypothesis that IBCA-NP is a stress inducer, we observed and counted the encystment frequency of H. lacustris by the nanoparticle treatment and acetate supplementation. This study must be informative to design further research on the enhancement of astaxanthin production by IBCA-NP exposure.

2. Materials and methods

2.1 The microalgae species
H. lacustris NIES-144 (Chlorophyceae, Volvocales, Chlorogonia) were obtained from the Microbial Culture Collection at National Institute of Environmental Studies (NIES, Tsukuba, Japan).

2.2 Isobutyl-cyanoacrylate nanoparticles (IBCA-NPs)
CHIKAMI MILTEC INC (Kochi, Japan) supplied the nanoparticles made of poly-isobutyl-cyanoacrylate (IBCA-NPs) [17]. The nanoparticles were supplied as a 1% (w/v) suspension in water containing 1.0% (w/v) dextran 60,000 (041-30525; Wako Pure Chemical Industries, Osaka, Japan). The zeta potential of iBCA-NPs was $-0.67 \pm 0.164$ mV. The average diameter of the IBCA-NPs after being suspended in C medium for 6 hours was $198.33 \pm 2.80$ nm. The nanoparticles were monodispersed with the PDI value was 0.2666.

2.3 Cultivation condition
The cells were cultivated under constant fluorescent light (84 µmol photons · m$^{-2}$ · s$^{-1}$) with gentle shaking in C medium (pH 7.0), which is a recommendation of NIES. The cells were cultivated for 20 days until the cells entered the stationary phase marked by a 50% transformation of the total cells to resting-phase. The treatments of nanoparticle exposure and acetate supplementation were conducted for stationary-phase cells under the same conditions, such as light, aeration, and medium.

2.4 Exposure of nanoparticle to culture
Cells were pelleted by centrifugation of 3000 g for 5 min, and then resuspended in new C medium at
the same volume. IBCA-NPs were added to the culture at final concentrations of 100 mg · L⁻¹ for 14 days. The observation of the changes in the cell morphology was conducted after 4, 7, 10, and 14 days of nanoparticles exposure. The observation was conducted triplicate for more than 100 cells.

2.5 Acetate supplementation
The addition of acetate was suggested to give a stress condition for H. lacustris [15, 18]. The stationary-phase cells were pelleted by centrifugation of 3000 g for 5 min, then resuspended in the C media that supplemented with sodium acetate at the final concentration of 25 mM, and continuously cultivated for 14 days. The observation of the changes in the cell morphology was conducted after 4, 7, 10, and 14 days after the treatment. The observation was conducted triplicate for more than 100 cells.

2.6 Fluorescence brightener 28 staining
Calcofluor white staining was aimed to investigate if the cell wall contains chitin or cellulose [19]. A stock solution of Fluorescent Brightener 28 (F3543, Sigma-Aldrich). (1 mg • mL⁻¹ in H₂O) was added to the cell culture (final concentration 0.04 mg/mL). Samples were kept in the dark for 10 min preceding the observations. Stained cells were observed without a washing step.

2.7 Microscopic observation
The observations of morphological changes were conducted by bright-field observations using an Olympus IX71 (Olympus, Japan). Fluorescence observations for checking the calcofluor stained cell were made using an Olympus IX71 (Olympus, Japan) with a combination of appropriate mirror units.

2.8 Statistical analysis
In this research, the cell stress responses were showed by the different stages of cell morphology. The ratio of cellular morphological stages between treatments were compared using one-way analysis (ANOVA) of variance followed by Tukey’s test as post hoc multiple comparisons. The differences between means were considered statistically significant when the p-value was < 0.05.

3. Results

3.1 Different cell wall composition of H. lacustris in green vegetative-motile and non-motile-stage
To investigate the presence of chitin or cellulose in the cell wall, we employed the fluorescent dye staining to H. lacustris cells on the green vegetative-motile stage and non-motile-stage cells. Fluorescence brightener 28 (Sigma-Aldrich) specifically binds to chitin or cellulose [19]. In this assay, the blue fluorescence is emitted from the cell wall if it contains chitin or cellulose, while only red fluorescence emitted by chlorophylls is observed if the cell wall contains neither of them.

In the vegetative-motile phase, the cell only emitted red fluorescence without a blue-fluorescence in the ring structure. Contrastingly, the non-motile-phase cell colored in pinkish-red that resulted from the overlaid color of pure-red autofluorescence from chlorophylls and blue fluorescence from calcoflour white-stained cell wall with blue-fluorescence in ring structure at the edge (figure 1). This result suggested the presence of chitin or cellulose in the cell wall of a non-motile cell but not in a green vegetative-motile cell. Therefore, cell wall composition changes must be accompanied by the morphological transformation from a green vegetative motile cell into palmella.

3.2 Effect of IBCA-NP to cell morphology of H. lacustris
H. lacustris has a similar life cycle with other Volvocales microalgae species. Interestingly, there are four distinct stages of cellular morphologies of H. lacustris that affected by the life cycle and environmental stress. The vegetative stage consists of a green vegetative motile cell called macrozooid (zoospore), while the green vegetative non-motile one is called palmella. The macrozooid (figure 2a) is a biflagellate cell with an elliptical or a spherical shape, while the palmella (figure 2b) is a big
spherical shape with a thick cell wall. Macrozooid is predominant in lag- and log-phase of cell growth under favorable culture conditions [16]. In the stationary phase, some portion of cell transforms into the palmella stage preceded by losing the flagella, expanding the cell size, and arranging an amorphous multilayered structure in the inner region of the cell wall. Moreover, the transformation of macrozooid to the palmella is mostly induced by the unfavorable culture condition or environmental stress [8, 15].

Figure 1. Fluorescence brightener 28 staining of *H. lacustris* in the stage of a green vegetative motile cell (a) and palmella cell (b). The red color of the cell was from the autofluorescence of chlorophyll, while the blue color of the cell wall was from Fluorescence brightener 28 binding on of chitin or cellulose contained in the cell wall. The pure red-colored cell indicated the absence of chitin or cellulose in the cell wall composition. The line indicated as 10 µm.

*H. lacustris* begins the encystment process when the cells are exposed to continuous environmental stress. During encystment, the palmella-stage cells transform into intermediate-phase cells then further into aplanospores. The intermediate-phase cells are the astaxanthin-accumulated cell described by the appearance of red pigment in the center of cells (figure 2c). Aplanospores are the enlarged red-colored cyst cell covered by a thick cell wall (figure 2d) [15-16].

Figure 2. The light microscopic observation of the morphology of *H. lacustris* on the different stages of encystment: (a) the green vegetative motile cells, (b) palmella, (c) the intermediate-phase cells, and (d) aplanospores. The line indicated as 10 µm.

The addition of IBCA-NP into the stationary-phase cell culture of *H. lacustris* caused the gradual morphological changes from palmella into aplanospores (figure 2). The predominant cell stage was changed throughout nanoparticle exposure. The number of palmella cells was decreased alongside with the increment of the intermediate-phase cell at the longer time of IBCA-NPs exposure (figure 3a).
Palmella was the predominant after four days of NP treatment, and the ratio reached 75.8% of total cells, while the ratio of intermediate-phase cells was 22.2%. The ratio of palmella cells after exposure to nanoparticles for 7, 10, and 14 days was 69.6%, 51.4%, and 52.42%, respectively. The ratio of intermediate-phase cells after exposure to nanoparticles for 7, 10, and 14 days was 30.39%, 43.75%, and 46.77%, respectively. Some cells were changed into aplanospores after 10- and 14-days exposure to IBCA-NP with a ratio of 3-4% of the total cells. In control, i.e., without nanoparticles addition, most of the cells were in the palmella stage with a ratio of 85%, irrespective of the extended treatment duration (figure 3b). The results suggested that the longer time of nanoparticle exposure triggered the encystment on *H. lacutris*.

**Figure 3.** The ratio of each cell stage in *H. lacutris* following exposure of IBCA-NP (a) and control (b). Cells were exposed to IBCA-NP at a concentration of 100 mg · L⁻¹. The cell stages ratio was calculated as the number of the cell on each cell stages observed out of 100 intact cells. Results are shown as mean ratios (± SE) from three independent experiments.

3.3 Comparison of the effect of IBCA-NP exposure and acetate supplementation on the encystment process in *H. lacutris*

To investigate whether IBCA-NP was efficiently worked as a stress enhancer that leads the encystment process, we compared the effect of IBCA-NP exposure and acetate supplementation in *H. lacutris*, especially on the encystment process that associated with carotenogenesis. Both of IBCA-NP treatment and acetate addition could induce the encystment process described by the appearance of spherical shape and red-colored cells.

The encystment was faster when the cell culture was supplemented with acetate than when the cell culture was added with IBCA-NPs (figure 4). For example, the ratio of intermediate-phase cells reached to 60% after 4-days supplementation of acetate, while the ratio of intermediate-phase only reached to 13.59% after 4-days supplementation of IBCA-NP exposure. After 14-days of acetate treatment, the ratio of intermediate-phase and aplanospores cells reached to 51% and 22.75%, respectively, from the intact cells. The treatment of 14-days IBCA-NP addition induced the morphological transformation to intermediate-phase and aplanospores with a ratio of 33.3% and 6.14%, respectively. Besides, the acetate supplementation caused a higher cell mortality ratio compared to the IBCA-NP treatment. The cell mortality ratio of acetate supplementation was 27%, while the cell mortality ratio of IBCA-NP was limited to 13% (data not shown).

4. Discussion

*H. lacutris* (Volvocales) is a freshwater unicellular green alga that primarily found in a temporary freshwater pool. This algae species is well-known as an alternative natural source of astaxanthin, making the increment number of researches for optimizing the condition for astaxanthin production and cell growth. The environmental stress is suggested as the inductive condition for carotenogenesis, the process for producing astaxanthin. Boussiba *et al* [2] reported that high light intensity, salt stress,
phosphate starvation, and nitrogen limitation induced the encystment followed by astaxanthin accumulation.

![Figure 4](image-url)

**Figure 4.** The ratio of each cell stage in *H. lacustris* following exposure of IBCA-NP and acetate supplementation after 4-days (A) and 14-days treatment (B). The cells were exposed to IBCA-NPs at a concentration of 100 mg·L⁻¹. For the acetate supplementation, the cells were cultured in C medium with 25 mM acetate. The cell stages ratio was calculated as the number of the cell on each cell stages observed out of 100 intact cells. Results are shown as mean ratios (±SE) from three independent experiments. Asterisks denote significant differences between the strains at a given time (one-way analysis of variance, *p < 0.05, **** p < 0.0001).

The environmental stress was suggested could induce the ROS generation to lead the astaxanthin accumulation. A study reported that the inhibition of glutamine synthase activity showed a similar effect to nitrogen starvation that inhibited the cell division accompanied by encystment and astaxanthin accumulation [2-3, 9, 11]. Moreover, a study showed that four types of active oxygen generators could enhance astaxanthin accumulation. The addition of methylene blue, methylene viologen, AAPH, and H₂O₂ at the very low concentration could stimulate the enhancement of carotenogenesis on *H. lacustris*. However, the excessive supplementation of active oxygen generators remarkably reduced the carotenoid formation and led the cell mortality [18].

In this study, the acetate supplementation caused rapid morphological changes in *H. lacustris*. Around 20% of living cells were transformed into aplanospores after 14-days by addition of acetate to the cell culture at the final concentration of 25 mM. Kobayashi [15, 18] reported that the acetate addition at the final concentration of 45 mM induced the morphological change from the biflagellate vegetative cell to aplanospores. *H. lacustris*, which was cultured in an acetate-modified medium, entered the cyst stage in the shorter period compared to the cells that cultured in the basal medium. Moreover, supplementation of ferrous and acetate into the basal medium enhanced the carotenogenesis and cell growth. The study suggested that the addition of acetate caused the high C/N ratio that induced stress condition possibly caused by the relative nitrogen deficiency.

The exposure of IBCA-NPs to *H. lacustris* showed a similar effect with the treatment of acetate supplementation in which green vegetative motile cells entered to encystment process. However, the ratio of aplanospores induced by nanoparticle addition was lesser than that induced by acetate treatment. This result suggested that IBCA-NPs could be acted as stress inducer that stimulated the encystment accompanied by carotenogenesis in *H. lacustris*.

Our previous study showed that IBCA-NPs could induce the ROS generation to lead the cell mortality in *C. reinhardtii* that have a close phylogenetic relationship with *H. lacustris*. That study
suggested that the collision of IBCA-NPs to the cell wall worked as the first trigger of ROS generation that finally led to cell mortality [17]. In this study, we observed the adsorption of FITC-labelled IBCA-NPs on the cell wall of *H. lacustris* (data not shown), but unexpectedly this interaction did not cause cell death. The adsorption of IBCA-NPs on the *H. lacustris* cell wall seems to be working as an inducer of the limited amount of ROS generation. However, the induced ROS level was not large enough to induce cell death, but only good enough to cause morphological transformation and astaxanthin accumulation. Further studies are essential to detect the activated signal pathways by ROS that lead to morphological transformation and astaxanthin accumulation in *H. lacustris*.

Moreover, it is essential to find the optimized condition of nanoparticle addition in order to enhance the astaxanthin accumulation and minimize the risk of cell mortality. The statistical measurement of carotenoid and astaxanthin amount is also essential to provide direct proof of the increment of astaxanthin caused by nanoparticle exposure.

5. Conclusion
The IBCA-NP exposure to *H. lacustris* could induce the encystment or morphological changes associated with carotenogenesis. The effect of IBCA-NP on the encystment process of *H. lacustris* was similar to the effect of acetate supplementation. It is suggested that IBCA-NP could be work as a stress enhancer that induces ROS accumulation. Further studies, such as the investigation of the signal pathways that connect nanoparticle activated signals, ROS generation, and carotenogenesis, are essential to understand the basic mechanism of secondary metabolite generation.

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