

# Enhancement of astaxanthin production using *Haematococcus pluvialis* with novel LED wavelength shift strategy

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**Abstract** *Haematococcus pluvialis* is a green microalga of particular interest, since it is considered the best potential natural source of astaxanthin, which is widely used as an additive for natural pigmentation. In addition, astaxanthin has recently garnered commercial interest as a nutraceutical, cosmetic, and pharmaceutical. However, producing astaxanthin from *H. pluvialis* necessitates separation with distinctive culture conditions, dividing between the microalgae growth and the astaxanthin production stages. Light-emitting diodes (LEDs) have emerged as a replacement for traditional light sources, and LED applications are now rapidly expanding to multiple areas in fields such as biotechnology. However, further detail application into microalgae biotechnology remains limited. In this study, we have attempted to establish new protocols based on the specific wavelength of LEDs for the cultivation and

production of astaxanthin using *H. pluvialis*. Specifically, we applied red LEDs for microalgae cell growth and then switched to blue LEDs to induce astaxanthin biosynthesis. The result showed that astaxanthin productions based on a wavelength shift from red to blue were significantly increased, compared to those with continuous illumination using red LEDs. Furthermore, additional increase of astaxanthin production was achieved with simultaneous application of exogenous carbon with blue LED illumination. Our approach based on the proper manipulation of LED wavelengths upon *H. pluvialis* cell stages will enable the improvement of biomass and enhance astaxanthin production using *H. pluvialis*.

**Keywords** LED · Wavelength shift · *Haematococcus pluvialis* · Astaxanthin · Carbon source

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## Introduction

Ketocarotenoid astaxanthin ((3S-3'S)-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione) is one of the most important secondary metabolites with a high price (approximately US\$2500 kg<sup>-1</sup>) (Lorenz and Cysewski 2000). Due to its high antioxidant activity, it can be applied in many industrial applications such as the nutraceutical, pharmaceutical, and food industries. There are two main routes for obtaining astaxanthin: natural astaxanthin and chemically synthesized astaxanthin. Natural astaxanthin has several advantages over chemically synthesized astaxanthin, as natural astaxanthin exhibits much stronger antioxidant activities for health benefits and is safer for human consumption. Although astaxanthin has already been found in various microorganisms and marine animals, unicellular green microalgae *Haematococcus pluvialis* is known as the richest natural source for astaxanthin. Consequently, as a natural source, the freshwater microalga *H. pluvialis* has

received considerable attention for the production of ketocarotenoid astaxanthin (Ambati et al. 2014; Boussiba and Vonshak 1991).

*H. pluvialis* has distinctive life cycle, completely divided between its green motile and its reddish non-motile resting stages, which is called cyst. Under favorable environmental conditions, *H. pluvialis* cells actively divide and swim in its medium with two flagella. This stage could be categorized as the vegetative growth stage. However, upon exposure to unfavorable environmental conditions, the cells of *H. pluvialis* undergo a dramatic transformation to the resting phase called cyst. At that stage, *H. pluvialis* cells completely stop cell division and accumulate a thick resistant cell wall (Boussiba 2000; Kakizono et al. 1992). The biosynthesis of astaxanthin in *H. pluvialis* is strictly limited to the cyst stage, thereby coping with any oxidative stresses generated from a wide variety of unfavorable environments (Choi et al. 2002; Kang et al. 2007). Because the life stages of *H. pluvialis* for astaxanthin production completely differ from the actively multiplying motile stage, a two-stage process that divides growth and production stages has been widely accepted for *H. pluvialis* cultivation.

It was determined that light is the most significant factor in inducing astaxanthin biosynthesis in *H. pluvialis*. Despite the critical roles of light in inducing astaxanthin biosynthesis, limited information is available on the further development of astaxanthin production systems, based on efficient light illumination. In this regard, there is no doubt that light-emitting diodes (LED) must be considered as a next generation light source, replacing traditional light sources for astaxanthin production via *H. pluvialis* cultivation. Contrary to classical tubular fluorescence lamps, LED light makes it possible to narrow illumination to a specific wavelength with low power consumption. Additionally, LEDs are suitable for photo-bioreactors (PBR) for mass cultivation and production of microalgae, including *H. pluvialis*, due to their small chip size and long duration (Zhao et al. 2011). Due to these advantages, we must seek ways to apply LEDs to the field of microalgae cultivation and valuable bio-chemical production, such as astaxanthin production using *H. pluvialis*.

Because LEDs have remarkable advantages for generating particular wavelengths in microalgae cells, it will be necessary to determine the particular LED wavelength as a mean for the adequate manipulation of *H. pluvialis* metabolism to enhance the biomass productivity and astaxanthin production. However, we still have limited reports regarding LED applications in the field of microalgae biotechnology, including *H. pluvialis* cultivations. To date, few studies have focused on the simple deployment of LED illuminations for microalgae growth without knowing details about the microalgae biological response to specific LED wavelengths.

In this study, we investigated the effects of specific wavelength LEDs on *H. pluvialis* biology including growth, cell physiology, and astaxanthin biosynthesis. Since the majority of photosynthesis depends on light wavelengths mostly corresponding to both red and blue wavelengths, in particular, we focused on LED wavelengths matched with either red or blue illumination. It turned out that the red and blue wavelengths have a specific influence on *H. pluvialis* biology. Biomass productivity and astaxanthin production was significantly increased upon red and blue LED illumination, respectively. Our results suggested that adequate manipulations of LED wavelengths could lead to increasing biomass and astaxanthin productivity. Furthermore, simultaneous application of additional carbon with blue LED illumination could further increase the biosynthesis of astaxanthin. Here, we report the operational data of LED wavelengths for *H. pluvialis* cultivation and enhanced astaxanthin production.

## Materials and methods

### Strain and culture condition

*Haematococcus pluvialis* was obtained from the UTEX Culture Collection (UTEX2505). All cultures were performed in an OHM medium composed of KNO<sub>3</sub>, 0.41 g; Na<sub>2</sub>HPO<sub>4</sub>, 0.03 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.246 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.11 g; Fe(III)-citrate H<sub>2</sub>O, 2.62 mg; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.011 mg; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.012 mg; Cr<sub>2</sub>O<sub>3</sub>, 0.075 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.98 mg; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.12 mg; SeO<sub>2</sub>, 0.005 mg; biotin, 25 µg; thiamine, 17.5 µg; and B<sub>12</sub>, 15 µg per 1 liter of deionized distilled water. Inoculums were prepared from 5-day-old culture cultivated in OHM medium. The initial cell density was adjusted to be about  $7 \times 10^5$  cells mL<sup>-1</sup>. During all cultivations, sterilized–filtered air was supplied at a flow rate of 100 mL min<sup>-1</sup>.

Five-day cultures were inoculated into 120-mL OHM medium in 250-mL Erlenmeyer flasks at  $25 \pm 0.5$  °C under a 20-h light/4-h dark illumination cycle at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  of either red (630–665 nm) or blue ( $\lambda_{\text{max}} = 430\text{--}465$  nm) LEDs. For further experiments, cells were cultivated in the same conditions under red LEDs (approximately  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), and then one group of samples (three replicates) was shifted into blue LEDs (approximately  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the sixth day. After 2 days, some of cultivations were exposed to increased light intensity, at about  $100\text{--}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ , except the control or the experimental groups for a comparison. For the experiment setups of the concomitant applications of both blue LEDs and carbon source, certain amount of acetate (2 % w/v) was added, along with blue light illumination at the light intensity of either 50 or  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

## Astaxanthin analysis

Astaxanthin was analyzed by the spectrophotometric method after extraction with 90 % acetone. First, a 1-mL well-distributed sample was taken from the flask, then centrifuged at 13,000 rpm for 5 min, and washed twice with distilled water. After that, cells were disrupted by bead beating using a cell disruptor with 0.6-mm acid-washed beads for 2 min. One milliliter of 90 % acetone was added to extract pigments. After centrifugation at 13,000 rpm for 10 min, supernatant was collected and absorbance was measured at 474 nm with a spectrophotometer. The content of astaxanthin (mg/L, ppm) ( $C_A$ ) was calculated using the following equation:  $C_A = (A_{474} - 0.0831) / 0.1426$ .

To verify the amount of astaxanthin further, HPLC analyses were also employed. HPLC was carried out using a Shimadzu LC (Shimadzu, Kyoto, Japan) system equipped with a CBM-20A system controller, a LC-20AD pump, a DGU-20A<sub>3R</sub> degasser, and a SPD-20A UV-Vis detector. The chromatographic conditions were YMC Carotenoid Column (4.6 × 250 mm, 5 μm), and the mobile phase used for gradient elution was composed of methanol as system A, t-butylmethylether as system B, and 1 % phosphoric acid aqueous as system C. The progress of HPLC was monitored at the wavelength of 474 nm.

## Growth measurements

The number of cells was determined via direct counting with a hemocytometer under an OPTINITY microscope. The dry cell weight was measured by filtering the algal suspension through a pre-dried and pre-weighed, 0.45-μm cellulose nitrate membrane filter paper and drying in an oven at 80 °C for 24 h. The size of *H. pluvialis* was measured with OPTINITY microscope camera from more than 100 randomly selected microalgae cells.

## Statistical analysis

All experiments were performed with three replications. The SigmaPlot 10.0 program was used for all statistical analyses.

## Results

### The effect of red wavelength on the *Haematococcus pluvialis* cell growth

To examine the different effects of specific wavelengths on growth, *H. pluvialis* was cultivated under different light wavelengths. Blue LEDs (450 nm) and red LEDs (660 nm) were selected to determine the major effect of wavelength on *H. pluvialis* biology. Interestingly, specific wavelengths could

indeed affect *H. pluvialis* biology, thereby affecting growth (Fig. 1). For each light source, the growth patterns in the logarithmic phase was totally independently different. *H. pluvialis* cell numbers were significantly influenced by the light wavelength, since *H. pluvialis* produced higher cell numbers under red LEDs. The cell number of *H. pluvialis* grown under red LEDs was nearly 150 % higher than under blue LEDs at the tenth day (Fig. 1). It was concluded that red LEDs could promote *H. pluvialis* growth by enhancing cell divisions.

### The effect of blue wavelength on the *Haematococcus pluvialis* astaxanthin content and cell size

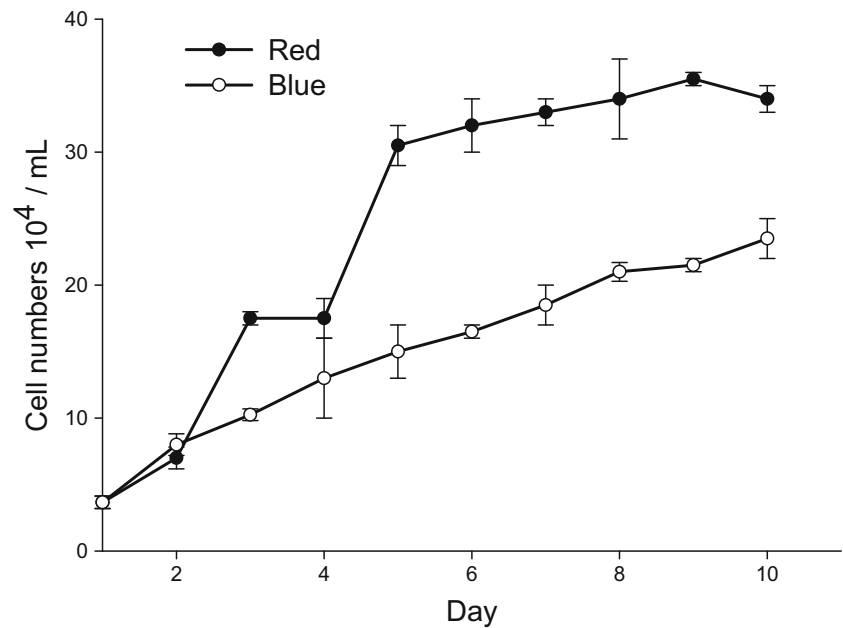
Apart from the conclusion we had above, it also meant that the growth of *H. pluvialis* was hampered by the blue LED illumination. Since blue LEDs seemed to cause stress conditions in *H. pluvialis*, it will be natural to question whether blue LEDs could increase astaxanthin biosynthesis. To examine this, we thoroughly compared the astaxanthin content. Consistent with our hypothesis, astaxanthin production in *H. pluvialis* cultivated under blue LEDs is higher than that under red LEDs (Fig. 2a). We observed consistently increasing astaxanthin production in more than three independent experimental replications, compared to those under red LEDs (Fig. 2a). An increase of approximately greater than 50 % in astaxanthin production was observed when cultivated under blue light. Our data clearly indicates that the blue wavelength could be one of the various induction factors for astaxanthin biosynthesis.

Observation under a dissecting microscope clearly revealed that the cell morphology, such as the cell size, was increased upon blue wavelength illumination. The *H. pluvialis* cells cultivated under blue LEDs were larger than those cultivated under red LEDs (Fig. 2b). Measurement of cell size revealed that cells grown under blue LEDs exhibited an approximately 15–20 % increase in diameter, compared to those under red LEDs. In addition, we detected the typical phenotypic characteristics of the absence of flagella with a reddish hue, representing the accumulation of astaxanthin (data not shown). All observations (e.g., lack of flagella with reddish color and increased cell size) recognized under blue wavelength illumination represent the development of the non-motile resting stage called cyst, which is accompanied with astaxanthin biosynthesis.

### Innovative approach to enhance productivities of *Haematococcus pluvialis*

From the above results, we could discover novel results regarding the phenotypic characteristics of *H. pluvialis* upon illumination by different LED light wavelengths. Obviously, red light enhances cell division, giving rise to higher cell

**Fig. 1** The effect of two different wavelength LEDs on *H. pluvialis* cell number using red (black circle) and blue (white circle) LEDs



numbers, whereas blue light definitely causes stress in *H. pluvialis* cells, thereby triggering astaxanthin biosynthesis. Since each specific wavelength of light stimulates particular characteristics in *H. pluvialis* biology (e.g., increased cell numbers or enhanced astaxanthin production), we then decided to utilize these cellular features upon specific LED wavelengths into biotechnological applications. We hypothesized that different *H. pluvialis* cell stages possibly demanded different wavelengths and the overall productivity of *H. pluvialis* could be further increased if the appropriate wavelength were applied at the proper cell stage.

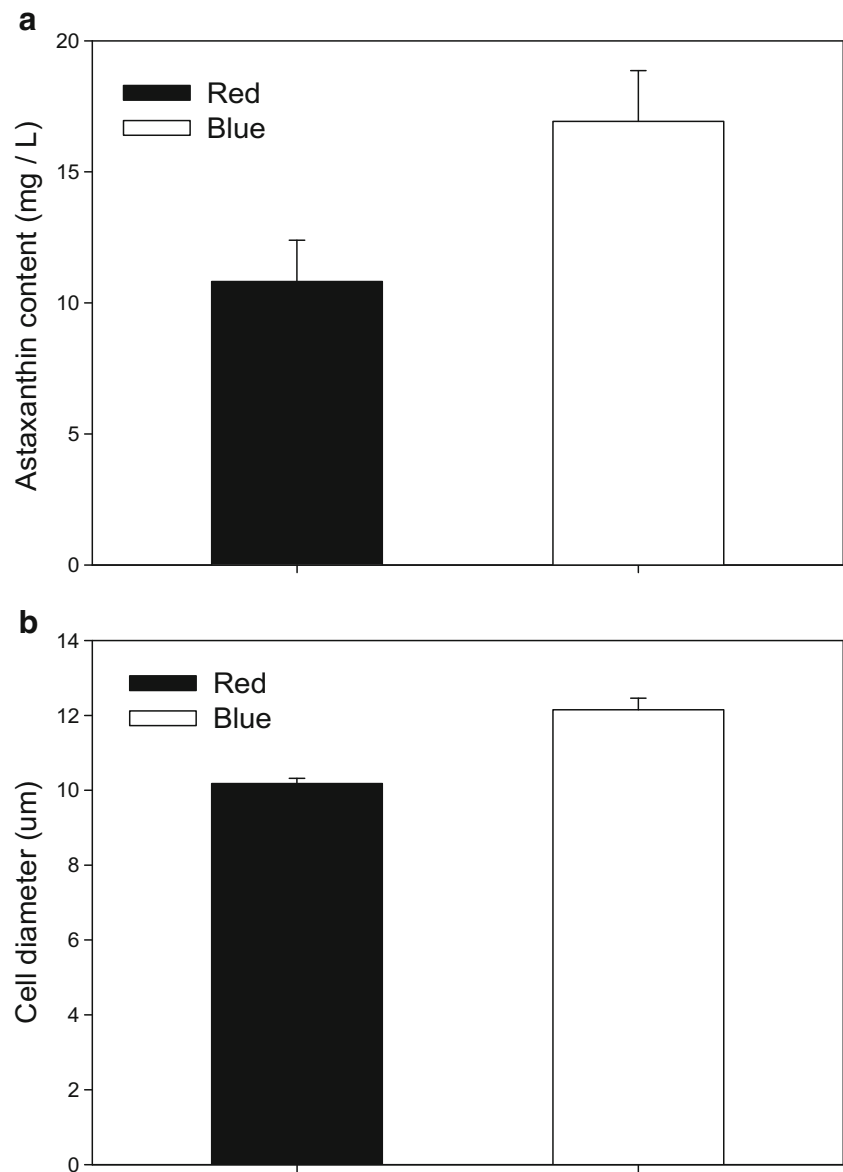
Based on the above results, the red wavelength gives rise to greater numbers of *H. pluvialis* cells, which might match the period for the vegetative stage of cultivation. However, blue wavelength light must be categorized as an inducing factor of astaxanthin production, due to its stressor effect in *H. pluvialis* cells. Therefore, we could reason a putative two-stage process, divided into growth and production stages illuminated by red and blue wavelength LEDs, respectively. To test our proposed process, we adopted the adequate manipulation of LED wavelength illumination, depending on *H. pluvialis* cell stage. The experiments were performed via the shift of light wavelength at different times in *H. pluvialis* cultivation. The experiments consisted of two different sets. The first set of experiments was divided on the wavelength illuminations: continuous red light as a control (R); red light first, then shifted to blue light (R–B). Moreover, the additional set of experiments was also consistent with the first set of experiment but with increased light intensity: red light with increased light intensity (R–I); red light shifted into blue light with increased intensity (R–B–I).

#### Change of astaxanthin content under wavelength shift

Before the change in light wavelength, all experimental setups showed a similar extent of astaxanthin production (Fig. 3). However, when we changed the wavelength to blue after 6 days, a discrepancy between the R and R–B groups became apparent. Then, R–B showed a significant increase of astaxanthin compared to those of the R group, and these trends continued until the end of cultivation (Fig. 3). Astaxanthin contents were increased by approximately 50–62 % with the appropriate deployment of wavelength shift from red to blue, compared to those under the simple continuous illumination of red wavelength. Our data indicated that the appropriate manipulation of wavelength in consideration of its influence on *H. pluvialis* biology could increase astaxanthin production.

In addition, whether a simultaneous wavelength shift along with a stepwise increase of light intensity could further increase in astaxanthin production was also tested. To do so, we gradually increased the light intensity from 50 to 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in one group of the experiment (R–B–I), during the period of cultivation as shown in the above graph (Fig. 3). As a comparison, the red light was included with the same extent of increased light intensity (R–I). As expected, the astaxanthin content steeply increased with the combination of blue wavelength illumination and high irradiance (Fig. 3). The results clearly indicate that our novel strategy based on an appropriate wavelength shift for *H. pluvialis* biology improved astaxanthin production and a further increase was achieved with the help of the simultaneous application of blue wavelength illumination and high irradiance.

**Fig. 2** The effect of two different wavelength LEDs on astaxanthin production and *H. pluvialis* cell morphology. **a** Astaxanthin content grown under either red (black bar) or blue (white bar) LED with three biological replications. **b** Cell diameters grown under either red (black bar) or blue (white bar) LED with three biological replications



### Concomitant applications of carbon source with blue light illumination

Since carbon source is generally required to induce astaxanthin biosynthesis, we next examined whether additional carbon source, simultaneously applied with the blue wavelength illumination, could enhance astaxanthin production further. To do so, first, various carbon sources, including glucose and acetate, were tested for selecting the best carbon to induce astaxanthin biosynthesis. Based on that, acetate could be chosen for subsequent experiments due to the fact that acetate displayed the best activity to enhance astaxanthin production (Table. 1). Then, we thoroughly examined the combinatory effect of both blue LEDs and acetate for astaxanthin biosynthesis. As expected, there was a significant increase of astaxanthin production when both blue illumination and

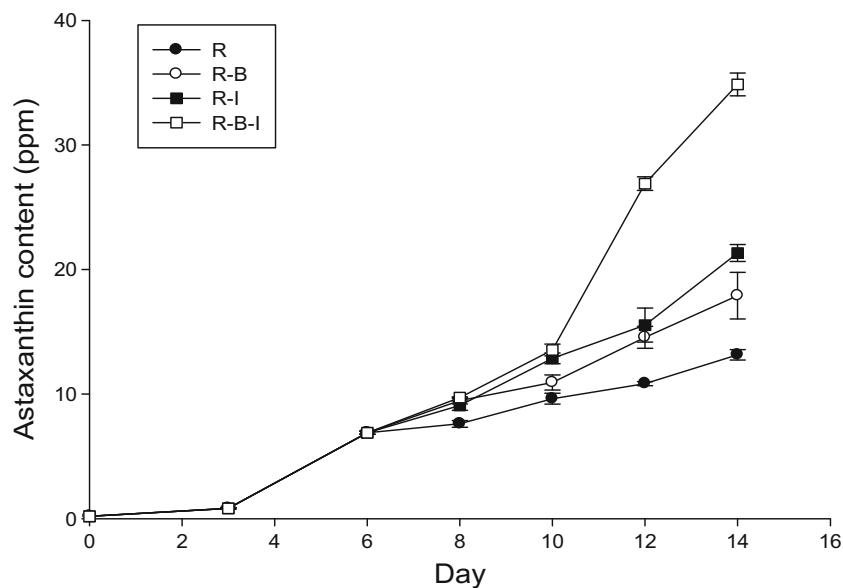
acetate were simultaneously applied (Fig. 4). These increases were consistently observed regardless of low or high light intensity indicating synergistic effect of acetate and blue wavelength illumination.

### Discussion

To facilitate astaxanthin production, it will be indispensable to develop efficient inductive systems that cause stress to *H. pluvialis* cells. The beneficial effects of astaxanthin on human health are reported by researchers, which drive for more intensive studies on astaxanthin biosynthesis (Hussein et al. 2006; Kidd 2011). To date, various environmental factors on astaxanthin biosynthesis have been reported: high irradiation (Kobayashi et al. 1992), nitrogen deficiency



**Fig. 3** Employment of the wavelength-shifting strategy to enhance astaxanthin production; experiments consisted of three different conditions of light wavelength illumination: continuous red light as a control (R, black circle); red light first, then shifted to blue light (R-B, white circle); red light with increased light intensity (R-I, black rectangle); and red light shifted to blue light with increased intensity (R-B-I, white rectangle)



(Kakizono et al. 1992), phosphate deficiency (Harker et al. 1996), magnesium deficiency (Fábregas et al. 2003), acetate addition, ferrous addition (Kobayashi et al. 1991), and salt addition (Kobayashi et al. 1997). All of these factors and conditions could be utilized for biotechnological processes using *H. pluvialis* by inducing astaxanthin biosynthesis.

In this study, the effect of a specific light wavelength on *H. pluvialis* biology was examined, specifically with red or blue wavelength illumination. Red LEDs seemed helpful for maintaining the vegetative stage of *H. pluvialis*, by generating greater cell numbers. However, blue LEDs continuously gave rise to lesser cell numbers. We speculated that blue LED wavelength could have put *H. pluvialis* in a stress condition, so that it could delay *H. pluvialis* cell division. Furthermore, *H. pluvialis* exhibits a distinctive morphology with the cessation of cell divisions and the formation of a thick, resistant cell wall during the process of astaxanthin biosynthesis. In this regard, we also investigated whether the unique morphology of *H. pluvialis*, tightly linked with astaxanthin production, could be evident upon blue LEDs or not. As expected, a significant difference in *H. pluvialis* morphology was noticed upon blue wavelength illumination, along with enhanced astaxanthin production. Our results clearly indicate that blue wavelength illumination could help induce astaxanthin

production. Because blue light has high energetic wavelength, it is likely that *H. pluvialis* cells could receive greater stress under blue light, compared to those under red light. In response to this stress caused by blue light illumination, *H. pluvialis* cells accumulate astaxanthin and develop a thick cell wall.

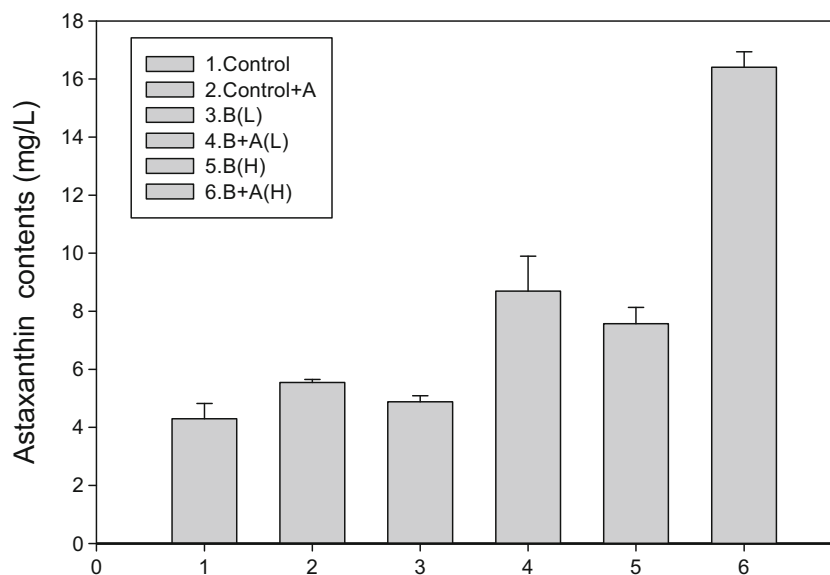
Based on the above results, the red wavelength gives rise to greater numbers of *H. pluvialis* cells, which might match the period for the vegetative stage of cultivation. However, blue wavelength light must be categorized as an inducing factor of astaxanthin production, due to its stressor effect in *H. pluvialis* cells. Therefore, we could reason a putative two-stage process, divided into growth and production stages illuminated by red and blue wavelength LEDs, respectively (Fig. 5). Since the two-stage process dividing the growth and production stages has been widely accepted for the process of astaxanthin production using *H. pluvialis* cultivation, it was conceived that red and blue wavelengths must be well matched with the growth and production stages. With our novel strategy of proper wavelength shift, higher astaxanthin production was successfully achieved.

Most notably, previously, we demonstrated that blue wavelength could increase cell size, whereas red wavelength resulted in a small-sized cell with active division in *Chlorella vulgaris* (Kim et al. 2014). The morphological characteristics upon either blue or red light between *H. pluvialis* and *C. vulgaris* were similar each other suggesting the consistent effect of specific wavelength illumination across different microalgal species. However, due to the largely divergent biology between *H. pluvialis* and *C. vulgaris*, the wavelength strategy must be opposite each other. For example, blue light first and then switch to red illumination was helpful for enhancing *C. vulgaris* productivity, whereas red light first and then switch to blue illumination improved astaxanthin

**Table 1** The effect of different carbon source on *H. pluvialis* astaxanthin accumulation

Additional carbon source (w/v)	Astaxanthin content (ppm)
Glucose (2 %)	19.8036 ± 1.2272
Glucose (4 %)	19.1725 ± 1.6363
Acetate (2 %)	25.9397 ± 1.771
Acetate (4 %)	22.2236 ± 1.5259

**Fig. 4** The effect of fluorescent or blue LEDs in different light intensity on astaxanthin production with or without acetate: control; control + A, control with acetate; B(L), blue light; B + A(L), blue light with acetate; B(H), blue light with increased intensity; and B + A(H), blue light with increased intensity plus acetate



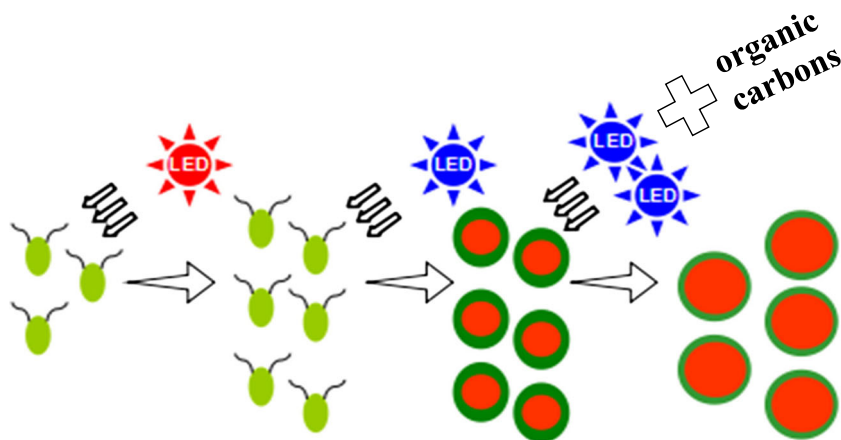
production in *H. pluvialis*. Consequently, our data suggest that special care must be made whenever employing wavelength shift strategy, because each of the specific microalgal species possesses and displays a completely different biology from each other.

It has been reported that organic carbon could increase astaxanthin production in *H. pluvialis* by promoting carbon assimilation, which in turn leads to the induced cells with thick cell wall. Therefore, we also examined the possibility of a further increase in astaxanthin production via the simultaneous treatment of both organic carbons and blue light illumination. Prior to the experiment, several organic carbons were screened for the most effective carbon source for astaxanthin induction, and it turned out that acetate (2 %) is the most effective organic carbon for the induction. Subsequently, simultaneous application of both acetate (2 %) and blue wavelength was performed and evaluated, compared to the other experiments. Interestingly, the concomitant applications of blue wavelength as well as additional carbon source

could lead to a further increase in astaxanthin production. We speculated that blue wavelength illumination definitely causes cellular stress in *H. pluvialis* and acetate addition probably helps increase cell size with cyst formation, thereby enhancing the capacity of astaxanthin accumulation further. Hence, we successfully demonstrated that the concomitant applications of blue wavelength as well as additional carbon source such as acetate could be one of the most powerful inductive factors to astaxanthin production in *H. pluvialis*. Our results will have a significant impact on the future development of microalgae biotechnology for astaxanthin production using *H. pluvialis*.

Taken together, we could propose the following strategy for astaxanthin production in *H. pluvialis*. (1) The proper wavelength shift from red LEDs (well matched with green motile stage) to blue LEDs (for red cyst stage) could be employed to enhance the astaxanthin production. (2) Blue illumination could be deployed with the concomitant application of carbon source, e.g., acetate, to increase the productivity further (Fig. 5).

**Fig. 5** The schematic illustration of the strategy developed in this study



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#### Compliance with ethical standards

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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