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Non-motile cell development and astaxanthin accumulation in *Haematococcus pluvialis* under different light spectra

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ABSTRACT: Light plays a crucial role in influencing the growth of *Haematococcus pluvialis* and its production of astaxanthin. Previous studies have shown that non-motile cells of *H. pluvialis* exhibit higher light tolerance compared to motile cells, and cultivating non-motile cells as the predominant cell type in the red stage can significantly enhance overall astaxanthin productivity. However, despite some progress has been made in inducing the formation of non-motile cells through high light intensity, the specific light spectra responsible for inducing both non-motile cells formation and astaxanthin accumulation remain largely unknown. In this study, we explored the impact of three different light spectra on the formation of non-motile cells, cellular morphology, pigment composition, and chlorophyll a fluorescence parameters in the green stage of *H. pluvialis*. Additionally, we assessed the astaxanthin accumulation, dry weight, mortality rate, and cyst diameter during the induction period. The results indicate that light spectra had a significant impact on the growth and development as well as physiological processes of *H. pluvialis* cells. Compared to red and white light, blue light proves more conducive to promoting non-motile cells formation and enhancing astaxanthin accumulation. The findings make a significant contribution to the development of optimized method for cultivating *H. pluvialis* and enhancing astaxanthin productivity in factory settings.

KEYWORDS: *Haematococcus pluvialis*, cell division, light-emitting diodes, light spectra, astaxanthin

INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-Dione), a red ketocarotenoid, is highly esteemed for its robust antioxidant properties and boasts widespread utilization across diverse industries including nutraceuticals, aquaculture, cosmetics, food, and feed [1]. Due to the substantial market potential of natural astaxanthin, its efficient production has become a primary concern in industrial contexts. *Haematococcus pluvialis*, a unicellular member of *Chlorophyceae*, is widely acknowledged as a leading contender for natural astaxanthin production [2], rendering it invaluable for commercial endeavors [3].

The life cycle of *H. pluvialis* is complex, consisting of two phases based on cell morphology and physiology: the motile phase and the non-motile phase [4]. During the motile phase, *H. pluvialis* cells possess a thick wall and two flagella, primarily undergoing cell proliferation [5]. Under favorable conditions, these cells divide quickly and appear green , leading to this stage being termed the "green stage" [6]. In contrast, during the non-motile phase, *H. pluvialis* cells possess a thick wall and lack flagella. Under stressful conditions, cell proliferation is impeded, causing motile cells to transition into non-motile cysts [5, 6]. This transformation involves the loss of flagella, further thickening of secondary cell walls, and accumulation of astaxanthin pigment, which imparts a red color to the cells, hence this stage is referred to as the "red stage" [7]. In actual production, stressors such as high light intensity, high salinity, and nitrogen or phosphorus deficiency are usually employed to induce astaxanthin accumulate, resulting in the formation of red non-motile aplanospores [8, 9].

The characteristics of light (such as spectra, intensity, and duration) have long been recognized as the primary abiotic factor influencing the metabolism and development of microalgal cells under photoautotrophic conditions [10]. This is because photosynthesis is the main process through which microalgae obtain energy for their growth and survival. Although sunlight is the most cost-effective option, artificial light remains economically viable for the production of high-value products using microalgae biomass [11]. Compared to traditional artificial light sources such as incandescent and gas-discharge lamps (e.g., fluorescent lamps), light-emitting diodes (LEDs) can produce light with a selected peak wavelength and adjust light intensity by changing the current, providing advantages in terms of luminous efficiency, power requirements, heat generation, and directionality [12, 13].

With the development of LED lighting technology, researchers have been able to conduct more in-depth studies on how different light qualities influence the growth and development of microalgae [13]. Among various light spectra, blue and red wavelengths play a crucial role in the photosynthesis, thereby influencing

the development and metabolism of photosynthetic organisms [14]. It has been reported that red and blue light can increase the ratio of fucoxanthin to Chlorophyll a and Chlorophyll c in *Laminaria hyperborea* [15]. Furthermore, several studies have consistently shown that green microalgae such as *Chlamydomonas reinhardtii* and *Dunaliella salina* exhibit larger cell sizes under blue light compared to red light [16, 17]. However, it was observed that the growth rate and biomass composition of microalgae showed species-specific changes when cultivated under different light colors. For example, some studies have indicated significant variations in the maximum growth rates of photoautotrophic or heterotrophic *Nannochloropsis* under different light wavelengths, with blue light being particularly favorable for their proliferation compared to red and white light [18]. Conversely, high-intensity red light significantly enhances biomass production in *H. pluvialis* while blue light promotes the accumulation of astaxanthin [19]. Despite recognizing the critical role of light in the growth and development of *H. pluvialis*, our understanding of how different spectra of light affect non-motile cell formation, cell growth, and astaxanthin accumulation within microalgae-based systems is still limited.

In this study, aimed at enhancing astaxanthin production by *H. pluvialis*, we investigated the impact of three different light spectra on the formation of non-motile cells, physiological processes, and astaxanthin accumulation. This study not only offers novel insights into the effects of light spectra on the formation of *H. pluvialis* non-motile cells but also highlights the potential for developing more efficient cultivation techniques to augment astaxanthin production from these cells.

MATERIALS AND METHODS

Algal strain and inoculum preparation

The *H. pluvialis* strain (CCMA-451, Genbank accession number MG847145) utilized in this study was obtained from the Center for Collections of Marine Algae of Guangdong Ocean University. Seed culture was maintained in the Bold Basal Medium (BBM) [20] growth medium (Solarbio, Beijing, China) in bubble column reactors with an inner diameter of 4.5 cm under aeration (CO_2/air, v/v 1%). Temperature was maintained at 25 ± 1 °C. White LEDs, consisting of blue LED and yellow fluorescent material, were utilized as the light source. Continuous light was provided with intensity set at 30 µmol photons/m^2/s.

Algal cells that were cultivated for about 5 days and in exponential phase were used for inoculation. The inoculation density was 0.5×10^6 cells per ml and the initial culture volume was 700 ml. The impact of three different light (including a narrow band of blue light with wavelength centered around 465–475 nm, a narrow band of red light with wavelength centered around 640–650 nm, and white LED light) on the vegetative growth of *H. pluvialis* were then investigated. Different light spectra are emitted by LED square light panels (Xiamen Top-Succeed Photobiology Technology, Co., Ltd., Xiamen, China). During cultivation, light intensity was set to 30 µmol photons/m^2/s and continuous illumination was provided. The temperature was maintained at 25 ± 1 °C. For aeration, a mixture of CO_2 and air (1%, v/v) was fed into the culture medium from a tube extending to the bottom of each reactor. During cultivation, the columns were covered with a piece of black cloth to avoid the influence of ambient light.

Determination of cell concentration and dry cell weight

To determine cell numbers, samples were initially fixed with Lugols iodine solution (Solarbio), followed by counting using a Neubauer improved cell counting chamber under an Olympus BX53 light microscope (Olympus, Tokyo, Japan).

Dry weight (DW) was determined every two days following the method outlined by Li et al [21]. Briefly, 10 ml algal suspension were filtered through a pre-weighed (m_1) filter paper (47 mm, 1.2 µm, Whatman GF/C). The filter paper was then dried overnight at 90 °C until a constant weight was achieved (m_2). The DW per one liter algal culture was calculated using Eq. (1).

$$\mathrm{DW} = (m_2 - m_1) \times 10^3/10 \tag{1}$$

Analysis of cell morphology and photosynthetic pigment

Microscopic observation and measurement: the morphological changes of algal cells were examined every 12 h using an Olympus BX53 light microscope and the microphotographs were captured with an Olympus DP74 digital camera. Given the irregular shape of motile cells, cell size is characterized by protoplast length and measured using Olympus application software with an internal reticle scale. A minimum of 300 cells per group were utilized for cell size statistics.

Photosynthetic pigment analysis: The pigments were extracted following the method described by Pruvost et al [22]. In brief, 1 ml of algal solution underwent centrifugation at a speed of 12,000 rpm for 5 min. The supernatant was then discarded, and 1.5 ml of methanol was added to the precipitate. Intracellular pigments were extracted at room temperature and in darkness until the cells became colorless. Finally, the absorbance of the extract was measured at 480, 652, and 665 nm. Total chlorophyll (chl) content was calculated using Eqs. (2), (3), and (4) as established by Ritchie [23]. Carotenoid content was determined using Eq. (5) as established by Strickland and Par-

sons [24].

$$[\text{chl-a}]\,(\mu\text{g/ml}) = -8.096\times OD_{652} + 16.5169\times OD_{665} \quad (2)$$

$$[\text{chl-b}]\,(\mu\text{g/ml}) = 27.4405\times OD_{652} - 12.1688\times OD_{665} \quad (3)$$

$$\text{Total chl} = [\text{chl-a}] + [\text{chl-b}] \quad (4)$$

$$[\text{carotenoid}]\,(\mu\text{g/ml}) = 4\times OD_{480} \quad (5)$$

where OD_{480}, OD_{652}, and OD_{665} were the absorbance of extracts at 480, 652, and 665 nm respectively.

Analysis of astaxanthin content

The concentration of astaxanthin (AC) was determined using the photometric method outlined by Li et al [25]. Initially, algal cells were harvested from a 10 ml sample by centrifugation at 3000 rpm for 2 min. Subsequently, the cells were treated with a solution of 5% (w/v) KOH in 30% (v/v) methanol at 75 °C for 10 min. The resulting pellet was then mixed with 25 ml acetic acid and subsequently extracted with 5 ml of DMSO (Solarbio) for 10 min in a water bath maintained at 75 °C. Following centrifugation at 10,000 rpm for 5 min, the supernatant was collected. This extraction process was repeated iteratively until the pellet was devoid of color. The extraction procedure was repeated several times until the pellet became colorless. Finally, the absorbance of the extract was measured at 492 nm. Astaxanthin content was determined using Eq. (6) as established by Sun et al [26].

$$\text{AC}\ (\mu\text{g/ml}) = 4.5\times OD_{492}\times \text{Va}\ (\text{ml})/10\ (\text{ml}) \quad (6)$$

where OD_{492} was the absorbance of extracts at 492 nm and Va was the volume of extracts.

Astaxanthin content increase rate were calculated with Eq. (7).

$$\text{AC}\ (\%) = (AC_t - AC_0)\times 10^{-3}/DW_t \times 100\% \quad (7)$$

where AC_t and AC_0 were the astaxanthin content on day t and day 0.

Measurement of chlorophyll fluorescence parameters

The chlorophyll fluorescence parameters of the photosystem II of fresh *H. pluvialis* green cells were measured using a Dual-PAM 100 chlorophyll fluorometer (Walz Company, Effeltrich, Germany), following the method described by Dummee et al [27]. Prior to measurement, the samples underwent a 15 min dark adaptation. After dark adaptation of the microalgal cells, the slow kinetics were determined and various chlorophyll a fluorescence parameters including F_v/F_m (intrinsic or maximum efficiency of photosystem II), NPQ (non-photochemical quenching), and ETR (linear electron transport rate) of the microalgal cells were obtained.

Statistical analysis

Each exposure experiment was independently repeated three times, and the data were recorded as the mean with standard error (SE). Statistical analyses were performed using the Spearman correlation analysis (SPSS19.0).

RESULTS

Effect of light spectra on the cellular morphology and formation of non-motile cells

Light spectra significantly influenced changes in cellular morphology. Under the light microscope, the morphology of motile cells was observed after exposure to three different light spectra, as shown in Fig. 1a. Exposure to monochromatic blue light for 24 h induced a transformation of some *H. pluvialis* cells from ellipsoidal or pear-shaped to spherical. By 48 h, the number of spherical cells increased, accompanied by the appearance of a small amount of orange-red pigment in the central region of the cells. Continuous irradiation for 72 h resulted in a further increase in the orange-red pigment in the central region, although some dead or damaged cells were also observed. Interestingly, not all cells that contain red pigment exhibit flagellum loss. Similarly, exposure to red light for 48 h led to a transformation of a small number of *H. pluvialis* cells from pear-shaped to spherical; by 72 h, some cells exhibited a small amount of orange-red pigment in the central region. In contrast, no significant changes in cell morphology were observed after 72 h of white light irradiation.

Furthermore, a significant difference in non-motile cell numbers was observed among the three light spectra groups, with the highest count recorded in the blue light group, followed by the red light and then the white light group (Fig. 1b). After 72 h of culture, non-motile cell counts in the blue light group reached 13.0×10^4 cells/ml, significantly higher than those observed in red light and white light groups, indicating the effective promotion of *H. pluvialis* non-motile cell formation by blue light (Fig. 1c). Additionally, the percentage of non-motile cells was calculated, revealing that both blue and red light can moderately enhance the formation of *H. pluvialis* non-motile cells to some extent. We observed that blue light resulted in the highest percentage of non-motile cells at $23.47\pm4.4\%$, which is significantly higher compared to white light ($0.75\pm0.5\%$) and red light ($7.1\pm1.1\%$). This suggests that blue light effectively stimulates the formation of non-motile cells in *H. pluvialis* within a short period (Fig. 1d).

Effect of light spectra on the photosynthetic pigment of *H. pluvialis* green cells

The level of chlorophyll a, chlorophyll b, chlorophyll a/b ratio, and carotenoids was analyzed every 12 h

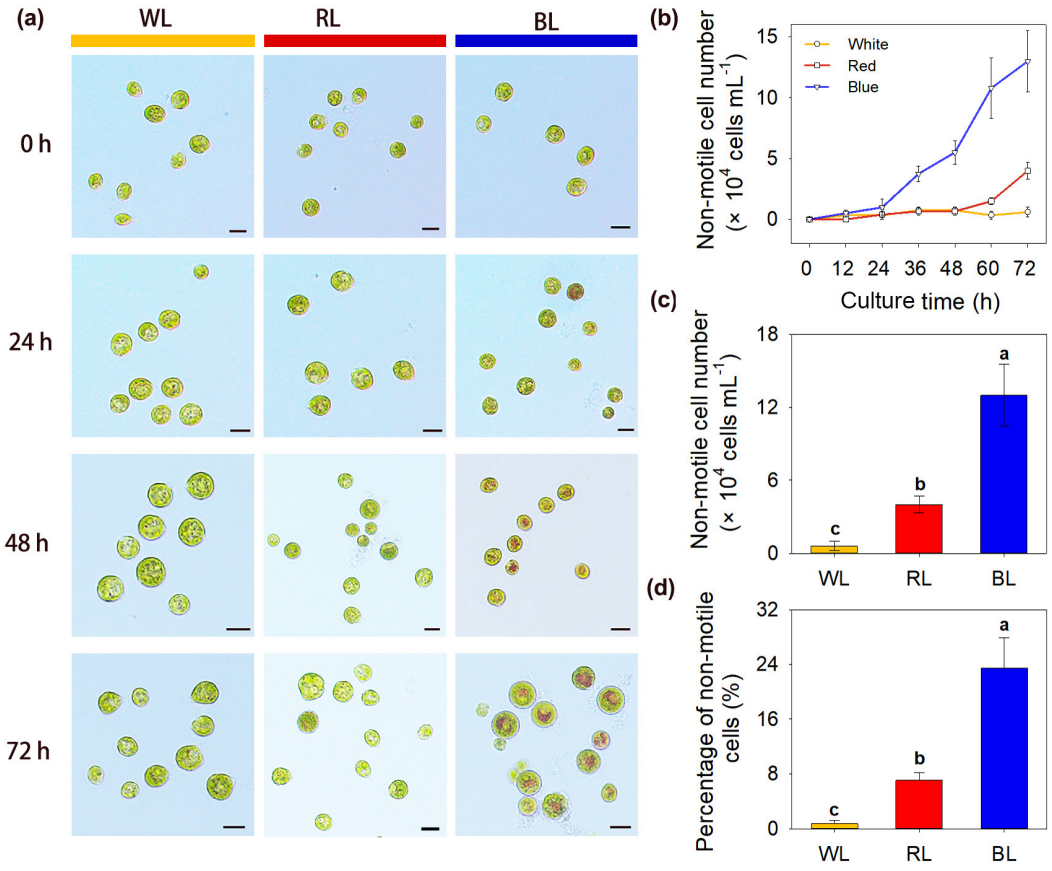

Fig. 1 Effect of light spectra on the morphology and formation of non-motile cells. The cell morphological changes (a), the number of non-motile cells (b), non-motile cell number obtained at the end of cultivation (c), and percentage of non-motile cell number at the end of cultivation (d) under different quality of light treatment (WL – white light; RL – red light; BL – blue light). In (b–d), results were presented as mean ± SE of the measurements of three biological replicates. In (c,d), the same lowercase letter means insignificant difference, while different lowercase letters mean significant difference. Results were presented as mean ± SE of the measurements of three biological replicates. The scale bar = 10 μm.

to verify the impact of different light spectra effects on pigment synthesis and modulation during the early stage of non-motile cell cultivation. The production of chlorophyll a and chlorophyll b exhibited a significant increase with prolonged cultivation time under both white and red light. However, exposure to blue light initially led to an increase in both chlorophyll a and chlorophyll b production, followed by a subsequent decline (Fig. 2a). After 72 h of cultivation, the chlorophyll a and chlorophyll b levels in *H. pluvialis* green cells grown under white light reached 14.42 ± 0.14 μg/ml and 5.95 ± 0.17 μg/ml, respectively. These values were slightly higher than those observed under red light (12.33 ± 0.15 μg/ml and 4.95 ± 0.036 μg/ml). Compared to blue light conditions, there was an approximate increase of 43.62% for chlorophyll a and 38.98% for chlorophyll b (Fig. 2a,b).

Among all three different light spectra, the ratio of chlorophyll a/b of *H. pluvialis* cells showed an increasing trend within 36 h of cultivation period. However, as time progressed, we observed a subsequent decrease in the ratio of chlorophyll a/b under white and blue light, with values slightly lower than the initial starting points. The results suggested that during the initial stage of cultivation, the three wavelengths of light stimulation facilitate the synthesis and accumulation of chlorophylls within *H. pluvialis* green cells, thereby resulting in an elevation in the chlorophyll a/b ratio. However, subsequent light stimulation induces chlorophyll degradation, leading to a reduction in the chlorophyll a/b ratio (Fig. 2c).

Changes in carotenoids content throughout the cultivation period are shown in Fig. 2d. A significant difference in carotenoid content was observed among the three different light spectra, with white light being the most favorable for the content of carotenoids in *H. pluvialis*, followed by red light and blue light. After 72 h of cultivation, the carotenoid content in *H. plu-*

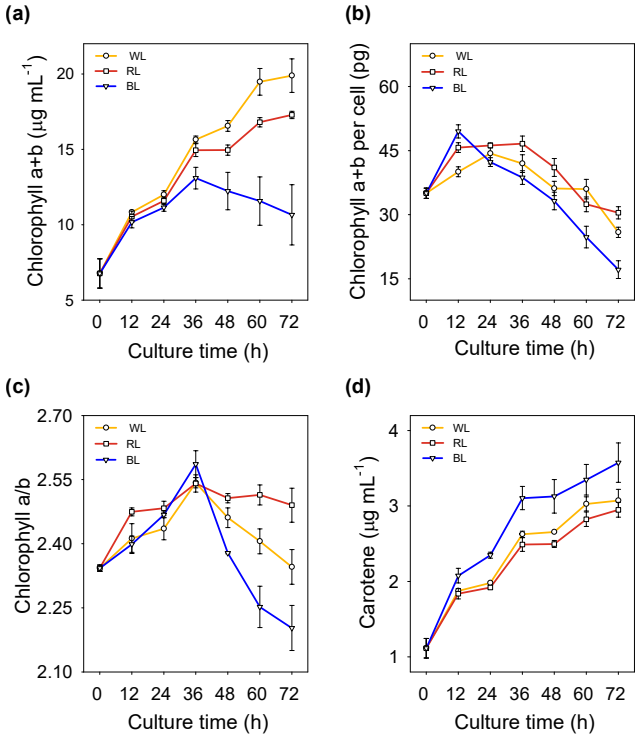

Fig. 2 Changes in pigment content under different spectra of light exposure. (a) Chlorophyll a content. (b) Chlorophyll b content. (c) Chlorophyll a/b ratio. (d) Carotene content. In (a–d), WL – white light; RL – red light; BL – blue light. Results were presented as mean ± SE of the measurements of three biological replicates.

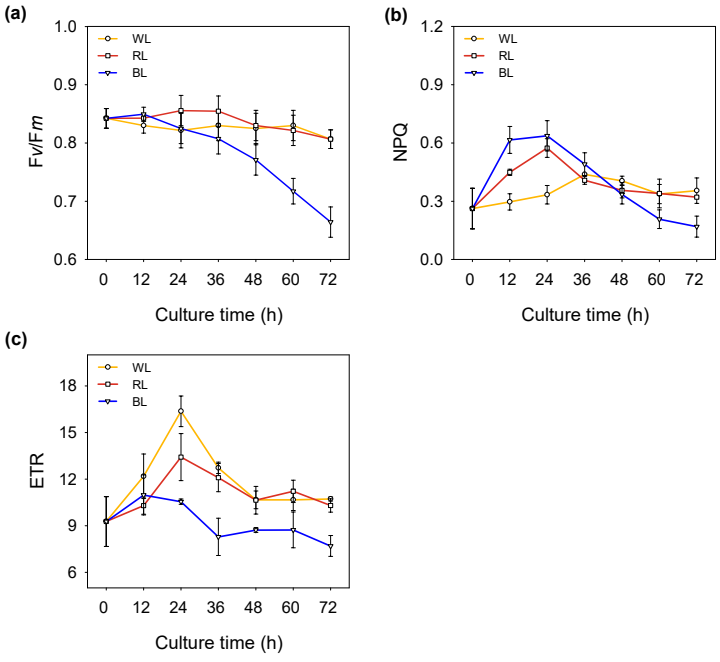

Fig. 3 Changes in chlorophyll fluorescence parameters under different spectra of light exposure. (a) F_v/F_m. (b) NPQ. (c) ETR. In (a–d), WL – white light; RL – red light; BL – blue light. Results were presented as mean ± SE of the measurements of three biological replicates.

vialis green cells grown under white light reached 6.72 ± 0.22 µg/ml, which is slightly higher than that observed under red light (5.87 ± 0.065 µg/ml). This represents an approximate increase of 29.8% compared to the levels seen under blue light (Fig. 2d).

Effect of light spectra on the chlorophyll fluorescence parameters of *H. pluvialis* green cells

The chlorophyll fluorescence parameters, including F_v/F_m, NPQ, and ETR, were analyzed to characterize the response of *H. pluvialis* green cells under different light spectra (Fig. 3). The F_v/F_m of *H. pluvialis* is influenced by the spectral composition of light in its environment, and its F_v/F_m also exhibits variations under different light spectra (Fig. 3a). Among all three different light spectra, F_v/F_m of *H. pluvialis* cells showed no significant difference within 24-h cultivation period. However, after 24 h of cultivation, the F_v/F_m of *H. pluvialis* exposed to blue light gradually decreased with increasing cultivation time, dropping from 0.82 to 0.66. In contrast, the F_v/F_m of *H. pluvialis* in red light and white light groups exhibited fluctuations but remained stable within the range of 0.80–0.84 (Fig. 3a). The NPQ exhibited an initial increasing trend followed by a subsequent decline as the cultivation time increased under three different light conditions (Fig. 3b). In the blue light group, the NPQ reached its peak at 24 h of cultivation and subsequently exhibited a gradual decrease. The NPQ in red light group reached its maximum value after 24 h of cultivation and then decreased, remaining stable and basically unchanged in the 48–72 h cultivation period (Fig. 3b). The trends of ETR of *H. pluvialis* green cells with cultivation time and light spectra were consistent with those of NPQ. Notably, the ETR of *H. pluvialis* green cells was significantly lower in the blue light group compared to both the white light and red light during the cultivation period (Fig. 3c).

Effects of light spectra on the growth of *H. pluvialis* green cells

Among the three light spectra investigated, blue light was the most favorable for the division of *H. pluvialis* green cells, followed by white light, and red light (Fig. 4a). By the tenth day of cultivation, the cell number of *H. pluvialis* cultivated under the blue light condition reached 53.6×10^4 cells/ml, marking a 28.3% increase compared to those under red light (38.5×10^4 cells/ml). However, the cell number of *H. pluvialis* cultivated under white light, albeit marginally lower than that under blue light (51.3×10^4 cells/ml), exhibited no statistically significant variance.

Conversely, concerning the accumulation of dry cell weight in *H. pluvialis* green cells, red light emerged as the most favorable among the three light spectra studied, followed by blue light and white light (Fig. 4b). By the tenth day of cultivation, the dry cell weight attained under red light reached 1.12 ± 0.055 g/l, surpassing that cultivated under white light (0.87 ± 0.01 g/l) by approximately 22.6% and slightly exceeding that under blue light (1.0 ± 0.03 g/l)(Table 1). It is noteworthy that during the initial stages of cultivation, the accumulation of dry cell weight in *H. pluvialis* remained relatively consistent across the three different light spectra. However, as the cultivation progressed, the dry cell weight under red light exhibited a pronounced increase, outpacing that under the other two light spectra.

Effect of light spectra on the astaxanthin production of *H. pluvialis* green cells

The alterations in astaxanthin concentration among different light spectra are shown in Fig. 5. Astaxanthin accumulation displayed a consistent upward trajectory with prolonged culture duration under all light spectra (Fig. 5a,b). Noteworthy disparities in astaxanthin concentrations were discerned among the three distinct light spectra, with blue light exhibiting the most propitious environment for astaxanthin accumulation in *H. pluvialis*, succeeded by white light and red light (Fig. 5a). The maximum astaxanthin concentration values of all three groups appeared at the end of the 10-day cultivation period. Under blue light conditions, the maximum astaxanthin concentration reached 18.58 ± 0.24 mg/l, which was about 30.52% increase compared to those cultivated under white light (12.91 ± 2.36 mg/l) (Fig. 5c). However, the maximum concentration of astaxanthin in *H. pluvialis* cultivated under red light was slightly higher than that obtained under white light (14.16 ± 1.09 mg/l), although this difference was not statistically significant (Fig. 5c). Furthermore, the astaxanthin content of the three cultures at the culmination of cultivation was computed (Fig. 5b,d). The astaxanthin content at the conclusion of cultivation exhibited no significant variance between the red light and white light groups, registering values of 0.14 ± 0.0093% and 0.12 ± 0.005%, respectively. In contrast, the astaxanthin content attained under blue light was significantly higher than that obtained under the other two light spectra, with a value of 0.26 ± 0.039% (Fig. 5d and Table 1).

DISCUSSION

H. pluvialis cultivation requires mutually exclusive conditions for maximum growth and astaxanthin content, as rapid growth needs favorable conditions while astaxanthin accumulation necessitates stress conditions. The two-stage strategy has resolved the conflict between these conditions but not effectively addressed massive cell death during the red stage, leading to low overall astaxanthin productivity [5, 28]. In recent years, researchers have made significant progress in searching for advanced cultivation strategies for

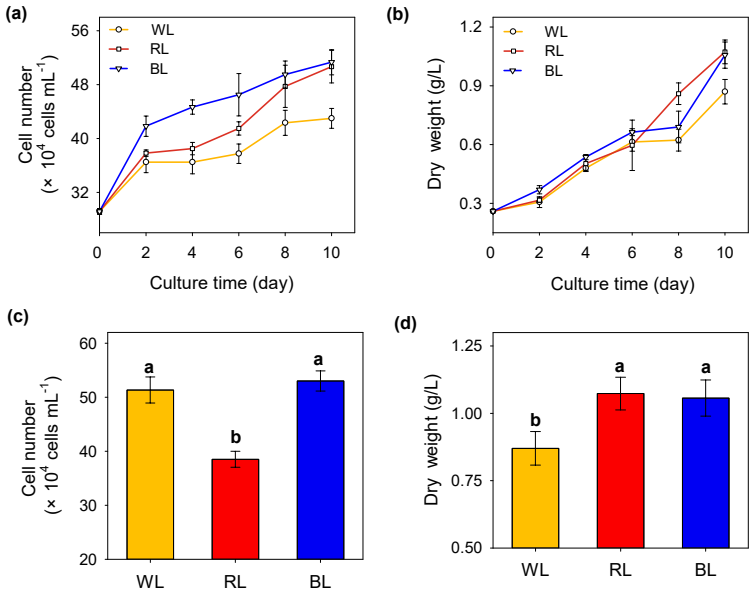

Fig. 4 Effects of light spectra on the growth of *H. pluvialis* green cells. (a) Growth curves based on cell number; (b) growth curves based on dry cell weight; (c) cell number obtained at the end of cultivation; (d) dry cell weight obtained at the end of cultivation. Results were presented as means ± SE of the measurements of three biological replicates. In (c,d), the same lowercase letter means insignificant difference, while different lowercase letters mean significant difference. In (a–d), BL – blue light; RL – red light; WL – white light. Results were presented as mean ± SE of the measurements of three biological replicates.

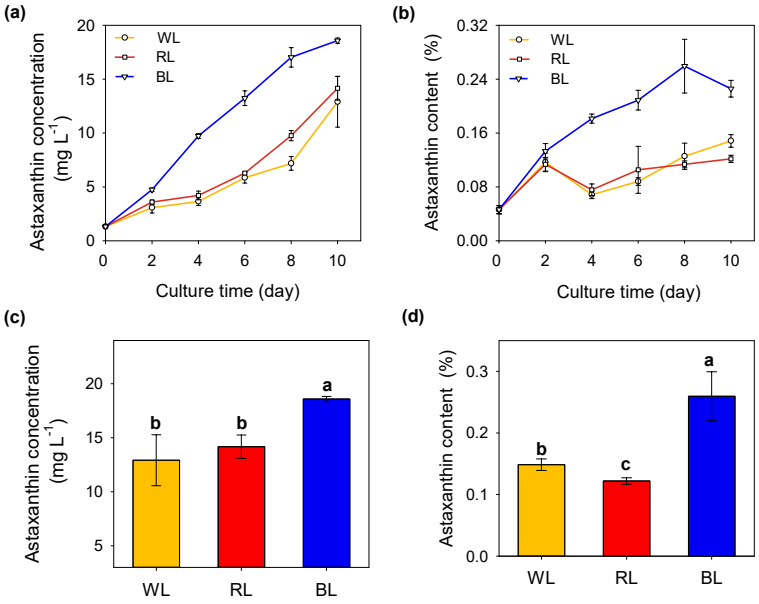

Fig. 5 Effect of light spectra on the astaxanthin production of *H. pluvialis* green cells. The astaxanthin concentration (a), astaxanthin content (b), astaxanthin concentration obtained at the end of cultivation (c), and astaxanthin content obtained at the end of cultivation. Results were presented as mean ± SE of the measurements of three biological replicates. In (c, d), the same lowercase letter means insignificant difference, while different lowercase letters mean significant difference. In (a–d), BL – blue light; RL – red light; WL – white light. Results were presented as mean ± SE of the measurements of three biological replicates.

Table 1 The maximum value of biomass, astaxanthin concentration, astaxanthin productivity, astaxanthin content, the percentage of non-motile cells, daily percentage growth rate of non-motile cells, cell mortality, and cell size of treatment groups exposed to different light spectra.

Parameters	Light spectra		
	White	Red	Blue
Dry weight (g/l)	0.87 ± 0.062	1.12 ± 0.055	1.06 ± 0.03
Astaxanthin concentration (mg/l)	14.16 ± 1.09	12.91 ± 2.36	18.58 ± 0.24
Astaxanthin productivity (mg/l/d)	1.42 ± 0.67	1.54 ± 0.30	2.46 ± 0.13
Astaxanthin content (%)	0.12 ± 0.005	0.14 ± 0.009	0.26 ± 0.039
Non-motile cells (%)	31.16 ± 1.67	26.33 ± 2.21	42.00 ± 2.46
Growth rate of non-motile cells (%/day)	3.12 ± 0.17	2.63 ± 0.22	4.20 ± 0.25
Cell mortality rate (%)	3.34 ± 1.31	5.77 ± 1.33	6.26 ± 1.65
Cell size (μm)	24.76 ± 0.39	28.18 ± 0.64	19.76 ± 0.39

The maximum value of each parameter was presented in the table. All data were obtained on day 10 of the culture stage.

H. pluvialis to achieve efficient and stable production of astaxanthin. For instance, Li et al [29, 30] reported that incorporating non-motile cells as the dominant cell type during the red stage resulted in a remarkable reduction in cell mortality by over 70%, accompanied by a significant increase in biomass and astaxanthin content by up to 2.1-fold and 3.5-fold, respectively. These findings suggest that the substantial decrease in cell mortality may be attributed to a higher proportion of non-motile cells during the red stage. In this study, we examined morphological changes and proportions of non-motile cells in *H. pluvialis* exposed to different light spectra to determine their impact on divergence in morphology, development, and physiological process. The results showed that blue light is more effective than red or white light at inducing transformation from green motile cells into red non-motile cells and promoting astaxanthin accumulation. This finding is further supported by previous research indicating that non-motile cells demonstrate greater resilience to stressful conditions compared to motile cells [31]. Thus, by inducing motile cells to transform into non-motile cells with higher tolerance and astaxanthin productivity before entering the red stage, the contradiction between astaxanthin accumulation and high cell mortality is expected to be effectively solved [30].

Currently, there is a relative lack of research on the optimal conditions for inducing motile cells to transition into non-motile cells. Li et al [30] reported that phosphorus deficiency can enhance the formation of non-motile cells, achieving a proportion of 40.5% after a 9-day induction period with a mortality rate of 9.4%. In this study, we demonstrated that continuous exposure to blue light also promotes the formation of non-motile cells, reaching a proportion of 42.0% after 10 days of induction. The induction efficiency is comparable to that observed under phosphorus deficiency conditions; however, the mortality rate is significantly lower at only 6.2%. Furthermore, Li et al [30] additionally demonstrated that the addition of NaCl can also enhance the formation of non-motile cells and its efficacy is directly proportional to NaCl concentration. When adding NaCl at a concentration of 0.1%–0.2%, the induction efficiency is relatively low (less than 16% non-motile cells); however, increasing the NaCl concentration to over 0.4% results in a higher observation of non-motile cells (up to 86%), but also leads to a death rate exceeding 10%. According to our results, when blue light was used to induce the formation of non-motile cells, the proportion of non-motile cells reached 23.5% after 72 h of induction, which was significantly higher compared to the addition of 0.1%–0.2% NaCl. Taken together, compared to phosphorus deficiency and the addition of NaCl, blue light is more beneficial for promoting the formation of non-motile cells.

Several studies have demonstrated that red light is the optimal wavelength for the growth of most algal species, including *Spirulina platensis* [32], *Chlorella vulgaris* [33] and *Botryococcus braunii* [34]. The promotion of cell proliferation by red light can be attributed to its higher conversion efficiency of chlorophyll in the red spectrum range, enabling effective transformation of light energy into stable chemical energy through the electron transport chain and subsequent storage within the organism [35]. It is important to note that not all algae respond in the same way to light wavelengths. Recent studies have shown that blue light can also significantly contribute to promoting cell division in *Nannochloropsis* sp. and *H. pluvialis* when compared with pure red or white light [18, 19]. Consistent with these findings, our study also revealed significant differences in cell numbers among the three main monochromatic lights (i.e., white, red, and blue LEDs). The results indicate that selecting blue light as the optimal option can achieve the greatest cell numbers compared to white and red light. In accordance with our study, Asuthkar et al [36] found that blue light was better than the other lights due to an observed 0.5 specific growth rate while red and white light showed only 0.21 and 0.24 respectively in *Chlorella*

pyrenoidosa. Shih et al [37] observed that the blue light doubled the algal biomass than the white light and others . These present data indicates that the growth rate and biomass composition in microalgae grown under different color light exhibit species-specific variations. This phenomenon may be attributed to the varying pigment concentrations found in certain algae, which serve as an adaptive mechanism for photoautotrophs to optimize their response to environmental conditions [38].

Our observation supports previous studies indicating that fast cell division and large cell size cannot be achieved simultaneously [19, 39]. In this study, we investigated the effects of different light spectra on cell division rates and cellular characteristics. Interestingly, *H. pluvialis* cells dividing under blue light exhibited a faster rate of division compared to those cultivated under red and white lights (Fig. 4). However, these fast-dividing cells also displayed smaller sizes, lower dry weight per cell, decreased F_v/F_m, NPQ, and ETR, and reduced photosynthetic pigment content (Table 1, Fig. 2, and Fig. 3). This suggests an inherent limitation in achieving both rapid cell division and larger cell size simultaneously. One possible explanation for this phenomenon is the energy demand of cell division which requires a significant amount of energy from the cells' reservoirs [40, 41]. Consequently, when cells divide at a slower pace they can allocate more resources towards accumulating storage compounds. This inverse relationship between chemical energy storage and energy expenditure for normal cellular functions during the cell cycle have been previously observed [36, 42]. These findings shed light on the intricate balance between cellular growth and reproduction. While rapid proliferation may be advantageous in certain situations where quick cell expansion is necessary, it comes at the cost of reduced individual cellular size and metabolic activity. On the other hand, smaller cells can invest more resources into building up their internal reserves such as astaxanthin which is believed to play a crucial role in defense response during stressful conditions.

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