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Influence of inoculum size, CO_2 concentration and LEDs on the growth of green microalgae *Haematococcus pluvialis* Flotow

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Abstract:

Green microalgae *Haematococcus pluvialis* is best known for astaxanthin production. The cultivation of *H. pluvialis* involves two main phases, namely green vegetative stage and cyst stage with astaxanthin accumulation. In fact, the growth of *H. pluvialis* in the vegetative stage is one of the most important parts in the entire cultivation process. The aim of the study is to investigate the influence of temperature, inoculum size, CO_2 concentration and light emitting diodes (LEDs) on the specific growth rate, cell density and dry weight of *H. pluvialis* in the vegetative stage. Results indicated that the temperature from 25 to 28^{0}C and the inoculum size from 3×10^4 cells.ml^{-1} were suitable for the growth of the studied strain. Illumination with red light LEDs (630 nm) at 80 µE.m^{-2}.s^{-1}, the highest specific growth rate (µ) was 0.197 day^{-1} and the maximal density was of 2.87×10^5 cells.ml^{-1}. A concentration of 5% CO_2 (v/v) was the optimal dose for the growth of this strain. Under the condition of both 5% CO_2 and illumination with red light LEDs at 80 µE.m^{-2}.s^{-1}, the specific growth rate was 0.242 day^{-1} and the cell density was 4.28×10^5 cells.ml^{-1}. Illumination with only blue LEDs (430 nm) at 120 µE.m^{-2}.s^{-1} stimulated the astaxanthin accumulation with a maximum content at 2.36 µg.ml^{-1}.

Keywords: **cell density, dry weight, growth rate, *Haematococcus pluvialis* Flotow.**

Classification number: **3.4**

Introduction

Astaxanthin, a red-orange carotenoid pigment, is a powerful biological antioxidant that occurs naturally in a wide variety of living organisms, especially in green microalgae *Haematococcus pluvialis* [1-3]. Astaxanthin is widely used in various areas such as as feed supplements for aquatic animals, cosmetics, nutraceutical and pharmaceutical industries [4-7]. The life cycle of *H. pluvialis* consists of four cell stages: vegetative growth, encystment, maturation or red stage, and germination. In the production of astaxanthin, *H. pluvialis* is cultivated mainly in the vegetative growth and the red stages [1, 8]. In facts, the vegetative growth is the most important stage in the entire cultivation process of *H. pluvialis*. In the phototrophic cultivation, aside from nutritional components, temperature, lighting condition and CO_2 concentration are essential factors effecting photosynthesis and cell growth [9]. Recently, LEDs have emerged as a replacement for traditional light sources, and they have rapidly expanded to multiple areas including cultivation of microalgae [10, 11]. The physiological effects of light with a specific range of wavelength on photosynthetic cells have been extensively studied by using combinations of special light sources [12, 13]. Although many reports have mentioned the conditions for cultivating *H. pluvialis* for astaxanthin [12-16], no standard procedure can be applied due to the complexity of cell life cycle, different strains, cultivation conditions and technical parameters. This study investigates the combination effect of inoculum size, CO_2 concentration and illumination condition for the cultivation of the green microalgae *H. pluvialis* Flotow. In addition, the study also attempts to determine the suitable conditions for the improvement of cell density, biomass and astaxanthin content for the large-scale production of this strain.

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Material and methods

Microalgal strain and stock culture preparation

Haematococcus pluvialis Flotow was obtained from the culture collection at the University of Göttingen (Germany). The stock culture was prepared by cultivating the purified microalgae in a 100-ml basal medium consisting of $Ca(NO_3)_2.4H_2O$, 15.0 mg; KNO_3, 10.0 mg; β-Na_2glycerophosphate, 5.0 mg; $MgSO_4.7H_2O$ 4.0 mg; vitamin B12, 0.01 µg; biotin 0.01 µg; thiamine HCl, 1.0 µg; PIV metals, 0.3 ml; Tris aminomethane, 50.0 mg; and distilled water, 99.7 ml. The PIV metals consisted of $FeCl_3.6H_2O$, 19.6 mg; $MnCl_2.4H_2O$, 3.6 mg; $ZnSO_4.7H_2O$, 2.2 mg; $CoCl_2.6H_2O$, 0.4 mg; $Na_2MoO_4.2H_2O$, 0.25 mg; $Na_2EDTA.2H_2O$, 100 mg; and distilled water, 100 ml. The medium was adjusted to pH 7.5 and autoclaved at 121^{0}C for 15 min [2]. *H. pluvialis* was cultured at room temperature from 25 to 28^{0}C and light intensity of roughly 50 $\mu E.m^{-2}.s^{-1}$ from fluorescent light with a light/dark cycle of 16:8 h. Agitation was completed by manually shaking the flasks thrice a day.

Specific growth rate and dry weight measurement

The specific growth rate was calculated in a duration of 48h in the logarithmic phase by using the following equation: $\mu = (\ln DWt - \ln DW_0)/t$ [17]. Biomass productivity ($g.l^{-1}.day^{-1}$) was calculated as follows: $(DW_t - DW_0)/t$, where DW_t and DW_0 denote the dry weights of day t and day 0, respectively. Doubling time was calculated by ln2/µ.

The dry weight was measured through the gravimetric method [9]. Briefly, a 10-ml cell suspension was centrifuged at 5,000 g for 10 min. The pellet was rapidly washed in distilled water by centrifugation, dried overnight on a Petri dish at 60°C and subsequently weighted. The control was medium only.

To investigate the relationships among optical density (OD), cell density and dry weight, the suspension culture in exponential growth (at 2.10^5 $cells.ml^{-1}$) was diluted to different cell densities by serial dilutions. The dry weight was also calculated based on the correlation between the OD of cell suspension at 750 nm and dry weight [13, 18]. The obtained data were used to plot based on the correlation among OD values and the dry weights of different dilution folds. The doubling time in the logarithmic phase was also estimated by counting the cell number.

Influence of temperature

Temperature is a key factor that directly affects the specific growth rate of *H. pluvialis*. In this study, *H. pluvialis* cells were cultured in a 500-ml flask placed in a water bath at different temperature points (18, 20, 23, 25, 28, 30 and 32^{0}C) and monitored via a Hailea HC-150A chiller system and air conditioner. The cell suspension was illuminated with 40W fluorescent lamps at 35 $\mu moles.m^2.s^{-1}$. The effect of temperature on the growth of *H. pluvialis* was characterized by a specific growth rate in the logarithmic phase.

Influence of inoculum size

The effect of inoculum size on the growth of *H. pluvialis* was investigated in 200 ml of different initial biomass densities from 0.5×10^4 to 4×10^4 $cells.ml^{-1}$. Stock culture was used to prepare cells for inoculum experiments. The stock culture was centrifuged at 2,500×g for 5 min, and the collected cells were resuspended in basal medium with initial cell densities that vary from 0.5×10^4 to 4×10^4 $cells.ml^{-1}$ at 25°C. The effect of inoculum size on the growth of *H. pluvialis* was studied at a volume of 200 ml. The optimal seeding size was recorded based on the specific growth rate.

Influence of CO_2 supply

The influence of CO_2 on the growth of *H. pluvialis* was evaluated by measuring the growth rate and the change of pH at different concentrations: control (CO_2 in air 0.04%), 1.0, 2.5, 5.0, 7.5 and 10.0% (volume air/volume culture/min). CO_2 was introduced to the culture medium by using a stainless needle with a 0.6 mm diameter. The flow of CO_2 was monitored through a CO_2 meter. The parameters of specific growth rate and pH were measured.

Influence of light sources

Three light sources were used in this study, namely 40W fluorescent lamps, 25W red 630 nm LEDs and 25W blue 430 nm LEDs. The intensity of light sources was adjusted by maintaining the distance from the light source to the cultivation flasks and monitored through a light intensity meter (Teslo 545). Different combinations of these light sources, including red LEDs, blue LEDs and a combination of both red LEDs and blue LEDs and fluorescent lamps, were investigated.

Measurement of pigments

The chlorophyll concentration was determined spectrophotometrically using the extinction coefficients in 100% methanol as described by Wellburn [19] and Lichtenthaler and Wellburn [20]. Briefly, a 2ml cell suspension was centrifuged at 2,500×g for 10 min, and the supernatant was discarded. Two ml of methanol were subsequently added to the pellet and mixed thoroughly.

The samples were mixed and incubated in a water bath at 70°C for 10 min and then centrifuged at 2,500×g for 10 min. The liquid was read spectrophotometrically at different wavelengths (653, 665, 649 and 470) to determine chlorophyll a (1), chlorophyll b (2) and carotene (3).

$$\text{Chlorophyll a } (C_a) = 15.65 \times A_{666} - 7.34 \times A_{653} \quad (1)$$

$$\text{Chlorophyll b } (C_b) = 27.05 \times A_{649} - 5.32 \times A_{665} \quad (2)$$

$$\text{Carotene} = (1{,}000 \times A_{470} - 2.86 \times C_a - 129.2 \times C_b) \times 221 \quad (3)$$

where: A_x is the absorbance at x wavelength, and C_a and C_b represent the concentrations of chlorophyll a and chlorophyll b ($\mu g.ml^{-1}$), respectively.

Removal of chlorophyll: the cell pellets of *H. pluvialis* in the cyst stage were treated with 5% KOH in 30% methanol and incubated at 70°C for 5 min to remove chlorophyll. After being washed with distilled water, cell pellets were used for astaxanthin extraction and quantitative analysis [1].

Astaxanthin extraction and analysis: Chlorophyll-removed cells were treated with 2N hydrochloric acid (HCl) and incubated at 70°C. The samples were centrifuged at 2,500×g for 10 min at 4°C, and the supernatants were removed and washed twice with distilled water. The cells were treated with methanol for 1 h and subsequently centrifuged as described. The supernatants were used for estimating the extractable astaxanthin (as carotene). In this study, astaxanthin content was calculated by using a calibration curve constructed via pure astaxanthin purchased from Sigma Aldrich (CAS Number 472-61-7). Pure astaxanthin was prepared in absolute methanol in different concentrations from 10 to 100 $\mu g.ml^{-1}$ and measured at 470 nm. All of the steps in the astaxanthin extraction and sample preparation were carried out in dark [21]. Data on astaxanthin content calculated from the calibration curve were also compared with the data computed from equation (3) to adjust for rapid estimation in experiments.

Data analysis

In this study, data were analysed from three replicates and represented as mean values with standard deviations (SDs). Graphs were plotted using Microsoft Excel 2016. Analysis of variance was applied to assess the significant differences among the groups. Tukey's test was used to verify all of the pairwise differences among the means of the experiment groups.

Results and discussion

Influence of temperature

To determine the effect of temperature on the growth of *H. pluvialis*, a serial of cell suspensions at 3.10^4 $cells.ml^{-1}$ in the green vegetative state was inoculated at 18, 22, 25, 28, 30 and 32°C under fluorescent light at 50 $\mu E.m^{-2}.s^{-1}$ with a dark/light cycle of 8:16 h. The results indicated that the specific growth rates in the logarithmic phase increased when temperature increased from 18 to 27°C. However, a decrease in the specific growth rate was observed when the temperature was higher than 28°C. In this study, cells were unable to grow when the temperature was over 30°C (Fig. 1).

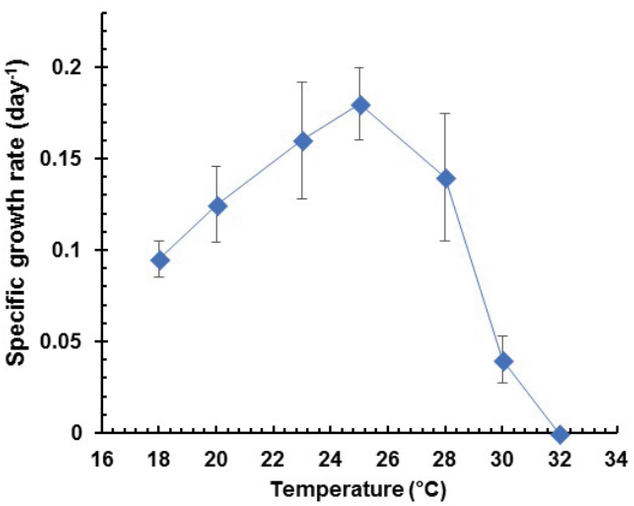

Fig. 1. Influence of temperature on the specific growth rate of *H. pluvialis*. Cell suspensions were cultured under fluorescent light at 50 $\mu E.m^{-2}.s^{-1}$ with a dark/light cycle of 8:16 h. The specific growth rate was measured in the logarithmic phase for 72h from days 10 to 14 after inoculation.

The optimal temperature for the growth of this *H. pluvialis* was 25°C, and the specific growth rate (μ) under this condition in the logarithmic phase was 0.18±0.025 per day or 0.0075 h^{-1}, which was equivalent to the doubling time of 3.85 days or 84h. Studies revealed that the specific growth rate of *H. pluvialis* was affected by many cultivation factors and varied from 0.007 to 0.05 per h [1, 17, 22, 23]. Depending on strains, most studies reported that the optimal temperature for *H. pluvialis* strains was within the range of 25 to 28°C [1-3, 13].

Technically, the best method for cell density measurement is through a haemocytometer, but it is time consuming and inconvenient. Based on the findings of Chekanov about the correlation between OD_{750}, cell density and dry weight [13], a relationship among cell density, OD_{750} and dry weight was established (Fig. 2). A linear correlation emerged between OD_{750} and dry weight in the logarithmic phase (Fig. 2, inner graph).

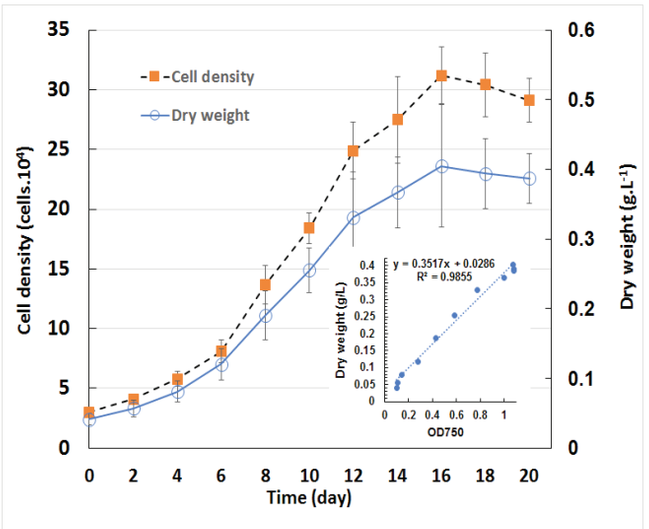

Fig. 2. Relationship between cell density and dry weight. Outer graph: relationship between cell density and dry weight over 20 days. Inner graph: correlation between dry weight and OD_{750}. *H. pluvialis* was inoculated at 3×10^4 cells.ml^{-1} and cultured for 20 days at 25^{0}C with light intensity of 50 µE.m^{-2}.s^{-1} with a dark/light cycle of 8:16 h and continuously bubbled with air at 10% (v/v).

Influence of inoculum size

All of the cell suspensions were cultured at 25°C in a 500-ml flask with a dark/light cycle of 8:16 h and bubbled with air (10% v/v). Data revealed that cell density increased proportionally with the initial biomass density. The highest cell density was observed at approximately 2.874×10^5 cells.ml^{-1} (equivalent to 209.13 mg.ml^{-1}) when inoculum size was 3×10^4 cells.ml^{-1}. At a lower inoculum size, the maximum cell density was reduced significantly, and the duration from the time point of inoculation to the stationary phase was extended (data not shown). In the stationary phase, the morphology of the cells changed slowly from green active to inactive without flagella. No significant difference was observed between two initial biomass densities of 3×10^4 cells.ml^{-1} and 4×10^4 cells.ml^{-1} (Table 1). This result indicated the probability that a higher initial biomass density was not better at all times. The outcome might be due to the mutual shading of cells in the culture, the frequency of light/dark cycle and the total length of light period during which individual cells were exposed to incident light intensity [17, 22]. Several studies have reported that the higher initial biomass density, the higher frequency of light/dark cycle and the shorter the light exposure time individual cells receive from each light/dark cycle [17]. This result suggested that the initial biomass density of 3×10^4 cells.ml^{-1} was suitable for biomass production in this study.

Table 1. Effect of inoculum size on the growth of *H. pluvialis*.

Inoculum size ($\times10^4$ cells.ml^{-1})	0.5	1.0	2.0	3.0	4.0
Max cell density ($\times10^4$ cells.ml^{-1})	16.12±1.81 $^{(a)}$	21.63±2.51$^{(b)}$	25.16±3.05 $^{(c)}$	28.74±3.11 $^{(d)}$	24.69±2.17 $^{(c)}$
Max specific growth rate (day^{-1})	0.138±0.019 $^{(a)}$	0.153±0.022$^{(b)}$	0.184± 0.024$^{(c)}$	0.197±0.021$^{(d)}$	0.182±0.023 $^{(c)}$
Doubling time in log phase (day^{-1})	5.07±0.41$^{(a)}$	4.57±0.35$^{(b)}$	3.80±0.26$^{(c)}$	3.55±0.35$^{(d)}$	3.84±0.43$^{(c)}$
Duration in vegetative stage (day)	16±2$^{(a)}$	14±2$^{(b)}$	12±2$^{(c)}$	11±2$^{(d)}$	12±2$^{(c)}$
pH at stationary phase	9.12±0,46$^{(a)}$	9.10±0,64$^{(a)}$	9.06±0,59$^{(a)}$	8.91±0,47$^{(b)}$	8.87±0,52$^{(b)}$

Note: means that share the same superscript are not significantly different at $p<0.05$.

Effects of LED lights on the growth and astaxanthin production

Several studies have demonstrated the effect of red and blue LEDs on the growth and astaxanthin accumulation in *H. pluvialis* [14-16]. In this study, different light sources were used separately or in combination (Fig. 3).

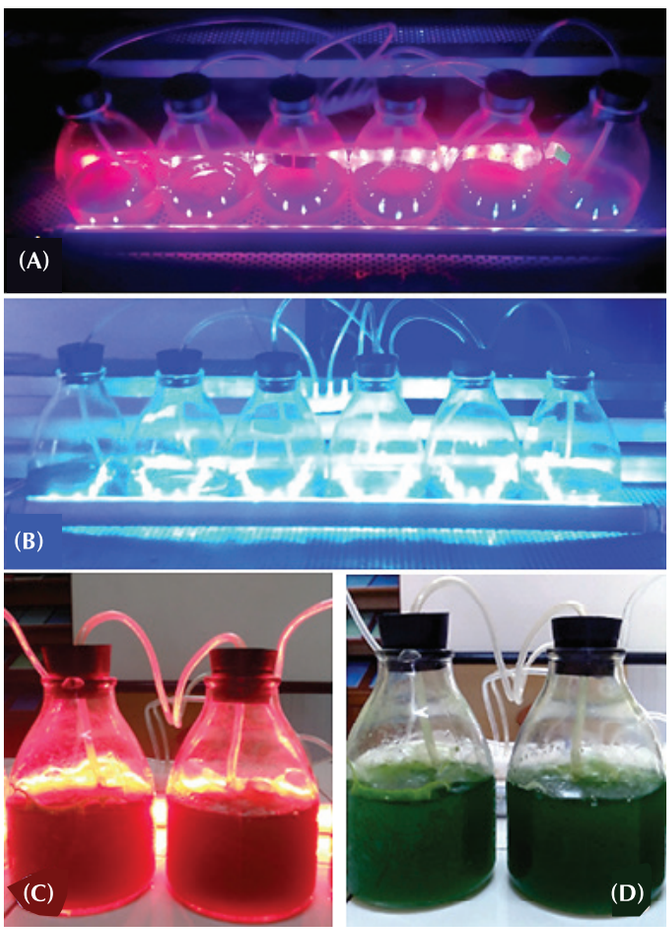

Fig. 3. Effect of LEDs on the growth and cell density of *H. pluvialis*: (A) Experiments with both red LEDs and blue LEDs at 50 µmol.m^{-2}.s^{-1}; **(B)** Experiments with only blue LEDs at 50 µmol.m^{-2}.s^{-1}; **(C)** Experiments with only blue LEDs at 50 µmol.m^{-2}.s^{-1}; **(D)** Cell suspension illuminated by fluorescent lamp at 50 µmol.m^{-2}.s^{-1}.

In the first four days, no difference in cell density was observed among treatments. However, the density increased significantly from days 6 to 14 (Fig. 4). The red LEDs evidently exhibited a positive effect on the growth of *H. pluvialis*, whereas the blue LEDs did not promote the growth of these microalgae. The specific growth rate was affected by the intensity of light (Table 2). No significant difference emerged between a combination of red LEDs with fluorescent light and only red LEDs. Data analysis indicated that cell density increased significantly in the case of red LEDs.

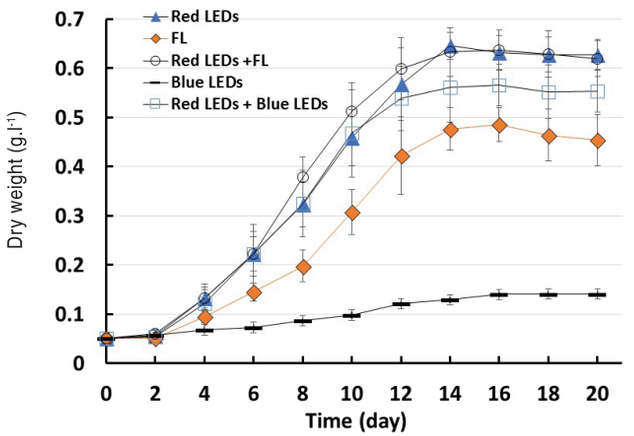

Fig. 4. Effect of light source on the growth of *H. pluvialis*. Inoculation density was 3.10^4 cell.ml^{-1} in all experiments. Red LEDs: 25W red LEDs, 630 nm (50 µmol.m^{-2}.s^{-1}); Blue LEDs 25W, 430 nm (50 µmol.m^{-2}.s^{-1}); FL: 40W fluorescent lamp (50 µmol.m^{-2}.s^{-1}); Red LEDs + FL (using both red LEDs and fluorescent lamp); Red LEDs + Blue LEDs (using both red LEDs and blue LEDs).

Although illumination with blue LEDs did not significantly promote the growth of cells, the number of immotile cells increased rapidly, and cells changed into mature cysts within 12 days (Table 2). Harvested cells in the cyst state were extracted after removing the chlorophyll. In this study, astaxanthin was extracted from cells illuminated with red LEDs and blue LEDs after 14 and 16 days of illumination, respectively (Table 2). Morphological analysis revealed that red LEDs did not significantly stimulate astaxanthin accumulation, and the cells were in the resting (cyst) stage without cell division after 14 days of illumination (data not shown). In case of illumination with blue LEDs at 120 µmol.m^{-2}.s^{-1} for 16 days, the carotene content was 2.36±0.46 µg.ml^{-1} or 0.5% dry weight (Table 2 and Fig. 5). Several attempts were undertaken to stimulate the accumulation of astaxanthin at different temperatures in combination with treatment with blue LEDs. The accumulation of astaxanthin by blue LED treatment was likely to be more efficient when temperature was within the range of 25 to 27^{0}C (data not shown). This effect was significant only if the high intensity of blue LEDs was applied (120 µmol.m^{-2}.s^{-1}, Fig. 5). A higher temperature (above 28^{0}C) indicated an adverse effect and a significant decrease in the number of cells. Several studies underscored the similar effect of LED light [10-13]. In addition, the accumulation of astaxanthin required a prolonged stimulation period of 16 days (Fig. 5). It suggested that illumination by using blue LEDs at 430 nm induced astaxanthin production. However, the application of blue LEDs only for the stimulation of astaxanthin production was inefficient. Additional investigations that use other factors such as chemical reagents are necessary to obtain better results.

Table 2. Effect of light intensity on the growth of *H. pluvialis*.

	Light sources and intensity					
	Red LEDs			*Blue LEDs*		
Light intensities (µE.m^{-2}.s^{-1})	50	80	120	50	80	120
Max cell density (10^4cells.ml^{-1}) (*)	31.84±4.12$^{(a)}$	35.42±4.42$^{(b)}$	35.67±4.67$^{(b)}$	6.31±0.71$^{(c)}$	4.45±0.50$^{(d)}$	2.26±0.315$^{(e)}$
Max specific growth rate (day^{-1})	0.188±0.02$^{(a)}$	0.196± 0.023$^{(b)}$	0.187±0.021$^{(ab)}$	0.052±0.012$^{(c)}$	0.039±0.011$^{(d)}$	-0.013±0.001$^{(e)}$
Doubling time (day^{-1})	3.52±0.41$^{(a)}$	3.34±0.26$^{(a)}$	3.32±0.46$^{(a)}$	13.4±1.50$^{(b)}$	18±2.06$^{(c)}$	-
Highest astaxanthin content (µg.ml^{-1})	(**)	(**)	0.12±0.02$^{(a)}$	0.25±0.03$^{(b)}$	1.05±0.02$^{(c)}$	2.36±0.46$^{(d)}$
% motile cells	75.13±6.71$^{(a)}$	65.26±6.12$^{(ab)}$	62.15±7.97$^{(b)}$	10.26±1.12$^{(c)}$	6.14±0.84$^{(d)}$	0
% cysts	4.53±0.31$^{(a)}$	5.71±0.41$^{(ab)}$	6.25±0.52$^{(b)}$	20.25±4.12$^{(c)}$	20.11±3.95$^{(c)}$	60.16±4.12$^{(e)}$

Notes: means that share the same superscript are not significantly different at $p<0.05$. The initial biomass density was 3.10^4 cell.ml^{-1} in all experiments. A 200-ml cell suspension was cultured in a 500-ml flask with a dark/light cycle of 8:16 h and continuously bubbled with filtered air at 10% (v/v); (*) data were measured on day 14 with red LEDs or day 16 with blue LEDs (see also Fig. 4); (**) negligible.

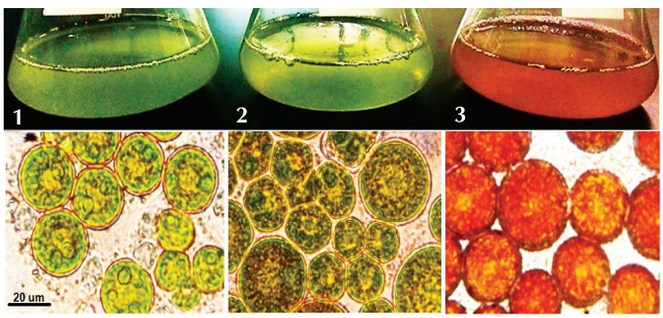

Fig. 5. Effect of LEDs on astaxanthin accumulation in *H. pluvialis*. Cells in the cyst stage and astaxanthin accumulation after treatment with blue LEDs at 50 µmol.m^{-2}.s^{-1} (1), 80 µmol.m^{-2}.s^{-1} (2) and 120 µmol.m^{-2}.s^{-1} (3) for 16 days at 27°C.

Effects of CO_2 concentration

CO_2 is a critical factor that affects the growth of microalgae by providing a carbon source for photosynthesis. As a rule, high cell density and synchronization in the vegetative stage are the most important parameters in the two-stage cultivation of *H. pluvialis*. In this study, cell

suspensions were cultured at 25^{0}C and illuminated by red LEDs at 80 µmol.m^{-2}.s^{-1} in the presence of different CO_2 concentrations 0.04 (air/control), 1.0, 3.0, 5.0, 7.5 and 10% (v/v). The influence of CO_2 on the growth parameters of *H. pluvialis*, including pH, cell density, specific growth rate, doubling time and pigment contents, was investigated. The results are presented in Table 3.

Table 3. Effect of CO_2 on growth parameters.

CO_2 concentration (%, v/v)	0.04 (*)	1	2.5	5	7.5	10
Initial pH	7.5	7.5	7.5	7.5	7.5	7.5
pH at log phase	8.33±0.23$^{(a)}$	8.11±0.22$^{(a)}$	7.61±0.26$^{(b)}$	6.95±0.21$^{(c)}$	6.63±0.22$^{(cd)}$	6.43±0.21$^{(d)}$
pH at stationary phase	9.50±0.31$^{(a)}$	8.87±0.38$^{(ab)}$	8.25±0.33$^{(b)}$	6.68±0.24$^{(c)}$	6.16±0.21$^{(cd)}$	5.75±0.28$^{(d)}$
Maximum cell density (10^4 cells.ml^{-1})	25.79±3.45$^{(a)}$	31.46±4.16$^{(ab)}$	36.46±3.25$^{(bc)}$	42.79±4.65$^{(c)}$	41.91±6.37$^{(c)}$	33.52±3.19$^{(b)}$
Maximum specific growth rate (day^{-1})	0.196±0.015$^{(a)}$	0.213±0.016$^{(ab)}$	0.231±0.011$^{(b)}$	0.242±0.022$^{(b)}$	0.236±0.021$^{(b)}$	0.208±0.026$^{(ab)}$
Doubling time in log phase (day^{-1})	4.89±0.46$^{(a)}$	3.28±0.43$^{(b)}$	3.03±0.24$^{(bc)}$	2.89±0.33$^{(c)}$	2.96±0.26$^{(c)}$	3.36±0.44$^{(c)}$
Maximum chlorophyll in log phase (µg.ml^{-1})	8.77±1.02$^{(a)}$	12.28±1.56$^{(b)}$	16.38±2.06$^{(c)}$	18.2±1.92$^{(c)}$	18.5±2.06$^{(c)}$	12.4±1.54$^{(b)}$
Maximum carotene in stationary phase (µg.ml^{-1})	1.57±0.20$^{(a)}$	2.07±0.17$^{(bc)}$	2.44±0.26$^{(cd)}$	2.81±0.26$^{(d)}$	2.93±0.41$^{(cd)}$	2.87±0.37$^{(cd)}$

(*) Notes: CO_2 from air (control). Cell density was measured by OD_{750} on the day after inoculum at 3×10^4 cell.ml^{-1}. Means that share the same superscript are not significantly different at $p<0.05$.

Experiments with different CO_2 concentrations and red LEDs at 80 µmol.m^{-2}.s^{-1} were conducted. The growth of *H. pluvialis* increased with the elevation of CO_2 concentration. An elevation of CO_2 concentration from 0.04% (air) to 5% promoted cell growth, and the highest density of approximately 4.279±5.65×10^5 cells.ml^{-1} was reached on day 14 at 5% CO_2 (Table 3 and Fig. 6). Under this condition, pH in the logarithmic phase was nearly neutral at 6.95±0.21 and decreased slightly to 6.68±0.24 at the stationary phase (Table 3). This outcome revealed that the supply of CO_2 at 5% in basal medium stabilized the pH value throughout the culture period. As previously stated, pH was strongly dependent on the buffer capacity of the media [3, 18, 22-24]. A lower concentration of CO_2 at 0.04% (air), 1% and 2.5% was insufficient, and the pH of the medium became more alkaline over time. A higher concentration of CO_2 (7 and 10%) resulted in a significant reduction of pH and a specific growth rate (Table 3 and Fig. 6). No significant difference emerged between two concentrations of CO_2, 5.0 and 7.5% (v/v). A slight difference of all the investigated parameters between two concentrations of CO_2, 2.5 and 5.0% (v/v) was observed. The concentration of 5% CO_2 was likely to be more suitable for the growth of this strain.

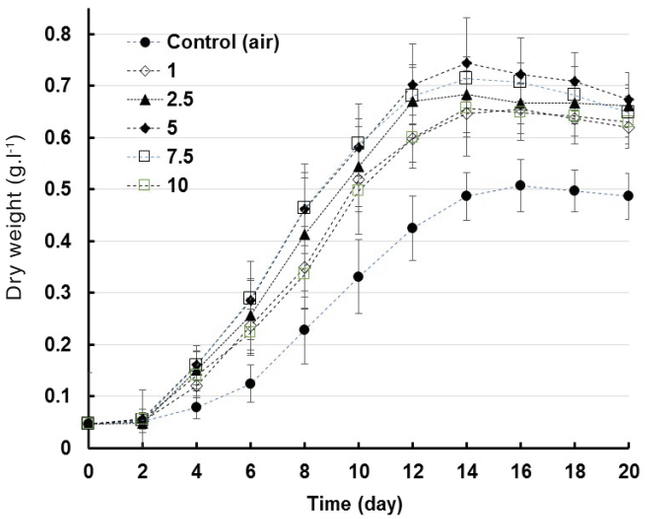

Fig. 6. Effect of CO_2 and red LEDs on the growth and cell density.

Numerous studies on both indoor and outdoor systems similarly emphasized that a higher concentration of CO_2 reduced pH at the stationary phase [18, 23, 24]. The reduction of pH value denoted a significant effect on the growth of *H. pluvialis* (Table 3). In addition, the doubling time was shortened significantly when 5% or 7.5% of CO_2 was applied. This result suggested that a concentration of 5% CO_2 was optimal for the culture of this strain. The concentration of 10% CO_2 did not exhibit a positive effect on the growth and chlorophyll content. However, data analysis indicated that the elevation of CO_2 concentration significantly increased carotene content (Table 3). An illumination by using blue LEDs at 120 µE.m^{-2}.s^{-1} for 16 days stimulated astaxanthin accumulation in the study strain with maximum content (calculated based on β-carotene) of approximately 2.93±0.41 µg.ml^{-1} and 2.81±0.26 µg.ml^{-1} at concentrations of CO_2 7.5% and 5%, respectively. Data analysis revealed the lack of a significant difference between two concentrations of CO_2 5% and 7.5% CO_2 in a light/dark cycle of 16:8 h. Therefore, the concentration of CO_2 at 5% should be used for large-scale production due to economic issues. In comparison with published data, the results obtained in this study were also matched with the findings of several studies [18, 22-24].

Conclusions

In this study, the optimal condition for the growth of *H. pluvialis* Flotow was at a temperature of approximately 25^{0}C and the inoculum size at 3×10^4 cells.ml^{-1}. Under the condition of red light LEDs (630 nm) at 80 µE.m^{-2}.s^{-1}, the highest specific growth rate and maximal density were 0.196.day^{-1} and 3.56×10^5 cells.ml^{-1}, respectively. A concentration of 5% CO_2 (v/v) was the optimal dose

for this strain. Under the conditions of both 5% CO_2 and illumination with red light LEDs at 80 µmol.m^{-2}.s^{-1}, the maximal specific growth rate was 0.242±0.022, the doubling time was 2.89 days or 69.36 hours and the cell density reached approximately 4.28×10^5 cells.ml^{-1}. Illumination by using blue LEDs at 120 µE.m^{-2}.s^{-1} for 16 days in a light/dark cycle of 16:8 h stimulated astaxanthin accumulation in the study strain with a maximum content of roughly 2.81±0.26 µg.ml^{-1}.

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