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Red and Blue Photons Can Enhance the Production of Astaxanthin from *Haematococcus pluvialis*

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The unicellular green alga, *Haematococcus pluvialis*, accumulates the highest level of astaxanthin among known astaxanthin-producing organisms. Light is the most important factor to induce astaxanthin by *H. pluvialis*. Blue and red LEDs, whose λ_{max}'s are 470 and 665 nm, respectively, were used for internally illuminated light sources. Fluorescent lamps were also used for both internal and external illumination sources. The astaxanthin levels in these various lighting systems were analyzed and compared each other. The cultures under internally illuminated LEDs accumulated 20% more astaxanthin than those under fluorescent lamp. Furthermore, LEDs generated much less heat than the fluorescent lamps, which gives one more reason for the LEDs being a suitable internal light source for astaxanthin induction. The results reported here would lead novel designs of photobioreactors with improvements of illumination methods for high level of astaxanthin production. The maximum astaxanthin concentrations as well as the astaxanthin yield per supplied photon were increased by at least 20% when blue or red LEDs were supplied.

Key Words: astaxanthin, *Haematococcus pluvialis*, internal illumination, light emitting diodes (LEDs), photobioreactor

INTRODUCTION

The growing demand for natural products for the health food and beauty products markets has attracted the interest for microalgal biotechnology during the last two decades. Algal biotechnology is an emerging field with various high-valued bioactive compounds. There have been considerable interests in the production of many clinically and medically important biochemicals from algae, especially those cannot be synthesized chemically (Lee 1997).

The history of the commercial use of algal cultures spans about 60 years with various applications (Myers *et al.* 1951; Burlew 1953). One of the most obvious characteristics of the algae is their colors, each phylum has its own particular combination of pigments and an individual color. In view of commercial utilization of algae, algal pigment, especially the carotenoids, play an important role. A red carotenoid, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), has attracted a tremendous commercial interest as nutraceuticals and pharmaceuticals due to its antioxidant activity (Guerin *et al.* 2003). The antioxidant activity of astaxanthin is approximately ten times greater than other carotenoids, such as zeaxanthin, lutein, canthaxanthin, and β-carotene; 500 times greater than α-tocoperol (Orosa *et al.* 2001; Park and Lee 2001). Astaxanthin has long been used as a food colorant for fish and, to a lesser extent, for poultry (Lorenz and Cysewski 2000). The pink flesh characteristic of wild salmon and trout occurs in farmed fish if this pigment is added to their feed. A green alga *Haematococcus pluvialis* is considered as one of the best producers of astaxanthin. This photosynthetic microorganism requires different environments for vegetative growth and for production of astaxanthin. In order to induce astaxanthin from *H. pluvialis*, stressed conditions are needed in light intensity, nutrient level, metal ions, temperature, and/or pH. Among these, the light such as light intensity, light quality, and light delivery and distribution are undoubtedly the most important factors in the astaxanthin accumulation (Park and Lee 2001; Choi *et al.* 2002; Kim *et al.* 2006).

Numerous factors must be taken into consideration when selecting light sources for photoautotrophic cultures: light intensity and quality, life expectancy and electrical efficiency, price and availability, *etc*. For the light sources of internal irradiation, a few more factors must be also considered: weight and volume characteris-

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tics, degree of heat emission, emission of photosynthetically inactive radiation such as UV and IR, insulating or submersible characteristics, *etc*. Among these, degree of heat emission is the most critical factor in choosing an internal light source, not only because many light sources emit enough heat to affect the microalgal growth rate, but also because the cooling or blocking the heat in internally illuminated photobioreactors is difficult and not economical. Light-emitting diodes (LEDs) have numerous advantages as a light source for photosynthetic cultures and their small volume and weight characteristics make the LEDs a perfect light source for internally illuminated photobioreactors (PBRs) (Lee and Palsson 1994).

In this study, in order to produce high-level of astaxanthin, *Haematococcus pluvialis* was cultivated under LEDs and fluorescent lamps in bubble column photobioreactors and the efficiencies of different light sources were compared.

MATERIALS AND METHODS

Strain and culture conditions

The unicellular green alga *Haematococcus pluvialis* UTEX 16 was purchased from the Culture Collection of Alga at the University of Texas at Austin and was cultivated photoautotrophically in the modified Bold's basal medium (MBBM), whose composition consisted of 246.5 mg L^{-1} of $NaNO_3$, 24.99 mg L^{-1} of $CaCl_2 \cdot 2H_2O$, 73.95 mg L^{-1} of $MgSO_4 \cdot 7H_2O$, 4.98 mg L^{-1} of $FeSO_4 \cdot 7H_2O$, 74.9 mg L^{-1} of K_2HPO_4, 175.57 mg L^{-1} of KH_2PO_4, 25.13 mg L^{-1} of NaCl, 49.68 mg L^{-1} of $C_{10}H_{16}N_2O_8$ (EDTA), 1.57 mg L^{-1} of $CuSO_4 \cdot 5H_2O$, 1.19 mg L^{-1} of $Na_2MoO_4 \cdot 2H_2O$, 11.13 mg L^{-1} of H_3BO_3, 1.44 mg L^{-1} of $MnCl_2 \cdot 4H_2O$, 8.83 mg L^{-1} of $ZnSO_4 \cdot 7H_2O$, 0.49 mg L^{-1} of $Co(NO_3)_2 \cdot 6H_2O$, 6.06 mg L^{-1} of MoO_3, 30.86 mg L^{-1} of KOH, and 0.98 mg L^{-1} of H_2SO_4 in distilled water. A single colony of cells grown on the agar was inoculated into 120 mL medium in a 250 mL Erlenmeyer flask, after adjusting the initial pH to 6.5 ± 0.5. The inoculated flasks were incubated at 25°C under continuous shaking (175 rpm) and irradiated at 40 ± 2 μE m^{-2} s^{-1} with fluorescent lamps (Model FL 18D, OSRAM Korea Co., Ansan, Korea). The experiments were performed in 2 L bubble column PBRs containing 2 L of MBBM media at 0.2 VVM aeration with 5% CO_2 gas and 95% air under constant continuous light intensity of 40 ± 2 μE m^{-2} s^{-1} at column surface. Compact fluorescent lamps (Model DULUX L®, OSRAM Korea, Ansan, Korea) were used for all the external illumination of PBRs. The temperature and pH were kept at 25°C and 6.5 ± 0.5 during cultivation time. Cultures were inoculated at a cell density of 10^5 cells mL^{-1} with the cells in exponentially growing phase.

Construction of photobioreactors

The 2 L bubble column PBRs were made of Pyrex glass tubes with 90 mm in diameter (OD) and 580 mm long. The bottoms were modified into cone-shape to reduce the cell sedimentation. In the center of each PBR, there was small inner tube (28 mm OD × 500 mm long) to host either a fluorescent lamp or a LED array. The top of the PBR was plugged with silicon stopper with 3 ports: medium inlet, gas outlet, and sampling. The gas inlet was at the end of the tapered bottom.

Measurement of cultures

The cell number, average cell size, cell size distribution, and fresh weight were measured using a Coulter counter (Model Z2, Beckman Coulter, Inc., Fullerton, CA, U.S.A.) after diluting the samples with an electrolyte solution (Isotonic Diluent, Hematronix, Inc., Benicia, CA, U.S.A.) and converted by AccuComp® software (ver. 2.01, Beckman Coulter, Inc.) before exporting to Microsoft® Excel program (ver. 2002, Microsoft Co., Redmond, WA , U.S.A.) to calculate the total fresh weight and the average cell size.

Astaxanthin concentration was analyzed by a spectrophotometer (Model HP8453B, Hewlett-Packard, Waldbronn, Germany) after extracting with acetone and centrifuging at 3,000 rpm for 10 min using the calibration curves discussed below. Synthetic astaxanthin (Product number A9335, Sigma-Aldrich Co., St Louis, MO, U.S.A.) was used for calibration and the concentration was calculated by the following equation for astaxanthin concentration of less than 10 mg mL^{-1}: astaxanthin concentration (in mg L^{-1}) = 0.0045 × OD_{475} (Park and Lee 2001).

Light sources and measurement of light intensity

Three different internal light sources were used: thin fluorescent lamps (Model FL-18X-W, Feelux Lighting Co., Ltd., Yangju, Korea), blue (λ_{max} 470 nm, Model ULP-UB36A, U-Jin LED Co., Ltd., Koyang, Korea) and red LEDs (λ_{max} 665 nm, Model LN261CALUR, Panasonic, Matsushita Electric Industrial Co., Ltd, Kyoto, Japan). For external illumination of PBRs, brighter compact fluorescent lamps (Model DULUX L®, OSRAM Korea, Ansan, Korea) were used. As shown in Fig. 1, the emission spectra of these light sources are very distinctive

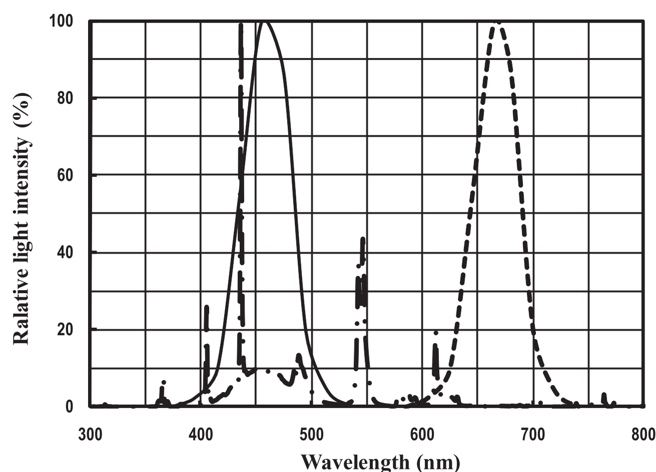

Fig. 1. Emission spectra of blue (λ_{max} 470 nm) and red LEDs (λ_{max} 665 nm) as well as fluorescent lamp. Blue LED (—); Red LED (·····); Fluorescent lamp (-·-·-).

each other. The light intensities of external fluorescent lights were adjusted by altering the number of fluorescent lamps and the distance from the photobioreactor. The light intensity of internal fluorescent light was controlled using a fluorescent light controller (Model SKD-1000, SK Tech, Seoul, Korea). The LEDs were powered by DC power supplies (Model GP-4303D, LG Innotek, Seoul, Korea) and thus the light intensities of LEDs were adjusted by varying the supplied voltage. The light intensities were measured using a quantum sensor (Model LI-190SA, LI-COR, Inc., Lincoln, NE, U.S.A.).

RESULTS AND DISCUSSION

Measurement of temperature from internal light sources

As discussed earlier, the most important factor for using internal irradiation is the heat from the light source. Then, the steady state temperatures in different internal light sources were measured and the results are listed in Table 1. The electrical efficiencies of LEDs are much higher than fluorescent lamps and the surface temperature of the LEDs was much cooler than that of fluorescent light (Table 1). When the light sources were inserted into the inner tube, the temperature was dropped in all cases due to the large volume of culture media and the forced convection by air conditioner and fans. However, the amount of heat generated from a fluorescent lamp was high enough to affect the cell growth. When the light intensity of fluorescent light was adjusted to that of LEDs (20 μE m^{-2} s^{-1}, which is about one-seventh of the full intensity of the fluorescent lamp), both

Table. 1. Measurement of temperature from internal light sources

Internal light types	Temperature at surface of light source (°C)	Average temperature of the inner tube during cultivation (°C)
Blue LEDs (20 μE m^{-2} s^{-1})	27 ± 0.5°C	24 ± 0.5°C
Red LEDs (20 μE m^{-2} s^{-1})	28 ± 0.5°C	25 ± 0.5°C
Fluorescent lamp (135 μE m^{-2} s^{-1})	42 ± 0.5°C	31 ± 0.5°C
Fluorescent lamp (20 μE m^{-2} s^{-1})	31 ± 0.5°C	26 ± 0.5°C

Room temperature: 23 ± 0.5°C

the surface temperature of fluorescent light and the temperature of the inner tube were comparable to those of LEDs (Table 1). As a result, a fluorescent lamp that is generally known for their high electric-to-light energy conversion efficiency may not be suitable for internal illumination at its full power. The experiments afterwards used internal fluorescent lamps as a control at 20 μE m^{-2} s^{-1} and thus the temperature effect could be neglected.

Light penetration as a function of the morphology of the cells

Microalgae are so efficient that they can absorb all the photons that hit them even though they cannot use all the absorbed photons in photosynthesis. This will cause mutual shading in higher density cultures: the cells shielded from light by other cells cannot see the light. In order for all the cells to go through photosynthesis, enough photon should penetrate deep into the culture. However, the photosynthetic photon flux (PPF) cannot be increased to infinity to overcome this mutual shading because supplying so much light (i) may not be economically favorable; (ii) may cause other problems such as heat and spatial efficiency; and (iii) can damage algal metabolism by photoinhibition (Lee 1999; Park and Lee 2000). The same problems would be observed during the cultivation of *H. pluvialis*. Especially after the induction of astaxanthin, light distribution in bubble column photobioreactor drastically has changed by cell concentration, cell size, and pigment. Life cycle of *H. pluvialis* is very complex and is not clearly understood yet. One of the simplest life cycles has 4 stages: (i) vegetative cell growth; (ii) encystment (vegetative to immature cyst cells); (iii) maturation (immature to mature cyst cells);

Table. 2. Maximum astaxanthin concentration and maximum astaxanthin concentration per supplied photons under different light types. Note the definition of the abbreviations

Internal light (μE m^{-2} s^{-1}) + External light (μE m^{-2} s^{-1})	Max. astaxanthin concentration (mg L^{-1})	Max. astaxanthin concentration per supplied photons (mg L^{-1}/μE s^{-1})
Blue LED (20) + FL (300) = BF	345.3	12.2
Red LED (20) + FL (300) = RF	342.1	12.1
FL (20) + FL (300) = FF	278.9	9.9
None + FL (320) = FL	271.4	9.6
Blue LED (20) + None = BN	53.5	30.4
Red LED (20) + None = RN	53.1	30.1
FL (20) + None = FN	44.5	25.2

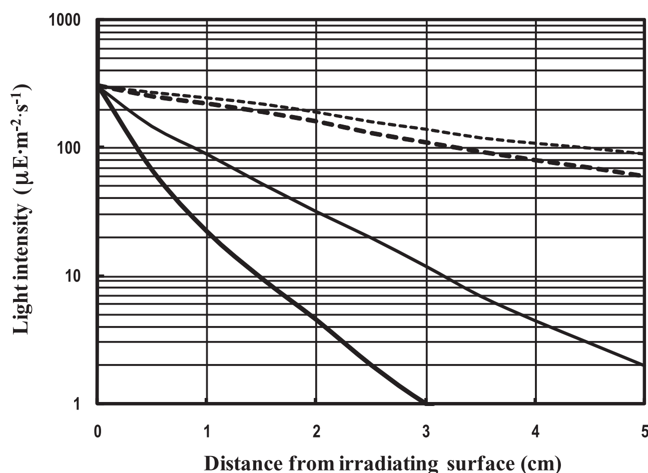

Fig. 2. Comparisons of light penetration depth between vegetative cells and mature cyst cells in the bubble column photobireactors. Mature cyst cell: 5 × 10^5 cell mL^{-1} (**—**), 5 × 10^4 cell mL^{-1} (**····**), Vegetative cell: 5 × 10^5 cell mL^{-1} (—), 5 × 10^4 cell mL^{-1} (·····).

(iv) germination (mature cyst to vegetative cells) (Kobayashi *et al.* 1992). Since vegetative cells undergo a dramatic morphological change to mature cyst cells are dramatic, the light penetration or the volume of photic zone of one stage will be a function of cell cycle. The diameter of a typical mature cyst cell is normally 10 μm bigger than that of a typical vegetative cell. This change alone will affect the light penetration depth since the size of the cells is the main factor of package effect (Geider and Osborne 1987). Furthermore, the dramatic size change is accompanied by the change of dominant pigment (from chlorophylls to astaxanthin). Consequently, the light intensity at the center of a photobioreactor with small green vegetative cells can be more than 20 times higher than that with large red cyst cells at the same cell concentration. For example, the light intensities at the center of a photobioreactor with 5 × 10^4 and 5 × 10^5 cells mL are 90 and 2 μE m^{-2} s^{-1}, respectively, for vegetative cells and 60 and 0.1 μE m^{-2} s^{-1}, respectively, for cyst cells (Fig. 2). However, the light penetration of the culture of vegetative cells is shorter when compared with the penetration of the culture of cyst cells with the same fresh weight (data not shown). These results suggest that a more sophisticated control of light intensity is required to increase the efficiency of supplied light for astaxanthin production.

Effects of blue and red lights with white light on astaxanthin production

As described earlier, light is the most important factor to induce astaxanthin in *H. pluvialis*. Among the properties of the light, spectral quality and intensity seem to be more critical than other properties of light energy for algal growth and metabolism (Lee and Palsson 1994). LEDs can serve as an ideal light source for algal growth due to its advantages: (i) narrow spectral output, which can overlap with the absorption spectra of microalgae; (ii) high electric-to-light conversion efficiency, which generates less heat; (iii) no emission outside of photosynthetically active radiation (PAR), such as ultraviolet and infrared regions, which makes the light delivery system simpler; (iv) small weight and volume characteristics, which makes LEDs to be incorporated into virtually all types of PBRs for both internal and external light sources; (v) many other advantages, such as long life expectancy, solid state, safe (powered by low DC voltage), extremely short rise and fall time (Park and Lee 2000).

The control culture of *H. pluvialis* was cultivated under 320 μE m^{-2} s^{-1} of light intensity from fluorescent lamp. Three cultures with the same total light intensity were also performed but with 300 μE m^{-2} s^{-1} of light intensity from external fluorescent lamps and with 20 μE m^{-2} s^{-1} light intensity from three different internal light sources. The other three PBRs had only internal light sources with

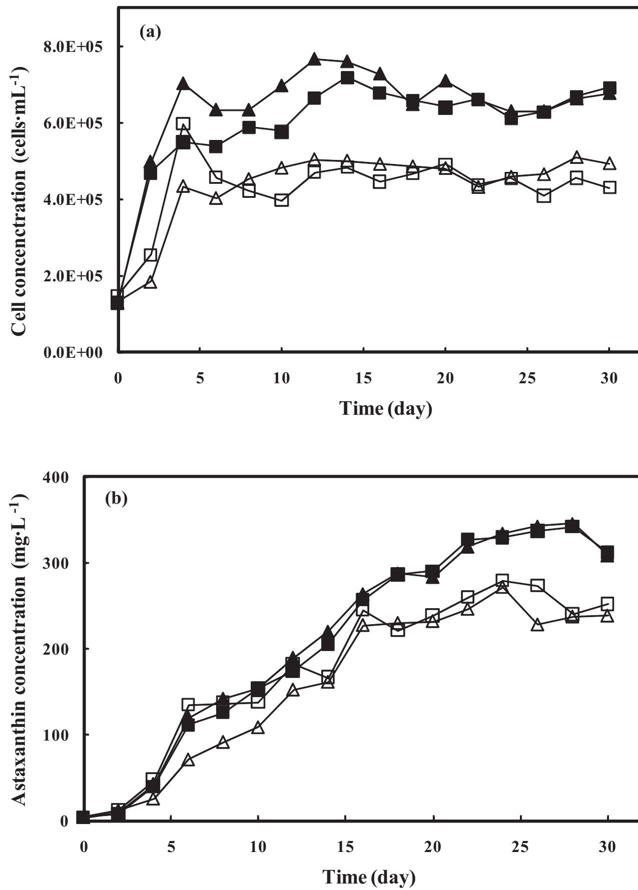

Fig. 3. Time profiles of (a) cell concentrations and (b) astaxanthin concentrations under various combinations of 320 μE m^{-2} s^{-1} of illumination. All the light intensities are in μE m^{-2} s^{-1} and the first term represents the internal light source and intensity and the latter is for the external light source. Blue LED 20 + FL 300 (▲; BF); Red LED 20 + FL 300 (■; RF); FL 20 + FL 300 (□; FF); None + FL 320 (△; FL).

the intensity of 20 μE m^{-2} s^{-1} (Table 2). All the PBRs had 2 L of fresh MBBM media.

Fig. 3a shows the cell growth profiles of *H. pluvialis* under the same total light intensity of 320 μE m^{-2} s^{-1} with different internal light sources (Table 2). Cell concentrations of all four cases increased sharply at initial stage till day 4, and then maintained the roughly same cell concentration in later part of the culture due to the depletion of nutrients. These profiles of cell concentration were almost identical, except one interesting aspect. The cell under blue LEDs + FL (▲ in Fig. 3; BF) and red LEDs + FL (■ in Fig. 3; RF) showed slightly higher than white light (□ and △ in Fig. 3; FF and FL, respectively), regardless of the location of the light source. If a small portion of white light were illuminated internally (□ in Fig. 3; 20 μE m^{-2} s^{-1} from an internal fluorescent lamp and 300 μE m^{-2} s^{-1} from external fluorescent lamps), the profiles of cell concentration were identical with FL (△ in Fig. 3; all 320 μE m^{-2} s^{-1} from external fluorescent lamps with empty inner tube). However, if a small portion of blue or red lights were illuminated internally (▲ and ■ in Fig. 3), the cell concentrations were increased about 50% over the white light control, either FF or FL. Maximum cell density of BF was around 7.5 × 10^{5} cells mL^{-1} (6.5-7.0 g L^{-1} of fresh weight), and that of RF was in the same range. However, the maximum fresh weights of FF and FL were much lower at a little over 5 g L^{-1}. This clearly suggests that red or blue photons, whose wavelengths fall on the absorption spectra of chlorophylls, have higher efficiency than non-overlapping spectral sources. Furthermore, the light intensities of LEDs are still somewhat low for high-density photoautotrophic cultures but the efficiency of blue LEDs have been jumped recently and LEDs can be suitable light sources.

Confirming results were observed in the production of astaxanthin (Fig. 3b). The maximum astaxanthin levels of BF and RF (▲ and ■, respectively in Fig. 3b) were about 340 mg L^{-1} at day 28, which was over 20% higher than those of control (□ and △ in Fig. 3b). These values are summarized in Table 2. To clarify the effect of red and blue light, a rough astaxanthin yield parameter was calculated by the ratio of the total produced astaxanthin to the total photons supplied per unit time, as listed in the right column of Table 2. Again, this result clearly suggests that red and blue lights are more efficient in stimulating astaxanthin synthesis because the astaxanthin yield per photon was a little over 20% higher in RF and BF, even though the total number of supplied photons was identical in all four cases (Table 2). Consequently, supplementing relatively small quantity of red and blue light to white light can enhance the production of astaxanthin by 20%.

Effects of blue and red lights without white light on astaxanthin production

To further examine the effectiveness of blue and red light on astaxanthin accumulation, cells were also cultivated under low intensities of internal light without external illumination. Three PBRs were setup with only internal light sources with the intensity of 20 μE m^{-2} s^{-1} (Table 2). The light intensity of 20 μE m^{-2} s^{-1} was not enough to support the high density culture or to fully induce astaxanthin. The cell didn't grow very well in all three cases (refer Fig. 4a) and the astaxanthin concentration did not increase till day 15 (refer Fig. 4b). The astaxanthin level, however, started to increase to a certain

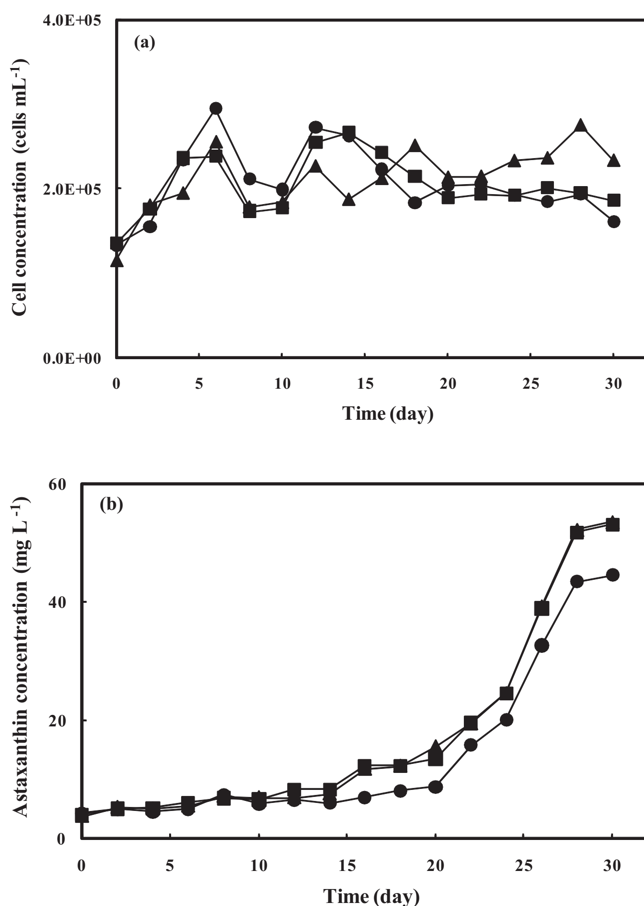

Fig. 4. Concentration profiles of (a) cell concentration and astaxanthin concentration under 20 μE m^{-2} s^{-1} of internal illumination by fluorescent lamp and LEDs (blue and red). Blue LEDs (▲); Red LEDs (■); Fluorescent lamp (●).

degree after day 15, probably after consuming nitrogen source. Astaxanthin concentrations under blue LEDs (▲ in Fig. 4) and red LEDs (■ in Fig. 4) were over 50 mg L^{-1} (Table 2) whereas that under fluorescent lamp (● in Fig. 4) was again about 20% lower (Table 2). Astaxanthin concentration profile looks different from the previous experiment (Fig. 3), but the trends of 20% increase with blue and red lights were the same. Astaxanthin yield per supplied photon was also about 20% higher with colored lights (Table 2). There have been a couple of reports that the blue LEDs could enhance the astaxanthin formation (Park and Lee 2001; Lababpour *et al.* 2004). Though there was a report on increased transcript levels of carotenoid biosynthesis genes under both blue and red light conditions (Steinbrenner and Linden 2003), this may be the first report that the red light can actually enhance the production of astaxanthin in *Haematococcus*. Furthermore, as far as the yield of astaxanthin per photon is concerned, internal illumination is much more efficient than external illumination (Table 2). Conclusively, LEDs with the emission spectra that overlap with the absorption spectra of algae can be a perfect light source for internal illumination for enhancing astaxanthin accumulation form *H. pluvialis*.

In Conclusions, few systematic investigations have reported on the effect of the quality of light on astaxanthin production from *H. pluvialis*. The results reported here clearly showed that the internal illumination method using LEDs has numerous advantages, such as reduction of power consumption, and higher astaxanthin accumulation with better yield. In addition, LEDs have other merits as an internal light source such as low heat generation, small volume characteristics, and low voltage of power.

The cells grown under blue or red light supplement showed 20% higher cell concentration (cells mL^{-1}) as well as astaxanthin concentration than those grown under white light, regardless of the location of the white light sources. The maximum astaxanthin levels (cells mL^{-1}) of under blue or red light supplement were also about over 20% higher than those of control as summarized in Table 2. Consequently, the astaxanthin yield per supplied photon is also consistently higher with the cells grown under colored light supplement. Interestingly, exactly the same trends were observed when the experiment was repeated without white light. Even if there is no white light, astaxanthin concentrations under low intensities of blue LEDs and red LEDs only were again 20% higher than that under internal white light without external white light. As with the previous experiment, the astaxanthin yield per photon was higher with colored light. Conclusively, LEDs can be a perfect light source for internal illumination for enhancing astaxanthin accumulation form *H. pluvialis* and improving light regime inside photobioreactor.

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