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Astaxanthin Production by *Haematococcus pluvialis* under Various Light Intensities and Wavelengths

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Abstract The key factors for high-density *Haematococcus pluvialis* cultures and conditions for astaxanthin induction were examined to maximize astaxanthin production. Light intensity was found to be the most important factor, and thus experiments were carried out using different light sources and intensities. A high cell density of over 2.7 g/l was obtained at 75 μE/m^2/s, whereas a much lower cell concentration (<1.0 g/l) was obtained with lower light intensities (15–30 μE/m^2/s). A high light intensity and the supplement of 470 nm photons had a more dramatic effect on the final astaxanthin concentration and per cell astaxanthin content. A maximum astaxanthin concentration of 6.5 mg/l was obtained at a light intensity of 160 μE/m^2/s, whereas only 1.3 and 0.7 mg/l were obtained at 30 and 15 μE/m^2/s, respectively. A supplement of 470 nm photons enhanced the carotenoid and chlorophyll formation.

Key words: Astaxanthin, *Haematococcus pluvialis*, light intensity, blue and red light, LEDs

The commercialization of microalgal biotechnology has been accelerated due to the vast potential of microalgae as a source of valuable metabolites, such as proteins, carbohydrates, fatty acids, enzymes, antibiotics, pharmaceuticals, bioactive compounds, vitamins, and biofuels. The applications of microalgae have been extended to agriculture and environmental industries as biofertilizers and in the bioremediation of soils [14]. The recent development of related techniques, such as natural product screening systems, cultivation methods, separation and purification techniques, as well as the availability of high-density large-scale photobioreactors, have also expedited the commercialization processes of microalgal biotechnology in various fields [3, 27, 28]. Examples of successful commercialization include *Chlorella* and *Spirulina* for functional foods, and *Dunaliella* for β-carotene production [3, 4].

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is a natural algal product that has recently received increasing interest due to its use as a preferred pigment in feeds for farmed fish and other marine animals [24]. This red ketocarotenoid also serves as a vitamin source for the poultry industry, a colorant, natural preservative, and food additive in the food industry, a superior antioxidant to α-tocopherol (vitamin E) and β-carotene in the cosmetic industry [11-13, 30], an antiaging reagent as a precursor of vitamin A, an anticancer agent through singlet oxygen quenching, and an immunomodulator in the pharmaceutical industry [2, 23, 25, 29]. There are several algal strains that produce astaxanthin [8, 22]. Despite the accumulation of a high level of astaxanthin in *H. pluvialis* [10, 15, 16, 18, 33], the low growth rate, thick cell wall, and low cell density restrict this excellent producer of astaxanthin from commercial uses. Recently, a number of research groups have studied large-scale cultivation, scale-up techniques, and the design and operation of photobioreactors for the commercial production of astaxanthin from *H. pluvialis* [5, 7, 20, 26, 32, 34].

Accordingly, the current study investigated the effects of light intensities and colored light on cell growth and astaxanthin accumulation using light intensities from 0 (dark conditions) to 160 μE/m^2/s, and light sources from a cool white fluorescent lamp and LEDs (470 and 680 nm).

Materials and Methods

Strain and Culture Conditions

The green microalga, *Haematococcus pluvialis*, Flotow, Volvocales, Chlorophyceae, (*H. lacustris*, UTEX 16) was obtained from the University of Texas Culture Collection (Austin, TX, U.S.A.). *H. pluvialis* was cultivated in a proteose-peptone medium consisting of $NaNO_3$ 250 mg/l,

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$CaCl_2 \cdot 2H_2O$ 25 mg/l, $MgSO_4 \cdot 7H_2O$ 150 mg/l, K_2HPO_4 75 mg/l, KH_2PO_4 175 mg/l, NaCl 25 mg/l, and proteose-peptone 1 g/l in distilled water. The experiments were carried out in 250-ml Erlenmeyer flasks containing 120 ml of the culture medium, after adjusting the initial pH to 6.4. The seed culture was grown at 20°C under continuous shaking (175 rpm) and irradiated with a white fluorescent lamp at a light intensity of 60 $\mu E/m^2/s$. The light intensities examined were dark conditions, 15, 30, 60, 75, 90, and 160 $\mu E/m^2/s$, and colored lights were supplemented by LEDs (470 and 680 nm).

Measurement of Cell Concentration and Cell Size Distribution

The cell concentration and cell size distribution were measured using a Coulter Counter (model Z2, Coulter Electronics, Inc., Hialeah, FL, U.S.A.) after the culture samples were diluted with an electrolyte solution, Isoton® II (Coulter Electronics, Ltd., Hong Kong) to about 10^4 cell/ml. The data from the Coulter Counter were converted by AccuComp® software and exported to Excel to calculate the cell numbers and cell size distributions. Information on the cell cycle stages, cell growth kinetics, and morphological characteristics was obtained from these data.

Extraction of Pigments

One milliliter of the culture sample was centrifuged at 10,000 rpm for 10 min and the supernatant was removed. The collected algal cells were resuspended with 1 ml acetone and the cell walls were broken by a specially-designed tissue homogenizer for 2 min with 12 strokes. Two milliliters of acetone were added and the sample was stored at 4°C in a refrigerator for 20 min to extract the pigments. These steps were repeated until the color of the cell debris became white or colorless. The supernatant that contained the pigments was then harvested after removing the cell debris by centrifugation at 10,000 rpm for 10 min.

Analysis of Pigments

The chlorophyll and astaxanthin concentrations were analyzed using a spectrophotometer (model HP8453B, Hewlett Packard, Waldbronn, Germany). The chlorophyll concentration was calculated using a previously reported equation [1]: chlorophyll $a=(12.7\times A_{663})-(2.69\times A_{645})$, chlorophyll $b=(22.9\times A_{645})-(4.64\times A_{663})$. The astaxanthin concentration was calculated with a calibration curve using synthetic astaxanthin (A9335, Sigma Chemical Co., St Louis, MO, U.S.A.) as the standard. For an astaxanthin concentration of less than 10 mg/l, the following calibration was used: astaxanthin concentration (mg/l)=0.0045 OD_{475}.

More quantitative and qualitative measurements of astaxanthin were determined using an HPLC (Younglin Instrument Co., Anyang, Korea) with two M930D pumps and a M730D photodiode array (PDA) detector. A reversed-phase C18 column (300×3.9 mm; 5 mm, Waters Corp., Milford, MA, U.S.A.) was chosen for the separation and analysis of the extracted pigments. For a better resolution between the astaxanthin and the other pigments, a gradient procedure with two solvents was introduced: solvent A (dichloromethane:methanol:acetonitrile:water=5:85:6:4 v/v) and solvent B (22:28:6:4 v/v). The gradient procedure was as follows: 100% of A for 8 min; a linear gradient from 0 to 100% of B for 6 min; and 100% of B for 24 min. The flow rate was set at 1.0 ml/min. Chromatographic peaks for astaxanthin were observed at 480 nm as reported earlier [32].

Measurement of Light Intensity and Power

The colored light was acquired using 470 nm LEDs (DigiKey, Thief River Falls, MN, U.S.A.) and 680 nm LEDs (Quantum Devices Inc., Barneveld, WI, U.S.A.). The light intensities of the fluorescent lights were changed by changing the number of cool white fluorescent lamps, the distance from the samples, and by the addition of a reflector. The LEDs were powered by DC power suppliers and the supplemental light intensities were controlled by adjusting the supplied voltage.

The light intensity was measured using a quantum sensor (LI-190SA, LI-COR, Lincoln, NE, U.S.A.).

RESULTS AND DISCUSSION

Effect of Light Intensities on Cell Growth

There are many known factors that affect the astaxanthin accumulation in *H. pluvialis*: nutrient limitation or supplement (in iron, manganese, nitrogen, and potassium), salt stress [6, 18, 19], plus other environmental factors, such as excess oxygen stress [17], high light intensity [13, 18, 34], blue light [18], dark conditions, and high temperature [9, 31]. Among these factors, light intensity was found to have the most effect on the *H. pluvialis* cultures. The cell division, cell cycle and thus morphological change of the cells, carotenoid formation and concentration, and chlorophyll concentration of *H. pluvialis* were all affected by the light intensity.

Figure 1 shows the cell growth curves under various light intensities. All the data points represented in Fig. 1 were the average of triplicate tests, and the standard deviation of each average is represented by an error bar. The cell concentrations were calculated from the total cell number and the average cell size from the Coulter Counter after multiplying the proper calibration factor to convert the total cell volume (=cell number × average cell size) to a dry cell weight. As expected, the culture kept in the dark did not grow (represented by ● in Fig. 1). However, an optimal light intensity was found for cell growth. Light

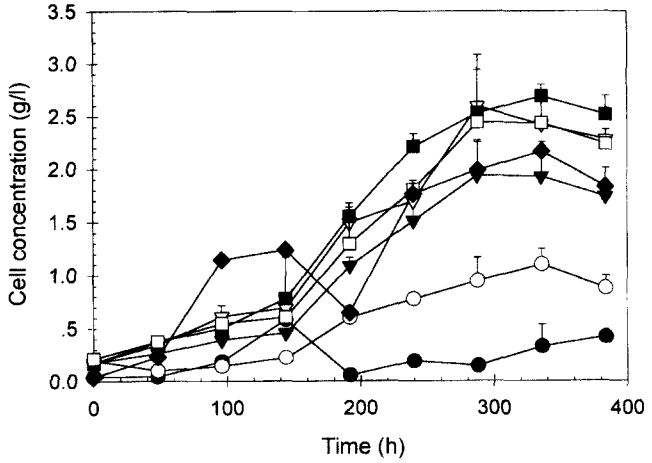

Fig. 1. Effect of light intensity on cell growth.
Growth curves under dark conditions (-●-); under 15 μE/m²/s (-○-); 30 μE/m²/s (-▼-); 60 μE/m²/s (-▽-); 75 μE/m²/s (-■-); 90 μE/m²/s (-□-); and 160 μE/m²/s (-◆-).

intensities between 60–90 μE/m²/s (represented by ▽, ■, and □) supported the cells the best, while lower light intensities from 15–30 μE/m²/s (○ and ▼) or a higher light intensity of 160 μE/m²/s (◆) could not match the growth under the optimal light intensity. The highest cell concentrations under different light intensities were 1.1, 1.9, 2.2, and 2.7 g/l under 15, 30, 60, and 75 μE/m²/s, respectively. The highest cell concentration obtained was 2.7 g/l, which is one of the highest among previously reported values.

Effect of Light Intensities on Pigment Production

The optimal light intensity for astaxanthin accumulation was different from that for cell growth (Fig. 2A), thereby suggesting that a two-stage culture may be the best way to produce astaxanthin on a commercial scale [3, 12, 24]. As shown in Fig. 2A, the effect of light intensity was distinct with different light intensities. For low light intensities from 0–30 μE/m²/s (●, ○, and ▼ in Fig. 2A), the astaxanthin formation or accumulation was also very low. For this regime of light intensity, both the cell growth rate and astaxanthin formation rate were not acceptable. Further statistical analysis (ANOVA) revealed that the astaxanthin accumulation rate under medium light intensities from 60–90 μE/m²/s (represented by ▽, ■, and □) was 6 times higher than that under a low light intensity of 15 μE/m²/s (○ in Fig. 2A). However, the astaxanthin and cell concentrations did not exhibit a strong correlation, when considering that the middle light intensity regime was optimal for *H. pluvialis* growth, as described in the previous section (refer Fig. 1). The highest astaxanthin concentration of 6.5 mg/l was observed at 340 h after inoculation under a higher light intensity (160 μE/m²/s, marked with ◆ in Fig. 2A), and this astaxanthin

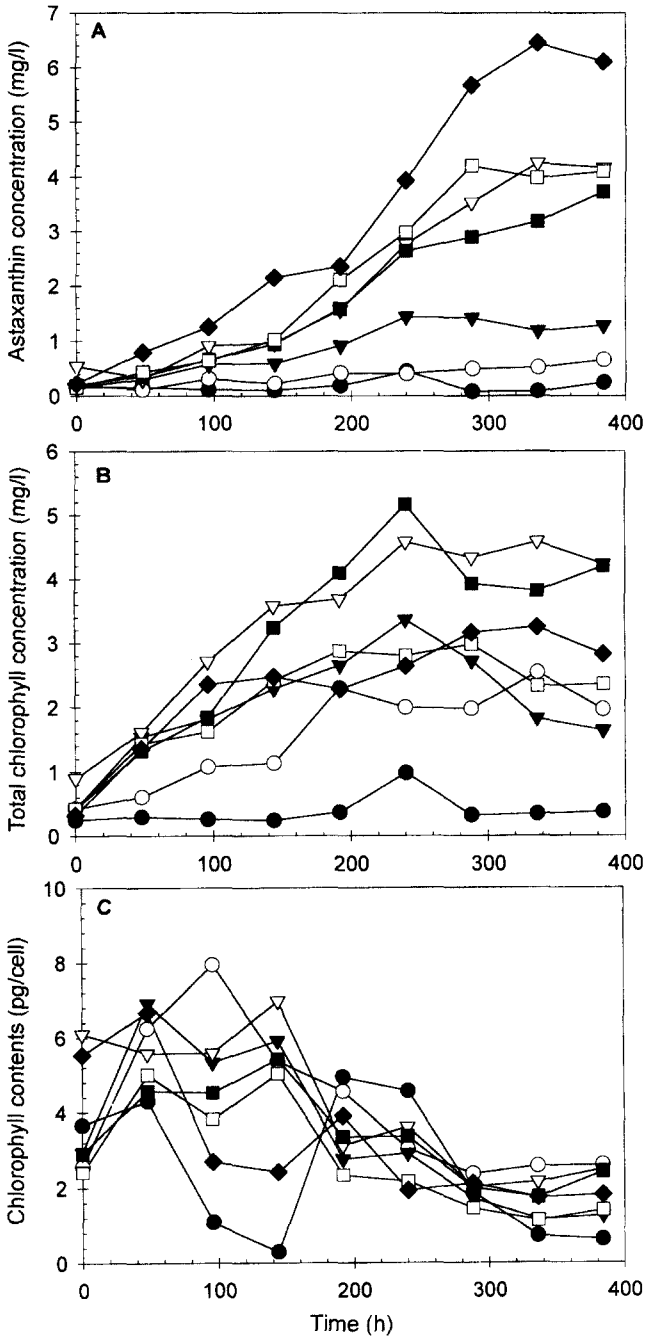

Fig. 2. Pigments concentration profiles under various light intensities.
A. Astaxanthin concentration (mg/l); B. Total chlorophyll concentration (mg/l); C. Chlorophyll contents per cell (pg/cell). Data under dark conditions (-●-); under 15 μE/m²/s (-○-); 30 μE/m²/s (-▼-); 60 μE/m²/s (-▽-); 75 μE/m²/s (-■-); 90 μE/m²/s (-□-); and 160 μE/m²/s (-◆-).

concentration was more than 10 times higher than that under 15 μE/m²/s.

The differences in the astaxanthin content per cell under different light intensities were a clear indicator of the effect

Table 1. Astaxanthin contents per cell under different light intensities.

Light intensity ($\mu E/m^2/s$)	Astaxanthin contents per cell (pg/cell)	Relative contents
0	0.19	1.00
15	0.53	2.77
30	0.76	3.93
60	2.00	10.40
75	1.46	7.60
90	1.93	10.07
160	3.47	18.06

of light intensity on the *H. pluvialis* cultures (Table 1). For an easier comparison, the relative contents were calculated in Table 1, which are the normalized astaxanthin content per cell to the value obtained under dark conditions. The astaxanthin concentration (mg/l) under 160 $\mu E/m^2/s$ was about 60% higher than that under 60 $\mu E/m^2/s$. However, this ratio increased to over 80% on a per cell basis when using the data in Table 1. As such, this data also suggested that using two-stage *H. pluvialis* cultures would be more practical and efficient, since each stage could be optimized separately for cell growth and astaxanthin production, respectively.

In contrast, the total chlorophyll concentration (=chlorophyll *a*+chlorophyll *b*) exhibited different behavior (Fig. 2B). Total chlorophyll concentrations were higher (around 4.5 mg/l) at 60 and 75 $\mu E/m^2/s$ (represented by ▽ and ■, respectively), while all the other chlorophyll concentration profiles were roughly within the same range of 2.0–3.0 mg/l (15, 30, 90, and 160 $\mu E/m^2/s$, marked by ○, ▼, □, and ◆, respectively). One interesting observation was that the final cell concentration (Fig. 1) seemed to be a close function of the total chlorophyll (Fig. 2B): The higher the cell concentration, the higher the total amount of chlorophyll and vice versa. However, the chlorophyll contents per cell, which can serve as a viability parameter of microalgae [21], provided other information on the experiment. Although the chlorophyll content per cell was similar in all cases, there was also a rough trend that the total astaxanthin concentration was inversely proportional to the chlorophyll contents per cell (Fig. 2C).

Effect of Light Intensity on Cell Size Distribution

The cell size distribution was also affected by the light intensity. Four sets of Coulter Counter data are compared in Figs. 3A and 3B. The cell size of *H. pluvialis* increased dramatically as the cells accumulated astaxanthin. Figure 3A is a histogram of the cells under 0, 15, 60, and 160 $\mu E/m^2/s$ during the earlier phase of the culture (at 96 h). It clearly shows that the main peak shifted to a larger size as the light intensity increased. For example, the cells under 15 $\mu E/m^2/s$ (···· in Figs. 3A and 3B) had a peak near 60 μm^3/cell, whereas the peaks under 60 and 160 $\mu E/m^2/s$

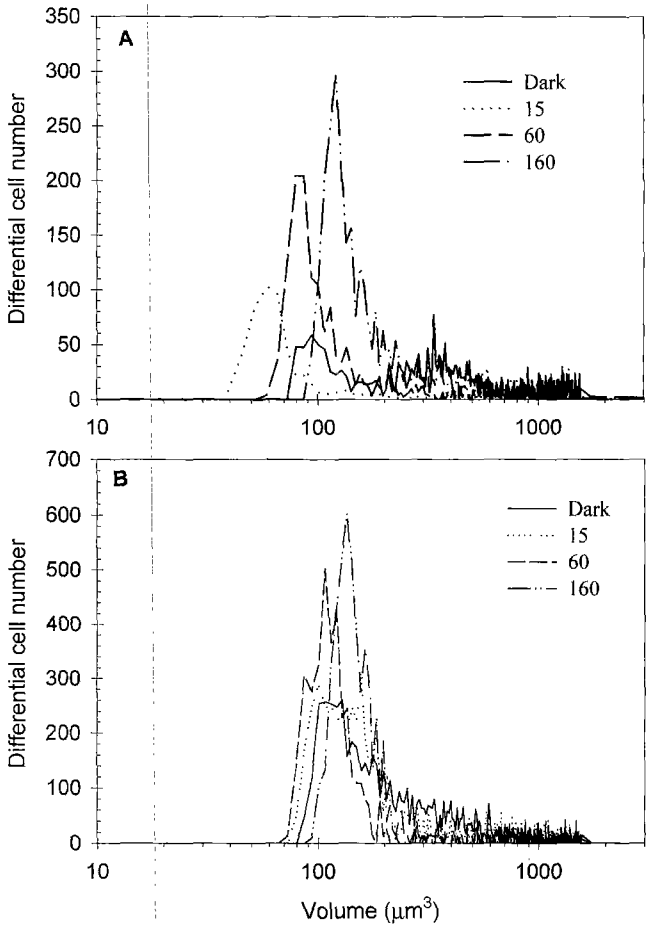

Fig. 3. Comparison of per cell volume distribution under various light intensities. A. After 96 h of cultivation; B. After 336 h of cultivation.

(— and –··, respectively, in Figs. 3A and 3B) moved to 80 and 102 μm^3/cell, respectively, thereby indicating an increase in the average cell size. Again, a higher light intensity stimulated astaxanthin accumulation and thus increased the astaxanthin productivity. As the *H. pluvialis* cultures entered into the stationary phase, all the cultures started to accumulate astaxanthin. The histogram peaks decreased in the stationary phase (Fig. 3B at 336 h), however, the same trend that a higher intensity produced more cells in larger volumes was observed in the later phase of the culture. Consequently, although a higher intensity stimulated astaxanthin production, there would seem to be an optimal light intensity for growth. Accordingly, this data again suggested that two-stage cultures would be beneficial for high-yield astaxanthin production.

Effect of Colored Lights on Cell Concentration and Astaxanthin Production

The effect of colored lights on the astaxanthin production by *H. pluvialis* was also investigated. The algae were

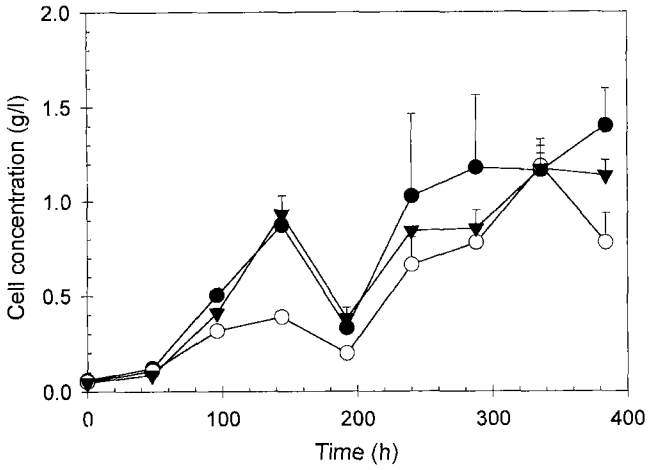

Fig. 4. Effect of different light sources on cell growth.
Growth curves under fluorescent lamp only (-○-); under fluorescent lamp supplemented with 470 nm (-●-); under fluorescent lamp supplemented with 680 nm (-▼-).

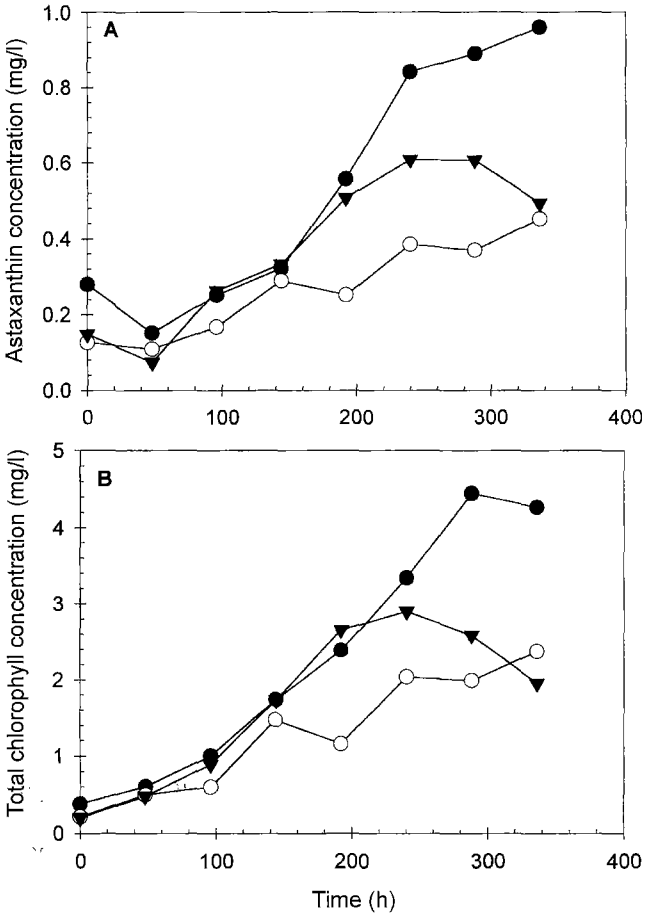

Fig. 5. Pigments concentration profiles under different light sources.
A. Astaxanthin; B. Total chlorophyll concentration (mg/l). Data under fluorescent lamp only (-○-); under fluorescent lamp supplemented with 470 nm (-●-); under fluorescent lamp supplemented with 680 nm (-▼-).

cultivated under a 15 μE/m²/s light intensity from fluorescent light with supplements of 470 or 680 nm photons from LEDs. As shown in Fig. 4, the supplements of blue or red lights significantly affected the cell growth. As expected from the results in the previous section, the cell growth under 15 μE/m²/s of fluorescent light (marked by ○ in Fig. 4) was unsatisfactory. However, the poor growth under the low light conditions was significantly improved by supplementing a small amount of blue (● in Fig. 4) or red (▼) light. The supplement of blue light produced a more drastic effect than the supplement of red light. When considering an even lower intensity of the supplemented colored light, 0.1 μE/m²/s, the improvement was much higher than expected. The average cell concentration with a blue light supplement was almost twice that without a supplement, and this result was very stimulating for optimizing the astaxanthin production by *Haematococcus*.

More interesting results were obtained as regards to the astaxanthin concentration (Fig. 5A). The total astaxanthin concentration (mg/l) with a supplement of blue light (● in Fig. 5A) was over 2 times more than that without a supplement. In addition to the higher cell concentration with a blue light supplement, the astaxanthin content per cell (pg/cell) with a blue light supplement was also 1.6–2.4 times higher than that without a supplement. The red light supplement also affected the astaxanthin content per cell, which was 1.3–1.5 times higher than that without a supplement. Kobayashi *et al.* [18] also reported that blue light enhances astaxanthin production when using filtered fluorescent light (although they did not use blue light as a supplement). The current results showed that only a small amount of blue light was necessary to exploit the blue light phototaxis of *Haematococcus*.

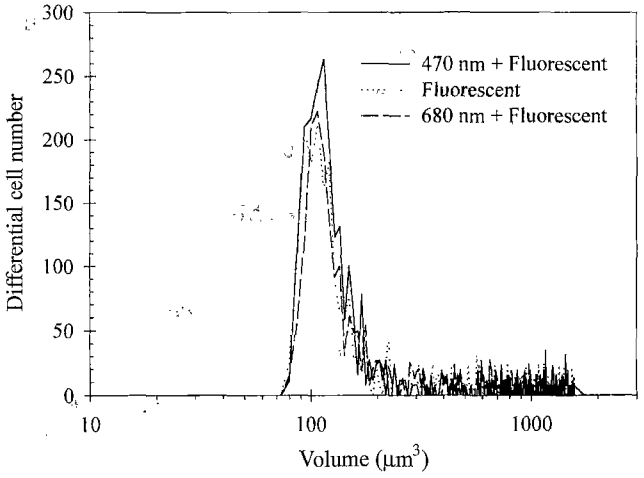

Fig. 6. Comparison of per cell volume distribution under different light sources after 228 h of cultivation.

The profiles of the total chlorophyll concentration (mg/l) exhibited a similar trend with the total astaxanthin concentration (Fig. 5B), and the profiles of the chlorophyll content per cell (pg/cell) was also almost identical in all cases (data not shown). The cell size analysis using a Coulter Counter revealed similar distributions regardless of a colored light supplement. Figure 6 represents the cell size distribution histogram at 228 h.

In conclusion, the supplement of a small amount of colored light significantly increased the astaxanthin productivity without affecting the cell cycle. Although a more detailed study is required to optimize the astaxanthin production in *H. pluvialis*, the results reported in this paper show a potential for a commercial process.

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