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**ASTAXANTHIN PRODUCTION BY *Haematococcus pluvialis*
UNDER ILLUMINATION WITH LEDS**

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Abstract

The photosynthetic microalga *Haematococcus pluvialis*, a potential source of astaxanthin, was cultivated under illumination with LEDs emitting red (λ_{\max} = 625 nm), green (λ_{\max} = 525 nm), blue (λ_{\max} = 470 nm), blue-purple (λ_{\max} = 410 nm) and purple (λ_{\max} = 380 nm) light and a fluorescent lamp, and the effects of wavelength on cell growth and astaxanthin accumulation were studied. LEDs emitting light of short wavelengths (380 – 470 nm) were found to induce astaxanthin accumulation of up to 5 – 6% per dry-cell, although the induction caused the suppression of cell growth. From these results, we proposed a new strategy of cultivating *H. pluvialis* under illumination with red LEDs without inducing a high level of astaxanthin accumulation, and then switching to illumination with blue LEDs at a high light intensity to induce a high level of astaxanthin accumulation.

Keywords : Astaxanthin, Light emitting diode, Wavelength, Accumulation of astaxanthin

1. Introduction

Many kinds of photosynthetic bacteria and microalgae produce unique bioactive materials, such as pigments, proteins, fatty acids and vitamins. In cultivation of these photosynthetic cells, the design of photobioreactors for effective illumination is essential to reduce the production cost. While sunlight is the cheapest light source available, its light intensity fluctuates diurnally and seasonally, and its energy intensity is limited. To attain controlled cultivation conditions and productivity, electric illuminating devices that convert energy to light with high efficiency and emit light with favorable physiological effects on photosynthetic cells must be used in photobioreactor systems. Light emitting diodes (LEDs) have narrow light emission spectra, high conversion efficiency and low heat emission. The physiological effects of light with a specific range of wavelength on photosynthetic cells have been widely studied by using combinations of special light sources and filters. LEDs can be useful light sources for this purpose, because a wide variety of LEDs emitting from red to purple light are now available.

The photosynthetic microalga *Haematococcus pluvialis* is a potential source of astaxanthin, which is used as a feed additive for the pigmentation of cultured salmon and egg yolk and also as an antioxidative drug [1]. It is reported that a morphological change of *H. pluvialis* from the green

vegetative cells moving with flagella to red resting cyst cells without flagella enhances astaxanthin production, and that astaxanthin accumulation could be induced by high temperature, deficiency of nutrients [2], high light intensity [3], supplemental blue light [4] and the addition of oxidative supplements to media [5]. These results show that astaxanthin production by *H. pluvialis* is enhanced under conditions of stress. A specific range of incident light wavelength may affect the production of pigments such as astaxanthin and can contribute to the stress acting against photosynthetic cells.

In this work, *H. pluvialis* was cultivated under illumination with LEDs emitting red ($\lambda_{\text{max}} = 625 \text{ nm}$), green ($\lambda_{\text{max}} = 525 \text{ nm}$), blue ($\lambda_{\text{max}} = 470 \text{ nm}$), blue-purple ($\lambda_{\text{max}} = 410 \text{ nm}$) and purple ($\lambda_{\text{max}} = 380 \text{ nm}$) light. The effects of wavelength on the growth rate and astaxanthin accumulation in cells were studied.

2. Materials and methods

2.1 Microorganism and cultivation conditions

H. pluvialis NIES 144, which was obtained from Microbial Culture Collection in National Institute for Environmental Studies (Tsukuba, Japan), was grown aerobically under illumination by LEDs or a fluorescent lamp in Kobayashi's basal medium (pH 6.8), containing 1.2 g/l sodium acetate, 2

g/l yeast extract, 0.4 g/l L-asparagine, 0.2 g/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [6]. After precultivation in a 200-ml flask set in a glass-sided water bath under illumination at $3.8 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ with a fluorescent lamp, *H. pluvialis* was cultivated at 20°C in a glass vessel 6.5 cm high, 5.0 cm wide and 2.6 cm deep with a working volume of 55 ml. The light intensity is expressed as the number of photons passing through a unit area in a unit time, namely, as the photon flux, $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$. In two cultivation runs, to avoid nutrient deficiency and/or accumulation of wastes, cells were replaced several times into fresh medium after separation by centrifugation at 3,500 g for 5 min.

The surface of the vessel was illuminated from one side at the light intensities of 2.8, 8.0 and $12.0 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ with a panel of red, green, blue, blue-purple or purple LEDs or a fluorescent lamp. On the LED panels (5 cm x 5 cm), 85 LED lamps were arranged in 10 rows and 9 columns. The ranges and maxima of wavelengths emitted by the LEDs are shown in **Table. 1**. The LEDs have narrow emission spectra, while the fluorescent lamp emitted a wider range of wavelengths with the main peak in the green band.

2.2 Measurement of cell concentration and size

Since *H. pluvialis* shows a morphological change from green

vegetative cells moving with flagella to red resting cyst cells without flagella during cultivation, the absorbance and spectra of cell suspensions, and thus the correlation between cell weight and the absorbance at a specific wavelength, change with time. The change in spectra of cell suspensions with cultivation time is shown in **Fig. 1 (a)**. Overall, the absorbance from 300 nm to 1100 nm decreased with cultivation time, but the absorbance at 680 nm, attributed to chlorophyll a as shown in **Fig. 1 (b)**, decreased markedly. This was caused by accumulation of astaxanthin inside cells. On the other hand, chlorophyll a and astaxanthin show very low absorbance at 750 nm. When the slopes of the cell concentration vs. OD₆₈₀ were plotted against the difference in absorbance at 680 nm and 750 nm normalized by the absorbance at 680 nm during the course of cultivation (1 – 20 d), an approximately linear relationship was found between them, and this led to the following experimental equation.

$$\text{Dry cell weight} = [- 4.2 \times \{(\text{OD}_{680} - \text{OD}_{750})/\text{OD}_{680}\} + 1.4] \times \text{OD}_{680}$$

The values of the dry cell weight estimated by the above equation were compared with measured values in cultures illuminated with the LEDs and fluorescent lamp and showed good agreement throughout cultivation with the R² value of 0.968. Therefore, in this work the dry cell weight was estimated from the values of OD at 680 and 750 nm by use of the above equation. The spectra of cell suspensions were measured with a spectrophotometer (UV 1600, Shimadzu, Kyoto, Japan), and the dry-cell

weight was determined after drying at 80°C for 50 h.

The size, number and color of cells were observed with a microscope.

2.3 Measurement of astaxanthin content in cells

To determine the astaxanthin concentration in cultured *H. pluvialis*, samples of 0.5 ml of cell suspensions were removed daily from the culture vessel and centrifuged at 7,500 g for 10 min. The cell precipitate was resuspended in 0.5 ml of methanol and mixed with 0.40 g of silica particles (particle size = 0.2 – 1 mm, Kanto Chemical, Tokyo, Japan). To extract astaxanthin from cells, the mixture was vigorously mixed with a vibrator for 10 min and centrifuged for 10 min. The supernatant was divided into three portions. The precipitate was extracted again with 0.5 ml methanol to determine the remaining amount of astaxanthin.

The first portion of the supernatant was used for measurement of the spectrum. The second portion was applied to a HPLC system (LC-10, Shimadzu) equipped with a reverse phase column (Cosmosil 5C18-MS-II, 4.6 x 150 mm, nacalai tesque, Kyoto, Japan). The mobile phase was methanol with a flow rate of 1 ml/min, and the absorbance of the effluent solution was measured at 470 nm and 680 nm with a photodiode array detector (SPD-M10A, Shimadzu). The third portion (0.5 ml) was mixed with 0.1 ml of a methanol solution of NaOH (5 mM NaOH), kept overnight

under nitrogen in darkness at room temperature for saponification of astaxanthin esters and applied to the HPLC column [7, 8] under the same conditions as above. The concentration of astaxanthin was determined by use of a calibration curve obtained with authentic free astaxanthin.

3. Results and Discussion

3.1 Analysis of astaxanthin extracted from cells

In the first extraction step (section 2.3 above), over 90 % of colored products were extracted into methanol. **Figures 2 (a) and (b)** show chromatograms of extracted samples before and after saponification. Many peaks attributed to astaxanthin esters disappeared after saponification, and a single main peak of free astaxanthin was observed in Fig. 2 (b) [8].

3.2 Effects of incident wavelength and intensity on growth rate and astaxanthin production

Table 1 shows the specific growth rate, dry cell weight, specific maximum production rate of astaxanthin and astaxanthin concentration in fermentation broth after 8 d under illumination at $2.8 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$ with LEDs emitting different wavelengths and the fluorescent lamp. High cell and astaxanthin concentrations were obtained under illumination with

LEDs emitting shorter wavelengths. The fluorescent lamp shows a low intensity of emission in this range and thus was ineffective for growth of cells and astaxanthin production. Green LEDs ($\lambda_{\text{max}} = 525 \text{ nm}$) were also ineffective for growth of cells, because the absorption efficiency of chlorophyll in this range of wavelength is low.

The effectiveness of illumination at 380 – 470 nm for astaxanthin production was confirmed by the fact that the color of these fermentation broths changed from green to red at 4 – 5 d, while the broth illuminated with red LEDs or the fluorescent lamp remained brownish green.

Figures 3 (a) (b) and (c) compare the growth curves of *H. pluvialis* under illumination with blue LEDs, red LEDs and the fluorescent lamp at light intensities of 2.8, 8.0 and 12.0 $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$. For each light source the growth rate in the logarithmic phase was almost independent of the light intensity, showing that the light intensity was above saturation level. The specific growth rates at the initial stage were 0.026 h^{-1} for all light sources, but the subsequent decrease in growth rate differed depending on the intensity and wavelength of the light source. Under illumination with the red LEDs or the fluorescent lamp at 2.8 $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$, the cell concentration reached a plateau earlier than at 8.0 $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ because of light attenuation by cells. At 12.0 $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$, however, a slight delay of the cell growth was observed.

Figures 4 (a) (b) and (c) show the concentration of astaxanthin in fermentation broth for the same runs as in Figure 3. The astaxanthin concentration increased with time and reached around 13 $\mu\text{g/ml}$ for the red LEDs and fluorescent lamp at the light intensity of $8.0 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$. Under illumination with the blue LEDs, the concentration increased to 16 $\mu\text{g/ml}$ at the light intensity of $8.0 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$, and at $12.0 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$ it increased to 25 $\mu\text{g/ml}$ (astaxanthin content 6.5% of dry-cell weight) after the cell concentration reached the plateau. This concentration is four times those previously reported [4, 6]. This high productivity is attributed to the induction of morphological change and accumulation of astaxanthin by light of short wavelength and high intensity, although the induction caused the suppression of cell growth.

These results led us to examine the following regimen for high-efficiency astaxanthin production with *H. pluvialis* under LED illumination. In the first stage cells are grown under illumination with red LEDs at a relatively low intensity and with replacement of medium to avoid induction of morphological change. After cell concentration reaches a predetermined value, astaxanthin production can effectively be induced by illumination with blue LEDs at a high light intensity (above $10 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$).

Figures 5 (a) and (b) show the cell growth and the astaxanthin concentration in cultures illuminated first with red LEDs ($8.0 \mu\text{mol-photon}$

$\text{m}^{-2} \text{s}^{-1}$, 0 – 103, 140 or 171 h), then with blue LEDs ($11 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$). As controls, the results for illumination with red LEDs only and for cultivation without the replacement of medium are also shown. As shown in these figures, after the change from red to blue LEDs at 103 h, the astaxanthin concentration of cells rapidly increased above $22 \mu\text{g/ml}$ (solid triangles, broken line), corresponding to 6% of dry-cell weight. Red cyst cells were not observed before 100 h, and the percentage of green cells was above 70% in cultures illuminated with red LEDs. On the other hand, change of illumination from red to blue LED at 171 h, where no green cells were observed, did not induce a high level of accumulation (crosses, chain line).

In the runs shown by solid and open squares keys, in which the medium was replaced three times, the cell concentration reached above 0.8 mg/ml . After the third replacement, the percentage of green cells remained above 90%. The accumulation of astaxanthin was induced immediately after the start of illumination with blue LEDs at 140 h and increased to at least $42 \mu\text{g/ml}$ and 5.5% of dry-cell weight. In comparison, the accumulation of astaxanthin in the culture illuminated throughout with red LEDs started later, after 180 h, and was lower.

This work has shown that light of different wavelengths emitted by LEDs has different effects on cell growth and astaxanthin production by *H. pluvialis*. Red LEDs operated at a relatively low light intensity are suitable

for cell growth with replacement of medium; and LEDs emitting short wavelengths (380 – 470 nm) can induce the morphological change of *H. pluvialis* and enhance the accumulation of astaxanthin. Illumination of photobioreactors with LEDs emitting a suitable wavelength might be a useful means to control the physiological condition of cells. The strategy of utilizing the response of cells in photobioreactors to illumination with LEDs emitting different wavelengths is a promising method for astaxanthin production by *H. pluvialis*, and studies on high-concentration culture are now under way.

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References

- [1] Lorenz RT, Cysewski GR. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. TIBTech 2000;18: 160–167.
- [2] Fábregas J, Dominguez A, Garcia-Álvarez D, Lamela T, Otero A. Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies. Biotechnol. Lett 1998;20: 623-626.
- [3] Kobayashi M, Kakizono T, Nishio N, Nagai S. Effects of light

intensity, light quality, and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis*. J Ferment Bioeng 1992;74: 61–63.

[4] Park EK, Lee CG. Astaxanthin production by *Haematococcus pluvialis* under various light intensities and wavelengths. J Microbiol Biotechnol 2001;11: 1024–1030.

[5] Kobayashi M, Kakizono T, Nagai S. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Appl Environ Microbiol 1993; 59: 867–873.

[6] Kobayashi M, Kakizono T, Nagai S. Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. J Ferment Bioeng 1991;71: 335–339.

[7] Yuan J.P, Chen F. Chromatographic separation and purification of trans-astaxanthin from the extracts of *Haematococcus pluvialis*. J Agric Food Chem 1998;46: 3371–3375.

[8] Yuan J.P, Chen F. Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. J Agric Food Chem 1999;47: 31–35.

Figure captions

Figure 1 Change in absorbance accompanying by morphological change of *Haematococcus pluvialis*

(a) Absorption spectra of cell suspensions at different days

(b) Absorption spectra of astaxanthin and chlorophyll a

Figure 2 Chromatograms of HPLC before and after saponification of extracted astaxanthin

(Sample: cells cultivated for 182 h under illumination with blue LEDs at $8.0 \mu\text{mol-photon m}^{-2} \text{s}^{-1}$, analytical method: Cosmosil 5C18-MS-II, measured wavelength: 470 nm, mobile phase : methanol, 1 ml/min)

Figure 3 Growth curves of *Haematococcus pluvialis* illuminated with

(a) blue LEDs (b) red LEDs (c) fluorescent lamp.

Figure 4 Astaxanthin concentration in fermentation broth of

Haematococcus pluvialis

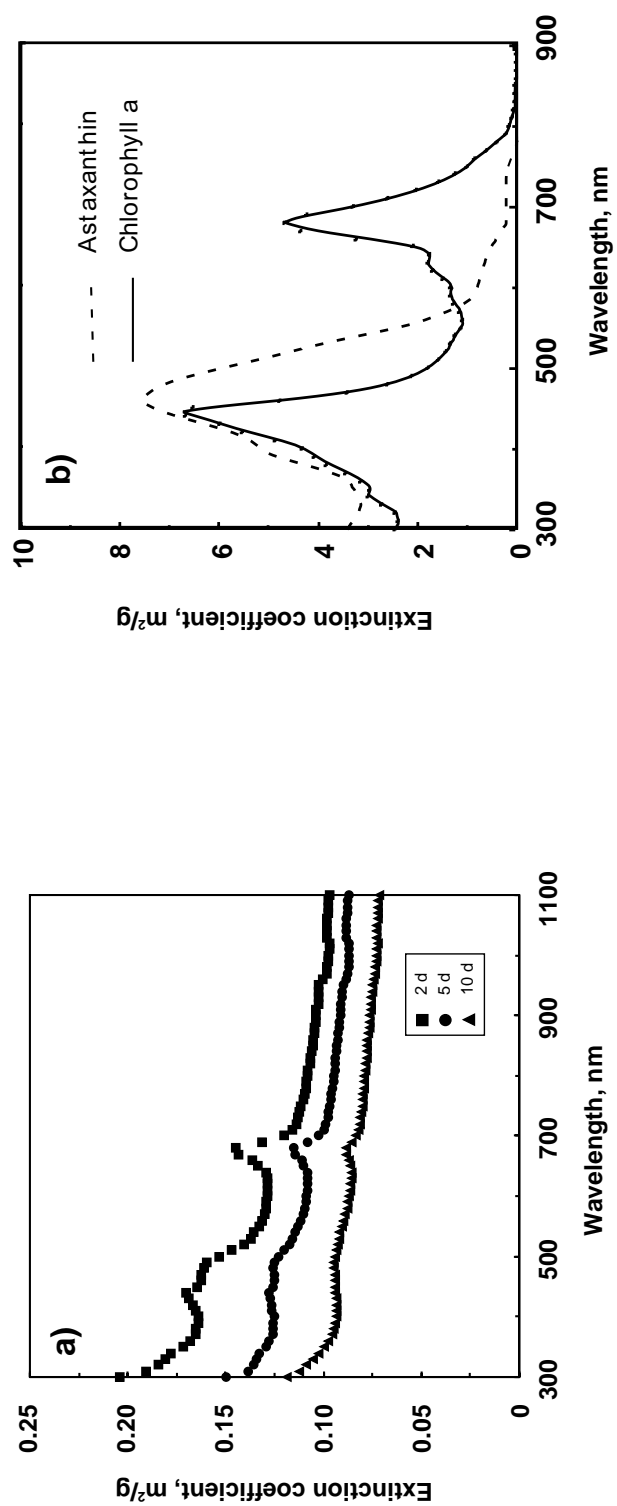
(a) blue LEDs (b) red LEDs (c) fluorescent lamp.

Figure 5 Growth curves (a) and astaxanthin concentration (b) under illumination with red and blue LEDs

Table 1 Effects of wavelength on cell growth and astaxanthin production

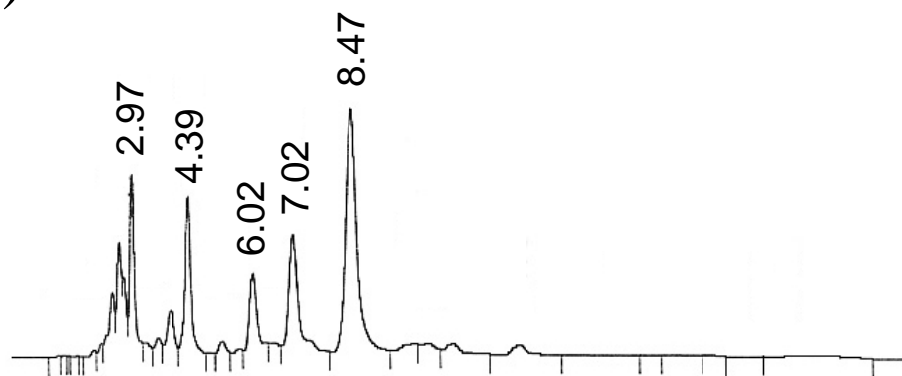
Light source	Sp. growth rate in exponential phase (1/h)	Highest cell concentration (mg dry-cells/cm³)	Maximum sp. production rate of astaxanthin (μg/(mg dry-cells·h))	Highest astaxanthin concentration (μg/cm³)
Purple LEDs (300 – 460 nm; λ _{max} 380 nm)	0.026	0.495	0.353	9.3
Blue-purple LEDs (340 – 500 nm; λ _{max} 420 nm)	0.027	0.546	0.222	–
Blue LEDs (390 – 550 nm; λ _{max} 470 nm)	0.026	0.480	0.285	14
Green LEDs (430 – 610 nm; λ _{max} 525 nm)	0.016	0.178	0.021	–
Red LEDs (560 – 700 nm; λ _{max} 625 nm)	0.025	0.465	0.255	5.1
Fluorescent lamp (400 – 740 nm; λ _{max} 550 nm)	0.023	0.273	0.232	3.0

(Incident intensity of light was adjusted at 2.8 μmol photons/(m²·s))

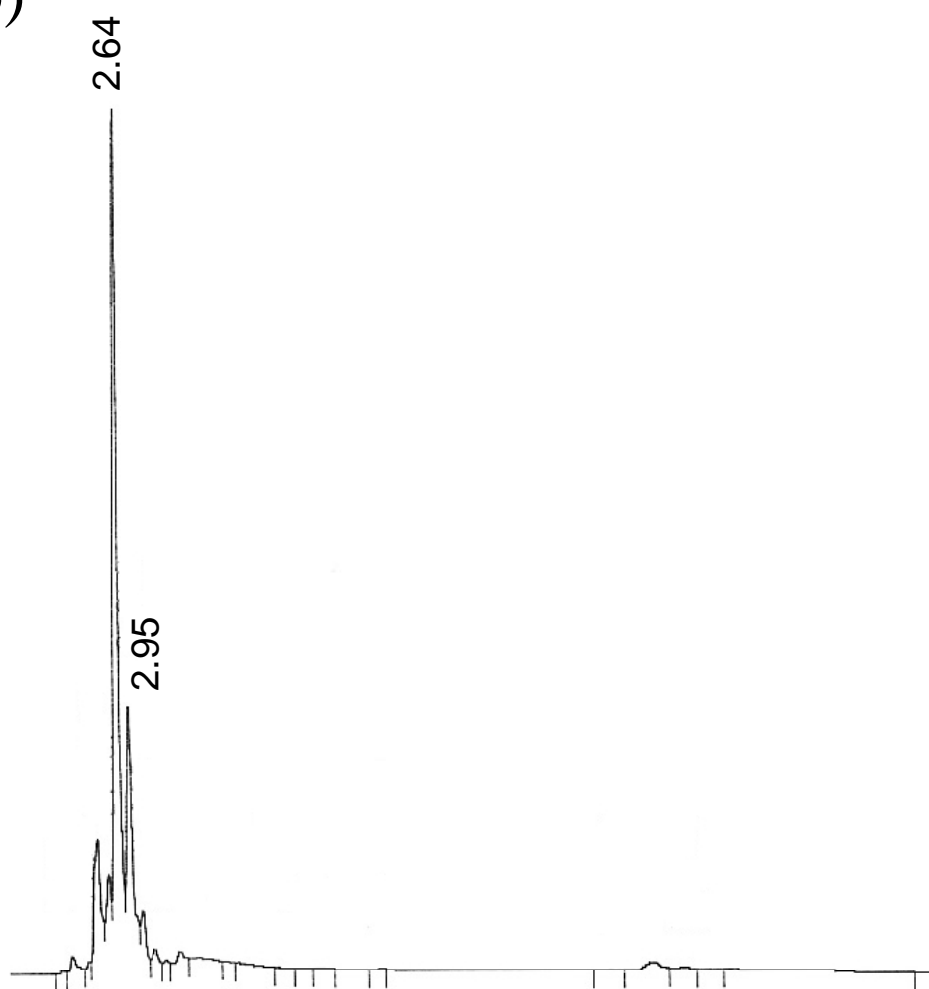


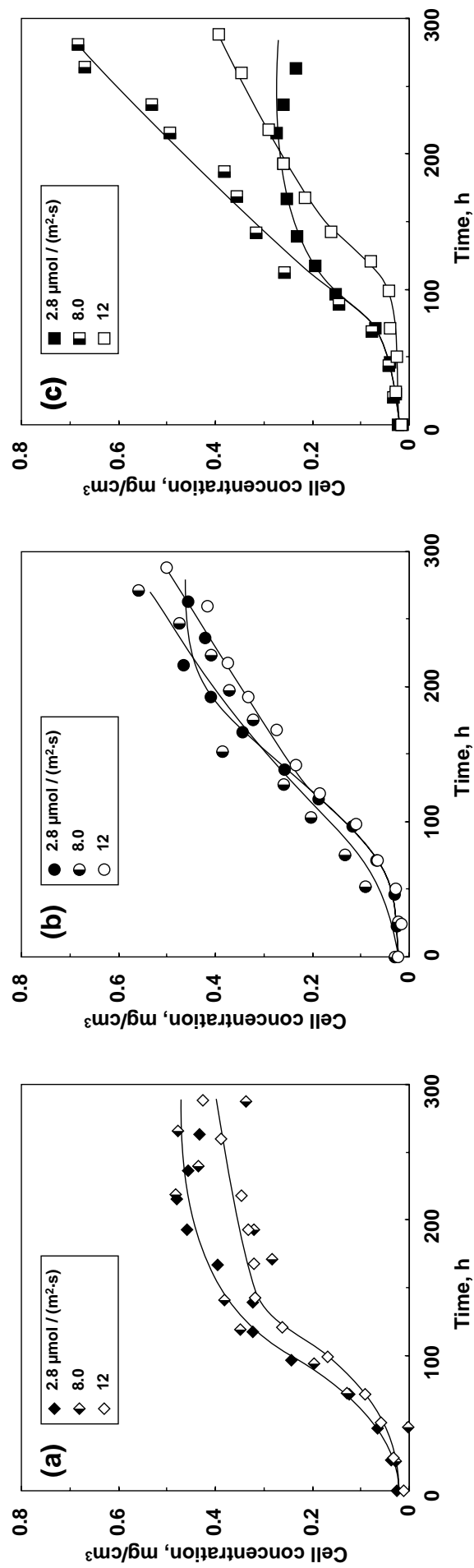
T. Katsuda et al. Figure 1

(a)

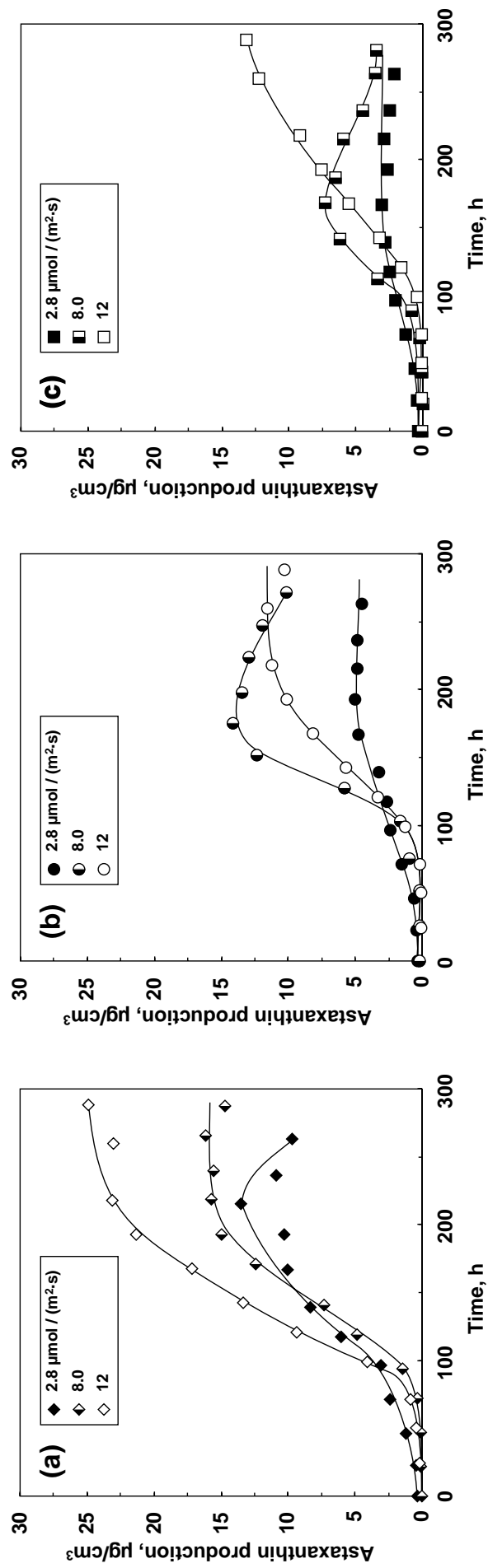


(b)

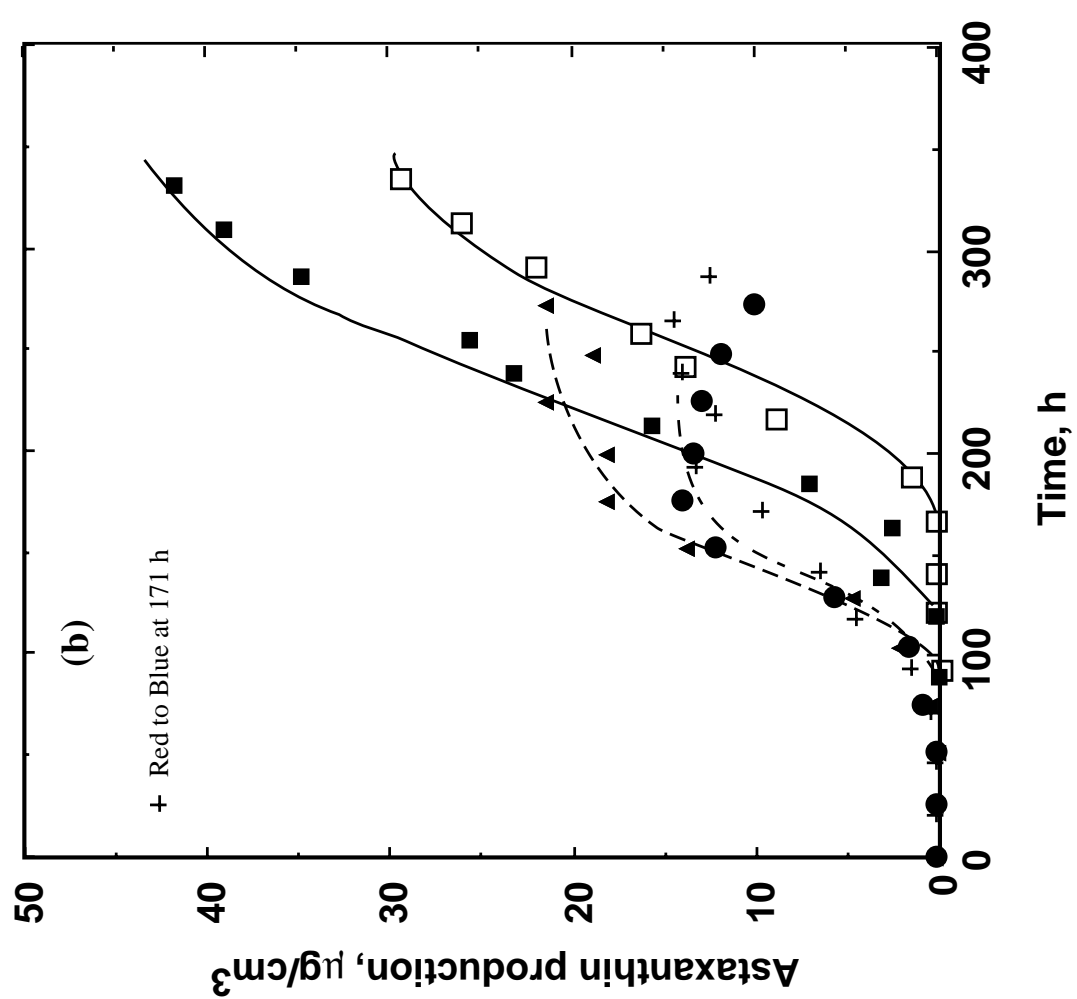
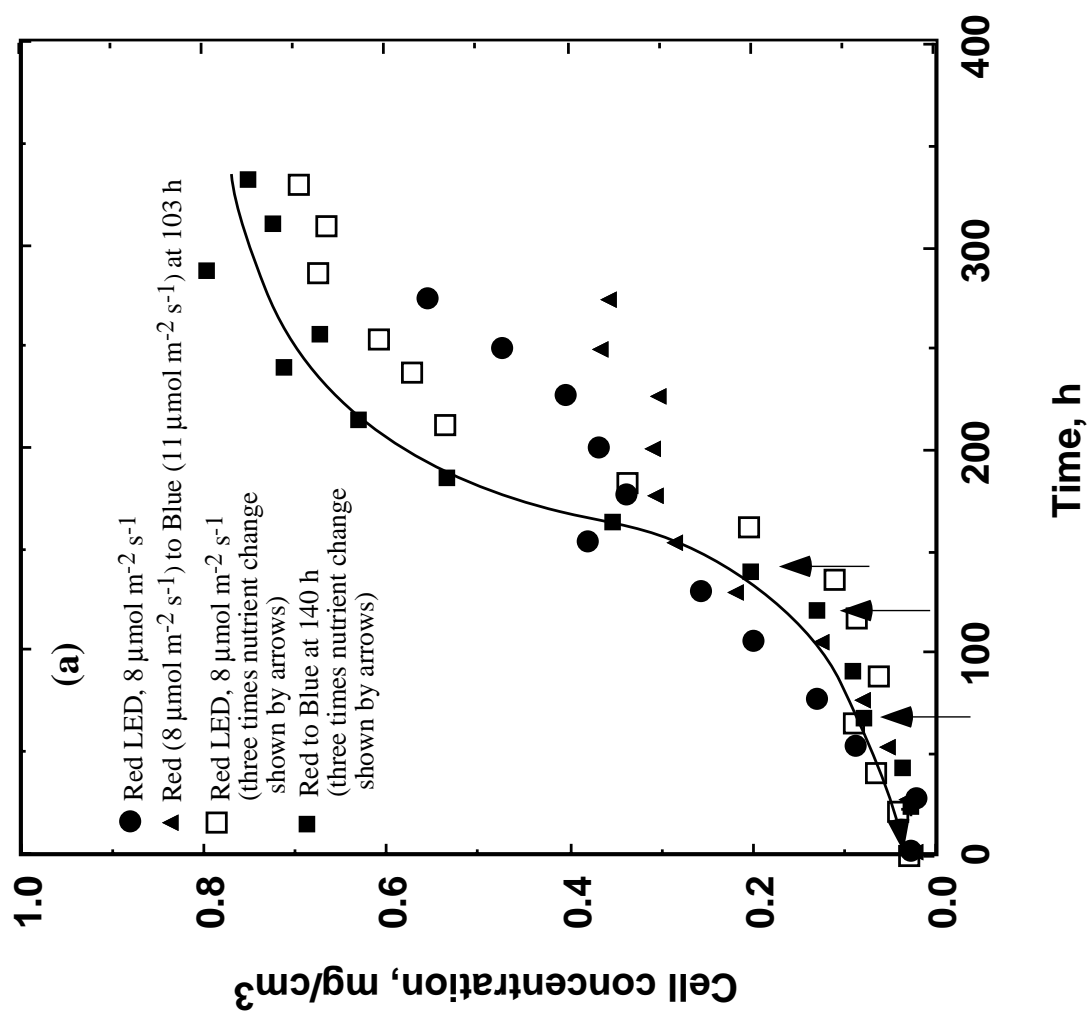




T. Katsuda et al. Figure 3



T. Katsuda et al. Figure 4



T. Katsuda et al. Figure 5