Astaxanthin Production by *H. pluvialis* in Sequential Batch Followed by Fed-Batch Culture Illuminated by LED Lamps

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ABSTRACT

In this work, a sequential batch followed by fed-batch and medium replacement were attempted to increase the cell concentration in *H. pluvialis* cultures. Also, illumination with LEDs was performed for induction of astaxanthin. The cell concentration reached to about 0.5 mg-dry cell/cm^3 in the batch run; 0.8 mg-dry cell/cm^3 and 1.5 mg-dry cell/cm^3 in the three and seven times replacement runs; and 0.9 mg/cm^3 in the sequential batch followed by fed batch run, respectively. The final concentrations of astaxanthin were higher in the medium replacement (about 65 μg/ cm^3) and sequential batch followed by fed-batch (about 60 μg/ cm^3) runs than the batch run (about 30 μg/ cm^3). Illumination by red switched to both sides blue LEDs induced the astaxanthin accumulation up to 75 μg/ cm^3, the highest value reported for astaxanthin production. The results show that higher astaxnthin concentrations were attained by illumination with blue LEDs and the medium replacement or sequential batch followed by fed-batch runs.

INTRODUCTION

Astaxanthin, a red ketocarotenoid, is widely used in food, cosmetic, pharmaceutical and medical applications due to its high antioxidant activity (Leorenz *et al.*, 2000). The green photosynthetic microalgae *Haematococcus pluvialis* is considered a good source for production of natural astaxanthin. Since different culture conditions are required for the production of green vegetative cells of *H. pluvialis* and the accumulation of astaxanthin in the red cysts, a two-stage production process has been proposed (Fábregas *et al*., 1998). In the first step, cells grow vegetatively and then stop growing under some stress conditions and start to accumulate astaxanthin. Deficiency of the nutrients and accumulation of the metabolite wastes in the culture medium cause depression on the cell growth, and therefore reaching to high cell concentration is difficult in batch culture. Two alternatives to overcome these limiting parameters on cell growth are medium replacement and fed-batch cultivation. Another problem in the astaxanthin production process is low astaxanthin accumulation in the *H. pluvialis* cultures. It is affected mainly by illumination strategies in photobioreactors and nutrient conditions. Previous study showed that blue LEDs are effective for accelerating encystment and concomitant synthesis of astaxanthin in *Haematococcus*

(Katsuda *et al*., 2004). Switching of light illumination from red to blue had significant effects on the accumulation of astaxanthin in the *Haematococcus* cultures. LED lamps, which have special characters, such as providing narrow wavelengths and low heat emission, are alternatives as light sources in photobioreactors for effective induction of astaxanthin. In this work, medium replacement and sequential batch followed by fed batch cultivation; and illumination with red LEDs switched to one side or both sides blue LEDs were attempted to increase the cell concentration and astaxanthin production in *H. pluvialis* cultures.

MATERIALS AND METHODS

Microorganism and cultivation conditions

H. pluvialis NIES 144 from Microbial Culture Collection of the National Institute for Environmental Studies (Tsukuba, Japan) were grown aerobically in a 75-cm^3 rectangular vessel with 6.5 cm high, 5.0 cm wide and 2.6 cm deep under illumination with LEDs (Toyoda Gosei Co. Ltd.) or fluorescent lamps (National FL 20-SSEX-D/18). The twice concentrated of Kobayashi's basal medium (pH 6.8), containing 0.24 w/v% sodium acetate, 0.4 w/v % yeast extract, 0.08 w/v % L-asparagine, 0.04 w/v % $MgCl_2 \cdot 6H_2O$, 0.002 w/v% $FeSO_4 \cdot 7H_2O$ and 0.004 w/v% $CaCl_2 \cdot 2H_2O$ was used for cultivation (Lababpour, *et al*., in press). After precultivation of the *H. pluvialis* in a 200-cm^3 Erlenmeyer flask set in a glass-sided water bath under illumination at 3.8 μ mol-photon m^{-2} s^{-1} with a fluorescent lamp, *H. pluvialis* was inoculated at 0.03 mg-dry cell/cm^3 in the vessel with the working volume of 50 cm^3. The temperature was kept at 20°C. In some cultivation runs, the 10 times concentrated culture medium was added or cells were replaced several times into fresh medium.

The flask was illuminated with a panel of red or blue LED lamps from one or two sides with the photon flux of 8.0 or 12.0 μ mol-photon m^{-2} s^{-1} at the surface of the vessel. On the LED panels (5 cm x 5 cm), 85 LED lamps were arranged in 10 rows and 9 columns. The LEDs have narrow emission spectra, while the fluorescent lamp emitted a wider range of wavelengths with the main peak at about 550 nm.

Measurement of cell concentration

Since *H. pluvialis* shows a morphological changes from small green cells (vegetative cells) to the larger red cells (cyst cells), which accumulate astaxanthin, the correlation between the absorbance of cell suspensions and dry-cell weight changes with time. Depending on the change in the spectra of cell suspensions during cultivation, we correlated the dry-cell weight to the difference of the absorbance at 680 nm and 750 nm normalized by the absorbance at 680 nm, as shown in the following equation (Katsuda, *et al*., 2004).

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

The spectra of cell suspensions were measured with a spectrophotometer (UV 1600,

Shimadzu), and the dry-cell weight was determined after drying at 80°C for 50 h. The values of the dry cell weight estimated by the above equation showed good agreement with measured values throughout cultivation with the R^2 value of 0.968. The size, number and color of the cells were also measured with a microscope (NIKON ECLIPSE 80i).

Measurement of astaxanthin concentration in cells

Methanol was used for extraction of astaxanthin from the cells. Samples of 0.5 cm^3 of cell suspensions were removed from the culture vessel, centrifuged at 7,500 g for 10 min and cell precipitate was resuspended in 1.0 cm^3 of methanol. To break cells and extract astaxanthin, 0.40 g of silica particles (particle size = 0.2 – 1 mm, Kanto Chemical) was added to the mixture and vigorously mixed with a vibrator for 10 min and then centrifuged for 10 min at 3000 g.

The supernatant (1.0 cm^3) was mixed with 0.1 cm^3 of a methanol solution of NaOH (5 mM NaOH) and kept overnight under nitrogen in darkness at 20°C for saponification of astaxanthin esters (Yuan, *et al.*, 1998). The astaxanthin concentration was measured by an HPLC system (LC-10, Shimadzu) equipped with a reverse phase column (Cosmosil 5C18-MS-II, 4.6 x 150 mm, nacalai tesque). The mobile phase was methanol with a flow rate of 1 cm^3/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 concentration of astaxanthin was determined by use of a calibration curve obtained with authentic free astaxanthin.

RESULTS AND DISCUSSION

Effects of medium replacement

H. pluvialis was inoculated at the concentration of 0.03 mg-dry cell/cm^3 in the 75-cm^3 vessels containing 50-cm^3 medium and cultivated at 20°C by illumination with red LEDs. After the thired day, nutrients were supplied by replacement of separated cells into fresh medium daily. The medium was replaced three times or seven times, and the results were compared with batch run.

The cell concentration and astaxanthin production of *H. pluvialis* in the batch run and three and seven times medium replacement runs are shown in **Figures 1 and 2**. In the batch run, the cell concentration increased to about 0.5 mg-dry cell/cm^3. In the three and seven times replacement runs, the final cell concentration was higher than that in batch run and reached to 0.8 and 1.5 mg-dry cell/cm^3. The cell number continued to increase in the cultures in which medium was replaced. They remain green with two flagellate, and the average cell size was relatively smaller than the batch culture. On the other hand, in the

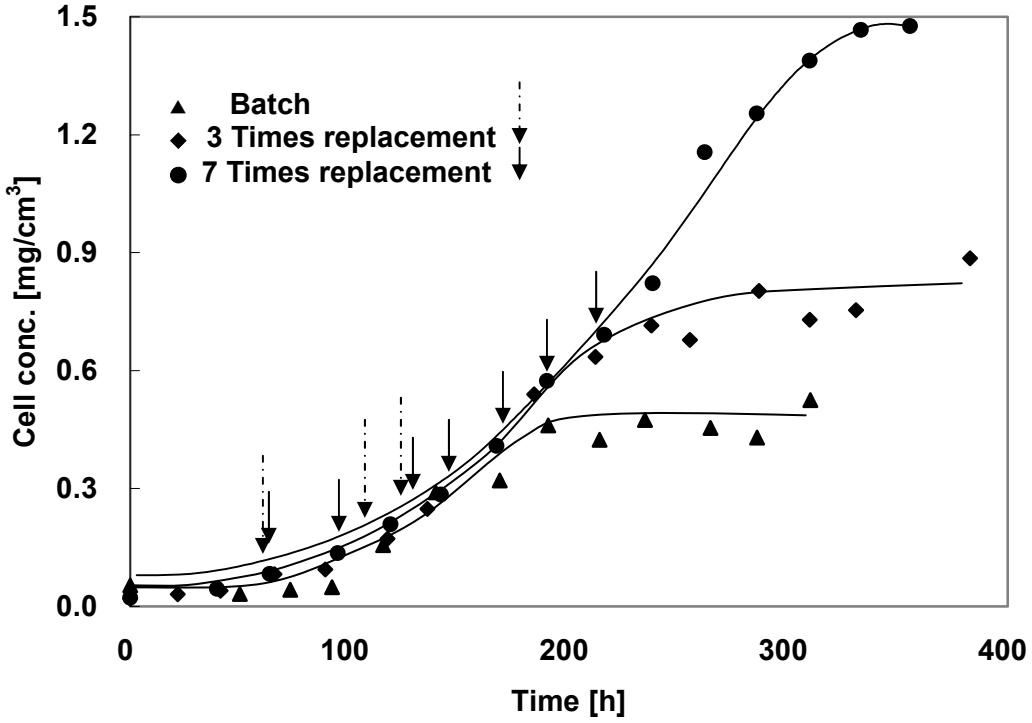

Figure 1 Cell concentrations in the batch and medium replacement runs.

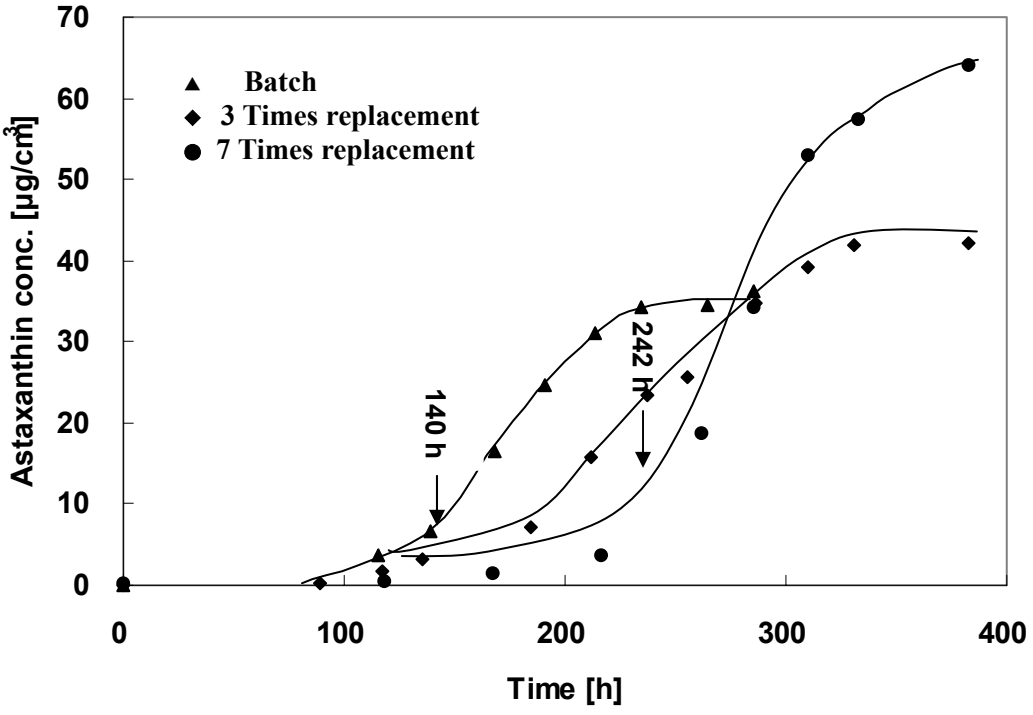

Figure 2 Astaxanthin concentrations in the batch and medium replacement runs (Illumination with blue LED for batch; switch illumination from red to blue at 140 h for three times replacement; and at 242 h for seven times replacement).

batch culture, the percentage of the green cells decreased after 100 h (data not shown).

The highest astaxanthin concentration was obtained in the seven times replaced culture, but the highest astaxanthin content was obtained in batch run. The highest astaxanthin concentration obtained by medium replacement was 65.0 μ g/cm^3.

Effects of fed-batch

H. pluvialis was inoculated at the concentration of 0.03 mg-dry cell/cm^3 in the 75-cm^3 vessel containing 50-cm^3 medium and cultivated at 20°C by illumination with red LED lamps. The nutrients were supplied by adding 5 cm^3 of ten times concentrated of Kobayashi's medium two times at 4 and 5th days.

The cell concentration and astaxanthin production of *H. pluvialis* in the batch and batch followed by fed batch runs are shown in **Figures 3 and 4**. In the batch run, the cell concentration increased to about 0.60 mg-dry cell/cm^3. In the batch followed by fed-batch runs, the cell concentration was higher than those in batch run and reached to 0.9 mg-dry cell/cm^3.

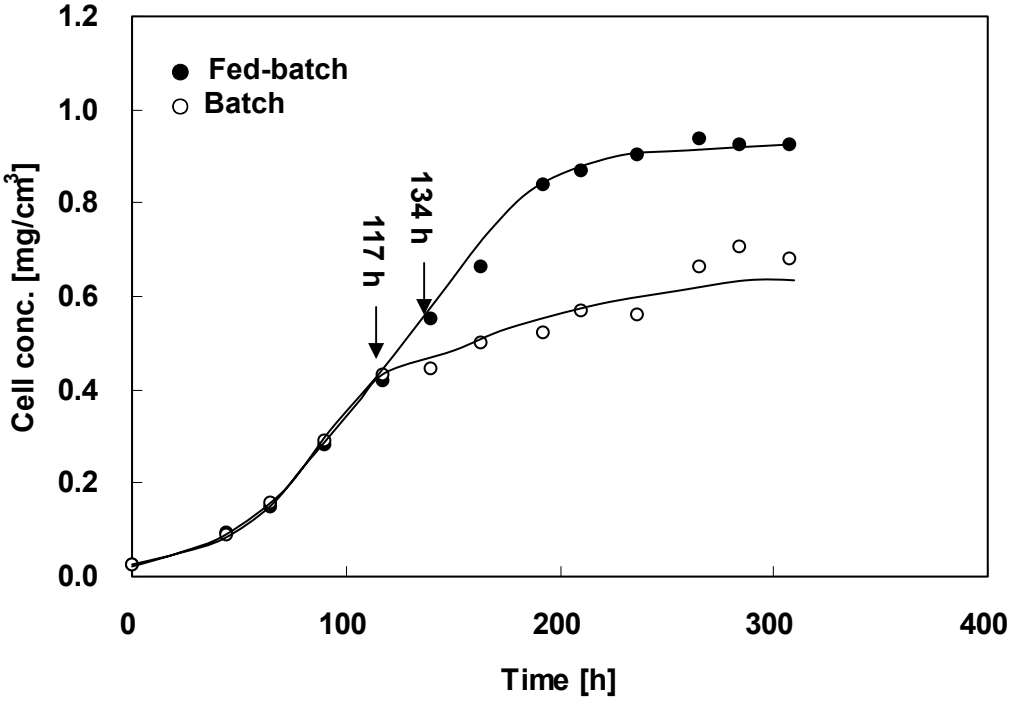

Figure 3 Cell concentrations in batch and fed-batch runs.

The astaxanthin concentration increased to about 27 μ g/cm^3 in batch run and 60 μ g/cm^3 in the batch followed by fed-batch run.

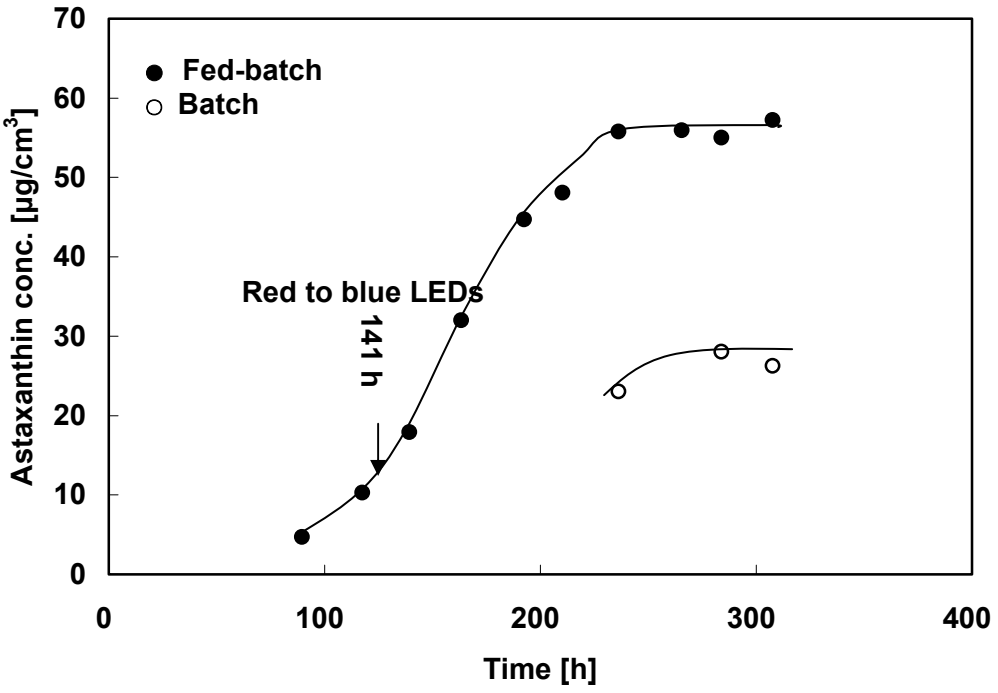

Figure 4 Astaxanthin concentrations in batch and fed-batch runs

Induction of astaxanthin accumulation by blue LED lamps

H. pluvialis, inoculated at 0.03 mg-dry cell/cm^3, was cultured in the glass vessel containing 50- cm^3 medium under illumination with red LEDs at 8.0 μ mol photons m^{-2} s^{-1} for cell growth and switched to one or two sides illumination by blue LEDs at 12 μ mol photons m^{-2} s^{-1} for induction of astaxanthin when the medium replacement was stopped and cells were still in the logarithmic phase of growth. This did not cause reduction in the cell number of the cultures and stimulated a rapid increase in astaxanthin accumulation and the cell size.

The cell concentration and astaxanthin production of *H. pluvialis* with one or two sides illumination with blue LEDs are shown in **Figures 5 and 6**.

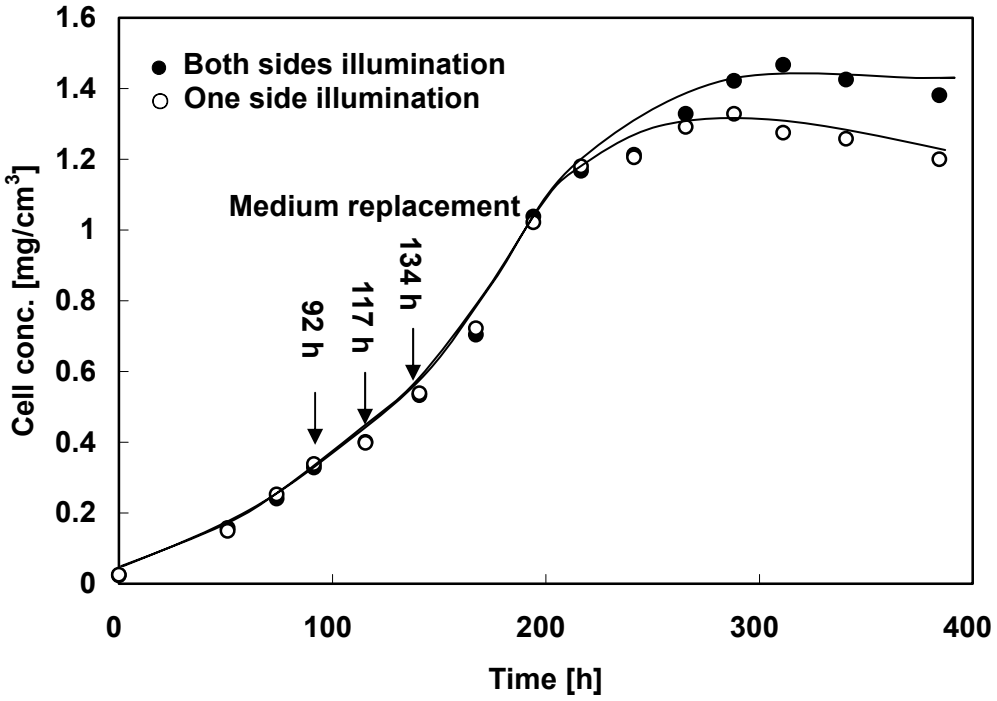

Figure 5 Cell concentrations in medium replacement runs illuminated by red switched to one side or both sides blue LEDs.

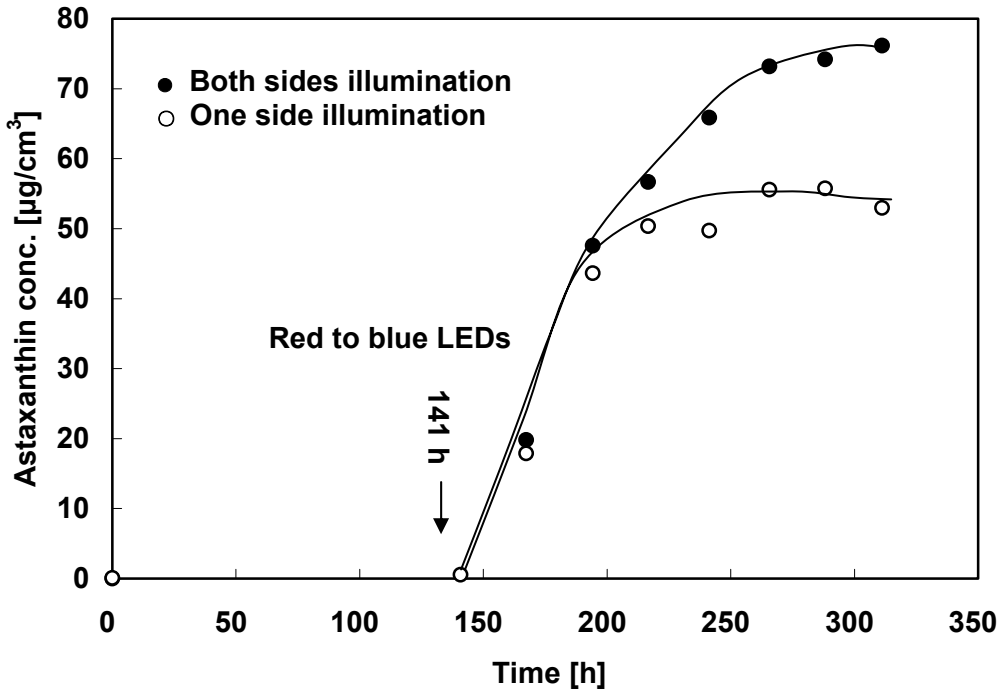

Figure 6 Astaxanthin concentrations with one or two sides illumination

The astaxanthin concentration reached up to 75 μ mol-photons m^{-2} $s^{-1}$by both side illuminations with blue LEDs. The astaxanthin concentration was lower in the case of one side illumination and reached up to 55 μ mol photons m^{-2} s^{-1}.

CONCLUSIONS

This investigation showed that sequential batch followed by fed batch and medium replacement are good strategies for increasing the cell concentration and that change of red LED lamps for cell growth to both sides illumination with blue LED lamps was effective in stimulating astaxanthin accumulation in *Haematococcus* cultures without death of the cells.

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