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Multistage Operation of Airlift Photobioreactor for Increased Production of Astaxanthin from *Haematococcus pluvialis*

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An internally radiating photobioreactor was applied for the production of astaxanthin using the unicellular green alga *Haematococcus pluvialis*. The cellular morphology of *H. pluvialis* was significantly affected by the intensity of irradiance of the photobioreactor. Small green cells were widespread under lower light intensity, whereas big reddish cells were predominant under high light intensity. For these reasons, growth reflected by cell number or dry weight varied markedly with light conditions. Even under internal illumination of the photobioreactor, light penetration was significantly decreased as algal cells grew. Therefore, we employed a multistage process by gradually increasing the internal illuminations for astaxanthin production. Our results revealed that a multistage process might be essential to the successful operation of a photobioreactor for astaxnthin production using *H. pluvialis*.

Keywords: Astaxanthin, *Haematococcus pluvialis*, photobioreactor, multistage process

For more than a half century, numerous attempts have been made to exploit microalgae as food, feed, lipids, vitamins, pigments, fertilizers, and other chemical sources. Many microalgae have been investigated and recommended for these purposes. For example, *Chlorella* and *Spirulina* species have been grown for health food, *Dunaliella salina* for β-carotene, and *Haematococcus pluvialis* for astaxanthin [1]. Of these algal species, the fresh-water unicellular microalga *H. pluvialis* has received considerable attention for the production of a highly valued ketocarotenoid, astaxanthin [20]. Although some plant, bacteria, and yeast are able to biosynthesize astaxanthin, *H. pluvialis* showed the best capacity to accumulate astaxanthin [1].

Astaxanthin [(3*S*-3'*S*)-dihydroxy-β,β-carotene-4,4'-dione] has been of great commercial interest owing to its high price (approximately US$ 2,500 kg^{-1}) [10, 20]. Astaxanthin is mainly used as pigmentation sources in salmon, shrimp, lobster, and crayfish. Because many living species could not synthesize astaxanthin *de novo*, it should be continuously supplied in the feed of aquaculture [11, 21]. Furthermore, several reports showed the potential application of astaxanthin in medicines, because of the powerful antioxidant feature [19, 23].

The life cycle of *H. pluvialis* consists of completely different phases; a green motile phase and a red nonmotile resting phase. Under favorable environmental conditions, *H. pluvialis* displays green motile cells with two flagella. However, upon exposed to unfavorable environmental conditions, the life mode of *H. pluvialis* is rapidly shifted into the resting phase, called a cyst, with the cessation of cell division and the formation of a thick resistant cell wall [2, 4, 18]. Accumulation of astaxanthin in *H. pluvialis* is strictly limited to the cyst stage, thereby coping with oxidative stress generated from a wide variety of unfavorable environments [17]. Because the life phase of *H. pluvialis* for astaxanthin production is completely different from the actively multiplying motile phase, a two-stage process dividing the growth and production stages has been widely accepted for *H. pluvialis* cultivation [20].

Most commercial productions using photosynthetic microalgae a employ open pond systems, owing to lower

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capital and operation costs. However, the photobioreactor has been of particular interest for the production of valuable microalgal biochemicals, because of the increased productivity [19]. In this context, the application of a photobioreactor for astaxanthin production might have an advantage over the open pond system. However, there has been little report on the application of photobioreactors for astaxanthin production using *H. pluvialis*.

In this study, we operated photobioreactors equipped with internal illuminations for astaxanthin production in *H. pluvialis*. Growth and astaxanthin production were tested under various extents of internal illuminations. Based on these data, we suggest the multistage process, instead of the previously recommended two-stage process, for further facilitating astaxanthin production using *H. pluvialis*. Here, we report on the operational data of photobioreactors and propose the putative multistage process for enhancing astaxanthin production in *H. pluvialis*.

Materials and Methods

Algal Strain and Inoculation

The unicellular green alga *H. pluvialis* LB 16 was obtained from the culture collection of algae at the University of Texas, Austin, TX, USA. *H. pluvialis* was grown in an optimal *Haematococcus* medium (OHM) [8], composed of KNO_3, 0.41 g; Na_2HPO_4, 0.03; $MgSO_4 \cdot 7H_2O$, 0.246 g; $CaCl_2 \cdot 2H_2O$, 0.11 g; Fe(III)-citrate H_2O, 2.62 mg; $CoCl_2 \cdot 6H_2O$, 0.011 mg; $CuSO_4 \cdot 5H_2O$, 0.012 mg; Cr_2O_3, 0.075 mg; $MnCl_2 \cdot 4H_2O$, 0.98 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.12 mg; SeO_2, 0.005 mg; biotin, 25 g, thiamine, 17.5 g; and B12, 15 g, per one liter of deionized distilled water.

For seed culture, 250 ml flasks with 100 ml of medium were used for the cultivation on a shaker rotated at 180 rpm and 22°C. Air was bubbled into the flask culture along with shaking in order to supply sufficient aeration. Continuous illumination was supplied at an average light intensity of 50 E m^{-2} s^{-1} with 20 Watt warm white fluorescent tubes (Korea General Electric Co., Korea). The inoculation was conducted by extracting 200 ml from the seed culture in the middle of the linear growth phase (OD_{550} = 0.2, Cell number = 20× 10^4 cells/ml, DW = 0.2 g/l) and inoculating directly into 5 l of OHM medium for the photobioreactor operation.

Design and Operation of Photobioreactor

We constructed the photobioreactor with internal illuminations to take advantage of the efficient distribution of light (Fig. 1). The diameter and height of the photobioreactor were 15 cm and 45 cm, respectively. The H/D (height to dimension) ratio and working volume of the photobioreactor were 3.33 and 5.2 l, respectively. A draft tube with 10 cm diameter was implemented for efficient circulation. The air-sparger was located at the bottom of the reactor, and 5% (v/v) carbon dioxide was applied to the photobioreactors at a flow rate of 2 l/min after humidification. The temperature was adjusted *via* a double-jacket cylindrical tube connected with a water bath controlled at 22°C. A maximum of seven fluorescent tubes could be integrated into the photobioreactor, and each fluorescent tube used for internal illumination was shielded with a glass tube (Fig. 1).

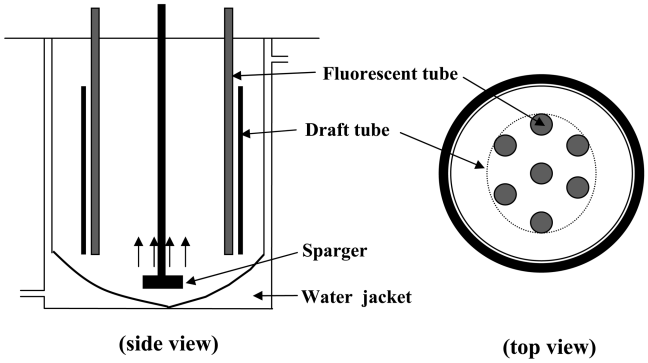

Fig. 1. Schematic presentation of an internally illuminated photobioreactor.

Analytical Methods

For astaxanthin analysis, cells of an appropriate stage were harvested by centrifugation (3,000 ×*g*, 3 min) and then treated with a solution of 5% KOH in 30% methanol to destroy chlorophyll. Centrifugation followed, and the remaining pellet was extracted with DMSO after adding 0.07 ml of acetic acid to recover the astaxanthin in a 75°C water bath for 5 min. This last step was repeated if necessary until the cell debris was totally white. The absorbance of the extracts was determined at 492 nm, and the amount of astaxanthin was calculated according to Davies [6]. The dry weight was measured by filtering an aliquot of the algal suspension through a pre-dried and pre-weighed 0.45 μm cellulose nitrate membrane filter (Whatman, USA) and drying in an oven at 80°C for 10 h. The number of cells was determined by cell counting with an improved Neubauer hemocytometer. The light intensity was measured using a quantum sensor (LI-190SA; Licor, USA) connected to a readout instrument (L-1000; Licor, USA). Absorption spectra were scanned using a spectrometer (Genesys5; Spectronic, USA).

Results

Photobioreactor Operation Using Low or High Internal Irradiances

A photobioreactor built with internal illuminations was operated with a supply of 5% carbon dioxide and illuminated with different extents of light intensity by manipulating the numbers of fluorescent tubes. Operations with one, two, or seven fluorescent tubes of photobioreactors were performed in batch cultures (Fig. 2, 3, and 4). The result of lower light irradiance using one fluorescent tube could reach the highest cell numbers (approximately 55 × 10^4 cells/ml). However, cell numbers were significantly decreased along with the increase of light intensities; maximum 25 × 10^4 cells/ml from two fluorescent tubes and 13 × 10^4 cells/ml using seven fluorescent tubes were obtained from the photobioreactor operation (Fig. 2, 3, and 4). Based on these data, we concluded the cell numbers of *H. pluvialis* were reversely correlated with light intensities, possibly due to light inhibition on the cell divisions of *H. pluvialis*.

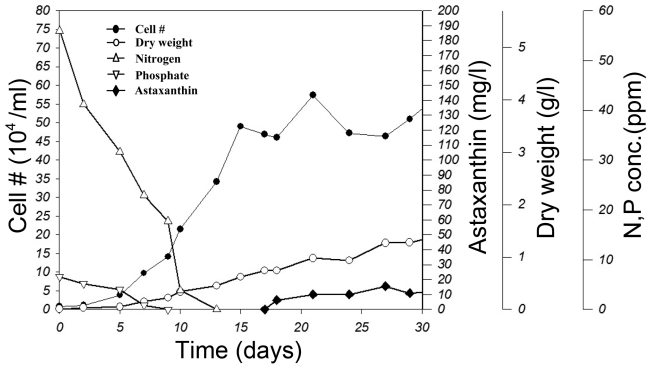

Fig. 2. Changes in cell numbers, dry weight, astaxanthin production, and nutrient (N or P) concentrations during the batch culture of a photobioreactor using one fluorescent tube.

In contrast, we obtained seemingly contradictory results in the growth based on dry weight, as it showed different patterns with cell numbers. The highest dry weight (2.5 g/l) was achieved in the operation using two fluorescent tubes, and it was decreased in both low and high light exposures (1.2 g/l from one internal illumination and 1.5 g/l from seven internal illuminations) (Fig. 2, 3, and 4). It is certain that the growth inhibition by high irradiance using seven fluorescent tubes accounts for lower biomass productivity (1.5 g/l). Thus, we compared more precisely by taking cell numbers into consideration for biomass productions. We employed a different unit of measurement (g/cell), and then it was evident that the dry weight per cell of *H. pluvialis* is proportional to light intensity (approximately 0.022×10^{-7} g/cell from one fluorescent tube; 0.1×10^{-7} g/cell from two fluorescent tubes; 0.1154×10^{-7} g/cell was obtained from the photobioreactor operation using seven fluorescent tubes). A possible explanation for this observation is that *H. pluvialis* cells were gradually transformed into big and heavy cyst cells to tolerate stress conditions caused by the exposure of high irradiance.

To help in understanding the deviations between cell number and dry weight according to light intensities, we

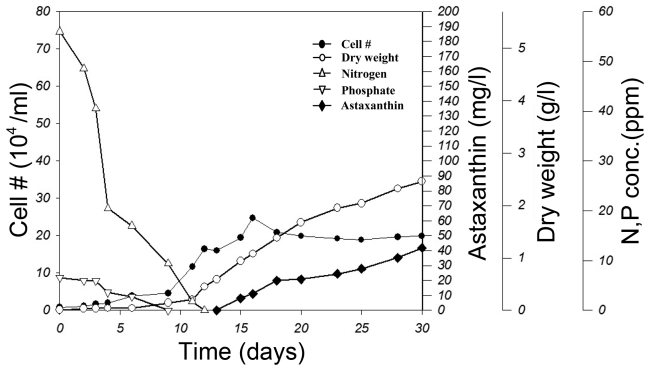

Fig. 3. Changes in cell numbers, dry weight, astaxanthin production and nutrient (N or P) concentrations during the batch culture of a photobioreactor using two fluorescent tubes.

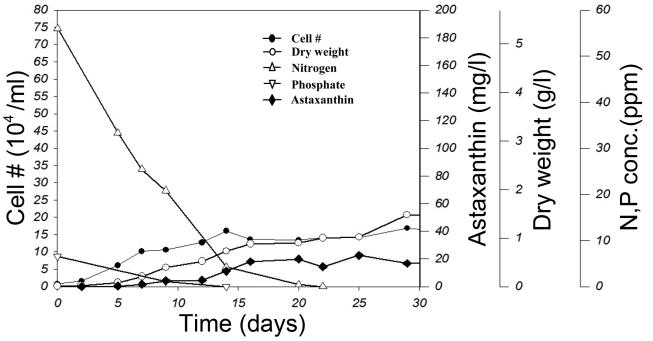

Fig. 4. Changes in cell numbers, dry weight, astaxanthin production, and nutrient (N or P) concentrations during the batch culture of a photobioreactor using seven fluorescent tubes.

also illustrated different progressions of *H. pluvialis* morphologies under one or two fluorescent tubes (Fig. 5A). The different life-cycle progressions with distinct morphologies under one or two fluorescent tubes were verified by plotting correlation graphs between optical densities (OD) and dry weights (Fig. 5B). The correlation curve was significantly altered by increasing light intensity, as much higher dry weights could be obtained at a certain OD value under the two fluorescent tubes, compared with those under one fluorescent tube (Fig. 5B). The difference in the correlations

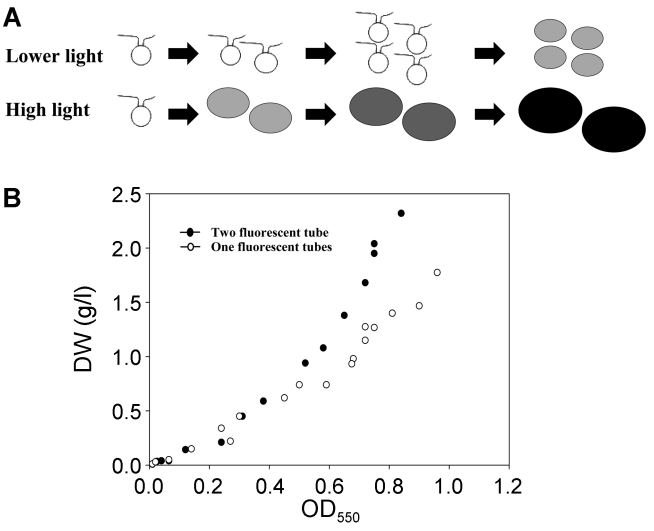

Fig. 5. Progressions of *H. pluvialis* morphologies under one or two fluorescent tubes.

A. Schematic drawing of the different extent of the *H. pluvialis* morphological progressions illustrating the contradiction between cell number and dry weight according to light intensities. Higher light intensity facilitates transformations of small green cells with two flagella into red bigger cysts. White and dark tones indicate green and red colors of *H. pluvialis*, respectively. **B**. Correlation graphs between optical density and dry weight under the different light intensities. The correlation curve was shifted by high light intensity, indicating that distinct progression in cell morphologies of *H. pluvialis* indeed occurred with different light conditions. Optical densities and dry weights were obtained during the operations of the photobioreactors described in Fig. 2 and 3.

under one or two fluorescent tubes clearly reflects the fact that there were significant changes in morphological features by increasing light conditions.

Nitrogen and phosphate consumption curves indicated that their concentrations were declined sharply, as with increase of biomass (Fig. 2, 3, and 4). However, the depletion times of nitrogen and phosphate were significantly delayed under the high irradiance, because of the growth inhibition (Fig. 4). Interestingly, when we compared dry weight and cell numbers at the time points of nitrogen or phosphate depletion, they varied significantly with the conditions of irradiances in the photobioreactor. For example, with the same concentration of nitrogen (58 ppm), 0.5 g/l and 35×10^4 cell/ml were obtained under one fluorescent tube, whereas 0.8 g/l and 13×10^4 cell/ml were achieved under seven fluorescent tubes. Based on these data, we concluded the biomass productivities consuming certain amounts of nitrogen and phosphate (yield coefficients) were significantly affected by light irradiances, most likely due to carbon-based enlargement, called encystment, of *H. pluvialis*. These data again provide evidence that the life phases of *H. pluvialis* cells are remarkably altered depending on the extent of light illuminations.

Likewise, we also noticed similar results in astaxanthin production. The highest production of astaxanthin (35 mg/l) was observed under the middle intensity of lights (two fluorescent tubes), and it was significantly reduced by both lower and higher light exposures (approximately 10 mg/l from one internal illumination, and 20 mg/l from seven internal illuminations) (Fig. 2, 3, and 4). However, when we converted the volumetric productivity of astaxanthin (mg/l) into a cellular unit basis (mg/cell), it is clear that astaxanthin biosynthesis is positively related with light intensity; approximately 0.182×10^{-7} mg/cell of astanxnthin from one fluorescent tube, 1.4×10^{-7} mg/cell from two fluorescent tubes, and 1.54×10^{-7} mg/cell from seven fluorescent tubes could be obtained. These data are consistent with previous reports demonstrating the significant role of high irradiance for enhancing astaxanthin production in *H. pluvialis* [5, 12, 13].

Under the microscope, we could also confirm distinct differences in the cellular status of *H. pluvialis* depending on light conditions. A high density of cells was present exclusively as small motile green stage under lower light exposure, whereas a low concentration of big cyst cells with deep red hue were predominant under high light intensity (data not shown). Taken together, our data indicated that light is the most important factor influencing the cellular status of *H. pluvialis*.

Development of Multistage Photobioreactor by Increasing Internal Irradiances Gradually

We employed an internal illumination system on the photobioreactor, as it is one of the most efficient methods for delivering light energy into algal culture [22, 25]. However, as algal cell density is increased, significant decrease of light passing through the algal suspension has been reported [24, 26]. In this regard, we tested the penetration of light into *H. pluvialis* suspension cultured in the photobioreactor using one fluorescent tube. We noticed the significant attenuation of light penetration, as with the increase of algal density (Fig. 6). After 13 and 24 days, more than 75% and 95% decreases of light penetration were observed, respectively (Fig. 6). Apparently, our result indicated insufficient light distribution for the growth and astaxanthin production of *H. pluvialis,* as algal density increased. Moreover, the light penetration was noticeably reduced as the life stage of *H. pluvialis* was changed into the immature reddish encystment. Approximate cell densities that could block half of the illumination passing into algal suspension (4.6 cm light path length) were 12×10^4 cell/ml in the green algal suspension, and 5×10^4 cell/ml in the early reddish *H. pluvialis* suspension (data not shown). Owing to the requirement of intensive light for astaxanthin production in *H. pluvialis* [5, 12, 13], light attenuations should be considered for the process of the *H. pluvialis* astaxanthin production.

Considering both the light attenuation phenomena and two-stage process for *H. pluvialis* cultivation, it is likely that the photobioreactor with multistage of gradually increasing illuminations could provide the alternative solution for further enhancing the efficiency of astaxanthin production. Therefore, we operated the photobioreactor with gradually increased numbers of fluorescent tubes, along with the growth of biomass. Particularly, we increased the fluorescent

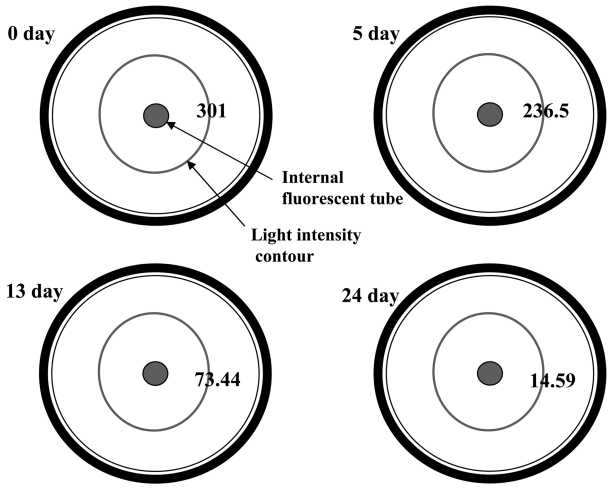

Fig. 6. Attenuation of light penetration into the photobioreactor with one fluorescent tube.
The light intensity contour line shows the significant reduction of light intensity along with the growth of *H. pluvialis*. Numbers on the light contours indicate light intensity ($\mu mol/m^2s$) after passing through *H. pluvialis* culture (4.6 cm depth of photobioreactor) at the appropriate time points.

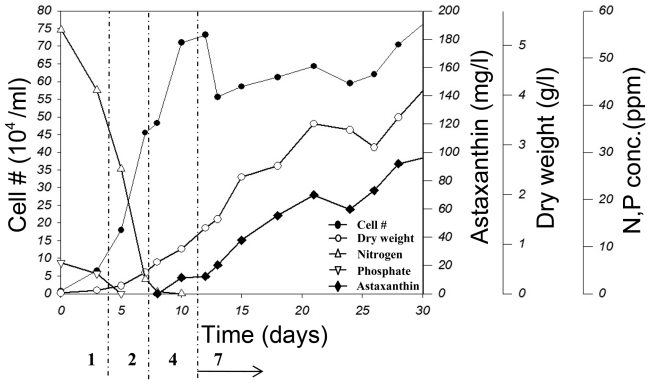

Fig. 7. Changes in cell numbers, dry weight, astaxanthin production, and nutrient (N or P) concentrations during the multistage batch culture of a photobioreactor by gradually increasing the fluorescent tubes.
The number of fluorescent tubes applied to the photobioreactor is shown below the figure.

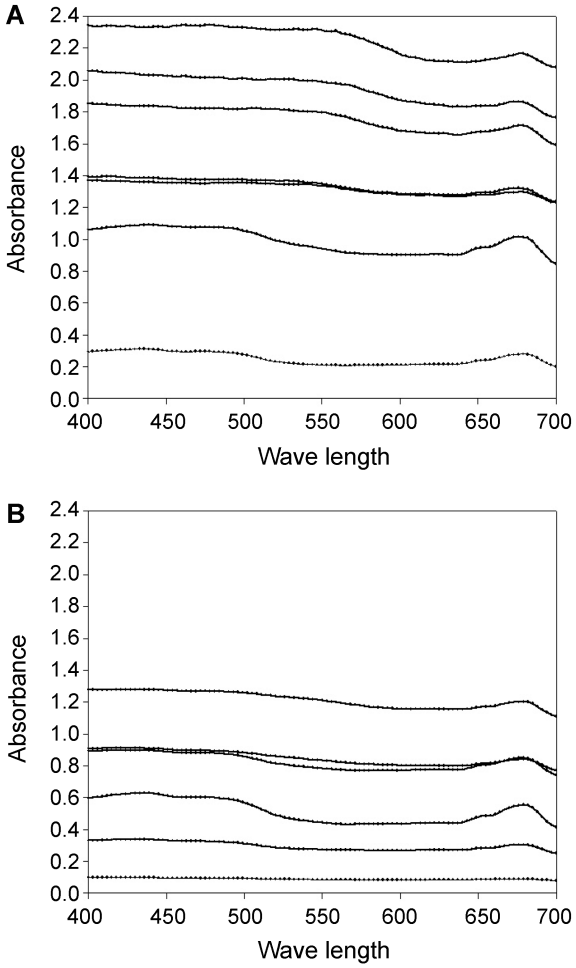

Fig. 8. Absorption spectra of *H. pluvialis* suspensions with stepwise increased fluorescent tubes (**A**) and one fluorescent tube (**B**).
Absorbance was scanned and measured after 5, 10, 15, 21, 28, and 45 days later. Multistage operation led to significant increase of absorbance, compared to those under one fluorescent tube.

tubes whenever the biomass based on the dry weight was doubled, up to seven fluorescent tubes (Fig. 7). One, two, four, and then seven fluorescent tubes were applied at appropriate time points, comprising each of different stages for the multistage photobioreactor.

As expected, the multistage photobioreactor showed the best performance in terms of biomass and astaxanthin production (Fig. 7). Around 2- to 3-folds increase in biomass growth and 3- to 10-folds in astaxanthin production were obtained (Fig. 7). For example, we could achieve significantly increased biomass (75×10^4 cell/ml and 4.2 g/l) and astaxanthin production (100 mg/l). In addition, we tested with absorption spectra, which indicate the degree of algal growth as well as cellular status of *H. pluvialis* by generating unique peaks of chlorophyll or astaxanthin. We observed clear chlorophyll (430 nm and 670 nm) and astaxanthin peaks (500 nm), which appeared at the earlier time points in the multistage cultivation (Fig. 8). Compared with those of one fluorescent tube, absorbances of multistage were also approximately doubled (Fig. 8). Consequently, our data evidently showed that the maximum production of biomass and astaxanthin of *H. pluvialis* could be achieved through the multistage process by increasing the light intensity stepwisely.

DISCUSSION

Applications of internally illuminated photobioreactors for production of astaxanthin using *H. pluvialis* were tested. We obtained significantly different results according to light conditions in the photobioreactor, as the life cycle of *H. pluvialis* was influenced markedly by the light intensity [16, 17]. Small green cells were predominant under the lower light intensity, whereas reddish large cells were widespread under the high light intensity. Moreover, cell division of *H. pluvialis* was significantly suppressed by the high irradiance, as its mode of life was rapidly shifted into the encystment in response to high irradiance. Therefore, cell numbers were increased with one fluorescent tube, whereas high dry weights could be obtained with two fluorescent tubes (Fig. 2 and 3). In this regard, our data suggested that it should be necessary to consider the life cycle of *H. pluvialis* carefully for any future applications.

Although a high intensity of illumination showed the best performance for cellular astaxanthin production, there was significant reduction of the volumetric productivity due to the light inhibition to the biomass production (Fig. 4). Considering the two-stage process for *H. pluvialis*, operational data using seven fluorescent tubes must be an example of the second-stage photobioreactor. Our results showed that the production stage alone, without the

prerequisite of growth stage, could not reach the satisfactory levels of astaxanthin production. Consequently, our data supported the notion that it should be necessary to divide the optimal growth and inductive astaxanthin production stages for the *H. pluvialis* cultivation [9, 20].

It has been widely accepted that nutrient depletions trigger astaxanthin biosynthesis in *H. pluvialis* [3, 7, 14]. Consistent with these reports, astaxanthin biosynthesis could occur only after the starvation of nitrogen and phosphate in the culture media (Fig. 2, 3, and 7). Interestingly, unlike the other operational results, astaxanthin accumulations could be initiated under seven fluorescent tubes, even when nitrogen or phosphate was not completely depleted (Fig. 4). Light is the most significant factor affecting astaxanthin biosynthesis in *H. pluvialis* [5, 12, 13]. Thus, it is conceivable that light intensity using seven fluorescent tubes was enough to trigger astaxanthin biosynthesis, even though there are still available nutrients for *H. pluvialis* (Fig. 4).

An internally illuminated photobioreactor has been invented for efficient light distribution into the photobioreactor, because light has been regarded as a limiting factor for growth of microalga [22, 25]. However, even the employment of internal illuminations is not sufficient for the delivery of light energy, because light penetration is significantly decreased along with the growth of algal cells (Fig. 6). Moreover, light is crucial for inducing secondary carotenoid astaxanthin in *H. pluvialis* [5, 12, 13], but light penetration was further decreased as *H. pluvialis* cells were transformed into the immature cyst stage (data not shown). Thus, light attenuation effects must be taken into account for achieving the optimal production of astaxanthin using *H. pluvialis*.

In order to address light attenuation effects, a gradual increase of light intensity was conceived and its excellence for the photobioreactor operation using *H. pluvialis* was confirmed (Fig. 7). So far, a two-stage photobioreactor for *H. pluvialis* was widely accepted as an appropriate approach [9, 20]. However, in this study, we proposed that multistage processes might be the alternative or more suitable approach for astaxanthin production using *H. pluvialis*, in consideration of the light attenuation. Gradually increased internal illuminations correspond to the modification of the two-stage process with a multistage photobioreactor. Photobioreactors with one and two fluorescent tubes could be classified into the growth stage, whereas four and seven fluorescent tubes might fit into the category of the production stage (Fig. 7). The multistage approach for the production of astaxanthin must be valid, as it was proven by the superior amount of astaxanthin productivity (Fig. 7). While more study will be necessary to further advance the efficiency of the photobioreactor, the multistage process will be fundamental to the future development of the astaxanthin production process using *H. pluvialis.*

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