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Improved Production of Astaxanthin from *Haematococcus pluvialis* Using a Hybrid Open–Closed Cultivation System

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Abstract: *Haematococcus species* are rich sources of the antioxidant astaxanthin and have good potential for carbon dioxide reduction. A variety of culture systems for these microalgae are currently in development, but clearly profitable approaches have yet to be reported. Open outdoor culture is currently the only feasible culture system for producing large amounts of biomass. In this study, based on laboratory results, the cultivation of *Haematococcus* was divided into two stages: a green stage characterised by cell growth, and a red stage characterised by astaxanthin accumulation. For mass culture, we adopted a hybrid open–closed pond system for astaxanthin production. The open culture system was shown to produce approximately 50 kg (dry weight) of biomass per culture at an average rate of 0.51 g L^{-1}, with 0.52 µg mL^{-1} of astaxanthin content in a 12 -m^3 water tank. As large amounts of microalgal bioproducts are in high demand, inexpensive open outdoor culture methods should be adopted as an alternative to costly closed photobioreactors. Although the levels of biomass and astaxanthin production were found to be 30% lower in the field than in the laboratory in this study, the basic data obtained in this research may be useful for lowering astaxanthin production costs.

Keywords: *Haematococcus*; biomass; open outdoor culture; astaxanthin; photobioreactor

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1. Introduction

Microalgae are highly efficient photosynthetic unicellular organisms that efficiently generate chemical energy from solar energy [1]. The mass cultivation of microalgae is highly valued for the bioproduction of carotenoids (e.g., β-carotene, astaxanthin, lutein, phycoerythrin, and phycocyanin), polysaccharides, enzymes, pigments, hydrocarbons, antibiotics, sterols (e.g., sitosterol, stigmasterol, 24-ethylcholesterol, campesterol, and 24-propylidene-cholesterol), proteins (e.g., microcolin A, cyanovirin-N, and superoxide dismutase), polyunsaturated fatty acids (e.g., γ-linolenic acid, docosahexaenoic acid, sitosterol, eicosapentaenoic acid, and stigmasterol), vitamins (e.g., A, C, and E), and biomass and sugars for biofuel production. Microalgal biomass is also used as a component of food supplements, animal fodder, and fertiliser [2–4]. Microalgal cultivation for various purposes has been reported in a number of countries, including the USA, China, Japan, Taiwan, India, Australia, Denmark, Canada, the Netherlands, France, Chile, and Israel [4–9]. The commercial mass cultivation of microalgae began with the culture of *Chlorella* in the early 1960s in Japan. In the early 1970s, *Arthrospira* cultivation and harvesting systems were developed in Mexico. By 1980, 46 companies were involved in the production of microalgae (particularly *Chlorella*) in Asia, where more than 1000 kg of microalgae was manufactured per month. In 1986, *Dunaliella salina* became the third major microalga to be industrially cultivated, with

the introduction of mass production systems in Australia. Later, in the 1990s, Japan became the foremost Asian country to authorise the consumption of *Haematococcus-derived* astaxanthin as a common food or food supplement. Following this, the use of astaxanthin as a food additive started in Europe in 1995 and in North America in the late 1990s. The market for astaxanthin as a food supplement grew and became available in Malaysia, Indonesia, and Thailand during 2004–2006, in Singapore in 2012, and in the Philippines in 2018 [10]. Several plants were established in the USA, India, and Australia to produce astaxanthin from *Haematococcus pluvialis* [11]. Commercially, astaxanthin is produced from *H. pluvialis* by the following companies: Cyanotech Corporation (USA), AstaReal Inc. (USA), Fuji Chemical Industry (Japan), Beijing Ginko Group (China), Algatechnologies Ltd. (China), Parry's Pharmaceuticals (India), etc. [12]. The mass cultivation of microalgae has also focused on biofuel production [13].

The xanthophyll astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) is a 40-carbon tetraterpene with linked isoprene units; it is composed of two terminal β rings and a linear polyene chain. The characteristic pink and red colours of astaxanthin are determined by its 11 conjugated double-bond systems. Astaxanthin was initially derived in 1938 from lobsters [14]. Chemically synthesised astaxanthin is abundantly available commercially with low production costs but is utilised mainly in the poultry and aquaculture industries as a pigmentation source and has not been authorised for human consumption. These products have 20 times lower antioxidant activity than natural astaxanthin. Therefore, there is high demand for the natural form of astaxanthin, which has not been met due to the arduous procedures required for its manufacture [15–17]. The astaxanthin market was valued at USD 1.0 billion globally in 2019 and is projected to increase at a compound annual growth rate of 16.2% to USD 3.4 billion by 2027 [18]. Natural astaxanthin has very powerful antioxidative properties, which have applications in the nutraceutical, cosmetic, and pharmaceutical industries [17,19]. The antioxidant capacity of natural astaxanthin is 500 and 38 times greater than that of vitamin E and β-carotene, respectively [20]. These agents are important bioactive components in antiaging serums, sunblock creams, and beauty products, and are also provided as oral dietary supplements at a recommended dose of 4 mg per day. They also have anti-gastric, anti-cancer, anti-inflammatory, anti-diabetic, anti-obesity, ocular-protective, and immune response-boosting properties, and many studies are currently underway to explore their immense potential for biological applications [17,18,21–23].

Natural sources of astaxanthin include marine bacteria, fungi, higher plants (*Adonis* species), krill, and microalgae, such as *Chlorococcum* spp., *Chlorella zofingiensis*, *H. pluvialis*, *Dunaliella salina*, etc. Among these, the green alga *H. pluvialis* is regarded as the most abundant source of natural astaxanthin on the market [17,19,21,22,24,25]. *Haematococcus pluvialis consists of* green ovoid–spherical biflagellate cells containing chloroplasts; they are motile and swim freely in the initial green vegetative stage. In the subsequent intermediate stage, the cells turn orange–green within 7–10 days, and astaxanthin begins to accumulate with the development of a thick red cell wall containing the encystment system. Following these stages, the red stage appears within 11–14 days under unfavourable growth conditions, in which the cell size increases, flagella are lost, and orange–red astaxanthin droplets accumulate rapidly in the perinuclear cytoplasm [15,22]. Under such unfavourable conditions, *H. pluvialis* accumulates up to 4% dry weight (DW) of astaxanthin for resistance against oxidative stress and photoinhibition [9]. Astaxanthin accumulation in a shorter time has also been attempted using high-light irradiation and specific inhibitors of cytochrome P450 enzymes. Although these results have not been conclusive, further research may lead to further clarification [26].

Generally, large-scale cultivation of microalgae is achieved using one of two types of systems: open systems, in which microalgae are cultured in an open environment, and closed systems, in which they are grown in photobioreactors or closed vessels [27]. Open-pond cultivation allows the extensive production of microalgal biomass in a natural environment. These ponds are categorised as raceway ponds, unmixed ponds, shallow

lagoons, circular ponds, and inclined ponds based on their architectural features and modes of operation. Among these types, raceway ponds are the most popular due to their simplicity, low cost, ease of operation and maintenance, low power demands, and utilisation of solar energy [28–30]. Various systems are employed for stirring and blending the culture and avoiding sedimentation in open raceway ponds; these include paddle-wheels, propellers, impellers, air lifts, Archimedes screws, and water jets. Some ponds also feature a clear plastic cover that reduces their vulnerability to contamination from airborne dust particles. Covering the ponds with plastic allows them to retain heat in colder climates, whereas cooling systems allow temperature adjustment in hotter climates [30]. However, open-pond systems require physiochemical supervision and are susceptible to the risk of biological contamination, mainly from birds, as well as drastic climatic changes, heavy metal and microplastic pollution, and biomass yield and water losses due to evaporation. Absolute isolation from the external environment, continuous water supply to compensate for water loss, high cell density, contamination avoidance, and harvest system improvements can minimise the drawbacks and maximise the operational effectiveness of open-pond systems for natural astaxanthin production at large scales [1,6,29,31]. Photobioreactors or closed microalgae cultivation systems also have diverse designs (e.g., tubular, fermenter-like, or laminar reactors). The benefits of closed systems include less contamination, reduced carbon dioxide loss, a regenerative cultivation environment, better regulation of the cultivation parameters, and improved yield. However, closed systems have several major drawbacks, including difficulties in construction, maintenance, scaling up, operation, overheating, and biofouling, as well as high costs [32–34]. According to [35], a combination of an outdoor photobioreactor and a raceway pond can be used to produce astaxanthin from *H. pluvialis* with more commercial benefits than chemically synthesised astaxanthin.

A number of factors must also be taken into consideration for the feasible mass production of microalgae such as *H. pluvialis* to achieve high astaxanthin yield, including the accessibility of nutrients (e.g., phosphorous, potassium, nitrogen, magnesium, and zinc); oxygen; light intensity; temperature; pH; inorganic carbon, carbon dioxide, and salinity levels; bioreactor dimensions; mixing; dilution rates; harvesting frequency; nutrition modes; genetic modifications; and supplementation of chemicals or growth factors [5]. Light is the prime factor regulating microalgal growth, as it controls the photosynthesis, growth, reproduction, and metabolism of microalgae. Importantly, excessive cell density can prevent the penetration of light and thus diminish the rate of photosynthesis. The use of nitrogen-deficient nutrient medium also plays a key role in astaxanthin accumulation, as nitrogen is a necessary nutrient for enzymatic activity and growth in *H. pluvialis* [34,36]. Apart from projects related to *Chlorella*-derived food and dietary supplements, the development of microalgal mass cultivation systems is in its initial stages in South Korea, principally targeting applications in the biofuel industry [37].

In this study, we established an efficient, innovative, economically viable hybrid open–closed mass cultivation system for *H. pluvialis* to fulfil semi-industrial demands for natural astaxanthin in South Korea, while reducing contamination. We investigated various parameters for achieving optimum astaxanthin production. We analysed the effects of varying light intensities on cell density, biomass, chlorophyll content, and astaxanthin accumulation. We discuss the application of the proposed hybrid open–closed *H. pluvialis* mass cultivation system as a cost-effective, profitable method for the large-scale bioproduction of natural astaxanthin. Many laboratory-based studies have been conducted on *Haematococcus* due to its unique ecological characteristics; however, there have been very few field trials. Therefore, we cultured *Haematococcus* at the laboratory scale and applied the same methods to the field.

2. Materials and Methods

2.1. Microalgal Strain and Culture

For the experimental culture of *Haematococcus*, we used different media. For the green stage, which is characterised by cell growth under favourable conditions, the cells were

grown with the use of Jaworski's medium (JM) [38]; for the red stage, which is characterised by astaxanthin accumulation under stressed conditions, the cells were grown with the removal of nitrogen source ingredients from JM. In this study, *Haematococcus pluvialis* (LIMS-PS-1354) was obtained from the Library of Marine Samples at the Korea Institute of Ocean Science & Technology, Geoje, Korea. This culture was maintained in Jaworski's medium (JM) at 21 °C ± 1 °C under a 12 h:12 h light:dark cycle, with a photon flux density of 37 μmol m^{-2} s^{-1}.

2.2. Design and Construction of a Hybrid System for H. pluvialis Mass Cultivation

The developed hybrid culture system was connected to two culture tanks. The first was a square tank for the growth of the green stage of *H. pluvialis*, which consisted of a greenhouse covering, a temperature control system, and a filtered air supply. The second tank was a system designed in our laboratory, consisting of a greenhouse composed of sunlight-controlled material and an air agitation system for the astaxanthin accumulation stage. A schematic diagram of this culture system is shown in Figure 1.

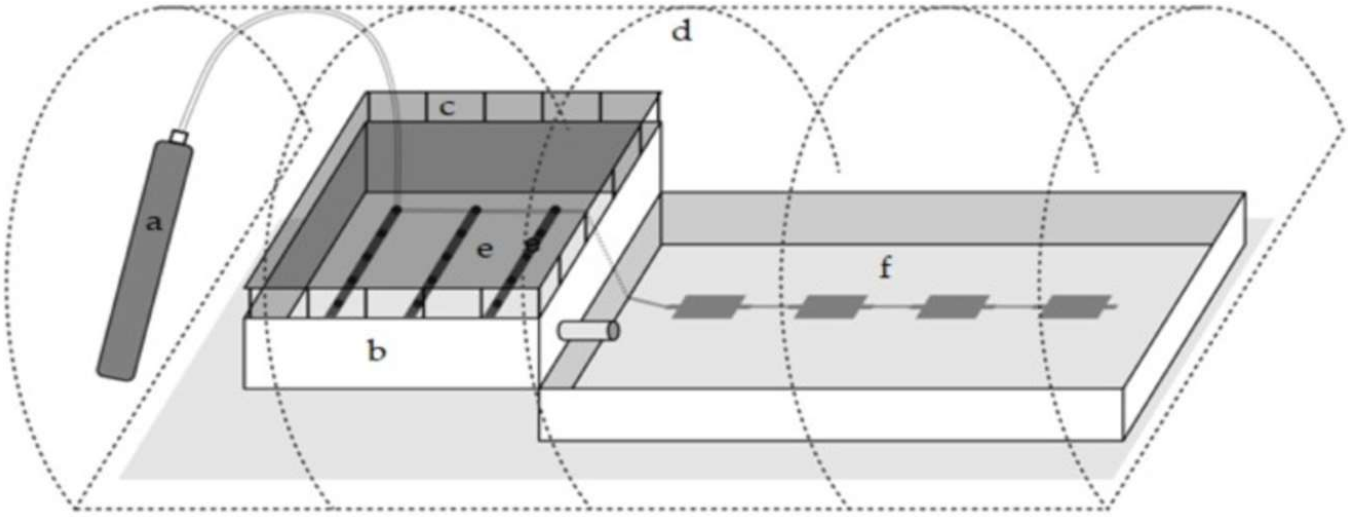

Figure 1. Schematic diagram of the hybrid microalgal culture system. (a) Air pump; (b) green vegetative culture tank; (c) temperature controller; (d) greenhouse covering allowing sunlight-penetration control; (e) filtered air diffuser; (f) culture tank for astaxanthin accumulation.

In detail, the hybrid mass cultivation system consisted of a square aquarium with a double shading screen, constructed using a greenhouse covering to control light penetration. The total culture volume of the tank was 750 m^3 (10 m × 15 m × 5 m). An automatic temperature control system was used to maintain green vegetative phase cultures at a temperature of 18 °C ± 2 °C. A screen that controls sunlight was used to maximise growth by maintaining a photon flux density of 25 ± 5 μmol m^{-2} s^{-1}. Natural air was supplied after passing through a 20 μm mesh. The concentration of carbon dioxide in the atmosphere was maintained at 400 ± 50 ppm. To accumulate astaxanthin, water tanks with a total area of 3000 m^3 (10 m × 60 m × 5 m), with greenhouse covering to adjust the incoming sunlight to a photon flux density of 160 ± 50 μmol m^{-2} s^{-1}, were maintained in a nitrogen-depleted state by dilution through adding water. The actual culture volume was 120 m^3 (10 m × 60 m × 0.2 m).

2.3. Conditions for H. pluvialis Growth (Nitrogen-Depleted Conditions)

The preparation of JM was performed according to our previous research [39]. For the nitrogen-depletion condition experiments, nitrogen sources $Ca(NO_3)_2 \cdot 4H_2O$ and $NaNO_3$ were removed from the JM medium under laboratory conditions. *Haematococcus pluvialis* cells were concentrated using a centrifuge and cultured. In the field, the nitrate concentra-

tion of the microalgal culture was measured using a spectrophotometer; when nitrate levels decreased by 40% compared to the initial medium at a wavelength of 235 nm, the culture was transferred to a large tank for astaxanthin accumulation. The system was diluted with water to a ratio of 1:10 for culture. The fluorescence light intensity was varied at 15, 30, 60, 90, 160, and 220 μmol m^{-2} s^{-1} and the effects on growth and astaxanthin accumulation were examined. The temperature was maintained at 18 °C ± 2 °C.

2.4. Measurement of Light Intensity

In laboratory experiments, light intensity was controlled according to the distance between fluorescent lights and the culture pond and measured using a light meter (Fluke-941; Fluke Corp., Everett, WA, USA). In the field, sunlight intensity was adjusted using multilayered polyolefin vinyl.

2.5. Estimation of Cell Density

Cell density was estimated using a haemocytometer with an improved Neubauer chamber (Marienfeld Superior, Lauda-Königshofen, Germany) and a light microscope with a 20× objective lens (BX53; Olympus, Tokyo, Japan). Absorbance values of supernatants were read using an ultraviolet–visible light (UV-vis) spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan). To determine the dry weight (DW) biomass of samples, 3 mL culture samples were centrifuged at 2000× *g* for 5 min. The cell pellets were dried at 105 °C in a preweighed aluminium dish until constant weight was maintained for 24 h, cooled to room temperature in a desiccator, and weighed.

2.6. Extraction and Estimation of Chlorophyll (Chl) and Total Carotenoid Contents

To estimate carotenoid contents, *H. pluvialis* cells (10 mL) were centrifuged for 15 min at 4500× *g* in 15 mL conical tubes. The supernatant was removed, and carotenoids were extracted with the addition of 10 mL of 99.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Darmstadt, Germany), boiling for 10 min at 55 °C, and vortexing for 30 s to disrupt the cells using the vortex GENIE 2 (Scientific Industries, Inc., Bohemia, NY, USA). Centrifugation was repeated once again, and the absorbance of the pooled supernatant was read at 480, 665, and 649 nm. The final pellet was white in colour. Finally, the carotenoid contents were determined as follows [40]:

$$\text{Chl } a \text{ } (C_a) = 12.19\ A_{665} - 3.45\ A_{649}$$

$$\text{Chl } b \text{ } (C_b) = 21.99\ A_{649} - 5.32\ A_{665}$$

$$\text{Carotenoid } (\mu g\ mL^{-1}) = (1000\ A_{480} - 2.14\ C_a - 70.16\ C_b)/220.$$

To estimate the amounts of accumulated astaxanthin, 1 mL samples were centrifuged for 10 min at 1700× *g*. The pellets were treated with 5% (*w*/*v*) KOH solution and further diluted with 30% (*v*/*v*) methanol. The chlorophyll was then denatured for 10 min at 70 °C, followed by centrifugation for 10 min at 3500× *g*. The supernatants were acidified by adding 100 μL of glacial acetic acid. DMSO (5 mL) was then added to the solution, which was heated for 15 min at 70 °C. The samples were once again centrifuged, and the supernatants were pooled; the astaxanthin contents of the supernatants were determined by measuring the absorbance at 490 nm as follows [41]:

$$\text{Astaxanthin } (mg\ L^{-1}) = [4.5 \times A_{490} \times (V_a/V_b)],$$

where V_a and V_b are the volumes of DMSO and microalgal samples, respectively. The pigments were quantified spectrophotometrically as described previously.

2.7. Cell Growth Calculation

The specific growth rate was calculated according to [42], using the formula below:

$$\mu/d = (\text{In } X_2 - \text{In } X_1)/t_2 - t_1;$$

where X_2 = Biomass concentration at t_2 and X_1 = Biomass concentration at t_1.

2.8. Statistical Analysis

Cell density, biomass, and pigment data are presented as means ± standard deviation. Differences between pairs of samples were evaluated using the paired Student's *t*-test and those among three or more groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using SPSS v22 software (IBM Corp., Armonk, NY, USA). For all analyses, significance was evaluated at a level of $p < 0.001$.

3. Results

3.1. Haematococcus pluvialis Growth Curve

In the laboratory, *H. pluvialis* growth was evaluated in a limited environment using filtered natural air for 15 days under a light intensity of 25 µmol m^{-2} s^{-1}. The cell density of *H. pluvialis* increased steadily throughout the experimental period from the initial inoculum of $3.14 \pm 0.52 \times 10^4$ cells mL^{-1} to $19.18 \pm 0.55 \times 10^4$ cells mL^{-1} at the end of the experiment (day 15), representing a growth rate of 0.12 day^{-1} during 15 days of culture. Cells grew rapidly from day 3 to day 6, with a growth rate of 0.36 day^{-1}, and grew slowly thereafter (Figure 2). There were significant differences in the growth rates among culture days at *p*-values < 0.001. The F-value was 314.464.

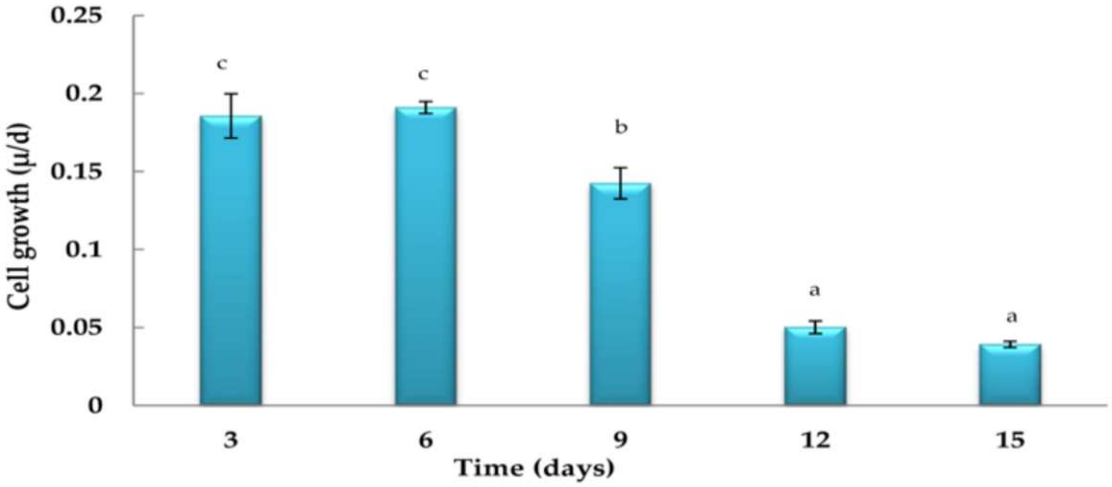

Figure 2. Growth curve of *Haematococcus pluvialis* under an artificial *photon flux density of* 25 µmol m^{-2} s^{-1} ($n = 5$). Data are represented as mean ± SD (error bars). Bars with different letters indicate significant differences in the growth rates among culture days at *p*-values < 0.001.

3.2. Effects of Varying Light Intensities on H. pluvialis Growth under Nitrogen-Depleted Conditions

Figure 3 shows a comparison of growth curves for *H. pluvialis* cultured in nitrogen-depleted medium under six different light intensity levels. Cell growth occurred for 3 days at all light intensity levels and then decreased rapidly. The maximum cell density was 8.98×10^4 cells mL^{-1} at a photon flux density of 60 µmol m^{-2} s^{-1}. On day 3, *H. pluvialis* exposed to light intensity levels of 60, 90, 160, and 220 µmol m^{-2} s^{-1} began to turn red, indicating the astaxanthin accumulation stage. Red dots began to appear on day 6 of culture at a photon flux density of 15 µmol m^{-2} s^{-1}. Most cells had turned red by the end of the experiment (day 15) at all light intensity levels. The cell density readings showed significant differences at *p*-values < 0.001.

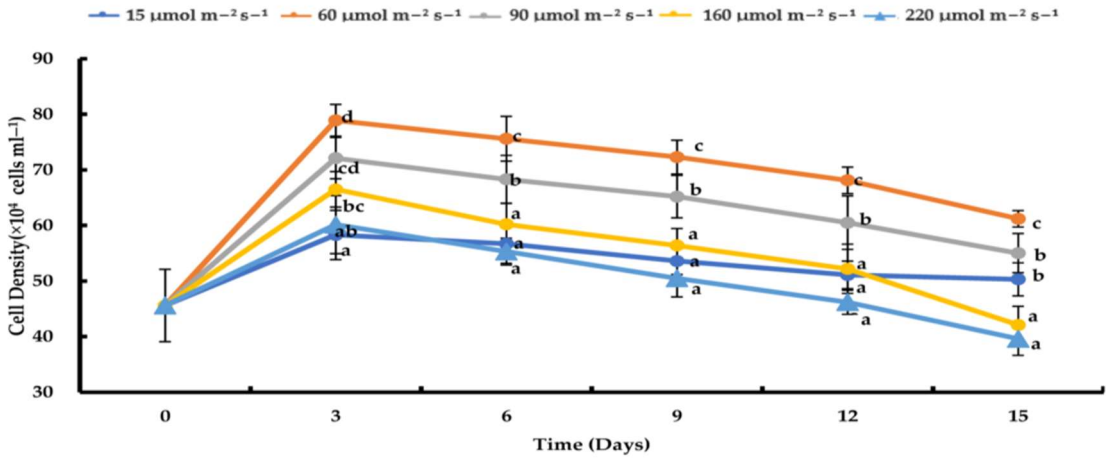

Figure 3. Comparison of *H. pluvialis* growth curves under nitrogen depletion and varying light intensity conditions (n = 5). Data are represented as mean ± SD (error bars). Bars with different letters indicate significant differences in the cell density among culture days at p-values < 0.001.

3.3. Effects of Varying Light Intensities on H. pluvialis Biomass under Nitrogen-Depleted Conditions

After culture under nitrogen-depleted conditions at various light intensity levels for 15 days, *H. pluvialis* was harvested and the DW was measured (Figure 4). Biomass was highest when *H. pluvialis* was grown at a light intensity of 160 µmol m^{-2} s^{-1} (0.841 ± 0.020 g L^{-1}) and lowest at 15 µmol µmol m^{-2} s^{-1} (0.332 ± 0.014 g L^{-1}). Cells developed a red colour with increased volume, indicating the astaxanthin accumulation stage, at all light intensity values except 15 µmol m^{-2} s^{-1}. The biomass readings showed significant differences at p-values < 0.001. The F-value was 190.590.

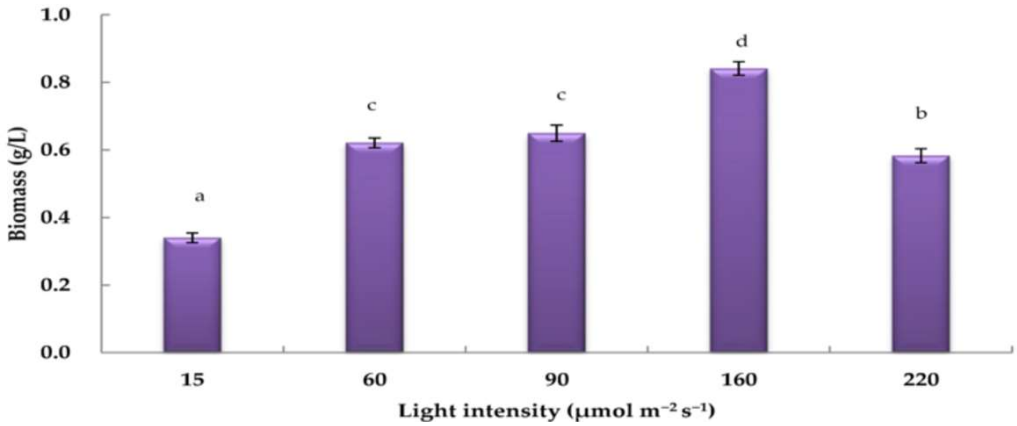

Figure 4. Comparison of *H. pluvialis* biomass after 15 days of culture under nitrogen-depleted conditions at different light intensity levels (n = 5). Data are represented as mean ± SD (error bars). Bars with different letters indicate significant differences at p < 0.001.

3.4. Effects of Varying Light Intensities on H. pluvialis Chl a, Chl b, and Carotenoid Contents under Nitrogen-depleted Conditions

After *H. pluvialis* culture under nitrogen-depleted conditions, Chl *a*, Chl *b*, and carotenoids were extracted and their contents compared according to light intensity. Chl *a* and Chl *b* and total carotenoid levels were highest in *H. pluvialis* grown at a light intensity of 160 µmol m^{-2} s^{-1}; however, there were no significant differences in Chl *a* and Chl *b* levels at other light intensity levels. Significant differences were detected among light intensity levels for Chl *a* (F = 20.101), Chl *b* (F = 17.806), astaxanthin (F = 521.241), and total carotenoid contents (F = 137.990) at p-values < 0.001 (Figure 5).

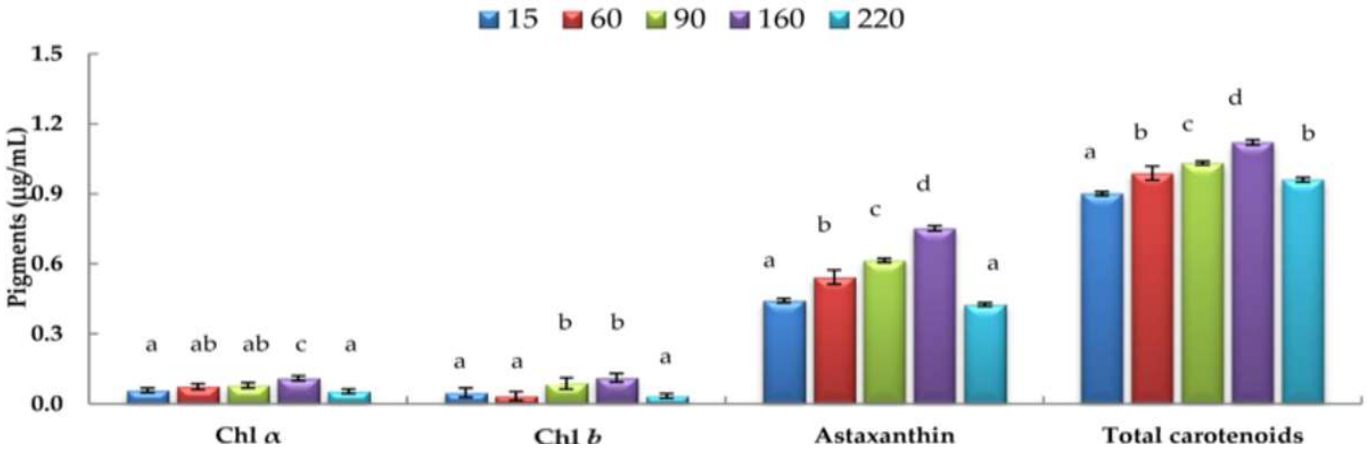

Figure 5. Chlorophyll (Chl) *a*, Chl *b*, astaxanthin, and total carotenoid levels under nitrogen-depleted conditions and varying light intensities. Data are represented as mean ± SD (error bars). Bars with different letters indicate significant differences at *p*-values < 0.001.

3.5. Effects of 25 μmol m^{-2} s^{-1} Light Intensity on Cell Density

In the outdoor culture system developed in this study, *H. pluvialis* was cultured in an autotrophic environment at a light intensity of 25 μmol m^{-2} s^{-1} for 15 days. *Haematococcus pluvialis* cell numbers increased with time from the initial inoculum of 3.56 ± 0.47 × 10^4 cells mL^{-1} to 12.01 ± 0.28 × 10^4 cells mL^{-1} by day 15. Cells grew rapidly from day 6 to day 9, reaching a growth rate of 0.15 day^{-1}, and then grew more slowly, reaching a growth rate of 0.08 day^{-1} on day 15, with significant differences among days at *p*-values < 0.001. (Figure 6). The F-value was 319.607.

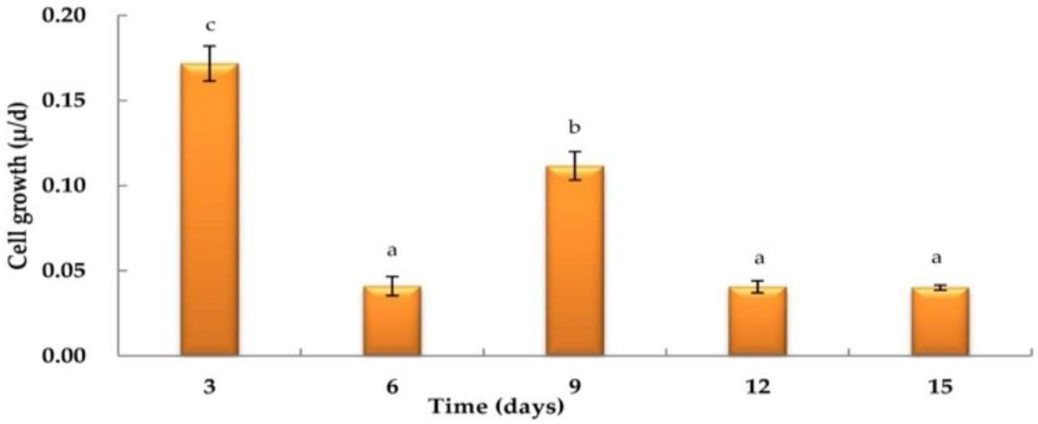

Figure 6. Growth curve of *H. pluvialis* in the proposed outdoor culture system, with sunlight adjusted to a light intensity of 25 μmol m^{-2} s^{-1}. Data are represented as mean ± SD (error bars), $n = 5$. Bars with different letters indicate significant differences at *p*-values < 0.001.

3.6. Effects of 160 μmol m^{-2} s^{-1} Light Intensity on Cell Density

To verify the effects of a light intensity of 160 μmol m^{-2} s^{-1}, which produced the highest astaxanthin content among light intensity levels in laboratory culture, cell numbers were measured after culturing *H. pluvialis* under nitrogen-depleted conditions in the outdoor culture system. Similar to the laboratory results, cells grew well until day 3, after which the growth rate decreased or cells did not grow. The maximum cell density (5.92 × 10^4 cells/mL) was reached on day 6 and decreased rapidly thereafter. There were significant differences in the *H. pluvialis* growth rate among culture days in the outdoor culture system at *p*-values < 0.001 (Figure 7). The F-value was 314.464. On day 3, most cells began to turn red.

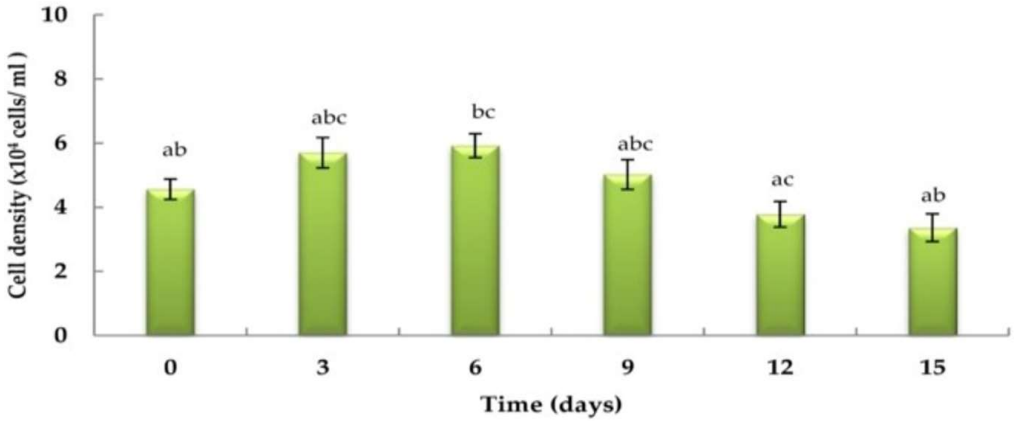

Figure 7. Cell density values for *H. pluvialis* in outdoor culture under nitrogen-depleted conditions exposed to sunlight at 160 µmol m^{-2} s^{-1}. Data are represented as mean ± SD (error bars), $n = 6$. Bars with different letters indicate significant differences at p-values < 0.001.

Figure 8 shows the results of mass cultivation of *H. pluvialis* in the hybrid open–closed pond system.

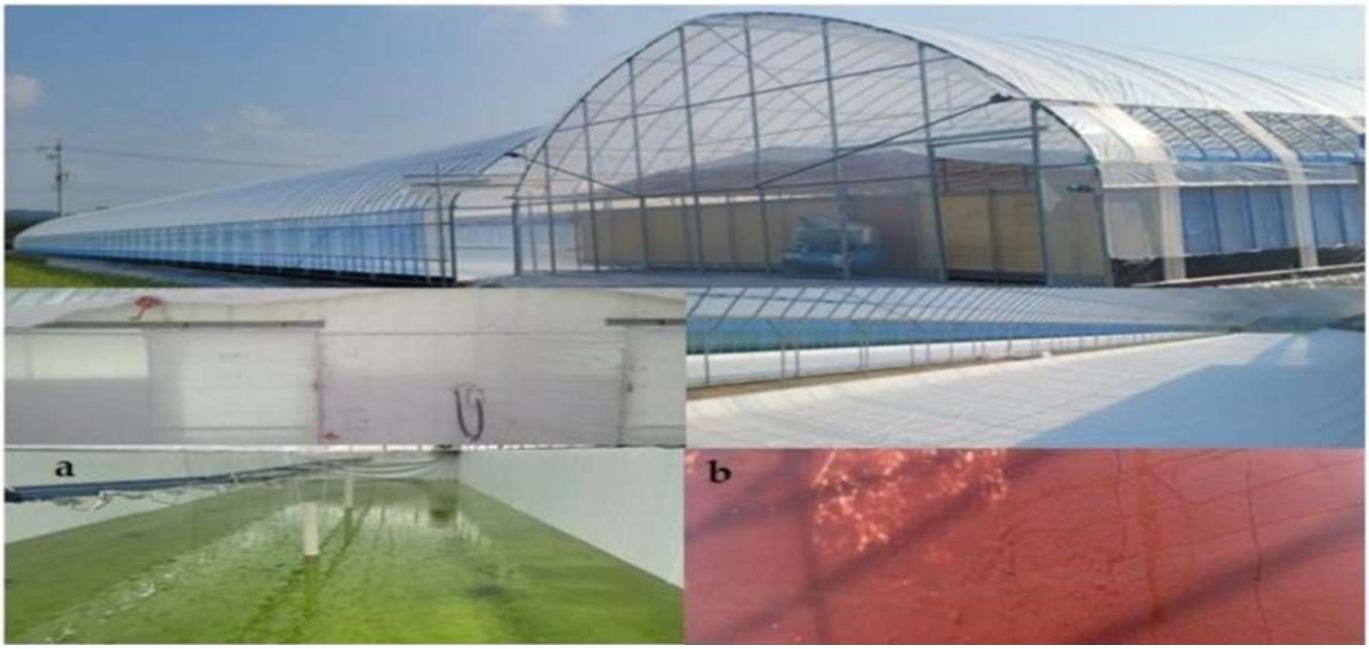

Figure 8. Mass culture of *H. pluvialis* under conditions optimised for the promotion of astaxanthin accumulation under nitrogen depletion conditions at a sunlight intensity of 160 µmol m^{-2} s^{-1} ((**a**) green stage cultivation in the hybrid culture system, (**b**) red stage cultivation in the hybrid culture system).

3.7. Biomass and Chl a, Chl b, Total Carotenoid, and Astaxanthin Contents Produced under Laboratory Culture and Hybrid Outdoor Mass Culture Conditions

Haematococcus pluvialis from the outdoor culture system was harvested, and the biomass and Chl *a*, Chl *b*, total carotenoid, and astaxanthin contents were analysed and compared with those from laboratory experiments (Table 1). Although the same number of cells were inoculated in both the laboratory and outdoor cultures, the biomass was 30% lower, Chl *a* and Chl *b* contents were 20% lower, total carotenoid content was 25% lower, and astaxanthin content was 30% lower in the outdoor culture system than in the laboratory culture system after 15 days.

Table 1. Biomass, Chl *a*, Chl *b*, total carotenoid, and astaxanthin contents produced in the laboratory and hybrid outdoor culture systems (n = 5).

	Laboratory Culture	Outdoor Culture
Biomass (g L^{-1} DW)	0.71 ± 0.020 *	0.51 ± 0.130
Chl *a* (μg mL^{-1})	0.110 ± 0.010	0.090 ± 0.012
Chl *b* (μg mL^{-1})	0.112 ± 0.024	0.090 ± 0.014
Total carotenoid content (μg mL^{-1})	1.125 ± 0.011 *	0.841 ± 0.240
Astaxanthin content (μg mL^{-1})	0.752 ± 0.014 *	0.521 ± 0.171

* $p < 0.001$ (Student's *t*-test).

4. Discussion

The light source and wavelength played important roles in improving astaxanthin and biomass yield during the cultivation of *H. pluvialis*. Although various colours of light are used for microalgal cultivation, mainly red light (600–700 nm) and blue light (400–500 nm) are utilized [43]. The red and blue wavelengths of visible light assist in cell division at the vegetative stage and cell maturation at the astaxanthin accumulation stage, respectively. Blue light is more important, as it passes through clear water and regulates metabolic pathways. Astaxanthin accumulation and dry biomass were higher when red and blue light-emitting diodes (LEDs) were used together, compared to their separate use at an intensity of 100–120 μmol m^{-2} s^{-1}. A comparative study of monochromatic red and mixed red/blue light similarly showed that red/blue light at a 2:1 ratio produced higher biomass [44–46]. The use of blue LEDs (440 nm) enhanced the biosynthesis of astaxanthin in *H. pluvialis* [47].

This study examined the growth of *H. pluvialis* cultures in the green and red phases separately. In the green stage, inorganic nutrients were provided for growth; as low light intensity was preferred, the light intensity was fixed at 25 μmol m^{-2} s^{-1}. Microalgae generally stop dividing at light intensity levels below 60 μmol m^{-2} s^{-1} [48], whereas *Haematococcus* cells divided at lower levels in this study. High light intensity triggers the formation of astaxanthin in microalgae. Lighting frequency, duration, intensity, and spectral composition direct the growth and biosynthesis of compounds in microalgae [32]. Astaxanthin accumulation in *Haematococcus* requires cultivation under stress conditions such as nutrient deficiency or exposure to high light intensity. As nitrogen depletion is also involved in the induction process, photoinhibition is highly relevant as it depends on the de novo synthesis of the D1 protein [49].

However, our results also suggested that these light intensity effects have an upper limit. Biomass and carotenoid contents were approximately 15% higher at a light intensity of 160 μmol m^{-2} s^{-1} than at 220 μmol m^{-2} s^{-1}. A previous study reported that growth and astaxanthin production were increased at 170 μmol m^{-2} s^{-1} [50]; in another, an organic carbon source was added and open culture was performed under light intensity conditions of 150 and 300 μmol m^{-2} s^{-1} [51]. Bacterial contamination is inevitable in outdoor microalgal culture [52], and the risk increases when organic carbon is added to the medium [53]. However, the number of bacterial cells in water has been shown to be a function of phosphorus and nitrogen concentrations [54]. Bacterial growth is more accurately described by its high affinity for nutrients that are readily available in the culture medium [55]. Therefore, astaxanthin accumulation in *H. pluvialis* under nitrogen depletion conditions should be studied further. Interestingly, the values of Chl *a* and Chl *b* were found to be similar in this study. According to [56], red light (600–700 nm) and blue light (420–450 nm) are absorbed by chlorophyll. Therefore, the mechanism behind this needs to be further explored in future research.

5. Conclusions

In this study, we conducted field and laboratory experiments under the same conditions. In the laboratory, to reduce contamination during *H. pluvialis* growth, we minimised contact with the outside environment and used temperature-controlled tanks. In an outdoor

experiment to promote astaxanthin accumulation, *H. pluvialis* was also grown in a 120 m^3 water tank under a light intensity of 160 µmol m^{-2} s^{-1} as in the laboratory experiment. However, both the biomass and astaxanthin contents were approximately 30% lower in the outdoor culture system than in the indoor culture system. Various factors such as light periodicity, light source, water temperature, and external pollution likely affected the outdoor culture system, in comparison to the indoor culture system. Our open culture system produced approximately 50 kg of biomass per culture at an average rate of 0.51 g L^{-1} DW in a 120 m^3 water tank.

Although a higher temperature would be preferred to reduce production costs, we performed the mass cultivation of *H. pluvialis* at 18 °C ± 2 °C, as the National Institute for Environmental Studies (NIES) in Japan reported that the optimum temperature for *H. pluvialis* growth is 20 °C [57]. Using sunlight, a natural source of light, is beneficial in terms of reducing capital expenditure. However, its intensity is not constant and varies with changes in weather [58]. As a solution to this drawback, we applied a multilayered polyolefin vinyl screen in our open–closed pond system to maintain a consistent light intensity of 25 ± 5 µmol m^{-2} s^{-1}.

The industrial application of algae to produce astaxanthin has several limitations, which can directly affect the final price of the product. As large amounts of energy are required, inexpensive open outdoor culture systems should be adopted as an alternative to costly closed photobioreactors. Moreover, astaxanthin production rates must be increased through additional research on the effects of light periodicity, changes in nutrient levels, and water temperature. Under increasing consumer demand for natural products derived from microalgae, the proposed system may also be used for the mass cultivation of various other types of microalgae.

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