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A novel drying film culture method applying a natural phenomenon: Increased carotenoid production by *Haematococcus* sp

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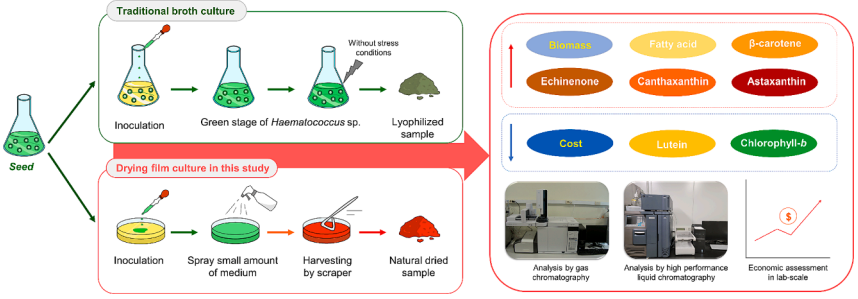
HIGHLIGHTS

- The drying film culture method induces significant cell morphological alteration.
- The drying film culture method significantly affects biomass production.
- The drying film culture method significantly impacts carotenoid concentration.
- The drying film culture system reduces water and medium use.

GRAPHICAL ABSTRACT



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ABSTRACT

Low productivity and high cost remain major bottlenecks for the large-scale production of *Haematococcus* sp. This study explored biomass production and carotenoid accumulation in *Haematococcus* sp. (KCTC 12348BP) using drying film culture. The broth-cultured strain (3.2×10^6 cells/mL, 0.83 ± 0.02 mg/mL for a 21 d culture) was cultured under various conditions (different inoculum volumes and mist feeding intervals) in waterless agar plates at 28 ± 0.5 °C, under fluorescent light (12 h light–dark cycle) for 1 month. The maximum biomass obtained was 17.60 ± 0.72 g/m^2, while the maximum astaxanthin concentration was 8.23 ± 1.13 mg/g in the culture using 1 mL inoculum and 3 d feeding interval. Drought stress in drying film culture effectively induced the accumulation of carotenoids from β-carotene, facilitating the production of canthaxanthin via the astaxanthin biosynthesis pathway. This cost-effective culture system can increase the biomass and carotenoid pigment production in *Haematococcus* sp.

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1. Introduction

Haematococcus, a genus of freshwater unicellular microalgae belonging to the class Chlorophyceae, is known to accumulate large amounts of astaxanthin and fatty acids (FAs) under stress conditions (Boussiba, 2000; Guiry & Guiry, 2022). Based on the external growth environment, members of the *Haematococcus* genus exhibit four distinct cell types, namely macrozooids (or zoospores), palmelloids (or coccoids), hematocysts (or aplanospores), and microzooids (Han et al., 2013). For instance, under favorable environments, macrozooids exhibit motility with the help of two flagella (Hagen et al., 2002). In contrast, under unfavorable environmental conditions, such as nutrient depletion, drought, or high light intensity (Aflalo et al., 2007; Kobayashi et al., 1997), macrozooids lose their flagella and transform into palmelloid cells with enlarged protoplasts. At this stage, a small amount of astaxanthin begins to accumulate in the cytoplasm, giving rise to greenish-red intermediate cells (Jannel et al., 2020). If unfavorable environmental conditions prevail for a prolonged period, palmelloid cells transform into non-motile red hematocysts (Li et al., 2019). Mature hematocysts can accumulate FA-esterified astaxanthin up to 3 %–5% of their dry weight (Oslan et al., 2021). Astaxanthin biomolecules accumulate in FA-associated extraplastidic lipid globules (Grünewald et al., 2001). Notably, carotenogenesis resulting in astaxanthin accumulation requires active synthesis of FAs (Nishshanka et al., 2022).

The endogenous levels of abscisic acid (ABA) are elevated under abiotic stress conditions, such as drought and high salinity (Hauser et al., 2011; Kobayashi et al., 1997). In addition, ABA content controls the morphological changes of cells from a vegetative to a cystic state and enhances carotenogenesis (Kobayashi et al., 1997). This process represents a natural microalgal defense system that induces increased carotenogenesis, encystment, and ABA biosynthesis to protect cells from oxidative damage caused by abiotic stress (Hauser et al., 2011; Kobayashi et al., 1997).

Astaxanthin is a high-value secondary metabolite and a member of the xanthophyll family of carotenoid pigments (Mularczyk et al., 2020). These pigments have a unique molecular structure with 13 conjugated bonds, a hydroxyl group (–OH), and a ketone group (=O) on each ring, at both ends (Brotosudarmo et al., 2020). Astaxanthin displays greater antioxidant activity than β-carotene, canthaxanthin, zeaxanthin, lutein, and α-tocopherol (Miki, 1991). In particular, it neutralizes free radicals and scavenges photosynthetic reactive oxygen species (Shah et al., 2016; Song et al., 2022). Astaxanthin is used in various applications in different industries, such as cosmetics, pharmaceuticals, food additives, feed additives, and aquaculture (Jiang et al., 2020; Shah et al., 2016). In addition to algae, natural astaxanthin can also be obtained from krill, fungi, bacteria, and yeast; however, most of these sources are unsuitable for mass due to their astaxanthin content being low (less than 1 % of their dry weight) (Shah et al., 2016; Zhu et al., 2022). *Haematococcus pluvialis* is a natural biological source with the greatest astaxanthin content, reaching > 3 % of its dry weight (Patil et al., 2022). In addition, the use of synthetic astaxanthin as a raw food material is not allowed in the European Union or the United States due to safety concerns, which is expected to increase the demand for natural astaxanthin (Liaqat et al., 2023). Moreover, due to its high production cost, the market price of natural astaxanthin is estimated to reach USD 2,500–7,000 per kg (Molino et al., 2018).

In general, industrial-scale *Haematococcus* cultures are conducted in two phases, either in a closed photobioreactor or in an open raceway pond under photoautotrophic culture conditions (Aflalo et al., 2007; Han et al., 2013). The culture in the first phase is performed under normal conditions for cell proliferation and biomass accumulation. In the second phase culture, astaxanthin accumulation is induced under various stressful conditions (Boussiba, 2000; Li et al., 2020). However, *Haematococcus* mass culture systems are associated with many challenges. One of these challenges is the high level of contamination by other species, for which no effective solution has been found (Liu et al., 2014; Mularczyk et al., 2020). In addition, the large-scale production of biomass and astaxanthin from *Haematococcus* cells is limited owing to morphological changes, slow growth rates, and high sensitivity to hydrodynamic stress (Liu et al., 2014; Shah et al., 2016). Moreover, infrastructure (e.g., photobioreactor systems, biomass drying, and astaxanthin extraction) and essential resources for *Haematococcus* biomass production (e.g., water, culture medium, and electricity) are highly cost-dependent (Li et al., 2011; Li et al., 2020; Shah et al., 2016). Therefore, it is necessary to develop innovative culture systems to improve the large-scale culture of *Haematococcus* by reducing raw material costs and avoiding expensive photobioreactor systems.

In this study, a novel selective fed-batch drying film culture method was designed for the repeated culture of *Haematococcus* in dry agar plates per unit of time. This was achieved by spraying small amounts of medium to produce highly concentrated cell biomass and carotenoid pigments. Drought stress occurring naturally by evaporation of standing water was used as a stress factor for carotenoid accumulation in *Haematococcus* sp. KORDI03. This study aimed to assess the feasibility of the strain culture system by comparing the production of biomass, carotenoid pigments, and FAs, and the cost-effectiveness between the conventional and drying film culture methods at the laboratory scale.

2. Materials and methods

2.1. Chemicals and reagents

Acetone [high-performance liquid chromatography (HPLC)-grade], used for extracting dried biomass, was purchased from Tedia (Fairfield, OH, USA). For saponification, cholesterol esterase from *Pseudomonas fluorescens* (≥10,000 units/g protein) was purchased from Sigma–Aldrich (Saint-Louis, MO, USA). For the HPLC solvent, water and methyl alcohol (99.9 % purity) were purchased from Samchun Pure Chemical (Seoul, Republic of Korea); *tert*-butyl methyl ether (≥99.8 % purity) was purchased from Sigma–Aldrich. Astaxanthin (≥97.0 % purity), echinenone (≥95.0 % purity), canthaxanthin (≥95.0 % purity), β-carotene (≥95.0 % purity), and lutein (≥95.0 % purity) standards were purchased from Sigma–Aldrich. The chlorophyll-b (90 % purity) standard was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Microalgal strain and culture medium

Haematococcus sp. KORDI03 (KCTC 12348BP) was collected from standing water (Kim et al., 2015) and sub-cultured in modified Bold's basal medium (Sigma–Aldrich) at 28 °C ± 0.5 °C under a 12 h light:12 h dark cycle with 71 ± 2 μmol photons m^{-2} s^{-1} (Li-250A, Li-COR, Lincoln, NE, USA) for 21 d. The agar medium was prepared by mixing 15 g Bacto agar powder (BD Diagnostics, Franklin Lakes, NJ, USA) with 1 L distilled water and 20 mL of modified Bold's basal medium, followed by autoclaving, and dispensing 25 mL of the medium into each 90 mm Petri dish (SPL Lifesciences, Pocheon, Republic of Korea) (63.59 cm^2). The broth culture medium was prepared by mixing 4 L distilled water with 80 mL of modified Bold's basal medium in a 5 L Erlenmeyer flask.

2.3. Culture conditions

To determine the optimum culture condition for the drying film culture (experimental), various combinations of inoculum concentrations (3.2×10^6 cells/mL; 0.83 ± 0.02 mg/ mL) at different volumes (0, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 mL) over 1–5 d feeding intervals (1, 2, 3, 4, and 5 d) were used to create 45 experimental culture groups. The feeding interval was established by spraying 1 mL broth medium (i.e., mist feeding) with compressed air using a sprayer (Dia sprayer, Model No. 570). A sprayer was used to apply the medium as uniformly as possible to the *Haematococcus* cells on the agar medium. A broth medium volume of 1 mL was determined as the minimum amount that

could be applied to the entire agar medium area in preliminary experiments. The broth culture (control) was inoculated with 1 mL inoculum (3.2×10^6 cells/mL, 0.83 ± 0.02 mg/mL). All culture experiments were conducted in duplicates at 28 ± 0.5 °C, under a 12 h light:12 h dark cycle with 94 ± 3 μmol photons $m^{-2}s^{-1}$ for 30 d.

2.4. Analytical methods

2.4.1. Drying rate, biomass cell density, and harvesting

The moisture content in the agar medium was measured by comparing the difference in weight of the medium before and after weighing each day, using an analytical balance (ME204; Mettler Toledo, Columbus, OH, USA) (Agbede et al., 2020) (see Supplementary Material). The mass (g)–time (d) data obtained from the experiment were converted to moisture content (g)–drying time (d) data. The moisture content in the agar medium was calculated based on the equation: $Y_t = (X_t - X_d)/X_d$, where Y_t denotes the moisture content (g water/g dry matter) and X_t and X_d are the mass (g) of the microalgal sample at any time (t) and mass (g) of the whole dried sample, respectively.

After 30 d of culture, 20 mL of distilled water was sprayed on the surface of the agar medium of each experimental group, and the agar was scraped using a scraper to create a mixture. A small fragment of agar medium from the mixture was separated using a 60 μm mesh filter (Samjee Tech, Anyang, Republic of Korea). The final biomass samples were obtained by centrifuging the filtered biomass mixture at 1,967 × *g* for 10 min at 4 °C. To measure the change in biomass cell density of the control, 20 mL samples of microalgal cultures were collected at 3 d intervals from the start of culture and passed through a glass fiber filter (GF/C, Whatman, Maidstone, UK), and then dried at 60 °C in a drying oven (VS-1202D3; Vision Scientific Co., Bucheon, Korea) for 24 h before measuring the biomass cell density (g/L; Lee et al., 2021).

All the harvested wet biomass was frozen at −50 °C in a deep freezer (SBD-520; Jungjin deep freezer, Goyang, Korea) and lyophilized for 2 d in a freeze-dryer (FDTA-45; Operon, Gimpo, Korea). The dry biomass samples were homogenized using a mortar and pestle. Finally, the powdered samples were stored at 4 °C in a refrigerator in the dark.

The biomass production of the experimental group is expressed as the dry weight (g) of biomass sample per unit of area (63.59 cm^2) of the agar medium, and was converted to g/m^2 units. Unit conversion was essential to compare biomass production between broth (g/L) and drying film culture (g/m^2) in this study. The biomass yield of the culture obtained for 25 mL of the agar medium used for plating was converted to g/L (see Supplementary Material).

2.4.2. Extraction and determination of carotenoid pigment content

Carotenoids from *Haematococcus* sp. biomass were extracted using a method described by Do et al. (2019) with slight modifications. For carotenoid extraction, a 90 % acetone solution (0.5 mL) was added to 4 mg of dried sample. Cells were disrupted by sonication at 40 amp for 1 min (20 kHz; QSonica Q700 sonicator, QSonica LLC., Newtown, CT, USA) using 10 s pulse-on and pulse-off intervals to obtain the extract, which was then centrifuged at 2,655 × *g* for 10 min at 4 °C to obtain the supernatant. This process was repeated until the cell precipitate appeared clear. The final mixed extract was centrifuged at 10,621 × *g* for 10 min at 4 °C and stored in a –20 °C freezer in the dark. The levels of astaxanthin in the extract from *Haematococcus* sp. cells were measured using the method described by Li et al. (2012). The degree of astaxanthin accumulation in all experimental groups was determined by measuring the absorbance at 530 nm using a spectrophotometer (Optizen POP, Mecasys, Daejeon, Korea). Astaxanthin concentration was calculated using the standard curve equation $y = 0.0536x + 0.016$, where y is the optical density value and × is the astaxanthin concentration (g/mL). Unit conversion was performed based on the equation $y = [A \times B]/D$, where y, A, B, and D represent astaxanthin concentration (g/mg or mg/g), astaxanthin concentration (g/mL), solvent volume (mL), and dry cell weight (mg), respectively.

According to screening test results, saponification was essential before HPLC to quantitatively compare the contents of the experimental groups, which had the highest biomass production and astaxanthin concentration, with those of the control group. To convert the ester form of free astaxanthin, saponification was performed using a method described by Kim et al. (2008) with slight modification. Three milliliters of the extract was mixed with 2 mL of 0.05 M Tris-HCl buffer, and the mixture was preheated for 2 min at 37 °C and subjected to reaction for 45 min following the addition of cholesterol esterase to hydrolyze the carotenoid esters at 37 °C (Jacobs et al., 1982). After completion of the reaction, 0.44 g anhydrous sodium sulfate and 4 mL petroleum ether were added to the mixture, stirred for 30 s, and centrifuged at 1,967 × *g* for 10 min at 4 °C to retrieve the petroleum ether layer. The retrieved layer was transferred to a clean test tube to volatilize the solvent within the extract and dissolve the saponified extract in acetone-methanol before HPLC analysis.

A Waters New Alliance E2695 (Waters, Singapore) HPLC system equipped with a Waters 2998 PDA detector and YMC carotenoid C30 column (5 μm, 250 × 4.6 mm; YMC, Wilmington, NC, USA) was used for HPLC analysis. A reverse gradient system was employed for an effective separation of the extracted carotenoid components in the mobile phase. Mobile phase A was composed of 100 (v/v) methanol, while mobile phase B was composed of methanol, methyl *tert*-butyl ether, and water in a ratio of 7.0:90.0:3.0 (v/v). A series linear-gradient system from B to A at a flow rate of 1.0 mL/min was employed, with elution starting with 0 % B and proceeding with 0 %–100 % B (0–90 min). The detection wavelengths of the PDA detector were set at 250 and 700 nm, and chromatographic peaks were measured at 474 nm to facilitate astaxanthin detection (Wang et al., 2018). To express the pigment concentration of the sample in milligrams per gram, the peak area corresponding to the pigment in the sample with the same retention time as that of the standard pigment was converted and quantified by comparing it with a standard calibration curve.

2.4.3. Determination of ABA and nitrate concentrations

Intracellular ABA was extracted according to the method described by Sulochana and Arumugam (2016). The concentration of ABA in *Haematococcus* biomass was measured using a plant hormone ABA ELISA kit (EK799009; AFG Scientific, Chicago, IL, USA) according to the manufacturer's instructions. To measure the nitrate concentration in the agar medium, the latter was immersed in 20 mL of distilled water, autoclaved at 121 °C for 15 min, and treated with *Pseudomonas atlantica* agarase (Sigma-Aldrich). All samples were filtered through 0.22 μm vacuum filtration units (Sartolab® RF 50, Sartorius, Goettingen, Germany). The nitrate concentration was measured at 410 nm using a UV/VIS spectrophotometer (HS-3700, Humas, Korea) following the manufacturer's instructions.

2.4.4. Comparison of FA profiles

The protocol described by Garcés and Mancha (1993) was used to pretreat dried *Haematococcus* biomass samples. A gas chromatograph (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) was equipped with a capillary column (DB-23, 120 mm × 0.25 mm × 0.25 μm, Agilent Technologies), and the carrier gas, helium, was supplied. The injector temperature was 250 °C; the injection volume was 1 μL, and the detector temperature was 280 °C. The programmed oven had an initial temperature of 80 °C (hold time of 1.5 min, run time of 1.5 min), and Ramp 1 (rate of 30 °C/min, temperature of 110 °C, hold time of 2 min, run time of 4.5 min), Ramp 2 (rate of 15 °C/min, temperature of 200 °C, hold time of 8 min, run time of 18.5 min), Ramp 3 (rate of 1 °C/min, temperature of 215 °C, hold time of 8 min, run time of 41.5 min), and Ramp 4 (rate of 2 °C/min, temperature of 250 °C, hold time of 3 min, run time of 62 min) were set. FA composition and concentration were confirmed by comparison with authentic standards (Supelco 37 FAME mix; Supelco, Bellefonte, PA, USA) with known retention times.

2.4.5. Morphological analysis

Haematococcus was examined under a light microscope (Eclipse Niu, Nikon Co., Tokyo, Japan) and imaged using a camera (Digital sight DS-Fi2, Nikon Co., Tokyo, Japan). Cell size was calculated using an image analyzer (NIS-Elements BR 4.30.00, Nikon Co.) (see Supplementary Material). The morphological variations (cell color, drying, and biomass) were determined using a scanner (Epson Perfection V700 photo, Seiko Epson Co., Nagano, Japan) (see Supplementary Material).

2.5. Statistical analysis

All experiments were performed in duplicate, and a one-way analysis of variance of all experimental data was conducted using the GraphPad Prism 8.0.2 software (version 8.0.2; GraphPad Software, Inc., La Jolla, CA, USA). The Tukey's test at a significance level of 95 % was used to compare results ($p < 0.05$).

3. Results and discussion

3.1. Optimal condition for drying film culture

3.1.1. Biomass production using drying film culture

As the establishment of optimal incubation conditions was necessary for *Haematococcus* sp. culture at high concentrations using the drying film culture method, various combinations of inoculum volume and feeding intervals were compared to determine their effects on cell growth. Biomass production varied from the lowest value (1.36 ± 0.54 g/m^2) when 5 mL inoculum and a feeding interval of 5 d were used to the highest value (17.60 ± 0.72 g/m^2) when 1 mL inoculum and a feeding interval of 1 d employed (Fig. 1). Interestingly, biomass production in the experimental group using 1 mL inoculum at each feeding interval except for the 1 d interval, was significantly higher than that under other conditions ($p < 0.05$). Furthermore, a comparison of the biomass production relative to inoculum volume ranges confirmed that the 1 d feeding interval group displayed considerably higher biomass production than groups under other conditions, regardless of inoculum volume. Under incubation conditions wherein 1 mL inoculum with the 1 d feeding interval was used, cell growth on the agar plate was highly effective. A comparison of the effects of the initial inoculum volume on the final biomass cell density in the drying film culture revealed that higher initial inoculum volumes resulted in lower biomass production, indicating that reduced biomass production did not occur proportionally with an increase in inoculum volume. Additionally, the decrease in the growth rate for the various inoculums was most likely limited due to the absorption of key nutrients, such as oxygen, owing to the high cell density (Zhang et al., 2002). However, as the feeding interval decreased, biomass production increased owing to the continuous nutrient supply. Moreover, the reduction in the thickness of the agar medium intensified as the feeding interval increased (experimental group with > 2 d of feeding interval); this was attributed to the drying of the agar plate. These results indicated that a periodic supply of nutrients is more crucial than the initial *Haematococcus* cell density for the production of high biomass levels under the conditions of the artificial culture system used in this study.

3.1.2. Astaxanthin accumulation using drying film culture

Next, the concentration of astaxanthin in *Haematococcus* sp. cultured under various combinations of inoculum volume and feeding intervals was determined. An increase in the volume of the feeding medium did not induce sufficient drought stress and thus, the astaxanthin content did

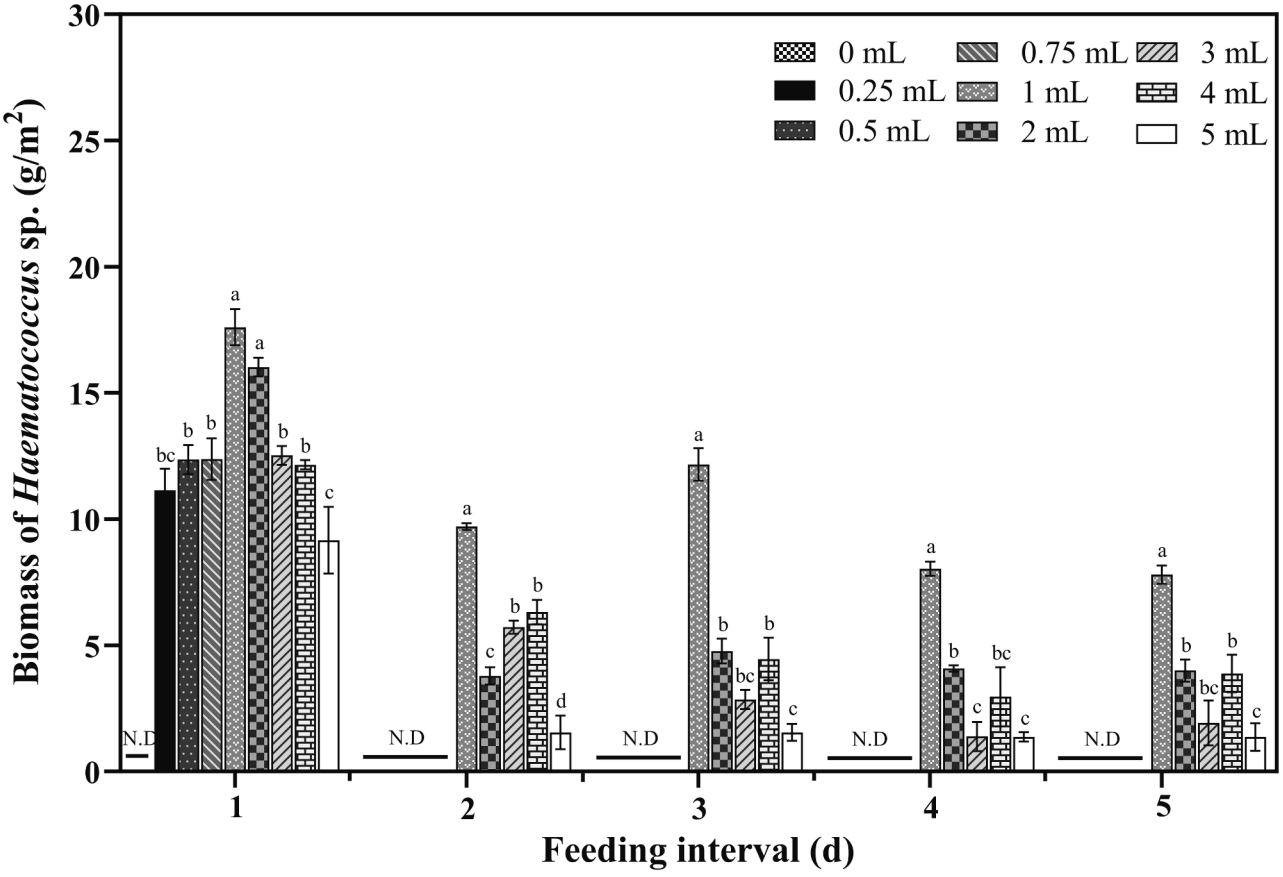

Fig. 1. Final biomass content per unit of area in the dry weight (g) of *Haematococcus* sp. under various culture conditions (45 experimental groups using varying combinations of feeding intervals and inoculum volumes). ND: not detected. The assays were performed in duplicate and different letters in the same row indicate significant differences, identified by Tukey's test ($p < 0.05$).

not differ significantly from that in the broth culture. Therefore, the minimum volume (1 mL) that could cover the entire surface of the agar plate was determined based on the feeding interval. The astaxanthin concentration in dry cells varied between 1.23 ± 0.13 mg/g (3 d feeding interval and 5 mL inoculum) and 8.23 ± 1.13 mg/g (3 d feeding interval and 1 mL inoculum) (Fig. 2). Of note, astaxanthin concentration in the group using 1 mL inoculum was considerably higher than that in the other groups except for those with 5 d feeding intervals (Fig. 2). Astaxanthin concentration did not differ significantly between groups using 1 mL inoculum and 2 or 3 d feeding intervals ($p > 0.05$). Moreover, astaxanthin concentration in the experimental group using a feeding interval of 2–3 d was significantly higher than that in all other experimental groups ($p < 0.001$). An inoculum volume of 1 mL and a feeding interval of 2–3 d was confirmed to be the optimal condition for increasing the concentration of astaxanthin in the drying film culture. Drought stress induced by these culture conditions enhanced astaxanthin biosynthesis. Furthermore, appropriate drought stress for the feeding intervals used herein (2–3 d) increased the rate of conversion of cells from green cells to red cysts. It also had the most remarkable effect on the concentration of astaxanthin. In conclusion, the degree of drought stress according to the feeding interval in the drying film culture is an important factor for the accumulation of high concentrations of astaxanthin.

3.2. Carotenoid pigment concentrations

Table 1 shows the HPLC and chromatogram results of the carotenoid extracts from *Haematococcus* cells (see Supplementary Material). Experimental group 1, which received a 1 mL inoculum volume and was subjected to a 1 d feeding interval, exhibited the highest biomass production, whereas experimental group 2, which received a 1 mL inoculum volume and used a 3 d feeding interval, demonstrated the highest astaxanthin concentration. Broth culture conditions were set as the control. Using standard carotenoid solutions led to the identification of six resolved peaks. The concentration of chlorophyll-b differed significantly from that of the control, exhibiting a rapid decrease to 0.34 ± 0.03 and 0.35 ± 0.02 mg/g in experimental groups 1 and 2, respectively ($p < 0.001$). The concentration of β-carotene in *Haematococcus* cells was 5.15 ± 0.64 mg/g in experimental group 2, which was significantly higher than that in other groups ($p < 0.01$). However, no significant difference was detected between experimental group 1 and the control group. In contrast, the concentration of echinenone and canthaxanthin was 2.20 ± 0.43 and 0.96 ± 0.13 mg/g in experimental group 1, respectively, and 20.53 ± 1.61 and 6.77 ± 0.64 mg/g in experimental group 2, respectively. The above concentrations were equal to or considerably higher than those under control conditions. The concentration of free astaxanthin was 6.21 ± 0.45 mg/g in experimental group 2, which was significantly higher than that in other groups ($p < 0.001$). However, the concentration of free astaxanthin was not significantly different between experimental group 1 and the control group ($p > 0.05$). Overall, the 3 d feeding interval enhanced the accumulation of free astaxanthin by approximately 6.5 times that of the control. Interestingly, the level of lutein, which was the most abundant carotenoid in the control, was decreased under drying film culture conditions

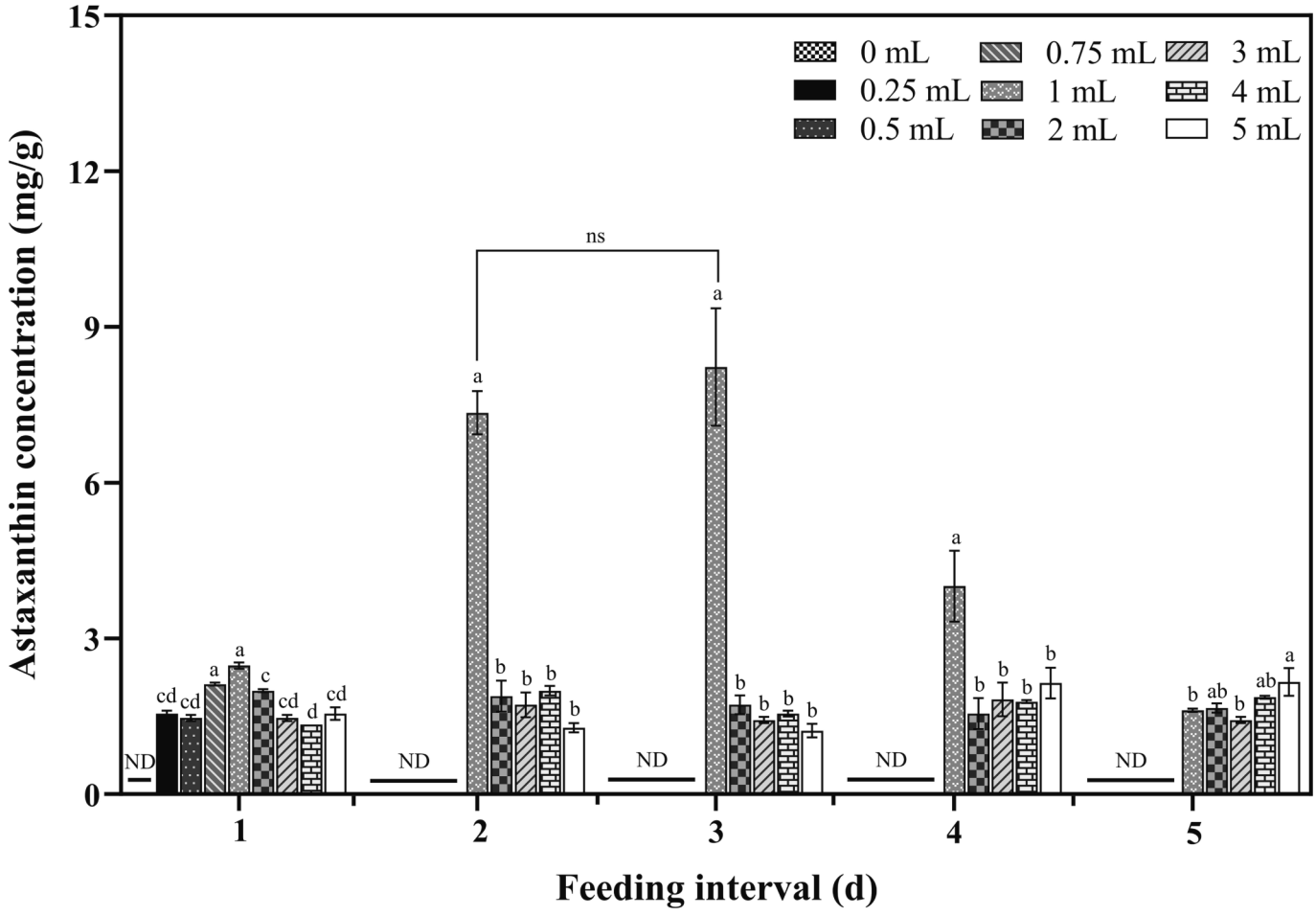

Fig. 2. Astaxanthin concentration in *Haematococcus* sp. under various culture conditions (45 experimental groups using varying combinations of feeding intervals and inoculum volumes). ND: not detected. The assays were performed in duplicate and different letters in the same row indicate significant differences, identified by Tukey's test ($p < 0.05$).

Table 1
Comparison of pigment concentrations in extracts from *Haematococcus* cells cultured under broth and drying film conditions using high-performance liquid chromatography.

Identification	Control[a]	Experimental group 1[b]	Experimental group 2[c]
Culture duration	30 d		
Total volume (sum of initial + additions)	5,000 mL (5,000 + 0 mL)	55 mL (25 + 30 mL)	35 mL (25 + 10 mL)
g/area (m^2) or g/L	0.63 ± 0.01 g/L	10.56 ± 0.43 g/m^2	7.30 ± 0.38 g/m^2
Chlorophyll-b (mg/g)	3.71[a] ± 0.86	0.34[b] ± 0.03	0.35[b] ± 0.02
Free astaxanthin (mg/g)	0.96[b] ± 0.15	0.86[b] ± 0.12	6.21[a] ± 0.45
Lutein (mg/g)	7.26[a] ± 1.04	0.77[b] ± 0.15	1.52[b] ± 0.25
Canthaxanthin (mg/g)	1.02[b] ± 0.17	0.96[b] ± 0.13	6.77[a] ± 0.64
Echinenone (mg/g)	1.64[b] ± 0.14	2.20[b] ± 0.43	20.53[a] ± 1.61
β-carotene (mg/g)	3.45[b] ± 0.25	2.48[b] ± 0.31	5.15[a] ± 0.64
Total pigment conc. (mg/g)	18.04[b] ± 0.24	7.60[c] ± 0.63	40.28[a] ± 1.63

The assays were performed in duplicate and different letters in the same row indicate significant differences, identified by Tukey's test ($p < 0.05$).
[a] Pigment concentration in broth culture.
[b] Pigment concentration in culture using 1 mL inoculum volume and 1 d feeding interval.
[c] Pigment concentration in culture using 1 mL inoculum volume and 3 d feeding interval.

(Table 1).

Astaxanthin, a secondary carotenoid, and lutein, a primary carotenoid, are synthesized from the common precursor lycopene through different metabolic pathways using β-carotene and α-carotene, respectively, as intermediates (Jin et al., 2017). In this metabolic pathway, drought stress in the drying film culture differentially affects the accumulation of lutein and astaxanthin, resulting in enhanced astaxanthin biosynthesis and decreased lutein accumulation (Del Campo et al., 2004). Therefore, the total pigment concentration in the control was relatively higher than that in experimental group 1, in which astaxanthin synthesis was induced and lutein concentration was decreased (Table 1). To compare the difference in moisture content of the agar medium during the culture period, the experimental group was compared with the agar medium (control) without inoculation with *Haematococcus* sp. KORDI03 (see Supplementary Material). Moisture forms the main component of the microalgal biomass; therefore, comparison with the control was essential (Cheng et al., 2013). Although the amount of moisture evaporated from the agar medium differed between the experimental and control groups, a highly similar drying tendency was observed. Experimental group 2 showed a considerably higher rate of moisture loss in the agar medium than experimental group 1 did during the cultivation period. More specifically, on the 30th d of culture, the water content of experimental group 2 was markedly lower than that of experimental group 1 (see Supplementary Material). In addition, the concentration of ABA in *Haematococcus* cells differed significantly between experimental groups 1 and 2 ($p < 0.001$), with significantly higher levels observed in cells with enhanced carotene production (Fig. 3). Consequently, the drought level according to the mist feeding interval in the drying film culture affected the biosynthesis of the stress indicator ABA. The concentration of nitrate in the agar medium was measured to confirm the occurrence of nitrate deficiency, which is known to induce astaxanthin accumulation in *Haematococcus* cells (Scibilia et al., 2015). However, nitrate accumulation was also observed in the experimental groups because of agar medium drying and continuous medium mist feeding (Fig. 3). In particular, the change in the concentration of nitrate before and after cultivation was the highest in experimental group 1, which had been subjected to a short mist feeding interval. Consequently, the concentration of nitrate in the agar medium was considered to not act as a stress factor for astaxanthin accumulation in *Haematococcus* cells; however, further studies are required to determine the difference between conventional media mist feeding and simple water mist feeding in terms of their ability to induce drought stress levels in this culture system.

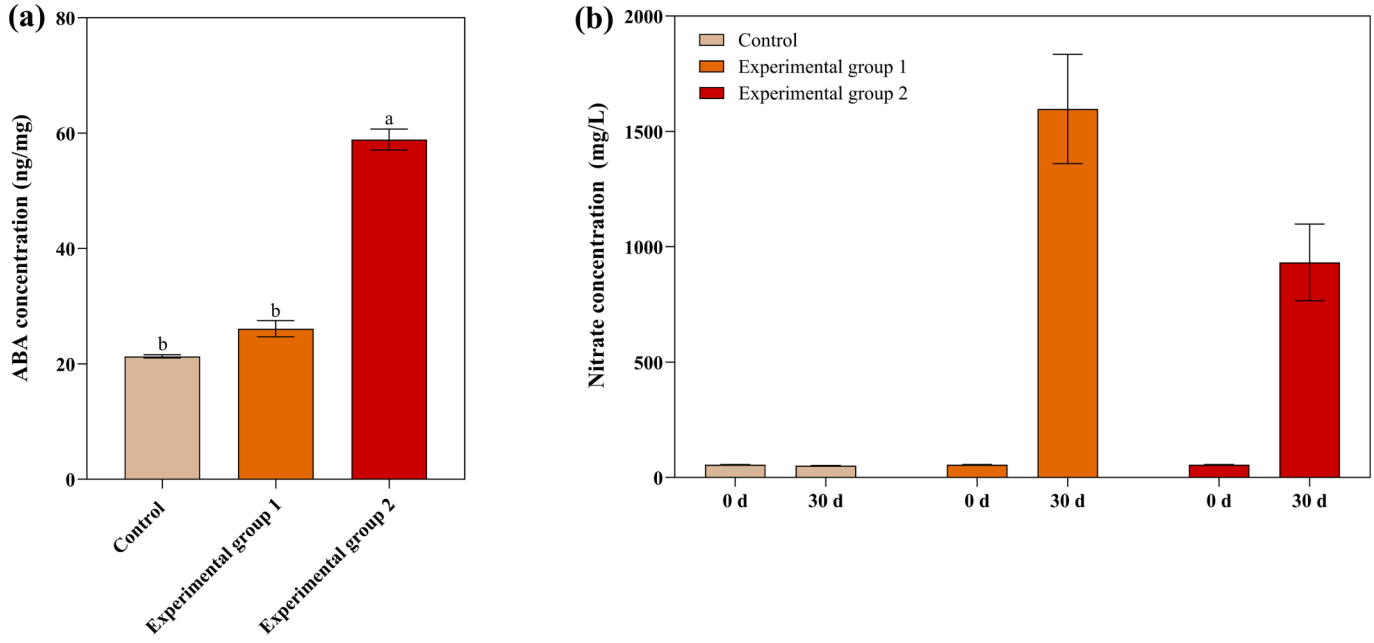

Fig. 3. Comparison of (a) abscisic acid (ABA) content in the *Haematococcus* sp. biomass and (b) nitrate concentration in the culture medium, including broth culture (control), 1 mL of inoculum volume and 1 d feeding interval (experimental group 1), and 1 mL of inoculum volume and 3 d feeding interval (experimental group 2). The assays were performed in duplicates; different letters in the same row indicate significant differences identified by Tukey's test ($p < 0.05$).

3.3. Cell morphology

The morphological characteristics of the *Haematococcus* sp. were investigated using optical microscopy and scanner-based image analysis. From 0 d of culture, immobile intermediate cells were rarely observed in the experimental groups, and their number gradually increased. At 3 d of culture, the color of the biomass film in the agar plate of each experimental group (1 d feeding interval and 1 mL inoculum, 3 d feeding interval and 1 mL inoculum) was green, and motile vegetative green cells were observed (see Supplementary Material). In experimental group 2 (3 d feeding interval and 1 mL inoculum), the color of the biomass film was orange, and conversion to orange cysts at 30 d of culture was observed in most cells; however, a few cells with red cysts were observed (see Supplementary Material). In experimental group 1 (1 d feeding interval, 1 mL inoculation), the color of the biomass film was yellow green; the most common cell morphology was intermediate, and no motile cells were observed (see Supplementary Material). Overall, the level of drying of the film in the agar plate considerably affected alterations in *Haematococcus* sp. cell morphology and carotenoid accumulation.

3.4. FA profiles

Fig. 4 shows the FA (mg/g) profiles of *Haematococcus* cells under drying film and broth culture conditions. FA biosynthesis was greatly activated in experimental group 2 (Fig. 3). In particular, the total FA concentration in experimental group 2 was 284.53 ± 3.93 mg/g, approximately 4.57 times higher than that in the control (61.62 ± 4.17 mg/g). Of note, approximately 95 % of astaxanthin molecules in *Haematococcus* are in the ester form, and FAs are considered important metabolic precursors for astaxanthin esterification (Holtin et al., 2009; Hoys et al., 2021; Yu et al., 2021). Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6), and-linolenic acid (C18:3n3) were the main FA components in *Haematococcus* cells. Previous studies reported that these are the most common FAs esterified with astaxanthin (Breithaupt, 2004; Chen et al., 2015). Interestingly, oleic acid, a monounsaturated FA, was present in the highest proportion in *Haematococcus* cells, with a significantly higher concentration in experimental group 2 than in other groups ($p < 0.001$). These findings suggest that astaxanthin ester accumulation in *Haematococcus* cells under stress conditions is accompanied mainly by oleic acid synthesis (Zhekisheva et al., 2002). Additionally, increased levels of oleic acid (C18:1n9), linoleic acid (C18:2n6), and α-linolenic acid (C18:3n3) may associated with the esterification of astaxanthin (Chen et al., 2015; Yu et al., 2021). These results suggested that drought stress conditions enhanced the production of FAs, a major component of the astaxanthin esters of *Haematococcus* sp. KORIDI03 in this study.

3.5. Benefits of the drying film culture with mist feeding

The total fixed capital and operating costs based on the final biomass cell density were compared between the broth and drying film cultures. The biomass cell density of the group using 1 mL inoculum volume and 1 d feeding interval in this study was 4.47 ± 0.18 g/L. The total unit production cost of biomass was $38.66 ± 2.20 per gram. The final biomass cell density of the control was 0.63 ± 0.01 g/L, with the total unit production cost of the biomass being $327.51 ± 7.35 per gram. Both final biomass cell densities were converted to 1 g/L, and the results were then calibrated (Table 2) to summarize the fixed capital and operating costs in the drying film and broth culture processes, respectively. This experiment was conducted at a laboratory scale, focusing on the phenomena of repeated drying and replenishment of water in the

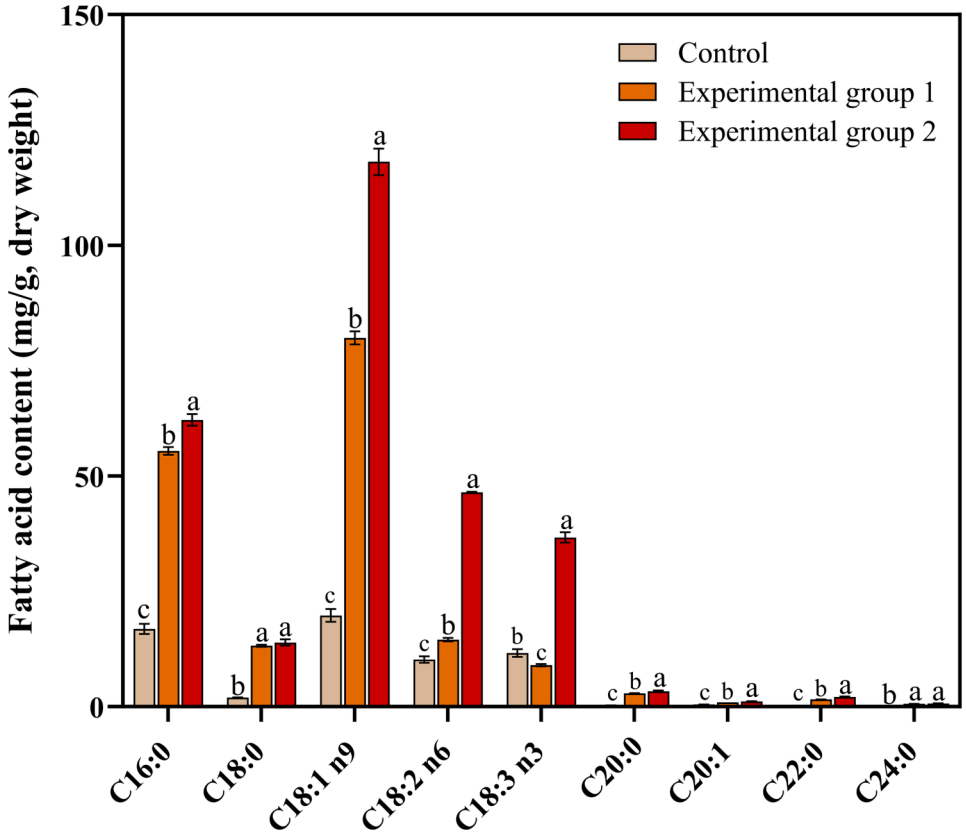

Fig. 4. Comparison of fatty acid profiles of *Haematococcus* sp. biomass cultured under various conditions, including broth culture (control), 1 mL inoculum volume and 1 d feeding interval (experimental group 1), and 1 mL inoculum volume and 3 d feeding interval (experimental group 2). The assays were performed in duplicate and different letters in the same row indicate significant differences, identified by Tukey's test ($p < 0.05$).

Table 2
Comparison of the unit costs of broth and drying film cultures. Input parameters and their values are presented as used for cost analysis. All input parameters are set based on laboratory standards using the biomass yield for drought and broth conditions as determined for each culture.

Culture types and parameters			Broth culture (control)				Drying film culture (experimental)			
Classification			Unit costs ($)	Quantities	Units	Total costs ($)	Unit costs ($)	Quantities	Units	Total costs ($)
Fixed capital cost	Culture systems	Growth chamber	50.00	1.00	set	50.00	50.00	1.00	set	50.00
		Light system	8.99	1.00	set	8.99	8.99	1.00	set	8.99
		Control system (air, temperature, and humidity)	70.00	1.00	set	70.00	70.00	1.00	set	70.00
Total fixed capital costs ($)			128.99				128.99			
Operating cost	Erlenmeyer flask (5 L)		33.09	1.00	EA	33.09	–	–	EA	–
	Petri dish		–	–	EA	–	0.18	9.00	EA	1.62
	Water usage[a]		0.48	1.59	L	0.76	0.48	0.22	L	0.11
	Electricity		0.94	39.60	kWh	37.22	0.94	39.60	kWh	37.22
	Labor[b]		1.52	1.00	hour	1.52	1.52	2.00	hour	3.04
	Chemicals in medium		2.20	1.59	L	3.50	2.20	0.22	L	0.48
	Agar usage		–	–	g	–	0.04	3.30	g	0.13
	Sprayer		–	–	EA	–	0.90	1.00	EA	0.90
	Filter		1.07	1.00	EA	1.07	–	–	EA	–
	Air tube		0.13	1.00	set	0.13	–	–	set	–
Total operating costs ($)			77.29				43.51			
Biomass production			0.63 ± 0.01 g/L				4.47 ± 0.18 g/L			
Total production cost ($/g)[c]			327.51 ± 7.35				38.66 ± 2.20			

[a]Water usage = $9.7 distilled water per 20 L.
[b]Labor = $5,000 per year (one employee).
[c]Total production cost = (Total capital cost + Total operating cost)/Biomass production.
Note. Both final biomass cell densities were converted to 1 g/L, and the results were then calibrated. Table 2 summarizes the details of the fixed capital and operating costs in the drying film and broth culture processes.

natural environment where *Haematococcus* are found. As each culture experiment (drying film and broth culture) was conducted using lab-scale culture systems, the total fixed capital and operating costs were set under the same conditions. The culture experiment was also performed at the same endpoint in the growth chamber. However, drying film culture and broth culture are entirely different culture methods that focus on area (solid) and volume (liquid) for biomass production, respectively. Therefore, when comparing the culture methods, environmental conditions (aeration, distance from the illumination, and mist feeding) had to be excluded; thus, an accurate comparison between broth culture and drying film culture inevitably had some limitations. The cost of water usage was converted from a unit of $9.70 per 20 L of distilled water to a base unit of $0.48 per 1 L. Of note, the cost of water usage in the broth culture was approximately 6.9 times higher than that of the drying film culture. The labor cost was also converted from annual labor to hourly labor cost. Each culture preparation process took approximately 1 h, and the sprayer injection time measured approximately 2 min for the drying film culture. The cost of chemicals for the medium of the broth culture was approximately 7.3 times higher than that of the drying film culture. However, the agar usage cost was $3.30 per gram solely for the drying film culture. Consequently, a considerable difference in nutrient cost with water usage was identified based on the OPEX data. These culture experiments were performed at a laboratory scale, whereas differences may occur in industrial-scale *Haematococcus* sp. cultures. Nevertheless, considering the advantages of the final biomass cell density and its water and medium usage, the *Haematococcus* culture conditions proposed in this study are expected to increase both biomass and pigment productivities and substantially reduce production costs at an industrial scale. However, research on drying film culture is still in its infancy, and several limitations must be overcome for its application at the industrial scale. First, applying the same amount of water to the medium on the industrial scale would warrant a larger area than that in broth culture. Hence, several alternatives, including a cabinet structure that stacks culture plates to limit the unit area and increase the height, are necessitated for improvement. Moreover, optimal conditions of humidity and temperature must be identified to minimize moisture evaporation from the agar medium. These improvements will allow the agar medium to be reused several times by harvesting the film biomass from the agar plate surface, further reducing water use (such as by mist feeding). A suitable processing method is also needed to harvest the produced biomass and extract astaxanthin. Further investigations of different stress combinations based on drought stress are also needed to minimize the accumulation time of astaxanthin.

4. Conclusions

In this study, a novel drying film culture method was developed for *Haematococcus* sp. cultures. The drying film culture method significantly improved biomass production and carotenoid pigment concentration under conditions of varying inoculum volumes and mist-feeding intervals. The proposed drying film culture method is a simplified culture process that effectively reduces the production cost compared with that of broth culture and may be developed at the industrial scale for the cost-effective culture of *Haematococcus* sp. Consequently, the proposed drying film culture method has the potential to optimize biomass and carotenoid pigment production in *Haematococcus*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Additional data will be made available by the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129827.

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