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Rapid Induction of Astaxanthin in *Haematococcus lacustris* by Mild Electric Stimulation

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Abstract: Efficient induction of astaxanthin (AXT) biosynthesis remains a considerable challenge for the industrialization of the biorefinement of the microalga *Haematococcus lacustris*. In this study, we evaluated the technical feasibility of photosynthetic electrotreatment to enhance AXT accumulation in *H. lacustris*. The AXT content of *H. lacustris* electrotreated at an optimal current intensity (10 mA for 4 h) was 21.8% to 34.9% higher than that of the untreated control group, depending on the physiological state of the initial palmella cells. The contents of other carotenoids (i.e., canthaxanthin, zeaxanthin, and β-carotene) were also increased by this electrotreatment. However, when *H. lacustris* cells were exposed to more intense electric treatments, particularly at 20 and 30 mA, cell viability significantly decreased to 84.2% and 65.6%, respectively, with a concurrent reduction in the contents of both AXT and the three other carotenoids compared to those of the control group. The cumulative effect of electric stimulation is likely related to two opposing functions of reactive oxygen species, which facilitate AXT biosynthesis as signaling molecules while also causing cellular damage as oxidizing radicals. Collectively, our findings indicate that when adequately controlled, electric stimulation can be an effective and eco-friendly strategy for inducing targeted carotenoid pigments in photosynthetic microalgae.

Keywords: *Haematococcus pluvialis*; *Haematococcus lacustris*; astaxanthin; electric treatment; oxidative stress; reactive oxygen species; carotenoid

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1. Introduction

Photosynthetic microalgae are valuable renewable resources for a diverse range of industrial applications, including food, nutraceuticals, pharmaceuticals, animal feed, biofuels, and biochemicals [1,2]. Astaxanthin (AXT), a prominent secondary ketocarotenoid pigment found in microalgae, has gained considerable commercial importance in the health food supplementation, cosmetics, and aquaculture industries owing to its potent antioxidant bioactivity and distinctive reddish coloration [3–5]. Furthermore, AXT exhibits substantial potential for therapeutic effects, including cardiovascular protection, immune modulation, antiaging, and anticancer effects [6,7].

Haematococcus lacustris (formerly referred to as *H. pluvialis* [8]) is a rich source of AXT (~5% of the dry cell weight) and is extensively cultivated to meet growing market demands [9]. This microalga accumulates AXT during its physiological and morphological transformation from vegetative cells to dormant cysts under unfavorable culture conditions [10]. AXT biosynthesis is linked to a complex defense mechanism that aids microalgal cells in the management of oxidative stress [11]. Various stress-based approaches, including intense light irradiance [12], high salinity [13], nitrogen deficiency [14], elevated

temperature [15], phytohormone supplementation [16], mechanical compression [17], and hydrostatic pressurization [18], have been employed to enhance AXT production. However, the development of an environmentally friendly and effective AXT induction strategy remains a substantial challenge in industrial microalgal biotechnology [6].

Electric stimulation techniques have been effectively applied to enhance the biosynthesis of various organic products in individual electroactive, acetogenic, and methanogenic microorganisms, as well as in mixed cultures [19,20]. This process is potentially sustainable because it uses renewable electrical energy at ambient pressure and temperature, thereby eliminating the need for chemical additives. The resulting renewable electricity-based chemicals (referred to as e-chemicals [21]) include ethanol, butanol, 3-hydroxypropionic acid, 1,3-propanediol, isopropanol, formic acid, butyric acid, and acetate. However, research on microalgae in this context remains limited, and the effect of electric treatment varies considerably based on the species and physiological cell status.

H. lacustris exhibits a complex life cycle consisting of five distinct cell types: red dormant cysts, germinating cysts, green biflagellate cells, green palmella cells, and greenish-brown palmella cells [22,23]. Kim et al. [24] subjected biflagellate cells of *H. lacustris* to a strong current intensity (100 mA) for a brief duration (1 min), resulting in a considerable increase (20%) in cell number density, although this treatment did not cause alteration in the AXT content compared to that of untreated controls after subsequent photosynthetic cultivation for seven days. Conversely, Fitriana et al. [25] continuously electrotreated *H. lacustris* biflagellate cells at a relatively low intensity (30 mA) for an extended period (two days), resulting in a 36.9% higher AXT content (6.0 mg/g cell) than that of the untreated cells. Aerobic electrochemical conditions [26] can generate reactive oxygen species (ROS), which act as signaling molecules to help microalgal cells adapt to stress and activate AXT biosynthesis [3]. However, severe cell damage occurs in electrotreated cells, including cell-wall and cytoplasm separation along with the extracellular secretion of biomaterials, which is likely due to the excess generation of oxidizing radicals triggered by electrotreatment and a significant drop in pH through water electrolysis [25].

To address these challenges, this study aimed to investigate the technical feasibility of mild electrotreatment (30 mA or below) to stimulate AXT accumulation in *H. lacustris* while maintaining cell stability under photosynthetic culture conditions. To this end, we utilized the greenish-brown palmella cells of *H. lacustris* containing previously synthesized AXT intermediates [18]. We found that very mild-intensity, short-duration treatment conditions yielded AXT accumulation approaching the maximum reported yields without any associated toxicity. Moreover, we propose an AXT induction mechanism based on microscopic observations and ROS analyses.

2. Materials and Methods

2.1. Microalga and Culture Conditions

H. lacustris NIES-144 was acquired from the National Institute for Environmental Studies (NIES), University of Tokyo, Tokyo, Japan, and cultivated under photosynthetic conditions in NIES-C medium (pH 7.5) [16]. Details regarding the medium composition and sterilization method are provided in Supplementary File S1.

For the seed culture, a single colony of *H. lacustris* grown on an agar plate was inoculated into 100 mL NIES-C medium in a 250 mL Erlenmeyer flask fitted with a porous silicon stopper and cultured for 30 days in a shaking incubator at 25 °C and 150 rpm [25]. The culture was exposed to continuous illumination at 80 ± 10 μmol photon/m^2/s emitted by white light-emitting diode (LED) lamps (ISF-7100RF, Jeiotech, Daejeon, Republic of Korea).

Using a flask culture as the inoculum, the main culture for electrical stimulation was established in a Pyrex-glass bubble-column photobioreactor (bc-PBR) with a height of 28 cm, an inner diameter of 4.5 cm, and a working volume of 400 mL. The NIES-C medium was supplemented with 5 mM sodium phosphate buffer (pH 7.0) for the bc-PBR culture. The inoculum concentration was approximately 0.2 g/L based on dry cell weight (DCW) measurements (see Section 2.6 for details). The bc-PBR was continuously supplied with

a gas mixture of 5.0% (v/v) CO_2 and air at a flow rate of 200 mL/min from the bottom of the reactor using a mass flow control system [18]. The bc-PBR was illuminated with a light intensity of 200 ± 10 µmol photon/m^2/s using three LED lamps positioned in front of the bc-PBR. The bc-PBR culture was maintained in a temperature-controlled room at approximately 25 °C.

2.2. Electric Stimulation of AXT Biosynthesis

Figure 1 illustrates the photosynthetic electrochemical system used to stimulate AXT biosynthesis in *H. lacustris*. The system comprised a two-chamber H-type electrochemical reactor, a power supply, two white LED lamps, and aeration control. The custom-made H-type reactor consisted of two glass bottles (working volume: 200 mL; Corning, Armonk, NY, USA) linked by a glass tube and separated by a proton-exchange membrane (PEM, PFSA D 125-U; Fuel Cell Store, Bryan, TX, USA), as described by Kim et al. [27]. The PEM was positioned in the middle of the tubes and firmly clamped. We used plain carbon rods (diameter, 6 mm; length, 50 mm; Shinsung Carbon, Gwacheon, Republic of Korea) connected to titanium wires (diameter, 1 mm; length, 12 cm; Seoul Titanium, Seoul, Republic of Korea) using an electroconductive silver paste (Elcoat P-100, Cans, Tokyo, Japan). The distance between the cathode and anode was maintained at 15 cm.

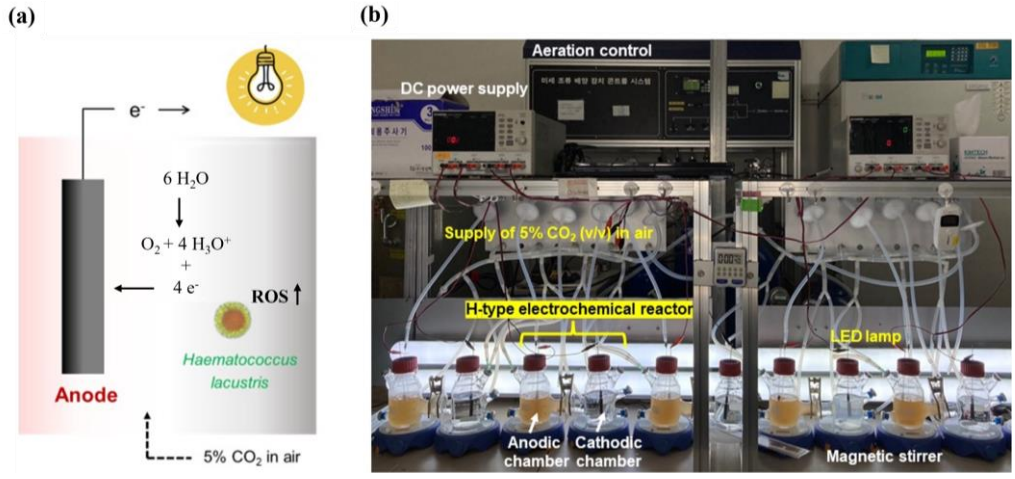

Figure 1. (**a**) Schematic diagram of photosynthetic electrotreatment and (**b**) illustration of the photosynthetic two-chamber electrochemical cell system used in this study, equipped with a power supply, light source, magnetic agitation, and aeration control to stimulate astaxanthin accumulation in *Haematococcus lacustris*. The diagram was modified from Fitriana et al. [25]. DC, direct current; LED, light-emitting diode; ROS, reactive oxygen species.

In previous experiments using the same microalgal strain, severe cell damage was induced even at a low current intensity of 3 mA in the cathodic chamber of an electrochemical system [25]. Therefore, in this study, mild electrical stimulation of the microalga was performed only in the anodic chamber. For the electric stimulation experiment, greenish-brown palmella cells of *H. lacustris* (0.8 ± 0.1 g/L), collected from a 4-day bc-PBR culture, were directly transferred to the anodic chamber of the H-type electrochemical reactor under clean bench conditions. A sterile NIES-C medium fortified with 11.2 mM phosphate buffer (pH 7.0) was introduced into the cathodic chamber to prevent substantial pH fluctuations. Currents of 4.8 ± 0.3, 10.0 ± 0.6, 20.1 ± 0.8, and 29.9 ± 0.4 mA were applied to the microalgal cells for up to 12 h by adjusting the voltage to 2, 4, 6, and 8 V, respectively, using a direct current power supply (GPD-43035, GWINSTEK, New Taipei City, Taiwan), as outlined by Choi et al. [28]. For convenience, each current value was designated as 5, 10, 20, or 30 mA on the main scale included in the relevant figures. Cyclic voltammograms of the carbon electrodes in the presence and absence of *H. lacustris* were obtained using

a single-chamber electrochemical reactor with three electrodes [25]. Notably, different oxidation and reduction current densities were observed in the microalgal cells.

To maintain the metabolic activity of the photosynthetic microalgae during the electrochemical stimulation experiments, continuous CO_2 and light supply were provided. Both anodic and cathodic chambers were aerated with 5.0% CO_2 (v/v) in air at a flow rate of 40 mL/min through stainless-steel needles (size 21G, LK Lab Korea Co., Namyangju, Republic of Korea) after being filtered using a 0.2 µm polyvinylidene fluoride air filter (Biofact Co., Daejeon, Republic of Korea), incorporating the same aeration control system as used for microalgal cultivation (see Section 2.1). Additionally, both chambers were independently stirred at 250 rpm using magnetic stirrers (Color Squid, IKA, Staufen, Germany) with polytetrafluoroethylene magnetic bars (size 30 × 8 mm; Bel-Art Products, Wayne, NJ, USA) to prevent the *H. lacustris* cells from settling. The electrochemical reactor received illumination with a light intensity of 200 ± 10 µmol photon/m^2/s in the same temperature-controlled room (approximately 25 °C) used for bc-PBR culture. During electrostimulation experiments for up to 12 h, the pH of the microalgal solution ranged from 6.9 to 5.9, and no marked changes in the cell number density of *H. lacustris* were observed (not shown). This indicates that the total cells remained at approximately 0.16 g during the electrotreatment, the same as the initial inoculum.

2.3. Cell Morphology and Viability Analyses

The morphological characteristics of *H. lacustris*, such as color, size, internal structure, and pigmentation, were assessed using an Axiolab bright-field microscope equipped with a digital camera and Zen 3.2 software (Carl Zeiss, Jena, Germany). The color of algal cells can change due to the considerable migration of AXT-rich oil globules when exposed to high-level illumination for more than 15 min [29]. Therefore, to avoid artificial changes in cell color, algal cells were observed under a light microscope immediately after sampling. Cell viability (%) after electrochemical treatment was evaluated using an improved Neubauer hemocytometer (Marienfeld, Lauda-Königshofen, Germany) after staining with trypan blue dye (Sigma Aldrich, St. Louis, MO, USA), which can penetrate the cytoplasmic membranes of damaged microalgal cells [18,30,31]. The comprehensive staining protocol is provided in Supplementary File S2.

2.4. ROS Measurement

Intracellular ROS levels of microalgal cells were assessed using the cell-permeable fluorogenic probe method employing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Sigma Aldrich) [32,33]. Briefly, 1 mL of *H. lacustris* solution from the electrochemical reactor was transferred to an amber 1.5 mL Eppendorf tube, centrifuged at 10,000× g for 5 min (Legend Micro 17R; Thermo Fisher Scientific, Waltham, MA, USA), and washed with 0.1 M phosphate buffer saline (PBS; pH 7.0). Next, 990 µL of 0.1 M PBS and 10 µL of DCFDA were added to the microalgal cells and incubated in the dark at 37 °C for 60 min (VS-8480; Vision Scientific Co., Daejeon, Republic of Korea). The mixture was intermittently vortexed during incubation (FINE VORTEX; FINEPCR, Gunpo, Republic of Korea). Microalgal cells were harvested and washed twice with 0.1 M PBS (pH 7.0). After centrifugation, 100 µL of the supernatant was transferred to a 96-well plate (SPL Life Sciences, Pocheon, Republic of Korea) for analysis using a fluorescence spectrophotometer (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA). The fluorescence of the sample was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, and the average intensity was used to indicate the ROS level.

2.5. Carotenoid Quantification

Following electrotreatment, *H. lacustris* cells were collected by centrifugation at 1952× g for 10 min (Combi R515; Hanil Science Co., Gimpo, Republic of Korea), washed twice with distilled water, freeze-dried using a lyophilizer for 48 h (FD8508; IlShinBioBase Co., Daejeon, Republic of Korea), and stored at −22 °C until further analysis. The cellular

contents of AXT and other carotenoids (i.e., canthaxanthin, zeaxanthin, and β-carotene) in *H. lacustris* were analyzed using bead beating–based solvent extraction and subsequent high-performance liquid chromatography (HPLC), according to recently published protocols [16,18]. Approximately 2 mg of lyophilized *H. lacustris* cells were mixed with 1.0 g of glass beads (1.5 mm diameter; Daihan Scientific, Gangwon, Republic of Korea) and 1 mL of extraction solution (dichloromethane/methanol [1:1, *v*/*v*] containing 0.025 M NaOH). *H. lacustris* cells were mechanically disrupted using a FastPrep-24 bead-beater (6 m/s for 30 s for three cycles; MP Biomedicals, Irvine, CA, USA). Subsequently, the *H. lacustris* extract was saponified, filtered, and analyzed using HPLC. Details of the methods and HPLC conditions are provided in Supplementary File S3.

2.6. Other Analytical Methods

The DCW was gravimetrically determined by filtering 4 mL of the microalgal broth through a Whatman glass microfiber filter (GF/C, diameter 47 mm; Buckinghamshire, UK). The filter was washed with purified water and dried overnight at 70 °C [10]. The light intensity was measured using a quantum photometer (LI-250A, Li-Cor Inc., Lincoln, NE, USA), and the pH was measured using a pH meter (HM–30R, DKK-TOA Co., Tokyo, Japan). The electric current was measured using a digital multimeter (DT 4282, HIOKI E.E. Co., Nagano, Japan).

2.7. Statistical Analysis

Plotting and statistical data analyses were conducted using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA). The results derived from independent duplicate reactors are expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) was determined using two-tailed *t*-tests.

3. Results and Discussion

3.1. Morphological Changes during 10 mA Electrotreatment for 12 h

In this study, greenish-brown palmella cells of *H. lacustris* cells collected from a 4-day-old photobioreactor culture were subjected to continuous electrotreatment at a current intensity of 10 mA for 12 h under photosynthetic conditions. Fitriana et al. [25] reported significant cell damage in the green flagellate *H. lacustris* upon exposure to a higher current intensity of 30 mA for 2 days, resulting in a marked decrease in pH to 2.3 despite the use of a phosphate buffer. Therefore, a relatively modest current intensity of 10 mA, which is generally recommended for electrochemical bacterial research [28], was employed to ensure the metabolic stability of the photosynthetic microalgae. This approach was combined with the use of NIES-C medium supplemented with a phosphate buffer under continuous illumination at a light intensity of 200 μmol photon/m^2/s, as optimized by Mahadi et al. [18].

Figure 2 illustrates the microscopic morphological changes in *H. lacustris* during the photosynthetic electrotreatment at 10 mA. At 12 h, the untreated control microalgal cells had retained their initial greenish-brown pigmentation. Conversely, cells electrotreated for 4 h showed increased size and color intensity of the reddish globules located at the center of the cytoplasm. This indicated that electrical stimulation induced a rapid accumulation of AXT compared to that in control cells. However, the overall color intensity of the treated cells gradually decreased after 4 h of electrotreatment, together with a distinct separation of the cytoplasmic content from the cell wall, especially at 12 h. These observations suggest that excessive and prolonged exposure to electrical stress can hinder the viability of *H. lacustris*, as previously observed after electrotreatment at 30 mA [25].

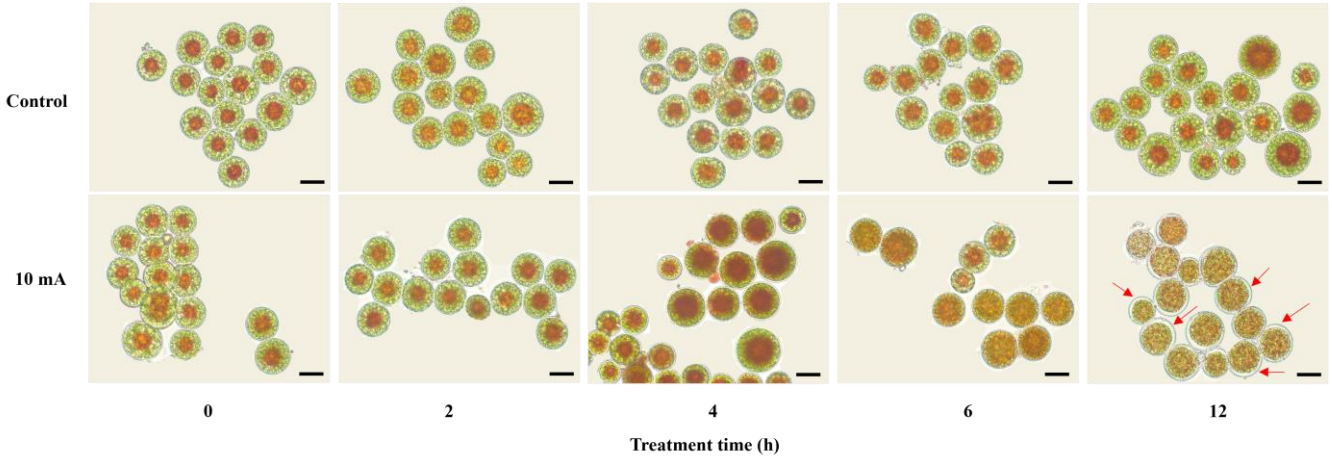

Figure 2. Time-course profiles of microscopic features in *Haematococcus lacustris* electrotreated for 12 h at 10 mA. Red arrows in the 12 h image show the separation of the cytoplasm from the cell wall. Scale bar: 20 μm.

3.2. AXT Accumulation and Cell Viability during 10 mA Electrotreatment for 12 h

Figure 3 shows the quantification of cellular AXT content determined by HPLC analysis. Untreated microalgal cells maintained AXT levels (7.6 ± 0.2 mg/g DCW) consistent with their initial content (7.6 ± 0.7 mg/g DCW) after 12 h. In contrast, microalgal cells treated with 10 mA for 4 h reached a maximum AXT content of 10.2 ± 0.4 mg/g DCW, showing a significant increase of 34.9 ± 4.3% over that of the untreated controls. However, similar to the microscopic observations, a progressive decrease in AXT content was observed in *H. lacustris* after 6 and 12 h of prolonged electrotreatment, declining to 7.1 ± 0.3 and 4.1 ± 0.7 mg/g DCW, respectively.

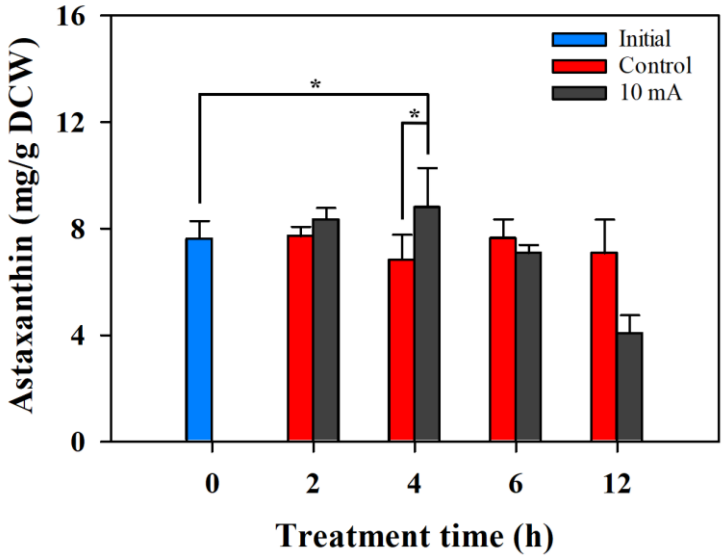

Figure 3. Time-course profiles of astaxanthin content in *Haematococcus lacustris* electrotreated for 12 h at 10 mA. The error bars represent the standard deviations of four samples. * $p < 0.05$. DCW, dry cell weight.

To evaluate the effect of electrochemical stress on *H. lacustris* during 10 mA electric treatment for 12 h, cell viability was assessed by trypan blue dye staining [18,31]. Within the range of 2–4 h, the electrotreated microalgal cells consistently maintained cell viability rates close to 100%, similar to those of the untreated controls (Supplementary Figure S1). However, after 6 and 12 h of electrotreatment, the cell viability gradually decreased to 96.9 ± 0.7% and 89.2 ± 1.1%, respectively. This decline may be associated with the mor-

phological perturbations observed during these treatment durations (Figure 2). Overall, the combined results from HPLC and cell viability assessments suggested that very mild electrical stimulation, particularly at 10 mA for 4 h, effectively triggered AXT biosynthesis in *H. lacustris* without detrimental effects on cell viability.

3.3. Effect of Current Intensity on AXT and Other Carotenoids

Algal carotenoids are classified into carotenes and xanthophylls. Carotenes contain only hydrocarbons in their structure (e.g., β-carotene), while xanthophylls are oxygenated carotenoids, which contain various functional groups such as hydroxy (zeaxanthin) and keto (AXT and canthaxanthin) groups [34]. AXT is closely tied to the formation of β-carotene, a primary precursor that undergoes enzymatic transformations involving intermediates during carotenogenesis. In *H. lacustris*, the trajectory from β-carotene to AXT involves a biochemical route via a canthaxanthin intermediate. Zeaxanthin is also synthesized as a primary carotenoid, similar to β-carotene [35,36].

Figure 4a–d illustrate the relative content (%) of AXT and other carotenoid pigments (i.e., β-carotene, zeaxanthin, and canthaxanthin) in electrotreated *H. lacustris* under different current intensities (5, 10, 20, and 30 mA) compared to that of the untreated controls. The treatment time was fixed at 4 h based on the results from the previous 10 mA experiment (Figure 3). Increasing the current intensity from 5 to 10 mA led to a parallel upward trend in the levels of the three other carotenoids, similar to the AXT pattern. At 10 mA, the relative contents of AXT, canthaxanthin, zeaxanthin, and β-carotene in electrotreated cells were 121.8 ± 4.2%, 123.4 ± 7.0%, 111.1 ± 2.9%, and 116.1 ± 5.0%, respectively, representing the highest percentage increases compared to the contents of untreated cells. This indicates that a carefully tuned electrostimulation strategy can potentially stimulate multiple enzymatic pathways for the production of desired carotenoids in microalgae.

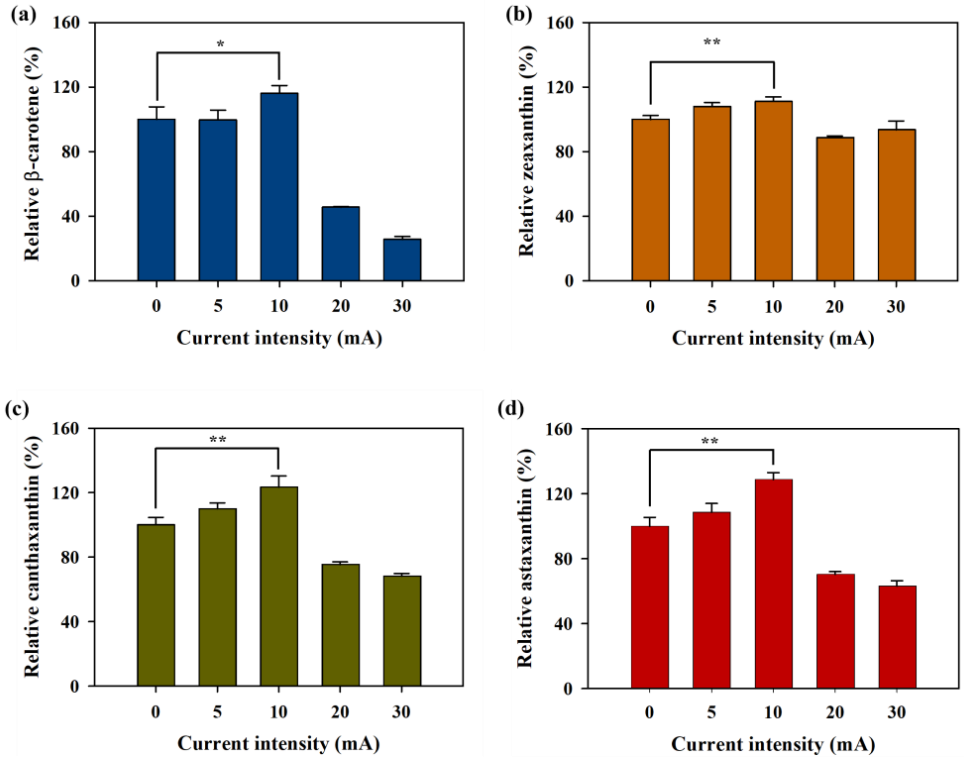

Figure 4. Effect of current intensity on the relative contents of β-carotene (**a**), zeaxanthin (**b**), canthaxanthin (**c**), and astaxanthin (**d**) of *Haematococcus lacustris* electrotreated for 4 h under different current intensities (5, 10, 20, and 30 mA) compared to those of the untreated controls. The relative content (%) of each carotenoid pigment is presented compared to that of the untreated control (100%). The error bars represent the standard deviations of four samples. * $p < 0.05$ and ** $p < 0.005$.

However, at higher currents of 20 and 30 mA, the contents of all quantified carotenoids in *H. lacustris* gradually decreased with increasing current intensity compared to those of the untreated controls (Figure 4a–d). This trend corresponds to the time-dependent profile of AXT biosynthesis shown in Figure 3 and aligns with the previously reported build-up of neutral lipids upon electrotreatment in *Chlorella* [28]. These findings suggest that a threshold exists at which the positive effects of electrostimulation on microalgae are inhibitory. Therefore, to maximize AXT electrostimulation, it is crucial to properly manage the electrochemical conditions while simultaneously ensuring the vitality of the microalgae. This necessitates a comprehensive exploration of various bioelectrochemical engineering aspects, such as determining the ideal duration and magnitude of the electric current; selecting appropriate operation modes such as continuous, intermittent, or gradual applications; ensuring effective supplies of CO_2 and light; devising optimal electrode materials and structures; and even considering the use of exogenous electron mediators.

Notably, under identical electrotreatment conditions of 10 mA for 4 h, the relative enhancement in AXT content differed significantly in different experiments: 34.9 ± 4.3% (Figure 3) and 21.8 ± 4.2% (Figure 4d) compared to that of their respective untreated control groups. This variability could potentially be attributed to the utilization of initial greenish-brown palmella *H. lacustris* cells that exist in distinct developmental and physiological states. Consistent with this speculation, the actual AXT content differed between the two populations (7.6 ± 0.7 and 3.5 ± 0.7 mg/g DCW, respectively). This observation suggests that the effect of electrostimulation may be dependent on the inherent biological characteristics of *H. lacustris*, including its metabolic vigor and pigment content (i.e., carotenoids and chlorophyll pigments), which warrants further research.

3.4. Effect of Current Intensity on Cell Morphology, Viability, and ROS

Figure 5a shows the cell viability rate of *H. lacustris* at various current intensities (5, 10, 20, and 30 mA) after 4 h of electrotreatment. As expected, microalgal cells subjected to very mild currents of 5 and 10 mA maintained high cell viability rates, ranging from 98.1% to 99.1%, which were similar to those of the untreated controls (99.0%). However, at current intensities of 20 and 30 mA, the cell viability rates significantly decreased to 84.2% and 65.6%, respectively.

To evaluate the impact of 4 h of electrotreatment at different currents (5, 10, 20, and 30 mA) on AXT biosynthesis and cell damage, the relative generation of ROS in electrotreated *H. lacustris* cells was compared with that in untreated cells (Figure 5b). As the current intensity increased from 5 to 20 mA, the relative ROS level (%) in the electrotreated microalgal cells progressively increased from 17.1% to 34.9% above those in the untreated controls. However, at the highest intensity of 30 mA, the ROS content decreased to a level similar to that of the control group.

Based on these results, we propose an ROS-mediated induction hypothesis to explain the enhanced AXT accumulation in *H. lacustris* cells during electrotreatment. Given the prevailing electrochemical conditions coupled with a continuous air supply, various ROS species, including H_2O_2, ·OH, O_3, and $\cdot O_2^-$, were likely generated [26]. Notably, ROS can exert dual and contrasting effects on photosynthetic metabolism in microalgae. In particular, ROS can function as effective signaling molecules that promote the synthesis and accumulation of lipids and secondary carotenoid pigments such as AXT [37,38]. However, when present at excessive concentrations, ROS can damage crucial cellular components, including lipids, proteins, and DNA, potentially leading to cell death [39,40]. Our results suggest that *H. lacustris* has evolved to synthesize AXT in response to oxidative damage, which acts as an antioxidant and efficiently scavenges ROS [41].

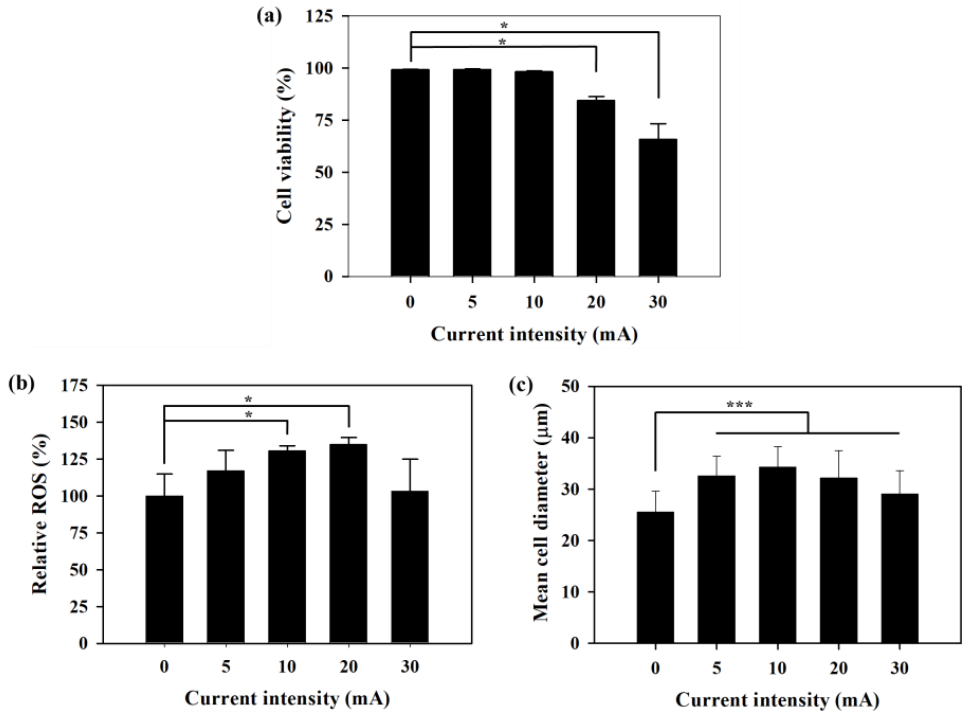

Figure 5. Effect of current intensity (5, 10, 20, and 30 mA) on the relative cell viability (**a**), reactive oxygen species (ROS) content (**b**), and mean cell diameter (**c**) of *Haematococcus lacustris* after electrotreatment for 4 h. The relative value (%) is presented compared to that of the untreated control (100%). The error bars for (**a**,**b**) represent the standard deviations of four samples, and those for (**c**) represent at least 100 cells randomly measured in two independent electrochemical experiments. * $p < 0.05$ and *** $p < 0.001$.

The relationship between AXT synthesis and ROS concentration in *H. lacustris* is complex. The concurrent increase in carotenoid production (Figure 4) and ROS levels (Figure 5b) in the microalgal cells treated with 5–10 mA compared to those of the controls suggests that ROS might promote the synthesis of AXT and the three other carotenoids. However, at higher ROS levels, specifically 20 mA, a threshold for cell damage was reached, leading to reduced AXT biosynthesis (Figure 4) and cell viability (Figure 5a). Notably, at the most stressful current of 30 mA, a lower ROS level was observed compared with that of the untreated control. This decrease could be attributed to the severe disruption of cellular homeostasis and essential enzyme systems, including esterases, involved in the measurement of ROS. Various biochemical analyses, including the determination of photochemical activity and the ratio of chlorophyll to carotenoid pigments [42], are required to understand the physiological and biochemical responses of microalgae during electrotreatment. Multiple factors, including ROS formation, acidification, and electrolysis of medium components, can affect the electrostimulation of microalgae. Furthermore, it is also necessary to elucidate the exact bioelectrochemical mechanism through additional studies, such as those involving the external addition of hydrogen peroxide.

Figure 5c shows the changes in the mean cell diameter of *H. lacustris* after 4 h of electrotreatment at different current intensities (5, 10, 20, and 30 mA). In normal photosynthetic cultures, beyond approximately 20 days, *H. lacustris* palmella cells subjected to nutrient limitation and aging gradually undergo volumetric expansion, accumulate AXT, and eventually transform into dormant cysts [16]. In this study, the mean cell diameter of untreated control cells (25.5 ± 4.1 μm) remained unchanged compared to that of the initial cells (25.1 ± 4.2 μm) owing to the short 4 h incubation duration.

Notably, all electrotreated microalgal cells exhibited a significant increase in mean cell diameter, irrespective of the current intensity, compared with that of the control group. The mean cell diameter peaked at 34.2 ± 4.0 μm (34.3% higher than that of the control) at

10 mA. However, the microalgal cells treated at higher current intensities of 20 and 30 mA had smaller mean cell diameters than those treated with lower intensities of 5 and 10 mA. This reduction in volume may be associated with microscopic morphological damage, such as the extracellular release of intercellular materials through damaged cell walls. Cell wall damage may be related to reduced cell viability, as determined by staining with trypan blue [18,30,31] (Figure 5a).

The increase in the mean cell diameter (Figure 5c) or volume expansion of the microalgal cells under beneficial electrotreatment conditions may be related to the enhanced AXT content (Figure 4). Notably, AXT in *H. lacustris* is predominantly present in lipid bodies, mainly in the form of mono- and diesters with high fatty acid contents [43,44]. Similar concurrent increases in cell size and neutral lipid content have been reported in microalgae exposed to oxidative stress; however, the underlying mechanisms remain unclear. Yilancioglu et al. [45] observed an increased biovolume in *Chlamydomonas reinhardtii* in response to oxidative stress caused by H_2O_2, correlating with an elevated lipid content. Choi et al. [28] reported simultaneous increases in cell size and neutral lipid content in *Chlorella* after a similar 4 h electrotreatment. To gain deeper insight into the morphological transformations, including changes in cell size and cell wall structure, occurring in *H. lacustris* during electrostimulation, more extensive biochemical investigations are necessary.

4. Conclusions

Mild electric treatment at 10 mA for 4 h effectively induced rapid AXT accumulation in *H. lacustris*, particularly during the greenish-brown palmella stage, resulting in a significant improvement of 21.8% to 34.9% AXT compared to the level in the untreated controls. However, prolonged or excessive electrotreatment resulted in a reduction in the content of AXT and three other carotenoids (canthaxanthin, zeaxanthin, and β-carotene). Electrotreatment at higher intensities of 20 and 30 mA significantly decreased cell viability to 84.2% and 65.6%, respectively, and caused distinctive morphological alterations, including a decreased intensity of the red color and damaged cell walls. Under physiologically mild electrotreatment conditions, the elevated levels of ROS in electrotreated microalgal cells relative to those in the control are likely associated with enhanced AXT accumulation in *H. lacustris*.

Supplementary Materials: The following supporting information can be downloaded from https://www.mdpi.com/article/10.3390/app132312959/s1, Supplementary File S1: NIES-C medium preparation; Supplementary File S2: Cell viability analysis; Supplementary File S3: Carotenoid quantification; Figure S1: Cell viability of *Haematococcus lacustris* after 10 mA electrotreatment over 12 h.

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