Research Article

Enhancement of β-Cryptoxanthin Production in Three Different Green Microalgae Species Using an Innovative Red LED Wavelength Shift Approach

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Abstract
β-Cryptoxanthin is a natural carotenoid pigment with several important functions for human health, including antioxidant, provitamin A, and anticancer activities. Microalgae could be a potential source to produce β-cryptoxanthin instead of its production from plants. In this research study, the effect of the red light-emitting diode (LED) at wavelengths 620 to 750 nm and different intensities of 50, 100, 200, and 300 μmol/m^2.s was evaluated for the second phase of the two-stage microalgae cultivation with three microalgae species: *Scenedesmus obliquus*, *Coelastrum morus*, and *Chlorococcum* sp. The results were focused on biomass production, β-cryptoxanthin, and total carotenoid contents to select the microalgae strain that could produce a high amount of β-cryptoxanthin under optimized light conditions. Red LED with an intensity of 200 μmol/m^2.s resulted in the biomass production of 5.23 ± 0.27 g/L, 5.72 ± 0.25 g/L, and 5.70 ± 0.17 g/L in three microalgae species of *Scenedesmus obliquus*, *Coelastrum morus*, and *Chlorococcum* sp., respectively. In addition, when compared with 50 μmol/m^2.s red LED, the highest β-cryptoxanthin content was obtained from the condition of the red LED at 100 μmol/m^2.s for *S. obliquus* (229.22 ± 5.11 μg/g DCW) and 200 μmol/m^2.s for *C. morus* (311.01 ± 4.75 μg/g DCW) and *Chlorococcum* sp. (383.68 ± 6.63 μg/g DCW). The applied approach to the proper manipulation of LED color, wavelengths, and intensities in this study will enable the improvement of biomass and enhance β-cryptoxanthin production in three tested microalgae species.

Keywords: β-Cryptoxanthin, Bioactive compound, LED Artificial light, Microalgae, Stress conditions

1 Introduction

In recent years, microalgae have become a main source of high-value carotenoid products used as functional food products and food supplements [1]. Some outstanding commercial microalgae for carotenoid production are lutein from *Desmodesmus* sp., astaxanthin from *Haematococcus pluvialis*, and β-carotene from *Dunaliella salina* [2]–[5]. Microalgae cultivation has replaced the production of carotenoid by plant because of their fast growth rate, high yield per unit area, less land use, potential cultivation in non-agricultural land, and so on [6]. However, their current biomass yield and carotenoid production efficiency still required improvement so the production cost would be reduced [7]. Therefore, the development of microalgae cultivation for high biomass and carotenoid yield is very challenging for the industrial scale production of the carotenoids.

This research presents one of the highly effective carotenoids, β-cryptoxanthin. Beta-cryptoxanthin ($C_{40}H_{56}O$) is a bioactive compound, also known as a xanthophyll carotenoid, almost like β-carotene in chemical structure and bioactivity. Despite having an additional hydroxyl group at the third carbon atom of the β-ring, β-cryptoxanthin exhibits greater polarity than β-carotene. Conjugated double bonds or chromophores in the structure of β-cryptoxanthin (Figure 1) allow it to absorb light and give plants color and photoprotection [8]–[10]. Beta-cryptoxanthin is converted to vitamin A in human serum and tissues, and it exhibits higher bio-accessibility and bioavailability than lycopene and β-carotene [11], [12]. β-Cryptoxanthin has been found to possess strong antioxidant qualities, it has also been shown to have bioactivity against cancer, diabetes, and liver disorders, reduce neuropathic pain, stimulate immunity, reduce blood pressure, and prevent bone loss [13]–[19]. β-Cryptoxanthin is also found in only some fruits and vegetables. The highest concentration of β-cryptoxanthin was detected in butternut squash at 34.71 μg/g sample [20], [21]. Commercially available natural β-cryptoxanthin is the product from the extraction of satsuma mandarin orange (18.00 μg/g sample). Interestingly, several microalgae, including *Scenedesmus obliquus*, *Spirulina maxima*, and *Chlorella vulgaris* could produce β-cryptoxanthin at 23.76, 20.13, and 15.05 μg/g dry weight, respectively [22], [23].

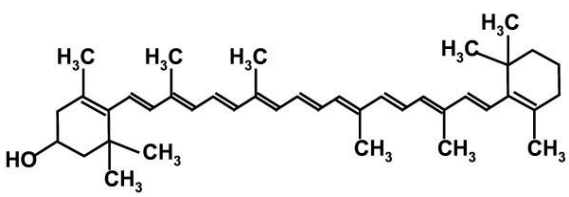

Figure 1: Chemical structure of β-cryptoxanthin.

A two-stage cultivation strategy is a generic countermeasure to maximize cell development and the creation of valuable molecules. The first stage of the strategy is devoted to optimizing biomass production, while the second stage is reserved for the accumulation of carotenoids under varying stress conditions [24]. Another important reason for using microalgae to produce carotenoids is that microalgae have strong protective defense capability against stressful environmental conditions for their cells [25]. Stress conditions such as high light intensity, temperature, and salinity, as well as the limitation of nitrogen and phosphate, can enhance the synthesis of carotenoid and other bioactive compounds in microalgae cells [26]. Light intensity and spectrum are the major factors that effectively enhance microalgae growth curing the cultivation [24]. White, blue, and red LED were applied to enhance both biomass and β-cryptoxanthin in the two-stage culture of microalgae [27], [28]. Ma *et al*. [29] reported that blue, and red light-emitting diode (LED) light irradiation in satsuma mandarin (*Citrus unshiu* Marc.) showed that β-cryptoxanthin accumulation was induced by red light, not by blue light.

Three microalgal strains, *Scenedesmus obliquus*, *Coelastrum morus*, and *Chlorococcum* sp. were reported as high-potential strains for carotenoids production [30], [31] and *S. obliquus* showed high production of β-cryptoxanthin [23]. Our previous study reported that the use of red LEDs could potentially enhance the production of β-cryptoxanthin by three microalgae, *Scenedesmus obliquus*, *Coelastrum morus*, and *Chlorococcum* sp., approximately 29.43–33.27% higher than the use of white and blue LEDs at the same light intensity [32]. The production of β-cryptoxanthin in microalgae cultivation was confirmed with the standard liquid chromatography-high resolution mass spectrometry (LC-HRMS/MS). In this study, we aimed to enhance the production of biomass and β-cryptoxanthin with increasing red LED light intensity. The experiment started by investigating the effects of red LED with different light intensities on the production of biomass, total carotenoids, and β-cryptoxanthin in the second stage of the two-stage cultivation by three microalgae strains. The information obtained could be used for microalgae cultivation to obtain higher β-cryptoxanthin production yields, which will enable the food industry to produce a more competitive product in terms of production cost.

2 Materials and Methods

2.1 *Chemicals*

Standard of β-cryptoxanthin (≥ 97%) was procured from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) and potassium hydroxide (KOH) were obtained from Sigma Aldrich, USA. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sisco Research Laboratories Pvt. Ltd., India. The following materials were obtained from LAB-SCAN (Gliwice,

Poland): ammonium acetate, acetonitrile, methyl tert-butyl ether (MTBE), methanol (MeOH), petroleum ether, and diethyl ether. MeOH and MTBE were of the high-performance liquid chromatography (HPLC) grade while all other reagents and chemicals used were analytical grade.

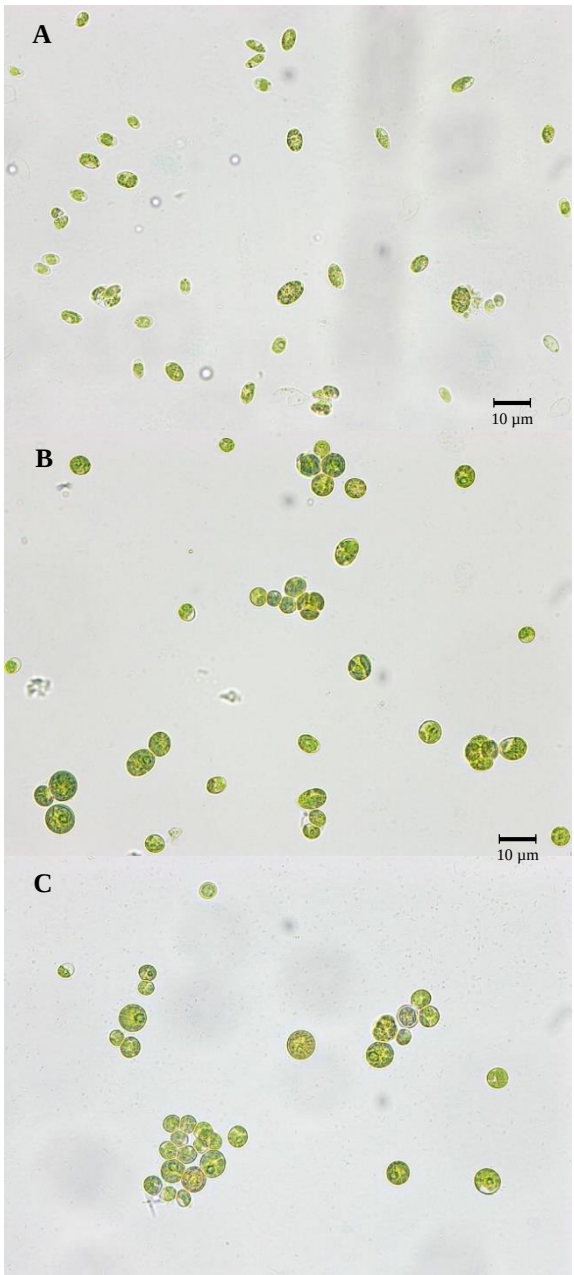

Figure 2: Three microalgae strains used for the experiment, *S. obliquus* (A), *C. morus* (B) and *Chlorococcum* sp. (C).

2.2 *Microalgae strains and biomass production*

Three strains of microalgae, *Scenedesmus obliquus* strain TISTR 8522, *Coelastrum morus* strain TISTR 8566 and *Chlorococcum* sp. strain TISTR 8266 were kindly provided by the algae library of Thailand Institute of Scientific and Technological Research (TISTR). The morphology of three microalgae is presented in Figure 2. Each strain was pre-cultured at 25 °C under 10 µmol/m^2/s of cool white light in 80 mL of synthetic BG11 liquid medium at pH 7 in a 250 mL Erlenmeyer flask and shaken reciprocally at 120 rpm for 5 days before the transfer to further biomass production.

Each microalgae strain was cultivated to produce cell biomass for the first stage cultivation in a laboratory bottle (1000 mL) with 800 mL of BG11 medium, using a system consisting of a rubber stopper with a glass tube, an air stone and a 0.22 µm polytetrafluoroethylene polymer (PTFE) filter. The cultivation was carried out with aeration of 0.1 air volume per culture volume per minute (vvm) for 14 days under 50 µmol/m^2/s of fluorescent cool white light, a photoperiod of 12:12 (dark: light cycle illumination), at 25ºC. The cell biomass was harvested by centrifugation (Hermle Z206A, Germany) with 9,000 g for 15 min at 10ºC. The cell pellets were transferred to the fresh BG11 for further experiments.

The biomass was measured by a dry cell weight (DCW) method. Whatman GF/C filter papers (47 mm diameter, 1.2 µm pore size), which contain the sample were dried in a hot air oven for one hour at 80°C and overnight at 60°C. After 30 minutes in a vacuum desiccator, filter sheets were removed, and their empty weights were calculated with an analytical balance. Until constant weights were achieved, the drying and weighing processes were repeated. Pre-weighed and pre-dried filter papers were used to filter samples of homogenized cultures [33].

2.3 *Effect of light intensity on total carotenoids and β-cryptoxanthin production*

The second stage of the two-stage cultivation was investigated in this experiment. Light intensity for β-cryptoxanthin production was determined at 50, 100, 200, and 300 µmol/m^2/s of red LED light has a wavelength of 620 to 750 nm. The inoculum at 1 g/L was used for the cultivation of all microalgae strains. The temperature was controlled at 25 °C. Three microalgae growths were measured by dry weight methods. The microalgae cell was harvested after 8

days of cultivation. The cultivation was performed in triplicate. The cell pellets were subsequently washed with distilled water before freeze-drying at –50 °C, pressure around –175 μmHg for 24 h with a freeze-dryer (LSCplus, Germany). Then, the freeze-dried samples were kept in a freezer at –20 °C.

2.4 *Carotenoids extraction and total carotenoid content analysis*

β-cryptoxanthin and other carotenoid compounds were carefully extracted from each freeze-dried sample (200 mg) using a mortar and pestle, ethyl acetate, and methanol. The supernatant was centrifuged at 9,000 g for 15 minutes at 10 °C until it became colorless [34]. The extract was concentrated at 30 °C using a vacuum rotary evaporator (BUCHI R-114, Fawil, Switzerland) after being filtered through a 0.22 μm polyethylene membrane. The concentrated extract was subsequently suspended in a 1:1 v/v petroleum ether/diethyl ether mixture and saponified for 16 h at room temperature using 10% (w/v) methanolic KOH. Washing the sample with 10% (w/v) sodium chloride eliminated the alkali.

The volume of the extract was made up of petroleum ether and measured for its absorbance at 450 nm by a spectrophotometer. Total carotenoid content (μg/g) was calculated using the following formula (Equation (1)):

$$\text{Carotenoid content} = [(A \times V \times 10^4)] / [A_{1cm}^{1\%} \times P] \quad (1)$$

where A = absorbance; V = total extract volume (mL); P = sample weight (g); $A_{1cm}^{1\%}$=2592 (β-carotene extinction coefficient in petroleum ether). Finally, the carotenoid extract was flushed with N_2 and kept at –20 °C in the dark until further analysis [35].

2.5 *β-Cryptoxanthin identification and content analysis*

Identification of β-cryptoxanthin from the algal cell was performed with the standard β-cryptoxanthin, using Dionex Ultimate 3000 RSLC system liquid chromatography-high resolution mass spectrometry (LC-HRMS/MS) following the method described by Chuechomsuk *et al*. [32]. The LC-HRMS/MS installed with an orbitrap mass analyzer system (QEXACTIVE plus, Thermo Fisher Scientific, Germany) was used for the analysis. Each compound was separated into samples by running with a Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 μm Particle size, Thermo Fisher Scientific) at 37 °C. The carotenoid extract from the freeze-dried sample was suspended in methanol and filtered with a 0.22 μm polyethylene membrane before analysis by LC-HRMS. The mobile phase consisted of 3 mM ammonium acetate in methanol /water (70:30, v/v; mobile phase A) and 3 mM ammonium acetate in acetonitrile/diethyl ether (99:1, v/v; mobile phase B). The linear gradient was programmed as follows: 0:00–0:20 min 100% A, 3:50–15:50 min 100% B, and 15:75–20:00 min A. The flow rate was set as 0.5 mL/min with an injection volume of 20 μL.

The content analysis of β-cryptoxanthin was performed by the high-performance liquid chromatography, HPLC (KNAUER Model AZURA, Berlin, Germany) with a system consisting of a pump (AZURA P 6.1 L), a diode array detector (AZURA DAD 2.1 L,) and a C30 YMC column 5 μm, 250 × 4.6 mm (YMC America, Inc.). The mobile phase is composed of methanol (mobile phase A) and methyl tert-butyl ether (mobile phase B) in a linear gradient. A linear gradient was applied (95:5 to 70:30 in 30 min, to 50:50) in 20 min. The sample injection volume was 15 μL and the mobile phase flow rate was set at 0.9 mL/min. The detection was performed at a wavelength of 450 nm [23]. The processing of chromatogram data was performed using the Clarity Chrom software (KNAUER, Berlin, Germany).

2.6 *Statistical analysis*

The results were reported as the mean ± SD. IBM SPSS software (SPSS Inc.) version 28 for Windows, one-way analysis of variance (ANOVA) and post-hoc Duncan's test with p-value < 0.05 were used to determine the significance of the variables. A minimum of three replications were conducted for each experiment.

3 Results and Discussion

3.1 *Biomass production*

Light intensity for the growth at the first stage of each microalgal strain was set at 50 μmol/m^2.s of fluorescent cool white light. The accumulation of the biomass production of *S. obliquus*, *C. morus*, and *Chlorococcum* sp. at 14 days was 2.67 ± 0.11 g/L, 2.35 ± 0.14 g/L, and 2.50 ± 0.11 g/L, respectively. The biomass production of *S. obliquus*, *C. morus*, and

Chlorococcum sp. from each cultivation using red LED with different light intensities at 50, 100, 200, and 300 µmol/m^2.s are presented in Figure 3. The inoculum at 1 g/L was used for the cultivation of all microalgae strains. A significant increase of the microalgae biomass at 8 days of cultivation with the increase of red LED light intensity from 50 to 300 µmol/m^2/s was observed. Interestingly, *C. morus*, and *Chlorococcum* sp. cultivated under 200 µmol/m^2.s of light intensity yielded the highest biomass at 5.72 ± 0.25 g/L, and 5.70 ± 0.17 g/L, respectively, while *S. obliquus* also indicated the highest biomass at 5.23 ± 0.27 g/L with the same light intensity. On the contrary, when the light intensity went beyond 200 µmol/m^2.s and reached 300 µmol/m^2.s, the lower biomass of each microalga was obtained.

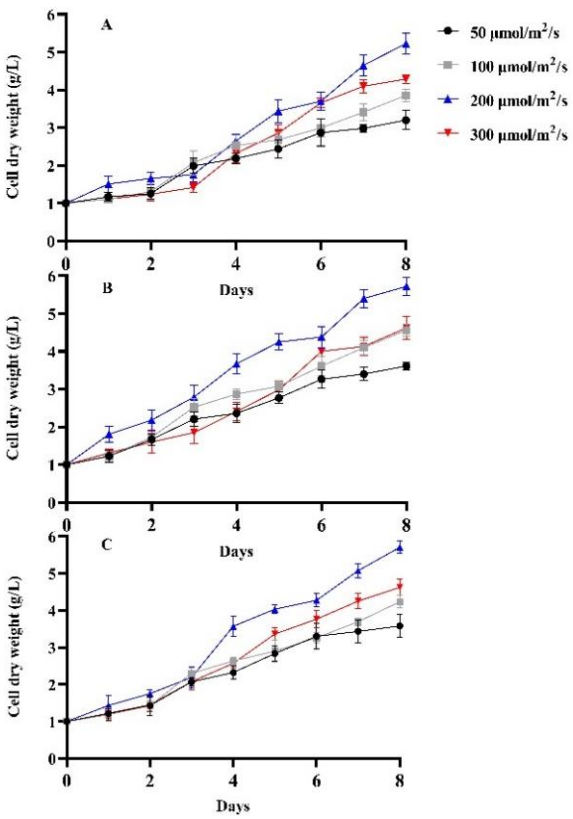

Figure 3: Cell dry weight obtained during the cultivation of *S. obliquus* (A), *C. morus* (B) and *Chlorococcum* sp. (C) after using red LED with different light intensities.

This might be due to a phenomenon known as photoinhibition [36]. Either low lighting or excessive lighting could result in limited growth of the microalgae. At low light levels, photosynthesis increases almost linearly with increasing lighting. Nevertheless, an additional increase in lighting does not result in a higher rate of photosynthesis in a region of saturated light intensities. The photosynthetic system can be harmed by excessive light exposure to microalgae, which could hinder and lower the rate and efficiency of photosynthesis [37], [38].

Photoinhibition's molecular mechanisms are quite complex. Cells that are photo inhibited may produce more reactive oxygen species (ROS) than usual. ROS is a crucial component of microalgae metabolism and a natural consequence of their respiration. Numerous parameters, including cell size and shape, cell density, development stage, light intensity, and temperature, influence ROS levels. ROS at low concentrations can act as signaling molecules that can stimulate the growth and reproduction of algae cells. However, ROS at high concentrations is harmful to cellular components and exhibits inhibitory effects on microalgae growth [39], [40].

3.2 *Total carotenoid content*

Carotenoids are one of the main compounds contributing to antioxidant capacity in microalgae. Figure 4 presents the effect of increasing the red LED light intensity on carotenoid production with three microalgae strains. An increase in the red LED light intensity for the cultivation of *S. obliquus*, *C. morus*, and *Chlorococcum* sp. led to a higher total carotenoid content of all strains. However, total carotenoid contents from *Chlorococcum* sp. cultivation with 50, and 100 µmol/m^2.s (2.83 ± 0.30, and 2.89 ± 0.16 mg/g DCW) were not significantly different from that of *S. obliquus* cultivation with 50 µmol/m^2.s (3.05 ± 0.30 mg/g DCW). In addition, the carotenoid content of *S. obliquus* (4.51 ± 0.15 mg/g DCW), and *C. morus* (4.78 ± 0.12 mg/g DCW) was not significantly different when the algae were exposed to 200 µmol/m^2.s light intensity. Increasing the light intensity to 300 µmol/m^2.s could significantly increase total carotenoid content in *C. morus* (8.31 ± 0.14 mg/g DCW), *S. obliquus* (6.40 ± 0.32 mg/g DCW), and *Chlorococcum* sp. (5.58 ± 0.36 mg/g DCW).

One of the primary mechanisms of photoprotection is based on the capacity of carotenoids, particularly those linked to a xanthophylls group, to engage in reversible light-dependent reactions known as non-photochemical quenching, which results in the dissipation of excess excitation energy of chlorophylls as heat [41], [42]. Additionally,

carotenoids in cells can filter light, reducing the quantity that passes through a photosynthetic mechanism. Furthermore, carotenoids lead to the chemical or physical inhibition of O_2 generated in the photosystem II reaction center, the primary location for O_2 production, when exposed to high light intensities. It has been observed that when microalgae cells are exposed to bright light for an extended period, they exclusively accumulate specific carotenoids. [37], [43].

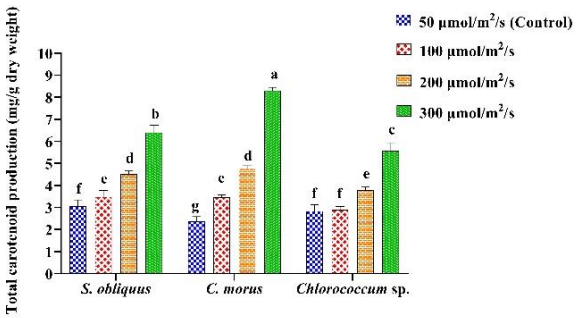

Figure 4: Total carotenoid production by *S. obliquus*, *C. morus*, and *Chlorococcum* sp. using red LED with different light intensities. Different letters in each bar mean significant differences at $p \leq 0.05$.

3.3 *Optimization of the β-cryptoxanthin production*

The carotenoid extract from all microalgae identified by LC-HRMS/MS exhibited the same fragment patterns as the β-cryptoxanthin standard. The fragment ions of beta-cryptoxanthin identity were molecular ion $[M]^+$ 552.4326, characteristic ion $[M-92]^+$ 460.3699 generated from carotenoids polyene (isoprene skeleton chain) losing a hydroxylated group and toluene (C_7H_8), identical ion m/z 119.0858 and elimination/cleavage of hydrocarbon at polyene (isoprene skeleton chain) of carotenoids. The results were the same as our previous report that β-cryptoxanthin can be produced by all three microalgae.

The HPLC chromatograms of the carotenoids in the extracts of each microalga cultivated with red LED light at 50 µmol/m^2.s are presented in Figure 5. Peak 16 in the chromatograms with retention time (RT) at 19.65 min was identified as β-cryptoxanthin. Additionally, Figure 6 presents the β-cryptoxanthin content from each microalga under different light intensities indicating that the production by *S. obliquus* was significantly increased from 193.49 ± 5.67 µg/g DCW to 229.22 ± 5.11 µg/g DCW when the light intensity was increased from 50 µmol/m^2.s to 100 µmol/m^2.s. In contrast, the increased light intensity from 200 µmol/m^2.s to 300 µmol/m^2.s conversely decreased its β-cryptoxanthin content. The same behavior was observed with the *Chlorococcum* sp. and *C. morus*. The highest β-cryptoxanthin content at 383.86 ± 6.63 µg/g DCW was obtained from the *Chlorococcum* sp. cultivated by 200 µmol/m^2.s of red LED. However, β-cryptoxanthin content from *C. morus* (311.01 ± 4.75 µg/g DCW) with 200 µmol/m^2.s was not significantly different from β-cryptoxanthin content from *Chlorococcum* sp. (311.68 ± 9.18 µg/g DCW) with 100 µmol/m^2.s.

Increasing the threshold of the red LED light intensity for the microalgae cultivation in our research revealed a much higher β-cryptoxanthin content compared to previously reported studies. For instance, *Scenedesmus obliquus*, *Spirulina maxima*, and *Chlorella vulgaris* could produce 23.76, 20.13, and 15.05 µg of β-cryptoxanthin/g dry weight, respectively [22], [23]. The content of β-cryptoxanthin produced in our study was also higher than the main source of β-cryptoxanthin reported [20], [21], such as butternut squash (34.71 µg/g sample) and satsuma mandarin orange (18.00 µg/g sample). This might be because photosynthetic organisms are exposed to high light intensity, then the total content of carotenoids, particularly those involved in the xanthophyll cycle will increase. However, above a certain threshold of light intensity, the content of specific xanthophylls like β-cryptoxanthin may decrease. This could be due to the conversion of these xanthophylls into other forms as part of the xanthophyll cycle. For example, under strong light, the enzymatic conversion of violaxanthin to zeaxanthin occurs, which helps dissipate excess energy. This process might reduce the content of other xanthophylls, including β-cryptoxanthin. Also, it is important to note that the response to light intensity can vary among different species of microalgae and other photosynthetic organisms. Thus, the specific changes in carotenoid and xanthophyll content can highly depend on the organism and its environmental conditions [44], [45]. Therefore, the type and intensity of light should be selected to suit the cultivation of each microalga.

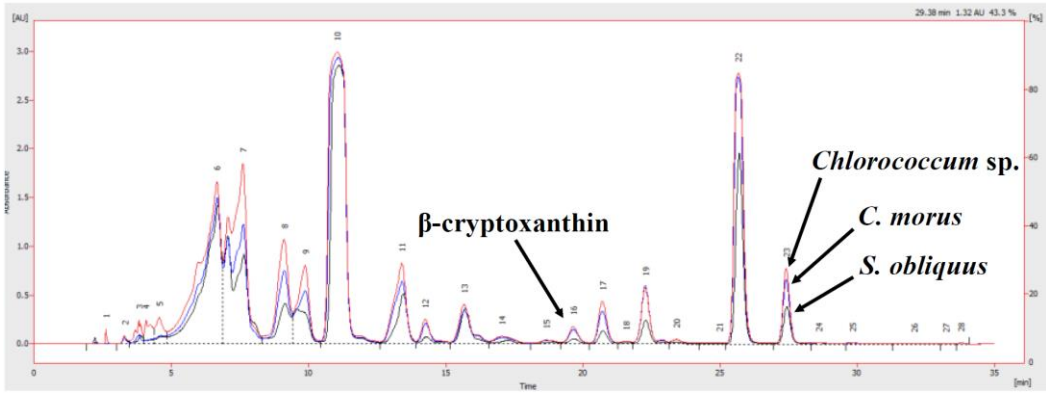

Figure 5: Chromatogram of compounds detected in the extract prepared from the cultivation of *S. obliquus* (black line), *C. morus* (blue line) and *Chlorococcum* sp. (red line) with red LED lights at 50 µmol/m^2.s. Peak 16 was identified as β-cryptoxanthin.

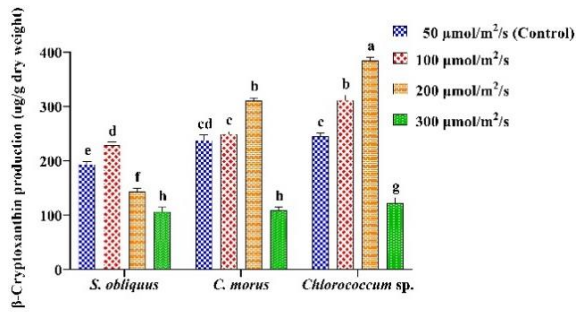

Figure 6: Beta-cryptoxanthin production in the cultivation of *S. obliquus*, *C. morus*, and *Chlorococcum* sp. using red LED with different light intensities. Different letters in each bar mean significant differences at *p*-value ≤ 0.05.

4 Conclusions

A second stage of a two-stage cultivation strategy using the red LED at different light intensities was investigated by the cultivation of three microalgae strains. In summary, this study discovered that the increased red LED light intensity could enhance biomass production, total carotenoid content, and β-cryptoxanthin content. The highest β-cryptoxanthin content was obtained from the condition for each microalga as *S. obliquus* 229.22 ± 5.11 µg/g DCW, *C. morus* 311.01 ± 4.75 µg/g DCW and *Chlorococcum* sp. 383.68 ± 6.63 µg/g DCW. However, extended exposure to light for microalgae could damage the photosynthetic system through photo-oxidative processes, which limits photosynthesis and increases ROS and reduction. Therefore, oxidative stress has the potential to greatly enhance the microalgae species' economic attributes. We have managed to get the ideal equilibrium between the development and growth of β-cryptoxanthin content in three different microalgae strains.

In our further study, we plan to use the microalgae as a new source for β-cryptoxanthin production. This approach will address the sustainability concern and align with the goals of developing eco-friendly and economically viable processes. Some research showed that the innovation of cultivating microalgae in food processing wastewater greatly reduced the cost of wastewater treatment compared to a conventional approach in terms of lower carbon emissions, energy consumption, and chemical usage while producing microalgae biomass, which can benefit low-cost fertilizer, bioactive compounds, bioplastic applications, and biofuel production. For example, the feasibility of agri-food waste such as corncob, banana peels, and onion residues as potential biostimulants for the lab-scale cultivation of *Chlorella vulgaris* [46]–[48]. We have planned to use the microalgae as a new source for β-cryptoxanthin production in our future work. This approach will address the sustainability concern and align with the goals of developing eco-friendly and economically viable processes. The other factors influencing carotenoids, especially beta-cryptoxanthin, such as medium composition, salinity, and temperature will be further investigated.

Acknowledgments

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Author Contributions

S.C.: methodology, software, data curation, formal analysis; N.B.: methodology, data curation; S.K.: methodology; M.S.K.: review & editing; B.L.: review & editing; B.T.: supervision, funding acquisition, formal analysis; V.R.: conceptualization, supervision, funding acquisition, writing – original draft, review & editing.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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