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Photoperiod influenced the growth and antioxidative responses of *Chlorella vulgaris*, *Isochrysis galbana*, and *Tetraselmis chuii*

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ABSTRACT

Microalgae are rich in natural antioxidants, with occurrence and quality depending primarily on the species of microalgae and the conditions of cultivation. Light and photoperiod affect microalgae accumulation of antioxidants. This study examined the growth, biomass, and antioxidant responses of microalgae *Chlorella vulgaris* (UMT-M1), *Isochrysis galbana* (CB), and *Tetraselmis chuii* (CT) grown in 30 ppt F/2-enriched seawater and exposed to 12:12 and 24:0 hours light/dark cycles at 24°C ± 2°C. Overall cell density, wet biomass, chlorophyll a, chlorophyll b, carotenoids, and ascorbic acid content of UMT-M1, CB, and CT increased upon continuous light exposure for 8 days ($p < 0.05$). Constant light exposure induced α-tocopherol accumulation in UMT-M1 and CT microalgae but not CB. Results indicated that as a response to continuous light treatment, UMT-M1, CB, and CT may exert natural antioxidant protection mechanism.

INTRODUCTION

Microalgae contain lipids, carbohydrates, proteins, pigments, essential fatty acids, antioxidants, and vitamins (Mobin *et al*., 2019; Nethravathy *et al*., 2019; Safafar *et al*., 2015), where all bioactive compounds are potentially useful in pharmaceutical and nutraceutical applications (Mobin *et al*., 2019). In particular, antioxidant compounds have been exploited for use in human healthcare and therapeutic product formulation, and in this sense, natural microalgae antioxidants are considered a valuable resource compared to synthetic antioxidants obtained from chemical processes (Safafar *et al*., 2015; Smerilli *et al*., 2019; Tan *et al*., 2020). The synthetic antioxidants have raised many human health concerns and controversies; for instance, they trigger liver damage and carcinogenesis (Hamidi *et al*., 2020). Contrary to artificial antioxidants, microalgae are the ultimate choice to meet the incremental demands in the perspective of human population growth as they are fast growing with high biomass, aimed at complementing the antioxidant production from traditional plants (Barkia *et al*., 2019; Sansone and Brunet, 2019).

Microalgae are photosynthetic organisms that use light, carbon dioxide, and water to produce food in the form of biological macromolecules like proteins, lipids, and carbohydrates (Morales *et al*., 2019). Light and photoperiod affect the growth and yield of microalgae during cultivation (Ahmad *et al*., 2020; Zhang *et al*., 2019). In addition, the period of light and dark exposure until the saturation point at which the maximal photosynthetic rate is reached may control cellular contents (Darvehei *et al*., 2018; Sirisuk *et al*., 2018), including chlorophyll and antioxidants (Maroneze *et al*., 2016). Light promotes the synthesis of various bioactive compounds (Chandra *et al*., 2016; Mobin *et al*., 2019; Ye *et al*., 2017) in which they play a pivotal role in suppressing the development of reactive oxygen species (ROS) from molecular oxygen (O_2) in chloroplasts, as well as mitochondria, apoplasts,

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and peroxisomes (Noshi *et al*., 2016), with superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) (Assunção *et al*., 2017; Khorobrykh *et al*., 2020). Constant illumination may cause an imbalance between ROS output and its scavengers (Khorobrykh *et al*., 2020). Excessive ROS development promotes oxidative damage to lipids and other molecules in cytosols or within chloroplasts, and in this regard, microalgae provide a wide array of antioxidants to combat oxidative stress (Mobin *et al*., 2019; Szymańska *et al*., 2017). Antioxidants consist of enzymatic (superoxide dismutase, peroxidase, and catalase) and nonenzymatic groups (water-soluble vitamin C, lipid-soluble vitamin E, and quenchers such as β-carotene) (Assunção *et al*., 2017; Yu *et al*., 2017). Antioxidant production generally depends on the form and period of stress as well as the species of microalgae (Szymańska *et al*., 2017; Yu *et al*., 2017).

Up until now, most of the research conducted with microalgae focused on biomass production (Che *et al*., 2019; Kato *et al*., 2019; Ren *et al*., 2020; Vendruscolo *et al*., 2019; Zhang *et al*., 2019) and determination of the cellular contents such as lipid (Che *et al*., 2019; Kato *et al*., 2019; Medved *et al*., 2020; Ren *et al*., 2020; Vendruscolo *et al*., 2019; Zhang *et al*., 2019), fatty-acid (Che *et al*., 2019; Kato *et al*., 2019; Vendruscolo *et al*., 2019), chlorophyll (Gonzalez-Camejo *et al*., 2019; Patel *et al*., 2019; Vendruscolo *et al*., 2019), protein (Medved *et al*., 2020; Vendruscolo *et al*., 2019; Zhang *et al*., 2019), and carbohydrate content (Chong *et al*., 2019; Kato *et al*., 2019; Medved *et al*., 2020) upon the variations in the light and dark exposure. The role of photoperiods in the development of antioxidants and their responses to microalgae, however, is poorly understood. The goal of this study was to determine the effect of the photoperiod on growth and antioxidant responses of three species of marine microalgae, *Chlorella vulgaris*, *Isochrysis galbana* (CB), and *Tetraselmis chuii* (CT), commonly found in tropical brackish and marine environments. This evaluation is important to extend the knowledge of optimizing the culture conditions of microalgae with high antioxidants producing capacity.

MATERIALS AND METHODS

Microalgae stock and inoculum culture

Chlorella vulgaris (UMT-M1), CB, and CT obtained from Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Terengganu, Malaysia, were grown with F/2 medium containing $NaNO_3$ (0.88 mM), NaH_2PO_4 (0.036 mM), $Na_2EDTA.H_2O$ (0.012 mM), $FeCl_3.6H_2O$ (0.012 mM), $MnCl_2.4H_2O$ (0.91 µM), $ZnSO_4.7H_2O$ (0.077 µM), $CoCl_2.6H_2O$ (0.042 µM), $Na_2MoO_4.2H_2O$ (0.026 µM), $CuSO_4.5H_2O$ (0.039 µM), thiamine-HCl (0.30 µM), biotin (8.84 nM), and cyanocobalamin (0.37 nM) (Guillard, 1975; Guillard and Ryther, 1962) at 30 ppt salinity and pH 8.0 ± 0.2. The cultures were incubated at 24°C ± 2°C under constant illumination (2,000 lux) using 6,500 K daylight white light emitting diode lamp. The aeration Hailea HAP-120 pump (Hailea Group Co., China) was supplied with an airflow rate of around 120 l/minutes of air under the pressure of 0.018 MPa, filtered through a 0.22 µm Minisart® Sartorius syringe filter to avoid contamination. The cultivation of stock culture was first introduced in 100 ml of the liquid medium in a 250 ml flask before upscaling to 500 ml took place. The inoculum cultures were upscaled to 450 ml of the liquid medium in 500 ml conical flasks and harvested at 1×10^6 cells/ml prior to use in experiments. Microalgae were tested for purity and density and subsequently inoculated into separate flasks for use in photoperiod experiments.

Photoperiod treatment

Microalgae inoculated from the stock cultures were grown under two photoperiod conditions; one was given continuous illumination (24:0 hours light/dark cycles) and the other 12 hours light (12:12 hours light/dark cycles), respectively. The scaled-up inoculum was culture and treated accordingly until the end of the exponential phase (8 days). The 12 hours light (12:12 hours light/dark cycles) was controlled with a 24-hour programmed electrical timer (MS1144, Eurosafe, Malaysia). Experiments were conducted in three batches of cultivation with three biological replications for each batch, followed by three technical replicates in individual biological replicate. The detailed experimental setup of continuous and 12 hours light was shown in Figure 1.

Determination of microalgae growth and biomass

Cell density

Cell density was estimated regularly for 13 days. Approximately 200 µl of the cultures was diluted with 800 µl of Lugol solution (White *et al*., 2014). 10 µl of the diluted solution was transferred to the Neubauer hemocytometer and the cells were counted using a compound microscope (Leica CME, Leica Microsystems GmbH, Germany) (Sahastrabuddhe, 2016). Cell densities were determined using the following formula:

$$\text{Cell density (cells/ml)} = \frac{\text{Total number of cells} \times \text{dilution factor}}{\text{Total number of squares} \times \text{Volume of the square at 0.01 mm depth}}$$

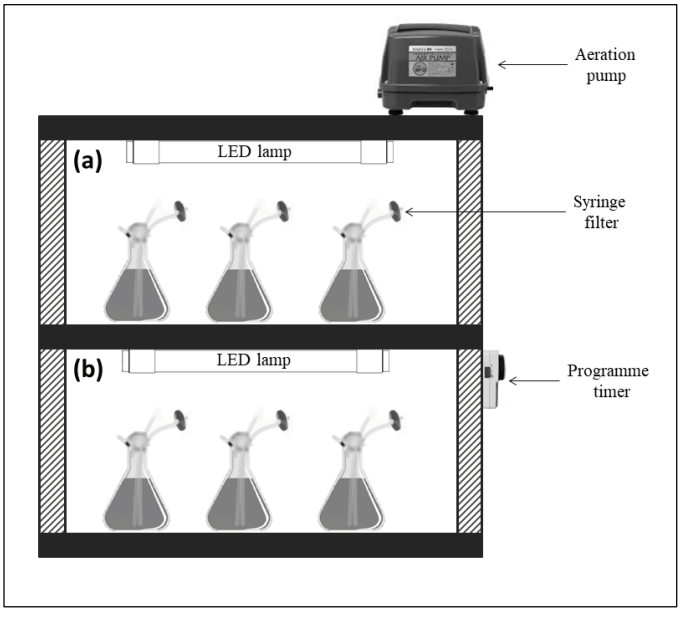

Figure 1. Experimental setup of (a) 24:0 hours L/D and (b) 12:12 hours L/D treatments.

Wet and dry biomass determination

The wet and dry biomass of microalgae were determined on day 8 (at the end of the exponential and early stationary phase). 50 ml culture was centrifuged 10 minutes with an Allegra X-30R Centrifuge, Beckman Coulter, Inc., Krefeld, Germany at 1,000 × g. The supernatant was discarded and wet microalgae paste was washed twice with the same amount of distilled water. Microalgae paste was weighed with a balance (ME204, Mettler Toledo, Switzerland) and the wet biomass was recorded in g/l. The wet paste was dried in the oven at 70°C ± 2°C for 24 hours until a constant weight was obtained, with the latter reported as dry biomass (g/l) (Kong *et al*., 2011). The remaining microalgae culture was centrifuged at 1,000 × g for 10 minutes and microalgae cells were harvested for antioxidant assays.

Determination of antioxidative assays

Chlorophylls and carotenoids contents determination

The content of chlorophylls and carotenoids was determined as essentially defined by Torres *et al*. (2014). Approximately 0.05 g of fresh microalgae cells was homogenized for 10 minutes with 1.5 ml of absolute methanol using an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Fisher Scientific, Schwerte, Germany) at 0°C–4°C. The homogenate was centrifuged (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Krefeld, Germany) at 9,168 × g for 10 minutes at 4°C. 200 µl of supernatant was transferred to a 96-well plate, with 200 µl of absolute methanol used as control. The plate was shaken in a microplate reader for 10 seconds (Varioskan™ LUX, Thermo Fisher Scientific, Vantaa, Finland). The chlorophylls and carotenoids absorbance were measured at 470, 653, and 666 nm (Lichtenthaler and Wellburn, 1983). The chlorophylls and carotenoids contents were calculated using the following formula:

Chlorophyll a, Chl a (mg/g FW)

$$= 15.65(A_{666}) - 7.34(A_{653}) \times \frac{V}{1{,}000 \times \mathrm{FW} \times d}$$

Chlorophyll b, Chl b (mg/g FW)

$$= 27.05(A_{653}) - 11.21(A_{666}) \times \frac{V}{1{,}000 \times \mathrm{FW} \times d}$$

Carotenoids (mg/g FW)

$$= \frac{1{,}000\ (A_{470}) - 2.86(\mathrm{Chl\ a}) - 129.2(\mathrm{Chl\ b})}{245} \times \frac{V}{1{,}000 \times \mathrm{FW} \times d}$$

where A is the absorbance value; V is the total extract volume; FW is the microalgal fresh weight; and d is the light path length.

Ascorbic acid content determination

Ascorbic acid content was determined following the procedures by Norhayati *et al*. (2016) with minor modifications. 1 ml of 10% trichloroacetic acid was applied to 0.05 g fresh microalgae cells and then homogenized for 10 minutes using an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Fisher Scientific, Germany) at 0°C–4°C. The homogenates were centrifuged (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Krefeld, Germany) at 9,168 for 10 minutes ×g in cold condition (4°C). 30 µl of the supernatant was then diluted to a total volume of 200 µl with distilled water and subsequently transferred to a flat bottom 96-well plate with 200 µl of distilled water used as a control (blank). 20 µl of 10% of the Folin-Ciocalteu reagent was applied. The plate was incubated in a microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, Vantaa, Finland) at 25°C for 10 minutes, where a blue color would be formed. The absorbance was measured at 760 nm (A_{760}) and the ascorbic acid value was calculated on a standard ascorbic acid curve (y = 0.022 × −0.0015) constructed at a concentration of 0–5 µg/ml. The content of ascorbic acid was calculated using the following formula:

$$\text{Ascorbic acid (mg / g FW)} = \left(\frac{A + 0.0015}{0.022}\right)\left(\frac{Vrm \times Veb}{d \times Ve \times \mathrm{FW}}\right)\left(\frac{\mathrm{df}}{1{,}000}\right)$$

where A is the absorbance value; Vrm is the total reaction mixture volume in well; Veb is the total extraction buffer volume; d is the light path length; Ve is the total extract volume in well; FW is the microalgal fresh weight; and df is the dilution factor.

α-Tocopherol content determination

α-Tocopherol content was determined using methods adapted by Norhayati *et al*. (2016) with minor modifications. Approximately 0.05 g of fresh microalgae cells was homogenized with 1.5 ml of absolute acetone in cold (0°C–4°C) for 10 minutes. The mixture was extracted with 0.5 ml hexane and then homogenized with an ultrasonic bath (Fisherbrand™ Elmasonic S 60H, Fisher Scientific, Germany) for 10 minutes at 0°C–4°C. Homogenates were then centrifuged (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Germany) for 10 minutes at 9,168 × g in cold condition (4°C). After centrifugation, the upper layer of the supernatant was removed and the hexane extraction was repeated twice. 25 µl of hexane extract was transferred to the flat bottom of the 96-well plate, with 25 µl of absolute ethanol used as control. Then 20 µl of 0.1% 3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine and 20 µl of 0.1% ferric chloride were added to the layer. 85 µl of absolute ethanol was applied to the microplate reader (Varioskan™, Thermo Fisher Scientific, Finland) for color production before the plate incubation at 25°C for 4 minutes. 10 µl of 0.2 M orthophosphoric acid was added to the mixture. The microplate was incubated at 25°C for another 10 minutes in the microplate reader prior to the 554 nm (A_{554}) absorbance measurement. The α-tocopherol value was calculated based on the standard curve (y = 0.0045 × +0.0046) constructed at a concentration of 0–5 µg/ml. The content of α-tocopherol was measured using the following formula:

$$\alpha\text{ - Tocopherol (mg / g FW)} = \left(\frac{A - 0.0046}{0.0045}\right)\left(\frac{Vrm \times Veb}{d \times Ve \times \mathrm{FW}}\right)\left(\frac{1}{1{,}000}\right)$$

where A is the absorbance value; Vrm is the total reaction mixture volume in well; Veb is the total extraction buffer volume; d is the light path length; Ve is the total hexane extract volume in well; FW is the microalgal fresh weight; and df is the dilution factor.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science software version 20. The mean comparisons were evaluated using a one-way analysis of variance

analysis. Multiple mean comparisons were determined by the Tukey test, while a comparison between 12:12 and 24:0 hours L/D cycles in each species was analyzed using a paired *t*-test sample. The differences were considered to be significant at $p < 0.05$. Bivariate (Pearson) correlations were conducted to evaluate the hypotheses of association between cell density, biomass (wet and dry), and antioxidative responses like chlorophyll a, chlorophyll b, carotenoids, ascorbic acid, and α-tocopherol.

RESULTS AND DISCUSSION

Effect of photoperiod on microalgae growth and biomass

Photoperiod affected the cell density and biomass of *C. vulgaris* (UMT-M1), CB, and CT during culture. The cell densities of UMT-M1, CB, and CT were higher in 24:0 hours L/D cycle than in 12:12 hours L/D cycle (Fig. 2), but only UMT-M1 and CT were significantly higher ($p < 0.05$) in 8 days of continuous light exposure (Table 1). The wet biomass obtained for UMT-M1, CB, and CT samples grown under constant light was higher ($p < 0.05$) compared to 12:12 hours L/D, observations consistent with Maroneze *et al*. (2016) analysis. It was discovered that, with constant illumination, *Scenedesmus obliquus*, a green microalga, achieved maximum cell density and biomass. Similar findings have been obtained with *Nannochloropsis* sp. when constant light was provided at 50 and 100 µmol m^{-2} s^{-1} (Wahidin *et al*., 2013) as well as *N. salina* and *Phaeodactylum tricornutum* at 250 µmol m^{-2} s^{-1} (Sirisuk *et al*., 2018). On the contrary, *I. galbana* reached optimum density when the culture was carried out with a 12:12 hours L/D cycle at 400 µmol m^{-2} s^{-1} (Che *et al*., 2019), suggesting that certain microalgae species do not need constant light to achieve maximum growth, as in the case of *Neochloris conjuncta*, *N. terrestris*, and *N. texensis* reported by Krzemińska *et al*. (2014). Interestingly, this study showed that the dry biomass of UMT-M1 grown with these two photoperiod cycles was significantly different, but this condition was not observed in CB and CT, which showed that dry biomass is associated with cellular water content, which is dependent on microalgae organisms, cell size, and length of light exposure (Chioccioli *et al*., 2014; Khoeyi *et al*., 2012).

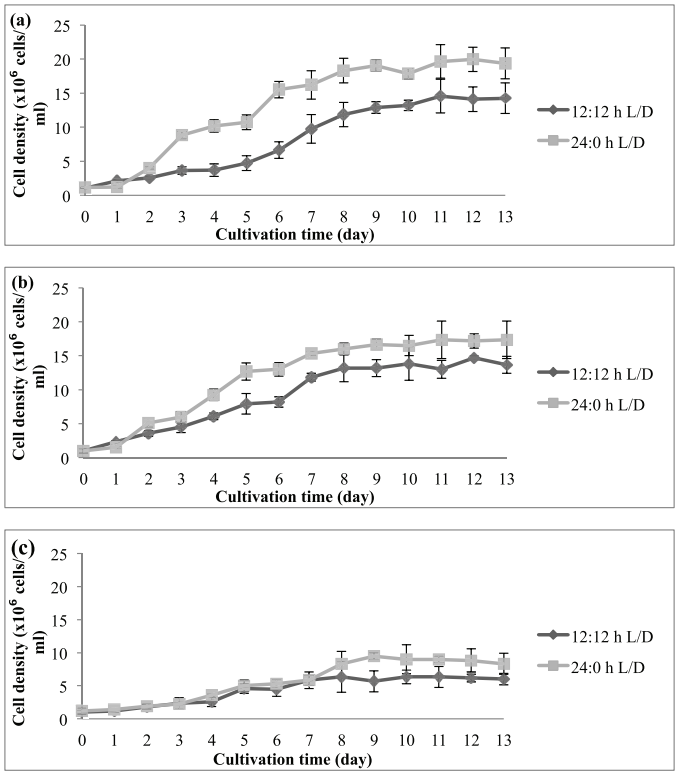

Figure 2. Cell density of (a) UMT-M1, (b) CB, and (c) CT cultivated under 12:12 and 24:0 hours L/D cycles. Data were reported as mean ± standard deviation ($n = 3$).

Under the light regime, the cellular water content can be affected by the size of cellular components such as nucleus, cytoskeleton, chloroplasts, and mitochondria (Aratboni *et al*., 2019; Chioccioli *et al*., 2014), as well as by the accumulation of photosynthetic-related biomass compounds such as starch, glycerol, and protein (Khan *et al*., 2018; Xu *et al*., 2016). The period of light exposure influences the growth and biomass of microalgae as they are parallel to the rate of photosynthesis and the metabolism of microalgae (Matos *et al*., 2017). Increasing the exposure to light may lead to an increase in the reproduction rate before the intensity of the saturation point is reached. Individual organisms vary in terms of light requirements, life cycles, and reproduction patterns. The culture conditions, therefore, have a major impact on the growth and biomass of microalgae (Krzemińska *et al*., 2014).

Effect of photoperiod on antioxidative responses

Chlorophylls, carotenoids, ascorbic acid, and tocopherol are major antioxidative response compounds. All these components effectively detoxify excessive ROS production (Papalia *et al*., 2019). Light absorption by photosynthetic microalgae occurs in chlorophyll a as well as accessory chlorophyll b and carotenoids

Table 1. Effect of photoperiod cycle on the wet and dry biomass of UMT-M1, CB, and CT.

Species	Photoperiod (L/D hour)	Cell density (cells/ml)	Wet biomass (g/l)	Dry biomass (g/l)
UMT-M1	12:12	$1.20 \times 10^7 \pm 1.80 \times 10^6$a,b*	2.735 ± 0.184a*	0.268 ± 0.023a,b*
	24:0	$1.87 \times 10^7 \pm 1.15 \times 10^6$c	4.536 ± 0.044b	0.460 ± 0.033c
CB	12:12	$1.32 \times 10^7 \pm 2.02 \times 10^6$a	2.618 ± 0.049a*	0.251 ± 0.010a
	24:0	$1.60 \times 10^7 \pm 8.66 \times 10^5$a,c	4.417 ± 0.124b	0.395 ± 0.065b,c
CT	12:12	$6.33 \times 10^6 \pm 2.31 \times 10^6$d*	2.505 ± 0.131a*	0.248 ± 0.020a
	24:0	$8.33 \times 10^6 \pm 1.89 \times 10^6$b,d	4.292 ± 0.278b	0.432 ± 0.100c

Data were reported as mean ± standard deviation ($n = 3$). Values with different superscript letters in a column represent significantly different at $p < 0.05$. The asterisk symbol (*) in a column represents a statistically significant difference between 12:12 and 24:0 hours L/D cycles between individual species.

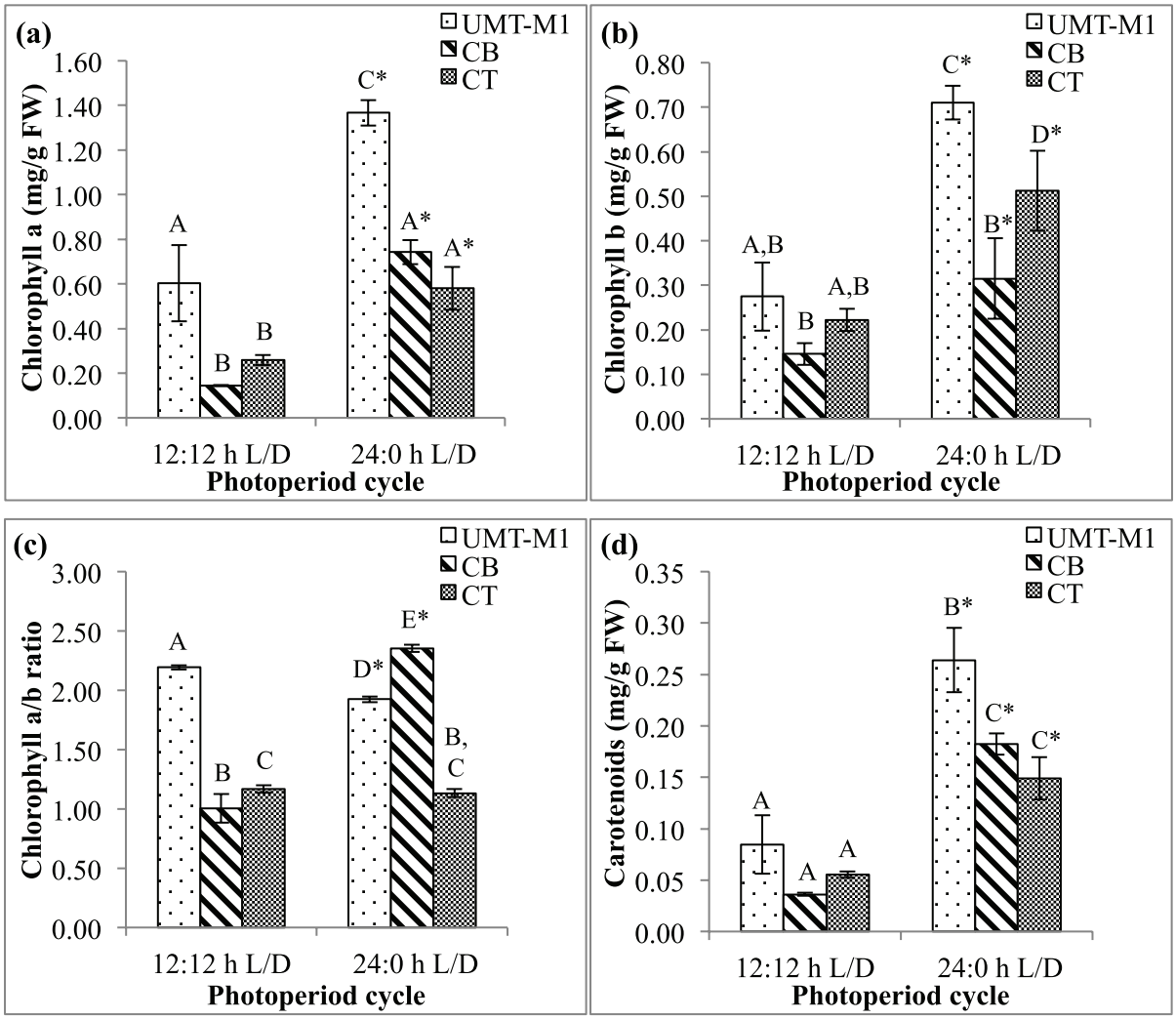

Figure 3. Chlorophyll a (a), chlorophyll b (b), chlorophyll a/b ratio (c) and carotenoids (d) contents of UMT-M1, CB, and CT cultivated under 12:12 and 24:0 hours L/D cycles. Data were reported as mean of repetitions ± standard deviation ($n = 3$). Values with different capital letters (A, B, C, and D) were statistically significantly different at $p < 0.05$. The asterisk symbol (*) indicates that there was a significant difference between 12:12 and 24:0 hours L/D cycles between individual species.

(Sirisuk *et al*., 2018). Figure 3 shows the content of chlorophyll a, chlorophyll b, chlorophyll a/b ratio, and carotenoids in UMT-M1, CB, and CT grown under different photoperiods. UMT-M1 contained the highest chlorophyll a (1.366 ± 0.057 mg/g FW), chlorophyll b (0.711 ± 0.038 mg/g FW), and carotenoids (0.264 ± 0.031 mg/g FW) but CB has the highest chlorophyll a/b ratio of three species. Interestingly, UMT-M1, CB, and CT chlorophyll a, chlorophyll b, and carotenoids were significantly induced ($p <$ 0.05) in 24:0 L/D compared to 12:12 hours L/D cycle. Compared to other microalgae species such as *Ankistrodesmus falcatus*, chlorophyll a and carotenoids were higher in cultivation with 12:12 hours L:/D cycle, whereas chlorophyll b remains higher in culture with constant light (George *et al*., 2014). In another study, the chlorophyll a of *S. obliquus* grown with 12:12 hours L/D cycle was higher than 24:0 L/D cycle but chlorophyll b content remained constant in both photoperiod conditions (Vendruscolo *et al*., 2019). In addition, chlorophyll a/b ratio decreased in UMT-M1 but increased in CB while CT remained constant when continuous light was provided. These findings suggested that continuous illumination promotes chlorophyll a and b synthesis as well as carotenoid content in UMT-M1, CB, and CT, but the chlorophyll a/b ratio varies in these species.

On an applied note, the efficacy of microalgal chlorophyll a to capture light photons varies by pigment arrangement, cell structure, and chloroplast composition (Sirisuk *et al*., 2018). The elevation of chlorophyll a content as observed in this current study may be due to the increasing number of photosynthetic units during light harvesting, whereas the rise in chlorophyll b content may have been linked to the increment of photoprotection pigment during light exposure (Levasseur *et al*., 2018). The increase in the chlorophyll a/b ratio indicates a relatively significant role for chlorophyll a in photosynthesis, whereas the decrease in the chlorophyll a/b ratio suggests that chlorophyll b appears to be highly functional in photosynthesis, which is closely related to the shift in chlorophyll composition in the light-harvesting complex of thylakoid membranes in the reaction center of a photosystem (Beneragama and Goto, 2010; Negi *et al*., 2016; Perrine *et al*., 2012; Ramaraj *et al*, 2013).

Carotenoids exist in chloroplast membranes and play a crucial role in light harvesting as well as in photo protecting photosynthetic systems against the illumination tension (Ma *et al*., 2018; Smerilli *et al*., 2019). Increasing carotenoids in UMT-M1, CB, and CT under continuous light are therefore parallel to their involvement in alleviating and assisting the photosynthetic pigment-protein complex (Sirisuk *et al*., 2018). Carotenoids can quench peroxides and singlet oxygen thus inhibits the ROS formation (Smerilli *et al*., 2019). A balance between light energy harvesting in chloroplast and energy use during carbon fixation is needed to adapt light strain (Levasseur *et al*., 2018).

Besides chlorophyll and carotenoids, photoperiod also affects microalgae ascorbic acid accumulation. The ascorbic acid content of UMT-M1, CB, and CT vary considerably with exposure to the photoperiod conditions tested in this study ($p < 0.05$) as portrayed in Figure 4. Notably, the ascorbic acid content of UMT-M1, CB, and CT was higher in continuous light culture with an accumulation of 1.460 ± 0.167, 0.924 ± 0.044, and 1.264 ± 0.086 mg/g *FW*, respectively. Our findings showed that ascorbic acid production was enhanced under continuous illumination, with similar results in *Desmonostoc salinum* and *S. quadricauda* (De Alvarenga *et al*., 2020; Zahra *et al*., 2017). However, some microalgae species like *Skeletonema marinoi* do not show a shift in ascorbic acid content on crops with different light period (Smerilli *et al*., 2019).

Ascorbic acid (vitamin C), a water-soluble vitamin with antioxidant properties, can be used to scavenge ROS development under light stress (Galasso *et al*., 2019). De Alvarenga *et al*. (2020) stated that microalgae can produce 4–9 times ascorbic acid under continuous light. Ascorbic acid elevation in this study is possibly correlated with the $^1O_2$ scavenging role as the photosynthesis process during light stress triggers scarce energy dissipation, thus intensifying $^1O_2$ development (Sharma *et al*., 2012; Smirnoff, 2015). Besides, ascorbic acid also acts as a substrate for antioxidant enzymes such as peroxidase, •OH radical electron donor, and violaxanthin deepoxidase cofactor (Rezayian *et al*., 2019; Smirnoff, 2015; Smerilli *et al*., 2019). Moreover, the increase in ascorbic acid may be attributed to the suppression of H_2O_2 in the ascorbate-glutathione pathway, which serves as a hydrogen donor to turn H_2O_2 into water and monodehydroascorbate molecules (Rezayian *et al*., 2019; Zhang *et al*., 2020). Ascorbic acid production in microalgae under stressors is still poorly recognized (Kováčik *et al*., 2017).

α-Tocopherol plays a crucial function in oxidative stress scavenger. A significant difference ($p < 0.05$) in α-tocopherol content was observed for UMT-M1 and CT in this study but not CB in 24:0 hours and 12:12 hours L/D photoperiod as shown in Figure 5. The α-tocopherol content of UMT-M1 and CT was higher in 24:0 hours L/D culture, with the accumulation of 12.534 ± 0.127 and 12.840 ± 0.546 mg/g *FW*, respectively. These findings were consistent with Durmaz (2007), where *Nannochloropsis* sp. accumulated high levels of α-tocopherol accumulated under continuous light exposure. Contrary to CB's outcome, *I. galbana* developed higher α-tocopherol in continuous light compared to 8:16 hours L/D period (Bandarra *et al*., 2003). Therefore, our findings suggest that continuous light stimulated α-tocopherol output in UMT-M1 and CT, but not in CB.

α-Tocopherol (vitamin E) is a lipophilic antioxidant in thylakoid membranes or plastid of microalgae (Papalia *et al*., 2019). The increase in α-tocopherol under continuous illumination can result from the defense of microalgae membrane lipids from photosynthesis-derived ROS, mainly in photosystem II (PSII) (Sharma *et al*., 2012). The protection mechanism uses scavenge $^1O_2$ resonance energy transfer (Papalia *et al*., 2019; Smerilli *et al*., 2019). It is also a well-known chain-breaking molecule among the four tocopherol groups (α-, β-, and β-tocopherols) and is capable of suppressing $^1O_2$, reducing $O_2^{\bullet-}$ and inhibiting lipid peroxidation by blocking ROS development involving inhibition of low-density lipoprotein oxidation (Galasso *et al*., 2019; Rezayian *et al*., 2019). Notwithstanding, α-tocopherol development in microalgae is rarely examined (Mudimu *et al*., 2017). Future studies should emphasize microalgal antioxidative responses under different

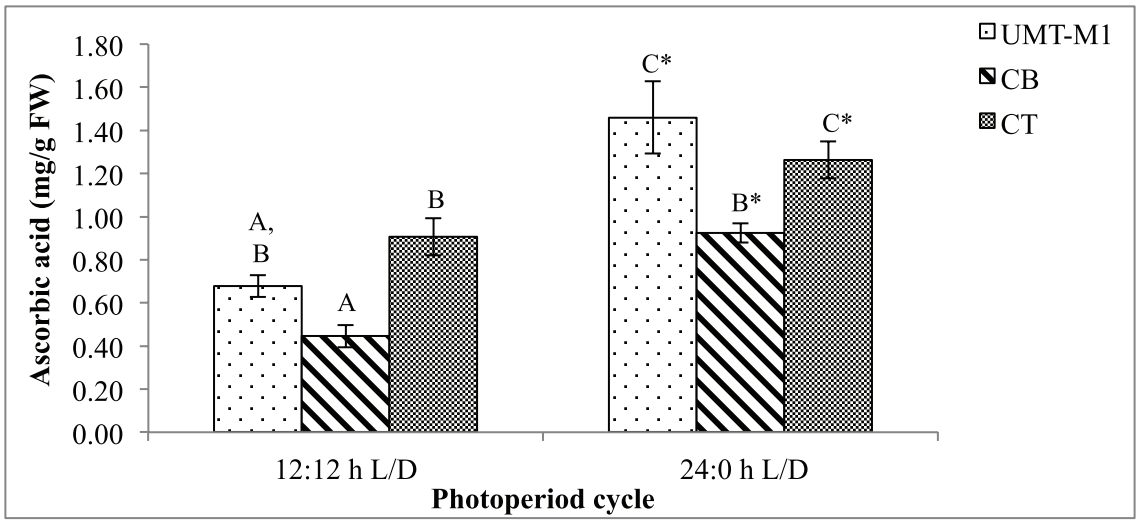

Figure 4. Ascorbic acid contents of UMT-M1, CB, and CT cultivated under 12:12 and 24:0 hours L/D cycles. Data were reported as mean of repetitions ± standard deviation ($n = 3$). Values with different capital letters (A, B, C, and D) were statistically significantly different at $p < 0.05$. The asterisk symbol (*) indicates that there was a significant difference between 12:12 and 24:0 hours L/D cycles between individual species.

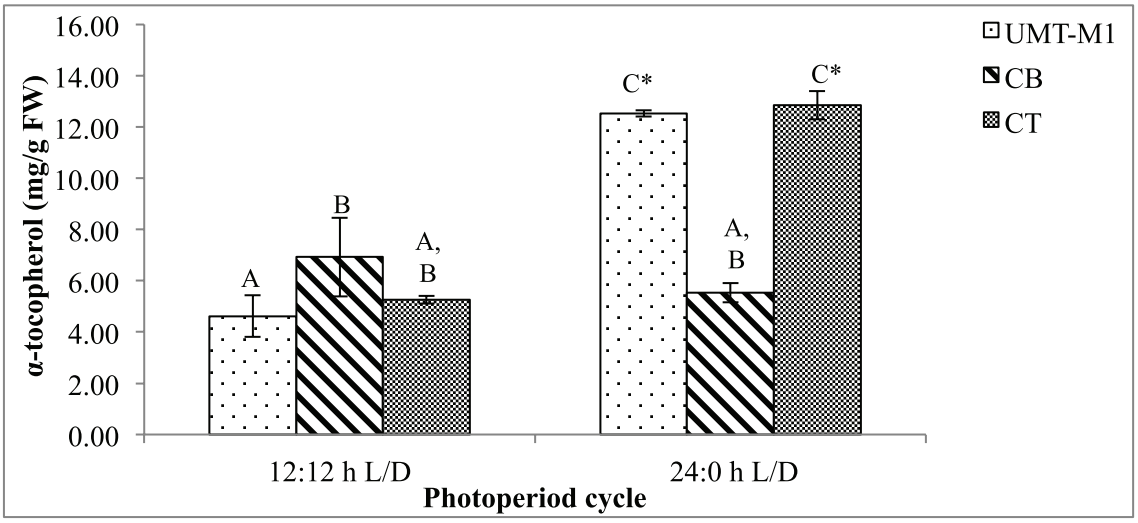

Figure 5. *α*-Tocopherol contents of UMT-M1, CB, and CT cultivated under 12:12 and 24:0 hours L/D cycles. Data were reported as mean of repetitions ± standard deviation (n = 3). Values with different capital letters (A, B, C, and D) were statistically significantly different at $p < 0.05$. The asterisk symbol (*) indicates that there was a significant difference between 12:12 and 24:0 hours L/D cycles between individual species.

Table 2. Pearson's correlation coefficients of cell density, biomass, and antioxidative responses.

Variables	Cell density	Wet biomass	Dry biomass	Chlorophyll a	Chlorophyll b	Carotenoids	Ascorbic acid	α-Tocopherol
Cell density	1							
Wet biomass	0.521*	1						
Dry biomass	0.435	0.896**	1					
Chlorophyll a	0.677**	0.754**	0.702**	1				
Chlorophyll b	0.401	0.770**	0.754**	0.891**	1			
Carotenoids	0.614**	0.893**	0.807**	0.950**	0.898**	1		
Ascorbic acid	0.191	0.757**	0.771**	0.750**	0.896**	0.825**	1	
α-Tocopherol	0.211	0.665**	0.707**	0.538*	0.810**	0.614**	0.753**	1

The asterisk symbol (*) in a column represents a statistically significant difference at the 0.05 level (2-tailed). The asterisk symbol (**) in a column represents a statistically significant difference at the 0.01 level (2-tailed).

environmental stressors to provide a practical understanding of cellular adaptation mechanisms and selection of the best stress-tolerant microalgae species for future pharmaceutical applications.

Correlation between cell density, biomass, and antioxidant responses

The relationship between cell density, biomass (wet and dry), and antioxidant responses (chlorophyll a, chlorophyll b, carotenoids, ascorbic acid, and α-tocopherol) with Pearson's correlation coefficient (R) was accessed as shown in Table 2.

Cell density showed a significant positive correlation of 0.521, 0.677, and 0.614, respectively, to wet biomass, chlorophyll a, and carotenoids. In comparison, there was no significant correlation between dry biomass cell density, chlorophyll b, ascorbic acid, and α-tocopherol. Due to cellular water content, microalgae cells are strongly affiliated, but the content varies by species, cell size, and light exposure (Chioccioli *et al*., 2014). Most unicellular microalgae have single or multiple chloroplasts per cell that contribute to the interactive division of microalgae cells and chloroplast (Sumiya, 2018), thus correlating with chlorophyll a and carotenoids output. Biomass (wet and dry) has a strong association with chlorophyll a and chlorophyll b, indicating that chlorophyll concentration serves as an indirect measurement of biomass concentration (Bauer *et al*., 2017).

Carotenoids demonstrated a strong positive correlation with cell density, biomass, and other antioxidant responses. Among them, two major associations were observed between carotenoids and chlorophyll a (R = 0.980, $p < 0.01$), as well as carotenoids and chlorophyll b (R = 0.898, $p < 0.01$), which show a direct link between chlorophylls and carotenoids in light-harvesting reactions and their protective function against oxidative stress (Sirisuk *et al*., 2018). Furthermore, chlorophylls, carotenoids, ascorbic acid, and α-tocopherol have a clear association, and it can be expected that they have a strong synergistic contribution to scavenge photosynthetic-derived ROS, as previously stated by Papalia *et al*. (2019). The results of these Pearson's correlation coefficients provide insight into the relationship between independent variables in this analysis, where most variables are highly interconnected, so it can be further manipulated to seek the best optimum growth conditions with high-value content.

CONCLUSION

Different microalgae species respond differently to photoperiod treatment. This study revealed continuous light exposure enhanced growth and antioxidative responses of *C. vulgaris*, *I. galbana,* and *T. chuii*. This study suggests that chlorophylls, carotenoids, ascorbic acid, and α-tocopherol have cooperative functions in quenching oxidative stress. *Chlorella vulgaris*, *I. galbana,* and *T. chuii* are good candidates for natural antioxidants to replace synthetic antioxidants in the industry, but more studies are needed to find ways to optimize the production of antioxidants from these microalgae.

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AUTHORS' CONTRIBUTIONS

YYS, HAZ, MEAW, and NY contributed to the conception and design of the work; NSY did the data acquisition, analysis, and data interpretation. NSY drafted the manuscript in consultation with NY; all authors discussed the results and critically reviewed the manuscript; and NY, YYS, MEAW, and HAZ were responsible for giving the final approval of the manuscript.

CONFLICT OF INTEREST

The authors declared that they do not have any conflicts of interest.

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