Antioxidative Responses of *Chlorella vulgaris* Under Different Growth Phases

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Abstract

Chlorella vulqaris is a unicellular microorganism that offers health benefits due to its concentrated antioxidant production. This microalga has received huge attention due to its natural antioxidative property as an alternative antioxidant source because of its rapid growth, easy and flexible culture. Research to date only focuses on the growth and antioxidant production in a selected growth phase, especially exponential and stationary phases; however, so far, limited reports on the production of antioxidants in all growth phases of *C. vulgaris*. Thus, this study determines the growth, the enzymatic (Catalase, CAT; Ascorbate Peroxidase, APX; and guaiacol peroxidase, gPOD) specific activities and the amount of the non-enzymatic antioxidants (α-tocopherol, ascorbic acid and carotenoids) of *C. vulgaris* in five growth phases. *Chlorella vulgaris* was cultured in F/2 medium at 25±2 °C under laboratory conditions. CAT specific activities were the highest at the exponential phase (1.50±0.08 units/mg protein), whereas APX and gPOD were induced at the lag phases of 37.13±4.93 units/mg protein and 1.31±0.03 units/mg protein, respectively. The amount of α-tocopherol was accumulated at the stationary phase (97.3±4.18 µg/g.fwt), whereas the highest amount of ascorbic acid (266.67±22.22 µg/g.fwt) and carotenoids (8.16±2.52 µg/g.fwt) were at the decline phase. Production of enzymatic and non-enzymatic antioxidants in the microalgae cells indicated that they efficiently scavenged reactive oxygen species (ROS) and converted them into less harmful substances. In addition, the production of these antioxidants in different growth phases can be used as a guideline to produce massive antioxidants, which can be commercialized in the food and pharmaceutical industries.

Keywords: *Chlorella vulgaris*, enzymatic antioxidants, non-enzymatic antioxidants, reactive oxygen species, growth phases

Introduction

Microalgae are a highly diverse group of mostly photosynthetic organisms found in almost all environments (Gerasimiuk, 2018; Singh & Patidar, 2018). These autotrophic unicellular or colonial microorganisms can proliferate in large quantities by the source of carbon dioxide, light energy and nutrients as basic requirements for their growth (Singh & Patidar, 2018). Like plants, they also undergo photosynthetic mechanisms and contribute approximately 40% of global photosynthesis (De Morais et al., 2015). These organisms can synthesize complex organic compounds with rich sources of structurally novel and biologically active metabolites and subsequently accumulate many primary and secondary metabolites of interest (Guedes et al., 2011). Increased demand for natural antioxidants as an alternative to synthetic antioxidants has drawn world attention in considering the use of microalgae as a potential source of natural antioxidant compounds. Some of these metabolites include pigments (e.g., carotenoids and chlorophyll), polyunsaturated fatty acids (PUFAs, e.g., the omega-3 or -6 fatty acids), polysaccharides, vitamins and sterols, which can be introduced as dietary supplements in human nutrition and animal feed (Aklakur, 2016; Guedes et al., 2011). In addition, excretion of bioactive molecules with anti-inflammatory, antibacterial, anti-UV, antifungal, anticancer, and/or antioxidant activities in microalgae may bring added value to cosmetics, pharmaceuticals, nutraceuticals or food products (Coulombier et al., 2020; Safafar et al., 2015; Sathasivam & Ki, 2018). Higher production, faster cultivation, processing and harvesting cycle, easier extraction, no lack of raw materials and the ability to be cultured on waste materials are among the advantages of using microalgae over the traditional plant (Gong & Bassi, 2016; Ruiz et al., 2016).

Chlorella vulgaris is a unicellular freshwater or marine microalga. It is a good source of protein (60%), lipids (20%), chlorophylls and β-carotenes, soluble vitamins, choline, dietary fiber and essential minerals

like iron, calcium, potassium, magnesium (Abdel-Karim et al., 2020) and phosphorous (Queiroz et al., 2011). These bioactive compounds are potentially useful in the food industry, aquaculture, pharmaceutical, and nutraceutical applications (Mobin et al., 2019). In the last decades, increased attention has been paid to the commercial and industrial potential of *C. vulgaris*. Among different compounds with functional properties, antioxidants are the most widely studied since the interest in finding new, safe, powerful antioxidants from natural sources keeps expanding. Nowadays, it appears that unicellular microalgae show to have promising potential as an alternative source of antioxidants. They are sustainable resources, have higher photosynthesis efficiency, easy to culture and occupy reduced space for their large cultivation at minimal cost (Sansone & Brunet, 2019; Tan et al., 2020).

The major group of antioxidants in microalgae is carotenoids (CAR). Still, there are also significant amounts of other antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR) as well as low molecular weight antioxidants, i.e., ascorbate (ASC), tocopherols (TOC) and glutathione (GSH) (Jaafar et al., 2020; Zhang et al., 2020). In addition, another type of antioxidant that has become increasingly explored in microalgae is a polyphenol (Levasseur et al., 2020). Carotenoids show a strong antioxidant effect that protects organisms against oxidative stress. Primary carotenoids are directly involved in photosynthesis while secondary carotenoids are present in the cells in response to different environmental factors. SOD is a primary defense antioxidant that catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen. The H_2O_2 produced needs to be further detoxified by CAT and PODs to water and oxygen. ASC, GSH and TOC played interrelated roles in interacting and detoxifying oxygen free radicals (like superoxide and hydroxyl radical) and thus contributed significantly to limit ROS production (Zhang et al., 2020). Polyphenols are a broad group of secondary metabolites and are considered powerful antioxidants. They suppress the generation of free radicals, thus reducing the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals (Tsao, 2010). Phenolic acids and flavonoids are examples of two major groups of polyphenols. Thus, due to the broad spectrum of antioxidants available, additional dietary intake of microalgae is required to limit or prevent certain health problems.

The concentrations of microalgal bioactive compounds vary widely depending on the original habitat, species, strains, and growth phases, which may influence the differences in the antioxidant responses of certain microalgae (Rahman et al., 2020). The microalgae growth phase is characterized into five phases including lag (initial cell metabolism adaptation to growth), exponential (rapid and maximum growth rate), declining growth rate (due to growth requirements for cell division being limited, causes a decline in cell density), stationary (limiting factor and the growth rate are balanced, resulting in relatively constant cell density) and death phase (cell density rapidly decreases due to depleted nutrient) (Lavens & Sorgeloos, 1996). Optimal harvesting of microalgae biomass for various industries is usually conducted at an exponential or stationary phase as these are the two phases with high cell biomass and overall compound productivity (Rahman et al., 2020). However, so far, the antioxidant production in microalgae is usually determined using samples harvested at either the exponential or stationary phase. Thus, the results mostly represent a specific growth phase without considering the differences in the antioxidant compound in all growth phases. Therefore, this study evaluated the fresh and dry weights, the enzymatic (CAT, APX, gPOD) as well as the non-enzymatic antioxidants (α-tocopherol, ascorbic acid and carotenoids) in *C. vulgaris* at different growth phases.

Materials and Methods

Microalgae Culture

Pure culture of *C. vulgaris* (UMT-M1) was obtained from the Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu (UMT). *C. vulgaris* was cultured in F/2 medium at 30 ppt salinity and pH 8.0. (Guillard, 1975; Guillard & Ryther,1962). The cultures were incubated at 25±2 °C under 24 hours photoperiod with 36 W fluorescent lamps in a growth chamber (Tomy CLE-305). The aeration using 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 initial cell density was 1.14×10^6 cells/mL and cultured in triplicates. Microalgae were tested for purity and density and subsequently inoculated into separate flasks for use in the experiments.

Determination of Cell Density

Cell density was estimated regularly for 21 days. Approximately 20 μL of sample from each replicate was transferred to the Neubauer hemocytometer and the cells were counted using a compound microscope (Leica CME, Leica Microsystems GmbH, Germany) (Sahastrabuddhe, 2016). Then, the growth curve was

plotted to determine the growth phases. Cell densities were determined using the following formula:

$$\text{Cell density}\left(\frac{\text{cells}}{\text{mL}}\right) = \frac{\text{Total number of cells x dilution factor}}{\text{Total number of squares x volume of the square at 0.01 mm depth}}$$

Determination of Fresh and Dry Weights

Fresh and dry weights were determined for each growth phase. 50 mL culture was centrifuged for 10 minutes with an Eppendorf 5810 centrifuge at 9,168 × g. The supernatant was discarded and the pellet was washed twice with distilled water. Microalgae pellet was weighed with a balance (ME204, Mettler Toledo) and the fresh weight was recorded in g/L. The fresh pellet was dried in the oven at 35 ºC for two days or until a constant weight was obtained. Then, the weight of the cells was recorded as dry weight (g/L) (Sostaric et al., 2009).

Enzymatic Antioxidants Assays

Catalase (CAT) specific activity was assayed according to a method by Claiborne (1985). Approximately 0.15 g of samples were sonicated with 1.0 mL of 50 mM phosphate buffer (pH 7.4) in a pre-chilled beaker at 0-4 °C for 10 minutes. The mixture was centrifuged at 9,168 x g and 4 °C for 10 minutes. The supernatant obtained was used as the enzyme extract. The reaction mixture consisted of 3 mL of reaction buffer (19 mM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0) and 100 µL of enzyme extract. The rate of changes in absorbance of the reaction mixture was monitored at 240 nm for 3 minutes using a spectrophotometer (Shimadzu UV-1601).

Ascorbate peroxidase (APX) specific activity was described according to the method of Nakano and Asada (1981). Approximately 0.15 g of sample was sonicated with 1.0 mL of 1 mM of ascorbic acid containing 100 mM of phosphate buffer (pH 7.0) in a pre-chilled beaker at 0-4 °C for 10 minutes. The homogenate was centrifuged (Eppendorf 5810) at 9,168 x g and 4 °C for 10 minutes. The reaction mixture consisted of 1.5 mL of 100 mM phosphate buffer (pH 7.0), 0.5 mL of 3 mM ascorbic acid, 0.1 mL of 3 mM EDTA, 0.3 mL distilled water and 0.2 mL of 1.5 mM hydrogen peroxide were added to 200 µL enzyme extract. Changes in absorbance were monitored at 290 nm for 3 minutes using a spectrophotometer (Shimadzu UV-1601).

Guaiacol peroxidase (gPOD) specific activity was assayed following the method of Agrawal and Patwardhan (1993). Approximately 0.15 g of sample was sonicated with 0.9 mL of 100 mM phosphate buffer (pH 7.0) in a pre-chilled beaker at 0-4 °C for 10 minutes. Then, the homogenate was centrifuged (Eppendorf 5810) at 9,168 x g and 4 °C for 10 minutes. The reaction mixture consisted of 1 mL of 50 mM phosphate buffer (pH 7.5), 1 mL of 20 mM guaiacol, 1 mL of 30 mM H_2O_2 and 200 µL enzyme extract. The changes in absorbance were monitored at 470 nm for 3 minutes using a spectrophotometer (Shimadzu UV-1601).

The protein content was determined following the Bradford method (1976). Approximately 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95% ethanol. Then, 100 mL of phosphoric acid was added and the mixture was diluted to 1.0 L with distilled water. The solution was filtered through filter paper and stored at 2-4 °C in light-proof bottles. About 100 µL of enzyme extract was added to 3 mL of Bradford's reagent. The absorbance was measured at 595 nm after 10 minutes. The protein concentration was calculated according to the standard curve prepared with 20 to 100 mg/mL BSA (Bovine Serum Albumin).

Non-enzymatic Antioxidant Assays

Ascorbic acid was assayed following the method of Jagota and Dani (1982). About 0.15 g of fresh sample was sonicated in 1.0 mL of 10% trichloroacetic acid and centrifuged (Eppendorf 5810) at 9,168 x g for 10 minutes at 4 ºC. Then, 1700 µL of distilled water and 200 µL of 10% Folin reagent were added. The mixture was gently swirled and left under dim light for 10 minutes. Then, the absorbance of the mixture was measured using a spectrophotometer (Shimadzu UV-1601) at 760 nm. A standard curve was prepared using ascorbic acid at various concentrations (0-60 µg/mL). The concentration of ascorbic acid in the samples was calculated based on the standard curve.

Approximately 0.15 g was ground up with 1.5 mL acetone and sonicated at 0-4 °C under dim light for the determination of α-tocopherol. The mixture was extracted with 0.5 mL hexane and then vortexed for 30 seconds. The mixture was centrifuged (Eppendorf 5810) at 9,168 x g for 10 minutes. After the centrifugation, the top layer was removed and the hexane extraction was repeated twice. About 0.5 mL of the hexane-extracts were added into 0.4 mL 0.1% (w/v) PDT (3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine, which was prepared in ethanol). Then, 0.4 mL of 0.1% (w/v) ferric chloride (prepared in ethanol) was added and made up to 3.0 mL with absolute ethanol. The mixture was gently swirled and left for 4 minutes for color development. After that, 0.2 mL of 0.2 M orthophosphoric acid was added to the mixture and left for 30 minutes at room temperature. The absorbance of the mixture was measured at 554 nm and the blank was prepared as the same method except

that absolute ethanol was used instead of the hexane extracts (Kanno & Yamauchi, 1977). A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 mg/ml). Then, 0.5 mL of α-tocopherol was added to the solution and the amount of α-tocopherol in the samples was calculated based on the standard curve (Hodges et al., 1996).

A total of 0.15 g of fresh sample was ground up with 3 mL of 80% (v/v) acetone and homogenized in a sonicator. The homogenate was centrifuged at 9,168 × g for 10 min and the absorbance of the supernatant obtained was measured at 663.2, 646.8 and 470 nm using a UV spectrophotometer (Shimadzu UV-1601). 80% acetone was used as a blank (Lichtenthaler, 1987). Carotenoid content was calculated using the formula:

$$Ca = 12.25A_{663.2} - 2.79A_{646.8}$$
$$Ca = 12.50A_{646.8} - 2.79A_{663.2}$$

$$Cx+c = \frac{1000_{A470} -_{1,8} 2Ca - 85.02Cb}{198}$$

where Ca = chlorophyll a (mg/L), Cb = chlorophyll b (mg/L), Cx+c = carotenoids (mg/L).

Statistical Analysis

The data obtained were analyzed using Statistical Package for the Social Sciences (SPSS) 25.0. The enzymatic and non-enzymatic activities of *C. vulgaris* in different growth phases were compared using One-way ANOVA, where the significance level was at *p*-value < 0.05.

Results and Discussion

Growth Curve

C. vulgaris is one of the microalgae species that is easy to cultivate and superior in growth (Mayers et al., 2018). The growth rate of microalgae can be measured either based on optical density or the number of cells. In this study, the hemocytometer counting chambers were used to determine the number of cells and to count the cell density. In this study, the growth curve of *C. vulgaris* is an important way to decide the harvesting time of microalgae cells for growth and antioxidative assays. *C. vulgaris* cells were cultured in F/2 medium using 30 ppt of natural seawater under laboratory conditions. *Chlorella vulgaris* completed the growth curve in 21 days. As shown in Figure 1, the *C. vulgaris* growth curve can be divided into five growth phases, i.e., lag, exponential, declining growth, stationary and death. These growth phases agreed with Mao et al. (2018) in *C. pyrenoidosa* and Ma'mun et al. (2022) in *C. vulgaris* obtained from a microalgae cultivation center in Sidoarjo, East Java, Indonesia.

On the first day of cultivation, an adjustment phase was observed where it showed an initial period of slow growth with a slight increase in cell density of 1.14 x 10^6 cells/mL. This lag phase is essential for the physiological adaptation of cell metabolisms, such as the production of enzymes required for growth and metabolism. Chen et al. (2011) reported that the cells in the lag phase begin to acclimate to the new medium after inoculation, resulting in a lower specific growth rate. At this stage, cells are more vulnerable to parameter changes such as temperature and light due to their high physiological activity in the cells (Sakarika & Kornaros, 2016). The growth of *C. vulgaris* cells increased exponentially from day two to day eight. The exponential or log phase is where the cell density elevates because rapid growth and cell division often occur. During this phase, the growth of microalgae reaches the maximum specific growth rate. However, the specific growth rate depends on species of algae, light intensity and temperature (Belaïdi et al., 2020). The declining growth phase occurs when the growth requirements for cell division are limited. Thus, it caused a decline in the cell density of microalgae starting at day nine of 1.42 x 10^7 cells/mL. At this point, it produces more toxic wastes in the medium and reduces the specific growth rate. This phase continued until the nutrient sources became limited. Li et al. (2016) highlighted that starvation of nitrogen could lead to chloroplast degradation, structural damage and reduction in photosynthetic efficiency. From day 10 to day 18, the growth of the cells started to enter the stationary phase, where the limiting factor and the growth rate were balanced, resulting in relatively constant cell density. The death phase begins after day 19 when the cell density rapidly decreases due to depletion of limiting factors such as nutrient deficiency, oxygen, overheating, pH alteration, and contamination

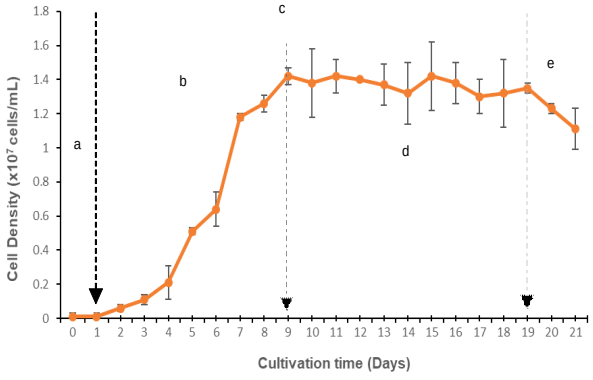

Figure 1. Growth phases of *C. vulgaris*: (a) lag, (b) log, (c) declining growth, (d) stationary, and (e) death phase. Data are mean ± standard error (n=3).

Table 1. Fresh and dry weights (g) of *C. vulgaris* at different growth phases

Growth stage	Fresh weight (g)	Dry weight (g)
Lag	0.52±0.08^{d}	0.01±0.01^{b}
Exponential	0.83±0.04^{b}	0.02±0.01^{b}
Declining	0.72±0.03^{c}	0.08±0.01^{a}
Stationary	1.00±0.15^{a}	0.08±0.01^{a}
Death	0.86±0.03^{a,b}	0.08±0.01^{a}

Note: Data shown are means ± SE (n=3). Means with the same letters in a column are not significantly different at $p<0.05$.

(Lee et al., 2015; Belaïdi et al., 2020). In this study, cells were harvested on days 1, 6, 9, 14 and 20 to represent the five growth phases: lag, log, declining, stationary and death phases.

The Fresh and Dry Weights of *C. vulgaris*

Table 1 shows the fresh and dry weights of *C. vulgaris* at different growth phases. This result showed that the biomass of *C. vulgaris* increased following the cultivation periods. The highest fresh weight of *C. vulgaris* was in the stationary phase (1.00±0.15 g), whereas the lowest fresh weight was in the lag phase (0.52±0.08 g). No significant difference was observed in the dry weight of *C. vulgaris* in the declining growth phase (0.08±0.01 g), stationary phase (0.08±0.01 g) and death phase (0.08±0.01 g). The least dry weight of *C. vulgaris* was in the lag phase (0.01±0.01 g).

In developing an optimal process for *C. vulgaris* biomass, two main aspects are usually considered, external factors such as temperature, light intensity, pH, aeration and agitation, and the selection of suitable nutrients. Nutrients can be the limiting factors affecting the quality and quantity of biomass. The fresh and dry weights of *C. vulgaris* were observed lower in the lag phase. Similar observations were reported by Kasan et al. (2020) and Zhu et al. (2019). They suggested that the lag phase is commonly observed when microalgae adapt to new cultivation conditions such as medium compositions, pH, and light. In this phase, the cell growth of microalgae increased slowly, causing low-fresh and dry microalgae biomass.

Furthermore, slow cell growth may trigger the production of enzymes and metabolites necessary for cell division. In the exponential phase, rapid growth occurs and usually reaches optimum growth. The cells may be divided into an unlimited number of cells, causing nutrient limitation in the medium. Not much variation was found in the later growth phases, where cell division and cell decline are constant and the cell's fresh and dry weights reach a plateau due to limited nutrient availability and other limiting factors (Nguyen et al., 2019).

Enzymatic Antioxidants in *C. vulgaris*

Enzymatic antioxidants prevent or delay the oxidation of other molecules by neutralizing reactive oxygen species (ROS) (Hasanuzzaman et al., 2020). These enzymes eliminate ROS by reducing the energy of free radicals or donating electrons to free radicals, constituting the first level of defense in the cell's antioxidant network (Roy et al., 2021). This study observed CAT, APX and GPOD activities of *C. vulgaris* in different growth phases.

Catalase (CAT) Specific Activity

Table 2 illustrates the CAT, APX and gPOD specific activities of *C. vulgaris* at different growth phases. It was observed that CAT specific activity was the lowest in the death phase of 0.16±0.01 units/mg protein, followed by the activity in the lag phase (0.48±0.06 units/mg protein), and stationary phase (1.02±0.13 units/mg protein). No significant difference in CAT activities in the exponential and decline phases of 1.50 ±0.04 and 1.46 ± 0.01 units/mg protein, respectively. CAT catalyzes the conversion of H_2O_2 to H_2O and O_2. The CAT specific activity showed lower activities in the lag phase due to the limited number of cells, thus

Table 2. CAT, APX and gPOD specific activities (units/mg protein) of *C. vulgaris* at different growth phases

Growth stage	CAT specific activity (units/mg protein)	APX specific activity (units/mg protein)	gPOD specific activity (units/mg protein)
Lag	0.48±0.06^{c}	37.13±2.85^{a}	1.31±0.02^{a}
Exponential	1.50±0.04^{a}	23.36±1.14^{b}	0.05±0.02^{b}
Declining	1.46±0.01^{a}	13.34±0.79^{c}	0.05±0.03^{b}
Stationary	1.02±0.13^{b}	16.02±2.63^{c}	0.05±0.03^{b}
Death	0.16±0.01^{d}	6.88±0.60^{d}	0.18±0.06^{c}

Note: Data shown are means ± SE (n=3). Means with the same letters in a column are not significantly different at $p<0.05$

reducing the cell metabolism. An increase in CAT activities during the exponential and early declining growth phase might be related to ROS accumulated within the organelles of *C. vulgaris*. Dmitrieva et al. (2020) pointed out that ROS are produced in the living cells as normal metabolism products from respiration, photosynthesis and catalyst reaction in peroxisomes and glycosomes.

Furthermore, it is indicated that more ROS production is due to rapid cell division and nutrient uptake by microalgae cells. Thus, higher CAT activities are required to detoxify the ROS build-up in the cells during these phases. Declining CAT specific activity in the stationary phase might be due to less cell division and depletion of nutrients in the medium. The lowest CAT activity in the death phase is due to the cells beginning to die and the substrate's low affinity, which is H_2O_2, thus depleting the CAT responses. Alternatively, microalgae have another protective system, including APX and gPOD, to eliminate ROS production (Gill & Tuteja, 2010).

APX and gPOD Specific Activity

Contrast results were observed for APX and gPOD activities, where both demonstrated the highest activities in the lag phase compared to other phases (Table 2). Acclimation of cells to a new environment may influence these results. At inoculation, microalgae cells adapted to the new environment. However, this process caused stress in the cells and elevated ROS production which triggered the synthesis of APX and gPOD. Both enzymes catalyze the reduction of H_2O_2 to H_2O. They differ from CAT in their requirement for an electron donor that subsequently becomes oxidized. APX, present in the stroma and thylakoids of chloroplasts, has a significantly lower affinity for H_2O_2 than CAT and uses ascorbate as a specific electron donor (Hasanuzzaman et al., 2019). gPOD is an essential group of peroxidases, which requires guaiacol to degrade the ROS. They are commonly found in cellular cytoplasm and apoplasm fractions involved in various processes in plant growth and development (Singh et al., 2019).

APX activity in the exponential phase (23.36±1.14 units/mg protein) also showed an increase due to rapid cell division and insufficient nutrients in the medium which caused stress in microalgae cells. This condition was also applied in the declining growth phase (13.34±0.79 units/mg protein) and stationary phase (16.02±2.63 units/mg protein). APX activity reached the lowest in the death phase (6.88±0.60 units/mg protein) owing to the microalgae cells began to die and reduced their metabolism activity. gPOD activity in the exponential (0.05±0.02 units/mg protein), declining growth (0.05±0.03 units/mg protein) and stationary phases (0.05±0.03 units/mg protein) demonstrated the lowest activity compared to the death phase. It can be suggested that CAT is more favorable compared to gPOD and APX to eliminate ROS in this current study, as observed by Gill and Tuteja (2010).

Non-Enzymatic Antioxidants in *C. vulgaris*

α-Tocopherol Concentration

The α-tocopherol concentrations were gradually increased and the maximum concentration was observed in the stationary phase (97.36±4.18 µg/g. fresh weight (fwt)) and was significantly reduced in the death phase (2.04±0.65 µg/g. fwt). No significant difference was observed between the exponential (62.24± 8.02 µg/g. fwt) and declining phase (73.55±14.15 µg/g. fwt) (Table 3). The results were consistent with the study by Mudimu et al. (2017). They also noticed that α-tocopherol content elevated from log to stationary phase in *Heamatococcus pluvialis*, *Coccomyxa* sp., *Nannochloropsis oculata*, and *Microchloropsis salina*. The increase in α-tocopherol in the stationary phase can result from the defense of microalgae membrane lipids from photosynthesis-derived ROS, mainly in photosystem II (PSII) (Sharma et al., 2012). Another reason is that *C. vulgaris* undergo nutrient stress (Wong, 2017). Different sources and concentrations of nitrogen and phosphorus in culture media also will affect the microalgae species. Other factors that contribute to the accumulation of α-tocopherol are the growth phase, culture conditions, extraction process and the harvest time (Maier & Pepper, 2014).

Tocopherols are located in the lipid bilayers of cell membranes. The most active antioxidant form is α-tocopherol, which is only synthesized in the chloroplasts of photosynthetic organisms. α-Tocopherol acts as an antioxidant through its ability to quench both singlet oxygen and lipid peroxides (Mallick & Mohn, 2000). Although α-tocopherol is located in the membranes and the hydrophilic vitamin C is located in the liquid phase, vitamin C can reduce the tocopheroxyl radical, thereby recycling the active form of tocopherol in the chloroplast (Seminario et al., 2017).

Ascorbic Acid Concentration

Elevated ascorbic acid concentration was found from the lag to the declining growth phases (145.42±9.96, 128.39±25.53 and 266.67±22.22 µg/g. fwt, respectively) as compared to other phases and the lowest concentration was in the death phase (30.15±11.86 µg/g. fwt) (Table 3). In this study, higher

ascorbic acid production is correlated with the physiological response to the inorganic nutrients' depletion such as nitrogen limitation which induced stress on the microalgae cells. Thus, higher ascorbic acid is needed to overcome ROS toxicity. In contrast, freshwater microalga, *Euglena gracilis* shows a decreasing pattern of ascorbic acid from the early log phase to the stationary phase (Hasan et al., 2017). Ascorbic acid will be easily affected by moisture, light, air, heat and metal ions. Ascorbic acid will act as a co-antioxidant when together with α-tocopherol. Ascorbic acid or vitamin C were found abundant in photosynthetic organisms including *C. vulgaris* (Barrita & Sánchez, 2013). They can reduce many ROS, present in both cytosol and chloroplast where it takes part in the ascorbate glutathione cycle to remove H_2O_2. They can also scavenge superoxide, hydroxyl radicals, and lipid hydroperoxides. In the chloroplast, vitamin C plays a crucial role in the regeneration of membrane-bound carotenoids and tocopherols, thereby protecting the photosynthetic apparatus (Mallick & Mohn, 2000).

Carotenoids Concentration

Various bioactive compounds found in microalgae play an essential role in protecting against oxidative stress and damage. Some of the most important and well-known antioxidants from microalgae include carotenoids (Safafar et al., 2015). Carotenoids are considered potent non-enzymatic antioxidants capable of protecting against photooxidative damage by converting the radical into a safer by-product. In this study, carotenoids yield was higher in the decline phase (8.16±2.52 units/mg protein) as compared to other phases (Table 3). Contrast results were observed by Rahman et al. (2020) where they spotted higher total carotenoids in the stationary phase of *Amphora montana, Isochrysis galbana*, *Phaeodactylum tricornutum*, *Chaetoceros gracilis*, and *Thalassiosira weissflogii*. They also suggested that the effect of the growth phase is mainly due to the nutrient availability factor, which affects the composition of the antioxidant compounds and, thus, the total antioxidant capacity of each algal strain. Higher carotenoids are also related to being growth stage-dependent, mainly due to the effect of nutrient depletion in the later growth stages (McClure et al., 2018). Differences in carotenoid composition across division and growth phases have also been reported by Guo et al. (2016). Comparing the data on carotenoid content with other studies must be made with caution since carotenoid content and composition are easily influenced by culture conditions such as nutrient availability and light intensity.

Notably, the generation of ROS is a fundamental process in *C. vulgaris* in response to changing environmental growth conditions, thus inducing oxidative stress. The equilibrium between the detoxification and generation of ROS is maintained by both enzymatic and non-enzymatic antioxidant defense systems. It is interesting to note that APX contributed the greatest enzyme activities compared to CAT and gPOD, hence suggesting that APX is the most favorable enzyme in *C. vulgaris* to diminish the higher production of ROS. The enhanced activities of APX and gPOD particularly in the lag phase aid in the rapid detoxification of H_2O_2 produced, so that a steady state level of this ROS is maintained (Hasanuzzaman et al., 2020). Removal of H_2O_2 by APX requires ascorbate as the principal electron donor of APX, therefore, the ascorbate concentration plays an important role in oxidative defense. Lower ascorbate, carotenoids and α-tocopherol concentrations in this study might be contributed by increased APX activity or it might be due to its participation in the ascorbate-glutathione oxidative pathway (Hasanuzzaman et al., 2019). It is proposed that antioxidants in this study, may function as synergists and act together to reduce ROS levels more effectively than single antioxidants as a response to different growth conditions.

Conclusion

Chlorella vulgaris exhibited different antioxidative activities at different growth phases. The highest activities of most antioxidants studied were observed

Table 3. α-Tocopherol, ascorbic acid and carotenoids concentration (µg/g.fwt) of *C. vulgaris* at different growth phases

Growth stage	α-Tocopherol (µg/g.fwt)	Ascorbic acid (µg/g.fwt)	Carotenoids (µg/g.fwt)
Lag	12.54±1.45^{c}	145.42±9.96^{b}	5.57± 0.12^{b}
Exponential	62.24±18.02^{b}	128.39±25.53^{b}	4.32±0.30^{c}
Declining	73.55±14.15^{b}	266.67±22.22^{a}	8.16±2.52^{a}
Stationary	97.36±4.18^{a}	67.87±21.16^{c}	5.53±1.24^{b}
Death	2.04±0.65^{d}	30.15±1.86^{c}	3.03±0.89^{d}

Note: Data shown are means ± SE (n=3). Means with the same letters in a column are not significantly different at $p<0.05$

in the stationary and declining growth phases except for APX and gPOD. The antioxidant enzymes and non-enzymatic antioxidants work in a coordinated fashion to inhibit the overproduction of ROS at different growth phases. The ROS produced, mainly H_2O_2 is further converted into H_2O by the enzymes CAT, APX, PODs and low molecular weight antioxidants. Molecular works should be carried out to confirm the right time to harvest the microalgae with the maximum quantity of the interested antioxidants prior to fulfilling the worldwide demands involving different areas such as live feeds, nutraceuticals, pharmaceuticals, food and wastewater treatments.

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Supplementary Material

Supplementary material is not available for this article.

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