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Analysis of Variations in Biomolecules during Various Growth Phases of Freshwater Microalgae *Chlorella* sp.

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Abstract

Background and Objective: *Chlorella* sp. is one of the most studied microalgae because it contains compounds with pharmacological properties, critical to human health. As diversity of the biomolecule changes with the growth of microalgae, it is essential to assess these changes during various growth phases for the targeted extraction of biomolecules. In this study, changes in biomolecules extracted from *Chlorella* sp. at various growth phases were analyzed using various solvent systems.

Material and Methods: *Chlorella* sp. was isolated from a freshwater pond and three solvents (ethanol, methanol and acetone) were used to extract phyto-compounds from biomasses from three different growth phases (exponential, early stationary and late stationary phases). Analysis **of** the fatty acids, total phenolic and flavonoid levels, and antioxidant activity were all performed on extracts from different growth phases.

Results and Conclusion: Total flavonoid concentration was highest during the late stationary growth phase in contrary to the early growth stages. In contrast, total phenolic content was highest in the exponential phase. Fatty acid profile revealed that the early growth phases of the algae included higher levels of omega-3 and omega-6 polyunsaturated fatty acids in comparison to the late stationary phase. The extract phyto-constituents varied in quantity and type during the microbial growth and were dependent on the solvent system. Correlations between the total phenolic content, fatty acids and DPPH free radical scavenging were positive, demonstrating that the quantity and type of phyto-constituents varied depending on the stages of algal growth. Additional investigations of *Chlorella* sp. can develop its potentials for commercial uses.
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1. Introduction

Microalgae are potentially rich sources of chemical products with uses in various industries such as food, cosmetics, pharmaceuticals, energy, and environmental industries [1,2]. Microalgae are prospective sources of nutritional supplements and food proteins due to their high protein concentration [3,4]. In recent years, microalgae have extensively been studied as sustainable sources of bioactive compounds such as proteins, fatty acids, vitamins, antioxidants, and pigments, as well as for the production of unique compounds such as phycotoxins and isotopically labelled organic chemicals [5,6].

Microalgae can be practical substitutes for the industrial production of antioxidants; given the global interest in switching from synthetic to natural sources of antioxidants [7]. Although microalgae are sources of diverse phyto-constituents, information on their antioxidant activity and purification of the target compounds are limited [1]. Nutraceuticals and food supplements from microalgae include well-developed global market worth of $3.4 billion in 2020 [8]. Secondary metabolites synthesized by algal species are commercially attractive compounds as several preclinical (*in vitro* and *in vivo*) and clinical studies have demonstrated their importance as potential therapeutics [9].

The antioxidant effects of phenolic compounds, a significant class of natural antioxidants, have widely been investigated. Studies have shown that microalgae are rich

sources of several classes of flavonoids such as isoflavones, flavanones and flavonols. Since microalgae may include unique phenolic compounds and have been shown to produce further complex phenolic compounds in several studies, it is important to identify phenolic chemicals in microalgae [10].

One of the most studied freshwater microalgae is *Chlorella*, which includes beneficial biological and pharmacological effects on human health. Due to its high biomass yield, cultivation flexibility, high protein content, unsaturated fatty acids, carotenoids, vitamins and other bioactive molecules, *Chlorella* sp. have widely been investigated as a part of bio-regenerative life support systems for space exploration [11]. Naturally, *C. vulgaris* includes antibacterial and antifungal characteristics due to the presence of various FAs, terpenes, phenols and halogenated aliphatic compounds [12]. *Chlorella* sp. can benefit the growing human population by providing biofuels, cosmetics, pharmaceuticals and foods, as well as other products. Clinical studies have demonstrated benefits of *Chlorella* supplements for lowering blood sugar levels, increasing hemoglobin levels, protecting the liver, boosting immunity and decreasing risk of oxidative stress [13].

Profile and content of bioactive molecules vary with the microbial growth phases and these variations are distinctive in lipid saturation and secondary metabolites. Phytoconstituents of *Chlorella* sp. have been assessed through significant studies; however, little has been carried out to access the biochemical substances during distinct growth phases [14]. Length of a microbial culture is always proportional with cost; therefore, assessing appropriate times to harvest microbial cultures for the production of the finest nutraceuticals can help microbial bioprocessing companies [15]. The current study was carried out to suggest appropriate harvesting times and extraction media for achieving the optimal yield of bioactive products, including polyunsaturated fatty acids (PUFAs), total phenolic and flavonoids. Moreover, this study analyzed bioactive molecules with the lipid profile of *Chlorella* sp. at three significant phases of the microbial growth and correlations of them with the antioxidative characteristics of the biomass.

2. Materials and Methods

2.1 Reagents

Aluminum chloride, ascorbic acid (vitamin C), Folin-Ciocalteu's reagent, pyrogallol, quercetin and sodium carbonate were purchased from Sigma-Aldrich, USA. Moreover, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Merck, Germany, and the analytical-grade solvents of acetone, ethanol and methanol from Fisher Scientific, India.

2.2 Microbial isolation

Water samples were collected from the Betana Wetland Area, Morang, Nepal (coordinates, 26°39'39.0"N 87°25'57.-9"E; altitude, 123 m) using falcon tubes with 10 ml of Bold's basal media (BBM) and brought to the laboratory on ice within 24 h of collection. Then, all the samples were filtered and washed with BBM. Samples were studied under microscope to assess if microalgal species were present. These were cultivated on agar plates using discontinuous streak plate method with cool-white light (110 µmol photons m^{-2} s^{-1}) and 16:8 h light:dark photoperiod. Individual colony was picked, inspected for morphological identification and recultured on agar plates until contamination-free single colony was achieved [15].

2.3 Molecular characterization

DNA was extracted and algal-specific primer (5'-CTGCGGAAGGATCATTGATTC-3') [16] and universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [17] were used to amplify the internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal DNA. In general, DNA was amplified using Dr. MAX DNA polymerase (Doctor Protein, Korea) and DNA Engine Tetrad 2 Peltier thermal cycler (BIO-RAD, USA). The PCR product was purified using multiscreen filter plates (Millipore, USA). Sanger sequencing of the purified PCR products was carried out (Macrogen, Korea) using ABI PRISM 3730XL analyzer (96-capillary type) and BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Forward and reverse ITS strands were trimmed and aligned using Sequencher DNA sequence analysis software v.5.4.6, (Gene Codes, USA). The most closely related species in the database were investigated by running a clean ITS sequence through the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) [18].

2.4 Assessment of the specific growth rate and mass culture

After verification of the unialgal species, starter cultures were prepared using 250-ml Erlenmeyer flasks containing 200 ml of BBM at 16:8 light:dark photoperiod, light intensity of 110 µmol photons m^{-2} s^{-1} and temperature of 25 °C ±2, while continuously monitoring for contaminants. Mass culture was carried out using 5.1 l (30 × 20 × 8.5 cm) tanks with 4 l of BBM, where aeration was facilitated via perforated silicon tubes at the bottom of the tank. Air was fed through 0.2-µm membrane filters at a rate of 1 l air l^{-1} culture min^{-1} (v v m). For the mass cultures, fresh growth media were added to the inoculum from the exponential phase at the ratio of 10:1, while maintaining the culture parameters. Each culture was monitored daily for cellular growth rate (GR) by counting the microalgae cells using hemocytometer and measuring the optical density (OD) of the culture at 750 nm. The GR was calculated using Eq. 1 [19]:

$$GR = \frac{\ln(N2 - N1)}{t2 - t1} \, d^{-1} \qquad \text{Eq. 1}$$

Where, N was the number of cells and t was the time of algal culture.

2.5 Biomolecule extraction

Algal biomass was centrifuged at 3,000× g for 10 min to harvest the biomass at three pre-assessed phases of exponential (5-d culture), early stationary (10-d culture) and late stationary (15-d culture) phases. The harvested biomass was spread onto Petri dishes to generate a thin film that was dried at 60 °C using hot air oven and weighed periodically until a constant mass was achieved. Dried biomass was scraped off, collected in Eppendorf tubes, sealed and stored at -20 °C. Extraction was carried out overnight using Soxhlet apparatus with various solvent systems as follows: 70% aqueous ethanol [20], 80% aqueous methanol [21] and 50% aqueous acetone [22]. Dried mass (1 g) was extracted with 250 ml solvent at various temperatures: 60 °C for ethanol, 70 °C for methanol and 50 °C for acetone. Rotary evaporator (Buchi R215, Switzerland) operating at 40 °C was used to concentrate the extracts. From the concentrated extracts, yield was calculated using Eq. 2:

$$\text{Yield (\%)} = \frac{\text{Weight of the obtained extract (g)}}{\text{Weight of dried biomass used for extraction (g)}} \times 100\% \quad \text{Eq. 2}$$

Stock solutions of the samples were prepared by weighing concentrated crude extracts, dissolving them in the respective solvents and storing them at -20 °C in dark vials. Working solutions of 200 µg ml^{-1} from each extract were prepared using appropriate dilutions of the stock solutions.

2.6 Estimation of total phenolic and flavonoid content

Folin-Ciocalteu method was used to spectrophotometrically assess the total phenolic content [23]. Calibration curve at 760 nm was created using pyrogallol as reference. In brief, 1 ml of the sample was mixed with 5 ml of 10% Folin-Ciocalteu reagent before incubation at room temperature (RT) for 5 min. Then, 4 ml of 7.5% (w v^{-1}) Na_2CO_3 solution were added to the mixture, vortexed and incubated for 2 h at RT in dark. Absorbance was measured at 760 nm. Pyrogallol equivalents in mg were used to express the total phenolic content per gram of dry extract. The total flavonoid content was calculated using aluminum chloride method and spectrophotometry. Briefly, 200 µl of the sample were mixed with 60 µl of 5% (w v^{-1}) $NaNO_2$ and 800 µl of distilled water (DW) and incubated for 10 min at RT in dark. After the first incubation, 60 µl of 10% (w v^{-1}) $AlCl_3$ were added to the mixture and further incubated for 5 min. Then, 400 µl of 1 M NaOH solution were added to the mixture and the total volume was adjusted to 2 ml by adding 480 µl of DW. Absorbance of the mixture at 415 nm was recorded after incubation at RT for 30 min in dark. To create the calibration curve at 415 nm (quercetin's max), quercetin was used as standard. Calculation of the total flavonoid content was carried out based on a previously published method [24].

2.7 Assessment of antioxidant activity

Using DPPH assay, free radical scavenging activity of the extracts to neutralize free radicals was assessed [25]. Decreases in absorption of the DPPH solution at 517 nm was measured by adding antioxidant using spectrophotometer. Ascorbic acid (vitamin C) was used as standard. Stock solution was prepared using various solvents of 20 mg ml^{-1}. A range of extracts concentrations (40-200 µg ml^{-1}) were prepared stock solution with ice-cold DPPH (0.1 mM) to achieve a final volume of 2 ml. After 30 min of RT incubation in dark, the reaction mixture absorbance was measured at 517 nm. Radical scavenging activity of the extracts was calculated by Eq. 3:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A^0 - (A1 - A0)}{A_0} \times 100\% \quad \text{Eq. 3}$$

Whereas was the sample absorbance with no DPPH solutions, A1 was the test sample absorbance after incubation with DPPH solution and Ao was the blank absorbance, which reacted with methanol instead of the sample.

2.8 Gas chromatography-mass spectroscopy

Methanol extracts were subjected to gas chromatography-mass spectroscopy (GC-MS) analysis. Derivatization of the extracts was carried out via reaction with acetyl chloride [26] and stored at -20 °C. A gas chromatography instrument [Agilent 7890A, USA] with an Agilent 5975 C mass selective detector and an HP-5MS GC column (5% phenyl methyl siloxane; Agilent 19091S-433, USA) with dimensions of 30 m (length), 250 m (internal diameter) and 0.25 m (film thickness) was used to analyze the extracts in the laboratory of Nepal Academy of Science and Technol-ogy.

An initial oven temperature of 250 °C was set for 1 min, which then increased to 280 °C at the rate of 30 °C min^{-1} and set for 2 min. Temperature increased to 300 °C at the rate of 15 °C min^{-1} and set for 15 min. Sample injection volume included 1 µl with split ratio of 10:1 and flow rate of helium carrier gas of 1.21 ml min^{-1}. Instrument was operated in electron impact mode with ionization voltage of 70 eV, setting the quadruple and ion source temperatures at 150 and 250 °C, respectively. Furthermore, MS scan range was set at 40-600 m z^{-1}. Chromatogram peaks were identified using MS data analysis for FA assessment. The FAs were expressed as proportions in the extract assessed through the area proportion of each peak. The FAs identified by GC-MS were grouped into various classes of FAs based on their degrees of saturation.

2.9 Statistical analysis

The mean of three independent replicate measurements ±SE (standard error) was used to express all results. Variations in extraction yield, phenol content, flavonoid content and antioxidant activity between the three growth phases and the three solvent systems were assessed using one-way ANOVA. Differences between the solvents and the

growth phases were assessed using Tukey's test at $p \leq 0.05$. Statistical analysis was carried out using R software v.3.6.1 and R Studio software v.1.4.1717.

3. Results and Discussion

3.1 Molecular characterization of the isolated microalgae sample

The BLAST search results showed that the isolated microalgae belonged to *Chlorella* genus. The isolates included 99.58% similarity with *Chlorella* LBA 50 strain (GenBank accession no. KT308085), 95.03% similarity with *C. sorokiniana* (GenBank accession no. KY229196) and 94.90% similarity with *C. vulgaris* (GenBank accession no. KP645229). Since sample was not identical to either *C. sorokiniana* or *C. vulgaris*, the current species was referred to as *Chlorella* under the GenBank accession number of MN161575.

3.2 Algal growth rate

Cell growth and division are triggered when the media contains sufficient food sources. When the culture reaches the stationary phase, several factors such as decreases of nutrients in media, CO_2 limitation and light limitation due to self-shading result in retardation of cell growth and conversion of fixed carbon to reserve compounds such as carbohydrates and lipids increases [27]. In the current study, the maximum cell density was reached on Day 12 (8.77 E + 07 cell ml^{-1} (Figure 1). Cultures reached an early stationary phase on Day 10 of growth, where the specific GR (μ) was 0.38 d^{-1}. After Day 10, cells entered the late stationary phase indicated by the retarded growth. These results were similar to the results reported by [15] for *Chlorella* sp. isolated from a pond in Kathmandu, Nepal, where the cells entered the late stationary phase after Day 10 of culture. However, the specific GR ($\mu = 0.24\ d^{-1}$) was lower than that in this study. *Chlorella* cultures were collected on Days 5, 10 and 15, corresponding to the exponential, early stationary and late stationary phases, respectively, using growth patterns (Figure 1) as references.

3.3 Total extractable component

Yield (wt. %) of the total extract during the growth phases of *Chlorella* sp. was higher in the late stationary phase (Day 15). The total yield value (wt. %) of all the extracts from 1 g of algal biomass is present in Figure 2. For all of the solvents used, constant increase in yield were seen, with the lowest value was seen during the exponential phase and the maximum during the late stationary phase. Based on the studies, biomass of the microalgal cells is impacted by the variation in cell size. The microalgal cell size is smaller during the early phase of their growth and the size increases during the late stationary phase. A bigger cell size results in higher extractable components [28]. Furthermore, increased yield with increasing culture time could be linked to lipid accumulation during later growth phases. The lipid content typically increases progressively in numerous microalgal species from logarithmic to stationary growth phases, resulting high yields of the extracts [29].

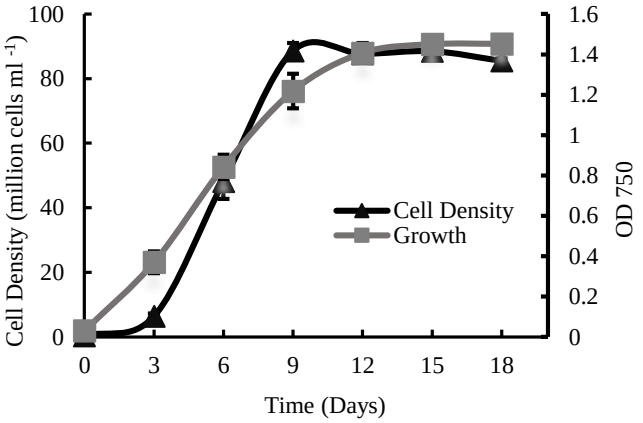

Figure 1. Growth curve of *Chlorella* sp. After 5 d of culture gradual increase in cell density and absorbance were observed (exponential phase). Growth gradually slowed down after 10 d of culture (early stationary phase) and decreases in cell density were seen after 15 d of culture (late stationary phase)

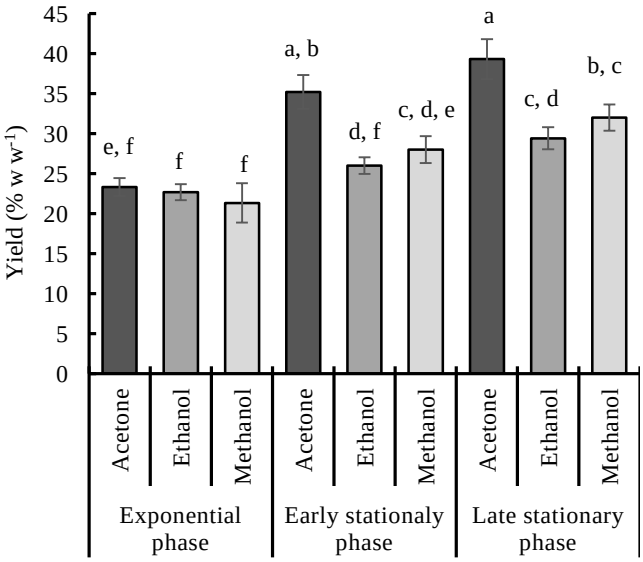

Figure 2. Yield values of the extracts from 1 g of each algal biomass at various solvents during various phases of growth curve. Data are presented as average of three replicates with error bars, indicating standard errors (means ±SEM, $n = 3$ per treatment group).$^{a\text{-}f}$Means with similar values are statistically similar, while means with no common letters are significantly different ($p \leq 0.05$), as analyzed using two-way ANOVA and TUKEY test

Technically, 50% acetone included the highest extract yield, compared to methanol and ethanol when used for the extraction in all the three stages of algal growth. This could be due to differences in the polarity index of the solvents. In a study on various beans, linear increases in the total extractable components were seen with increasing solvent polarity after the polarity index exceeded 4 [30]. Similarly, decreases in yields of extraction by various solvents was

orderly reported as 50% aqueous acetone > 75% aqueous methanol > 75% aqueous ethanol > 100% methanol > reverse osmosis water>100%ethanol>100% acetone [31]. Therefore, biomass extraction at the late stationary phase of algal culture with polar solvents (currently 50% acetone) could result in the maximum yield of the total extractable component.

3.4 Total phenolic and flavonoid contents

The total phenolic content in *Chlorella* extracts measured using Folin-Ciocalteu's reagent was represented as pyrogallol equivalent (R^2 = 0.9992). The total phenolic content in the extract varied with the extracting solvents, whereas no particular links were observed in the flavonoid content with the solvents (Figures 3 and 4). Methanol extract of *Chlorella* sp. demonstrated higher phenolic activities in all growth phases, compared to those ethanol and acetone did.

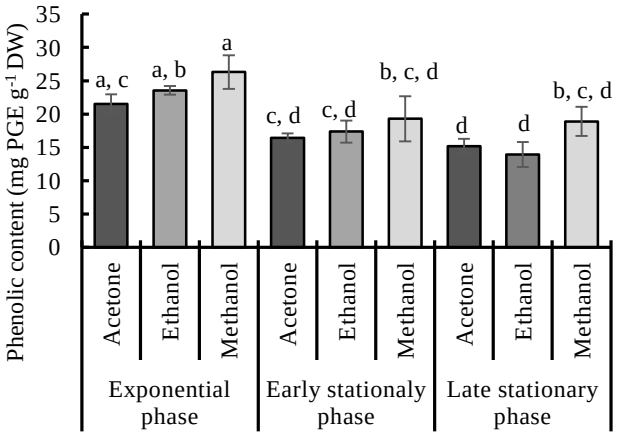

Figure 3. Total phenol content of the algal extracts at various growth phases expressed as mg of pyrogallol equivalent (PGE) per gram. Data are presented as average of three replicates with error bars, indicating standard errors (means ±SEM, n = 3 per treatment group). [a-d]Means with similar values are statistically similar, while means with no common letters are significantly different (p≤0.05), as analyzed using two-way ANOVA and TUKEY test

Positive correlations were observed between the anti-oxidant activity and phenol content, similar to a previous study [31]. In methanolic extract, the harvested biomass during the exponential phase included a phenolic content of 26 ±0.31 mg g^{-1} DW. A similar quantity of total phenolic content in methanol and acetone solvents and the lowest phenolic content in ethanol extract of *C. vulgaris* biomass was reported in a recent study [32], which was nearly eight folds less than that of the current study. Similar to the methanolic extract of *C. marina* reported to include the highest phenolic concentration in comparison to other solvents [33], the current finding suggests that the changes were due to the variations in polarity indices of the solvents. Although most studies have concluded that use of various solvents results in variations in total phenolic content, methods of use are important as well. Variables such as the solid-to-solvent ratio and extraction method, temperature and time help produce various results.

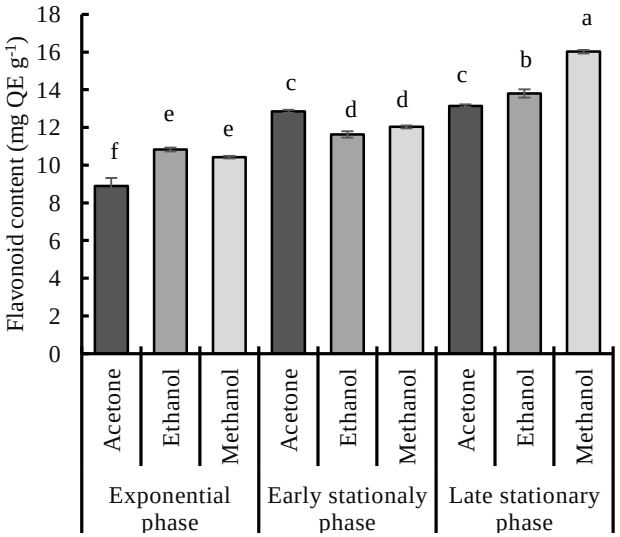

Figure 4. Total flavonoid content of the algal extracts at various growth phases expressed as mg equivalent of quercetin per gram dry sample. Data are presented as average of three replicates with error bars, indicating standard errors (means ±SEM, n = 3 per treatment group). [a-f]Means with similar values are statistically similar, while means with no common letters are significantly different (p<0.05), as analyzed using two-way ANOVA and TUKEY test

Regarding various growth phases of *Chlorella* sp., total phenolic content was maximum in the exponential phase, followed by the early and late stationary phases, respectively (Figure 3). Quantity of the total phenolics in small-size cells was significantly higher (p≤0.05) than that in big-size cells when phenolic compound productions were compared between various cells sizes [34]. Total phenolic content of the methanolic extract of *Chlorella* sp. during the exponential phase was higher than that of other extracts (26.31 ±2.51 mg g^{-1} DW). In a recent study, growth dependent phenolic content accumulation was reported in *C. sorokiniana* cultures, where phenolic content was the maximum (10.17 ±1.44 mg g^{-1} DW) on Day 4 (exponential phase) and was the minimum (6.79 ±1.76 mg g^{-1} DW) on Day 7 (stationary phase) of culture [35]. Similar findings were reported in a recent study [36], where total phenolic contents of the four microalgal species were higher in the early stationary phase, compared to that in the late stationary phase. Accumulation of phenolic compounds has been reported as growth dependent, where the nutrient limitation during the late exponential phase can decrease quantity of the total phenolic compounds [37].

A recent study has reported decreases in phenolic and flavonoid contents of *Phaseolus vulgaris* seed extracts with increasing polarity indices of the solvents [30]. In the present study, phenolic content decreased, except in the late stationary phase, and total flavonoids varied irrespective to the solvent polarity. In contrast, the total flavonoid content was different from that of phenolic content in various growth

phases of *Chlorella* sp., similar to the recent results [38] where the flavonoid content increased with the age of cultures, peaking at Day 25 of culture. From the respective growth phases, the total flavonoid content was highest in methanol extract in the late stationary phase (16.02 ±0.09 mg QE g^{-1}), acetone extract in the early stationary phase (12.85 ±0.08 mg QE g^{-1}) and ethanol extract in the exponential phase (10.83 ±0.09 mg QE g^{-1}) (Figure 4). Variation in flavonoid contents with respect to the solvents verifies the findings of [39], where hexane and ethyl acetate extracts of *C. pyrenoidosa* showed similar flavonoid contents, slightly lower than that in the aqueous extract.

3.5 Antioxidant assay (DPPH radical scavenging assay)

Technically, DPPH, a radical chromogen, is a stable free radical used to assess ability of antioxidants in samples to reduce these radicals from dark purple into yellow diphenyl picryl hydrazine by electron donation. Results are expressed as the absorbance decrease ratio of the DPPH radical solution. When incubated with the test sample, DPPH radicals are reduced into stable diamagnetic molecules leading to decreases in the absorbance of DPPH at 517 nm [40]. The antioxidant activities of *Chlorella* sp. extract with various solvents at various growth phases are present in Figure 5. Higher DPPH radical scavenging activities were seen in methanol extract of the exponential phase (R^2 = 0.9989). In early and late stationary phases, methanol and acetone extracts respectively showed higher DPPH radical scavenging activities (R^2 = 0.9761 and R^2 = 0.9996, respectively), compared to other solvents.

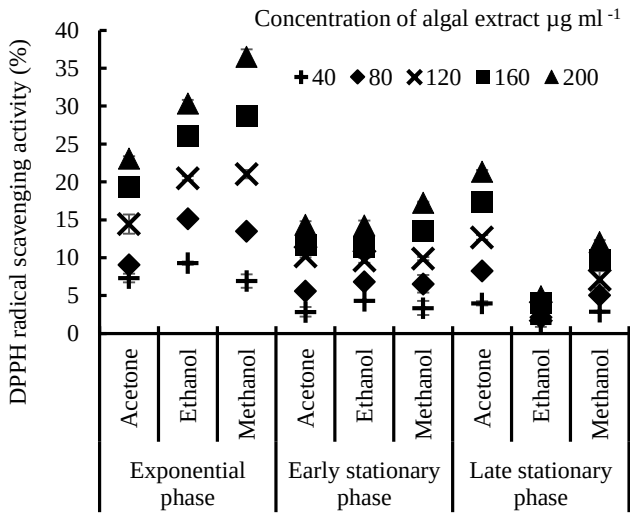

Figure 5. The DPPH radical scavenging activity (%) of *Chlorella* sp. extracts from biomass extracted at three various phases of growth (exponential, early stationary and late stationary phases), of various concentrations (40–200 µg ml^{-1}) extracted using three various solvents (acetone, ethanol and methanol). Values are presented as average of three replicates ±SD (standard deviation)

Comparing various growth phases, antioxidant activity of the extracts in all the solvents during their exponential phase was higher than that in early and late stationary phases. This is further supported by IC50 values. Quantity of the algal extract needed to scavenge 50% of the initial DPPH radicals is referred to as IC50 (half-maximal inhibitory concentration), which is demonstrated by decreases in the absorbance of DPPH radical solution (at 517 nm) to half of its initial value. A lower IC50 value, which shows that the extract is more effective at scavenging DPPH, suggests a higher antioxidant activity of the extract. During the exponential phase, at the concentration of 200 µg ml^{-1}, the maximum radical scavenging activity (36.52% ±0.98) was observed in methanol extracts with an IC50 value of 0.69 mg ml^{-1}, followed by ethanol extract (30.34% ±0.47) with an IC50 value of 0.86 mg ml^{-1}. In contrast, the radical scavenging activity was lowest (23.08% ±0.29) in acetone extracts with an IC50 value of 1.15 mg ml^{-1} (Table 1). Similar radical scavenging activities of *C. vulgaris* ethanol and methanol extracts, respectively 37.2 and 35.4%, and the lowest radical scavenging activity of acetone extract (23.5%) were reported in a recent study [41], similar to the present study. It has been reported that increasing polarity of the solvent during extraction positively affected free radical scavenging [42]. The highest inhibition rate was seen in methanol extract of the exponential and early stationary phases. However, acetone extracts from the late stationary phase, followed by methanol extracts, provided the highest inhibition rate. The late stationary phase extract showed the lowest overall antioxidant activity; within which, the ethanol extract included the lowest radical scavenging activity (4.92% ±0.35) with an IC50 value of 5.52 mg ml^{-1}.

Irrespective of the extraction solvent, constant decrease in antioxidant activity were observed with the aging of the culture, with the highest antioxidant activity in the exponential phase and the lowest one in the late stationary phase. In contrast, no peculiar drifts were seen regarding the solvents of extraction. Composition of the bioactive compounds, contributing to the antioxidant activity and accumulated in the cells, is growth-dependent and nutrient availability affects the growth phase in media. Hence, this affects the total antioxidant capacity of algal cells [35]. Mild decreases in antioxidant activity of the stationary phase were reported in a recent study, compared to the exponential phase of *C. sorokiniana* extracts [35]. Differences in the composition of antioxidant activity-exerting compounds, including carotenoids, have been reported in growth phases. Early growth variables such as light availability boost the presence of substances with the antioxidant activity (photoprotective carotenes such as carotene and zeaxanthin). In contrast, nitrate limitation decrease in the media majorly led to decreases in compounds contributing to the antioxidant activity at the end of the stationary phase [43,44].

Table 1. The IC50 values for the antioxidant activities of various extracts

IC_{50} (mg ml^{-1})	Acetone (mg ml^{-1})	Ethanol (mg ml^{-1})	Methanol (mg ml^{-1})
Exponential phase	1.15	0.86	0.69
Early Stationary Phase	1.71	1.97	1.45
Late Stationary Phase	1.15	5.52	2.15

3.6 Gas chromatography-mass spectroscopy analysis of fatty acids

In this study, PUFAs, monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) were the principal ingredients identified using GC-MS. Because of the relevance of these FAs in nutrition and medicine, they are the most researched phyto-constituents. In the present study, FAs in the methanol extracts of *Chlorella* sp. identified by GC-MS were grouped into various categories (Figure 6).

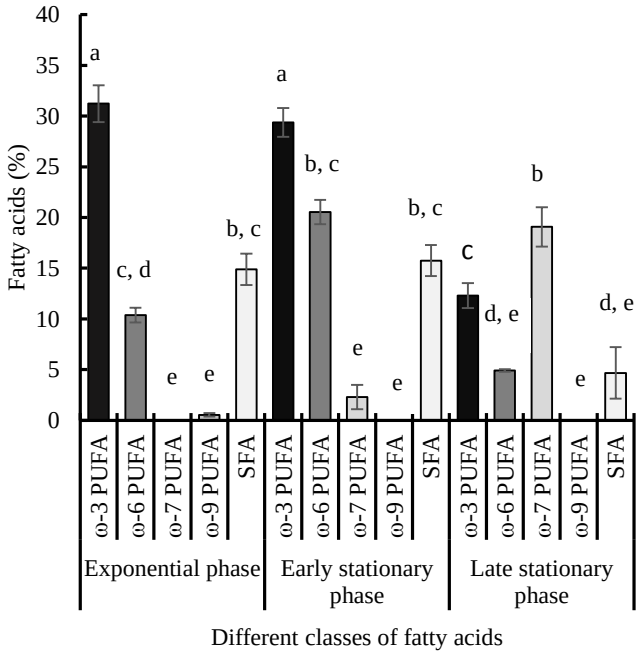

Figure 6. Major fatty acids in methanol extracts at various growth phases of microalgae; omega-3 and omega-6 as PUFAs, omega-7 and omega-9 as MUFAs and all of the saturated fatty acids (SFA) as one group. Proportional distribution of individual fatty acids is associated to the total content. [a-e]Mean values with similar letters are statistically similar, while mean values with different letters are significantly different using Tukey test ($p \leq 0.05$)

The ω-3 PUFAs, ω-6 PUFAs and SFAs were the most prevalent compounds in the lipid profile during the exponential and early stationary phases, whereas ω-7 MUFAs was the most prevalent compound in the late stationary phase. Concentrations of ω-3 FAs such as 7,10,13-hexadecatrienoic acid, 7,10-hexadecadienoic acid and 11,14,17-eicosatrienoic acid were significantly higher during the exponential and early stationary phases, compared to the late stationary phase. Furthermore, ω-3 FAs with the highest average peak value of 31% at the exponential phase decreased on periodic growth shifts of the culture and this was significantly low ($p \leq 0.05$) as 12% of the extract in the late stationary phase, ω-3 PUFAs included significant proportions in all phases of growth. Similar to the current findings, quantity of ω-3 FAs was higher in the early phase than in the late phase of *Gymnodinium* spp. [45] as the total ω-3 PUFA decreased with the aging of the culture.

In this study, ω-9 MUFAs were analyzed as the minimum available FA in *Chlorella* extract as its presence seemed to negligibly be detected (0.53%) during the exponential phase; however, this compound was not detectable in the other two phases. In contrast to ω-3 PUFAs, increases of ω-7 MUFAs were seen with increasing culture time. With detectable concentration of ω-7 PUFA (2%) in the early stationary phase, the peak area significantly increased to 19% during the late stationary phase ($p \leq 0.05$). A previously reported FA profile of microalgae showed increases in total MUFA concentration with the culture time [46]. Although the proportion of ω-6 FAs (e.g., 9,12-octadecadienoic acid and 11,14-eicosadienoic acid) increased from 10 to 20.5% during the growth phase shift from the exponential phase to the early stationary phase. Moreover, significant decreases were reported in their concentrations to 5% in the late stationary phase. In the current study, increases in ω-6 PUFAs were demonstrated until the early stationary phase, whereas ω-7 MUFAs increased to 19% (long chain ω-7 MUFAs were the highest FAs) at the late stationary phase. In a recent study, it was shown that *C. vulgaris* cultures in the early exponential phase included the highest levels of SFAs and PUFAs, whereas the total MUFAs were the lowest FAs. However, cultures in the stationary phase included the highest levels of long chain monounsaturated fatty acids (LCMUFAs), whereas SFAs and PUFAs gradually decreased [47]. This linear increase of ω-7 MUFAs can be result of from the time-dependent conversion of shorter chain fatty acids such as oleic acid and palmitic acid to longer chain fatty acids such as C20:1 [48]. The present results reveal higher PUFA concentrations in all growth phases compared to SFAs, similar to previously published results [46-48].

In fact, PUFAs are reported to include essential roles in cellular and tissue metabolism, maintaining membrane fluidity, electron and oxygen transports and, more importantly, lowering the risk of coronary heart diseases [49]. The ω-3: ω-6 ratio has become a benchmark for assessing the appropriate balance of these fats in diets [50]. Similarly, ω-3 and ω-6 PUFAs have been shown to regulate β-oxidation, gene expression, inflammation, synaptic plasticity, cell growth and pain [51]. The overconsumption of ω-6 over ω-3 has been shown to include adverse effects,

including chronic inflammatory diseases and cardiovascular diseases as ω-3 PUFAS are involved in anti-inflammatory reactions. In contrast, ω-6 PUFA derivatives are involved in pro-inflammatory reactions [52]. Therefore, it is critical to consume a variety of omega FAs in balance and it is not advised to consume a ω-3: ω-6 ratio higher than 1:10 in diets [53]. In all growth phases, the ratio of ω-3:ω-6 FAs in the extracts was higher than 1:1; of which, ω-3 was three folds higher than ω-6 in the exponential phase, showing that the *Chlorella* FA content during all phases was in the recommended range. Similarly, the UFA/SFA ratio is addressed as a measure of the nutritional quality of lipids. Increased high-density lipoproteins (HDL) and decreased low-density lipoproteins (LDL) with a UFA/SFA ratio greater than 1.5 in diets are reported to decrease the risk of heart diseases [54]. In the present study, the UFA/SFA ratio was highest (7.8) in the late stationary phase followed by 3.3 in the early stationary phase, suggesting the higher nutritional quality of *Chlorella* extracts.

3.7 Correlations between the antioxidant activities of phenolic and flavonoid contents and fatty acids

The current study has demonstrated the antioxidant characteristics of the algal extract, verifying the presence of phenolics, flavonoids and numerous classes of FAs in *Chlorella* sp. Further evidence that the biochemical composition of biomass changes with algal growth is linked to variations in antioxidant activity and phenolic and flavonoid contents during the growth phases. With changes in the growth time, cultures become older. Thus, essential elements such as nitrate and phosphate in the growth media change with factors, including culture pH and light availability, ultimately leading to differences in biochemical composition of the biomass [55]. The present data showed significant negative correlations between the antioxidant activity and total flavonoid concentration (R^2=0.8543) as well as significant positive correlations between the antioxidant activity and total phenolic content (R^2=0.8569). By chelating metal ions, limiting the production of free radicals and enhancing the body's antioxidant system, phenolic compounds act as antioxidants. Phenolic compounds include the potentials to donate an electron or a hydrogen atom to create stable radical intermediates [56]. It has been demonstrated that phenolic substances such as gallic acid, coumaric acid, protocatechuic acid and salicylic acid contribute to increasing the antioxidant activity of algal extracts [57,58].

Flavonoids, the largest group of phenolic compounds, include strong antioxidant activity as the flavonoids can scavenge the reactive oxygen species directly, chelate free radicals via hydrogen/electron transfer and even chelate the trace metal ions, including Fe^{2+} and Cu^{+}. However, the molecule free radical scavenging ability highly varies dependent on the classes. Molecules with di-OH substitution at C3' and C4' have the highest antioxidant characteristics, followed by those with a double bond between C2 and C3. Therefore, antioxidative characteristics of several flavonoid classes were reported less significant than others [59,60]. Negative correlations between the flavonoid content and antioxidant activity reveal that the phenolic compounds contributed to the antioxidant activity. In contrast, flavonoids in the samples did not include strong antioxidant activities. Thorough analysis of the antioxidant potential in *Chlorella* sp. revealed the presence of significant quantities of the simple phenolics and hydroxycinnamic acids [57], promoting use of *Chlorella* sp. for the production of algal-derived bioactive compounds.

Moderate correlations (R^2=0.5298) were observed between the content of ω-3 PUFAs and the antioxidant activity. In fact, FAs have been demonstrated to affect antioxidant activity of the algal extracts. Furthermore, PUFAs have been described to include the most substantial effect on the antioxidant capacity of the extracts, with linoleic acid (a ω-6 FA), α-linolenic acid (a ω-3 FA), eicosapentaenoic acid (an ω-3 FA) and docosahexaenoic acid (an ω-3 FA) as the most significant representatives [61]. In this study, GC-MS data showed the presence of various FAs in the extracts of *Chlorella* sp., with the exponential phase and early stationary phase extracts particularly rich in PUFAs. The antioxidant activity was further in the exponential and early stationary phases than that in the late stationary phase, similar to that of a previous study [37] suggesting that the ω-3 and ω-6 FAs in *Chlorophyta* strains included substantial effects on antioxidant activity. The antioxidant activity of PUFAs highly depends on the specific types of FAs [62] and dependence of FA composition was reported with the age of the culture. Thus, further studies on variables affecting the specific FAs and complete FA profiles during the specific growth phases can further increase the potentials of *Chlorella* sp. for commercial uses.

4. Conclusion

While most studies have focused on identifying presence of bioactive molecules in microalgae, only a few studies have devoted to analyzing differences in bioactive compounds with the microbial growth. The current study has shown that the freshwater microalga and *Chlorella* includes potential as a source of natural antioxidants with substantial phenolic and flavonoid contents as well as various FAs. In addition, this study has demonstrated that the antioxidant potential and production of compounds contributing to the antioxidant activity are growth-phase dependent. In conclusion, these results reveal that extraction of the selected high-value metabolites needs biomass harvesting at a certain stage of the microbial growth. This model of batch culture can further be optimized in continuous mode of bioreactors to validate the current results.

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6. Conflict of Interest

The authors report no conflicts of interest.

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تجزیه و تحلیل تغییرات در زیست‌مولکول ها در طول مراحل مختلف رشد ریزجلبک آب شیرین گونه کلرلا

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چکیده

سابقه و هدف: از آنجا که گونه کلرلا حاوی ترکیباتی با خواص دارویی است و در نتیجه برای سلامتی انسان اهمیت دارد، یکی از ریزجلبک‌هایی است که بیشترین مطالعات بر روی آن انجام شده است. از آنجا که تغییرات زیست‌مولکول با رشد ریزجلبک ها تغییر می‌کند، ارزیابی این تغییرات در طول مراحل گوناگون رشد برای استخراج هدفمند زیست-مولکول‌ها ضروری است. در این مطالعه تغییرات در زیست‌مولکول های استخراج شده از گونه *کلرلا* در مراحل گوناگون رشد با استفاده از سیستم‌های حلال گوناگون مورد تجزیه و تحلیل قرار گرفت.

مواد و روش ها: گونه کلرلا از حوضچه آب شیرین جداسازی و از سه حلال (اتانول، متانول و استون) برای استخراج ترکیبات گیاهی از زیست‌توده در سه فاز رشد مختلف (رشد نمای، فاز ساکن اولیه و فاز پایانی سکون) استفاده شد. تجزیه و تحلیل اسیدهای چرب، فنول کل و میزان فلاونوئید، و فعالیت ضداکسایشی همه بر روی عصاره حاصل از مراحل گوناگون رشد انجام شد.

یافته‌ها و نتیجه‌گیری: غلظت فلاونوئید کل بر خلاف فاز ساکن اولیه، در فاز پایانی سکون در بالاترین میزان قرار داشت. در مقابل، بیشترین محتوای فنول کل در فاز رشد نمایی اندازه‌گیری شد. پروفایل اسیدهای چرب نشان داد که فاز ساکن اولیه جلبک حاوی مقادیر بالاتر اسیدهای چرب غیراشباع امگا ۳ و امگا ۶ در مقایسه با فاز پایانی سکون است. ترکیبات گیاهی عصاره در طول رشد میکروبی از نظر کمیت و نوع متفاوت و به سیستم حلال وابسته بودند. همبستگی بین محتوای فنول کل، اسیدهای چرب و مهار رادیکال آزاد DPPH مثبت بود و نشان می‌دهد که مقدار و نوع ترکیبات گیاهی بسته به مراحل رشد جلبک متفاوت است. تحقیقات تکمیلی بر روی گونه کلرلا می‌تواند پتانسیل-های آن‌را برای کاربردهای تجاری توسعه دهد.

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واژگان کلیدی

- فعالیت ضداکسایشی
- فلاونوییدها
- ریزجلبک، غذادارو
- فنول‌ها
- اسیدهای چرب چند غیراشباعی

***نویسنده مسئول**

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