

Article - Biological and Applied Sciences

Comprehensive Growth Analysis to Detect Optimal Time Point for Carotenoid Production: A Comparative Study in *Spirulina subsalsa* and *Chlorella pyrenoidosa*

Sruthi Padmakumar Pillai¹

<https://orcid.org/0009-0008-6210-9315>

Sangeeta Atul Godbole^{2*}

<https://orcid.org/0009-0009-6734-1647>

Rutwik Ravindra Manda Thengodkar³

<https://orcid.org/0009-0000-6358-8807>

Deepali Dande Thengodkar⁴

<https://orcid.org/0009-0003-5765-9738>

¹University of Mumbai, Jai Hind College, Department of Botany, Mumbai, Maharashtra, India; ²University of Mumbai, Jai Hind College, Department of Botany, Mumbai, Maharashtra, India; ³Food and Nutrition lab, Cyanohealth Pvt. Ltd., Mumbai, Maharashtra, India; ⁴Food and Nutrition lab, Cyanohealth Pvt. Ltd., Mumbai, Maharashtra, India.

Editor-in-Chief: Paulo Vitor Farago

Associate Editor: Jane Manfron

Received: 24-Nov-2024; Accepted: 16-Jun-2025

*Correspondence: sangeeta.godbole@jaihindcollege.edu.in; Tel.: +91-98-20128476 (S.A.G.).

HIGHLIGHTS

- Maximum carotenoids are observed on day 12 for *Spirulina subsalsa*
- Maximum carotenoids are observed on day 27 for *Chlorella pyrenoidosa*
- *Spirulina* shows faster growth than *Chlorella* with slow and sustained growth
- Total protein, chlorophyll and carotenoids are higher in *Spirulina* than *Chlorella*

Abstract: This study investigated the growth dynamics of *Spirulina subsalsa* and *Chlorella pyrenoidosa* under standardized phototrophic cultivation conditions to determine the optimal harvest time for maximum carotenoid yield. Both species were maintained at temperature (28°C-35°C), relative humidity (52%-55%), light intensity (2.5-3.5 klx) and a photoperiod of (10:14 h) light/dark cycle. Growth dynamics were assessed by monitoring cell density, biomass productivity, protein content, chlorophyll and carotenoid content over a defined cultivation period. *S. subsalsa* showed rapid growth, reaching peak cell density with in a shorter duration of 12 days, compared to *C. pyrenoidosa* which showed longer duration of 27 days. The biomass productivity was higher (186.36 ± 0.029 mg/L/day) in *Spirulina* compared to *Chlorella* (12.98 ± 0.546 mg/L/day). *S. subsalsa* showed higher protein content on day 6 (33.634 ± 0.97 mg/g fresh weight (FW)), higher chlorophyll on day 8 (1.25 ± 0.034 mg/g FW) and higher carotenoid on day 12 (0.379 ± 0.003 mg/g FW) compared to *C. pyrenoidosa* with maximum protein (3.374 ± 0.039 mg/g FW), maximum chlorophyll (0.252 ± 0.002 mg/g FW) and maximum carotenoid (0.054 ± 0.001 mg/g FW) on day 27. The protein,

chlorophyll, and carotenoid levels showed decrease in both the algae at the end of the experiment. Harvesting *S. subsalsa* on day 12 and *C. pyrenoidosa* on day 27 will result in increased carotenoid yield and enhance the efficiency of carotenoid extraction. The study provides a base for sustainable large-scale cultivation of two microalgal species with targeted harvesting of natural carotenoids to meet industrial demand.

Keywords: microalgae; *Spirulina subsalsa*; *Chlorella pyrenoidosa*; standardized microalgal cultivation; comparative microalgal study; microalgal growth analysis; time-dependent metabolite synthesis; carotenoid production peak; optimal harvest time.

INTRODUCTION

Microalgae are rich in nutrients such as proteins, enzymes, fatty acids, minerals, vitamins, and pigments like carotenoids. Among these metabolites, carotenoids have selectively proved to be beneficial for human health, particularly in preventing chronic diseases [1]. Microalgae have been shown to produce a variety of carotenoids, including xanthophylls and carotenes, as a part of their normal growth metabolism [2]. Carotenoids like β -carotene, zeaxanthin, and lutein show strong antioxidant properties against reactive oxygen species (ROS), by protecting cells from oxidative damage [3–5]. Both raw extracts and purified β -carotene obtained from microalgae are observed to show immunomodulation, hepatoprotective activity, anti-inflammatory properties, anti-obesity and anti-cancer effects [6–10]. Xanthophylls like lutein and zeaxanthin play a role as macular pigments and accumulate in the cornea to protect the retinal membrane from the damage caused by blue light supporting visual clarity. Lutein pigment is also found to help with health conditions like Alzheimer's disease, diabetic retinopathy, retinal nerve diseases, cataracts and age-related macular degeneration [6,11,12]. Both chlorophyll and carotenoids are in high commercial demand. Chlorophyll plays its role as a natural colorants in food and pharmaceuticals. Other health promoting properties of chlorophylls include prevention of diabetes, viral and chronic inflammatory diseases, cardiovascular diseases, metabolic syndrome, tumors, nerve damage and ophthalmic diseases [13–15].

The global market value of carotenoids in 2024 was approximately \$2.1 billion USD as per the BCC Research reports and is expected to increase to \$2.9 billion USD by 2029 at a compound annual growth rate (CAGR) of 6.7% [16]. Though, there are large applications of carotenoids, the carotenoid market is largely dominated by chemically synthesized carotenoids contributing 80-90% where as 10-20% of carotenoids are derived from natural resources [17,18]. The rising awareness of consumers regarding food safety and their preference for plant derived bioactive compounds are increasing the demand for natural carotenoids [17].

Microalgae possess several advantages for carotenoid production such as lower nutrient requirements, shorter growth cycles and higher photosynthetic efficiency when compared to terrestrial plants [19]. Additionally, the synthesis of carotenoids (carotenogenesis), is stimulated by the presence of reactive oxygen species (ROS), produced under various stress conditions such as exposure to high light intensity, salinity, or high temperatures [6].

Though, microalgae shows huge biodiversity, only a few species have been investigated extensively for biotechnological applications [20–22]. Among these, the most studied are the species of *Arthrospira* (*Spirulina*) *platensis* and *Chlorella vulgaris*. Several studies have reported the growth performance of different species of *Spirulina* and *Chlorella* under varying environmental conditions [23,24]. It is necessary to identify and explore underutilized microalgal species such as *S. subsalsa* and *C. pyrenoidosa* to discover and optimize alternative sources for commercially valuable carotenoids and to detect novel bioactive compounds.

S. subsalsa is a cyanobacteria belonging to the order Oscillatoriales, which are found to be spiral, or coiled filamentous in morphology [25]. Some studies have investigated its ability to produce different bioactive metabolites and its role as a bioremediation agent [26,27]. Previous studies showed that high light intensity increased the production of antioxidant compounds like carotenoids and polyphenols in *S. subsalsa* [20]. A study also reported that, *S. subsalsa* attained optimal growth at 35°C with increased production of phycocyanin under green light [28]. *C. pyrenoidosa* is a single-celled, spherically shaped, fresh water growing green algae [29]. Previous findings acknowledge *C. pyrenoidosa* as a third-generation bioenergy source due to its remarkable photosynthetic efficiency [30]. *C. pyrenoidosa* is widely used for its various health benefits such as anti-inflammatory, antioxidant, anti-bacterial, anti-tumor and immune boosting properties due to its rich nutrient profile [30,31]. The rich carotenoid content of *C. pyrenoidosa* was found to play a major role in its antitumour activity [32].

Growth medium composition plays an important role on microalgal biomass production and accumulation of bioactive metabolites as variations based on nutrient supply significantly affects the carotenoid and chlorophyll pigment production. An increase in nitrate concentration in the growth medium was reported to boost β -carotene production in *S. platensis*, when grown under specific environmental conditions [33]. There

are no similar studies on *S. subsalsa*. Research on optimization of lutein production in *C. pyrenoidosa* included improved lutein biosynthesis using sodium bicarbonate (100–150 mM) for its cultivation as well as exposing the algae to a condition of nutrient starvation [34,35]. Extraction methods have been further developed to optimize yields from microalgal biomass. These include non-mechanical methods like thermal, chemical and enzymatic methods and mechanical methods such as pressurized systems, electric fields, microwave, ultrasonication and supercritical extraction [36]. Lutein and chlorophyll recovery was optimized using pulsed electric field (PEF)-assisted extraction [37]. However, large-scale, cost-effective extraction remains a challenge. The extraction of *Spirulina* and *Chlorella* biomass requires individual optimization due to its diverse growth characteristics. Before proceeding with analysis of carotenoid profiles of these algae, one would need to develop an efficient carotenoid extraction procedure. Identifying the optimal time point of carotenoid production during the growth cycle can boost carotenoid extraction and hence, is a primary step before starting the extraction process.

While there are studies on different carotenoids present in various microalgae, there is no comprehensive data on the full spectrum of carotenoids or their isomers in *S. subsalsa* and *C. pyrenoidosa*. Most existing research is either on single species of algae or different algal species belonging to same group, lacking comparative studies across different algal groups. The present study provides a comparative growth analysis of *S. subsalsa* (blue-green algae) and *C. pyrenoidosa* (green algae) to determine the optimal time of carotenoid accumulation. Both algae were cultivated under controlled conditions of temperature (28°C-35°C), relative humidity (52%-55%), and light intensity (2.5-3.5 klx), using different optimal growth medium for each alga. Zarrouk's culture medium was used for *S. subsalsa* and modified Bold's Basal Medium (BBM) for *C. pyrenoidosa*. Growth and biomass productivity of both the algae were assessed at various time points. Important biochemical parameters, including protein, chlorophyll, and carotenoid content, were also measured at specific intervals. The growth period promoting maximum carotenogenesis in both the algae was identified by quantifying the carotenoids produced during specific growth intervals using spectrophotometric assays.

MATERIAL AND METHODS

Media composition

The strain NCIM-5143 of *S. subsalsa* and NCIM-2738 of *C. pyrenoidosa* were obtained from the culture collection center of National Chemical Laboratory (NCL), Pune, India. Both the cultures were cultivated phototrophically. *S. subsalsa* was cultivated in Zarrouk's culture medium with following composition (g/L): NaHCO₃-16.8 g, NaNO₃-2.5 g, K₂HPO₄-0.5 g, K₂SO₄-1.0 g, NaCl-1.0 g, CaCl₂·2H₂O-0.04 g, MgSO₄·7H₂O-0.2 g, FeSO₄·7H₂O-0.01 g and 1.0 mL of A5 solution which was composed of H₃BO₃-2.86 g, MnCl₂·4H₂O-1.81 g, ZnSO₄·4H₂O-0.222 g, Na₂MoO₄-0.018 g, CuSO₄·5H₂O-0.079 g [38,39]. *C. pyrenoidosa* was cultivated in modified Bold's Basal Medium (BBM) with following composition (g/L): KNO₃-0.375 g, K₂HPO₄-0.08 g, MgSO₄·7H₂O-0.1 g, CaCl₂·2H₂O-0.0375 g, NaCl-0.015 g, FeSO₄-0.005 g, Na₂EDTA-0.0625 g [40].

Preparation of stock cultures of algal samples

Agar slopes of *S. subsalsa* NCIM-5143 and *C. pyrenoidosa* NCIM-2738 were obtained from National Chemical Laboratory (NCL) culture collection center. A loopful of *Spirulina* and *Chlorella* culture samples were transferred into 20 mL of fresh Zarrouk's culture medium and modified Bold's Basal Medium (BBM) respectively, in Erlenmeyer flasks (100 mL) each under aseptic conditions. The culture inoculums were scaled up every two weeks. The final culture volumes for *S. subsalsa* and *C. pyrenoidosa* were raised to 2 liters and 4 liters, respectively, using larger 3-liter and 5-liter glass bottles. Final volume of stock cultures were decided considering the maintenance and growth rates of both the algal samples. For the maintenance of stock cultures, a photobioreactor (dimensions = 33 cm width x 47 cm height x 43 cm breadth) was constructed to provide optimal growth conditions and a simple cost-effective agitator system (speed = 60 rpm) was developed using silicon bottle brush for efficient mixing of the culture medium. The main body of the photobioreactor was constructed using acrylic sheet (thickness = 3 mm) and the basal part consisted of aluminum sheet. Illumination was provided using natural white light LED strips. Four LED strips (5-meter long) of natural white light were used to surround the photobioreactor, with each strip interconnected in series. The LED strips were equally spaced to support uniform lighting (Figure 1). The growth conditions were maintained in the following range., temperature (28°C-35°C), relative humidity (52%-55%), and light intensity (2.5-3.5 klx) throughout the experimental period [24,41–44]. The cultures were maintained under a photoperiod of 10/14 hours (light/dark cycle) against the conventional 12/12 hour light/dark cycle, due to the institutes operating hours. Temperature, humidity, and light intensity were checked two times (morning and

evening) daily during the experiment using thermometer, humidity meter, and lux meter (Lutron LX-101A), respectively.



Figure 1. Lab-scale photobioreactor setup for the phototrophic cultivation of *S. subsalsa* and *C. pyrenoidosa* under controlled light and temperature conditions.

Preparation of experimental inoculum

The culture inoculum of *S. subsalsa* and *C. pyrenoidosa* were prepared and maintained in the photobioreactor to acclimate the cultures for growth curve experiment. The culture medium for both the algae was prepared by dissolving each chemical separately and then mixing them together to make a final volume of 1000 mL. This step was followed by sterilization of the culture medium using nylon membrane filters (size-0.2 μm , 47 mm). For the preparation of culture inoculum, 100 mL of the culture samples from *Spirulina* and *Chlorella* stock solutions were transferred into 1000 mL of fresh sterile Zarrouk's medium and modified BBM, respectively. The inoculum cultures were cultivated for about two weeks. For further growth curve experiments, these prepared live stocks were used as the inoculum. The experimental inoculums were prepared using multiple Erlenmeyer flasks (500 mL) each, by transferring 20 mL of the inoculum in to 200 mL of freshly prepared sterilized media. From these multiple flasks, a single flask was used to measure all the selected growth parameters per day to reduce the risk of sample contamination. The flasks were checked for similarity in size to reduce variability in growth outcomes. The consistency of the inoculum samples in multiple flasks were confirmed by measuring optical density (OD) at 680 nm. Manual agitation of the inoculum cultures were performed twice (morning and evening) everyday.

Growth analysis of *S. subsalsa* and *C. pyrenoidosa*

Important growth parameters like cell density, biomass productivity, total protein and pigment content (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid) were assessed on specific days. Measurements were taken on days (2, 4, 6, 8, 10, 12, and 14) for *S. subsalsa* and days (5, 10, 15, 20, 23, 25, 27, and 31) for *C. pyrenoidosa*. The days for analysis were selected based on the preliminary growth curve experiment results which were performed before standardizing the final protocols.

Determination of microalgal growth

Measurement of *S. subsalsa* and *C. pyrenoidosa* growth was performed by recording absorbance at 680 nm with the help of a UV/Vis spectrophotometer (Equiptronics EQ 822A) [45,46]. The cell concentration was determined for *C. pyrenoidosa* by cell count method using the Neubauer hemocytometer [47,48]. As *S. subsalsa* appears filamentous, traditional methods of cell count were not applicable. To address this, the trichome density of *S. subsalsa* for every experimental day was assessed by visually observing under a light microscope. *Spirulina* sample volume (10 µL) used to examine trichome density per experimental day was kept constant. The sample was well mixed prior to the analysis to avoid clumping and ensure uniformity. The images of *Spirulina* sample observed under the light microscope (40x magnification) were captured to document the trichome density. The dry weight (initial and final) of both the algae was measured to determine the biomass productivity at the end of the growth curve experiment (14th day - *Spirulina* and 31st day - *Chlorella*) [49,50]. Biomass productivity was calculated by using values for initial dry weight and final dry weight. Initial dry weight was determined on the day of experimental inoculation (day 0) by filtering 20 mL of inoculum samples (*S. subsalsa* and *C. pyrenoidosa*) through pre-weighed Whatman No. 3 filter paper followed by its rinsing with distilled water. Further process involved oven-drying (60°C) of the wet biomass until a constant weight was achieved. After desiccation of the dry biomass on pre-weighed filter paper, the dry weight of the samples were measured. This process was repeated at the end of growth experiment to measure the final dry weight of algal samples. The volumetric biomass productivity (P_{Biomass}) of algae was calculated [51].

Protein extraction and quantification

Total protein content was determined using the Lowry assay [52]. Bovine serum albumin (BSA) at a concentration range of 0.0625 to 2 mg/mL was used to prepare a calibration curve. The protein concentrations of *S. subsalsa* and *C. pyrenoidosa* for each experimental day were determined by plotting graphs of (absorbance against protein concentration) and the values were expressed as mg/g fresh weight (FW). For protein analysis, 0.1 g of the fresh *Spirulina* biomass was used. For protein analysis in *Chlorella*, initial tests with 0.1 g of fresh biomass showed absorbance values against the readable range. As a result, 0.5 g of fresh *Chlorella* biomass was used for the final experimental analysis to ensure accurate results. The algal samples were crushed in 3 mL of 0.1N NaOH using mortar and pestle. Subsequent mixture was centrifuged for 30 min at 2000 rpm and the supernatant was collected. After transferring 1 mL of the supernatant solution in to a 20 mL test tube, 5 mL of alkaline copper solution was added to it. The resulting solutions were left for incubation for 10 minutes at room temperature. This step was followed by addition of 0.5 mL Folin–Ciocalteu reagent (1:1 diluted). After incubating the mixture for 30 minutes, the values for absorbance were measured at 750 nm using a UV/Vis spectrophotometer (Equiptronics EQ 822A).

Pigment extraction and quantification

Arnon's method was used to quantify pigments from *S. subsalsa* and *C. pyrenoidosa* for each experimental day and the values were expressed as mg/g fresh weight (FW) [53]. The amount of fresh biomass used for pigment analysis for both the algae was consistent with the amount used for protein analysis. 0.1 g of *Spirulina* and 0.5 g of *Chlorella* fresh biomass was crushed in 95% ethanol using mortar and pestle and an ice bath. The resulting mixture was centrifuged at room temperature (27°C–30°C) for 30 minutes at 2000 rpm. The collected supernatant was checked for absorbance at the following wavelengths (480 nm, 510 nm, 645 nm, and 663 nm) using a UV/Vis spectrophotometer (Equiptronics EQ 822A). Pigment content was calculated using the following formula: Chlorophyll a (Chl_a) mg/g FW = $12.7 \times A_{663} - 2.69 \times A_{645} \times \text{volume of sample (mL)} / 1000 \times \text{weight of tissue (g)}$; Chlorophyll b (Chl_b) mg/g FW = $22.9 \times A_{645} - 4.68 \times A_{663} \times \text{volume of sample (mL)} / 1000 \times \text{weight of tissue (g)}$; Total Chlorophyll ($\text{Chl}_{\text{total}}$) mg/g FW = $20.21 \times A_{645} + 8.02 \times A_{663} \times \text{volume of sample (mL)} / 1000 \times \text{weight of tissue (g)}$; Total Carotenoids ($\text{Car}_{\text{total}}$) mg/g FW = $7.6 \times A_{480} - 1.49 \times A_{510} \times \text{volume of sample (mL)} / 1000 \times \text{weight of tissue (g)}$.

Statistical analysis

Data were analysed statistically using Microsoft Excel for standard deviation (SD) and standard error (SE) calculations. Python (SciPy library) was used for statistical tests. Experiments were carried out in triplicates (n=3). The results were reported as mean $\pm$ SE at 95% confidence. Differences between time points were analyzed using one-way analysis of variance (ANOVA), while pairwise comparisons were assessed using an independent *t*-test, with statistical significance set at $p < 0.05$.

RESULTS

Comparison of growth patterns of *S. subsalsa* and *C. pyrenoidosa* using turbidity and cell count measurements

Comparative growth analysis of *S. subsalsa* and *C. pyrenoidosa* was studied. Figure 2 presents the comparative growth analysis of *S. subsalsa* and *C. pyrenoidosa* against time, using turbidity by noting optical density (OD) measurements at 680 nm. Growth of *S. subsalsa* was also checked by observing trichome density on every experimental day. The images were captured after every visual inspection to document the trichome density of *S. subsalsa* for each experimental day as shown in Figure 3. Results of both methods concluded that *S. subsalsa* shows rapid growth within 14 days. The highest growth in *S. subsalsa* was observed on day 12 with a maximum OD of 2 ± 0.025 . A lag phase was observed in *S. subsalsa* during days 0 to 2 followed by an exponential phase during days 4 to 10. During days 12 to 14, growth was found to slow down with relatively stable OD, indicating stationary phase. In comparison, *C. pyrenoidosa* showed slower but sustained growth up to 31 days. The highest growth in *C. pyrenoidosa* was observed on day 31 with a maximum OD of 0.995 ± 0.017 . *C. pyrenoidosa* growth as analyzed for every experimental day using turbidity showed a stable increase in cell density from days 5 to 31. *C. pyrenoidosa* growth was also analyzed for every experimental day using cell count measurements. However, the cell count results for *C. pyrenoidosa* was maximum ($6.25 \times 10^6 \pm 14.974$ cells/mL) on day 27 followed by a decline on day 31 as seen in Table 1 and Figure 4. The difference in turbidity and cell count data of *C. pyrenoidosa* indicates interference of other factors like cell aggregation, release of extracellular substances, and cell debris to overall turbidity increase detected at the final day i.e. day 31 of *Chlorella* growth experiment. As a result, based on the cell count data, it can be concluded that *C. pyrenoidosa* strain NCIM 2738 shows a decline in its growth on day 31 of the growth experiments. Comparing the experimental results obtained for both the algae, it can be concluded that *S. subsalsa* shows rapid growth compared to *C. pyrenoidosa*.

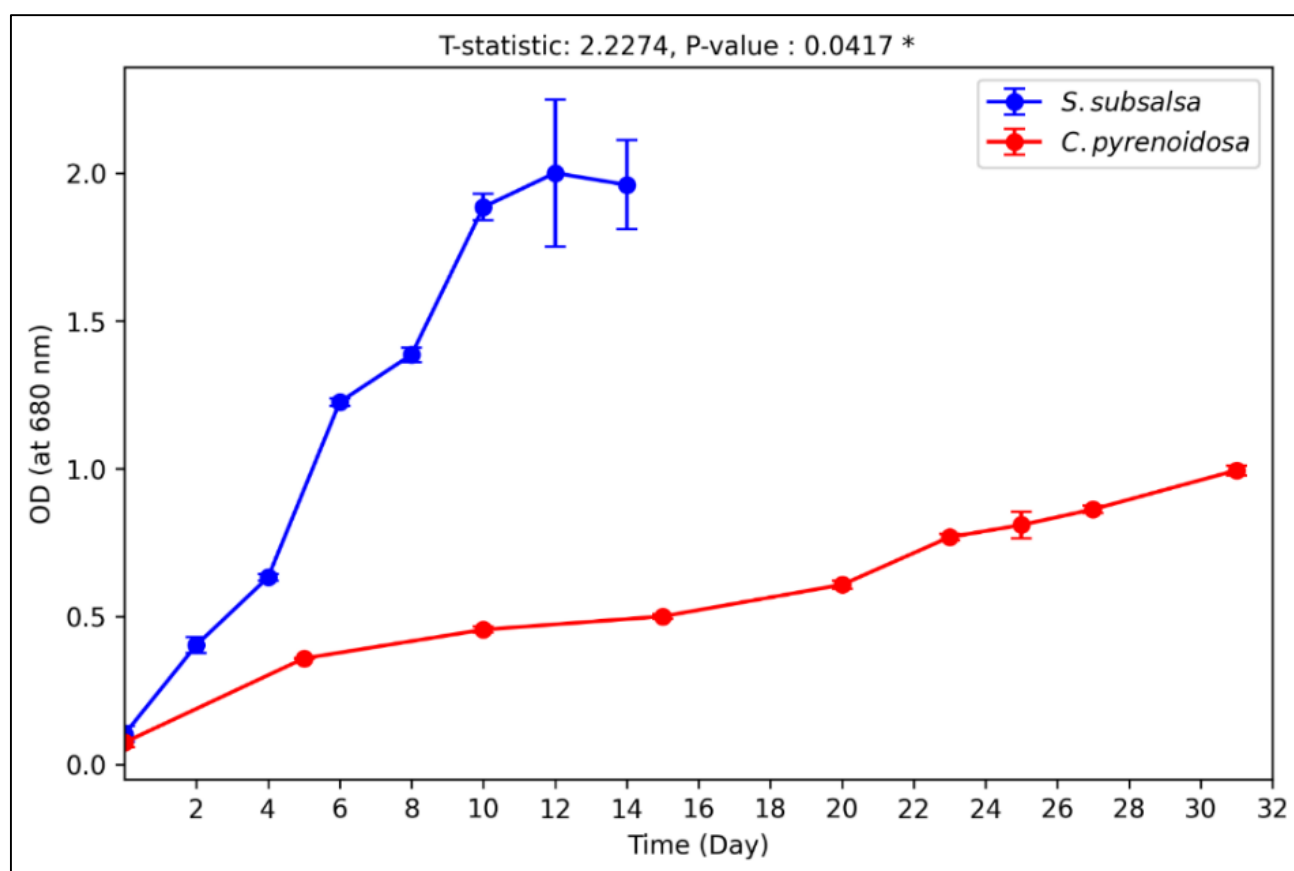


Figure 2. Comparative growth analysis of *S. subsalsa* and *C. pyrenoidosa*: Optical Density v/s Time. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence. Statistical significance was determined using an independent t-test. A single asterisk (*) indicates a statistically significant difference ($p < 0.05$) between species.

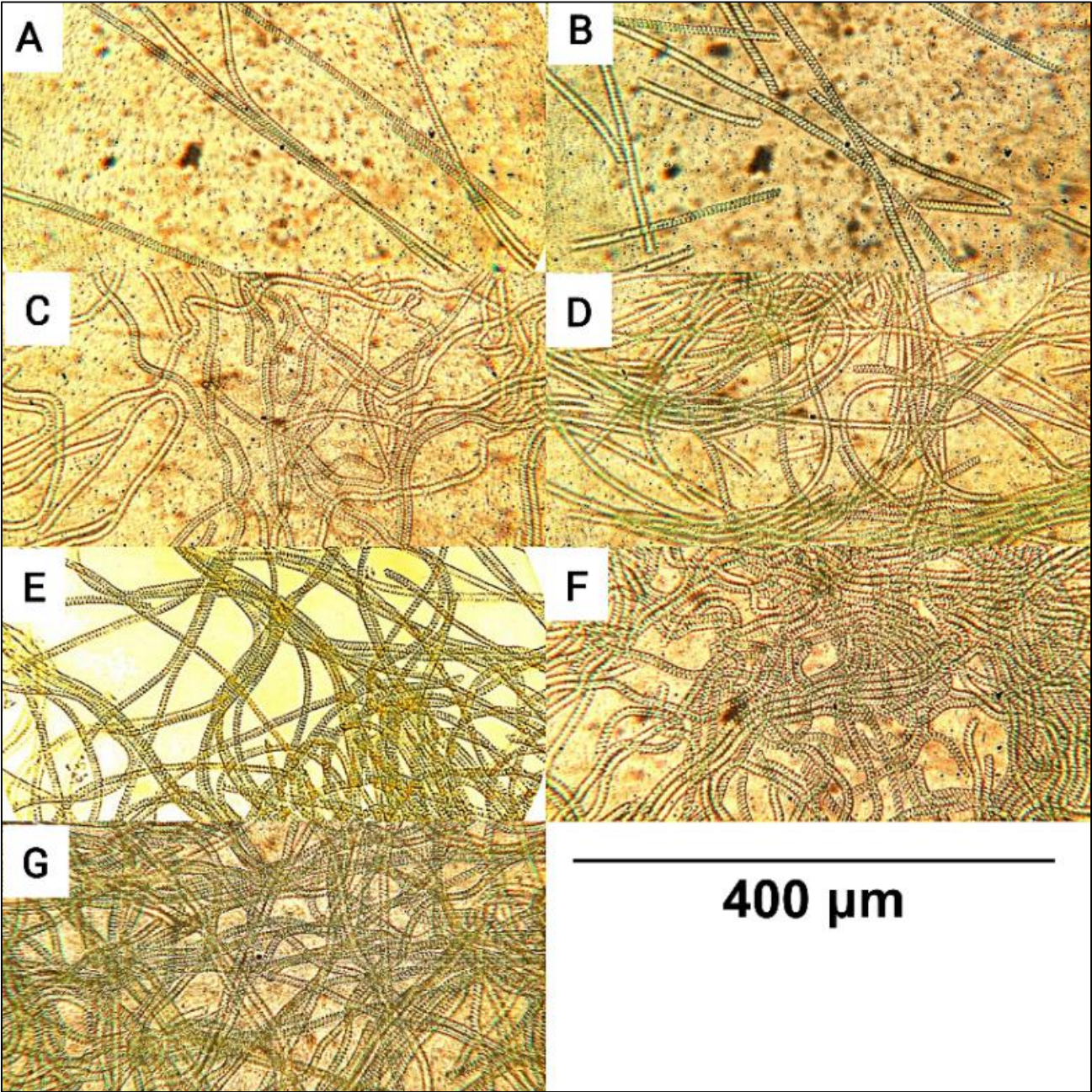


Figure 3. Growth analysis of *S. subsalsa* using images capturing trichome density under a light microscope (40x magnification) on different days (A, B, C, D, E, F and G corresponding to day 2, 4, 6, 8, 10, 12 and 14 respectively). These images represent multiple observations.

Table 1. Growth analysis of *C. pyrenoidosa*: Cell count v/s Time.

Time (Day)	Cell Count (x 10 ⁶ cells/mL)
0	0.683 ± 0.038
5	2.405 ± 0.047
10	3.913 ± 0.034
15	4.368 ± 0.044
20	4.515 ± 0.047
23	5.048 ± 0.044
25	5.750 ± 0.055
27	6.250 ± 0.037
31	4.335 ± 0.038

The data are the mean values of 3 replicates ± SE at 95% confidence.

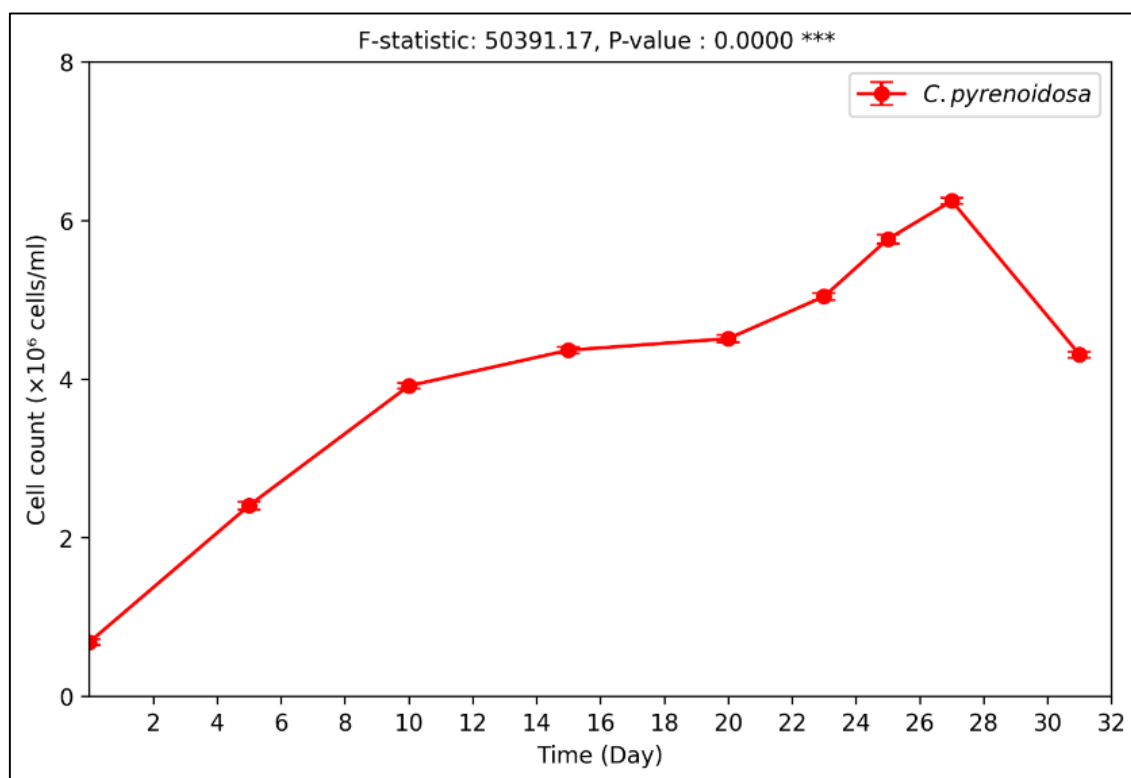


Figure 4. Growth analysis of *C. pyrenoidosa*: Cell count v/s Time. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence. Statistical analysis was performed using One-Way ANOVA. Three asterisks (***) indicate a highly significant difference ($p < 0.001$) between time points.

Comparison of biomass productivity (mg/L/day) in *S. subsalsa* and *C. pyrenoidosa*.

The growth of *S. subsalsa* and *C. pyrenoidosa* were also assessed by estimating the biomass productivity during the cultivation period as shown in Table 2. The biomass productivity was found to be higher in *S. subsalsa* (186.36 ± 0.029 mg/L/day) throughout the 14-day cultivation period compared to *C. pyrenoidosa* (12.98 ± 0.546 mg/L/day) throughout the 31-day cultivation day.

Table 2. Comparative Analysis of Biomass Productivity (mg/L/day) in *S. subsalsa* and *C. pyrenoidosa*.

Algal species	Biomass productivity (mg/L/day)
<i>S. subsalsa</i>	186.360 ± 0.029
<i>C. pyrenoidosa</i>	12.980 ± 0.546

The data are the mean values of 3 replicates $\pm$ SE at 95% confidence.

Comparison of protein (mg/g FW), chlorophyll (mg/g FW), and carotenoid (mg/g FW) contents in *S. subsalsa* and *C. pyrenoidosa*.

The total protein, total chlorophyll and total carotenoid contents were found to be considerably higher in *S. subsalsa* than *C. pyrenoidosa* during the experimental period as seen in Table 3. In *S. subsalsa*, the total protein content was maximum (33.634 ± 0.97 mg/g FW) on day 6 as shown in Figure 5, the total chlorophyll was maximum (1.25 ± 0.034 mg/g FW) on day 8 and the total carotenoid was maximum (0.379 ± 0.003 mg/g FW) on day 12 as given in Figure 6. In comparison, *C. pyrenoidosa* showed maximum amount of total protein (3.374 ± 0.039 mg/g FW) as seen in Figure 5. Maximum total chlorophyll (0.252 ± 0.002 mg/g FW) and total carotenoid (0.054 ± 0.001 mg/g FW) were also found to be present on day 27 in *C. pyrenoidosa* as seen in Figure 7. Towards the completion of growth cycle, the protein, chlorophyll and carotenoid contents were found to decline in both the algal species studied. Comparative analysis of changes in pigment contents over time in *S. subsalsa* and *C. pyrenoidosa* is shown in Figure 8.

Table 3. Comparison of Protein (mg/g FW), Chlorophyll (mg/g FW), and Carotenoid (mg/g FW) contents in *S. subsalsa* and *C. pyrenoidosa*.

Algal species	Time (Day)	Protein (mg/g FW)	Chl a (mg/g FW)	Chl b (mg/g FW)	Total Chl (mg/g FW)	Total Car (mg/g FW)
<i>S. subsalsa</i>	2	9.192 ± 0.568	0.151 ± 0.006	0.190 ± 0.002	0.342 ± 0.008	0.065 ± 0.014
	4	15.237 ± 0.784	0.222 ± 0.006	0.266 ± 0.014	0.487 ± 0.020	0.178 ± 0.003
	6	33.634 ± 0.970	0.521 ± 0.009	0.396 ± 0.020	0.917 ± 0.011	0.204 ± 0.003
	8	26.749 ± 1.131	0.746 ± 0.013	0.504 ± 0.040	1.250 ± 0.034	0.265 ± 0.005
	10	22.685 ± 0.710	0.704 ± 0.036	0.428 ± 0.049	1.132 ± 0.014	0.320 ± 0.004
	12	14.061 ± 0.248	0.489 ± 0.001	0.286 ± 0.011	0.775 ± 0.011	0.379 ± 0.003
	14	9.827 ± 0.316	0.270 ± 0.005	0.254 ± 0.009	0.524 ± 0.014	0.180 ± 0.002
<i>C. pyrenoidosa</i>	5	1.174 ± 0.092	0.007 ± 0.000	0.009 ± 0.002	0.015 ± 0.002	0.005 ± 0.000
	10	1.464 ± 0.024	0.013 ± 0.001	0.012 ± 0.001	0.025 ± 0.002	0.010 ± 0.000
	15	1.866 ± 0.075	0.029 ± 0.001	0.022 ± 0.001	0.050 ± 0.001	0.015 ± 0.000
	20	2.109 ± 0.075	0.046 ± 0.001	0.044 ± 0.001	0.090 ± 0.000	0.030 ± 0.000
	23	2.636 ± 0.076	0.078 ± 0.001	0.075 ± 0.001	0.153 ± 0.002	0.046 ± 0.000
	25	3.247 ± 0.016	0.134 ± 0.001	0.112 ± 0.006	0.245 ± 0.005	0.053 ± 0.002
	27	3.374 ± 0.039	0.124 ± 0.001	0.128 ± 0.001	0.252 ± 0.002	0.054 ± 0.001
	31	1.720 ± 0.069	0.032 ± 0.001	0.032 ± 0.002	0.064 ± 0.001	0.026 ± 0.000

The data are the mean values of 3 replicates ± SE at 95% confidence.

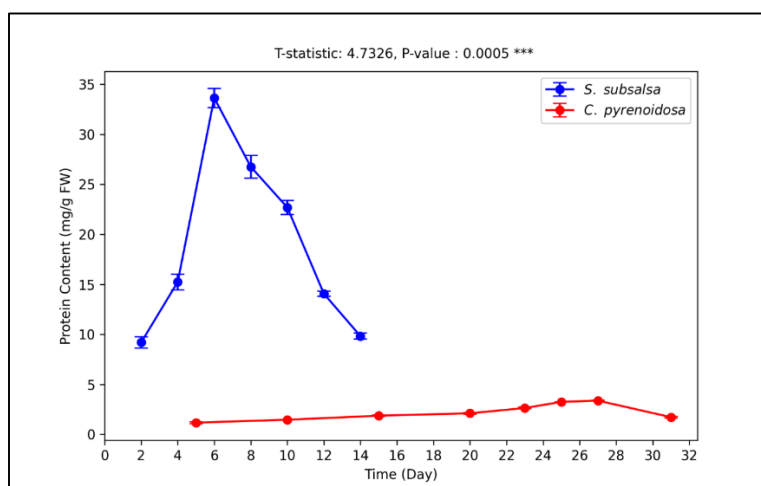


Figure 5. Comparative protein content (mg/g FW) in *S. subsalsa* and *C. pyrenoidosa*. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence. Statistical significance was determined using an independent t-test. Three asterisks (***) indicate a highly significant difference ($p < 0.001$) between species.

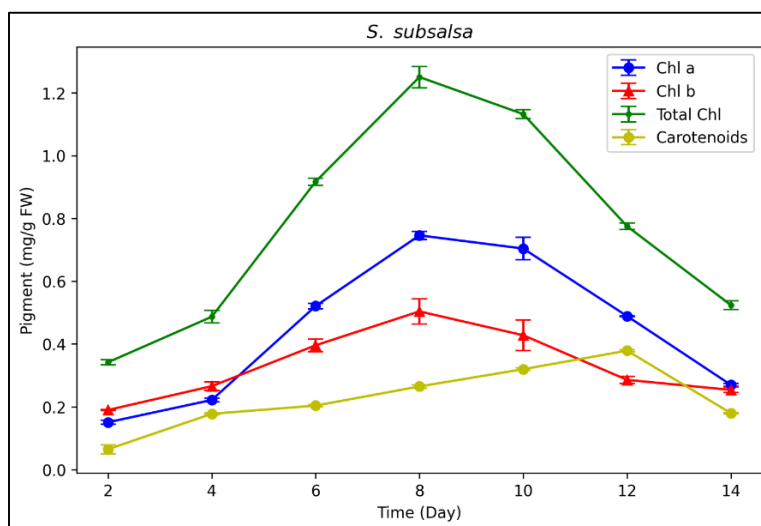


Figure 6. Changes in the following pigment contents (mg/g FW): Chlorophyll a, Chlorophyll b, Total Chlorophyll and Total carotenoid in *S. subsalsa*. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence.

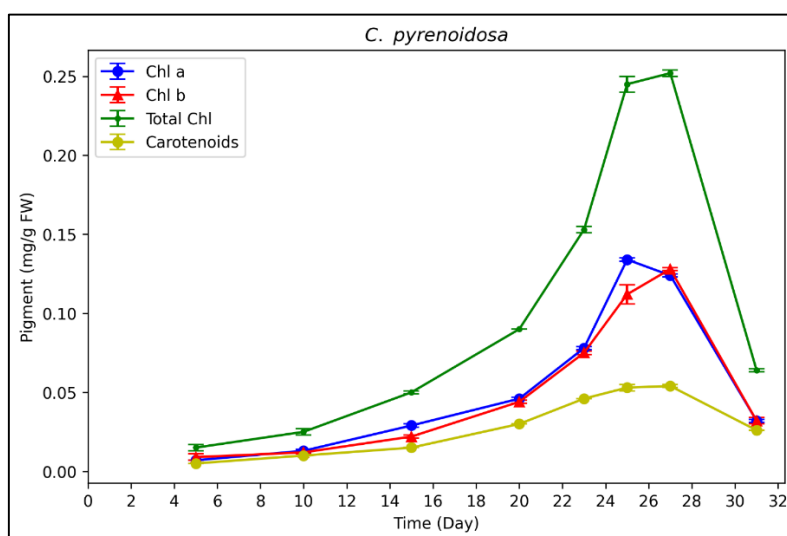


Figure 7. Changes in the following pigment contents (mg/g FW): Chlorophyll a, Chlorophyll b, Total Chlorophyll and Total carotenoid in *C. pyrenoidosa*. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence.

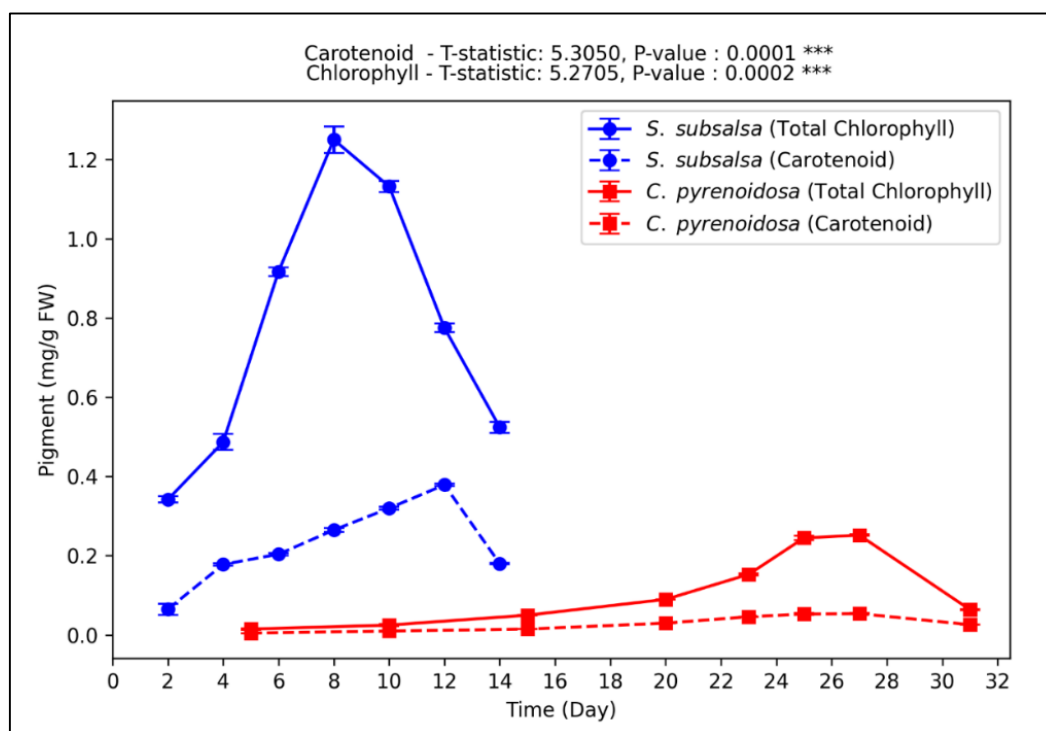


Figure 8. Comparative pigment contents (mg/g FW): Total Chlorophyll and Total carotenoid in *S. subsalsa* and *C. pyrenoidosa*. The data are the mean values of 3 replicates $\pm$ SE at 95% confidence. Statistical significance was determined using an independent t-test. Three asterisks (***) indicate a highly significant difference ($p < 0.001$) between species.

DISCUSSION

In this study, we observed distinct growth patterns and biochemical compositions in *S. subsalsa* NCIM 5143 and *C. pyrenoidosa* NCIM 2738. The turbidity of *S. subsalsa* showed a rapid increase, peaking on day 12, indicating a faster growth rate compared to *C. pyrenoidosa*, which continued to increase until day 27. This aligns with studies on other *Spirulina* strains, where rapid growth is observed in the early stages of cultivation [54,55]. The slower but sustained increase in turbidity and cell count (cells/ml) in *Chlorella* is consistent with its prolonged exponential phase observed in earlier research [56]. Biomass productivity (mg/L/day) was higher in *S. subsalsa* compared to *C. pyrenoidosa* during the experimental period, reflecting its faster growth rate. The protein content (mg/g FW) in *Spirulina* was highest around day 6, while *Chlorella* showed a peak in protein content around day 27. Pigment accumulation (mg/g FW) in both *Spirulina* and *Chlorella* followed distinct patterns. Total chlorophyll and carotenoid in *Spirulina* peaked on day 8 and day 12 respectively. This aligns with previous studies involving other strains of *Spirulina* (9). In contrast, *C. pyrenoidosa* showed maximum pigment content, including total chlorophyll and carotenoid on day 27. This delayed accumulation could be due to its extended growth phase, as reported in other *Chlorella* strains [57]. As per the observations, *S. subsalsa* showed significantly higher growth and production of compounds like protein, chlorophyll and carotenoid when compared to *C. pyrenoidosa*. Thus, *S. subsalsa* can be considered as a more favorable source than *C. pyrenoidosa* for extraction of carotenoids. However, as *S. subsalsa* shows rapid growth, its requirement for medium constituents also becomes higher than *C. pyrenoidosa*.

Collectively, *S. subsalsa* is desirable for faster production of carotenoids favouring short-term cultivations, whereas, *C. pyrenoidosa* can be considered for slow and sustainable carotenoid productions in long-term cultivations. Experiments on optimization of growth parameters could result in increased carotenoid synthesis in *C. pyrenoidosa*. Upcoming experiments will explore extraction and identification of carotenoids from *S. subsalsa* and *C. pyrenoidosa*.

Funding: This research received no external funding.

Acknowledgments: None

Conflicts of Interest: The authors declare no conflict of interest.

Data availability statement: Research data are available in the body of the manuscript.

REFERENCES

1. Cezare-Gomes EA, Mejia-da-Silva LDC, Pérez-Mora LS, Matsudo MC, Ferreira-Camargo LS, Singh AK, et al. Potential of Microalgae Carotenoids for Industrial Application. *Appl Biochem Biotechnol*. 2019 Jul;188(3):602–34.
2. Maltsev Y, Maltseva K, Kulikovskiy M, Maltseva S. Influence of Light Conditions on Microalgae Growth and Content of Lipids, Carotenoids, and Fatty Acid Composition. *Biology*. 2021 Oct;10(10):1060.
3. Park WS, Kim HJ, Li M, Lim DH, Kim J, Kwak SS, et al. Two Classes of Pigments, Carotenoids and C-Phycocyanin, in Spirulina Powder and Their Antioxidant Activities. *Molecules*. 2018 Aug;23(8):2065.
4. Konícková R, Vanková K, Vaníková J, Vánová K, Muchová L, Subhanová I, et al. Anti-cancer effects of blue-green alga *Spirulina platensis*, a natural source of bilirubin-like tetrapyrrolic compounds. *Ann Hepatol*. 2014 Apr 21;13(2):273–83.
5. Spínola MP, Mendes AR, Prates JAM. Chemical Composition, Bioactivities, and Applications of *Spirulina* (*Limnospira platensis*) in Food, Feed, and Medicine. *Foods*. 2024 Jan;13(22):3656.
6. Ambati RR, Gogisetty D, Aswathanarayana RG, Sarada B, Panduranga PN, Bo L, et al. Industrial potential of carotenoid pigments from microalgae: Current trends and future prospects. *Crit Rev Food Sci Nutr*. 2019 Jul 4;59(12):1880–902.
7. Wang J, Hu X, Chen J, Wang T, Huang X, Chen G. The Extraction of β -Carotene from Microalgae for Testing Their Health Benefits. *Foods*. 2022 Feb 10;11(4):502.
8. Martínez Andrade KA, Lauritano C, Romano G, Ianora A. Marine Microalgae with Anti-Cancer Properties. *Mar Drugs*. 2018 May;16(5):165.
9. Riccio G, Lauritano C. Microalgae with Immunomodulatory Activities. *Mar Drugs*. 2020 Jan;18(1):2.
10. Marcelino G, Machate DJ, Freitas K de C, Hiane PA, Maldonado IR, Pott A, et al. β -Carotene: Preventive Role for Type 2 Diabetes Mellitus and Obesity: A Review. *Molecules*. 2020 Jan;25(24):5803.
11. Chen JH, Chen CY, Chang JS. Lutein production with wild-type and mutant strains of *Chlorella sorokiniana* MB-1 under mixotrophic growth. *J Taiwan Inst Chem Eng*. 2017 Oct 1;79:66–73.
12. Zheng H, Wang Y, Li S, Nagarajan D, Varjani S, Lee DJ, et al. Recent advances in lutein production from microalgae. *Renew Sustain Energy Rev*. 2022 Jan 1;153:111795.
13. Sun D, Wu S, Li X, Ge B, Zhou C, Yan X, et al. The Structure, Functions and Potential Medicinal Effects of Chlorophylls Derived from Microalgae. *Mar Drugs*. 2024 Feb;22(2):65.
14. M. U. N, Mehar JG, Mudliar SN, Shekh AY. Recent Advances in Microalgal Bioactives for Food, Feed, and Healthcare Products: Commercial Potential, Market Space, and Sustainability. *Compr Rev Food Sci Food Saf*. 2019;18(6):1882–97.
15. Sun H, Wang Y, He Y, Liu B, Mou H, Chen F, et al. Microalgae-Derived Pigments for the Food Industry. *Mar Drugs*. 2023 Feb;21(2):82.
16. Global Carotenoids Markets Size and Industry Analysis 2029 [Internet]. [cited 2025 Mar 27]. Available from: <https://www.bccresearch.com/market-research/food-and-beverage/the-global-market-for-carotenoids.html>
17. Duan X, Xie C, Hill DRA, Barrow CJ, Dunshea FR, Martin GJO, et al. Bioaccessibility, Bioavailability and Bioactivities of Carotenoids in Microalgae: A Review. *Food Rev Int*. 2024 Jan 2;40(1):230–59.
18. Saini RK, Keum YS. Carotenoid extraction methods: A review of recent developments. *Food Chem*. 2018 Feb;240:90–103.
19. Ren Y, Sun H, Deng J, Huang J, Chen F. Carotenoid Production from Microalgae: Biosynthesis, Salinity Responses and Novel Biotechnologies. *Mar Drugs*. 2021 Dec 20;19(12):713.
20. Pistelli L, Del Mondo A, Smerilli A, Corato F, Sansone C, Brunet C. Biotechnological response curve of the cyanobacterium *Spirulina subsalsa* to light energy gradient. *Biotechnol Biofuels*. 2023 Feb 19;16(1):28.
21. Grama SB, Liu Z, Li J. Emerging Trends in Genetic Engineering of Microalgae for Commercial Applications. *Mar Drugs*. 2022 May;20(5):285.
22. Fernandes T, Cordeiro N. Microalgae as Sustainable Biofactories to Produce High-Value Lipids: Biodiversity, Exploitation, and Biotechnological Applications. *Mar Drugs*. 2021 Oct 14;19(10):573.
23. El-Sheekh M, Morsy H, Hassan L. Assessment of the optimum growth medium and the effect of different light intensities on growth and photosynthetic pigments of *Chlorella vulgaris* and *Scenedesmus arvernensis*. *Egypt J Bot*. 2019 Dec 30;0(0):0–0.
24. Soni RA, Sudhakar K, Rana RS. Comparative study on the growth performance of *Spirulina platensis* on modifying culture media. *Energy Rep*. 2019 Nov 1;5:327–36.
25. Bej S, Swain S, Bishoyi AK, Sahoo CR, Jali BR, Khan MS, et al. Biosynthesis, characterization and monitoring *in vitro* antibacterial efficiencies of AgNPs with filamentous cyanobacteria *Spirulina* sp. And *S. Subsalsa* against pathogenic bacteria. *J King Saud Univ Sci*. 2024 Sep 1;36(8):103336.
26. Jiang L, Pei H, Hu W, Ji Y, Han L, Ma G. The feasibility of using complex wastewater from a monosodium glutamate factory to cultivate *Spirulina subsalsa* and accumulate biochemical composition. *Bioresour Technol*. 2015 Mar 1;180:304–10.
27. Mazur-Marzec H, Błaszczuk A, Felczykowska A, Hohlfeld N, Kobos J, Toruńska-Sitarz A, et al. Baltic cyanobacteria – a source of biologically active compounds. *Eur J Phycol*. 2015 Jul 3;50(3):343–60.
28. Jiang L, Yu S, Chen H, Pei H. Enhanced phycocyanin production from *Spirulina subsalsa* via freshwater and marine cultivation with optimized light source and temperature. *Bioresour Technol*. 2023 Jun 1;378:129009.

29. Lian H, Wen C, Zhang J, Feng Y, Duan Y, Zhou J, et al. Effects of simultaneous dual-frequency divergent ultrasound-assisted extraction on the structure, thermal and antioxidant properties of protein from *Chlorella pyrenoidosa*. *Algal Res.* 2021 Jun 1;56:102294.
30. Wu Q, Ma Y, Zhang L, Han J, Lei Y, Le Y, et al. Extraction, functionality, and applications of *Chlorella pyrenoidosa* protein/peptide. *Curr Res Food Sci.* 2023 Jan 1;7:100621.
31. Zhang R, Chen J, Zhang X. Extraction of intracellular protein from *Chlorella pyrenoidosa* using a combination of ethanol soaking, enzyme digest, ultrasonication and homogenization techniques. *Bioresour Technol.* 2018 Jan 1;247:267–72.
32. Kubatka P, Kapinová A, Kružliak P, Kello M, Výbohová D, Kajo K, et al. Antineoplastic effects of *Chlorella pyrenoidosa* in the breast cancer model. *Nutrition.* 2015 Apr 1;31(4):560–9.
33. Hamidi M, Mohammadi A, Mashhadi H, Mahmoudnia F. Evaluation of effective environmental parameters on lipid, protein and beta-carotene production in *Spirulina platensis* microalga. *Results Eng.* 2023 Jun 1;18:101102.
34. Sampathkumar SJ, Gothandam KM. Sodium bicarbonate augmentation enhances lutein biosynthesis in green microalgae *Chlorella pyrenoidosa*. *Biocatal Agric Biotechnol.* 2019 Nov;22:101406.
35. Sampathkumar SJ, Srivastava P, Ramachandran S, Sivashanmugam K, Gothandam KM. Lutein: A potential antibiofilm and antiquorum sensing molecule from green microalga *Chlorella pyrenoidosa*. *Microb Pathog.* 2019 Oct 1;135:103658.
36. Pagels F, Pereira RN, Vicente AA, Guedes AC. Extraction of Pigments from Microalgae and Cyanobacteria—A Review on Current Methodologies. *Appl Sci.* 2021 Jan;11(11):5187.
37. Katsimichas A, Stathi S, Dimopoulos G, Giannakourou M, Taoukis P. Kinetics of pulsed electric fields assisted pigment extraction from *Chlorella pyrenoidosa*. *Innov Food Sci Emerg Technol.* 2024 Jan 1;91:103547.
38. ZARROUK C. [Contribution to the study of a cyanophyceae. Influence of various physical and chemical factors on the growth and photosynthesis of *Spirulina maxima*]. Thesis Univ Paris, France [Internet]. 1966 [cited 2024 Nov 2]; Available from: <https://cir.nii.ac.jp/crid/1571698599087861632>
39. Kumari A, Sharma V, Pathak AK, Guria C. Cultivation of *Spirulina platensis* using NPK-10:26:26 complex fertilizer and simulated flue gas in sintered disk chromatographic glass bubble column. *J Environ Chem Eng.* 2014 Sep;2(3):1859–69.
40. Mubarak M, Shaija A, Suchithra TV. Evaluation of ferric chloride and electroflocculation of *Chlorella pyrenoidosa* and reuse of the culture medium for subsequent cultures. *J Environ Chem Eng.* 2020 Feb;8(1):103612.
41. Coronado-Reyes JA, Salazar-Torres JA, Juárez-Campos B, González-Hernández JC. *Chlorella vulgaris*, a microalgae important to be used in Biotechnology: a review. *Food Sci Technol.* 2020 Nov 6;42:e37320.
42. Sharma R, Singh GP, Sharma VK. Effects of culture conditions on growth and biochemical profile of *Chlorella vulgaris*. *J Plant Pathol Microbiol.* 2012;3(5):1–6.
43. Ramlee A, Rasdi W, Abd ME. Microalgae and the factors involved in successful propagation for mass production. *J Sustain Sci Manag.* 2021;16(4):21–42.
44. Fagiri YMA, Salleh A, El-Nagerabi SAF. Impact of physico-chemical parameters on the physiological growth of *Arthrospira* (*Spirulina platensis*) exogenous strain UTEXLB2340. *African Journal of Biotechnology* [Internet]. 2013 [cited 2024 Sep 17];12(35). Available from: <https://www.ajol.info/index.php/ajb/article/view/135481>
45. Michael A, Kyewalyanga MS, Lugomela CV. Biomass and nutritive value of *Spirulina* (*Arthrospira fusiformis*) cultivated in a cost-effective medium. *Ann Microbiol.* 2019 Dec;69(13):1387–95.
46. Pandey A, Srivastava S, Kumar S. Development and cost-benefit analysis of a novel process for biofuel production from microalgae using pre-treated high-strength fresh cheese whey wastewater. *Environ Sci Pollut Res.* 2020 Jul 1;27(19):23963–80.
47. Ratowski P, Hawrot-Paw M. Production of *Chlorella vulgaris* Biomass in Tubular Photobioreactors during Different Culture Conditions. *Appl Sci.* 2021 Jan;11(7):3106.
48. Sarrafzadeh MH, La HJ, Seo SH, Asgharnejad H, Oh HM. Evaluation of various techniques for microalgal biomass quantification. *J Biotechnol.* 2015 Dec;216:90–7.
49. Yun HS, Kim YS, Yoon HS. Characterization of *Chlorella sorokiniana* and *Chlorella vulgaris* fatty acid components under a wide range of light intensity and growth temperature for their use as biological resources. *Heliyon.* 2020 Jul 1;6(7):e04447.
50. Zhu CJ, Lee YK. Determination of biomass dry weight of marine microalgae. *J Appl Phycol.* 1997 Apr 1;9(2):189–94.
51. Hempel N, Petrick I, Behrendt F. Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. *J Appl Phycol.* 2012 Dec 1;24(6):1407–18.
52. Lowry OliverH, Rosebrough NiraJ, Farr AL, Randall RoseJ. Protein measurement with the folin phenol reagent. *J Biol Chem.* 1951 Nov 1;193(1):265–75.
53. Arnon DI. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 1949 Jan;24(1):1–15.
54. Balaji S, Kalaivani T, Rajasekaran C, Shalini M, Vinodhini S, Priyadharshini SS, et al. Removal of heavy metals from tannery effluents of Ambur industrial area, Tamilnadu by *Arthrospira* (*Spirulina*) *platensis*. *Environ Monit Assess.* 2015 Jun;187(6):325.

55. Griffiths MJ, Garcin C, van Hille RP, Harrison STL. Interference by pigment in the estimation of microalgal biomass concentration by optical density. *J Microbiol Methods*. 2011 May;85(2):119–23.
56. El-Sheekh M, Sabagh S, Abou-El-Souod G, Elbeltagy A. The effect of different growth conditions on the biomass and chemical constituents of *Chlorella vulgaris*. *Exp Biol*. 2018 Aug 22;1:121–31.
57. Duppeti H, Chakraborty S, Das BS, Mallick N, Kotamreddy JNR. Rapid assessment of algal biomass and pigment contents using diffuse reflectance spectroscopy and chemometrics. *Algal Res*. 2017 Nov 1;27:274–85.



© 2025 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>)