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Enhanced carotenoid production in a novel *Chlorella vulgaris* isolate under nitrogen limitation: dynamics of pigment diversity across growth phases

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ABSTRACT

Chlorella vulgaris is of significant biotechnology interest with broad applications in the production of valuable compounds such as carotenoids. Although extensively studied, comprehensive investigations of its pigment composition under nutrient stress are limited. This study presents a detailed biochemical and physiological analysis of a new *C. vulgaris* isolate, focusing on pigment diversity across different growth stages and varying nitrogen levels. After morphological and molecular identification, the isolate was cultivated in BG11 medium supplemented with different $NaNO_3$ concentrations (1.5, 0.75, 0.375 and 0 g l^{-1}). Growth kinetics, photosynthetic activity and pigment profiles were monitored throughout the cultivation period. Using high-performance liquid chromatography, a total of 12 pigments were identified, including some not commonly found in *C. vulgaris*, such as fucoxanthin and 19′-butanoyloxyfucoxanthin. Moderate nitrogen limitation (0.375 g l^{-1}) led to a 43% increase in total carotenoid content without negatively affecting biomass yield or photosynthetic integrity. Under these conditions, levels of lutein, violaxanthin, fucoxanthin, zeaxanthin, *α*- and *β*-carotene were all enhanced, with lutein reaching a notably high content of 2.6 mg g^{-1} dry weight. Overall, our findings highlight the capacity of our new isolate to dynamically adjust its pigment composition in response to nutrient availability, particularly under nitrogen stress. This study provides detailed pigment data associated with different culture conditions supporting a cost-effective strategy for enhancing carotenoid yields and further substantiates the role of *C. vulgaris* as a sustainable source of functional ingredients for health and nutrition applications.

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Introduction

The interest in bioactive compounds from natural sources has grown significantly, positioning microalgae as a sustainable resource for numerous industries. Over the past two decades, the market for microalgal products has expanded rapidly (Zhang et al., 2017) and is expected to continue growing, driven by biotechnological advances, emerging applications across diverse sectors and rising consumer demand for natural and functional products. The global microalgae market, valued at USD 1.17 billion in 2023, is projected to reach USD 25.4 billion by 2033, with a compound annual growth rate (CAGR) of 8% (Behera et al., 2022; Pagnano et al., 2024). In particular, the food colourant sector exemplifies this trend, with natural pigments now accounting for over 80% of total market revenue (Fernandes et al., 2020). In 2016, the global food colours market was valued at USD 1.79 billion and was projected to reach USD 2.97 billion by 2025 (Grand View Research, 2019). This increasing demand underscores the need for alternative and sustainable sources of natural pigments, such as those derived from microalgae.

Compared to terrestrial plants, microalgae offer distinct advantages, such as high photosynthetic efficiency, rapid growth, short life cycles (Li et al., 2008) and the ability to cultivate them on non-arable land using wastewater or effluents as nutrient sources, eliminating competition with food crops (Da Silva Vaz et al., 2016). Given their rich content of bioactive compounds, microalgae can support a balanced diet

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and offer health-promoting benefits, including protection against chronic diseases (Da Silva Vaz et al., 2016; Gille et al., 2016). Among these compounds, carotenoids are major constituents and are especially valued in food applications (Gille et al., 2016). These C_{40} molecules, composed of eight isoprenoid units (Zhang et al., 2017), are divided into two main groups: carotenes (e.g. β-carotene and lycopene), which are hydrocarbons, and xanthophylls (e.g. lutein and zeaxanthin), which are oxygenated derivatives. Several carotenoids are widely used as natural colourants in the food industry, as antioxidants in cosmetic formulations and as feed additives in aquaculture and livestock industries (Zhang et al., 2017). Their use is preferred over synthetic alternatives due to the added health benefits as essential nutrients and antioxidants (Li et al., 2011). The broad utility and economic value of carotenoids drive the need for discovering more productive microalgal species and optimizing their yield.

Chlorella vulgaris is a key species in pigment research due to its relatively fast growth and high carotenoid content (Hynstova et al., 2018; Ördög et al., 2012). Moreover, its high photosynthetic efficiency, combined with the ability to thrive under extreme environmental conditions (Saad et al., 2019), makes *C. vulgaris* a cost-effective, high-quality source of carotenoids (Ru et al., 2020). This unicellular eukaryotic chlorophyte (Saad et al., 2019) is widely distributed across freshwater, marine and terrestrial habitats and was among the first microalgae cultivated at large scale for commercial purposes (Ru et al., 2020).

Various stress strategies have been employed to enhance carotenoid production in *C. vulgaris*, including nutrient limitation (e.g. nitrogen depletion; Zhang et al., 2017), environmental stressors (e.g. salinity and temperature; Arena et al., 2021) and combinations of multiple stress factors (Ali et al., 2021). Among these, nitrogen starvation is the most widely applied method due to its effectiveness, simplicity and low cost (Ördög et al., 2012). Several studies have demonstrated the impact of nitrogen stress on carotenoid biosynthesis in *Chlorella* species. For example, nitrogen limitation has been shown to promote carotenoid synthesis in *C. vulgaris* (Zhang et al., 2017) and enhance secondary carotenoid production in *C. sorokiniana* (Cordero et al., 2011). Similarly, Mao et al. (2018) reported that nitrogen starvation led to the highest astaxanthin productivity in *C. zofingiensis*, while Wu et al. (2007) found that nitrogen concentrations between 0.7 and 12 g l^{-1} favoured lutein production in heterotrophic *C. pyrenoidosa*.

However, most prior studies have focused either on optimizing total carotenoid yield or targeting specific pigments. To the best of our knowledge, *C. vulgaris* has not been comprehensively profiled for its carotenoid composition under any form of stress conditions. Much of the previous research on carotenoids and chlorophylls in *C. vulgaris* has been limited to mutant strains (Guardini et al., 2021; Schüler et al., 2020). Pantami et al. (2020) were the first to provide a detailed metabolite profile of a wild-type *C. vulgaris* cultured under standard conditions. Nevertheless, comprehensive profiling of pigments produced by *C. vulgaris* under stress conditions remains largely unexplored.

Although mutant strains of *C. vulgaris* have shown enhanced global pigment content and growth performance, increasing the production of high-value carotenoids in wild-type strains under controlled nutrient conditions, without compromising growth or photosynthetic efficiency, remains a significant challenge. Such optimization efforts should involve detailed carotenoid profiling (Guardini et al., 2021; Pantami et al., 2020; Schüler et al., 2020), as this is essential for detecting shifts in pigment composition and for tailoring cultivation conditions to enrich specific compounds suited for specialized food and nutritional products.

In this study, we isolated and identified a wild-type strain of *C. vulgaris* from wetlands of western Algeria. The strain was cultivated under both standard and nitrogen-depleted conditions and its carotenoid and chlorophyll content was comprehensively analysed by HPLC across different growth stages. Our results reveal a notable diversity of carotenoids, particularly xanthophylls, and confirm that *C. vulgaris* can be a rich natural source of lutein. Optimal nitrogen conditions for maximizing lutein production are discussed.

Materials and methods

Isolation and cultivation of microalgae

C. vulgaris was isolated in February 2016 from Gharabas Lake, also known as Lake Tlelat, in the wetlands of western Algeria (35°35'N, 0°25'W). The isolation and purification were carried out using a combination of serial filtrations and serial dilutions, following standard algal culturing techniques (Andersen & Kawachi, 2005).

Water samples were collected in clean containers and immediately filtered using Whatman filter paper (30 µm of pore size), to remove large debris. Approximately 50 of the filtrate was then enriched with 200 ml of f/2 medium (Guillard, 1975) and maintained at 20°C under a 24 h light photoperiod using white fluorescent lights, at 50 µmol photons m^{-2} s^{-1}. After 15 days of incubation, a second filtration was performed using Whatman filter paper (11 µm pore size), followed by a series of tenfold dilutions in sterile glass tubes, each containing 9 ml of f/2 medium. The final dilution was incubated at 25°C for 5 days then filtered through a 0.2 µm membrane filter and inoculated onto solid BG11 medium (supplemented with 1% agar). Culture plates were maintained at 25°C and 50 µmol photons m^{-2} s^{-1}.

A single algal colony was isolated after 15 days of incubation and subsequently cultured in liquid BG11 medium for molecular characterization. The resulting strain was maintained as a unialgal culture through regular sub-culturing in 250 ml Erlenmeyer flasks containing modified BG11 medium (Allen & Stanier, 1968; Andersen & Kawachi, 2005), incubated at 23°C under continuous illumination of 50 µmol photons m^{-2} s^{-1}.

Morphological identification by scanning electron microscopy (SEM)

The external morphology of the isolated *C. vulgaris* strain was examined using scanning electron microscopy (SEM). Cells harvested during the logarithmic growth phase were centrifuged (8000 × g, 5 min, 4°C), filtered onto 0.2 µm Millipore membrane filters and washed twice with 0.1 M sodium cacodylate buffer (pH 7.4). Cell fixation was carried out with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer for 15 min, followed by two buffer washes. Dehydration was performed through a graded ethanol series, with an overnight incubation in 70% ethanol at 4°C. Critical point drying was conducted using a Tousimis 780A dryer. Dried samples were mounted on copper stubs using carbon adhesive or silver paste, sputter-coated with a ~18 nm layer of gold/palladium alloy and examined with a JEOL JSM-6510LV low vacuum scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Molecular identification

Molecular identification was performed by targeting the nuclear small subunit ribosomal DNA (SSU rDNA) region using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3") and NS2 (5"-GGCTGCTGGCACCAGACTTGC-3'), yielding an expected amplicon of approximately 550 bp (Wu et al., 2001).

Microalgal biomass was harvested at the end of the exponential growth phase by centrifugation at 8000 × g for 10 minutes. The resulting cell pellet was flash-frozen in liquid nitrogen and stored at −80°C until nucleic acid extraction. Genomic DNA was extracted following the CTAB protocol (2% CTAB with β-mercaptoethanol and 100 µg ml^{-1} proteinase K) as described by Doyle & Doyle (1987), followed by RNase treatment to remove RNA contaminants.

PCR reactions were performed in 25 µl volumes containing 100 ng of genomic DNA, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 µl of 10X PCR buffer, 2.5 mM $MgCl_2$, 1 U of High-Fidelity DNA Polymerase Kapa HiFi (Kapa Biosystems, Wilmington, Massachuesetts, USA) and nuclease-free water. The thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes.

PCR products were purified using a GeneJET PCR Purification Kit (Thermo Fisher Scientific, Cat. No. K0701), according to the manufacturer's protocol, and subsequently sequenced. The obtained sequences were edited using BioEdit software (version 7.2) and compared against the NCBI nucleotide database using BLASTn to identify the closest taxonomic matches.

Characterization of pigment production

Vegetative growth stage

C. vulgaris cells in mid-logarithmic phase were used to inoculate pre-cultures at 1% (v/v) into 250 ml Erlenmeyer flasks containing 200 ml of BG11 medium. The medium was continuously aerated with filtered

air (0.22 µm Millipore filters) and the cultures were maintained at 26 ± 1°C under a continuous light regime (24 h light) at an intensity of 50 µmol photons m^{-2} s^{-1}, provided by 18 W cool-white fluorescent lamps, for a duration of 15 days. These vegetative cultures were subsequently used as inoculum for stress experiments.

Stress treatments

To investigate the effect of nitrogen limitation on carotenoid production, *C. vulgaris* was cultivated under four nitrogen conditions: a) nitrogen-free medium (0 g l^{-1} $NaNO_3$), b) medium with one-quarter the standard nitrate concentration (0.375 g l^{-1} $NaNO_3$), c) medium with half the standard nitrate concentration (0.75 g l^{-1} $NaNO_3$) and c) control medium with the standard nitrate concentration (1.5 g l^{-1} $NaNO_3$).

Each condition was prepared in triplicate using 500 ml Erlenmeyer flasks containing 350 ml of the respective medium. Cultures were maintained for 20 days at 26 ± 2°C, under continuous aeration and constant illumination (50 µmol photons m^{-2} s^{-1}) using cool-white fluorescent lamps arranged for uniform exposure (Supplementary fig. S1).

Growth monitoring and sampling

Daily samples (1 ml) were collected from each culture for cell density measurement and absorbance reading at 680 nm. Cell counts were determined using a Neubauer haemacytometer. Growth curves were plotted for each condition, and the exponential phase was determined accordingly. Samples for HPLC pigment analysis were collected at three key time points: Day 3 (initial phase), Day 13 (exponential phase) and Day 20 (stationary phase).

The specific growth rate (µ) during the exponential phase was calculated according to Krzemińska et al. (2014) using the following equation:

$$\mu = \frac{\mathrm{Ln}(\mathrm{Cf}/\mathrm{Ci})}{tf - ti}$$

Where *Cf* and *Ci* represent the cell concentrations at time points tf = 10 days and ti = 4 days, respectively.

Dry biomass determination

To quantify pigment content in mg g^{-1} dry weight (DW), the dry biomass of *C. vulgaris* cultures was measured at different growth phases. For each measurement, 5 ml of algal suspension was centrifuged at 7000 × g for 10 minutes. The pellet was resuspended in 10 ml of distilled water and centrifuged again under the same conditions to remove residual medium. The final cell pellets were transferred into pre-dried and pre-weighed aluminium dishes and subsequently dried at 80°C for 24 hours, following the method of Grama et al. (2014). After drying, the dishes were reweighed to determine the dry biomass.

Analysis of pigments by HPLC

High-Performance Liquid Chromatography (HPLC) was employed to analyse the pigment composition of *C. vulgaris* at different growth phases. For each sample, 1.0 ml of culture was centrifuged at 8000 × g for 10 minutes in 2 ml Eppendorf tubes. The cell pellets were flash-frozen in liquid nitrogen and stored at −80°C until analysis.

Pigments were extracted by transferring the frozen cell pellets into 15 ml Falcon tubes and resuspending them in 1 ml of acetone. After adding 20 µl of an internal standard (trans-β-Apo-8′-carotenal 3 ng μl^{-1}, Sigma-Aldrich) in each sample, the tubes were placed in an ice bath and cell suspensions were sonicated for 1.5 minutes using a sonication probe (50% amplitude, 0.5 cycle). Extracts were then centrifuged at 10 000 rpm for 10 minutes and the supernatants were subsequently collected and filtered through 0.2 µm syringe filters (Whatman ReZist, PTFE, 13 mm diameter). The extraction was repeated twice using 1 ml of fresh acetone and the supernatants from all extractions were pooled for each sample (3 ml in total). During the final extraction, the suspensions of microalgae in acetone were stored at −20°C overnight prior to centrifugation. All procedures were conducted under dim light to prevent pigment degradation, and only HPLC-grade solvents (Sigma-Aldrich) were used.

The analysis of pigments in microalgal extracts was carried out using an Agilent 1260 Infinity Binary Pump HPLC system (Agilent Technologies) equipped with a Poroshell 120 EC-C18 column (150 mm × 3 mm, 2.7 µm particle size; Agilent Technologies). Chromatographic conditions followed the protocol

described by Lagaria et al. (2017). Data acquisition and processing were performed using MassHunter WorkStation software (Agilent Technologies). Pigment concentrations were quantified using the internal standard calibration method.

Statistical analysis

Statistical analyses were performed using Statistica software (version 5.2). All results are presented as mean values ± standard deviation (SD) based on three biological replicates. Homogeneity of variances was assessed using Hartley's test. When variances were homogeneous ($p \leq 0.05$), differences among groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post hoc test. In cases of non-homogeneous variances ($p > 0.05$), non-parametric analysis was applied using the Kruskal – Wallis test, followed by the Mann – Whitney U-test for pairwise comparisons. Statistical significance was set at $p \leq 0.05$. In all graphical representations, histogram bars marked with different letters indicate statistically significant differences between groups.

Results

Morphological characterization of the isolated microalga

The morphological features of the isolated microalga strongly suggest its affiliation with the genus *Chlorella*, specifically *Chlorella vulgaris*. Light microscopy (Fig. 1) and scanning electron microscopy (SEM) (Fig. 2-4) revealed that the isolate is a unicellular, non-motile green alga, with cells that are spherical to slightly ellipsoidal and range from 2 to 10 µm in diameter. Notably, the cells display a relatively thick cell wall composed of two distinct layers: a thin outer layer and a more prominent inner layer (Fig. 4). Although these features are consistent with previous descriptions by Elumalai et al. (2011), we did not observe the irregular and folded surface texture reported in their SEM micrographs. Instead, our SEM analysis showed a smooth and uniform external surface without prominent ornamentation (Fig. 2-4), a characteristic also noted by Gärtner et al. (2015) in strain R-06/2. This distinct surface morphology may represent a unique ultrastructural feature of our isolate. Additionally, autosporulation was clearly visible under SEM, with four autospores per sporangium (Fig. 3), a reproductive feature known in *Chlorella* species (Yamamoto & Ishida, 1996), but rarely captured or emphasized in imaging studies.

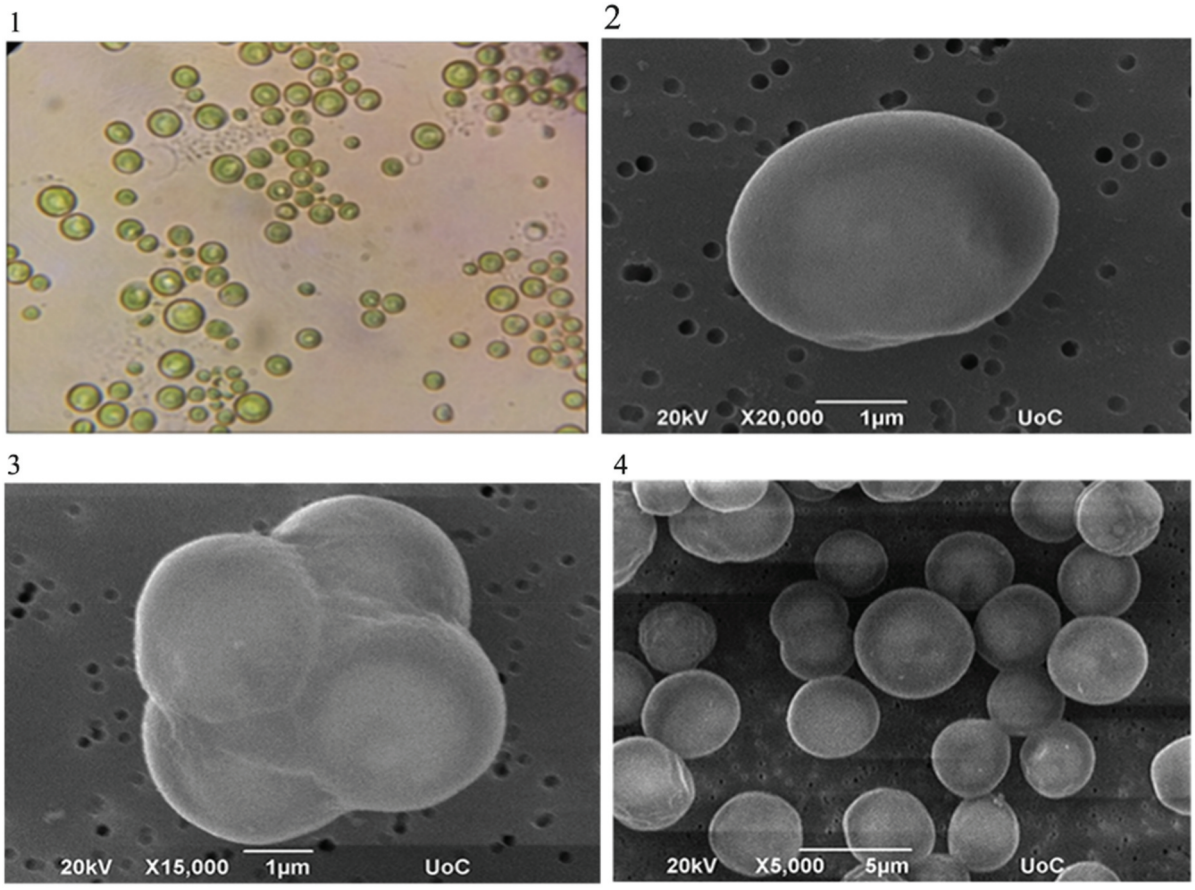
Molecular identification and phylogenetic analysis

Molecular analysis further confirmed the taxonomic identity of the isolate as the genus *Chlorella*. The NS12 region of the nuclear SSU 18s RNA gene was PCR amplified using the NS1 and NS2 primers producing a single 550 bp amplicon (Supplementary fig. S2) as expected (Wu et al., 2001). BLASTn analysis of the Sanger sequenced NS12 region returned as best hits multiple *C. vulgaris* sequences with 99.4% similarity. Based on both molecular and morphological evidence, the isolate was identified as *C. vulgaris* and designated as strain SR (the sequence was submitted to GenBank under the accession number OM864576).

Pigment profiling of Chlorella vulgaris *strain SR under standard conditions*

The pigment composition of *C. vulgaris* strain SR cultivated under standard conditions was analysed by HPLC, with the resulting chromatogram in Fig. 2 and the corresponding pigment composition in Table 1. Pigment identification was accomplished by matching the retention times and UV – visible absorbance spectra of the chromatographic peaks with those of authentic standards. These assignments were further validated through cross-referencing and previously published data (De Rosso & Mercadante, 2007; Patias et al., 2017; Rodrigues et al., 2015). Quantification was carried out using internal (trans-β-Apo-8′-carotenal) standard calibration curves. A total of 12 distinct pigments were identified in strain SR (Table 1).

The dominant chlorophylls were chlorophyll-a (143.1 fg $cell^{-1}$) and chlorophyll-b (52.1 fg $cell^{-1}$), while the major carotenoids were lutein (43.1 fg $cell^{-1}$), violaxanthin (11.1 fg $cell^{-1}$) and neoxanthin



Figs. 1-4. The isolated microalga *Chlorella vulgaris* under light micoscopy gr X 100; Figs 2-4. under scanning electron microscopy (SEM).

Table 1. Pigment content (fg cell^{-1}) of *Chlorella vulgaris* strain SR across different growth phases during its cultivation under standard conditions. Values are expressed as mean ± se (n = 3). Pigment identities correspond to peak assignments shown in Fig. 5.

Peak	Pigment	Start phase (fg cell^{-1})	Exponential phase (fg cell^{-1})	Stationary phase (fg cell^{-1})
1	Chlorophyllide *a*	3.0 ± 0.3	4.0 ± 0.5	2.0 ± 0.4
2	19′-Butanoyloxyfucoxanthin	0.32 ± 0.04	1.00 ± 0.02	0.18 ± 0.06
3	Fucoxanthin	2.6 ± 0.5	3.0 ± 0.4	1.0 ± 0.3
4	Neoxanthin	5.2 ± 0.7	12.8 ± 0.8	4.7 ± 0.6
5	Violaxanthin	8.4 ± 1.5	11.1 ± 0.6	4.2 ± 0.8
6	Antheraxanthin	0.37 ± 0.05	1.1 ± 0.1	0.7 ± 0.2
7	Zeaxanthin	0.41 ± 0.10	2.2 ± 0.3	1.4 ± 0.5
8	Lutein	26.0 ± 1.6	43.1 ± 2.0	19.2 ± 3.4
9	Chlorophyll *b*	39.0 ± 6.0	52.1 ± 3.5	18.2 ± 5.3
10	Chlorophyll *a*	138.3 ± 8.1	143.1 ± 5.3	45.2 ± 6.6
11	*α*-Carotene	3.0 ± 0.4	8.2 ± 0.3	2.3 ± 0.6
12	*β*-Carotene	4.2 ± 0.5	7.0 ± 0.3	3.1 ± 0.6
	Total carotenoids	54.3 ± 0.48	86.9 ± 1.8	35.4 ± 2.17

(12.8 fg cell^{-1}). These were followed by *α*-carotene (8.2 fg cell^{-1}) and *β*-carotene (7.0 fg cell^{-1}). Additional pigments were detected in trace amounts, including chlorophyllide *a* (4.0 fg cell^{-1}), fucoxanthin (3.0 fg cell^{-1}), zeaxanthin (2.2 fg cell^{-1}), antheraxanthin (1.1 fg cell^{-1}) and 19'-butanoyloxyfucoxanthin (1.0 fg cell^{-1}). The highest accumulation of carotenoids was observed during the exponential growth phase, as shown in Table 1 and Fig. 5a.

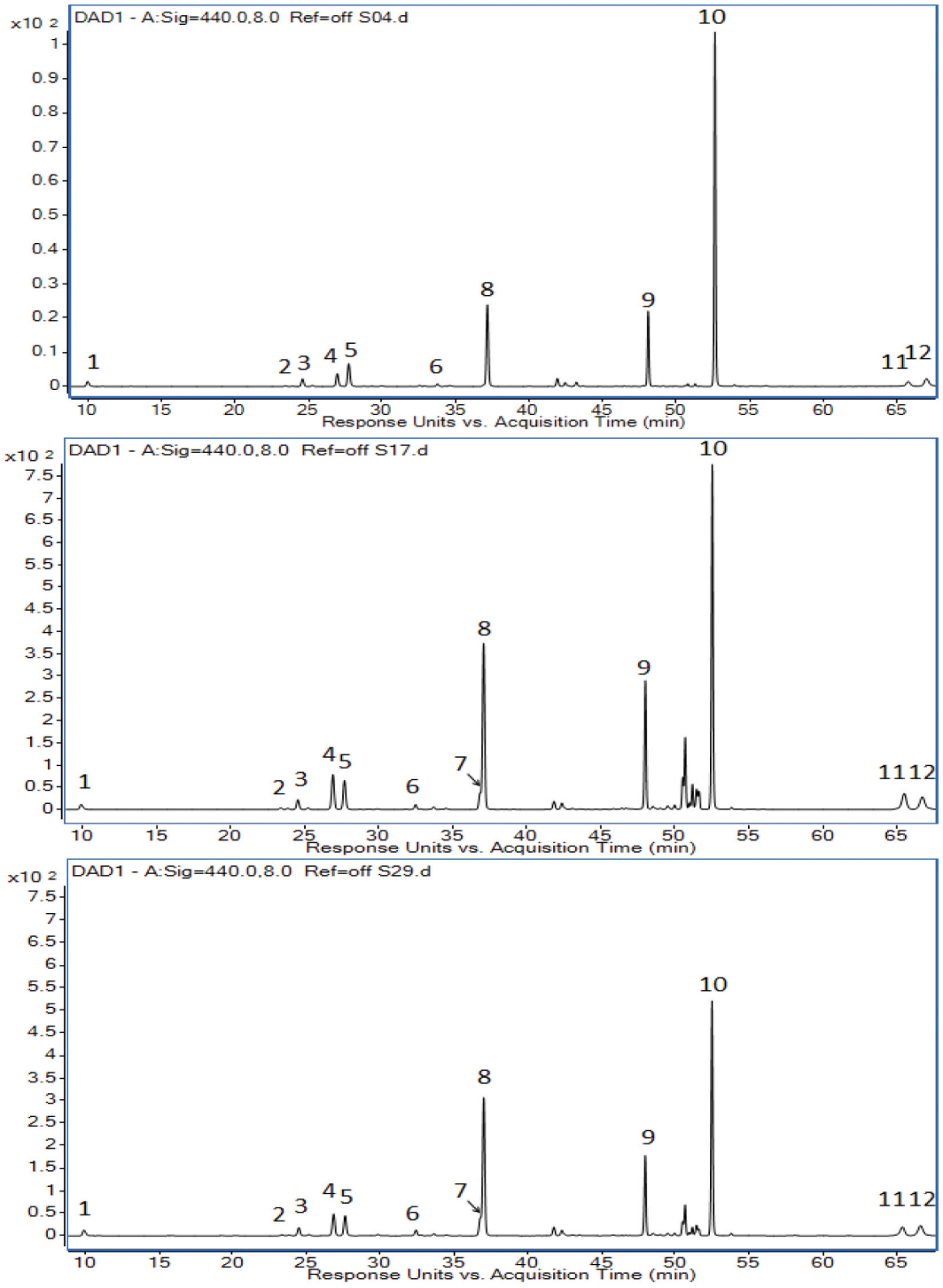


Fig. 5. HPLC chromatograms of carotenoid extracts from *Chlorella vulgaris* strain SR collected at the start (S04), exponential (S17) and stationary (S29) growth phases. Peaks were monitored at 440 nm. Identification of individual pigments corresponding to each peak are detailed in Table 1. Peak 1: chlorophyllide *a*. Peak 2: 19′- butanoyloxyfucoxanthin. Peak 3: Fucoxanthin. Peak 4: Neoxanthin. Peak 5: Violaxanthin. Peak 6: Antheraxanthin. Peak 7: zeaxanthin. Peak 8: Lutein. Peak 9: chlorophyll *b*. Peak 10: chlorophyll *a*. Peak 11: *α*-carotene. Peak 12: *β*-Carotene.

Effect of nitrogen limitation stress on growth

To assess the impact of nitrogen limitation on the growth of *C. vulgaris* strain SR, microalgae cultures were prepared in BG11 media with concentrations of sodium nitrate adjusted to 1.5 g l^{-1} (control; standard nitrogen concentration), 0.75 g l^{-1} (half-strength nitrogen), 0.375 g l^{-1} (quarter-strength nitrogen) and 0 g l^{-1} (nitrogen-free conditions).

Nitrogen deprivation led to a statistically significant decline ($p \leq 0.05$) in growth performance. The specific growth rate decreased from 0.26 ± 0.01 day^{-1} under control conditions to 0.20 ± 0.02 day^{-1} in nitrogen-free medium (Fig. 6a). In contrast, cultures grown in 0.375, 0.75 and 1.5 g l^{-1} $NaNO_3$ exhibited no statistically significant ($p \geq 0.05$) differences in growth rate suggesting that mild-to-moderate nitrogen limitation did not severely impair microalgal growth.

Maximum/final cell concentration at stationary phase indicated a differential response to nitrogen availability. The highest cell concentration (12.3×10^7 cells ml^{-1} on day 17) was recorded under ¼ nitrogen conditions (Fig. 6b), closely followed by the control culture (11.7×10^7 cells ml^{-1}). This similarity points to a potential compensatory or adaptive response of microalgal cells under moderate nitrogen limitation. In contrast, cultures grown without nitrogen supplementation exhibited the lowest cell density (3.4×10^7 cells ml^{-1}), confirming the growth-inhibitory effect of severe nitrogen deprivation.

Biomass accumulation of *C. vulgaris* further confirms the impact of nitrogen stress on cellular growth (Table 2). On day 3, biomass was low across all treatments (0.4–0.8 g l^{-1}), with higher nitrate levels showing a slight stimulatory effect. By day 13, biomass was markedly higher in cultures supplemented with 0.375 and 1.5 g l^{-1} $NaNO_3$ (2.07 and 2.53 g l^{-1}, respectively) compared to the 0 g l^{-1} NaNO3 treatment (1.2 g l^{-1}). At day 20, the highest biomass was observed at 1.5 g l^{-1} $NaNO_3$ (2.86 g l^{-1}), followed closely by 0.75 and 0.375 g l^{-1} NaNO3 treatments (2.5 and 2.27 g l^{-1}), whereas the nitrate-free culture remained lowest (1 g l^{-1}) which confirm that severe nitrogen deprivation markedly restricted cellular growth.

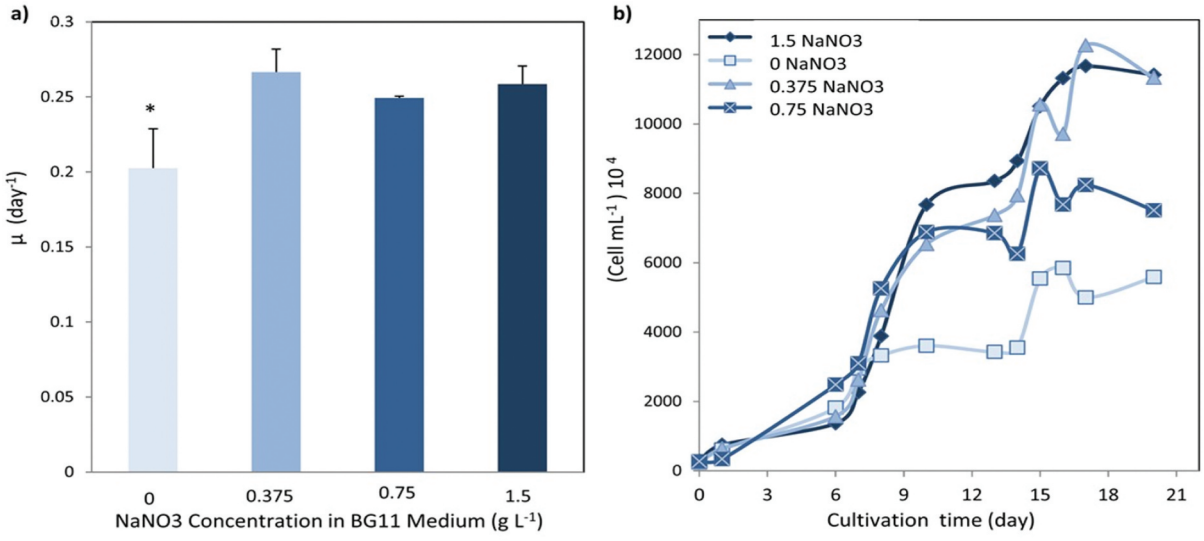


Fig. 6. (**a**) Specific growth rate (μ) of *Chlorella vulgaris* strain SR cultivated under different $NaNO_3$ concentrations. (**b**) growth curves of *Chlorella vulgaris* strain SR under different $NaNO_3$ concentrations. The values shown are means ± se (n = 3). *indicates statistical difference versus standard condition ($p < 0.05$, one-way ANOVA).

Table 2. Biomass of *Chlorella vulgaris* expressed in g l^{-1}, under varying $NaNO_3$ stress conditions and at different growth stages.

	Concentration of $NaNO_3$			
Growth stage	0 g l^{-1}	0.375 g l^{-1}	0.75 g l^{-1}	1.5 g l^{-1}
Day 3	0.4	0.4	0.5	0.8
Day 13	1.2	2.1	1.7	2.5
Day 20	1.0	2.3	2.5	2.9

Effect of nitrogen limitation on total carotenoid accumulation and photosynthetic activity

Nitrogen availability markedly influenced the phenotypic characteristics of the isolated strain. A progressive shift in culture colour from green to yellow and orange was observed with decreasing nitrogen concentrations, reflecting a physiological response to nitrogen limitation. This visible change indicates regulated biochemical adjustments and pigment remodelling within the cells, consistent with enhanced carotenoid accumulation under stress conditions (Campenni et al., 2013). As illustrated in Supplementary table S3, the culture colour progressively changed from day 4 (start phase) to 20 (stationary phase) as nitrogen levels decreased. Furthermore, clear differences were observed in the colouration of microalgal pellets collected on day 20 from cultures grown in 0 and 1.5 g l^{-1} $NaNO_3$ (Supplementary table S4).

HPLC analysis (Fig. 5a) demonstrated a pronounced impact of nitrogen limitation on total carotenoid accumulation in *C. vulgaris* strain SR, particularly during the exponential and stationary growth phases. Under the standard N condition, the isolated *C. vulgaris* strain SR reached maximum pigment concentrations of 2919 µg g^{-1} DW for total carotenoids, 4576 µg g^{-1} DW for chlorophyll *a* and 1735 µg g^{-1} DW for chlorophyll-*b* during the exponential phase (Supplementary table S2). These values are considerably higher than those reported for other *Chlorella* species (Damergi et al., 2017). Furthermore, under moderate nitrogen limitation (0.375 g l^{-1} $NaNO_3$), total carotenoid content was further increased. During the exponential phase, reducing nitrate levels in BG11 medium to one-quarter of the standard concentration (i.e. from 1.5 to 0.375 g l^{-1}) resulted in a significant increase ($p < 0.05$) in total cellular carotenoid content, reaching 124.6 fg $cell^{-1}$. This represents a 43.3% increase compared to the control culture (86.9 fg $cell^{-1}$) (Fig. 7b), and translates to an additional 1.9 mg g^{-1} DW of carotenoids in microalgal biomass relative to the full-strength nitrogen treatment (i.e. 4.84 versus 2.92 mg g^{-1}) (Supplementary table S2). No statistically significant differences in carotenoid content ($p \geq 0.05$) were observed among cultures grown at 0.75 and 1.5 g l^{-1} $NaNO_3$. In contrast, complete nitrogen deprivation (0 g l^{-1} $NaNO_3$) resulted in a significant reduction ($p \leq 0.05$) in total carotenoid content, compared to standard conditions (i.e. 86.9 fg $cell^{-1}$ to 3.3 fg $cell^{-1}$), indicating that carotenoid accumulation in *C. vulgaris* strain SR is stimulated only under moderate nitrogen limitation.

Under both standard and stressful nitrogen conditions, chlorophyll concentrations increased during the exponential phase and declined sharply during the stationary phase. Interestingly, no statistically significant differences ($p \geq 0.05$) were observed in total chlorophyll-*a* and chlorophyll-b content among the 0.375, 0.75 and 1.5 g l^{-1} $NaNO_3$ treatments (Fig. 7c, d), indicating that photosynthetic capacity was maintained under moderate nitrogen limitation. However, under complete nitrogen deprivation, the total content of chlorophyll-*a* and chlorophyll-*b* declined significantly ($p \leq 0.05$) (Figs. 7c, d), though both pigments remained detectable. This persistence implies that *C. vulgaris* retains a baseline level of photosynthetic capacity even under severe nitrogen stress.

Carotenoids profile

HPLC analysis revealed distinct patterns of pigment accumulation in *C. vulgaris* strain SR in response to varying nitrogen availability, with nine major carotenoids consistently detected across all stress treatments (Fig. 8). Carotenoid levels decreased significantly ($p \leq 0.05$) when nitrate concentration was reduced to 0 g l^{-1} (Fig. 8). In contrast, quarter-strength nitrogen conditions led to a marked increase in several carotenoids, including lutein, violaxanthin, fucoxanthin, zeaxanthin, α-carotene, and β-carotene, when compared to the control culture, during both the exponential and stationary phases. Regarding the exponential phase, cellular concentration of these pigments increased by 12% to 67% (Supplementary table S1), with lutein rising from 1.43 to 2.60 mg g^{-1} DW, violaxanthin from 0.34 to 0.55 mg g^{-1} DW, fucoxanthin from 0.11 to 0.16 mg g^{-1} DW, zeaxanthin from 0.06 to 0.10 mg g^{-1} DW, α-carotene from 0.27 to 0.39 mg·g^{-1} DW and β-carotene from 0.23 to 0.36 mg g^{-1} DW (Supplementary table S1). This substantial increase highlights the carotenogenic response of *C. vulgaris* to moderate nitrogen stress. Interestingly, carotenoid levels under the half-strength nitrogen treatment showed no substantial differences compared to the control, suggesting that this nitrogen limitation condition is insufficient to induce a significant carotenoid accumulation response.

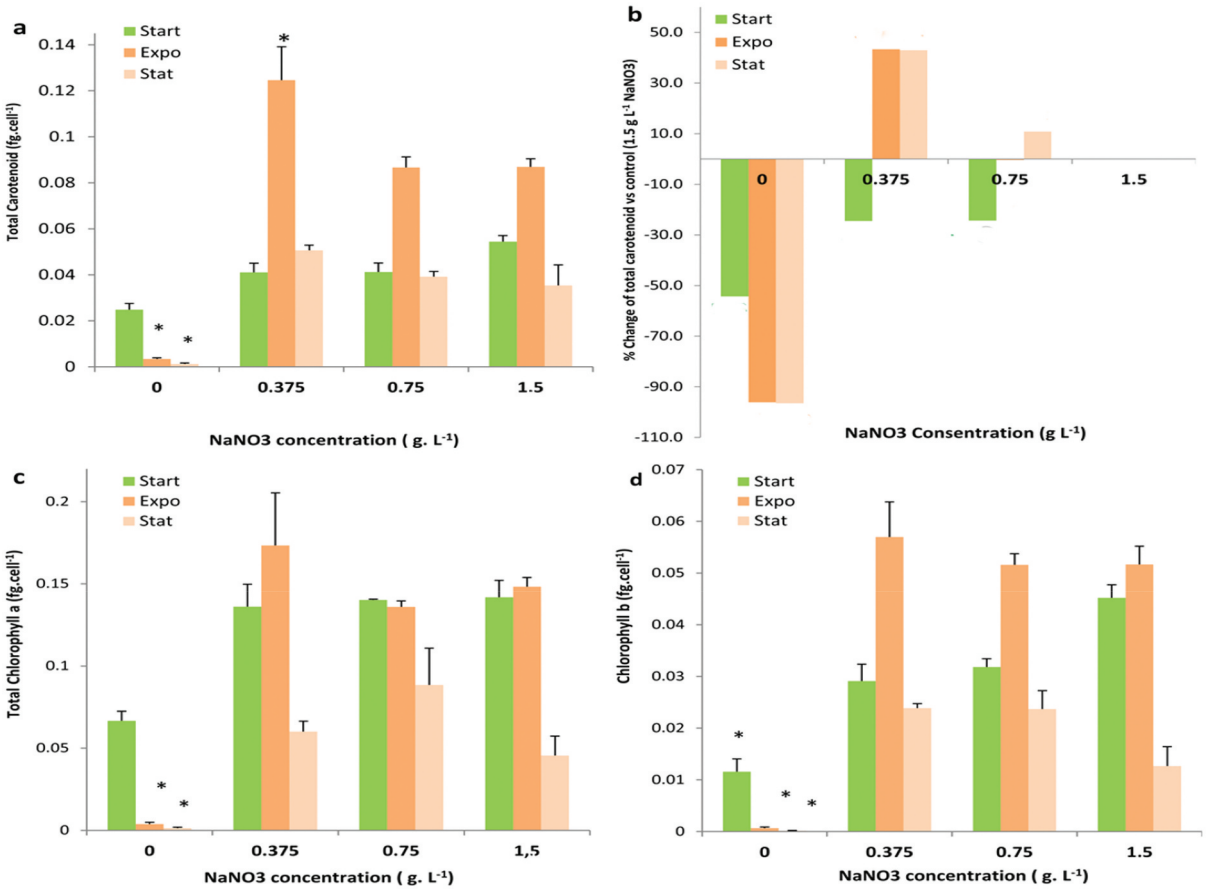


Fig. 7. (a) Total carotenoid accumulation per cell (fg cell^{-1}) in *Chlorella vulgaris* strain SR grown under different $NaNO_3$ concentrations, determined by HPLC using an internal standard method. (b) Percent change (%) in total carotenoid content per cell under different $NaNO_3$ treatments relative to the standard condition (1.5 g l^{-1} $NaNO_3$), which was used as control. (c, d) Chlorophyll a and chlorophyll b cellular content (fg cell^{-1}), respectively, in *Chlorella vulgaris* strain SR cultivated under different $NaNO_3$ concentrations values are expressed as means ± se (n = 3). Asterisks (*) indicate statistically significant differences compared to the control condition ($p < 0.05$).

Lutein was the predominant carotenoid detected in *C. vulgaris* across all stress treatments, showing the highest concentration and the most pronounced increase under nitrogen limitation. The maximum volumetric lutein concentration reached 4.5 ± 0.73 µg ml^{-1}, corresponding to a cellular content of 2.6 ± 0.09 mg g^{-1} DW during the exponential phase under quarter-strength nitrogen conditions, followed by violaxanthin, neoxanthin, α-carotene and β-carotene. Fucoxanthin and 19'-butanoyloxyfucoxanthin were unexpectedly detected in trace levels (Supplementary tables S1 and S2), despite being typically absent in *Chlorella* species (Jeffrey & Vesk, 1997; Jeffrey & Wright, 2006).

Overall, the optimal nitrogen concentration, characterized by the highest accumulation of valuable pigments, was attained at 0.375 g l^{-1} $NaNO_3$), demonstrating that controlled nitrogen limitation effectively enhances carotenoid yields without significantly ($p \geq 0.05$) impairing growth parameters (Fig. 6).

To our knowledge, no previous study has reported a complete quantitative and qualitative profiling of all major carotenoids in *Chlorella vulgaris*, including α-carotene, β-carotene, neoxanthin, violaxanthin, zeaxanthin and fucoxanthin, with precise concentrations expressed in standardized units (e.g. µg ml^{-1} or mg g^{-1} DW). Most available studies quantify only a subset of carotenoids or provide qualitative HPLC identification without exact concentration values. Table 3 presents a comparative summary of pigment yields obtained in the present *C. vulgaris* isolate cultivated under standard nitrogen conditions (1.5 g l^{-1} $NaNO_3$) and the optimal nitrogen concentration (0.375 g l^{-1} $NaNO_3$), alongside values reported for other *Chlorella* strains in different previous studies.

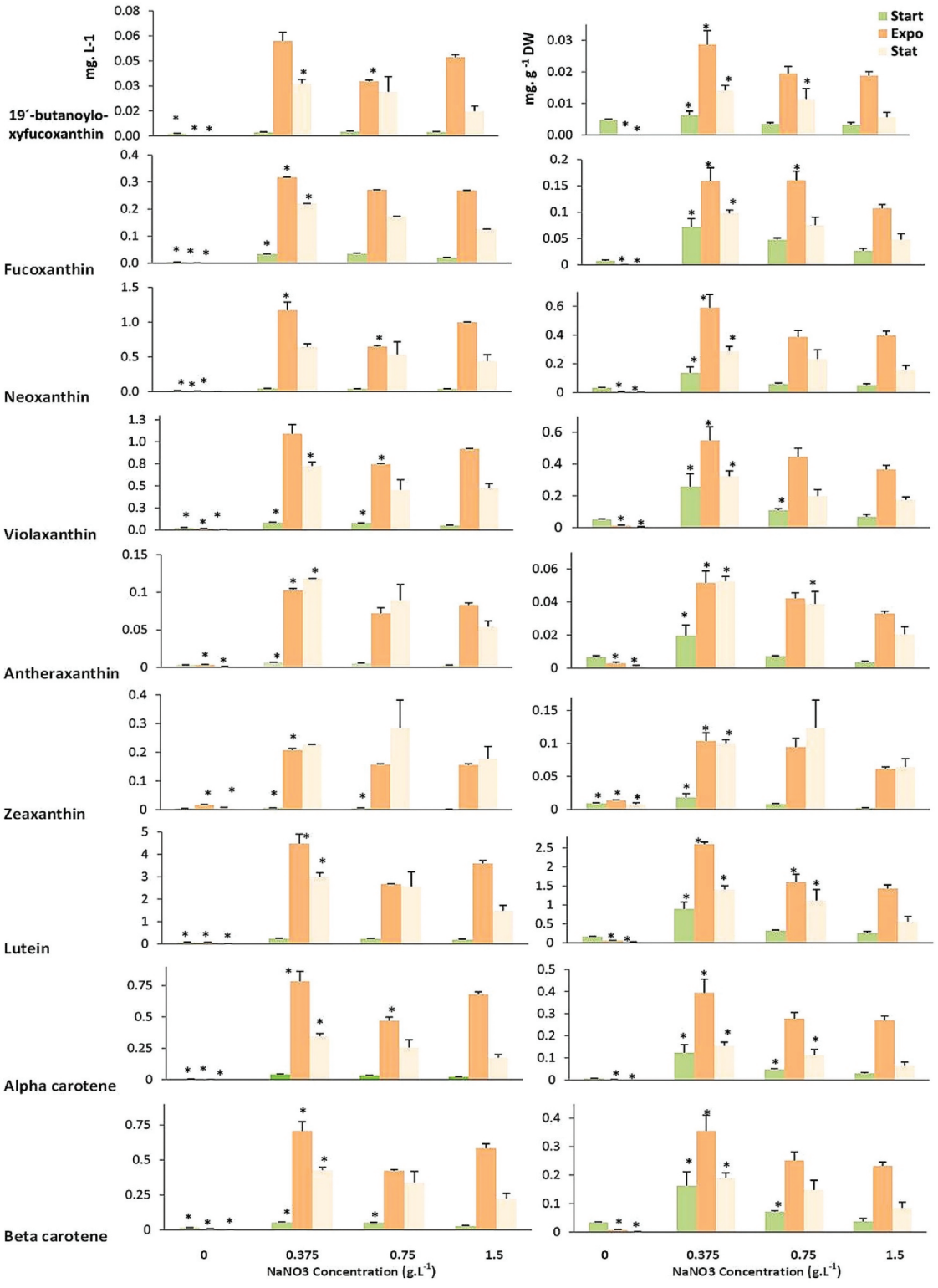


Fig. 8. Carotenoids profile of *Chlorella vulgaris* strain SR (mg l^{-1}) and content per unit of dry weight (mg g^{-1} DW) in response to different $NaNO_3$ concentrations in BG11 medium at different growth phases, by HPLC analyses. Results shown are means ± se (n = 3). * indicates statistical difference versus control for each growth phase ($p < 0.05$, one-way ANOVA).).

Discussion

Screening naturally diverse microalgal populations remains a classical and effective approach for identifying strains with superior metabolic capabilities (Dinpazhooh et al., 2022). Nitrogen limitation is a widely applied strategy to enhance pigment production in microalgae, as it induces distinct

Table 3. Pigment yields (μg g^{-1} DW) of *Chlorella vulgaris* strain SR under standard (1.5 g l^{-1} $NaNO_3$) and optimal (0.375 g l^{-1} $NaNO_3$) nitrogen conditions, compared with previously reported values for other *Chlorella strains*.

Pigment	*C. vulgaris* SR (control)[a]	*C. vulgaris* SR (N-limited)[b]	*Chlorella sorokiniana*[c]	*Chlorella* spp.[d]	*Chlorella* spp.[e]	*Chlorella sorokiniana*[f]	*Chlorella* spp.[g]
Chlorophyll-a	4576	8136	10130– 17 760	18 000–22 000	2016	–	–
Chlorophyll-b	1735	3205	3050 = 6230	5 000–7 000	147	–	–
19′-butanoyloxyfucoxanthin	19	29	–	–	–	–	–
Antheraxanthin	33	52	–	–	–	–	–
Fucoxanthin	107	160	280–450	–	–	Qualitative detection	–
Neoxanthin	399	590	–	–		–	Qualitative detection
Violaxanthin	366	549	20–30	–	–	–	Qualitative detection
Zeaxanthin	62	104	90–140	–	–	44.81	–
Lutein	1430	2600	1070–1760	7140	80–90	753	–
α-carotene	271	395	–	–	–	71.47	–
β-carotene	232	356	–	1013	–	156.21	–
Total carotenoids	2919	4836	1940–3270	8 000–9 000	417	–	60880

[a]Present study at standard nitrogen levels of 1.5 g l^{-1} $NaNO_3$; [b]Present study at 0.375 g l^{-1} $NaNO_3$; [c]Van Wagenen et al. (2015); [d]Safafar et al. (2016); [e]Damergi et al. (2017); [f]Fernandes et al. (2020); [g]Mary Leema et al. (2021).

photosynthetic and metabolic responses that alter pigment composition. Carotenoid biosynthesis in microalgae, including *Chlorella vulgaris*, occurs through the plastid-localized methylerythritol phosphate (MEP) pathway, which generates precursors such as geranylgeranyl pyrophosphate (GGPP). GGPP is converted into phytoene by the phytoene synthase (PSY), a rate-limiting enzyme, followed by desaturation and cyclization reactions catalysed by phytoene desaturase (PDS) and lycopene cyclase to form carotenoids like β-carotene and lutein (Ren, 2021). The activity of these enzymes depends on the availability of carbon precursors, reducing power (NADPH), ATP and nitrogen-containing cofactors. Under severe nitrogen depletion, *C. vulgaris* experiences a reduction in protein synthesis, including of PSY and PDS, reducing therefore the MEP pathway (Liu et al., 2022). Nitrogen deficiency also disrupts photosynthetic function by decreasing chlorophyll content and impairing electron transport, which lowers ATP and NADPH availability for carotenoid biosynthesis (Liu et al., 2022; Ren, 2021). Additionally, nitrogen starvation triggers metabolic reallocation in *C. vulgaris*, diverting carbon from secondary metabolites like carotenoids towards storage compounds such as lipids and carbohydrates, which support cell survival under nutrient-limiting conditions (Liu et al., 2022). While moderate nitrogen limitation can sometimes induce secondary metabolite production as a stress response, severe nitrogen deprivation suppresses carotenoid production via downregulation of PSY and PDS, diminished photosynthetic energy production, and the diversion of carbon flux towards survival pathways (Liu et al., 2022; Ren, 2021).

To date, numerous *C. vulgaris* strains have been isolated and some of them have been investigated for the large-scale production of carotenoids (Gong, 2017). Although several studies have previously investigated carotenoid accumulation in *Chlorella* species, most have focused on total carotenoid content or on a limited number of target compounds (Azaman et al., 2017; Chen et al., 2016; Minhas et al., 2016). More comprehensive investigations using advanced analytical techniques have examined pigment composition in *Chlorella sorokiniana* (Fernandes et al., 2020; Safafar et al., 2016; Van Wagenen et al., 2015). However, to the best of our knowledge, this study is the first to apply a detailed HPLC-based analytical approach to qualitatively and quantitatively characterize the carotenoid composition of a novel *C. vulgaris* isolate across different growth phases and nitrogen stress conditions. Our primary objectives were to investigate whether nitrogen limitation can enhance pigment biosynthesis in this microalgal strain and to identify the optimal nitrogen conditions that effectively balance growth with elevated carotenoid production.

Our study identified a rich carotenoid profile in *C. vulgaris* strain SR, with a clear predominance of primary carotenoids such as lutein, violaxanthin, neoxanthin, α-carotene and β-carotene, in accordance with previous findings on other strains of the same species (Da Silva Vaz et al., 2016; Gouveia et al., 1996;

Plaza et al., 2012). These carotenoids were accompanied by consistently higher levels of chlorophyll-a and chlorophyll-b, along with some minor pigments, such as fucoxanthin and antheraxanthin.

The carotenoid profile of strain SR differed markedly from that of other *Chlorella* species described in the literature. For example, Campenni et al. (2013) reported astaxanthin, canthaxanthin and echinenone in *C. protothecoides*, none of which were detected in our isolate under any N conditions and growth phase. On the other hand, we detected several xanthophylls not reported in that study.

In accordance with the findings of Safafar et al. (2015), we were able to resolve several minor carotenoids and their isomeric forms, including fucoxanthin and 19′-butanoyloxyfucoxanthin, owing to the enhanced chromatographic resolution provided by the 2.7 μm superficially porous particles of the Poroshell 120 HPLC column. However, we did not detect dihydro-lutein, as reported in their study, likely due to differences in strain genetics, cultivation conditions or extraction/analytical procedures. Similarly, Van Wagenen et al. (2015) employed UHPLC and identified a broader spectrum of carotenoids in *C. sorokiniana*, including astaxanthin, a pigment we did not observe in *C. vulgaris* strain SR. This absence is not surprising, as astaxanthin biosynthesis is typically restricted to species like *Haematococcus pluvialis* and is generally absent or minimal in *Chlorella* spp.

The detection of fucoxanthin is particularly noteworthy, as this pigment is commonly found in brown algae and diatoms, but it is rarely reported in green algae (Yalçın et al., 2021). This study is the first to document the presence of fucoxanthin in a *C. vulgaris* strain. Although uncommon, fucoxanthin has previously been identified in *C. sorokiniana* under specific culture conditions. For instance, Van Wagenen et al. (2015) detected fucoxanthin among other carotenoids when *C. sorokiniana was* cultivated under mixotrophic and cyclic autotrophic/heterotrophic modes. Similarly, Safafar et al. (2015) reported the presence of this carotenoid in *C. sorokiniana* cultivated on industrial wastewater, whereas Fernandes et al. (2020) unequivocally confirmed fucoxanthin presence in another *C. sorokiniana* strain using an advanced HPLC-PDA-MS/MS system, which provided structural verification via tandem mass spectrometry. Similarly, Soares et al. (2019) reported an HPLC peak in an extract of the freshwater green alga *Selenastrum bibraianum*, which was consistent with esterified fucoxanthin. These findings suggest that the synthesis of atypical pigments, such as fucoxanthin, though rare, is not without precedent. These comparisons emphasize the importance of analytical methodologies used in exploring inter-strain differences in pigment biosynthetic capacity.

These results further reinforce the taxonomic complexity and metabolic diversity within the *Chlorella* genus, as previously highlighted, the detection of low-abundance pigments such as fucoxanthin, along with the absence of other components like astaxanthin and dihydro-lutein, illustrates the strain-specific nature of carotenoid biosynthesis and its modulation by environmental and genetic factors. Further, Gärtner et al. (2015) noted that the simple morphology of the genus *Chlorella* often hampers accurate classification. While early taxonomic monographs relied on morphological characteristics to define type species, later physiological, biochemical and molecular studies have shown that *Chlorella* is a polyphyletic group, with species distributed across both the Trebouxiophyceae and Chlorophyceae classes. These findings are consistent with the work of Grama et al. (2014), who proposed that the phenotypic diversity observed in *Chlorella* may exceed the underlying genotypic diversity, pointing to the need for using integrative approaches in strain identification and functional characterization.

Previous studies have reported total carotenoid, chlorophyll-a and chlorophyll-b contents in *Chlorella vulgaris* of approximately 417, 2016 and 147 μg g^{-1} DW, respectively (Damergi et al., 2017). Our newly isolated *C. vulgaris* strain SR demonstrated substantially higher pigment levels under standard conditions (Supplementary table S2). Moreover, under moderate nitrogen limitation (0.375 g l^{-1} $NaNO_3$), total carotenoid content further elevated during the exponential growth phase, while no significant changes ($p \geq 0.05$) were recorded for chlorophyll-a and chlorophyll-b under this treatment. These enhanced performances reinforce the potential of *C. vulgaris* SR as a valuable resource for sustainable, large-scale production of pigment.

Across all nitrogen treatments, chlorophyll-a and chlorophyll-b peaked during the exponential phase but declined during the stationary phase as nitrogen stress intensified (Fig. 7c, d). This trend aligns with previous findings by Zhang et al. (2017) for *C. vulgaris* and by Ördög et al. (2012) for *C. minutissima*, where chlorophyll degradation under nitrogen limitation was interpreted as a strategic cellular response to recycle internal nitrogen reserves. Given that chlorophylls contain approximately 6.2% nitrogen by mass,

their breakdown provides a critical internal nitrogen source to support essential metabolic functions and maintain biomass production under nutrient-depleted conditions. These conclusions are further supported by Li et al. (2008), who demonstrated that nitrogen depletion in *Neochloris oleoabundans* redirected metabolic flux towards lipid biosynthesis following a reduction in chlorophyll content, and Farooq et al. (2022), who showed that *C. vulgaris* exhibited chlorophyll degradation and increased lipid accumulation at lower initial nitrogen levels.

Nitrogen limitation is a widely applied strategy to enhance pigment production in microalgae, as it induces distinct photosynthetic and metabolic responses that alter pigment composition. However, a major limitation of this approach is the concurrent reduction in growth rate and biomass productivity (Liefer et al., 2018; Valledor et al., 2014). Previous studies have shown that nitrogen deficiency disrupts cellular physiology by inhibiting cell division and photosynthetic processes, ultimately leading to reduced growth (Valledor et al., 2014).

In the present study, severe nitrogen deprivation had a detrimental effect on both cell growth and chlorophylls content. These findings are consistent with previous reports by Zhang et al. (2017) and Saad et al. (2019) who observed reduced cell proliferation and impaired photosynthetic performance in *Chlorella vulgaris* under extreme nitrogen limitation. In contrast, we did not detect any significant differences in growth rates between the standard conditions and mild or moderate nitrogen limitation). This suggests that within the specific concentration range, nitrogen availability did not impose a limiting constraint on growth. Farooq et al. (2022) also noted that higher initial concentrations of nitrogen do not necessarily result in higher growth rates. Nonetheless, there appears to be a critical nitrogen threshold below which growth is substantially reduced. This threshold likely varies depending on multiple factors, including the *Chlorella* strain, cultivation conditions and external stressors (Zarrinmehr et al., 2020). Overall, optimizing and carefully controlling nitrogen levels is crucial for sustaining physiological stability, maintaining robust growth and supporting high photosynthetic performance in *Chlorella* cultures.

Moderate nitrogen limitation (0.375 g l^{-1} $NaNO_3$) proved to be the most effective treatment for enhancing carotenoid synthesis during both the exponential and stationary phases, without adversely affecting growth rate or chlorophyll a and b accumulation (Fig. 7). Notably, the specific treatment led to a significant increase ($p \leq 0.05$) in the production of high-value carotenoids, including lutein, violaxanthin, fucoxanthin, zeaxanthin, α-carotene and β-carotene (Fig. 8). In contrast, complete nitrogen deprivation (0 g l^{-1} $NaNO_3$) resulted in a significant reduction ($p \leq 0.05$) in both total carotenoid content (Fig. 7a) and all individual carotenoids (Fig. 8), accompanied by a significant decline ($p \leq 0.05$) in growth rate (Fig. 6), during both the exponential and stationary phases, we also observed variations in carotenoid production between the early growth phase and the exponential/stationary phases under different nitrate concentrations (Figs 7 and 8). These outcomes align with the findings of Zhang et al. (2017), who reported that while moderate nitrogen limitation can enhance carotenoid accumulation, severe nitrogen deprivation may suppress carotenoid biosynthesis. Their study also emphasized that intracellular carotenoid level in *Chlorella vulgaris* varies dynamically over time depending on nitrogen availability, implying complex regulation of pigment metabolism under nutrient stress.

Across all stress treatments, lutein showed the most pronounced increase and was the dominant carotenoid accumulated in the isolated *Chlorella vulgaris SR*. This observation is consistent with previous studies, such as those of Gille et al. (2016) and Zhang et al. (2017), who also identified lutein as the principal carotenoid responsive to stress in *Chlorella* species. The highest volumetric and cellular lutein concentrations (4.5 µg ml^{-1} and 2.6 mg g^{-1} DW, respectively) were recorded during the exponential phase when nitrate concentration in the culture medium was reduced to 0.375 g l^{-1} (Supplementary tables S1, S2). These values are substantially higher than those reported in other studies, such as 0.753 mg g^{-1} DW by Zarrinmehr et al. (2020) and 80–90 µg g^{-1} DW by Damergi et al. (2017). Moreover, compared to marigold (*Tagetes erecta*, *T. patula*), the main commercial source of lutein with only 0.3 mg g^{-1} DW (Cordero et al., 2011; Deenu et al., 2013), our isolate demonstrates a significantly higher ($p \leq 0.05$) production capacity.

The high lutein yield observed in *Chlorella vulgaris SR*, combined with its rapid proliferation rate, reinforce the potential of this species as a valuable source for nutraceutical and functional food applications. Additionally, the concurrent presence of other bioactive carotenoids, including fucoxanthin, neoxanthin, violaxanthin, α-carotene and β-carotene, further enhances the overall nutritional profile and the market appeal of this microalgal strain, particularly when optimal cultivation conditions are applied. This study

provides the first comprehensive qualitative and quantitative characterization of carotenoids in a newly isolated *Chlorella vulgaris* strain across different growth phases and nitrogen regimes using an HPLC-based approach. Our findings demonstrate that moderate nitrogen limitation (0.375 g l^{-1} $NaNO_3$) represents an optimal condition that enhances the synthesis of high-value carotenoids, particularly lutein; without impairing growth or photosynthetic performance, whereas completes nitrogen deprivation negatively affects pigment biosynthesis and cellular proliferation. The detection of atypical carotenoids, including fucoxanthin and 19′-butanoyloxyfucoxanthin, further highlights the metabolic diversity of this isolate and its potential biotechnological relevance. These observations reinforce the need for integrative approaches combining genomics, transcriptomics, proteomics and metabolomics to unravel the regulatory mechanisms governing carotenoid biosynthesis in *C. vulgaris*. Such approaches will be essential to fully exploit the industrial potential of strain SR and to guide future optimization strategies for sustainable pigment production.

Culture conditions, particularly nitrogen availability, play a decisive role in directing the metabolic balance of *Chlorella vulgaris* towards either growth or high-value pigment biosynthesis. By adjusting nitrate concentrations, it is possible to shift cellular priorities from biomass production to enhanced carotenoid accumulation, revealing a finely regulated metabolic response to nutrient cues. Moderate nitrogen limitation was sufficient to stimulate the synthesis of key xanthophylls, most notably lutein, while preserving photosynthetic integrity, thereby achieving a favourable compromise between metabolic stress and physiological stability. The appearance of atypical carotenoids under specific conditions further illustrates the metabolic plasticity of this strain and the potential to unlock novel biochemical pathways through controlled environmental modulation. Overall, these findings highlight the power of strategic culture manipulation to steer microalgal metabolism towards targeted biochemical outputs and provide a foundation for future optimization using systems-level approaches.

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Availability of data and material

Data will be made available upon request.

Author contributions

R. Sebahi: conceived and designed the study, carried out the experimental work, formal analysis, data curation, investigation, visualization and prepared the original draft.; **F. Verret**: contributed to the conceptual design of the study and its methodology, supervised the project and assisted in manuscript editing; **M. Mandalakis**: was responsible for the extraction of pigments and their analysis by HPLC, including data processing and HPLC software handling, and assisted in manuscript editing; **M.B.B. Hamed**: contributed to the study conceptual development, funding acquisition and project supervision. All authors read and approved the final version of the manuscript.

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