



Effect of phosphorus and nitrogen levels and light emissions diodes illumination on growth and carotenoid synthesis in *Jaagichlorella luteoviridis*. Optimization of carotenoid extraction

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ABSTRACT

Modifying incident irradiance and colour can increase microalgal composition and productivity. The objective of this work was to evaluate the effects of LED light on *Heterochlorella luteoviridis*, recently renamed as *Jaagichlorella luteoviridis*, simulating circadian solar radiation. The influence of white, red, and blue light on the growth rate and biomass composition was studied. Prior to this, the optimal N/P ratio and initial nitrate concentration were investigated. Carotenoid extraction was also optimized for this microalga. To achieve complete carotenoid extraction, *J. luteoviridis* required a temperature of 60 °C and 20 % KOH. Furthermore, an N/P = 5 increased the carotenoid content by 10 % while an initial nitrate concentration of 10.59 mM exceeded the carotenoid productivity of the control by 48 %. An increase in white irradiance led to a higher biomass growth rate and protein content but lower carotenoid and lipid contents. In contrast, an increase in red light resulted in a higher growth rate, while blue light increased the high-value products content.

1. Introduction

Currently, >1100 different carotenoids have been identified, each having diverse structures and associated biological purposes [1]. Carotenoids are essential for human health but cannot be synthesized by animals, so they are mainly sourced through the diet [2]. Although carotenoids are natural biomolecules produced by plants, (micro)algae, yeast, fungi, and some bacteria, they can also be synthesized chemically. Nevertheless, natural pigments are healthier than synthetic pigments, in addition to the economic cost incurred by the latter [2]. The market for carotenoid pigments is expected to reach 2 billion US dollars by 2026, with a compound annual growth rate (CAGR) of 4.2 % [3]. Therefore, it is necessary to increase carotenoid production to meet this growing market demand.

Microalgae are photosynthetic microorganisms with a high carotenoid content [4]. The advantages of using microalgae as a pigment source are: (i) the possibility of obtaining multiple products through biorefining; (ii) their rapid growth rate; (iii) the ability to cultivate them without the need for fertile arable land, unlike plants; and (iv) their role

in the capture of CO₂ [5]. The microalgal growth rate and biomass composition can be modified by varying abiotic parameters such as limiting nitrogen and phosphorus concentrations and changing the nitrogen-to-phosphorus ratio (N/P), lighting, temperature, or salinity. Therefore, these parameters can be used to stimulate microalgal carotenoid productivity. For example, nitrogen depletion induced β-carotene accumulation by 50.6 % in *Dunaliella salina* [6]. Xu and Harvey (2019) observed that *D. salina* irradiated with red light increased the β-carotene content compared to white or blue light [7]. The high intensity of light also stimulated carotenoid productivity in *Haematococcus lacustris* after only a few hours of exposure, increasing especially with red light [8]. Furthermore, Pereira and Otero (2020) observed that the astaxanthin content increased with mixed red and blue light at 30 °C, increasing the yield by 50 % [8]. However, not all microalgae respond to abiotic stress in the same way.

Although the use of sunlight is economically feasible in the cultivation of microalgae, by using artificial light, it is possible to modulate the photon flux density, the photoperiod, and the light spectra, resulting in greater productivity of biomass and compounds of commercial interest

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such as polyunsaturated fatty acids and carotenoids [9–11]. The microalgal composition, modulated by abiotic conditions, is sensitive to changes in light intensity and spectral quality, which are responsible for substantial changes in the biomass compounds and biomass production [12–14].

Combining multispectral light-emitting diodes (LEDs) allows one to mimic incident light irradiation [15,16], which is especially useful when cultivating in a controlled light environment. Some of the advantages of LEDs include their long useful life, low heat generation, and highly efficient transformation of the energy they consume into light energy [17]. Atta et al. (2013) observed that a blue LED light system is more efficient than white fluorescent light when cultivating *Chlorella vulgaris*, resulting in an increased growth rate and lipid content [18]. Baidya et al. (2021) also observed that blue LED light was an efficient source of light energy for indoor *Chlorella ellipsoidea* cultures [19]. However, despite the positive results of LED light applications, there are few studies that investigate the effects of LED lighting on the cultures of most microalgae [16].

Pigments related to photosynthetic complexes absorb photons from different wavelengths, and because different microalgae have different photosynthetic pigment compositions, they have varied responses to distinct light compositions. Carotenoids absorb the blue-violet part of the spectrum and transfer it to chlorophyll. Therefore, some studies that have examined the effects of white, red, and blue light on the growth rate, lipids, and carotenoid composition [20].

Heterochlorella luteoviridis, or recently accepted name as *Jaagichlorella luteoviridis* (*J. luteoviridis*) [21], is a green microalga rich in carbohydrates ($0.41 \text{ g} \cdot \text{g}^{-1}$), protein ($0.29 \text{ g} \cdot \text{g}^{-1}$), lipids ($0.11 \text{ g} \cdot \text{g}^{-1}$), and calcium ($79 \text{ mg} \cdot \text{g}^{-1}$) [22]. Its carotenoid content is also relatively high ($3.48 \text{ mg} \cdot \text{g}^{-1}$), the main carotenoids being: lutein (approximately 40 % w/w), violaxanthin (14 %), and β -carotene (16 %) [22]. The high concentration of lutein makes *J. luteoviridis* an excellent source candidate for this pigment, as it is higher than that found in vegetables such as red pepper ($1.09 \mu\text{g} \cdot \text{g}^{-1} \text{ d.w.}$), lettuce ($0.95 \mu\text{g} \cdot \text{g}^{-1} \text{ d.w.}$), spinach ($0.92 \mu\text{g} \cdot \text{g}^{-1} \text{ d.w.}$), parsley ($0.87 \mu\text{g} \cdot \text{g}^{-1} \text{ d.w.}$) and kale ($0.72 \mu\text{g} \cdot \text{g}^{-1} \text{ d.w.}$) [23]. However, as far as it is known, there are few studies on *J. luteoviridis* even though it was commercialized in Europe prior to the 1997 and is not subject to Novel Food Regulation (EC) No. 258/97 [24].

Some of the xanthophylls present in *J. luteoviridis* biomass are ester-form, specifically lutein. Saponification is the most widely used method to release them, as it allows them to be removed, destroyed by chlorophylls and solubilize proteins and carbohydrates that could block the extraction [24]. KOH or NaOH are the saponification agents usually used. However, since the saponification conditions for each strain must be selected and the recovery solvent must be selected according to the carotenoid to be purified, optimization will be necessary to carry out following the method proposed by Cerón et al., 2018 [24], which was tested using different strains, and which used KOH as saponification agent and different solvents used. The use of a mixture of solvents, such as the tricomponent solution (hexane:ethanol:water), demonstrates generally good results for a sample with xanthophylls and carotenes.

This research aims to investigate the impact of LED light on the growth and high-value metabolites production of *J. luteoviridis* in batch cultures by manipulating various abiotic parameters. To achieve this, a photobioreactor system was designed to simulate circadian solar radiation and precisely control irradiance levels. The study evaluates several factors: (i) the effect of initial nitrate concentration on algal growth and high-value products accumulation; (ii) the influence of different nitrogen-to-phosphorus (N/P) ratios; (iii) the response of the algae to varying levels of white light irradiance in 2 L bubble columns; and (iv) the effect of different intensities of red light and combinations of blue and red light on growth and carotenoid synthesis. Furthermore, research includes the optimization of carotenoid extraction and quantification method to ensure reliable and reproducible results. This method was carried out for the first time for *J. luteoviridis*. This work will allow the selection of the most appropriate abiotic conditions to be implemented on an industrial

scale, with which the growth and/or production of high-value products from *J. luteoviridis* will be maximised. Moreover, it will provide the optimal conditions to maximize the carotenoid extraction from its biomass.

2. Materials and methods

2.1. Microorganism and culture medium

The marine microalgae *J. luteoviridis* BE002 was obtained from the Marine Biology Department of the Fluminense Federal University (Niterói, Rio de Janeiro, Brazil). Stock cultures were maintained photoautotrophically in 500 mL vessels containing 300 mL of culture. The inoculum was grown in a 1 L Erlenmeyer flask containing 800 mL of culture at $25 \pm 1^\circ\text{C}$ under a 12:12 h light–dark cycle with an incident PFD of $100 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. It was continuously bubbled with air at $0.5 \text{ v} \cdot \text{v} \cdot \text{min}^{-1}$. The culture was grown in modified f/2 medium using seawater [22].

2.2. Optimization of carotenoid quantification

The extraction and saponification of carotenoid from this microalga was optimized for the first time, while the characterization and quantification were performed according to the method of Cerón-García et al. [25]. Carotenoids were extracted from the freeze-dried biomass (5 mg), which had previously been macerated with aluminum oxide (5 mg) to break up the cell walls; in total, 10 mg were weighed in Pyrex glass tubes. For saponification, 1 mL of monophasic tricomponent solution was added and vortexed for 2 min. The tricomponent solution was composed of ethanol (96 %), hexane, and water in a ratio of 76:18:6 v/v/v. The potassium hydroxide concentration (KOH) was optimized by testing 10, 20, 30 and 40 % g KOH/ 100 g dry biomass. Subsequently, the temperature was also tested by immersing each tube in a water bath (Julabo® SW22, Julabo USA Inc.) at 25, 40, 60 and 80°C , where they were left for 2 min and vortexed every 30 s. Subsequently, the tubes were taken out, vortexed again for 30 s, and allowed to cool for 1 h at room temperature. Then, they were centrifuged at 12,000 rpm for 2 min (Mini Spin Plus, Eppendorf) and the supernatant was transferred to a vial ready for HPLC analysis.

2.3. Photobioreactors and culture mode

To maximize the *J. luteoviridis* growth rate, different initial nitrogen concentrations were tested ($650, 800, 900$ and $1000 \text{ mg} \cdot \text{L}^{-1}$ of NaNO_3 , which corresponded to 7.65, 9.41, 10.59 and 11.76 mM of NO_3^-), using the modified f/2 medium as the base with an N/P of 5, by also modifying the phosphorus concentration. Different molar N/P ratios were also tested (5, 10, 15 and 20), by fixing the nitrate concentration and modifying the phosphate concentration, were also tested to evaluate their influence on the microalgal growth rate and on the biomass composition. Both sets of experiments were performed in batch mode using spherical flasks with a working volume of 0.8 L at 25°C and $100 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ under a 12:12 h light/dark cycle.

To assess the influence of the LED light colour and intensity on the growth rate and biomass composition, experiments were conducted in batch mode culture using 2 L indoor glass bubble-column photobioreactors, each with a working volume of 1.8 L; the photobioreactors had an internal diameter of 0.07 m and a total height of 0.50 m. The culture was mixed by sparging the vessel with sterile filtered air from a compressor (Atlas Copco Airpower B.V.-GA7FF, Atlas Copco S.A.E., Madrid, Spain) at a flow rate of $0.5 \text{ v} \cdot \text{v}^{-1} \cdot \text{min}^{-1}$ via an inlet at the bottom of the column, while the pH was maintained in a range from 7.8 to 8.0 by the on-demand injection of carbon dioxide. The cultures were thermostated with cooling jackets, using water as the cooling agent at $25 \pm 1^\circ\text{C}$ and a refrigerated bath circulator (Thermo Scientific-Neslab RTE7, Thermo Scientific, Newington, N.H., United States). The

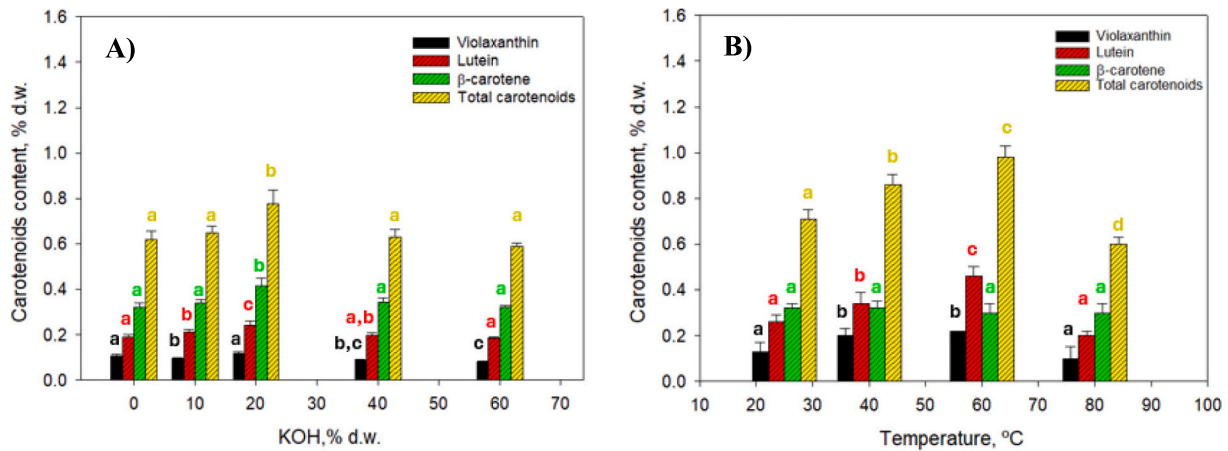


Fig. 1. Optimisation of carotenoid extraction from *J. luteoviridis* biomass for (a) the percentage of KOH per dry weight of biomass and (b) the temperature. Data points and columns are average values, and vertical bars are the standard deviations for the triplicate samples. Lowercase letters represent significant differences with $p < 0.05$. Different colours represent each compound. Black lowercase letters represent significant differences for violaxanthin, red for lutein, green for β -carotene and yellow for total carotenoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

photobioreactors were wrapped in a 7.5 m multicoloured LED strip (red, blue, green and warm white, collectively referred to as RGBW) (LEDs; Edison Opto Co., Taiwan), fixed horizontally around the entire bubble column, similar to those reported in a previous study on *Karlodinium veneticum* and *Amphidinium carterae* (López-Rosales et al., 2016; Molina-Miras et al., 2018).

The LED control unit allowed various illumination regimens, irradiance intensities, and colour compositions to be selected using Daqfactory software. A sinusoidal diel variation pattern of outdoor irradiance was imposed using Eq. (1) of López-Rosales et al. (2016):

$$I_0(t) = \begin{cases} I_{0\max} \sin\left[\pi \frac{t - t_{sr}}{t_{ss} - t_{sr}}\right] & \text{if } t_{sr} \leq t \leq t_{ss} \\ 0 & \text{otherwise} \end{cases} \quad (1)$$

where $I_{0\max}$ is the maximum irradiance occurring at midday inside the PBR filled with culture medium (without cells); t_{sr} is the sunrise time (8 h); and t_{ss} is the sunset time (20h).

Different levels of white irradiance ($I_{0\max}$) were tested (250, 500, 1000, 1500, 2000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) as well as different red intensities (700, 800, 900 and 1100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), along with four combinations of blue and red light (100 % blue, 75 % blue – 25 % red, 50 % blue – 50 % red, 25 % blue – 75 % red, 100 % red). When both colours were combined, the maximum irradiance emitted by the LED light was 700 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, so this was the combination of irradiance used during the blue-red light. All the sets of experiments were carried out by simulating the solar cycle, with a 12 h dark period, and all started at a biomass concentration of 0.1 $\text{g}\cdot\text{L}^{-1}$. The irradiance values were measured using a spherical quantum sensor (Biospherical QSL 100; Biospherical Instruments Inc., San Diego, CA, EUA).

2.4. Microalgal growth monitoring and analytical procedures

The biomass concentration was determined daily by measuring the absorbance at 750 nm [27] with a spectrophotometer (Helios Omega UV-VIS Spectrophotometer, Thermo Scientific, Horsham, England). The physiological state of the cells was determined by measuring the Fv/Fm using a fluorimeter (AquaPen-C AP-C 100, Photon Systems Instruments, The Czech Republic). Nutrient consumption was also analyzed by measuring the concentrations of nitrate and phosphate in the supernatant. To do so, samples were taken daily and centrifuged at 7500 rpm for 5 min (SIGMA 4 K15 Sartorius, Göttingen, Germany). The supernatant was kept apart and the biomass was washed with an aqueous 0.5 M solution of ammonium bicarbonate [28] and then lyophilized (TELSTAR

Cryonos-50, Madrid, Spain). Subsequently, the concentrations of nitrate and phosphate were quantified using the American Public Health Association's 4500-N and 4500-P method, respectively [29].

Regarding the biomass composition, the lipid content was determined gravimetrically from an extract obtained with chloroform:methanol (2,1) (v/v) according to the Kochert (1978) method [30]. Protein content in biomass was measured, as described by González-López et al. [31]. The carotenoids were analyzed by HPLC (Shimadzu High Performance Liquid Chromatograph SPD10AV) using a photodiode array detector and following the method proposed by Cerón-García et al. [25]. The carotenoids were quantified using the standard analysis curves of lutein and β -carotene provided by the Sigma Chemical Co. (St. Louis, MO, USA). The violaxanthin and zeaxanthin were purchased from DHI Lab Products (Hørsholm, Denmark). Each sample was analyzed in triplicate.

2.5. Kinetic parameters

The specific growth rate (μ , day^{-1}) was determined by dividing the slope of the biomass concentration profile by the average biomass concentration during the exponential growth phase (Eq. 2).

$$\mu = \frac{1}{C} \frac{dC}{dt} \quad (2)$$

The final biomass productivity (P_{Biomass} , $\text{mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) was calculated by dividing the final biomass yield ($\text{mg}\cdot\text{L}^{-1}$) by the culture period (day) (Eq. (3)). The final productivities of the high-value products (carotenoids, lipids, and proteins) were calculated by multiplying the biomass productivity by the percentage (in dry weight) of each compound.

$$P_B = \frac{C - C_0}{t - t_0} \quad (3)$$

The final productivities of the high-value products (carotenoids, lipids, and proteins) were calculated by multiplying the biomass productivity by the dry weight percentage of each compound (Eqs. (4), (5) and (6), respectively).

$$P_{\text{carotenoids}} = P_B \cdot \frac{\text{Carotenoids content}}{100} \quad (4)$$

$$P_{\text{lipids}} = P_B \cdot \frac{\text{Lipids content}}{100} \quad (5)$$

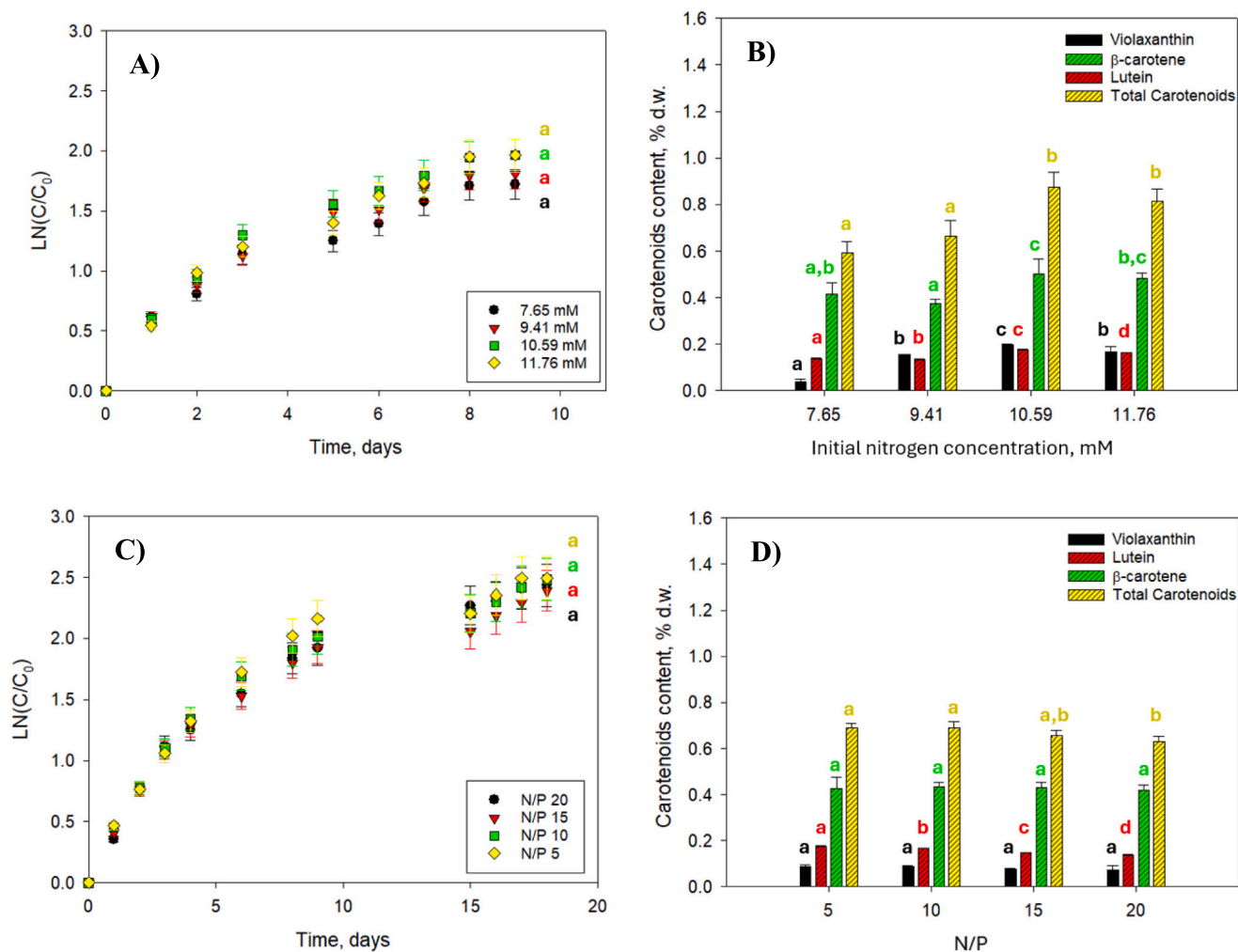


Fig. 2. (a) Variation in $\ln(C/C_0)$ and (b) the carotenoid content (% d.w.) for each initial nitrogen concentration in the culture and (c) variation in $\ln(C/C_0)$ and (d) the carotenoid content (% d.w.) for each N/P ratio in the culture. Data points and columns are average values, and vertical bars are the standard deviations for triplicate samples. Lowercase letters represent significant differences, with $p < 0.05$. Different colours represent each compound. In Fig. 2b and c, black lowercase letters represent significant differences for violaxanthin, red for lutein, green for β -carotene and yellow for total carotenoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$P_{\text{proteins}} = P_B \frac{\text{Proteins content}}{100} \quad (6)$$

2.6. Statistical analyses

A one-way analysis of variance (ANOVA) test was performed for the significant difference analysis and to evaluate the effects of each factor (the initial nitrate concentration, N/P ratio, the white irradiance level, and the combinations of red and blue light) on the growth rate and the productivity of the high-value compounds. Statistical analyses were performed using Statistica® software (12.0, Statsoft Inc., Tulsa, USA). Normality and homogeneity were evaluated using the Kolmogorov-Smirnov and Levene tests, respectively. When any of the samples were not homogeneous, the data (as a percentage) were transformed into arcsin ($x^{1/2}$). The results were analyzed using analysis of variance (ANOVA) and Tukey's test at a 95 % confidence level.

3. Results and discussion

3.1. Optimization of the carotenoid quantification

Fig. 1 shows the effect of the KOH concentration and temperature on carotenoid extraction, focussing on violaxanthin, lutein, β -carotene, and

the total carotenoid content in *J. luteoviridis*. The highest extraction of violaxanthin was achieved using 0 and 20 % d.w. of KOH, with no significant differences between them ($p > 0.05$). However, the highest amount of total carotenoids, β -carotene, and lutein was obtained using 20 % d.w. of KOH. As the KOH concentration increased, the amount of extracted carotenoids decreased, possibly due to degradation, as in the case of *Amphidinium carterae* Dn241EHU [32], and *Isochrysis galbana* and *H. lacustris* [25]. Regarding temperature, there was no significant influence ($p > 0.05$) on β -carotene extraction, while violaxanthin extraction was higher at 40 °C and 60 °C, reaching similar values at both temperatures. The highest amount of total carotenoids and lutein was obtained at 60 °C ($p < 0.05$), achieving >0.4 % d.w.; this decreased at 80 °C to an amount similar to that obtained at 25 °C. When the temperature increased, the resulting decrease in the carotenoid concentration may have been due to degradation, as in the case of *A. carterae* Dn241EHU and ACRN03 [32] or *I. galbana* [25], since carotenoid degradation can be caused by high temperature, KOH concentration, and/or contact time [33]. Therefore, the optimal carotenoid extraction conditions selected for *J. luteoviridis* were 20 % d.w. of KOH at 60 °C, achieving 1 % d.w. ($10 \text{ mg} \cdot \text{g}^{-1}$) of carotenoids contained in the biomass. This method improves carotenoid extraction compared to traditional methods, even when applying a moderate electric field in the presence of ethanol as a pretreatment followed by a diffusive step with ethanol as

Table 1

Growth rate (μ , day^{-1}) and biomass yield per gram of consumed nutrient (nitrate (Y_{B/NO_3} , g generated biomass $\cdot$ g $^{-1}$ consumed nitrate) and phosphate (Y_{B/PO_4} , g generated biomass $\cdot$ g $^{-1}$ consumed phosphate)) for each initial nitrate concentration and each nitrogen to phosphorus ratio.

Parameter	Modifying nitrate initial concentration, mM				Modifying N/P			
	7.65	9.41	10.59	11.76	20	15	10	5
μ , day^{-1}	0.252 ± 0.013^a	0.258 ± 0.015^a	0.278 ± 0.019^a	0.269 ± 0.013^a	0.244 ± 0.012^a	0.243 ± 0.017^a	0.257 ± 0.015^a	0.268 ± 0.013^a
Y_{B/NO_3} , g $\cdot$ g $^{-1}$	3.22 ± 0.30^a	2.11 ± 0.20^b	2.16 ± 0.20^b	1.56 ± 0.10^c	4.23 ± 1.20^a	$3.64 \pm 0.70^{a,b}$	$3.53 \pm 0.60^{a,b}$	2.57 ± 0.40^b
Y_{B/PO_4} , g $\cdot$ g $^{-1}$	4.93 ± 1.00^a	3.98 ± 0.70^a	3.97 ± 0.40^a	4.08 ± 0.40^a	20.43 ± 3.30^a	14.69 ± 2.30^b	10.71 ± 0.90^c	5.60 ± 0.70^d

the solvent, for which the maximum carotenoid content obtained was $1.21 \text{ mg} \cdot \text{g}^{-1}$ [34].

3.2. Effect of the initial nitrogen concentration and nitrogen-to-phosphorus ratio on cell growth and biomass composition

Fig. 2 shows the influence of the initial nitrogen concentration on $\text{Ln}(C/C_0)$ variation over time for the microalgae *J. luteoviridis* along with its carotenoid composition. The general trend was that the higher the initial nitrogen concentration, the higher the $\text{Ln}(C/C_0)$ reached at the end of the batch (Fig. 2a) - obtaining growth rates of 0.252, 0.258, 0.278 and 0.269 day^{-1} for initial nitrogen concentrations of 7.65 mM, 9.41 mM, 10.59 mM and 11.76 mM, respectively (see Table 1). However, there were no statistically significant differences between them ($p > 0.05$). Menegol et al. (2017) [35] also observed that the highest concentration of biomass of *J. luteoviridis* ($3.35 \text{ g} \cdot \text{L}^{-1}$, which corresponded to an $\text{Ln}(C/C_0)$ of 3.1) was obtained at the highest nitrogen concentration tested ($60 \text{ mg} \cdot \text{L}^{-1} \text{ N-NO}_3$, corresponding to 0.97 mM). The difference between the two works could be due to the irradiance value ($252 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ used by Menegol et al. (2017) [35] compared to $100 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ in this work) and the configuration of the photobioreactor. Although the nitrate concentrations used in this work seem extremely high (compared to the f/2 medium, a maintenance medium which has a concentration of $882 \mu\text{M}$), it is commonly used in microalgae cultures. For example, the optimal biomass concentration for *Nannochloropsis gaditana* was achieved at a nitrate concentration of 11.3 mM [36]; for *Scenedesmus rubescens*, the optimal lipid content was obtained at a nitrate concentration of 1000 mM [37], and *Chlorella sorokiniana* was cultured at 17.65 mM [38]. Furthermore, it can be observed that the biomass yield per gramme of intake of nitrate and phosphate (Table 1) was low and similar in all cases for both macronutrients. In the case of nitrate, the highest yield was obtained at 7.65 mM ($p < 0.05$), although the biomass concentration achieved was the lowest. For phosphate, there were no statistically significant difference ($p > 0.05$). This could be because both

nutrients were at high concentrations and so light that was the limiting factor.

In the case of the carotenoid composition (Fig. 2b) - the higher the initial nitrogen concentration, the higher the content obtained - although there was no significant difference between 10.59 mM and 11.76 mM ($p > 0.05$). The total carotenoid content increased by approximately 50 % compared to the initial nitrate concentration of 7.65 mM ; in the case of the violaxanthin content, it increased by 5 and 4 times, respectively. As is already known, when the irradiance is low, zeaxanthin is converted to violaxanthin [39], so the culture could be photolimited, as indicated by the low $\text{Ln}(C/C_0)$ value reached. It could also explain why zeaxanthin was not detected in this work. Menegol et al. (2017) [35] observed the same tendency for *J. luteoviridis*, with the carotenoid content increasing the higher the nitrate concentration.

Fig. 2c shows the influence of the nitrogen-to-phosphorus molar ratio (N/P) on the variation in $\text{Ln}(C/C_0)$ and the carotenoid composition in *J. luteoviridis* (Fig. 2d). The different N/P ratios did not influence this microalgae's growth rate ($p > 0.05$) after 20 days of culture (Fig. 2c), obtaining a growth rate of approximately 0.25 day^{-1} in all cases (see Table 1). This means that *J. luteoviridis* grows well when there is a low amount of phosphorus (i.e., a higher N/P). However, this is not the general tendency; for example, the biomass concentration of *N. gaditana* decreases with an increase in the phosphorus concentration (for an N/P lower than 60) [36,40]; similarly, the biomass concentration of *I. galbana* decreases at a phosphorus concentration below 0.2 mM [41]. In Table 1, one can see that, in the case of nitrate, the yield was higher for higher N/Ps although there was no difference in the biomass concentration (Fig. 2c). Therefore, this difference was due to the amount of intake nitrate, given that the same amount was used for all ratios. The N/P ratio had a significant influence on the biomass yield per phosphate gramme ($p < 0.05$), obtaining higher yields at higher N/Ps. However, in this case, it was due to the phosphate concentration being lower at higher N/Ps. Regarding the carotenoid content, one can observe that the N/P ratio also had no influence on violaxanthin or β -carotene (Fig. 2d).

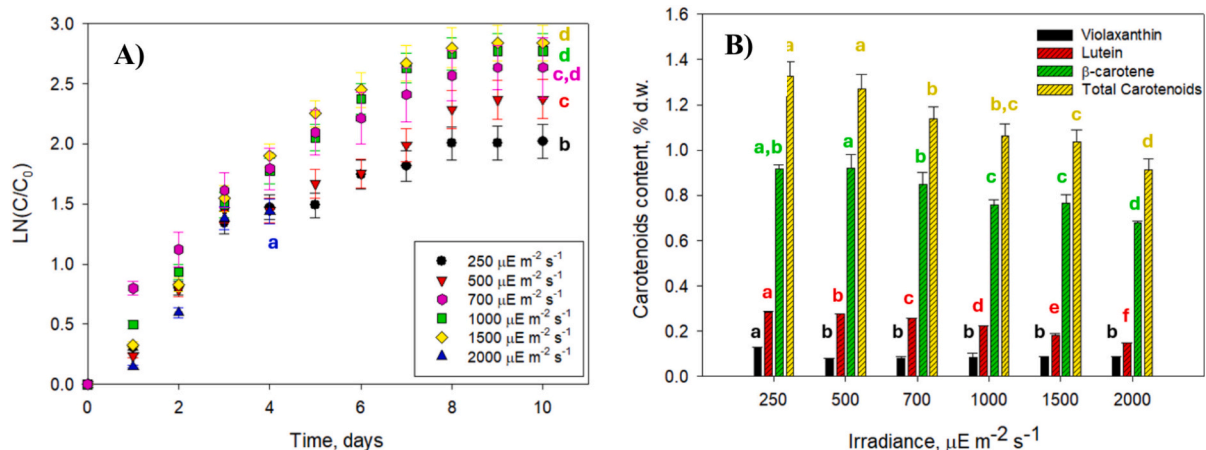


Fig. 3. (a) Variation in $\text{Ln}(C/C_0)$ and (b) the carotenoid content (% d.w.) for each initial white irradiance intensity. Data points and columns are average values, and vertical bars are the standard deviations for triplicate samples. Lowercase letters represent significant differences, with $p < 0.05$. Different colours represent each compound. In Fig. 3b black lowercase letters represent significant differences for violaxanthin, red for lutein, green for β -carotene and yellow for total carotenoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Influence of incident irradiance intensity and composition on biomass productivity, lipid and protein content, and their productivities^a.

Light	I _{0max} μE·m ⁻² ·s ⁻¹	μ, day ⁻¹	Total carotenoid content, % d.w.	Total lipid content, % d.w.	Protein content, % d.w.	Fv/Fm	P _{Biomass} mg·L ⁻¹ ·day ⁻¹	P _{Carotenoids} mg·L ⁻¹ ·day ⁻¹	P _{Lipids} mg·L ⁻¹ ·day ⁻¹	P _{Protein} mg·L ⁻¹ ·day ⁻¹
White	250	0.287 ± 0.029 ^a	1.33 ± 0.07 ^a	15.8 ± 0.11 ^a	43.7 ± 1.8 ^a	0.71 ± 0.01 ^a	83.00 ± 5.80 ^a	1.10 ± 0.14 ^a	13.11 ± 1.01 ^a	36.27 ± 4.09 ^a
	500	0.320 ± 0.020 ^a	1.27 ± 0.06 ^b	13.0 ± 0.57 ^b	45.3 ± 0.1 ^b	0.64 ± 0.01 ^b	123.40 ± 8.40 ^b	1.57 ± 0.19 ^b	16.04 ± 1.84 ^b	55.90 ± 3.90 ^b
	700	0.398 ± 0.028 ^b	1.14 ± 0.05 ^c	13.9 ± 0.40 ^c	48.1 ± 0.3 ^c	0.64 ± 0.02 ^b	157.50 ± 15.00 ^c	1.80 ± 0.26 ^{b,c}	21.97 ± 2.71 ^c	75.68 ± 7.78 ^c
	1000	0.406 ± 0.020 ^{b,c}	1.06 ± 0.05 ^{b,c}	14.9 ± 0.15 ^d	50.8 ± 0.6 ^d	0.63 ± 0.04 ^b	177.43 ± 9.20 ^d	1.89 ± 0.20 ^{b,c}	26.44 ± 1.65 ^d	90.13 ± 5.77 ^d
	1500	0.421 ± 0.025 ^{b,c}	1.04 ± 0.05 ^c	13.6 ± 0.26 ^c	51.1 ± 0.1 ^d	0.57 ± 0.00 ^c	193.00 ± 11.30 ^d	2.00 ± 0.22 ^c	26.25 ± 2.07 ^d	98.62 ± 5.79 ^d
	2000	0.374 ± 0.026 ^c	0.91 ± 0.05 ^d	10.20 ± 0.26 ^e	42.1 ± 0.6 ^e	0.54 ± 0.04 ^c	48.40 ± 3.40 ^e	0.44 ± 0.05 ^d	4.94 ± 0.48 ^e	20.38 ± 1.72 ^e
Red	700	0.518 ± 0.026 ^a	0.69 ± 0.04 ^a	12.1 ± 1.10 ^{a,b}	52.5 ± 1.06 ^a	0.69 ± 0.06 ^a	224.71 ± 15.74 ^a	1.62 ± 0.20 ^a	27.19 ± 2.05 ^a	117.97 ± 10.81 ^a
	800	0.554 ± 0.033 ^a	0.74 ± 0.04 ^a	11.3 ± 1.10 ^{a,b}	44.2 ± 0.9 ^b	0.73 ± 0.04 ^a	224.97 ± 14.30 ^a	1.67 ± 0.19 ^a	25.42 ± 4.25 ^a	93.44 ± 8.48 ^b
	900	0.556 ± 0.039 ^a	0.75 ± 0.04 ^a	11.0 ± 0.05 ^b	40.6 ± 1.0 ^c	0.72 ± 0.02 ^a	231.47 ± 13.00 ^a	1.75 ± 0.19 ^a	25.46 ± 1.55 ^a	95.98 ± 7.82 ^b
	1100	0.540 ± 0.027 ^a	0.78 ± 0.04 ^a	12.3 ± 0.15 ^a	41.0 ± 0.9 ^c	0.71 ± 0.06 ^a	230.17 ± 13.00 ^a	1.80 ± 0.20 ^a	28.31 ± 1.96 ^a	94.37 ± 7.59 ^b
	700	0.470 ± 0.023 ^a	1.09 ± 0.05 ^a	15.8 ± 0.20 ^a	55.1 ± 1.1 ^a	0.64 ± 0.08 ^a	163.13 ± 11.43 ^a	1.78 ± 0.22 ^a	25.77 ± 2.15 ^{a,b}	89.88 ± 8.18 ^a
	700	0.493 ± 0.030 ^a	0.99 ± 0.05 ^b	16.8 ± 0.85 ^b	59.3 ± 2.2 ^a	0.64 ± 0.10 ^b	181.00 ± 12.68 ^a	1.80 ± 0.22 ^b	30.41 ± 3.78 ^b	107.33 ± 11.82 ^{a,b}
25 % Red	700	0.517 ± 0.036 ^a	0.95 ± 0.05 ^b	13.9 ± 1.46 ^c	50.0 ± 0.9 ^b	0.62 ± 0.06 ^c	185.00 ± 12.50 ^a	1.76 ± 0.21 ^a	25.72 ± 4.62 ^{a,b}	92.50 ± 8.11 ^a
50 % Blue –	700	0.485 ± 0.024 ^a	0.76 ± 0.04 ^c	13.7 ± 0.09 ^d	55.0 ± 2.2 ^b	0.69 ± 0.04 ^a	177.50 ± 12.38 ^a	1.35 ± 0.17 ^b	24.32 ± 1.87 ^a	97.63 ± 11.02 ^{a,b}
25 % Blue –	700	0.518 ± 0.031 ^a	0.72 ± 0.04 ^c	12.1 ± 0.06 ^e	52.5 ± 1.1 ^c	0.69 ± 0.06 ^{b,c}	216.00 ± 15.13 ^b	1.56 ± 0.19 ^{a,b}	26.14 ± 1.97 ^{a,b}	113.40 ± 10.39 ^b

^a Dry weight (d.w.). The results are presented as means ± standard deviation R- Red and B – Blue. Different lowercase letters indicate significant differences between experimental groups ($p < 0.05$).

However, the content of lutein and total carotenoid decreased, by 20 % and 10 %, respectively, at a higher N/P (Fig. 3b), which means a lower phosphorus concentration. This tendency has also been observed in other microalgae, such as *Monoraphidium braunii* [42], whose carotenoid content of which decreased by 75 % after 9 days of culture with reduced phosphorus. Therefore, phosphorus has a significant influence ($p < 0.05$) on biomass quality, as it is an important macronutrient for cellular structure and for nucleic acid and protein synthesis [43].

3.3. Effect of irradiance level and colour composition on cell growth and biomass composition

White irradiance had a huge influence on Ln(C/C₀) variation and carotenoid composition in *J. luteoviridis* (Fig. 3). When it increased from 250 to 1500 μE·m⁻²·s⁻¹, the microalgae's growth rate likewise increased (Fig. 3a and Table 2), reaching a biomass concentration that was 2.3 times higher at 1500 μE·m⁻²·s⁻¹ than at 250 μE·m⁻²·s⁻¹ after 10 days of culture, with no significant differences ($p > 0.05$) between values of 700, 1000 and 1500 μE·m⁻²·s⁻¹. At these higher irradiances, the Ln(C/C₀) values were close to 3 (2.6, 2.8 and 2.9, respectively), the value obtained by Menegol et al. (2017) [35]. Above 1500 μE·m⁻²·s⁻¹, care must be taken since *J. luteoviridis* became photoinhibited at 2000 μE·m⁻²·s⁻¹, dying after 4 days of culture. However, the general tendency is that the growth rate and productivity increase along with irradiance. This was also found to be the case for *C. sorokiniana*, with irradiances ranging from 50 to 1000 μE·m⁻²·s⁻¹ [44], and for *Scenedesmus almeriensis*, where they ranged from 326 to 1625 μE·m⁻²·s⁻¹ [45]. However, the photoinhibition phenomena depends on both the incident irradiance and the light availability inside the culture. For example, in the case of *I. galbana*, a photoinhibition effect was observed above 1630 μE·m⁻²·s⁻¹ [46]. Therefore, *J. luteoviridis* should have a similar photoinhibition limit. On the other hand, the biomass quality increased at lower irradiance values (Fig. 3b and Table 2), increasing the carotenoid and lipid content by around 20 %, while the maximum protein content was found at 1000 and 1500 μE·m⁻²·s⁻¹. This tendency was also observed for *C. sorokiniana*, whose lipid content was greater at lower irradiances while the protein content increased at higher irradiances, up to 1000 μE·m⁻²·s⁻¹ [44]. The general tendency is that high light intensity increases the β-carotene content as excess irradiance exposure creates reactive oxygen species that could damage microalgae cells [47,48].

Table 3

Biomass yield per gram of nutrient consumed (nitrate and phosphate) for each white incident irradiance, red incident irradiance and combination of blue-red composition.

	Yield, g biomass g ⁻¹ consumed nutrient	
	Y _{B/NO3}	Y _{B/NO3}
White Incident irradiance, μE·m ⁻² ·s ⁻¹		
250	3.28 ± 1.20 ^{a,b}	5.10 ± 0.90 ^a
500	4.92 ± 1.70 ^a	9.50 ± 1.50 ^b
700	3.17 ± 1.10 ^{a,b}	5.20 ± 0.80 ^a
1000	3.38 ± 0.90 ^{a,b}	5.97 ± 0.90 ^a
1500	5.01 ± 1.40 ^a	7.83 ± 1.10 ^b
2000	1.40 ± 0.50 ^b	2.19 ± 0.40 ^c
Incident irradiance, μE·m ⁻² ·s ⁻¹		
700	3.17 ± 1.10 ^a	5.20 ± 0.80 ^a
800	2.75 ± 0.80 ^a	5.80 ± 0.90 ^a
900	2.80 ± 0.80 ^a	5.67 ± 0.90 ^a
1100	2.66 ± 0.80 ^a	5.51 ± 0.90 ^a
Combination of blue-red composition		
100 % Red	2.86 ± 0.80 ^a	5.87 ± 1.00 ^a
75 % Red – 25 % Blue	3.27 ± 1.00 ^a	6.40 ± 1.00 ^{a,b}
50 % Red – 50 % Blue	4.03 ± 1.40 ^a	6.61 ± 1.00 ^{a,b}
25 % Red – 75 % Blue	3.09 ± 0.90 ^a	6.50 ± 1.00 ^{a,b}
100 % Blue	4.19 ± 1.30 ^a	7.79 ± 1.20 ^b

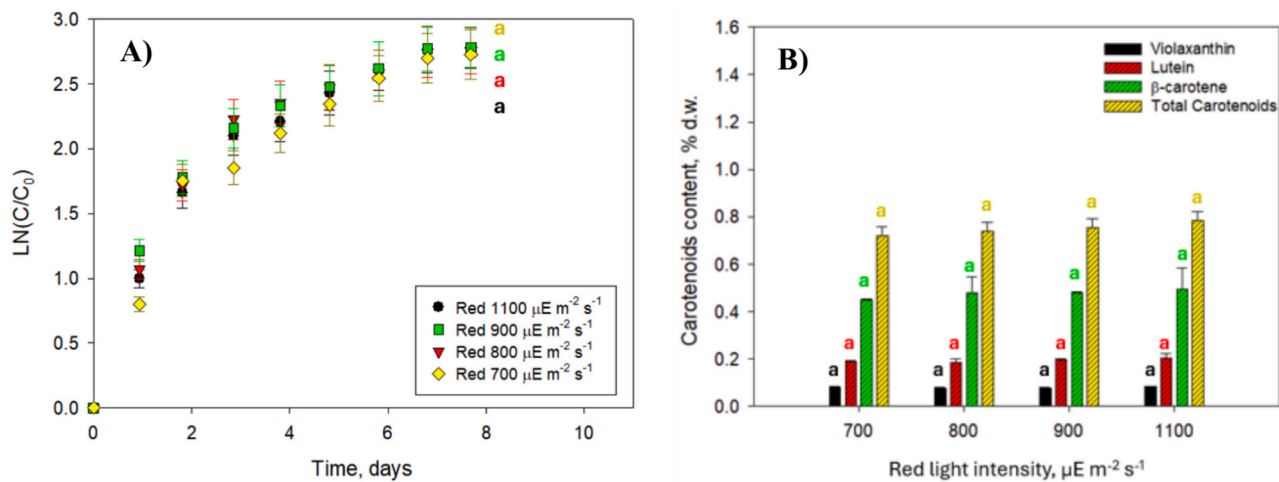


Fig. 4. (a) Variation in $\text{Ln}(C/C_0)$ and (b) the carotenoid content (% d.w.) for each intensity of incident red irradiance. Data points and columns are average values, and vertical bars are the standard deviations for triplicate samples. Lowercase letters represent significant differences, with $p < 0.05$. Different colours represent each compound. In Fig. 4b black lowercase letters represent significant differences for violaxanthin, red for lutein, green for β -carotene and yellow for total carotenoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, as β -carotene has a protective role, the synthesis of this carotenoid increases to protect itself [49]. Nevertheless, something similar occurred to *Rhodospirillum rubrum*, *Chlorella minutissima*, and *Nannochloropsis oceanica*, whose β -carotene content decreased using a light intensity $>800 \mu\text{E m}^{-2} \text{s}^{-1}$ [50]. This could be explained by carotenogenesis, which is greatly influenced by the light regime. At low light intensities, more light-harvesting molecules are required to guarantee an increased absorption cross section. In addition to chlorophylls, carotenoids can absorb light and convert it into energy that chlorophylls can use. Consequently, microalgae incorporate a larger concentration of light-harvesting pigments to adapt to low light intensities. Some carotenoids, namely primary carotenoids, have a tendency to degrade under high light intensities, while others prefer to accumulate to some extent as defence against cell damage [51]. Regarding lipid accumulation, to avoid photo-oxidative damage under stress, extra photoassimilates are efficiently consumed, leading to lipid buildup under high light intensities [52].

All cultures were healthy given that the F_v/f_m value was >0.5 (see

Table 2). Regarding the efficiency of nitrate intake, it was observed that this was the same at all irradiance levels, since the biomass generated per gramme of nitrate intake was constant in all cases (Table 3). However, the efficiency of phosphate intake increased along with irradiance, except at $2000 \mu\text{E m}^{-2} \text{s}^{-1}$, at which point the culture became photoinhibited.

Regarding red irradiance, there were no significant differences ($p > 0.05$) in the $\text{Ln}(C/C_0)$ variation achieved after 8 days of culture (Fig. 4a), nor was there a difference in the carotenoid content (Fig. 4b and Table 2) at the different irradiances levels assayed. This was also the case for the growth rate (Table 2) and the biomass yield per gramme of macronutrient intake (Table 3). However, the lipid content decreased by 10 % at 800 and 900 $\mu\text{E m}^{-2} \text{s}^{-1}$ while the protein content decreased by around 20 % at intensities $>700 \mu\text{E m}^{-2} \text{s}^{-1}$ (Table 2). In Table 2, one can see that the growth rate was higher when red light was used rather than white light. Moreover, in terms of the $\text{Ln}(C/C_0)$ variation using red (Fig. 4a) and white (Fig. 3a) light, one can observe that the stationary state was reached faster with red light (8 days) than with white light (10

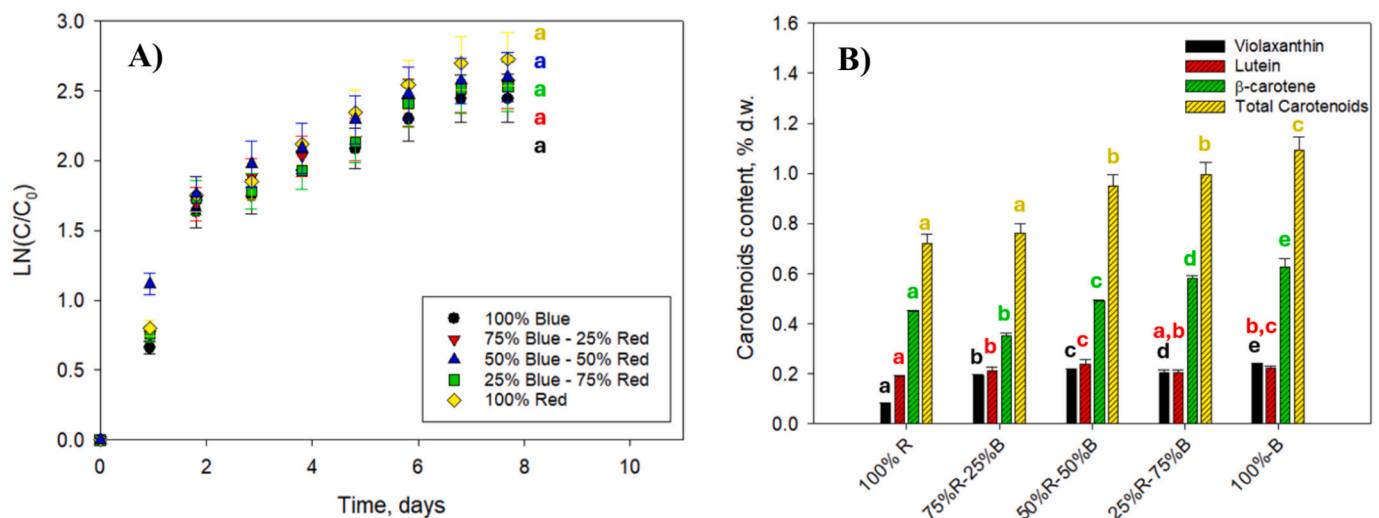


Fig. 5. (a) Variation in $\text{Ln}(C/C_0)$ and (b) the carotenoid content (% d.w.) for each blue-red light combination. Data points and columns are average values, and vertical bars are the standard deviations for triplicate samples. Lowercase letters represent significant differences, with $p < 0.05$. Different colours represent each compound. In Fig. 5b black lowercase letters represent significant differences for violaxanthin, red for lutein, green for β -carotene and yellow for total carotenoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

days). Furthermore, biomass productivity was higher with red light, as was the lipid and protein productivity (Table 2). The highest biomass concentration reached using red light compared to white light was also observed for *Tetrademus obliquus* and *Arthrospira platensis* [53]. This might be because the main pigments in most microalgal strains are chlorophylls, which can absorb red light more efficiently than other irradiances [54]. However, this does not occur in all microalgae; for example, *Porphyridium purpureum* grows well under white and green light, but there is no increase in biomass when exposed to red or blue light [55]; the same applies for *Chlorella vulgaris* [53,56]. Raqiba and Sibi [53] also observed that the lipid content increased with red light on *C. vulgaris*, *T. obliquus* and *A. platensis*, while the carotenoid content increased using white light. It could be because as carotenoids are yellow and orange in hue and absorb light at wavelengths between 400 and 550 nm in the solar spectrum, they also absorb blue and green regions of the spectrum. Therefore, red light does not have an effect on promoting its productivity.

Regarding the blue-red light combinations (Fig. 5), the greatest variation achieved in $\ln(C/C_0)$ after 8 days of culture was obtained with 100 % red light (Fig. 5a). However, there were no significant differences ($p > 0.05$) between the combinations used, with a growth rate of around 0.5 day^{-1} in all cases (Table 2). The carotenoid content followed the opposite trend, increasing as the blue content increased (Fig. 5b), achieving 30 % more total carotenoids using 100 % blue light than with 100 % red light. As was mentioned previously, carotenoids absorb blue and green light regions of the spectrum, so the blue light promotes its productivity. Violaxanthin stands out in particular since its content could be increased by 50 % by incorporating 25 % blue light. This increased to 70 % using 100 % blue light. In the case of β -carotene, its content increased by 30 % when 100 % blue light was used compared to 100 % red light. However, in the case of the photosynthetic pigments, their yield depends on the wavelength at which they absorb photons. The protein and lipid content also increased ($p > 0.05$) along with the percentage of blue light (Table 2), reaching higher values than those obtained with white light. Coward et al. [55] observed that in *P. purpureum*, the protein content increased along with blue light while the pigment concentration was lower in cells exposed to red light. Baidya et al. [19] reported that the β -carotene, protein, and lipid contents of *Chlorella ellipsoidea* increased with blue light while the minimum content was obtained with red light. On the contrary, Raqiba and Sibi [53] observed that the carotenoid content of *C. vulgaris*, *T. obliquus* and *A. platensis* increased more with red light than with blue light, while the lipid content was highest with blue light than when using red or white light. This increase in the lipid content could be because blue light favours enzymes that promote triglyceride accumulation [57].

4. Conclusions

Saponification and temperature must be selected for each strain for that reason *J. luteoviridis* required 60 °C and 20 % KOH to achieve total carotenoid extraction.

The carotenoid content of the biomass of *J. luteoviridis* was found to increase with the higher concentrations of nitrate and phosphate, converting in primary metabolites. White irradiance of up to $1500 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ stimulated the growth rate and protein content, while the carotenoid and lipid contents decreased. The intensity of the red light did not affect the growth rate or the carotenoid content, although the protein content decreased, which are associated to the growth. Nevertheless, when red light was combined with blue light, the growth rate increased along with the red-light percentage, while high-value products increased along with a higher blue-light content. Increasing the cell replication because their chlorophylls absorb this type of light achieving more photosynthetic level.

CRedit authorship contribution statement

T. Menegol: Writing – original draft, Methodology, Investigation, Conceptualization. **Y. Soriano-Jerez:** Writing – review & editing, Writing – original draft, Conceptualization. **L. López-Rosales:** Supervision, Investigation, Conceptualization. **F. García-Camacho:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **A. Contreras-Gómez:** Writing – review & editing, Writing – original draft, Formal analysis. **E. Molina-Grima:** Writing – review & editing, Conceptualization. **R. Rech:** Writing – review & editing. **M.C. Cerón-García:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2025.103936>.

Data availability

The authors do not have permission to share data.

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