Heterotrophic culture of *Scenedesmus almeriensis* for lutein productivity enhancement

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The green microalgae *Scenedesmus almeriensis* shows promise for natural carotenoid production, specifically lutein, a pigment inducing health benefits, often lacking in the human diet. In this study, *S. almeriensis* was successfully cultured heterotrophically for the first time. Heterotrophic biomass accumulation and pigment productivity were compared to a phototrophic culture, and a period of photoinduction followed the heterotrophic culture to stimulate carotenoid pigment synthesis. Under optimal glucose and sodium nitrate levels, the heterotrophic culture achieved a cell density of 22 g L^{-1}. Lutein and violaxanthin contents were 2.2 and 1.5 times higher in the phototrophic culture compared to the heterotrophic culture, while zeaxanthin content was 1.8 times higher in the heterotrophic culture. However, heterotrophic culture exhibited higher productivity for lutein, violaxanthin, and zeaxanthin, with 9.79, 2.08, and 2.46 mg L^{-1} d^{-1}, respectively. Additionally, the photoinduction stage enhanced carotenogenesis and increased productivity, reaching productivities of 11.52, 4.56, and 5.09 mg L^{-1} d^{-1} for lutein, violaxanthin, and zeaxanthin, respectively. These promising findings indicate the potential of *S. almeriensis* in heterotrophic culture for carotenoid production.

Microalgae | *Scenedesmus almeriensis* | Heterotrophy | Lutein | Two-stage

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1. Introduction

Lutein is a primary carotenoid that belongs to the xanthophyll group. It is found in photosynthetic organisms, specifically in leaves and fruits of higher plants and in vegetables, including kale, spinach, and corn, but also in microalgae (1). Since animals cannot synthesize lutein, it must be obtained directly from the diet. Once acquired, it is accumulated primarily in the eye, where it has protective functions against retinal oxidation (2). In addition to its benefits for eye health, lutein has also been studied for its potential to improve skin health and reduce the risk of certain types of cancer (3). Furthermore, lutein has been shown to cross the blood-brain barrier, a semi-permeable barrier that separates the brain from the circulatory system (4). This ability to cross the blood-brain barrier may be important for the benefits of lutein for brain health. Some studies have suggested that lutein may have a protective effect on the synapses by mitigating Reactive Oxygen Species (ROS), potentially reducing the risk of age-related cognitive decline and improving cognitive function (5). The current daily dietary intake of lutein among Europeans and North Americans stands at a mere 1.7 mg, which represents approximately 12% of the recommended 6-14 mg daily intake shown to be beneficial in reducing the risk of age-related diseases (6).

Currently, the commercial production of natural lutein is made from cultivating Marigold flowers (genus *Tagetes*). After a laborious flower harvest, a drying process of the petals is required to obtain a lutein-rich oleoresin with a final lutein productivity of 10.6 kg $hectare^{-1}$ $year^{-1}$ (1, 7). The rising demand for lutein, projected to reach a global market value of USD 491 million by 2029 (8), is causing concerns about the sustainability of this mode of production. The shortage of arable land and irrigation water, excessive use of pesticides and chemical fertilizers, and the need for specific climate conditions for growth are factors that are driving the exploration of alternative sources of production (1, 9).

Alternatives to Marigold exist to produce lutein. Artificial synthesis of lutein has been possible and synthetic biology tools have recently been proposed for the production of lutein from bacteria and yeast, microorganisms that do not produce this carotenoid naturally (10, 11). However, chemical synthesis of lutein and its precursors can only be obtained at very low yields (1-5%) and involving numerous steps (12, 13). On the other hand, despite the great potential for increasing lutein production using genetic engineering tools, low social acceptability and concerns about their adverse effects hinder its market.

Alternatively, microalgae are photosynthetic organisms naturally producing lutein at concentrations ranging from 0.1 to 1% of their dry weight (14). Their cultivation cycles are short and they can be grown biotechnologically in continuous mode all year long (15). Traditionally, microalgae are cultivated outdoors to take advantage of the sunlight they require for photosynthesis or are provided with artificial light under controlled conditions. Under this photoautotrophic mode of cultivation, (16) obtained 0.91% lutein in a Dry Weight basis (DW) culture of *Chlorella vulgaris*. Similar results were obtained with *Chlorella sorokiniana* (0.58% DW) (17), *Dunaliella salina* (0.7% DW) (18), *Parachlorella* sp. (1.18% DW) (14) and *Muriellopsis* sp. (0.54% DW) (19). However, in phototrophic cultures, where light is the only source of energy, accessibility to light is inversely proportional to cell concentration due to mutual shading of cells (20). Ultimately, limited light availability constrains the overall process productivity. On the one hand, it reduces the attainable microalgal density, and on the other, as light intensity increases and cell density rises, it lowers the cell pigment content.

To overcome this limitation, heterotrophic, mixotrophic

and two-stage cultivation strategies have been proposed, using different sources of organic carbon as energy and carbon source (21–25). (26) achieved a 42% enhancement in lutein productivity using 3 g L^{-1} of acetate as carbon source for a mixotrophic culture of *Chlorella sorokiniana* Mb-1. (23) cultured *Chlamydomonas* sp. JSC4 and increased lutein productivity by 60% under a two-stage process. (25) cultured *C. sorokiniana* under mixotrophic growth and achieved a biomass concentration and lutein content of 26.21 g L^{-1} and 5.01 mg gDW^{-1}, respectively, using sodium acetate as an organic carbon source.

Among the microalgal candidates for industrial lutein production, the genus *Scenedesmus* has been extensively studied for its potential to generate high biomass and pigment yield (27–29). Compared to species in the *Chlorella* group, *Scenedesmus* species have a weaker cell wall, according to (30), who reported half the concentration of rhamnose in the cell wall of *Scenedesmus* sp. compared to a strain of *Chlorella vulgaris*. The rhamnose concentration in the cell wall is directly related to wall rigidity in microalgae (31). This characteristic makes cell disruption and pigment extraction more accessible and cost-effective. Additionally, *Scenedesmus* cells are larger than *Chlorella*'s, which eases the harvesting process.

Within the *Scenedesmus* genus, the strain *Scenedesmus almeriensis* has been found to have high lutein content, with levels reaching up to 8.5 mg gDW^{-1} under phototrophic conditions (32). Several authors have positioned *S. almeriensis* as a species with potential for industrial cultivation for various reasons (27–29, 33, 34). In addition to containing one of the highest contents of lutein among microalgae, it is a species that withstands high cultivation temperatures (up to 35 °C), allowing its cultivation in areas with high solar incidence and high temperatures (35).

Still, while achieving a high cellular lutein content, a *S. almeriensis* phototrophic bioprocess would suffer from the above-mentioned light limitation. This article explores a new approach for lutein production with this strain: the heterotrophic regime. To the best of our knowledge, this is the first report of *S. almeriensis* being cultured under heterotrophic conditions. Although other microalgae species with the potential to produce lutein have been studied under heterotrophic conditions, the characteristics of *S. almeriensis* make it a potential candidate for exploring this mode of cultivation. Additionally, *S. almeriensis* has been proposed as an attractive candidate for nutrient removal during wastewater treatment (36). Hence, the behavior of this species under heterotrophic conditions contributes to a better understanding of the biology of the species with prospects for industrial use.

While this study primarily investigates lutein, the expression of other carotenoids has also been monitored. In fact, within the context of a biorefinery approach, the traditional single-product culture has been replaced by a multi-output biotechnological process. As a result, the levels of VAZ (Violaxanthin + Antheraxanthin + Zeaxanthin) cycle pigments, particularly zeaxanthin, are also reported in this study. Zeaxanthin is a natural carotenoid recognized for its importance in various aspects of human health, serving as a potent antioxidant and light filter. As with lutein, its market has increased in recent years. Zeaxanthin is generally used with other carotenoids, particularly lutein, so the simultaneous increase of its production presents advantages from the industrial point of view (37). Additionally, other fractions of biomass content are reported to provide a spectrum of opportunities within a biorefinery concept.

In this study, our initial focus was examining the growth and lutein production of *S. almeriensis* under heterotrophic conditions. Subsequently, we explored a two-stage culture approach to maximize lutein productivity. The first stage involved utilizing a glucose-rich medium to obtain a concentrated biomass culture, while the second stage involved introducing a light source to drive the cell to express their photosynthetic apparatus, hence lutein content, once the glucose was depleted from the medium. The findings showcased in this study indicate this species' prospective suitability for expanding industrial-level cultivation. This assertion stems from the significant biomass concentration achieved through heterotrophic culture, coupled with the augmentation of lutein synthesis during the photoinduction phase. As a result of these factors, the attained lutein productivities stand among the most remarkable reported to date. Although this species was recently renamed by (38) as *Tetradesmus almeriensis*, throughout this paper, reference will be made to the former name *Scenedesmus almeriensis* to facilitate comparison with previous work on this same species.

2. Materials and Methods

2 1. Strain and phototrophic culture conditions

A strain of *Scenedesmus almeriensis* was received as a kind donation from Prof. Francisco Gabriel Acien from the University of Almeria. The strain was grown on agar with nutrient medium and glucose as a routine procedure to check for bacterial contamination. During the process, it was found that *Scenedesmus almeriensis* grew in the absence of light, so the strain was preserved under two cultivation methods, phototrophic in B3N culture medium under artificial white light and heterotrophic in B3N culture medium added with 10 g L^{-1} of glucose and without a light source. The phototrophic culture was carried out in 250 mL flasks with 50 mL of B3N culture medium containing (per liter): $NaNO_3$ (750 mg), $MgSO_4$ $7H_2O$ (75 mg), NaCl (25 mg), K_2HPO_4 (75 mg), KH_2PO_4 (175 mg), $CaCl_2$ $2H_2O$ (25 mg), $ZnSO_4$ $7H_2O$ (8.82 mg), $MnCl_2$ $4H_2O$ (1.44 mg), MoO_3 (0.71 mg) $CuSO_4$ $5H_2O$ (1.57 mg), $CO(NO_3)_2$ $6H_2O$ (0.49 mg), H_3BO_3 (11.42 mg), EDTA (50.0 mg), KOH (31 mg) and $FeSO_4$ $7H_2O$ (4.98 mg) (39). Cultures were incubated on a shaker at 100 rpm, at 30 °C, under 60 µmolPhoton m^{-2} s^{-1} illumination provided by cool white LED lamps. Cultures were performed in biological triplicates.

2 2. Determination of glucose and nitrogen concentration for heterotrophic growth

In order to establish the strain affinity concerning glucose and nitrogen in the culture medium, tests were carried out at 100

rpm, 30 °C, without a light supply. To determine the maximum glucose concentration, B3N medium (containing 6 g L^{-1} sodium nitrate) was used and the amount of glucose was varied (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 g L^{-1}). To determine the noninhibitory nitrogen concentration, B3N medium (with 10 g L^{-1} of glucose) was used and the amount of sodium nitrate added was varied (0, 5, 7.5, 10, 15, and 20 g L^{-1}). Cultures were duplicated biologically.

2 3. Heterotrophic and two-stage culture conditions

Following the substrate affinity test, cultures were led on a maximal noninhibitory medium to increase biomass productivity before inducing the photosynthetic apparatus. For this, 25 mL of B3N culture medium at 4X concentration supplemented with 40 g L^{-1} of glucose was inoculated with 1 mL of *Scenedesmus almeriensis* previously subcultured every week under a heterotrophic regime. Given that the oxygen demand in the heterotrophic regime is high, the culture volume was low to ensure no limitation due to lack of aeration. Therefore, the sampling was limited to a minimal volume in order to measure only the cell concentration. Under these circumstances, the heterotrophic stage was maintained for seven days to guarantee the total consumption of glucose from the medium before starting the photoinduction stage, referred to as Late Photoinduction further on in the text.

Subsequently, the biomass was recovered, washed and resuspended in 50 mL of fresh B3N culture medium, which meant a dilution by half of the final heterotrophic concentration. This dilution was done for four reasons: i) to wash out any remaining glucose in the medium that could inhibit carotenoid synthesis (21); ii) to provide fresh nutrients, mainly nitrogen, to allow the formation of new proteins needed for the photosynthetic complex that hosts the carotenoids; iii) to allow daily sampling and monitoring of the evolution of the biomass and its pigments without exhausting the culture volume; and iv) to enable a higher average light irradiance in the dense culture. Then, the diluted samples were divided into two groups. The first group was exposed to a light intensity of 60 µmolPhoton m^{-2} s^{-1} to induce lutein synthesis and the rest of the culture conditions were maintained in the dark, as the previous heterotrophic culture.

Once the growth kinetics during the heterotrophic phase were determined, a second test was conducted (referred to as Immediate Photoinduction further on in the text). On this test, the photoinduction stage started immediately after the heterotrophic culture entered the stationary phase, only three days after the start of the heterotrophic cultivation. The results section compares the lutein content and productivity of the two tests, and the significance of the cells' metabolic state in responding to photoinduction is discussed. These tests were performed in biological triplicates.

2 4. Microalgal growth assessment

For all the cultures, flasks were sampled twice daily for growth monitoring. Additionally, during the photoinduction stage, the sampling included biomass recovery for pigment profiling. For this, each sample was centrifuged and the pellet was washed with distilled water and centrifuged again. The resulting biomass was stored at -20 °C in dark conditions until processing. The growth of *S. almeriensis* cells were tracked by measuring the absorption of samples at 750 nm using a UV/Visible spectrophotometer (Shimadzu UV-1800 spectrophotometer)(40). Samples were diluted to an optical density of 0.4 or less before recording the value. To determine the dry weight of the biomass, absorption values were recorded at various biomass concentrations, which were subsequently filtered and dried for 24 h at 100 °C and a calibration curve was developed linking absorbance to dry weight (10 points, ranging from 0.03 to 0.7 g L^{-1}, R^2=0.995). This curve yielded the equation (Eq. 1):

$$DW = 0.796 \times A_{750nm} - 0.0247 \quad \textbf{(1)}$$

where DW represents the dry weight biomass concentration in grams per liter (g L^{-1}), and A represents the total absorbance measured at 750 nm, using distilled water as blank.

The growth rate of the cultures under different glucose and sodium nitrate concentration were calculated in the exponential growth phase using the classical equation (Eq. 2):

$$\mu = \frac{ln(C_{t_2}) - ln(C_{t_1})}{t_2 - t_1} \quad \textbf{(2)}$$

2 5. Extraction of carotenoids from microalgae biomass

Lutein was extracted from biomass by adapting the method described by (41). In general, a wet pellet of known biomass (4-8 mgDW) was mixed with 1 mL of laboratory-grade inert sand (Fisher Scientific Code: 10132590) and 10 mL of 100% ethanol. Cell disruption was carried out in a high-speed benchtop homogenizer (MP Biomedicals FastPrep-24 5G Instrument, Fisherbrand, Waltham, MA, USA) at 6.5 m s^{-1} in two cycles of 30-second with a 60-second pause. The samples were then allowed to rest at room temperature in a rotator (Stuart Rotator SB3) at 10 rpm for 60 minutes. Finally, the samples were filtered through a 0.22 µm pore filter to separate particles before passing them through a High-Pressure Liquid Chromatograph (HPLC). All this protocol was conducted with light protection around the samples.

2 6. Pigment quantification

Pigments were quantified on an Ultima 3000 HPLC (Thermo Fisher Scientific) coupled with a UV detector. Separation was achieved on an Acclaim Polar Advantage II C18 column (4.6 x 150 mm, 3 µm, 120 Å) from Thermo Fisher Scientific. The column temperature was maintained at 30 °C. Pure methanol was the mobile phase. The flow rate was 0.5 mL min^{-1}, and the elution was set in isocratic mode. The injection volume was 5 µL, and the total run analysis was 40 min. Compounds were identified by comparing their retention time and UV-Vis spectra with standard solutions. UV-Vis spectra were recorded from 200 nm to 700 nm. Absorbance was recorded at 400, 450, 500, and 650 nm. Pigment quantifications were led using the area of the peaks in external

calibration for the most sensible of the recorded wavelength. External calibration concentrations ranged from 0.25 to 5 mg L^{-1}. Pigment standards and methanol were purchased from Sigma-Aldrich. Standards had a purity greater than 97%. The three pigments of interest (lutein, violaxanthin, and zeaxanthin) were reported systematically for each sample. "N.A." was used whenever a sample could not be detected or quantified.

2 7. Lipid, protein and carbohydrate quantification

Lipids were quantified gravimetrically. First, 100 mg of freeze-dried microalgae powder was resuspended in 10 ml of milliQ. Then, cells were homogenized using MP Biomedicals FastPrep42 bead miller. Lipids were extracted from lyzed cells following Bligh and Dyer protocol (42). The chloroform phase containing the lipids was then left to evaporate, and the solid residues were weighed.

For determination of the cells' protein content, 1.95 mg of freeze-dried microalgae was resuspended into 20 ml of water which was then analyzed by TOC-L CSH analyzer (Shimadzu) for total nitrogen quantification. Protein content was calculated from total nitrogen content using a correction factor derived for Chlorella sp., yielding the following equation (43):

$$Protein_{mg/l} = 4.78 \times TN_{mg/l} \quad \textbf{(3)}$$

5 mg of freeze-dried cells were homogenized before total carbohydrates determination using the anthrone blue method (44) (calibration curve realized twice at 630 nm using glucose, linearity range 0.05 g/L to 0.5 g/L, 5 points, $R^2 = 0.999$, with control to nullify potential chlorophyll contribution at 630 nm).

2 8. Statistical analysis

Statistical significance was assessed using the ANOVA test. When the null hypothesis was rejected ($p < 0.05$), data were further analyzed using Tukey's Honestly Significant Difference (HSD) test. The following results are presented as the average of the replicate (n = 3 unless stated otherwise), while the error bars account for the standard deviation.

3. Results

This study aimed to maximize lutein productivity from *S. almeriensis* cultured under heterotrophic conditions, compared to phototrophic conditions. This work generated data on cell density and the quantity of lutein, violaxanthin and zeaxanthin. The resulting data enabled a comparison of the carotenoid productivity between the two cultivation modes based on the difference in produced biomass. The subsequent sections describe the findings and discuss their implications for lutein production from *S. almeriensis*.

3 1. Glucose and nitrogen affinity and inhibition

Two separate tests were conducted independently to determine the carbon and nitrogen concentration to which the microalga *Scenedesmus almeriensis* is best adapted to grow and produce lutein. These experiments were conducted to determine the ideal concentration of glucose (on B3N medium supplemented with 6 g L^{-1} of sodium nitrate) and sodium nitrate (on B3N medium supplemented with 10 g L^{-1} of glucose). Figure 1a shows the growth rate of cultures at glucose concentrations ranging from 10 to 100 g L^{-1} in increments of 10 g L^{-1}. The results show that concentrations from 10 to 50 g L^{-1} belong to the same statistical group ($p < 0.05$), between 1.17 ± 0.02 and 1.42 ± 0.03 day^{-1} with a probably marginal inclusion of the culture under 50 g L^{-1} as discussed below. Concentrations higher than 50 g L^{-1} of glucose were detrimental to the growth rate, most probably due to an inhibitory effect of the substrate.

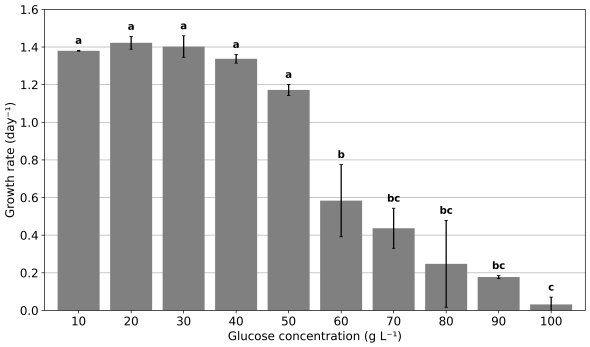
Since the objective was to obtain the highest biomass concentration, the 40 g L^{-1} glucose concentration was chosen, as the cultures at this concentration gave a similar growth rate compared to lower concentrations. Higher amounts of glucose showed increasing inhibition as the concentration was increased. Additionally, we can see in Figure 1b that after exponential growth started and up to day 4 of the culture, cultures with glucose concentrations from 10 to 40 g L^{-1} had similar biomass concentrations ($p = 0.440$). On the contrary, cultivation with 50 g L^{-1} of glucose exhibited a much shallower onset of the exponential phase, indicating that the substrate concentration is inhibiting faster growth. Ultimately, at higher concentrations, growth is not even observed within the initial 4-day period. Furthermore, Figure 1c shows the linear growth, where the trend concerning the glucose concentration is confirmed.

Considering that an increase in growth is expected due to glucose in the medium, an accelerated rate of nitrogen consumption is also anticipated, necessitating the determination of sodium nitrate concentration to prevent potential limitations or inhibitions. To achieve this, a parallel test was conducted with cultures on B3N medium with 10 g L^{-1} of glucose and the sodium nitrate content was varied at 0.75, 5, 7.5, 10, 15 and 20 g L^{-1}. Figure 2 shows the evolution of *S. almeriensis* growth rate with increased sodium nitrate concentrations. Also, one can see a reduction in growth rate at sodium nitrate concentrations above 7.5 g L^{-1}, so a concentration of 3 g L^{-1} was determined for subsequent experiments to support rapid biomass development.

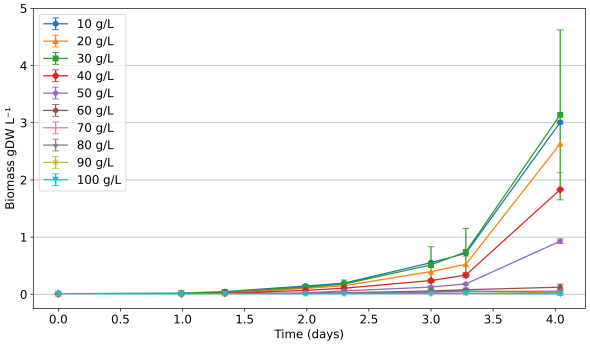
The combined effect of the glucose concentration at 40 g L^{-1} and sodium nitrate at 3 g L^{-1} was tested to confirm no adverse interaction between the two concentrations. The result showed an even higher growth rate (1.45 ± 0.011 day^{-1}) than the individual glucose and sodium nitrate tests.

3 2. Comparison between phototrophy and heterotrophy

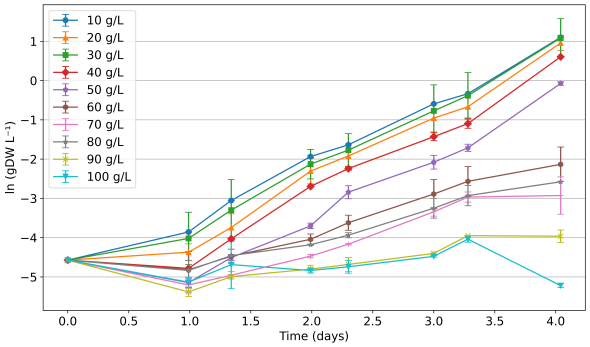
In addition to comparing biotechnological performance, this study also offered the opportunity to compare differences in *S. almeriensis* morphology induced by the different modes of cultivation. Figure 3 presents pictures of *Scenedesmus almeriensis* cells in cultures under phototrophy and heterotrophy conditions during the late exponential phase of each culture. As one can see, cells cultured with light (60 µmolPhoton



(a)



(b)



(c)

Fig. 1. Effect of different glucose concentrations on *S. almeriensis* using 6 g L^{-1} of sodium nitrate: (a) Growth rate (Different letters indicate differences between growth rates, p < 0.05), (b) Growth curves, and (c) Natural logarithm-transformed biomass concentration. Points are the average of duplicates.

m^{-2} s^{-1}) and under nutrient sufficiency exhibited the classical oval shape with pointed tips of this species as well as coenobia made of 2 and 4 cells, as also described by (35) and (38).

On the contrary, cells cultured under the heterotrophic condition with 10 g L^{-1} of glucose displayed a much rounder shape, with smoother tips and can only be found as single floating cells. The latter are also generally larger in volume compared with the cells grown under light. These characteristics are consistent with cell changes under heterotrophic cultures of species in the *Scenedesmaceae* family, like *Scenedesmus obliquus* ABC-009 (45), *Scenedesmus acuminatus* (46) and *Scenedesmus* sp. (47).

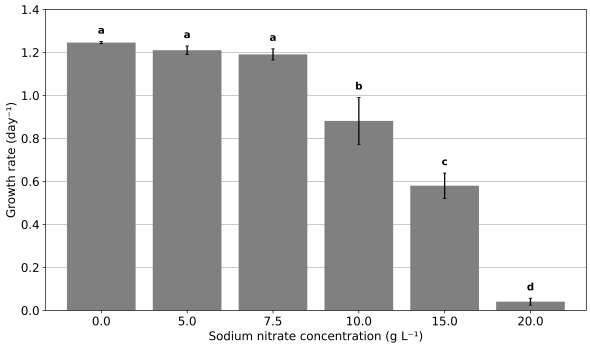


Fig. 2. Growth rate of *S. almeriensis* under different sodium nitrate concentrations. The concentration shown indicates the amount of sodium nitrate that was added to the regular B3N medium. Initial glucose concentration: 10g L^{-1}. Different letters indicate differences between growth rates, p < 0.05. n = 2

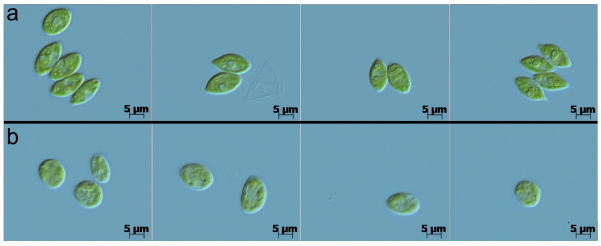


Fig. 3. *S. almeriensis* cells cultured under phototrophic (**a**) and heterotrophic (**b**) conditions. Pictures were taken from samples at the exponential growth phase of each cultivation mode. Cells growing in phototrophic culture were at 30 °C with 60 µmolPhoton m^{-2} s^{-1} of light intensity on B3N medium and at 100 rpm. Cells in heterotrophic culture were in the same conditions except that they had no light and the medium contained 10 g L^{-1} glucose. Both cultures had nutrient availability at the time of sampling.

3 3. Growth dynamic and lutein content

3.3.1. Phototrophic and heterotrophic comparison. Figure 4 shows the difference in cell concentration over time between cultures on phototrophic, heterotrophic and heterotrophic+photoinduction conditions. The culture on phototrophy reached a maximum biomass content of 1.47±0.18 g L^{-1} on day eleven after showing linear growth. In comparison, the heterotrophic culture had an accelerated growth during the first two days. On the third day, it reached the stationary phase with 22±0.8 g L^{-1} of biomass, a 15-fold increase in biomass content compared to day eleven on phototrophy mode. On the other hand, it can be observed that, after dilution of the heterotrophic culture by half with fresh B3N medium, the culture that was kept under dark conditions as a control did not show any growth (p=0.8783), which was to be expected, as it lacked energy source (neither glucose nor light). However, the culture that was transferred to light conditions to induce lutein synthesis was able to benefit from the low light intensity of 60 µmolPhoton m^{-2} s^{-1} to stimulate the growth of the culture (p=0.0009).

The carotenoid content of the cultures grown under phototrophic and heterotrophic conditions are presented in Figure 5. The phototrophically grown cells exhibited maximal lutein and violaxanthin contents after 11 days of culture, with values of 2.65±0.29 and 0.42±0.06 mg gDW^{-1}, respectively. Comparatively, cells cultured under heterotrophic conditions presented 1.43±0.04 and 0.3±0.03 mg gDW^{-1}

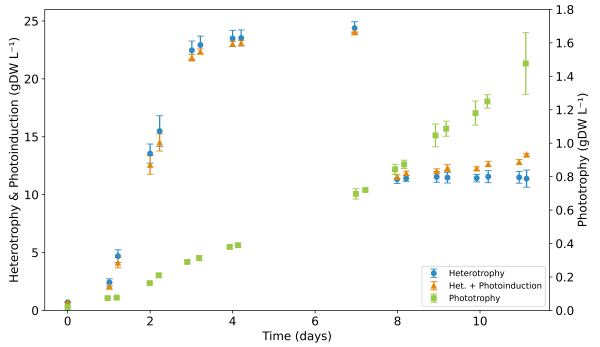


Fig. 4. Time course profile of *S. almeriensis* biomass concentration under different cultivation modes (n = 3).

of lutein and violaxanthin, respectively, at the end of the exponential growth phase (day 3). On the other hand, zeaxanthin content was the other way around, with a cell content 1.8 times higher in the heterotrophic culture (0.43±0.09 mg gDW^{-1} on heterotrophy and 0.25±0.005 mg gDW^{-1} on phototrophy, p = 0.006).

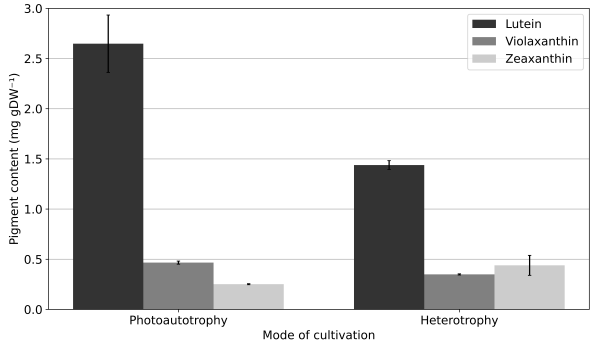


Fig. 5. Lutein, violaxanthin and zeaxanthin content of *S. almeriensis* under different culture regimes. For the phototrophic mode, the highest content was at day 11. For the heterotrophy mode, values were taken on day 3, corresponding to the end of the exponential phase.

3.3.2. Late and immediate photoinduction comparison. As explained before, a first photoinduction test was made starting the light treatment four days after the heterotrophic culture reached the stationary phase (Late Photoinduction). This delay was born of the conjunction of the will to ensure that glucose had been depleted and staff availability. In this first test, the lutein content reached a maximum value of 1.54±0.13 mg gDW^{-1} after 1.2 days of photoinduction, but 5.2 days after the heterotrophic culture reached the stationary phase (Figure 6). Despite the increase in lutein content due to photoinduction, the overall productivity is very low due to this long delay. Therefore, a second test was carried out to initiate photoinduction immediately after the heterotrophic culture reached the stationary phase (Immediate Photoinduction). On this second test, the lutein content reached a maximum of 1.62±0.13 mg gDW^{-1} after 10 hours of light treatment, an increase of 13.28% compared to the end of the heterotrophic culture.

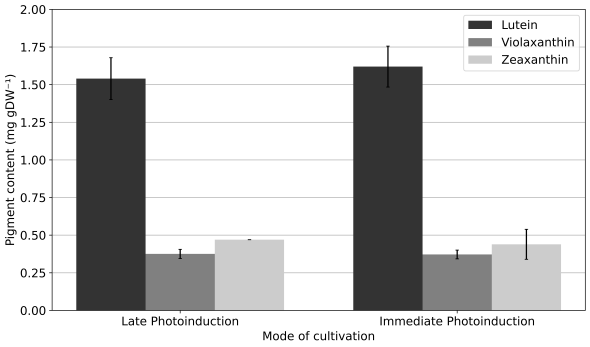


Fig. 6. Lutein, violaxanthin and zeaxanthin content of *S. almeriensis*. In the first test (Late Photoinduction), the photoinduction started four days after the heterotrophic culture reached the stationary phase. For the second test (Immediate Photoinduction), the photoinduction started immediately after the heterotrophic stationary phase. Carotenoid values are taken on day 8.2 for the Late Photoinduction and on day 3.3 for the Immediate Photoinduction, corresponding to the maximum content after each photoinduction started.

3 4. Lutein productivity

Despite the phototrophic culture having higher lutein content, the increased biomass productivity achieved through heterotrophic growth mode led to an equivalent increase in lutein productivity, both in heterotrophic and photoinduced cultures (Figure 7). Maximum lutein productivity under phototrophic culture was reached on day 11, with 0.34±0.04 mg L^{-1} d^{-1}. In turn, lutein productivity at day 7 of the heterotrophic culture (Long Heterotrophy) was 3.43±0.3 mg L^{-1} $d^{-}1$. The resulting biomass, diluted by half with fresh B3N medium and subjected to 60 µmolPhoton m^{-2} s^{-1} of light, showed a productivity of 2.09±0.15 mg L^{-1} d^{-1} after 1.2 days of light (Late Photoinduction).

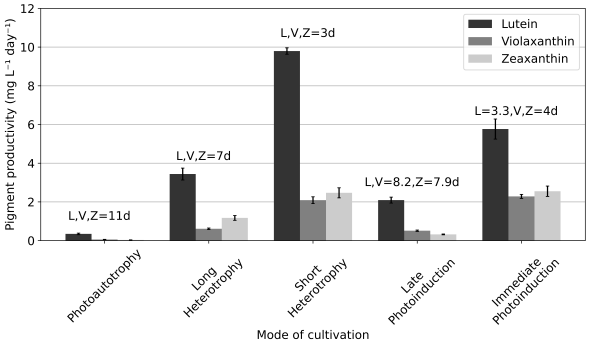


Fig. 7. Productivity content of *S. almeriensis* cultured under different cultivation modes. The culture duration (days) at which the maximal productivity was obtained is indicated for each pigment above every mode of cultivation (Lutein = L; Violaxanthin = V; Zeaxanthin = Z).

As stated earlier, the Late Photoinduction stage began four days after reaching the stationary phase of the heterotrophic culture. This long heterotrophic delay caused the calculated productivity to fall short of expectations, as several days went by that could have been avoided. Subsequently, for the Immediate Photoinduction test, the heterotrophic stage was stopped on day three after reaching lutein productivity of 9.79±0.17 mg L^{-1} d^{-1} (Short Heterotrophy), while the lutein productivity after dilution and 10 hours of photoinduction reached 5.76±0.51 mg L^{-1} d^{-1}.

4. Discussion

Different species of the genus *Scenedesmus* have been grown in a heterotrophic manner and have shown higher final biomass concentration than those obtained in illuminated cultures (47–49). However, this is the first report of *Scenedesmus almeriensis* grown without a light source. Therefore, the ideal glucose level had to be determined to take advantage of the glucose utilization capacity of this species without exceeding the limits that would induce growth inhibition.

In preliminary tests, *S. almeriensis* under a heterotrophic regime showed a biomass yield of 0.5 gDW per 1 g of glucose feed. This value is quite classical for microbial growth and demonstrates that *Scenedesmus almeriensis* grows well with glucose (50). These tests were carried out under 10 g L^{-1} of glucose. However, it was imperative to investigate the maximal glucose concentration within the growth medium to achieve maximal biomass productivity within a particular culture system. At the levels defined as optimal (40 g L^{-1} of glucose and 3 g L^{-1} of sodium nitrate), cells could fully utilize the organic substrate and grow at a growth rate of 1.45±0.011 day^{-1} while avoiding inhibitory effects. This value is twice as high than the one reported by (21) (0.74 day^{-1}) with *Scenedesmus incrassatulus* on heterotrophic conditions using 30 g L^{-1} of glucose as the carbon source. On the other hand, the defined concentration gives a C:N ratio of 32, which is higher than other reported studies. (46) tested different C:N ratios from 4 to 32 and reported the highest growth rate (1.03 day^{-1}) and maximum biomass concentration (220 g L^{-1}) using a rate of 12 on a culture of *Scenedesmus acuminatus*, however, they used urea as the nitrogen source, which is metabolized by microalgae in a faster way than sodium nitrate (51). As for the biomass production per unit of substrate (glucose) in other microalgae species, it is similar to the 0.5 yield found in this work. (21) reported a yield of 0.59 in *S. incrassatulus*, while (45) report 0.55 for *S. obliquus*. Similar observations have been made in other studies using different carbon sources ((46, 50, 52).

Given that the synthesis pathway for lutein in microalgae is closely linked to photoautotrophic biomass production (27), light appears essential to promote high lutein expression. However, low biomass production results from mutual shading between cells under photosynthetic growth, reducing lutein productivity. The biomass content obtained under phototrophic conditions is consistent with other reports for *Scenedesmus* species on phototrophic conditions on conventional culture systems (45, 47). Nevertheless, the extent of this statement is to be modulated, since short light path photobioreactor can also be a means to achieve dense culture (for example, 20 g L^{-1} in 7-8 days with *Chlorella* genus (53)) at the price of high areal requirement. On the other hand, the high biomass production of 22 g L^{-1} in the heterotrophic culture corresponds to an efficient utilization and conversion of substrate into biomass.

The growth that can be observed in the culture that was transferred to light conditions after having grown in heterotrophy is possibly due to the rapid adaptation to this new source of energy despite the low light intensity of 60 µmolPhoton m^{-2} s^{-1}. This light intensity was selected since, at low intensity, photosynthetic cells attempt to capture more light, developing their photosynthetic system, including the content of carotenoids that contribute to collecting more light. Additionally, it demonstrates that the photosynthetic system can be reactivated once the energy and carbon source changes. This has been previously reported by (47) in a comparison between phototrophic, heterotrophic and mixotrophic culture of *Scenedesmus* sp.

Although heterotrophic cultivation may suggest that photosynthetic pigments are unnecessary, studies have shown that some microalgae strains produce a certain amount of pigments, even under dark conditions. However, it should be noted that phototrophically grown microalgae typically exhibit substantially higher levels of carotenoid content. (22) reported an almost 5-fold lower amount of lutein in *Auxenochlorella protothecoides* in mixotrophic and heterotrophic cultures compared to a phototrophic culture, arguing that the presence of glucose in the medium may have had inhibitory effects on lutein synthesis. In a similar way, (45) reported almost three times more lutein in a *Scenedesmus obliquus* culture grown in phototrophy compared with heterotrophically grown cells. In this regard, the heterotrophic culture was expected to have a lower value than the phototrophic culture. Maximum lutein and violaxanthin content in the phototrophic culture is 2.2 and 1.5 times higher than in the heterotrophic culture ($p = 0.001$ for lutein and $p = 0.058$ for violaxanthin). This has been previously reported by (22), who demonstrated that the major enzyme encoding genes for carotenoid synthesis are downregulated during heterotrophic culture of *Auxenochlorella protothecoides*. On the other hand, zeaxanthin's higher values on the heterotrophic culture may be due to an antioxidant requirement, supplemented in the first instance by this carotenoid. Previous studies on *Chromochloris zofingiensis* showed that zeaxanthin could be overexpressed in response to stress when grown heterotrophically by (54), who reported an increase of 44 % in zeaxanthin after a stress stage using 10 mg L^{-1} of gibberellin acid-3 with high C/N ratio and NaCl concentration. These observations may be related to reports suggesting that an increased mitochondrial metabolic activity in yeast (55) and animal cells (56) gives rise to ROS generation, subsequently leading to enhanced antioxidant synthesis. In microalgae, mitochondria and chloroplasts are the main ROS generators (57). However, in the case of heterotrophic culture supplemented with exogenous glucose, it is possible that the increased metabolic activity of mitochondria leads to an overproduction of ROS, which will stimulate the onset of zeaxanthin synthesis in response to oxidative stress.

Although the yield of lutein in relation to the amount of glucose is not commonly reported, it is interesting to note that 7 mg of lutein per gram of glucose was obtained in the present work. Meanwhile, (21) and (45) only obtained 1mg of lutein per gram of glucose under similar conditions using *S. incrassatulus* and *S. obliquus*, respectively. These differences may be due to particular strategies of each species

to cope with culture under dark conditions by modifying the metabolic pathways for carotenoid synthesis. However, it is an aspect that requires further study to understand the underlying mechanism.

Since lutein content is lower in cells grown in darkness due to the reduction of photosynthetic complexes (24), two-stage culture strategies have been proposed in other *Scenedesmus* species (21, 45). This is also true for astaxanthin and β-carotene production in other microalgae species; however, the underlying mechanism differs from that of lutein synthesis and accumulation. Both cases aim to increase pigment productivity through two steps (24, 58, 59). In the first step, optimal culture conditions for biomass generation are favored, followed by a second stage that induces pigment synthesis and accumulation, resulting in high productivity. Nevertheless, in the case of astaxanthin and β-carotene, the second stage consists of stressing the cells to induce the synthesis of these carotenoids. In contrast, for lutein, the second stage consists of reactivating the photosynthetic capacity of the cells through the application of light. In this study, the 13.28 % increase in lutein content after 10 hours of photoinduction is an essential step toward increasing the overall lutein productivity. A similar approach is reported by (21), who increased total carotenoids by 40 % after 24 hours of photoinduction of a *Scenedesmus incrassatulus* heterotrophic culture.

From an industrial point of view, lutein content per unit of biomass is not the only indicator to evaluate the viability of a project. Although the lutein content must be taken into account to determine the energy input of the extraction process, overall lutein productivity is a critical value that includes time, which is fundamental in a feasibility analysis. Therefore, although the lutein content on the phototrophic culture was higher, the increased biomass productivity obtained under the heterotrophic mode resulted in an equivalent increase in lutein productivity. In this sense, the two schemes presented for high biomass production (Long and Short Heterotrophy) and the subsequent photoinduction (Late and Immediate Photoinduction) offered higher lutein productivity than those obtained under phototrophic conditions. The accumulation of biomass and lutein during three days in heterotrophy yielded a productivity 28 times higher than that obtained in phototrophy.

Comparing the two photoinduction strategies, there is an increase of 175.5 % in the Short Photoinduction compared to the Late Photoinduction. This increase in productivity, apart from the delay preceding the photoinduction, may be due to the fact that the metabolic state of *S. almeriensis* cells are more favorable to lutein synthesis when the light phase is induced immediately after the end of the heterotrophic growth phase. On the other hand, since the biomass obtained from the heterotrophic culture was diluted for the photoinduction stage, the productivity calculation only considers half of the biomass produced. Therefore, the total productivity could be considered 11.68 mg L^{-1} d^{-1} in relation to the initial biomass per liter. The productivity values (9.79 and 11.68 mg L^{-1} d^{-1}) are higher than other reports on the *Scenedesmus* genus and is comparable to results from *Chlorella* strains (see Table 1).

Moreover, it is essential to highlight that *Scenedesmus almeriensis* exhibits resilience to elevated irradiance and temperature levels and is characterized by weaker cell walls and larger cell sizes, which not only facilitate the harvesting process but also simplify the pigment extraction. These characteristics make this species an even more appealing choice for industrial applications. Additionally, the high cell productivity represents not only a way to increase lutein productivity but also delivers enough biomass to envision the valorization of the other fractions. The analysis of *S. almeriensis* biomass obtained from a heterotrophic culture has revealed a rich composition, showcasing its viability within the biorefinery paradigm. The combined constituents of carbohydrates (30 %), proteins (47 %), and lipids (17 %) offer a myriad of opportunities for extraction, processing, and utilization beyond a singular component such as lutein. This comprehensive composition does not differ much from that obtained by (35) in a phototrophic culture of this same species but reinforces the feasibility of biorefinery utilization and highlights the versatility and richness of microalgae biomass as a valuable resource in various industries and applications.

5. Conclusions

Our results demonstrate that culturing *Scenedesmus almeriensis* under heterotrophic conditions can lead to high biomass concentrations and increased biomass productivity. Although the lutein content in the phototrophic culture was higher, during the heterotrophic conditions the pigment content was not negligible and resulted in a higher lutein productivity than in the phototrophic culture due to the rapid increase in biomass content. Additionally, even though the photoinduction stage increased lutein productivity, the growth is minimal and further techno-economical analysis is needed to justify this phase. Future research should focus on optimizing the cultivation process under heterotrophic conditions to enhance lutein production by increasing biomass concentrations. Exploring novel nutrient supplementation strategies and fine-tuning light intensity during the photoinduction stage could potentially lead to even greater lutein yields. A comprehensive exploration of these avenues, coupled with rigorous techno-economic analyses, will be crucial in unlocking the full potential of *Scenedesmus almeriensis* as a valuable source of lutein.

Declarations

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Algal species	Cultivation condition	Max. biomass concentration (g L^{-1})	Max. lutein content (mg gDW^{-1})	Max. lutein productivity (mg L^{-1} d^{-1})	Ref.
Auxenochlorella protothecoides	Heterotrophic + photoinduction	6.9	4.9	12.36	(22)
Chlorella minutissima	Phototrophic		8	5.35	(60)
Chlorella prototecoides	Heterotrophic	20	9.1	10.57	(61)
Chlorella sorokiniana	Mixotrophic-phototrophic	6	11.2	8.25	(62)
Desmodesmus sp.	Phototrophic	3.9	5.05	3.56	(63)
Scenedesmus obliquus	Phototrophic	3.38	4.52	4.15	(64)
Scenedesmus obliquus	Phototrophic	2	3.5	0.8	(45)
Scenedesmus almeriensis	Phototrophic		5.4	4.77	(27)
Scenedesmus incrassatulus	Heterotrophic + photoinduction	5.4	1.4	3.1	(21)
Scenedesmus obliquus	Mixotrophic	12.8	1	4.96	(65)
Scenedesmus sp.	Phototrophic	2.8	6.4	2.3	(66)
Scenedesmus almeriensis	Heterotrophic + photoinduction	22	1.62	11.68	This study

Table 1. Overview of maximum biomass concentration, lutein content and lutein productivity for different microalgae species under phototrophic, heterotrophic and photoinduced cultures

Authors' contribution

All the authors designed and initiated the study. TJ and CCB led the experimental work. All the authors led the data analysis. CCB drafted the manuscript. TJ and VP corrected it. All authors approved the manuscript.

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Availability of data

The data that supports the findings of this study are available upon reasonable request from the corresponding author.

Conflict of interest

The authors declare that they have no conflicts of interest.

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