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RESEARCH

A two-stage cultivation strategy for the industrial production of the novel microalga *Chlorococcum amblystomatis*

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Abstract

This study presented the industrial-scale validation of a two-stage growth strategy for a poorly explored yet industrially relevant chlorophyte microalga, *Chlorococcum amblystomatis*, addressing the gap between laboratory research and practical application. By using cultures grown heterotrophically as inoculum for industrial-scale photoautotrophic reactors, this approach significantly accelerated scale-up. The proposed strategy reduced the overall scale-up time by approximately 5.5-fold compared to the conventional pipeline, without compromising productivity or biochemical quality. A maximum biomass concentration of 70.96 ± 0.35 g L^{-1} was achieved in 7-L benchtop fermenters under heterotrophic conditions, representing the highest value reported to date for this microalga. The biomass obtained presented a protein content of $32.42 \pm 2.30\%$ of dry weight, which increased to $54.55 \pm 2.04\%$ upon cultivation under photoautotrophic conditions, as well as polyunsaturated fatty acids, which increased from 50.12 ± 3.27 to $67.74 \pm 0.35\%$ of total fatty acids, with α-linolenic acid as the predominant fatty acid. Conversely, the saturated fatty acids content ($38.26 \pm 1.63\%$ of TFA) decreased to $22.37 \pm 0.02\%$ of TFA in the phototrophic stage. Pigment contents also increased significantly during the phototrophic stage, with chlorophylls rising from 10.04 ± 1.68 to 44.91 ± 5.11 mg g^{-1} and carotenoids reaching notable concentrations, including lutein (5.34 ± 0.10 mg g^{-1}), β-carotene (5.92 ± 0.39 mg g^{-1}), and neoxanthin (3.53 ± 0.75 mg g^{-1}). Among the industrial photoautotrophic systems tested, the closed tubular photobioreactors were the most suitable configuration for *C. amblystomatis* cultivation, achieving higher biomass productivity, enhanced protein content and pigment accumulation compared to open raceway ponds. Overall, this work demonstrates a scalable, efficient, and time-saving cultivation strategy for *C. amblystomatis*, supporting its potential for industrial biotechnological applications.

Keywords *Chlorococcum amblystomatis* · *Oophila* · Chlorophyceae · Two-stage cultivation · Industrial production · Heterotrophy · Photoautotrophy · Biomass characterization

Introduction

The rising global demand for food, feed, and biomaterials, driven by rapid population growth, is intensifying pressure on natural resources. Environmental issues such as water scarcity and climate change are closely linked to urbanization and conventional agriculture, which require extensive arable land and large freshwater inputs (Barrosde Medeiros et al. 2021; Vieira de Mendonça et al. 2021). Microalgal biomass offers an alternative source of proteins, essential fatty acids, and natural pigments for food and feed, providing a sustainable route to mitigate several of these challenges (Yu et al. 2024). These compounds have potential applications in diverse biotechnological fields aimed at developing

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sustainable solutions for current global challenges (Pandey et al. 2019; Hosseini et al. 2022).

Microalgae can be cultivated in industrial bioreactors, avoiding competition with conventional agriculture for freshwater resources and arable land. They also enable continuous or frequent harvesting and provide stable, predictable productivity, particularly when cultivated under controlled indoor conditions (Rumin et al. 2021). Their ability to grow in controlled reactors with a low areal footprint makes them attractive for different biotechnological applications (Barros de Medeiros et al. 2021; Rumin et al. 2021). Industrial-scale cultivation of microalgae is a promising area of research and development with applications across multiple sectors, including pharmaceutical, nutraceutical, bioremediation, agriculture and food and feed industries (García et al. 2017). However, large-scale microalgal production remains constrained by high operational costs, and the scale-up from laboratory cultures to industrial photobioreactors continues to be a laborious and time-consuming process that requires further technological optimisation (Barros et al. 2019).

The conventional photoautotrophic scale-up of microalgal cultures from laboratory to industrial photobioreactors remains challenging, as it is time-consuming, resource-intensive, and requires large production areas (Han et al. 2012; Barros et al. 2019). Furthermore, photoautotrophic growth is inherently dependent on weather conditions, namely light availability and temperature, imposing additional constraints on productivity and operational efficiency. Some microalgal species, however, can grow heterotrophically, using organic carbon sources to replace light-driven photosynthesis for energy and biomass production (Hu et al. 2018; Daneshvar et al. 2021). Heterotrophic cultivation offers several advantages comparing to the autotrophic process, including precise control and product consistency, reduced areal footprint (Smetana et al. 2017; Ruiz et al. 2022; Diaz et al. 2023), and the attainment of high cell densities, which can substantially reduce harvesting and downstream costs (Hu et al. 2018; Ruiz et al. 2022). Nevertheless, photoautotrophic cultivation remains attractive for enhancing the content of target bioactive compounds in microalgal biomass, namely proteins and photosynthetic pigments such as chlorophyll (Barros et al. 2019). Tubular photobioreactors (PBRs), as closed systems, require less land area, allow tighter control of culture parameters, minimise water evaporation, and reduce contamination risk, often resulting in higher productivities (Yen et al. 2019). In contrast, open ponds raceways (RWPs) are more susceptible to light limitation, temperature fluctuations, and contamination, but their lower construction and operational costs make them a cost-effective option for large-scale biomass production (Yen et al. 2019; Ruiz et al. 2022).

Additionally, improvements in microalgal production enhance resource efficiency, reduce environmental impacts, and promote process circularity, reinforcing the sustainability of microalgal bioprocesses (UNDP 2015).

Chlorococcum amblystomatis[1] is a freshwater microalgal species of particular interest due to its growth under both photo- and heterotrophic conditions (Beevi and Sukumaran 2014; Correia et al. 2023) and its nutritional composition which highlights its potential as an alternative nutrient source to meet the growing global demand for food and feed. Despite its limited exploration in the literature, this strain has demonstrated performance comparable to *Chlorella* spp., one of the most widely used industrial microalgal genera, while exhibiting advantageous biochemical features, including higher protein content, a favourable fatty acid profile, and carotenoid levels comparable to established producers (Conde et al. 2021; Correia et al. 2023), thereby reinforcing the importance of microalgal prospection as a critical step in identifying new strains with industrial potential (Chiriví-Salomón et al. 2024; Tirado et al. 2025).

Although advances in microalgal biotechnology, a persistent gap remains between laboratory- and pilot-scale studies and industrial application, as productivity and process stability often decrease during scale-up due to operational constraints and environmental variability (Guieysse and Plouviez 2024). This highlights the importance of the present study towards industrial validation and properly assesses real-world performance. In this context, *C. amblystomatis* cultures grown heterotrophically were used to inoculate industrial-scale photoautotrophic reactors, effectively combining the benefits of both trophic modes (Han et al. 2012; Barros et al. 2019). The integrated method offers several benefits. Firstly, it significantly reduces scale-up time and inherent costs by enabling the inoculation of multiple large-scale photobioreactors using smaller-scale reactors, i.e., laboratory-scale reactors, while maximising biomass productivity (Barros et al. 2019). Secondly, in the heterotrophic phase, the cultures are kept under axenic conditions, reducing contamination risks in the photoautotrophic phase and concomitantly increasing the accumulation of target compounds and improving biomass quality (Lu et al. 2024). This integrated approach thereby combines the fast biomass production through heterotrophic growth, with the enhanced biochemical profile provided by the photoautotrophic stage. The use of heterotrophically grown cells as inoculum for photoautotrophic cultivation has been emerged as an effective strategy to shorten seed culture time and ensure a reliable supply of biomass (Han et al. 2012; Barros et al. 2019; Ruiz et al. 2022).

In this study, *C. amblystomatis* was first cultivated under heterotrophic conditions and subsequently used as a

[1] *Chlorococcum amblystomatis* (F.D.Lambert ex N.Wille) N.Correia, J.Varela & Leonel Pereira has recently been renamed as *Oophila amblystomatis* F.D.Lambert ex N.Wille (Bishop and Garbary 2024).

concentrated inoculum for photoautotrophic industrial photobioreactors, aiming to enhance the biochemical quality of the resulting biomass. This approach established a two-stage cultivation strategy capable of accelerating industrial-scale microalgal production while improving biomass quality, with particular emphasis on increasing protein and pigment contents.

Materials and methods

Microalgal strain

The axenic culture of *Chlorococcum amblystomatis*, previously isolated in Pataias, Portugal, was retrieved from the Allmicroalgae culture collection (strain code AGF0066CA). Seed cultures were prepared from cryopreserved stocks stored in liquid nitrogen. A single cryovial was used to initiate the scale-up process.

Comparison of inocula obtained under different metabolic regimes

Inoculum of *C. amblystomatis*, grown under heterotrophic conditions, in 250-mL Erlenmeyer flasks, at 28 °C, in an orbital shaker (Agitorb 200IC, Norconcessus, Portugal) in the dark at 200 rpm, was used to seed 1-L bubble column reactors (5.6 cm inner diameter, height of 35 cm at a volume of 1 L), made of borosilicate glass (Normax, Portugal), with a working volume of 850 mL. Similarly, inoculum of the same strain, cultivated under autotrophic conditions, in 250-mL bubble column reactors (Normax), under continuous aeration (0.2-µm filtered air), at 23 ± 1 °C, under continuous LED irradiation (100 µmol photons m^{-2} s^{-1}), was also used to inoculate the 1-L bubble column reactors, to compare the autotrophic growth of *C. amblystomatis* with inocula obtained under different metabolic regimes.

Media optimised for this strain, both heterotrophic and autotrophic, previously described in Correia et al. (2023), were used in this trial. Briefly, the heterotrophic medium consisted of: 60 mM of $(NH_2)_2CO$; 10 mM of $NaH_2PO_4{\cdot}H_2O + K_2HPO_4$; 3 mM of $CaCl_2{\cdot}2H_2O$; 5.50 mM of $MgSO_4{\cdot}7H_2O$; 0.30 mM of $FeSO_4{\cdot}7H_2O$; 1.50 mM of $ZnSO_4{\cdot}7H_2O$; 0.02 mM of $Cl_2Co{\cdot}6H_2O$; 0.05 mM of $Na_2MoO_4{\cdot}2H_2O$; 0.85 mM of $MnSO_4{\cdot}H_2O$; 0.005 mM of $CuSO_4{\cdot}5H_2O$; 1 mM of H_3BO_3; 0.01 mM of $NiCl_2{\cdot}6H_2O$, glucose as the organic carbon source (initial concentration of 30 g L^{-1}), and 1 dose of heterotrophic vitamins mix (Thiamine-HCl (1.0 g L^{-1}), d-Biotin (0.015 g L^{-1}), cyanocobalamin (B12) (0.012 g L^{-1}), calcium pantothenate (0.030 g L^{-1}) and PABA (p-aminobenzoic acid) (0.060 g L^{-1}). Dose: 1.50 mL L^{-1}). While the autotrophic medium consisted of: 12 mM of $(NH_2)_2CO$; 0.50 mM of $NaH_2PO_4{\cdot}H_2O + K_2HPO_4$; 0.15 mM of $CaCl_2{\cdot}2H_2O$; 0.275 mM of $MgSO_4{\cdot}7H_2O$; 0.015 mM of $FeSO_4{\cdot}7H_2O$; 0.075 mM of $ZnSO_4{\cdot}7H_2O$; 0.001 mM of $Cl_2Co{\cdot}6H_2O$; 0.0025 mM of $Na_2MoO_4{\cdot}2H_2O$; 0.0425 mM of $MnSO_4{\cdot}H_2O$; 0.00025 mM of $CuSO_4{\cdot}5H_2O$; 0.05 mM of H_3BO_3; 0.0005 mM of $NiCl_2{\cdot}6H_2O$ and 1 dose of photoautotrophic vitamins mix (Thiamine-HCl (350 mg L^{-1}), d-Biotin (50 mg L^{-1}), cyanocobalamin (B12) (30 mg L^{-1}). Dose: 0.5 mL L^{-1}).

The cultures in the 1-L bubble column reactors, originated from both heterotrophic and autotrophic inocula, were grown under autotrophic conditions, inoculated at an initial concentration of 0.4 g L^{-1}, and were maintained at 23 ± 1 °C, under continuous aeration (0.8 L min^{-1}) and LED irradiation (100 µmol photons m^{-2} s^{-1}). Each assay was ended upon reaching the stationary phase.

Two-stage cultivation strategy

Inoculum of *C. amblystomatis*, cultivated under heterotrophic conditions, in 250-mL Erlenmeyer flasks, as described previously, was used to inoculate 7-L benchtop fermenters (5-L working volume) (New Brunswick BioFlo CelliGen115; Eppendorf AG, Germany), with the same culture medium optimised by Correia et al. (2023). Fermenters were operated in fed-batch mode, with glucose supply upon depletion. Growth parameters, such as temperature and pH were maintained at 28 ± 0.5 °C and pH 6.0 ± 0.5, respectively, with an air flow rate of approximately 1 vvm, and agitation ranging from 100 to 1200 rpm to ensure adequate oxygen supply throughout growth and to prevent biomass sedimentation.

To test the two-stage cultivation approach, three benchtop fermenters were used to inoculate each outdoor industrial photoreactor (tubular photobioreactors (PBR) and raceway ponds (RWP)) at Allmicroalgae's facilities between July and September of 2021, with average temperature of 21.5 °C, ranging between 14.6 and 28.5 °C, and light irradiance average of 23 MJ m^{-2} day^{-1}. Cultures started at an initial concentration of 0.5 g L^{-1}, and were grown using the industrial medium of Allmicroalgae, MNS, based on Guillard's F/2 medium, adapted to the local water, as described by (Barros et al. 2019). Additionally, cultures were aerated with 0.2-µm filtered air and the pH was maintained at 7.0 ± 0.5 using CO_2 pulse injection and temperature was maintained below 28 °C by a sprinkler-like irrigation system.

The PBR operated with a total working volume of 2.6 m^3, of which 1.8 m^3 corresponded to the culture circulating through a single-loop tubular system composed of 144 m of PMMA (polymethyl methacrylate) tubing with 5.6-cm internal diameter, constituting the photic area. Culture mixing and circulation were driven by a centrifugal pump (model CO 500/30, Lowara), operating at 1740 rpm and delivering a flow rate of 8.9 m^3 h^{-1} and a head pressure of 0.6 bar, resulting in an average culture velocity of approximately 1.0 m s^{-1}. After

completing the circulation loop, the culture was directed into a receiving vessel, entering from an elevated point and creating a "waterfall" effect that facilitated degassing.

The RWP consisted of open systems operated in a closed-loop configuration and driven by a paddlewheel system to ensure continuous circulation and prevent biomass sedimentation. The systems had a surface area of approximately 28 m^2 and consisted of channels 15 m in length and 2 m in width, with a 1-m channel width corresponding to the main circulation path. The channels had a culture depth of approximately 0.15 m and were composed of straight sections connected by 180° curved bends, ensuring a continuous recirculation loop. Mixing was provided by a single eight-flat-bladed paddlewheel, which maintained the hydrodynamic flow within the raceway at an average circulation velocity of approximately 0.30 m s^{-1}.

Growth assessment

Biomass growth was monitored by measuring dry weight (DW). Briefly, DW was determined by filtering a defined sample volume through 0.7-µm micro-glass fibre filters (GF/C 698; VWR, USA). The filters were rinsed with an equal volume of deionised water and dried at 120 °C using a moisture analyser (Kern & Sohn GmbH, Germany).

The specific growth rate (μ) was calculated by the quotient between the linearised biomass concentration (X) in g L^{-1}, over time (t), within the exponential phase. The rate was expressed in day^{-1}, according to Eq. (1):

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} \quad (1)$$

The volumetric biomass productivity (P) was determined as the difference in biomass concentration (X) in g L^{-1} over a defined time interval (t). Overall productivity was determined between day 0 and the onset of the stationary phase, whereas maximum productivity was calculated based on biomass accumulation within the exponential growth phase. Productivity was expressed in g L^{-1} day^{-1}, according to Eq. (2):

$$P = \frac{X_2 - X_1}{t_2 - t_1} \quad (2)$$

Areal biomass productivity (P_a) was determined by multiplying the volumetric biomass productivity (P) by the reactor working volume (V) in L, and dividing by the total ground area (A) in m^2, which includes non-photic areas in the case of tubular photobioreactors (PBR). Areal productivity was expressed in g m^{-2} day^{-1} as shown in Eq. (3):

$$P_a = P \times \left(\frac{V}{A}\right) \quad (3)$$

where PBR ground area was 26.5 m^2, whereas RWP 28 m^2.

Proximate composition

Protein content was determined by CHN elemental analysis using a Vario EL III analyser (Vario EL, Elemental Analyser System, GmbH, Germany). The nitrogen (N) content obtained was used to estimate protein content, with the conversion factor of 6.25, as reported for this strain (Correia et al. 2020).

Freeze-dried biomass samples were heated at 550 °C for 8 h in a muffle furnace. The ash content was determined by measuring the weight difference of the biomass before and after combustion.

The carbohydrate content was estimated as the difference between the remaining fraction and the measured protein, lipid, and ash contents.

Total lipidic content was determined gravimetrically after organic solvent extraction, following the Bligh and Dyer method (Bligh and Dyer 1959), with minor modifications (Pereira et al. 2011). Briefly, freeze-dried biomass was extracted with a chloroform:methanol:water mixture (1:2:0.8, v/v/v). The organic phase was separated by centrifugation, and a known volume was evaporated at 60 °C. The dried residue was weighed using an analytical balance (MSA36S-000-DH; Sartorius, Germany) and the lipid fraction was calculated based on the initial biomass weight.

Fatty acids profile

Fatty acid methyl esters (FAME) were determined according to the modifications described by Pereira et al. (2011) of the protocol of Lepage and Roy (1984). Briefly, freeze-dried biomass was mixed with methanol/acetyl chloride (20:1 v/v) solution and homogenised in a mixer mill (MM400, Retsch GmbH, Germany) with glass beads at 30 Hz. *N*-hexane was then added to the derivatisation vessels, which were incubated in a water bath at 70 °C for 60 min. Subsequently, water and *n*-hexane (1:4, v/v) were added and the mixture was homogenised using a vortex. The hexane phase was collected after centrifugation and transferred to new tubes, followed by the addition of anhydrous sodium sulphate (Na_2SO_4) to remove residual water. After filtration, samples were evaporated under a nitrogen gas flow and the residue was resuspended in gas chromatography-grade hexane.

FAME analysis was performed using a GC–MS system (Bruker SCION 456/GC, SCION TQ MS), equipped with a ZB-5MS capillary column (30 m × 0.25 mm of internal diameter, 0.25-µm of film thickness, Phenomenex), with helium as the carrier gas at a flow rate of 1.0 mL min^{-1}. The temperature program was set as follows: 60 °C for 1 min, increased at 30 °C min^{-1} to 120 °C, then 5 °C min^{-1} to 250 °C, and finally 20 °C min^{-1} to 300 °C. The injection temperature was maintained at 300 °C.

Pigment extraction and quantification

Chlorophyll *a* and *b* extraction and quantification were performed according to Ritchie (2008). Briefly, 10 mg of fresh biomass were mixed with 2 g of glass beads and extracted in 6 mL of 99% acetone for 10 min using a vortex mixer. The samples were then centrifuged at 2500 × *g* for 10 min, and the procedure was repeated until complete loss of colour in either the pellet or the supernatant. The supernatant was analysed by measuring absorbance at 630, 647, 664, and 691 nm, and chlorophyll concentrations were calculated using Eqs. 4 and 5:

$$Chl_a = -0.3319Abs_{630} - 1.7485Abs_{647} + 11.9442Abs_{664} - 1.4306Abs_{691} \quad (4)$$

$$Chl_b = -1.2825Abs_{630} + 19.8839Abs_{647} - 4.8806Abs_{664} - 2.3416Abs_{691} \quad (5)$$

The carotenoid content was determined following the method described by Couso et al. (2012). Briefly, 5–10 mg of freeze-dried biomass was resuspended in 1-mL methanol containing 0.03% butylhydroxytoluene and homogenised for 3 min in a mixer mill (MM400, Retsch, Germany) with 0.6 g of glass beads at 30 Hz. The samples were then centrifuged at 24000 × *g* for 3 min and the extraction procedure was repeated until complete loss of colour in either the pellet or the supernatant. The combined extracts were evaporated under a nitrogen flow and the pellet was dissolved in 1-mL of HPLC grade methanol and filtered (0.22 µm PTFE filter). Chromatographic analysis of carotenoids was performed using a Chromaster HPLC system (Hitachi, VWR, Portugal) equipped with a diode-array detector and a Purospher STAR RP-18 endcapped column (250 × 2.1-mm, 5-µm; Merck, Portugal). The mobile phase consisted of ethyl acetate (solvent A) and acetonitrile:water (9:1, v/v) (solvent B). The gradient program was as follows: 0–16 min, 0–60% A; 16–30 min, 60% A; 30–32 min, 100% A; and 32–35 min, 100% B. Carotenoid identification was carried out using Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, USA). Quantification was performed at 450 nm using calibration curves of neoxanthin, violaxanthin, lutein, and *β*-carotene standards (Sigma-Aldrich, Portugal). The injection volume was 100 µL.

Statistical analysis

Statistical analyses were performed using R software (version 3.6.1), within the RStudio IDE (version 1.2.1335). One-way ANOVA followed by Tukey's HSD post-hoc test multiple comparisons test with a confidence level ≥ 95%. For pairwise comparisons between independent groups, Student's *t*-test was used. Mean values and standard deviations were calculated from biological triplicates.

Results

Trophic shift assay

A preliminary assay was conducted to evaluate the impact of using a heterotrophically cultivated inoculum (1st stage) to grow this microalga photoautotrophically in bubble column reactors (2nd stage). The purpose was to verify whether the trophic shift would influence growth performance and potentially hinder or benefit the implementation of a two-stage cultivation approach.

The biomass volumetric productivities, specific growth rates and biomass production of the cultures grown under photoautotrophic conditions in 1-L bubble column reactors using inoculum obtained under different metabolic regimes are presented in Table 1 and Fig. 1. The experiment ended once the cultures reached the stationary phase.

Upon inoculation of the bubble column reactors with biomass derived from both heterotrophic and photoautotrophic cultures, no statistically significant differences were observed between the two conditions ($p \geq 0.05$). The biomass overall productivities registered values

Table 1 Maximum and overall volumetric productivities (g L^{-1} day^{-1}), specific growth rates (day^{-1}), and biomass concentration (g L^{-1}) of *Chlorococcum amblystomatis* grown in bubble column reactors with inoculum obtained under different metabolic regimes: photoautotrophic and heterotrophic conditions

Inoculum origin	Volumetric Productivity (g L^{-1} day^{-1})		Specific Growth Rate (day^{-1})	Biomass Concentration (g L^{-1})
	Overall	Maximum		
Photoautotrophic	0.14 ± 0.01	0.24 ± 0.03	0.27 ± 0.02	1.29 ± 0.07
Heterotrophic	0.14 ± 0.01	0.19 ± 0.05	0.24 ± 0.02	1.35 ± 0.05

No significant statistical differences were observed between conditions ($p \geq 0.05$). Values are presented as mean ± standard deviation ($n = 3$)

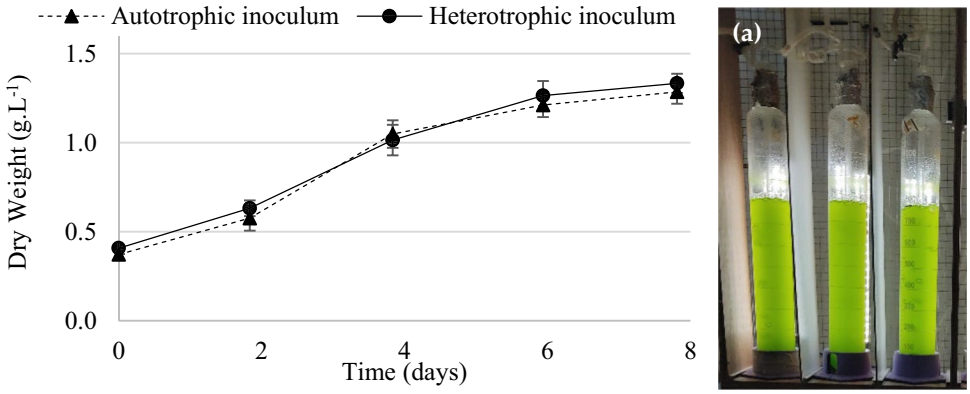

Fig. 1 Growth curves of *Chlorococcum amblystomatis* in bubble column reactors (**a**) with inoculum obtained under different metabolic regimes: photoautotrophic and heterotrophic conditions. Values are given as mean ± standard deviation ($n = 3$)

of 0.14 ± 0.01 g L^{-1} day^{-1} in both cases, while specific growth rates ranged from 0.24 ± 0.03 to 0.27 ± 0.02 day^{-1} and biomass concentration between 1.29 ± 0.07 and 1.35 ± 0.05 g L^{-1} (Table 1). These results strongly suggest that inoculum obtained under heterotrophic conditions can rapidly adapt to photoautotrophic conditions and effectively seed reactors without compromising growth performance (Fig. 1). This rapid adaptation is particularly relevant as it supports the feasibility of using dense heterotrophic cultures to initiate photoautotrophic systems without compromising biomass yield or growth rate.

Accordingly, a second experiment was conducted to validate these findings at industrial scale, in which heterotrophically grown inocula produced in benchtop fermenters were used to directly seed two different industrial cultivation systems: closed tubular photobioreactors (PBR) and open raceway ponds (RWP). This experimental setup aimed to assess whether the comparable growth performance observed at laboratory scale could be reproduced under outdoor operational conditions and in systems with different operational regimes. A schematic representation of the industrial scale-up of *C. amblystomatis* using photoautotrophic and heterotrophic routes is shown in Fig. 2.

The conventional photoautotrophic scale-up route, from cryovials to industrial reactors, typically requires approximately 66 days, whereas the heterotrophic route enables reaching the same target in only 12 days (Fig. 2), considering a minimum biomass concentration of 0.2 g L^{-1} to initiate the reactors and harvesting prior to the stationary phase in order to avoid productivity losses. This results were based on previous results (Barros et al. 2019; Correia et al. 2020, 2023) and internal unpublished data from the authors.

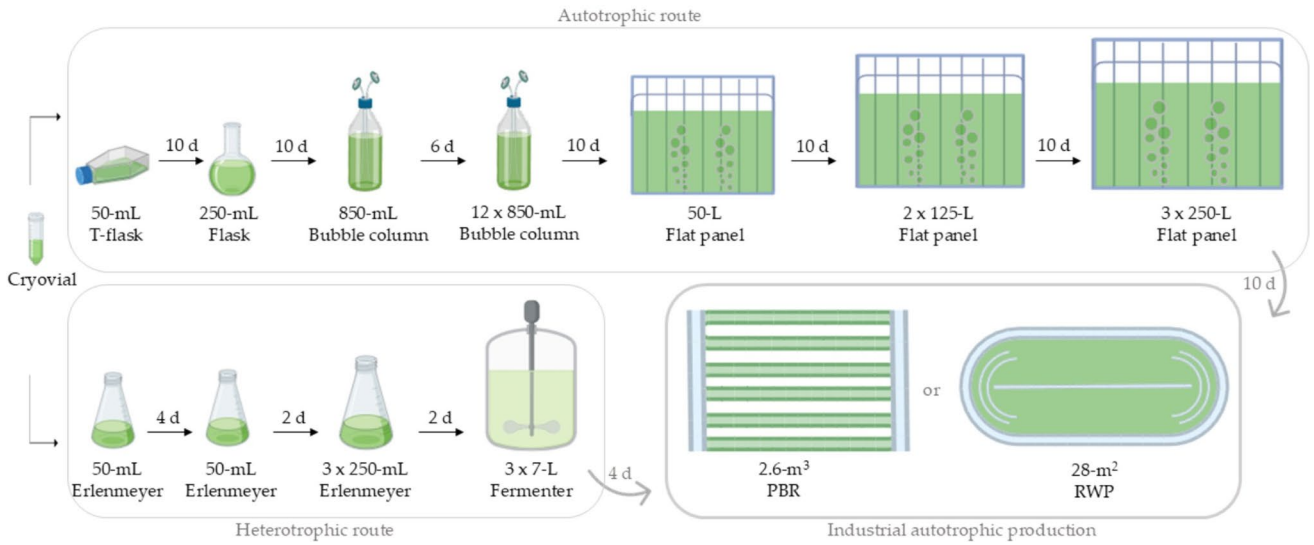

Fig. 2 Schematic representation of the industrial scale-up of *Chlorococcum amblystomatis* using photoautotrophic and heterotrophic routes for the inoculation of industrial photobioreactors

Two-stage cultivation strategy

The biomass volumetric productivities, specific growth rate and biomass concentration of cultures grown in each reactor until reaching stationary phase are described in Table 2. The growth curves of PBRs and RWPs are shown in Fig. 3.

As expected, cultivation in the PBRs resulted in higher biomass concentrations compared to RWPs (Table 2 and Fig. 3), reaching a maximum of 1.47 ± 0.16 g L^{-1}. Volumetric productivities ranged from 0.13 ± 0.02 to 0.17 ± 0.04 g L^{-1} day^{-1}, with a specific growth rate of 0.21 ± 0.03 day^{-1}, and areal productivity of 13.23 ± 1.88 g m^{-2} day^{-1} were obtained. In contrast, limited or negligible growth was observed in the RWPs. The lower and less uniform light availability, combined with reduced mixing efficiency and potential environmental fluctuations, likely contributed to the constrained growth performance under these conditions. Under markedly different growth conditions, heterotrophic cultivation in benchtop fermenters achieved substantially higher performance. Volumetric productivities ranged from 17.32 ± 0.74 to 44.46 ± 1.31 g L^{-1} day^{-1}, with a specific growth rate of 2.09 ± 0.11 day^{-1}, resulting in a much higher biomass concentration of 70.96 ± 0.35 g L^{-1}, in considerably less time (4 days) as shown in Figs. 2 and 4. From an industrial standpoint, this strategy presents important operational advantages. Heterotrophic cultivation in controlled fermenters enables the rapid generation of high-cell-density inocula, which can subsequently be used to directly seed large-scale photobioreactors. This approach highlights the potential to significantly shorten scale-up times, reduce contamination risks during the initial stages of outdoor cultivation, and improve overall process.

Proximate composition

To better understand the impact of this two-stage cultivation approach on biomass quality, the biochemical composition of biomass produced by heterotrophic and photoautotrophic cultivations was analysed at the point when cultures reached the stationary phase.

The growth and protein content were monitored throughout cultivation in the benchtop reactors (Fig. 4). Additionally, the content of proteins, lipids, carbohydrates and ashes of the biomass produced in each reactor is presented in Table 3.

During heterotrophic cultivation, protein content reached a maximum of $64.86 \pm 2.89\%$ of dry weight (DW) on day

Table 2 Maximum and overall volumetric productivities (g L^{-1} day^{-1}), specific growth rates (day^{-1}), areal productivities (g m^{-2} day^{-1}) and biomass concentration (g L^{-1}) of *Chlorococcum amblystomatis* grown under heterotrophic conditions in benchtop fermenters and under photoautotrophic conditions in tubular photobioreactors (PBRs) and open raceway ponds (RWPs), both inoculated with cultures grown in benchtop fermenters

Reactor	Volumetric Productivity (g L^{-1} day^{-1})		Specific Growth Rate (day^{-1})	Areal Productivity (g m^{-2} day^{-1})	Biomass Concentration (g L^{-1})
	Overall	Maximum			
Benchtop Fermenter	17.32 ± 0.74^{a}	44.46 ± 1.31^{a}	2.09 ± 0.11^{a}	-	70.96 ± 0.35^{a}
PBR	0.13 ± 0.02^{b}	0.17 ± 0.04^{b}	0.21 ± 0.03^{b}	13.23 ± 1.88^{a}	1.47 ± 0.16^{b}
RWP	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}	0.04 ± 0.01^{c}	2.14 ± 0.32^{b}	0.56 ± 0.09^{c}

Different letters indicate significant differences between reactors ($p < 0.05$). Values are given as mean ± standard deviation ($n = 3$)

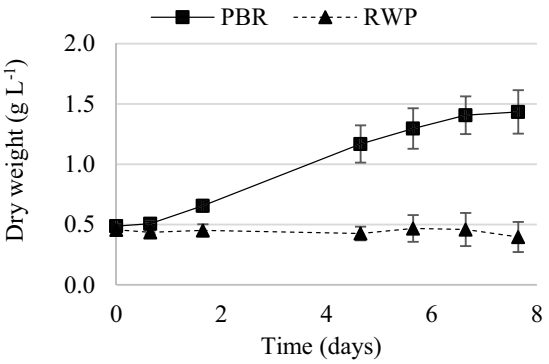

Fig. 3 Growth curves of *Chlorococcum amblystomatis* grown under photoautotrophic conditions in 2.6-m^3 closed tubular photobioreactors (PBR) (**a**) and 28-m^2 open raceway ponds (RWP) (**b**), seeded with heterotrophic inoculum. Values are given as mean ± standard deviation ($n = 3$)

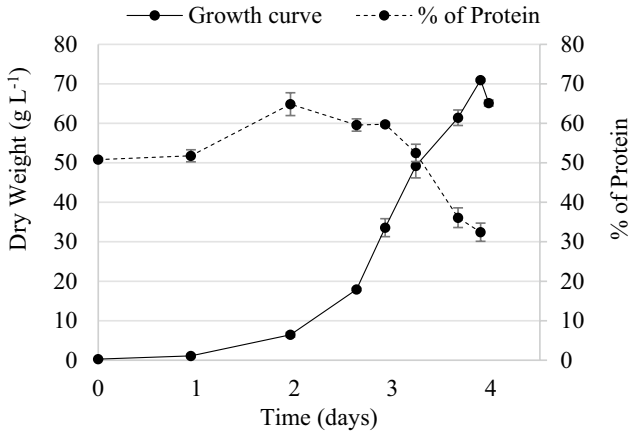

Fig. 4 Growth curves of *Chlorococcum amblystomatis* grown under heterotrophic conditions in 7-L benchtop fermenters as well as the protein content during the cultivation period. Values are given as mean ± standard deviation ($n = 3$)

2, followed by a progressive decline throughout the mid-exponential phase, ultimately reaching 32.42 ± 2.30% of DW (Fig. 4). This reduction in protein content coincided with active biomass accumulation, suggesting a shift in cellular resource allocation during rapid growth. In contrast, biomass obtained during the second growth, in outdoor photobioreactors, exhibited significantly higher protein contents ($p < 0.05$), compared to the final heterotrophic stage. The highest value was observed in the biomass grown in PBRs, reaching 54.55 ± 2.04% of DW (Table 3). This corresponds to a recovery of 22.13% in protein content during the photoautotrophic stage.

In line with the protein contents observed, carbohydrate content was significantly lower ($p < 0.05$) in cultures grown under photoautotrophic conditions. This pattern suggests an inverse relationship between protein and carbohydrate accumulation.

Ash content was significantly higher ($p < 0.05$) in biomass harvested from the RWP system, reaching 20.61 ± 2.78% of DW. This increase is consistent with the greater exposure to environmental contaminants and mineral salts typically associated with open cultivation systems. Furthermore, due to the negligible biomass growth observed in RWP, nutrient uptake was limited and the culture medium was not substantially consumed, thereby increasing the relative salt concentration in the biomass.

The lipid content was also significantly lower ($p < 0.05$) in the biomass grown in RWP (7.99 ± 2.42% of DW). This reduction may be associated with suboptimal growth conditions, including lower and less uniform light availability and temperature fluctuations, which can negatively impact photosynthetic efficiency and overall metabolic performance.

Fatty acids profile

The fatty acid profile was assessed through quantification of the fatty acid methyl esters (FAME), expressed as the percentage of total fatty acids (TFA) (Table 4).

Cultures grown under heterotrophic conditions in the 7-L benchtop fermenters were characterised by a high proportion of polyunsaturated fatty acids (PUFA), which accounted for 50.12 ± 3.27% of TFA. A small percentage of C20:1 and C20:0 was also detected (0.29 ± 0.03% and 0.53 ± 0.05% of TFA, respectively), which had not been previously reported for this strain. The dominant FAME under heterotrophic growth was C16:0, representing 34.37 ± 1.38% of TFA, followed by C18:2*n*−6 (LA), with 19.13 ± 1.94% of TFA. In contrast, biomass produced in industrial photoautotrophic systems the C18:3*n*−3 (ALA) as the predominant FAME, ranging from 29.58 ± 0.41% to 35.76 ± 0.58% of TFA, which was not detected in the biomass obtained from heterotrophic conditions. This indicates that the fatty acid profile also undergoes modifications during the transition from heterotrophic to photoautotrophic cultivation. In photobioreactors, C16:0 represented the second most abundant FAME (20.68 ± 0.32% – 22.83 ± 0.66% of TFA), followed by C16:4*n*−3 (17.77 ± 0.26% – 19.23 ± 0.32% of TFA), C16:1 (5.44 ± 0.32% – 8.70 ± 0.49% of TFA), C18:4*n*−3 (4.37 ± 0.25% – 5.12 ± 0.11% of TFA), LA (3.18 ± 0.12% – 5.60 ± 0.40% of TFA), C18:3*n*−6 (2.46 ± 0.03% – 3.24 ± 0.29% of TFA) and C18:1 (2.52 ± 0.09% – 2.57 ± 0.08% of TFA). Although C16:3*n*−3 and C16:2*n*−6 were not detected in biomass harvested from

Table 3 Proximate composition (%) of the biomass of *Chlorococcum amblystomatis* cultivated under heterotrophic and photoautotrophic conditions, in the different reactors: benchtop fermenters, closed tubular photobioreactors (PBR) and in open raceways ponds (RWP)

Reactor	Protein (%)	Lipids (%)	Carbohydrates (%)	Ash (%)
Benchtop Fermenter	32.42 ± 2.30^{c}	14.53 ± 0.07^{a}	47.53 ± 2.94 ^{a}	6.32 ± 1.57^{b}
PBR	54.55 ± 2.04^{a}	16.07 ± 2.88^{a}	20.64 ± 1.58^{c}	8.73 ± 0.42^{b}
RWP	44.92 ± 0.77^{b}	7.99 ± 2.42^{b}	26.48 ± 2.01^{b}	20.61 ± 2.78^{a}

Proteins, lipids, carbohydrates, and ashes are presented as the percentage of the biomass dry weight. Different letters indicate significant differences between reactors ($p < 0.05$). Values are given as mean ± standard deviation ($n = 3$)

Table 4 Fatty acid methyl esters (FAME) profile, presented in percentage of total fatty acids of *Chlorococcum amblystomatis* biomass obtained through cultivation in benchtop fermenters, closed tubular photobioreactors (PBR) and open raceway ponds (RWP)

FAME	Benchtop Fermenter (%)	PBR (%)	RWP (%)
C14:0	0.60 ± 0.05	n.d	n.d
C16:4*n*−3	12.38 ± 2.14^{b}	$17.77 \pm 0.26^{a,b}$	19.23 ± 0.32^{a}
C16:3*n*−3	1.39 ± 0.37^{a}	1.49 ± 0.08^{a}	n.d
C16:2*n*−6	4.08 ± 0.30^{a}	1.83 ± 0.07^{b}	n.d
C16:1	4.42 ± 0.09^{b}	8.70 ± 0.49^{a}	5.44 ± 0.32^{b}
C16:0	34.37 ± 1.38^{a}	20.68 ± 0.32^{b}	22.83 ± 0.66^{b}
C17:3	3.68 ± 0.31^{a}	$2.47 \pm 0.17^{a,b}$	2.00 ± 0.11^{b}
C18:4*n*−3	3.20 ± 0.43^{b}	$4.37 \pm 0.25^{a,b}$	5.12 ± 0.11^{a}
C18:3*n*−3	n.d	29.58 ± 0.41^{b}	35.76 ± 0.58^{a}
C18:3*n*−6	7.50 ± 1.11^{a}	3.24 ± 0.29^{b}	2.46 ± 0.03^{b}
C18:2*n*−6	19.13 ± 1.94^{a}	5.60 ± 0.40^{b}	3.18 ± 0.12^{b}
C18:1	10.37 ± 0.03^{a}	2.57 ± 0.08^{b}	2.52 ± 0.09^{b}
C18:0	2.76 ± 0.35^{a}	$1.69 \pm 0.34^{a,b}$	1.47 ± 0.09^{b}
C20:1	0.29 ± 0.03	n.d	n.d
C20:0	0.53 ± 0.05	n.d	n.d
Σ SFA	38.26 ± 1.63^{a}	22.37 ± 0.02^{b}	24.30 ± 0.57^{b}
Σ MUFA	11.62 ± 4.88^{a}	11.28 ± 0.41^{a}	7.96 ± 0.22^{a}
Σ PUFA	50.12 ± 3.27^{b}	66.35 ± 0.43^{a}	67.74 ± 0.35^{a}
Σ *n*−3	16.96 ± 2.70^{b}	53.21 ± 0.49^{a}	60.10 ± 0.15^{a}
Σ *n*−6	30.36 ± 2.85^{a}	10.67 ± 0.75^{b}	5.64 ± 0.09^{b}
Σ*n*−6/Σ*n*−3	1.84 ± 0.26^{a}	0.20 ± 0.02^{b}	0.09 ± 0.00^{b}
PUFA/SFA	1.31 ± 0.03^{a}	2.97 ± 0.02^{b}	2.79 ± 0.08^{b}

[1]n.d.: not detected

Different letters indicate significant differences between reactors ($p < 0.05$). Values are given as mean ± standard deviation ($n = 3$)

the RWP system, the overall FAME profiles obtained from RWP and PBR cultures were largely similar. Minor quantitative differences were observed between systems; however, the qualitative composition and relative abundance of the major fatty acids were consistent across both industrial photoautotrophic cultivation modes. Overall, the PUFA content remained the dominant fraction of total fatty acids under both heterotrophic and photoautotrophic conditions. However, notable changes in fatty acid distribution were observed between the two growth modes. PUFA levels increased from $50.12 \pm 3.27\%$ of total fatty acids under heterotrophy to 66.35 ± 0.43–$67.74 \pm 0.35\%$ under photoautotrophy. Despite this overall increase, the PUFA profile shifted markedly: heterotrophic cultures were mainly characterised by *n*−6 fatty acids, whereas photoautotrophic conditions favoured the accumulation of *n*−3 fatty acids.

Pigments profile

The content of carotenoids and chlorophylls of *C. amblystomatis* was evaluated (Table 5).

Biomass produced under heterotrophic conditions in benchtop fermenters showed reduced pigment content, which was subsequently restored during the second-stage of this cultivation strategy, in photoautotrophic reactors (Table 5). Cultures grown in fermenters presented significantly lower chlorophyll *a* and *b* contents (10.04 ± 1.68 mg g^{-1}) compared to biomass cultivated photoautotrophically in both in PBR and RWP ($p < 0.05$). Following the transition to photoautotrophic conditions, chlorophyll content increased significantly, reaching the highest value in biomass cultivated in PBRs (44.91 ± 5.11 mg g^{-1}) ($p < 0.05$). This represents a substantial recovery of photosynthetic capacity during the second stage of cultivation.

In the same way, cultures grown in RWPs showed significantly lower pigment content ($p < 0.05$) than those cultivated in PBRs, underscoring the influence of reactor configuration on biochemical composition.

Lutein and *β*-carotene were the most abundant carotenoids detected. Biomass cultivated in PBRs exhibited significantly higher concentrations of these compounds ($p < 0.05$), reaching 5.34 ± 0.10 mg g^{-1} and 5.92 ± 0.39 mg g^{-1}, respectively. Additionally, PBR-grown cultures showed a notable neoxanthin content (3.53 ± 0.75 mg g^{-1}), further indicating the enhanced development of the photosynthetic pigment complex under controlled photoautotrophic conditions.

Discussion

Heterotrophic cultivation of *Chlorococcum amblystomatis* was already reported in Erlenmeyer flasks, though with growth parameters considerably inferior to the values

Table 5 Pigment profile, in mg g^{-1}, of *Chlorococcum amblystomatis* biomass obtained from the cultivation in benchtop fermenter, closed tubular photobioreactor (PBR) and open raceway ponds (RWP)

Pigments	Benchtop Fermenter (mg g^{-1})	PBR (mg g^{-1})	RWP (mg g^{-1})
Neoxanthin	0.53 ± 0.06^{b}	3.53 ± 0.75^{a}	1.32 ± 0.07^{b}
Violaxanthin	0.15 ± 0.03^{b}	1.16 ± 0.11^{a}	0.46 ± 0.05^{b}
Lutein	1.17 ± 0.06^{c}	5.34 ± 0.10^{a}	2.37 ± 0.14^{b}
β-carotene	2.19 ± 0.30^{b}	5.92 ± 0.39^{a}	1.90 ± 0.19^{b}
Chlorophyll *a* and *b*	10.04 ± 1.68^{c}	44.91 ± 5.11^{a}	27.39 ± 0.86^{b}

Different letters indicate significant differences between reactors ($p < 0.05$). Values are given as mean ± standard deviation ($n = 3$)

presented in this work, namely global and maximum volumetric productivities of 0.19 and 0.60 ± 0.06 g L^{-1} h^{-1}, a specific growth rate of 0.05 ± 0.00 h^{-1} and a maximum biomass concentration of 10.53 ± 0.59 g L^{-1} (Correia et al. 2023). Scaling up to a larger reactor under automated control and fed-batch operation mode resulted in higher productivities and a final biomass concentration of 70.96 ± 0.35 g L^{-1}. To our knowledge, this represents the highest biomass concentration ever reported for this microalgal species. When compared to *Chlorella vulgaris*, a species widely cultivated under heterotrophic conditions and under the same reactor and operational settings used in the present study *C. amblystomatis* exhibited lower global productivity (17.32 ± 0.74 g L^{-1} day^{-1}, in contrast to 27.3 ± 6.8 g L^{-1} day^{-1}) and biomass concentration (70.9 ± 0.35 g L^{-1} compared with 174.5 g L^{-1}) (Barros et al. 2019). However, when compared with *Scenedesmus rubescens*, cultivated in the same reactor following medium optimisation to enhance growth, *C. amblystomatis* achieved superior performance, as *S. rubescens* reached only 8.63 g L^{-1} $day^{-1}$and 69.5 g L^{-1} (Santo et al. 2023). The improved performance of *C. amblystomatis* under the same controlled cultivation conditions highlights its potential as a robust and adaptable alternative for heterotrophic cultivation, when evaluated against well-established microalgae. This suggests that, with further strain improvement or process optimisation, *C. amblystomatis* may reach productivity levels these species while offering additional operational flexibility.

Moreover, *C. amblystomatis* is able to efficiently transition from heterotrophic to photoautotrophic growth without exhibiting any detrimental effects on growth performance. The rapid adaptation observed supports the feasibility of a two-stage cultivation strategy, in which concentrated heterotrophic inocula can be used to directly initiate photoautotrophic cultures. This approach presents clear advantages for industrial scale-up, particularly by reducing start-up times and improving overall process efficiency. Similar approach have been reported for *Chlorella* sp. in flat panel reactors (Barros et al. 2019), and 50-L open basin (Han et al. 2012) inoculated with both auto- and heterotrophic inocula. As described by Barros et al. (2019), no significant differences were observed when heterotrophic inocula were transferred to outdoor photoautotrophic conditions. Moreover, the overall biomass productivities (0.10 ± 0.01–0.11 ± 0.02 g L^{-1} day^{-1}) and final biomass concentrations (1.24–1.28 g L^{-1}) reported for *C. vulgaris* are slightly lower than those obtained for *C. amblystomatis* in the present study (of 0.14 ± 0.01 g L^{-1} $day^{-1}$and 1.29 ± 0.07–1.35 ± 0.05 g L^{-1}, respectively). These results indicate that *Chlorococcum* can be cultivated within a two-stage strategy with efficiencies comparable or even improved to those of industrially established microalgae, supporting its potential as a viable alternative for large-scale biomass production. Additionally, according to Han et al. (2012), the use of heterotrophic inoculum in a 50-L open basin (with a 15 cm depth) resulted in higher biomass productivities for *Chlorella pyrenoidosa*, *C. ellipsoidea*, and *C. vulgaris*, which were 1.91-, 1.51-, and 1.48-fold greater, respectively, than those achieved with photoautotrophic inoculum. These results further support the feasibility of using heterotrophically grown inocula to initiate photoautotrophic cultures without compromising or even improving growth performance.

Moreover, by uncoupling biomass production from light dependency, heterotrophic cultivation enables faster cell growth, under strictly controlled conditions, resulting in high-density inoculum that can be rapidly transferred to photoautotrophic systems, thereby reducing industrial-scale cultivation time from 66 to 12 days. Overall, the proposed two-stage cultivation strategy allows reducing the scale-up time 5.5-fold and, subsequently, the associated production costs, while minimising the risk of contamination, as supported by previous studies.

Whitin the industrial reactors, the highest growth performance was achieved in the closed tubular photobioreactor (PBR). The global volumetric and areal productivities observed in 2.6-m^3 PBR were higher than those previously reported for this microalga in the same reactor (with a 2.5 m^3 working volume), namely 0.09 ± 0.02 g L^{-1} day^{-1} and 8.65 ± 1.62 g m^{-2} day^{-1}, respectively, as well as the specific growth rate 0.13 ± 0.03 day^{-1} (Correia et al. 2020). However, it is noteworthy that the current experiment was conducted between July and September of 2021, whereas the previous study was carried out from January to June of 2018, under lower temperature and lower solar radiation. In addition, the *C. amblystomatis* strain used in the previous study was non-axenic 0030CN, also from Allmicroalgae culture collection, whereas in these trials, the axenic strain 0066CA was used. As reported in recent studies, inherent differences exist between axenic and non-axenic cultures, which can result in variations in growth performance and biochemical profiles, as the presence or absence of microbial consortia modulates nutrient availability, metabolic fluxes, and overall cellular physiology (Santos et al. 2026).

Raceway ponds generally exhibit lower performance compared to closed tubular photobioreactors due to inherent design and operational constraints, including less favourable hydrodynamic conditions resulting in limited mixing efficiency, reduced volumetric mass transfer coefficients for gas–liquid exchange, and suboptimal light distribution associated with longer optical path lengths (Yen et al. 2019; Chandola et al. 2026). Accordingly, lower productivities in raceway systems compared to PBRs were expected. These limitations are further accentuated by the relatively large cell size of *Chlorococcum* (approximately 15 μm) (Correia et al. 2020), which increases their susceptibility to sedimentation. This, combined with the suboptimal mixing regime, promotes cell aggregation and settling, thereby hindering homogeneous

suspension and further compromising biomass productivity in the open system. Indeed, some aggregation and settling of the cells were observed during cultivation, likely further compromising biomass productivity in the open system. A similar response of poorer performance in RWPs has also been reported for *Tetraselmis* (Raes et al. 2013), another microalga with cell dimensions comparable to those of *Chlorococcum*. These observations highlight the importance of reactor design for the successful cultivation of each microalgal species.

Regarding the biochemical profile of the biomass, these findings support the effectiveness of the two-stage cultivation strategy, as the protein content was restored from 32.42 ± 2.30% of DW at the time of photobioreactor inoculation to 54.55 ± 2.04% of DW at the end of the photoautotrophic growth stage. This value is consistent with that previously reported for the same species cultivated in PBRs using the conventional cultivation methods (55.72 ± 2.85% of DW) (Correia et al. 2020) and slightly higher than the protein content reported for *C. vulgaris* under a two-stage growth strategy (52.18 ± 1.30% of DW) (Barros et al. 2019). The recovery of protein content observed during the photoautotrophic stage can be explained by the metabolic reprogramming associated with the transition from heterotrophy to photosynthetic growth. During heterotrophic cultivation, organic carbon is primarily channelled towards rapid biomass accumulation and the synthesis of storage compounds. Following transfer to photoautotrophic conditions, the reactivation of photosynthetic metabolism promotes increased nitrogen assimilation to support chloroplast development, pigment synthesis and reconstruction of the photosynthetic apparatus. Consequently, cellular carbon partitioning shifts from storage metabolism towards the synthesis of nitrogen-containing compounds, including amino acids, enzymes and structural proteins, favouring protein accumulation and restoration of the biochemical profile (Jareonsin and Pumas 2021; Xiao et al. 2022; Lu et al. 2024; Levasseur and Pozzobon 2025). Similar metabolic responses have been reported during heterotrophy-to-phototrophy transition in *Chlorella* sp., where cultures initiated with heterotrophic inoculum exhibited lower protein content, which was subsequently restored during photoautotrophic cultivation. These cultures also showed higher carbohydrate and lower lipid contents initially, followed by modulation of both components during the photoautotrophic phase (Han et al. 2012). Nevertheless, protein recovery following trophic transition is not a universal response and varies substantially among species and cultivation strategies. According to Liyanaarachchi et al. (2021), the success of two-stage cultivation systems depends on the ability of microalgal cells to reconfigure carbon allocation and nitrogen assimilation pathways after the shift from heterotrophic to photoautotrophic growth. Factors such as irradiance, nutrient availability, stage duration, cultivation conditions and reactor configuration and scales can markedly influence the extent of biochemical restoration. Whereas several studies have reported only partial recovery of protein content after heterotrophic cultivation, others have demonstrated near-complete restoration through the reactivation of photosynthetic metabolism and associated biosynthetic pathways. The results obtained in the present study place *C. amblystomatis* among the latter group, demonstrating a strong capacity to restore its protein-rich phenotype while maintaining high biomass productivity, a characteristic that is particularly attractive for downstream biotechnological applications.

An inverse relationship between protein and carbohydrate accumulation may explain the lower carbohydrate content observed in cultures grown under photoautotrophic conditions. It has been pointed out by several authors that growth under heterotrophic conditions hinders protein production in microalgae, namely in chlorophytes, such as *Chlorella* sp. and *Chlorococcum* sp., which might favour carbohydrate accumulation instead (Barros et al. 2019). However, other authors have highlighted that this assumption is not necessarily true, as the protein/carbohydrate contents might be modulated by manipulating cultivation strategy, growth parameters and culture medium (Xie et al. 2017; Trovão et al. 2025). The highly variable protein contents, reported for *C. vulgaris*, for example, from 20 to 64% of DW, support this rationale (Lau et al. 2014; Cai et al. 2022; Trovão et al. 2024).

The FAME profiles obtained were consistent with those previously reported for cultures cultivated at laboratorial scale (Correia et al. 2023), as well as for cultures cultivated in the same reactor system using the conventional scale-up cultivation methods (Correia et al. 2020) with lipids predominantly composed of polyunsaturated fatty acids (PUFAs). However, cultures grown in heterotrophic reactors exhibited a lower PUFA content (50.12 ± 3.27% of total fatty acids, TFA) compared to autotrophically grown cultures (66.35 ± 0.43−67.74 ± 0.35% of TFA), at the expense of saturated fatty acids (SFA). Under heterotrophic growth, the main FAME was C16:0 (34.37 ± 1.38% of TFA), followed by C18:2*n*−6 (LA) (19.13 ± 1.94%), whereas in industrial photoautotrophic systems, C18:3*n*−3 (ALA) was predominant (29.58 ± 0.41% to 35.76 ± 0.58% of TFA). This indicates that the fatty acid profile is also influenced by the type of cultivation.

A similar cultivation-dependent shift in fatty acid distribution has been reported for other industrial chlorophytes. In *Chlorella* cultivated under photoautotrophic conditions, the total content of SFAs (22,5 ± 1.5%), MUFAs (11.2 ± 0.4%) and PUFAs (66.3 ± 1.5%) was very similar to reported in this study for *Chlorococcum*, and both containing ALA as the predominant FAME. For heterotrophically grown *Chlorella*, some differences were observed, with LA as the main FAME (32.7 ± 0.7%), followed by C16:0 with (23.7 ± 0.4%) (Maurício et al. 2023). These observations are consistent with the metabolic plasticity observed in *C. amblystomatis*, where

the transition from heterotrophic to photoautotrophic cultivation promoted both an increase in total PUFA content and a marked shift from an $n-6$- to a $n-3$ polyunsaturated fatty acid-dominated profile. In contrast, although *Scenedesmus acuminatus* exhibited significant differences in total FAME content between heterotrophic and photoautotrophic cultures during the initial stages of cultivation, with values of 7.2% and 13.3% DW, respectively, the FAME content of heterotrophic cells increased rapidly over time, reaching 42.56% DW by the end of cultivation. This value was only slightly lower than that observed in photoautotrophic cells (45.37% DW). Zhang et al. (2021) reported that both hetero- and photoautotrophic cultures showed profiles dominated by MUFAs (43.42–44.69% of TFA), while PUFAs represented only 25.78–26.63% of TFA at the end of cultivation, with no statistically significant differences between trophic modes. The fatty acid profile was largely composed of C18:1 (41.83–42.I3% of TFA) and C16:0 (25.82–26.95% of TFA), with only minor differences between cultivation strategies. Unlike *C. amblystomatis*, which undergoes substantial trophic-mode-dependent remodelling of its fatty acid profile, *S. acuminatus* maintains a relatively constant lipid composition, suggesting species-specific differences in carbon partitioning and fatty acid metabolism. These contrasting responses highlight the greater flexibility of *C. amblystomatis* to redirect lipid biosynthesis according to cultivation conditions, particularly regarding the balance between $n-6$ and $n-3$ polyunsaturated fatty acids.

Human body cannot synthesize essential PUFAs and must obtained them from diet. ALA, present in certain vegetable oils and animal fats, can be converted into long-chain $n-3$ PUFAs as EPA and DHA (Baker et al. 2016; Ahmmed et al. 2020). Microalgae, particularly *Chlorococcum*, provide these $n-3$ PUFAs without fishy taste or allergy risks, making them a practical alternative source.

The $\Sigma n-6/\Sigma n-3$ ratios were favourable low, which is important since $n-6$ PUFAs are pro-inflammatory and $n-3$ PUFAs are anti-inflammatory. The recommended ratio is between 4:1 and 1:1 to prevent several diseases and adverse effects on human health (Pereira et al. 2012; Gutierres et al. 2025). Overall, *C. amblystomatis* presents an interesting FAME profile, with optimal $\Sigma n-6/\Sigma n-3$ ratios, supporting its potential as a valuable source of high-value PUFAs for food and feed applications.

Pigment accumulation is another key parameter influenced by cultivation strategy. As pigments are primarily synthesised under photoautotrophic conditions (Morales-Sánchez et al. 2017; Hu et al. 2018), biomass produced under heterotrophic conditions exhibited significantly lower chlorophyll content (10.04 ± 1.68 mg g^{-1}) than biomass grown photoautotrophically in PBRs and RWPs. Pigment levels were restored during the second cultivation stage, reaching 44.91 ± 5.11 mg g^{-1} in PBR grown biomass, consistent with previously reported values for this microalga in the same reactor system using the conventional cultivation methods (40.24 ± 7.94 mg g^{-1}) (Correia et al. 2020), underscoring the benefits of the photoautotrophic stage. This recovery highlights the importance of light availability in promoting chlorophyll synthesis. This difference was expected, as pigment accumulation is strongly dependent on light availability. Photoautotrophic cultivation exposes cells to higher and more uniform light, promoting chlorophyll synthesis, whereas heterotrophic growth occurs in low-light conditions at high cell densities, further limiting light penetration. A similar relationship between trophic mode and pigment content has been reported for *S. acuminatus*, where photoautotrophically grown cells displayed higher chlorophyll and carotenoid contents than heterotrophically grown cells (25.03 vs 11.01 pg of chlorophyll per cell DW and 7.25 vs 3.65 pg of carotenoids per cell DW) (Zhang et al. 2021).

The lower pigment levels observed in RWPs are likely associated with suboptimal cultivation conditions, including variable light distribution and reduced light penetration in the deeper water layers, and cell sedimentation, which limit chlorophyll synthesis compared to the thin, well-illuminated tubes of PBRs (Guedes et al. 2011). Such constraints may affect the photosynthetic performance of *C. amblystomatis*.

Regarding carotenoids, lutein content (5.34 ± 0.10 mg g^{-1}) was comparable to previously reported values for this species under photoautotrophic cultivation (Correia et al. 2023), and higher than those observed for other species, such as *Muriellopsis* sp. (4.3 mg g^{-1}) and *Scenedesmus almeriensis* (4.5 mg g^{-1}) (Del Campo et al. 2007), even without an induction phase. Conversely, the β-carotene content (5.92 ± 0.39 mg g^{-1}) was more than fourfold higher than previously reported for this microalga cultivated in the same reactor configuration (Correia et al. 2020). Neoxanthin was also detected in cultures grown in PBR, with an interesting value of 3.53 ± 0.75 mg g^{-1}, representing a carotenoid of particular interest due to its reported anti-proliferative effects against human prostate cancer cells (Kotake-Nara et al. 2005). Chlorophylls and carotenoids are widely used as natural food colourants with remarkable antioxidant and anti-inflammatory properties, and the ability to prevent several diseases (Morales-Sánchez et al. 2017). Collectively, these findings reinforce the potential of *C. amblystomatis* as a promising candidate for industrial carotenoid production, especially considering the projected expansion of the global carotenoids market, expected to reach approximately US$ 2.9 billion by 2029 *(Global Carotenoids Markets Size and Industry Analysis 2029* 2025).

From an industrial perspective, the results obtained with *C. amblystomatis* highlight the practical significance of two-stage cultivation strategies for microalgal biomass production. By combining the high biomass productivity achieved under heterotrophic conditions with the improved biochemical composition associated with photoautotrophic growth, this approach addresses a major challenge in industrial microalgal cultivation, namely the trade-off between

biomass yield and biomass quality. The substantial recovery of protein content, pigment accumulation and fatty acid unsaturation observed during the photoautotrophic phase demonstrates that biomass quality can be enhanced without compromising the productivity advantages of heterotrophic cultivation. Similar responses have been reported for industrially relevant species such as *Chlorella* spp. and *Tetradesmus* spp., suggesting that *C. amblystomatis* possesses comparable potential for integration into industrial two-stage production processes. Furthermore, its ability to efficiently transition between trophic modes and maintain favourable growth performance highlights the versatility of this strain for cultivation schemes targeting high-quality biomass production.

Although the present results demonstrate the recovery of biomass biochemical composition under photoautotrophy, the definition of the most appropriate switching point could further improve process efficiency and biomass quality. In addition, further validation under larger-scale and more industrially representative systems would be important to confirm process robustness and scalability. Moreover, future studies could also evaluate the use of alternative, more complex and organic culture media, replacing synthetic media components with lower-cost substrates, including alternative carbon sources derived from waste streams, in addition to glucose, to further align the process with circular bioeconomy principles. Additionally, ensuring complete carbon (glucose) depletion prior to the transition to autotrophic cultivation should be systematically considered as a preventive measure to minimise the risk of bacterial contamination during scale-up.

Conclusions

This study demonstrated the strong potential of *Chlorococcum amblystomatis* as a robust microalga for industrial-scale biomass production. Heterotrophic fed-batch cultivation enabled very high cell densities (70.96 ± 0.35 g L^{-1}), representing the highest biomass concentration reported for this species to date. Importantly, the ability of *C. amblystomatis* to rapidly transition from heterotrophic to photoautotrophic growth without impairing performance supports the feasibility of a two-stage cultivation strategy, by using a concentrated inoculum obtained under heterotrophic conditions for the industrial-scale into photoautotrophic reactors. This approach represents a significant reduction of the scale-up time, as well as a significant improvement of the overall productivity of this bioprocess. Beyond process performance, the resulting biomass displayed a highly valuable biochemical profile, characterised by high protein content, a lipid fraction rich in polyunsaturated fatty acids, particularly the essential PUFA α-linolenic acid (ALA), resulting in favourable $\Sigma n{-}6/\Sigma n{-}3$ ratios. In addition, the recovery of chlorophylls and the accumulation of carotenoids such as lutein, β-carotene and neoxanthin further enhance its nutritional and biotechnological value. Importantly, this work addresses the gap between laboratory research and industrial application by validating a two-stage cultivation strategy at industrial scale while demonstrating the potential of a poorly explored yet industrially relevant microalgal strain. Overall, these results highlight *C. amblystomatis* as a versatile and promising microalga capable of combining efficient biomass production with the synthesis of high-value biomolecules. The proposed two-stage cultivation strategy therefore represents a promising approach for sustainable industrial production of nutrient-rich microalgal biomass for food, feed and nutraceutical applications.

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Declarations

Conflicts of interest The authors declare no competing interests.

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