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# Microalgal biomass production from brewery wastewater: a circular approach using *Chlorococcum amblystomatis* for sustainable valorisation

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**Abstract:** The integration of microalgal cultivation with wastewater treatment offers a sustainable strategy for resource recovery and environmental remediation. This study demonstrated the direct upcycling of brewery wastewater into *Chlorococcum amblystomatis* biomass, revealing strong metabolic robustness and intrinsic biotechnological potential, and providing a scientific basis for downstream valorisation pathways. Brewery wastewater did not exert toxic effects on microalgal cells, and urea supplementation significantly enhanced productivity, increasing maximum volumetric productivity by 138.3%, specific growth rate by 128.5%, and maximum biomass concentration by 21.7%. Although biomass concentration in supplemented wastewater ( $0.63 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ ) was lower than in the optimised medium ( $0.80 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ ), it remained comparable to the basal industrial medium ( $0.66 \pm 0.01 \text{ g} \cdot \text{L}^{-1}$ ), confirming its suitability as a cultivation substrate. Under outdoor flat-panel cultivation, growth performance in brewery wastewater was equivalent to that observed in the optimised medium, further supporting process scalability. *C. amblystomatis* exhibited high nutrient removal capacity, achieving  $70.5 \pm 6.4\%$  and  $92.6 \pm 1.5\%$  of nitrate-nitrogen and phosphate-phosphorus removals, respectively, confirming its effectiveness for wastewater bioremediation. Importantly, biochemical characterisation revealed remarkable stability across cultivation conditions, with consistently high protein content ( $59.86 \pm 1.88\%$ ), high omega-3 polyunsaturated fatty acids ( $28.47 \pm 3.19\%$  of  $\alpha$ -linolenic acid), and substantial carotenoid accumulation, including lutein ( $4.28 \pm 0.59 \text{ mg} \cdot \text{g}^{-1}$ ) and  $\beta$ -carotene ( $4.23 \pm 0.76 \text{ mg} \cdot \text{g}^{-1}$ ). Carbohydrate profiling revealed high content of arabinose ( $45.95 \pm 4.99 \text{ mol}\%$ ), suggesting the presence of polysaccharides with potential biotechnological relevance. Overall, *C. amblystomatis* combines efficient wastewater treatment with the production of high-value biomass, reinforcing its relevance within circular bioeconomy and supporting its further development for integrated environmental and biotechnological applications.

**Keywords:** Microalgae; Wastewater treatment; Brewery effluent; Water recovery; High value bioproducts

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## Introduction

Global water scarcity is expected to become one of the most critical social and economic challenges of the twenty-first century (Abdelfattah et al., 2022). The increasing demand for water across industrial sectors, including food and beverage, pharmaceuticals, and agriculture, together with the growing generation of wastewater, highlights the urgent need for more sustainable water management strategies (Ngo et al., 2022; Abdur Razzak et al., 2024). Rapid urbanisation and industrial activities have led to the production of large volumes of wastewater rich in organic matter and nutrients, often reflected in high chemical and biological oxygen demand (COD and BOD). When inadequately treated, these effluents pose significant risks to ecosystems and human health by promoting eutrophication through the excessive discharge of nutrients, particularly nitrogen and phosphorus. This nutrient enrichment stimulates uncontrolled algal and microbial proliferation, leading to oxygen depletion (hypoxia), disruption of aquatic ecosystems, biodiversity loss, and overall deterioration of water quality and usability (Abdelfattah et al., 2022; Fetisoa et al., 2024).

The high energy demand and reliance on chemical additives associated with conventional wastewater treatment processes have raised increasing concerns regarding their environmental and economic sustainability, despite their recognized effectiveness. Consequently, considerable attention has been directed toward the development of more sustainable and economically viable treatment alternatives for industrial effluents (Fetisoa et al., 2024; Pinar & Rodríguez-Couto, 2025). Among agro-industrial sectors, the brewing industry represents a major environmental challenge due to its intensive water consumption and the generation of approximately 3 to 10 L of effluent per litre of beer produced. Brewery wastewater is typically characterised by high concentrations of biodegradable organic matter, including sugars, ethanol, and volatile fatty acids, and high levels of carbon, nitrogen, and phosphorus, requiring adequate treatment before disposal into the environment (Amenorfenyo et al., 2019; Ferreira et al., 2019).

In this context, microalgae-based systems have emerged as promising and eco-efficient alternatives for wastewater remediation, owing to their capacity to assimilate nutrients and organic contaminants while producing oxygen through photosynthesis, thereby reducing aeration requirements and operational costs (Dias et al., 2023; Yu et al., 2024). In parallel, these systems enable the production of biomass that can be further valorised into high-value products, including biofertilisers, bioactive compounds, and environmental remediation agents (Acién et al., 2016; A. Ferreira et al., 2024). Importantly, food and beverage wastewaters, particularly brewery effluents, generally present lower toxicological and pathogenic risks than livestock-derived effluents, which may contain antibiotics, pathogens, and residual veterinary compounds. This lower-risk profile facilitates biomass upcycling for agricultural and biotechnological applications without significantly compromising biomass safety or quality (Ferreira et al., 2019, 2023; Bej et al., 2024).

Furthermore, recent studies have highlighted the potential of brewery wastewater as a sustainable and low-cost substrate for microalgal cultivation within circular bioeconomy frameworks, coupling nutrient recovery with the generation of valuable biomass while reducing the environmental burden associated with industrial effluents (Acién et al., 2016; Guignard et al., 2017).

Among the various microalgal species, *Chlorococcum amblystomatis* has emerged as a promising candidate for wastewater-based cultivation due to its tolerance to fluctuating environmental conditions, rapid growth rate, and ability to synthesise valuable biocompounds, such as proteins, fatty acids and pigments, under stress and nutrient-variable conditions (Conde et al., 2021; Correia et al., 2023). Given these characteristics, *C. amblystomatis* may represent a suitable candidate for brewery wastewater treatment and biomass valorisation. Therefore, the present study aimed to investigate the potential of *C. amblystomatis* for brewery wastewater treatment and subsequent biomass valorisation.

Specifically, microalgal growth performance in brewery effluent was evaluated, and the resulting biomass was characterised regarding its biochemical composition and potential for environmental applications. By integrating wastewater remediation with biomass production, this work contributes to the development of a more sustainable and circular approach for the management and upcycling of industrial effluents. To the best of the authors' knowledge, despite the growing interest in microalgae-based systems, only a limited number of studies have addressed their combined potential for integrated environmental remediation and agricultural applications. While the present study focuses on wastewater treatment and biomass biochemical characterisation, the valorisation of the resulting biomass for environmental and agricultural applications is explored in a complementary study. Within this framework, *C. amblystomatis* stands out as a highly promising yet still underexplored species.

## Materials and Methods

### Brewery wastewater characterisation

The brewery wastewater (BW) was supplied by Sociedade Central de Cervejas e Bebidas (SCC) (Vialonga, Portugal). The wastewater was pre-treated in a full-scale anaerobic BIOPAQ® IC reactor at the brewery's industrial wastewater treatment plant. The BW was stored at -17 °C until use and thawed at room temperature prior to experiments. Elemental analysis was performed using fresh samples. Additional characterisation, including nitrogen (ammonia and nitrates), phosphorus, and chemical oxygen demand (COD), was carried out after thawing and chlorine application for disinfection, both before and after microalgal cultivation. COD determination was carried out by the Open Reflux method – Method 5220-B (APHA, 2005). Ammonium (NH<sub>4</sub><sup>+</sup>) concentration was determined using a commercial test kit (Sera, Heinsberg, Germany), following the manufacturer's instructions. Absorbance was measured at 697 nm using a spectrophotometer (Genesys 10S UV–Vis), and concentrations were calculated based on a calibration curve previously established at Allmicroalgae. Nitrate (NO<sub>3</sub><sup>-</sup>) concentrations were determined using a Genesys 10S UV–VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA). A 0.1 mol L<sup>-1</sup> HCl solution was used as blank. Culture media and microalgal samples were centrifuged at 4500 × g for 15 min prior to analysis. Absorbance values were calculated as the difference between readings at 220 nm and twice the absorbance at 275 nm, and nitrate concentrations were determined by comparison with a calibration curve prepared using sodium nitrate. Phosphate concentration was determined using a similar commercial kit (Sera, Heinsberg, Germany), according to the manufacturer's protocol. Absorbance was measured at 916 nm using the same spectrophotometer, and quantification was performed using a previously established calibration curve.

To evaluate the efficiency of the microalgal-based treatment, the effluent was analysed after settling and filtration at the end of the cultivation period.

### Microalgal strain and growth conditions

*Chlorococcum amblystomatis* was obtained from the Allmicroalgae's culture collection (strain code AGF0066CA). All growth experiments were performed using a two-stage growth strategy, where heterotrophic culture was used to inoculate the autotrophic production systems.

To compare the growth performance of *C. amblystomatis*, the following culture media were tested: the optimised medium developed for this strain (Correia et al., 2023), Allmicroalgae's basal industrial medium (MNS), brewery wastewater without supplementation, and brewery wastewater supplemented with 4 mM nitrogen as urea.

Laboratory-scale trials were performed in 1-L bubble column reactors with a working volume of 0.8 L. Cultures were at an initial concentration of 0.4 g·L<sup>-1</sup>. The experiment

was conducted for 7 days at  $23 \pm 1$  °C under continuous LED irradiation of 100  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ , with continuous aeration using compressed air filtered through a 0.2- $\mu\text{m}$  membrane. The pH was maintained between 7.5 and 8.0 by controlled  $\text{CO}_2$  injection.

Scale-up to outdoor pilot-scale reactors was conducted as a proof of concept using laboratory-grown cultures to inoculate 50-L flat panel (FP) reactors. With an initial biomass concentration of 0.3  $\text{g}\cdot\text{L}^{-1}$ . The experiment was conducted over 7 days (from May 20th to May 27th, 2024). Cultures were aerated with 0.2- $\mu\text{m}$  filtered air, and pH was maintained between 6.5 and 7.0 by automated  $\text{CO}_2$  pulse injection. The average temperature was 18.5 °C and light irradiance average was 25  $\text{MJ}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ .

## Growth assessment

Growth of *C. amblystomatis* cultures under the different experimental conditions was monitored by daily sampling and analysis. Cell growth was assessed by measuring the optical density (OD) at 600 nm using a spectrophotometer (Genesys 10S UV–Vis). Dry weight (DW) was estimated based on a previously established calibration curve, described by Equation (1):

$$DW = 0.4914 \times OD_{600} + 0.2265 \quad (1)$$

The specific growth rate ( $\mu$ ) was calculated by the quotient of the linear regression of biomass concentration ( $X$ ) (in  $\text{g}\cdot\text{L}^{-1}$ ) over time ( $t$ ) within the exponential phase, expressed in  $\text{d}^{-1}$ , according to Equation (2):

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} \quad (2)$$

Biomass volumetric productivity ( $P$ ) was calculated as the difference in biomass concentration ( $X$ ) (in  $\text{g}\cdot\text{L}^{-1}$ ) over a given time interval ( $t$ ). Productivity was expressed in  $\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ , according to Equation (3):

$$P = \frac{X_2 - X_1}{t_2 - t_1} \quad (3)$$

## Elemental analysis, protein estimation and ash content determination

Biochemical composition analysis was performed on biomass harvested at the late exponential growth phase. The samples were freeze-dried prior to analysis.

Elemental analysis of carbon, hydrogen, and nitrogen was performed using a Vario EL III analyser (Elementar Analyser System GmbH, Hanau, Germany), following the manufacturer's instructions. Protein content was estimated from the measured nitrogen content using a conversion factor of 6.25, as reported for this strain (Correia et al., 2020).

Ash content was determined by measuring the weight difference before and after biomass combustion. Freeze-dried samples were heated at 550 °C for 4 h in a muffle furnace (LE062K17N1, Nabertherm, Germany).

## Carbohydrates analysis

Neutral sugar composition was determined following the method described by Ferreira et al., (2020). Briefly, a pre-hydrolysis of *C. amblystomatis* biomass (2 mg) was performed with 72% (w/w) sulphuric acid (200  $\mu\text{L}$ ) for 3 h at room temperature, followed by a hydrolysis with 1 M sulphuric acid (2.4 mL) for 2.5 h at 100 °C. After hydrolysis,

reduction and acetylation steps were performed. An internal standard (1 mg·mL<sup>-1</sup> of 2-deoxyglucose) was added (200 µL per sample), followed by neutralization with ammonium hydroxide. Monosaccharides were reduced by adding 100 µL of sodium borohydride (NaBH<sub>4</sub>; 15% w/v in 3 M NH<sub>3</sub>, Sigma-Aldrich) and incubating at 30 °C for 1 h. After cooling, excess borohydride was removed with glacial acetic acid. For acetylation, aliquots were transferred to reaction tubes, and 1-methylimidazole (450 µL) and acetic anhydride (3 mL) were added, followed by incubation at 30 °C for 30 min. The reaction was stopped in an ice bath by adding distilled water, and dichloromethane was used for phase separation. The organic phase containing the alditol acetate derivatives was washed repeatedly with distilled water, collected, and evaporated under vacuum. The alditol derivatives were re-dissolved in anhydrous acetone, evaporated again, and this step was repeated to ensure complete purification.

Alditol acetates were analyzed by gas chromatography with flame ionisation detection (GC-FID, Perkin Elmer Clarus 400, USA) using a DB-225 capillary column (30 m × 0.25 mm × 0.15 µm of film thickness, J&W Scientific, Folsom, CA). Samples were dissolved in anhydrous acetone, and 2 µL were injected. Injector and detector temperatures were set at 220 °C and 230 °C, respectively. The oven temperature program started at 200 °C, increased at 40 °C·min<sup>-1</sup> to 220 °C (held for 7 min), followed by a second slope of 20 °C·min<sup>-1</sup> to 230 °C (held for 1 min). The carrier gas (H<sub>2</sub>) had a flow rate set at 1.7 mL min<sup>-1</sup>. Monosaccharides were identified based on their chromatographic profiles by comparison with standards and quantified using the internal standard.

Uronic acids were quantified using the colorimetric 3-phenylphenol method (Blumenkrantz & Asboe-Hansen, 1973), with D-galacturonic acid used as standard for calibration. Following pre-hydrolysis as described for neutral sugars, the samples were hydrolysed in 2.4 mL of 1 M sulfuric acid for 1 h at 100 °C and then diluted (1:4, v/v). Briefly, 100 µL of each sample and standard was added to 1 mL of 200 mM boric acid prepared in 98% (w/w) sulfuric acid. The mixture was stirred and heated at 100 °C for 10 min. After cooling, 20 µL of *m*-phenylphenol solution (0.15% w/v in 0.5% NaOH) were added, and absorbance was measured at 520 nm. Carbohydrates were calculated by sum of neutral sugars and uronic acids content.

### Lipid extraction

Lipids were extracted from 25 mg of freeze-dried biomass based on Folch method as described in Lopes et al., (2026). Briefly, 2.5 mL of dichloromethane and 1.25 mL of methanol were added to each sample, followed by vortex mixing and incubation for 30 min on an orbital shaker. Extracts were centrifuged (Selecta JP Mixtasel, Abrera, Barcelona, Spain) at 2000 rpm for 10 min, and the supernatant was collected. The residual biomass was re-extracted twice as described above, and the combined supernatants were dried under a nitrogen (N<sub>2</sub>) stream.

The dried extracts were then re-dissolved in 2 mL of dichloromethane and 1 mL of methanol, vortexed, and mixed with 0.75 mL of Milli-Q water, followed by additional vortexing for 2 min. Phase separation was achieved by centrifugation at 660 × *g* for 10 min, and the organic phases were collected. The aqueous phase was re-extracted twice, as previously described, and the combined organic phases were again dried under a N<sub>2</sub> stream, transferred to pre-weighed vials and weighed to determine lipid content.

The total lipid content was calculated relative to the initial dry weight (DW) of the biomass.

### Fatty acids analysis

Fatty acid methyl esters (FAMES) were prepared from the total lipid extracts by alkaline transmethylation using a methanolic potassium hydroxide solution (2.0 M), as described by Lopes et al., (2026). A 2 µL aliquot of a hexane solution containing FAMES

and 1.0 µg·mL<sup>-1</sup> methyl nonadecanoate (Sigma, St. Louis, MO, USA), used as an internal standard, was injected into a gas chromatography–mass spectrometry (GC–MS) system (Agilent Technologies 8860 GC System, Santa Clara, CA, USA) equipped with a DB-FFAP column (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). The GC–MS system was coupled to a selective mass detector (Agilent 5977B). Helium was used as the carrier gas at a constant flow rate of 1.4 mL·min<sup>-1</sup>. The injector and detector temperatures were set at 220°C and 230°C, respectively, with an injection volume of 2 µL. The oven temperature program was as follows: 58 °C for 2 min, increased at 25 °C·min<sup>-1</sup> to 160 °C, followed by an increase of 2 °C·min<sup>-1</sup> to 210 °C, and finally 30 °C·min<sup>-1</sup> to 225 °C, maintained for 20 min. Data acquisition was performed with the software GCM 5977B/Enhanced MassHunter.

### Pigment extraction and quantification

Chlorophyll *a* and *b* were extracted and quantified according to Ritchie's method (Ritchie, 2008). Briefly, fresh biomass (10 mg) was extracted with 6 mL of acetone (99%) using glass beads, using a vortex mixer ELMI Intelli-mixer RM) for 10 min. The samples were then centrifuged at 2500 × *g* (HERMLE Z 400K) for 10 min, and the procedure was repeated until complete loss of colour in either the pellet or the supernatant. The supernatant was analysed using a spectrophotometer (Genesys 10S UV-VIS, Waltham, MA, USA) at 630, 647, 664, and 691 nm, and chlorophyll concentrations were calculated using Equations (4) and (5):

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

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

The carotenoid content was determined following the method described by (Couso et al., 2012). Briefly, 5–10 mg of freeze-dried biomass was extracted with 1 mL of methanol containing 0.03% butylhydroxytoluene and homogenised for 3 min using a mixer mill (MM400, Retsch GmbH, Germany) with glass beads at 20 Hz. Samples were centrifuged at 12000 × *g* for 6 min, and the extraction was repeated until complete loss of colour in the pellet or supernatant. Extracts were evaporated under nitrogen flow using a sample concentrator. The pellet was dissolved in 0.5 mL of high-performance liquid chromatography (HPLC) grade methanol and filtered through a 0.22 µm PTFE filter. Carotenoids were analysed using a Chromaster HPLC system (Hitachi, VWR, Portugal) equipped with a diode-array detector (5430 DAD, Hitachi, VWR, Portugal) 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), with the following gradient: 0–16 min, 0–60% A; 16–30 min, 60% A; 30–32 min, 100% A; and 32–35 min, 100% B. The injection volume was 100 µL. Carotenoids were identified using the Chromeleon Chromatography Data System (version 6.3, ThermoFisher Scientific, USA).

### Statistical analysis

All experiments were performed in triplicate (*n*=3). Results are presented as mean ± standard deviation for each experimental group. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to evaluate differences among the collected data.

GraphPad Prism software (version 10.4.2) was used for all statistical analyses, and differences were considered statistically significant when *p* < 0.05.

## Results

### Elemental composition of brewery wastewater

The given elemental composition of the brewery wastewater (BW) was evaluated and compared with the optimised culture medium (OPT) previously developed for *Chlorococcum amblystomatis*. The values are presented in Table 1. This comparison allowed a better understanding of the suitability of BW in relation to the nutritional requirements of this microalgal strain.

**Table 1** – Comparison of elemental composition of optimised medium (OPT) developed for *Chlorococcum amblystomatis* and brewery wastewater (BW).

| Element source                                                                       | OPT<br>mg·L <sup>-1</sup>                 | BW<br>(mg·L <sup>-1</sup> ) |
|--------------------------------------------------------------------------------------|-------------------------------------------|-----------------------------|
| Nitrogen source                                                                      | 720.72 (NH <sub>2</sub> ) <sub>2</sub> CO | 18.10 (NH <sub>3</sub> )    |
| NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O + K <sub>2</sub> HPO <sub>4</sub> | 73.27                                     | -                           |
|                                                                                      | P = 15.485                                | P = 14.977                  |
|                                                                                      | K = 9.230                                 | K = 79.191                  |
|                                                                                      | Na = 8.781                                | Na = 888.136                |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                                                 | 22.05                                     | -                           |
|                                                                                      | Ca = 6.012                                | Ca = 29.407                 |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                                                 | 67.84                                     | -                           |
|                                                                                      | Mg = 6.684                                | Mg = 10.873                 |
| FeSO <sub>4</sub> ·7H <sub>2</sub> O                                                 | 4.17                                      | -                           |
|                                                                                      | Fe = 0.838                                | Fe = 0.122                  |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                                                 | 21.57                                     | -                           |
|                                                                                      | Zn = 4.904                                | Zn < 0.001                  |
| Cl <sub>2</sub> Co·6H <sub>2</sub> O                                                 | 0.24                                      | -                           |
|                                                                                      | Co = 0.059                                | Co = 0.018                  |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O                                  | 0.60                                      | -                           |
|                                                                                      | Mo = 0.240                                | Mo < 0.001                  |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                                                  | 7.18                                      | -                           |
|                                                                                      | Mn = 2.335                                | Mn = 0.011                  |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                                                 | 0.06                                      | -                           |
|                                                                                      | Cu = 0.016                                | Cu < 0.001                  |
| H <sub>3</sub> BO <sub>3</sub>                                                       | 3.09                                      | -                           |
|                                                                                      | B = 0.541                                 | B = 0.180                   |
| NiCl <sub>2</sub> ·6H <sub>2</sub> O                                                 | 0.12                                      | -                           |
|                                                                                      | Ni = 0.029                                | Ni = 0.070                  |
| Vitamins Mix*                                                                        | 1 dose                                    | -                           |

\*Vitamins Mix: Thiamine-HCl (350 mg·L<sup>-1</sup>), d-Biotin (50 mg·L<sup>-1</sup>), cyanocobalamin (B12) (30 mg·L<sup>-1</sup>). Dose: 0.5mL·L<sup>-1</sup>.

According to the values presented in Table 1, the K concentration in the BW (79.191 mg·L<sup>-1</sup>) was considerably higher than that of the OPT (9.230 mg·L<sup>-1</sup>). A similar trend was observed for Ca, with values of 29.407 mg·L<sup>-1</sup> in the BW compared to 6.012 mg·L<sup>-1</sup> in the OPT. Furthermore, Na concentration in the BW was approximately 100 times higher than the value in OPT (8.781 mg·L<sup>-1</sup>), which could potentially inhibit microalgal growth performance.

### Cultivation in brewery wastewater

Growth performance of *C. amblystomatis* was analysed and compared between OPT, MNS (Allmicroalgae's industrial medium) and BW with and without nitrogen supplementation.

The biomass volumetric productivities, specific growth rates and biomass concentration of cultures grown with the different media, in 1-L bubble column, are described in Table 2 and growth curves are presented in Fig. 1.

**Table 2** - Maximum and overall volumetric productivities (g·L<sup>-1</sup>·d<sup>-1</sup>), specific growth rates (d<sup>-1</sup>), and maximum biomass concentration (g·L<sup>-1</sup>) of *Chlorococcum amblystomatis* grown in optimised medium (OPT), Allmicroalgae's industrial basal medium (MNS), brewery wastewater (BW) and brewery wastewater supplemented with urea (BW+U). Different letters indicate significant differences between conditions ( $p < 0.05$ ). Values are given as mean  $\pm$  standard deviation ( $n=3$ ).

|               | Volumetric productivity<br>(g·L <sup>-1</sup> ·d <sup>-1</sup> ) |                                | Specific growth<br>rate<br>(d <sup>-1</sup> ) | Maximum biomass con-<br>centration<br>(g·L <sup>-1</sup> ) |
|---------------|------------------------------------------------------------------|--------------------------------|-----------------------------------------------|------------------------------------------------------------|
|               | Overall                                                          | Maximum                        |                                               |                                                            |
| <b>BW</b>     | 0.02 $\pm$ 0.00 <sup>c</sup>                                     | 0.03 $\pm$ 0.01 <sup>c</sup>   | 0.07 $\pm$ 0.02 <sup>b</sup>                  | 0.52 $\pm$ 0.03 <sup>c</sup>                               |
| <b>BW + U</b> | 0.04 $\pm$ 0.00 <sup>b</sup>                                     | 0.07 $\pm$ 0.01 <sup>a,b</sup> | 0.16 $\pm$ 0.01 <sup>a</sup>                  | 0.63 $\pm$ 0.02 <sup>b</sup>                               |
| <b>MNS</b>    | 0.04 $\pm$ 0.00 <sup>b</sup>                                     | 0.05 $\pm$ 0.01 <sup>b,c</sup> | 0.11 $\pm$ 0.01 <sup>b</sup>                  | 0.66 $\pm$ 0.01 <sup>b</sup>                               |
| <b>OPT</b>    | 0.06 $\pm$ 0.00 <sup>a</sup>                                     | 0.09 $\pm$ 0.01 <sup>a</sup>   | 0.16 $\pm$ 0.01 <sup>a</sup>                  | 0.80 $\pm$ 0.02 <sup>a</sup>                               |

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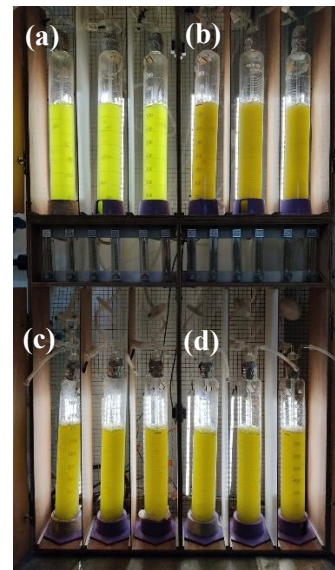
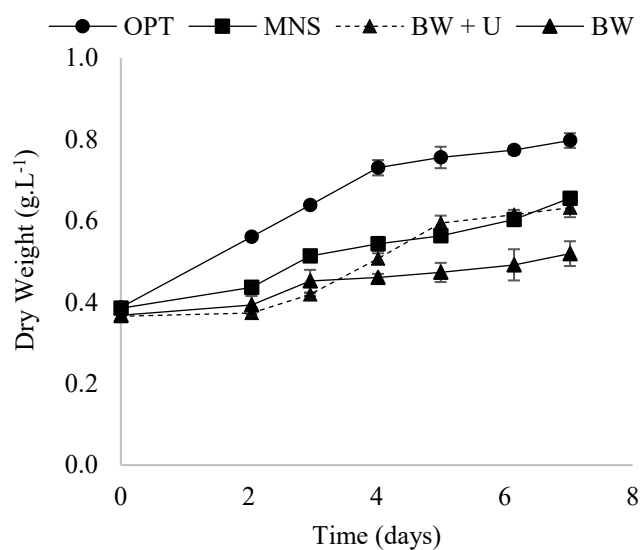


**Fig. 1** - Growth curves of *Chlorococcum amblystomatis* grown in 1-L bubble columns with: (a) optimised medium (OPT), (b) Allmicroalgae's industrial basal medium (MNS), (c) brewery wastewater (BW) and (d) brewery wastewater supplemented with urea (BW+U). Values are given as mean  $\pm$  deviation error bars ( $n=3$ ).

Supplementation of the brewery wastewater with urea (BW+U) resulted in significant improvements in growth performance compared to the non-supplemented wastewater (BW). According to the results presented in Table 2, increases of 92.9% in global volumetric productivity, 138.3% in maximum volumetric productivity, 128.5% in specific growth rate, and 21.7% in final biomass concentration were observed after supplementation.

Although biomass concentration obtained in cultures grown in BW+U ( $0.63 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ ) was lower than that obtained in cultures grown in the OPT (of  $0.80 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ ) ( $p < 0.05$ ), it was very similar to that achieved using the basal industrial medium (MNS) ( $0.66 \pm 0.01 \text{ g} \cdot \text{L}^{-1}$ ), as shown in Table 2. In fact, despite a longer lag phase observed in Fig. 1, cultures grown in BW+U showed increases of 33% in volumetric productivity and 42% in specific growth rate when compared with MNS (Table 2). The MNS medium is widely used for microalgal cultivation at Allmicroalgae, being adjusted according to the specific requirements of each species. For industrial-scale production, the use of a general, highly concentrated medium is often preferred because it simplifies logistics and can reduce operational costs.

The present results demonstrate that brewery effluent supplemented with urea can be successfully used for the cultivation of *Chlorococcum amblystomatis*, replacing the inorganic and more expensive industrial media without losses in productivity. This approach may contribute to reducing production costs while promoting wastewater reuse, supporting a more sustainable and circular microalgal production process.

### Pilot-scale outdoor validation

To better understand the impact of scale-up and the effect of outdoor conditions on biomass growth performance and biochemical composition, *C. amblystomatis* was cultivated in 50-L flat-panel photobioreactors. In order to assess the cultivation performance in brewery wastewater supplemented with urea (BW+U) relative to the maximum growth potential of *Chlorococcum amblystomatis*, only the optimised medium (OPT) was selected for comparison. The results obtained in OPT and BW+U conditions are presented in Table 3 and Fig. 2.

**Table 3** - Maximum and overall volumetric productivities ( $\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ ), specific growth rates ( $\text{d}^{-1}$ ), and maximum biomass concentration ( $\text{g}\cdot\text{L}^{-1}$ ) of *Chlorococcum amblystomatis* cultivated in flat panels with optimised medium (OPT) and brewery wastewater supplemented with urea (BW+U). No significant statistical differences were observed between conditions ( $p \geq 0.05$ ). Values are given as mean  $\pm$  standard deviation ( $n=3$ ).

|               | Volumetric productivity<br>( $\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ ) |                 | Specific<br>growth rate<br>( $\text{d}^{-1}$ ) | Maximum biomass<br>concentration<br>( $\text{g}\cdot\text{L}^{-1}$ ) |
|---------------|-------------------------------------------------------------------------------|-----------------|------------------------------------------------|----------------------------------------------------------------------|
|               | Overall                                                                       | Maximum         |                                                |                                                                      |
| <b>BW + U</b> | $0.04 \pm 0.01$                                                               | $0.07 \pm 0.03$ | $0.14 \pm 0.05$                                | $0.62 \pm 0.09$                                                      |
| <b>OPT</b>    | $0.05 \pm 0.01$                                                               | $0.08 \pm 0.01$ | $0.14 \pm 0.02$                                | $0.74 \pm 0.10$                                                      |

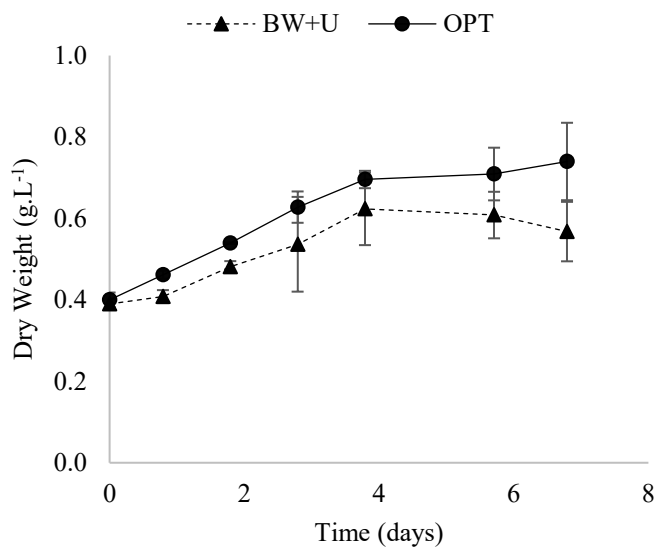


**Fig. 2** - Growth curves of *Chlorococcum amblystomatis* grown in 50-L flat panels (a) with optimised medium (OPT) and brewery wastewater supplemented with urea (BW+U). Values are given as mean  $\pm$  deviation error bars ( $n=3$ ).

The results obtained at pilot scale (Table 3) were very similar to those observed at laboratory scale (Table 2), indicating that the scale-up and outdoor cultivation conditions did not significantly affect the overall growth performance of *C. amblystomatis*.

Although BW+U exhibited slower growth during the first two days ( $p < 0.05$ ), no significant differences were observed between culture media during the subsequent growth stages (Fig. 2) or for the evaluated parameters (Table 3) ( $p \geq 0.05$ ).

### Brewery wastewater treatment after cultivation

To assess the effectiveness of microalgal-based wastewater treatment, the nutrient composition was determined before and after cultivation, focusing on nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), phosphate ( $\text{PO}_4^{3-}$ ), and chemical oxygen demand (COD). The results are shown in Table 4.

**Table 4** - Composition of brewery wastewater ( $\text{mg}\cdot\text{L}^{-1}$ ) before and after cultivation of *Chlorococcum amblystomatis* and respective removal percentage of nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), phosphate ( $\text{PO}_4^{3-}$ ), and chemical oxygen demand (COD). Different letters indicate significant differences between conditions ( $p < 0.05$ ). Values are given as mean  $\pm$  standard deviation ( $n=3$ ).

|                               | $\text{NO}_3^-$<br>( $\text{mg}\cdot\text{L}^{-1}$ ) | $\text{NH}_4^+$<br>( $\text{mg}\cdot\text{L}^{-1}$ ) | $\text{PO}_4^{3-}$<br>( $\text{mg}\cdot\text{L}^{-1}$ ) | COD<br>( $\text{mg}\cdot\text{L}^{-1}$ ) |
|-------------------------------|------------------------------------------------------|------------------------------------------------------|---------------------------------------------------------|------------------------------------------|
| Before microalgae cultivation | $410.0 \pm 5.4^a$                                    | $0.2 \pm 0.0^a$                                      | $29.8 \pm 0.4^a$                                        | $649.1 \pm 17.5^a$                       |
| After microalgae cultivation  | $120.9 \pm 26.2^b$                                   | $0.2 \pm 0.0^a$                                      | $2.2 \pm 0.4^b$                                         | $484.6 \pm 6.6^b$                        |
| Removal percentage            | $70.5 \pm 6.4$                                       | n.d.                                                 | $92.6 \pm 1.5$                                          | $25.3 \pm 1.0$                           |

<sup>1</sup> n.d.: not detected

The results demonstrated that after 7 days of cultivation,  $\text{NO}_3^-$  concentration decreased from  $410.03 \pm 5.4$  to  $120.9 \pm 26.2 \text{ mg}\cdot\text{L}^{-1}$ , corresponding to a nitrate–nitrogen ( $\text{N-NO}_3^-$ ) removal efficiency of 70.5%, while  $\text{PO}_4^{3-}$  concentration decreased from  $29.8 \pm 0.4$  to  $2.2 \pm 0.4 \text{ mg}\cdot\text{L}^{-1}$ , corresponding to a phosphate–phosphorus ( $\text{P-PO}_4^{3-}$ ) removal efficiency of 92.6%. In contrast, COD removal had an efficiency of 25.3%, corresponding to a reduction of  $164.5 \text{ mg O}_2\cdot\text{L}^{-1}$  (Table 4). Although an ammonium concentration of  $18.1 \text{ mg}\cdot\text{L}^{-1}$  (Table 1) was initially detected in the fresh sample, it was no longer measurable after thawing and chlorine application for disinfection, indicating its complete depletion or transformation.

### Proximate composition

Protein, lipid, carbohydrate, and ash contents were determined to characterise the biochemical composition of the biomass obtained from cultivation in flat panels with brewery wastewater supplemented with urea (BW+U), as well as to compare with the biomass produced under the reference condition, optimised medium (OPT). The results are presented in Table 5.

**Table 5** - Proximate composition (%) of the biomass of *Chlorococcum amblystomatis* cultivated in flat panels with brewery wastewater supplemented with urea (BW+U) and in optimised medium (OPT). Proteins, lipids, carbohydrates, and ash 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  $\pm$  standard deviation ( $n=3$ ).

| Reactor | Proteins<br>(%)    | Lipids<br>(%)      | Carbohydrates<br>(%) | Ash<br>(%)         |
|---------|--------------------|--------------------|----------------------|--------------------|
| BW + U  | $57.52 \pm 3.76^a$ | $11.26 \pm 0.58^a$ | $21.05 \pm 1.73^a$   | $9.64 \pm 1.36^b$  |
| OPT     | $59.86 \pm 1.88^a$ | $12.68 \pm 1.07^a$ | $16.81 \pm 1.16^b$   | $12.64 \pm 0.56^a$ |

Protein was the main macronutrient present in the biomass of *C. amblystomatis*, and no significant differences ( $p \geq 0.05$ ) were observed between the OPT and BW+U conditions, with protein contents of  $59.86 \pm 1.88$  and  $57.52 \pm 3.76\%$  of dry weight (DW), respectively (Table 5). Lipid content also showed no significant differences ( $p \geq 0.05$ ), with average values of  $12.68 \pm 1.07\%$  of DW for OPT and  $11.26 \pm 0.58\%$  of DW for BW+U. In contrast, carbohydrate content differed significantly between the two conditions ( $p <$

0.05), with biomass cultivated in BW+U presenting a higher percentage ( $21.05 \pm 1.73\%$  of DW) compared to OPT ( $16.81 \pm 1.16\%$  of DW). Ash content also showed significant differences ( $p < 0.05$ ), with higher values observed in OPT ( $12.64 \pm 0.56\%$  of DW) compared to BW+U ( $9.64 \pm 1.36\%$  of DW).

### Fatty acids profile

The fatty acid (FA) profile was assessed through quantification of the fatty acid methyl esters, expressed as the percentage of total esterified fatty acids (TEFA) (Table 6).

**Table 6** - Fatty acid profile, presented in percentage of total esterified fatty acids (TEFA) of *Chlorococcum amblystomatis* biomass cultivated in flat panels with brewery wastewater supplemented with urea (BW+U) and in optimised medium (OPT). No significant differences were observed between conditions ( $p \geq 0.05$ ). Values are given as mean  $\pm$  standard deviation ( $n=3$ ). Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

| Fatty acid                             | BW + U<br>(% of TEFA) | OPT<br>(% of TEFA) |
|----------------------------------------|-----------------------|--------------------|
| FA 14:0                                | $0.23 \pm 0.06$       | $0.23 \pm 0.12$    |
| FA 16:4 $\omega$ -3                    | $19.23 \pm 2.12$      | $18.05 \pm 1.85$   |
| FA 16:3 $\omega$ -3                    | $2.29 \pm 0.62$       | $2.77 \pm 0.27$    |
| FA 16:2 $\omega$ -6                    | $0.48 \pm 0.03$       | $0.53 \pm 0.08$    |
| FA 16:1 $\omega$ -7                    | $0.89 \pm 0.76$       | $0.87 \pm 0.34$    |
| FA 16:1                                | $1.52 \pm 0.51$       | $1.98 \pm 0.36$    |
| FA 16:0                                | $20.80 \pm 3.47$      | $23.07 \pm 1.23$   |
| FA 17:0                                | $0.12 \pm 0.11$       | $0.12 \pm 0.04$    |
| FA 18:4 $\omega$ -3                    | $5.78 \pm 1.48$       | $3.30 \pm 0.12$    |
| FA 18:3 $\omega$ -3                    | $23.69 \pm 3.78$      | $28.47 \pm 3.19$   |
| FA 18:3 $\omega$ -6                    | $3.25 \pm 0.81$       | $2.06 \pm 0.27$    |
| FA 18:2 $\omega$ -6                    | $2.87 \pm 0.42$       | $3.11 \pm 0.19$    |
| FA 18:1 $\omega$ -9                    | $1.85 \pm 0.29$       | $2.56 \pm 0.60$    |
| FA 18:1                                | $6.20 \pm 1.99$       | $6.62 \pm 0.61$    |
| FA 18:0                                | $10.80 \pm 6.64$      | $6.23 \pm 4.35$    |
| $\Sigma$ SFA                           | $31.96 \pm 3.40$      | $29.66 \pm 5.07$   |
| $\Sigma$ MUFA                          | $10.46 \pm 3.52$      | $12.04 \pm 1.35$   |
| $\Sigma$ PUFA                          | $57.58 \pm 5.20$      | $58.30 \pm 5.34$   |
| $\Sigma \omega$ -3                     | $50.98 \pm 4.83$      | $52.60 \pm 5.26$   |
| $\Sigma \omega$ -6                     | $6.60 \pm 0.48$       | $5.71 \pm 0.10$    |
| $\Sigma \omega$ -6/ $\Sigma \omega$ -3 | $0.13 \pm 0.01$       | $0.11 \pm 0.01$    |
| PUFA/SFA                               | $1.84 \pm 0.36$       | $2.05 \pm 0.47$    |

A total of fifteen fatty acids were identified in both *Chlorococcum amblystomatis* biomasses (Table 6). No significant differences ( $p \geq 0.05$ ) were observed between the two cultivation conditions (OPT and BW+U) for the individual fatty acids, indicating a robust, reproducible fatty acid profile, in this case independent of the utilised medium formulation. The dominant fatty acid in both conditions was FA 18:3 $\omega$ -3 ( $\alpha$ -linolenic acid, ALA), representing  $28.47 \pm 3.19\%$  of total esterified fatty acids (TEFA) in OPT and  $23.69 \pm 3.78\%$  in BW+U. The second and third most abundant fatty acid were FA 16:0, with values of  $23.07 \pm 1.23\%$  of TFA in OPT and  $20.80 \pm 3.47\%$  of TEFA in BW+U, followed by FA 16:4 $\omega$ -3, with  $18.05 \pm 1.85\%$  and  $19.23 \pm 2.12\%$  of TEFA in OPT and BW+U, respectively.

PUFAs represented 58.30% and 57.58% of TEFA in OPT and BW+U, respectively, while MUFAs accounted for 12.04±1.35% and 10.46±3.52%, and SFAs for 29.66±5.07% and 31.96±3.40% in OPT and BW+U, respectively.

## Pigments profile

Carotenoid and chlorophyll contents were also determined in *C. amblystomatis* biomass to enable the biochemical comparison between the two cultivation conditions. The results are presented in Table 7.

**Table 7** - Pigment profile (mg·g<sup>-1</sup>) of *Chlorococcum amblystomatis* biomass cultivated in flat panels with brewery wastewater supplemented with urea (BW+U) and in optimised medium (OPT). Different letters indicate significant differences between conditions ( $p < 0.05$ ). Values are given as means ± standard deviation ( $n=3$ ).

| Pigments                          | BW + U<br>(mg·g <sup>-1</sup> ) | OPT<br>(mg·g <sup>-1</sup> ) |
|-----------------------------------|---------------------------------|------------------------------|
| Neoxanthin                        | 0.62 ± 0.23 <sup>b</sup>        | 1.32 ± 0.27 <sup>a</sup>     |
| Lutein                            | 4.11 ± 0.26 <sup>a</sup>        | 4.28 ± 0.59 <sup>a</sup>     |
| $\beta$ -carotene                 | 4.23 ± 0.76 <sup>a</sup>        | 4.03 ± 1.04 <sup>a</sup>     |
| Chlorophyll <i>a</i> and <i>b</i> | 24.86 ± 4.25 <sup>a</sup>       | 23.07 ± 1.90 <sup>a</sup>    |

Chlorophyll content was similar in both cultivation conditions ( $p \geq 0.05$ ), with mean values of 23.07±1.90 and 24.86±4.25 mg·g<sup>-1</sup> of DW in OPT and BW+U, respectively.

The main carotenoids identified in *C. amblystomatis* were lutein and  $\beta$ -carotene. In the OPT condition, lutein and  $\beta$ -carotene contents were 4.28±0.59 and 4.03±1.04 mg·g<sup>-1</sup> of DW, respectively, while in BW+U the values were 4.11±0.26 and 4.23±0.76 mg·g<sup>-1</sup> of DW. No significant differences were detected between conditions ( $p \geq 0.05$ ).

Pigment analysis revealed that neoxanthin was the only pigment showing significant variation, with higher content observed in cultures grown in OPT (1.32±0.32 mg·g<sup>-1</sup>).

## Carbohydrates profile

The neutral sugars composition and total uronic acids content, determined after acid hydrolysis of *C. amblystomatis* biomass cultivated in optimized medium (OPT) and brewery wastewater supplemented with urea (BW+U), are presented in Table 8.

**Table 8** - Neutral sugars composition and total uronic acids content, presented as molar percentage (mol%) of *Chlorococcum amblystomatis* biomass cultivated in flat panels with brewery wastewater supplemented with urea (BW+U) and in optimised medium (OPT). Different letters indicate significant differences between reactors ( $p < 0.05$ ). Values are given as means ± standard deviation ( $n=3$ ).

| Sugars       | BW+U<br>(mol%)            | OPT<br>(mol%)             |
|--------------|---------------------------|---------------------------|
| Ribose       | 2.85 ± 0.52 <sup>b</sup>  | 4.84 ± 0.93 <sup>a</sup>  |
| Arabinose    | 45.95 ± 4.99 <sup>a</sup> | 44.58 ± 0.72 <sup>a</sup> |
| Xylose       | 6.69 ± 0.38 <sup>b</sup>  | 7.89 ± 0.41 <sup>a</sup>  |
| Mannose      | 6.88 ± 0.84 <sup>a</sup>  | 5.88 ± 1.28 <sup>a</sup>  |
| Galactose    | 23.11 ± 1.48 <sup>b</sup> | 26.47 ± 1.44 <sup>a</sup> |
| Glucose      | 8.86 ± 2.25 <sup>a</sup>  | 7.29 ± 4.00 <sup>a</sup>  |
| Uronic acids | 5.66 ± 3.83 <sup>a</sup>  | 3.06 ± 2.20 <sup>a</sup>  |

Beyond the differences observed in total carbohydrate content (Table 5), expressing the neutral sugar profile and uronic acid content on a molar percentage basis reveals significant differences in three sugars, namely galactose, xylose, and ribose. BW+U condition showed a lower molar percentage of these sugars compared to OPT. However, arabinose was the most abundant sugar in both conditions, accounting for  $44.58 \pm 0.72$  mol% in OPT and  $45.95 \pm 4.99$  mol% in BW+U, with no significant differences ( $p > 0.05$ ).

In biomass obtained under OPT conditions, the second most abundant monosaccharide was galactose ( $26.47 \pm 1.44$  mol%), followed by xylose ( $7.89 \pm 0.41$  mol%), glucose ( $7.29 \pm 4.00$  mol%), mannose ( $5.88 \pm 1.28$  mol%), ribose ( $4.84 \pm 0.93$  mol%), and uronic acids ( $3.06 \pm 2.20$  mol%). A similar profile was observed for BW+U, in which galactose was also the second most abundant sugar ( $23.11 \pm 1.48$  mol%), followed by glucose ( $8.86 \pm 2.25$  mol%), mannose ( $6.88 \pm 0.84$  mol%), xylose ( $6.69 \pm 0.38$  mol%), uronic acids ( $5.66 \pm 3.83$  mol%), and ribose ( $2.85 \pm 0.52$  mol%).

## Discussion

Brewery wastewater (BW) represents a valuable resource for microalgal cultivation due to its residual nutrient content and considerable water volume around the world, supporting its reuse within circular bioeconomy. The recovery and reutilisation of nutrients from industrial effluents not only reduces dependence on synthetic culture media but also mitigates the environmental risks associated with direct wastewater discharge, such as eutrophication and ecosystem imbalance (Ngo et al., 2022; Pinar & Rodríguez-Couto, 2025).

The composition of BW showed a clear imbalance in macronutrient availability compared to the optimised growth medium for *C. amblystomatis*. Nitrogen limitation was evident, indicating the need for supplementation to sustain higher biomass productivity, consistent with its role as a key growth-limiting nutrient in microalgal systems (Markou et al., 2019). The present results indicated that the composition of the brewery effluent did not show toxic effects on microalgal cells, providing suitable conditions for growth, with only a major adjustment requirement, namely nitrogen supplementation to sustain higher biomass production. This led to increases in 138.3% in maximum volumetric productivity, 128.5% in specific growth rate, and 21.7% in final biomass concentration (Table 2) compared to non-supplemented wastewater. Although biomass concentration, in laboratory trials, in supplemented wastewater ( $0.63 \pm 0.02$  g·L<sup>-1</sup>) was lower than in the optimised medium ( $0.80 \pm 0.02$  g·L<sup>-1</sup>), it was comparable to that obtained in the basal industrial medium (MNS) ( $0.66 \pm 0.01$  g·L<sup>-1</sup>). Furthermore, outdoor flat-panel cultivation resulted in comparable growth performance between the supplemented wastewater and the optimised medium ( $p \geq 0.05$ ), reinforcing the suitability of wastewater as an alternative cultivation medium for this microalga.

The MNS medium is widely used for microalgal cultivation at Allmicroalgae, being adjusted according to the specific requirements of each species. For industrial-scale production, the use of a general, highly concentrated medium is often preferred because it simplifies logistics and can reduce operational costs. The present results demonstrate that brewery effluent supplemented with urea can be successfully used for the cultivation of *Chlorococcum amblystomatis*, replacing the inorganic and more expensive industrial media. This approach may contribute to reducing production costs while promoting wastewater reuse, supporting a more sustainable and circular microalgal production process.

In agreement with the observed growth performance, *C. amblystomatis* exhibited a strong capacity for nutrient removal from brewery wastewater, particularly for nitrate and phosphate. Nitrogen and phosphorus are essential nutrients for primary production but, when in excess, contribute to eutrophication. (Guignard et al., 2017; Paerl et al., 2019).

The nitrate–nitrogen removal efficiency of  $70.5\pm6.4\%$  and phosphate–phosphorus removal efficiency of  $92.6\pm1.5\%$  (Table 4) demonstrate the strong performance of the microalgal treatment in nutrient remediation. Given the increasing concern regarding the depletion of global phosphorus reserves, phosphorus recovery from waste streams has emerged as a major priority within circular bioeconomy strategies (Yuan et al., 2012). In this context, the phosphorus removal efficiency achieved by *C. amblystomatis* was substantially higher than the 22% phosphorus removal reported by Ferreira et al. (2017) for *Tetrademus obliquus* (formerly *Scenedesmus obliquus*), cultivated in brewery effluent, and also exceeded the 69.32% total phosphorus removal reported for *Scenedesmus* sp. cultivated in aerobically digested brewery wastewater by Yirgu et al., (2023). Similarly, phosphorus removal efficiencies reported for *Chlorella vulgaris* ranged from 54–66% in brewery wastewater (Raposo et al., 2010) to approximately 90% in brewery wastewater (Darpito et al., 2014), while Gouveia et al., (2016) reported 92% phosphorus removal in urban wastewater. Therefore, the phosphate removal efficiency obtained for *C. amblystomatis* was comparable to or higher than those reported for other well-established chlorophyte microalgae, reinforcing the strong potential of this species for phosphorus recovery and brewery wastewater bioremediation applications.

Despite the observed decrease in nitrogen and phosphorus, the nitrate concentration ( $120.9\pm26.2\text{ mg}\cdot\text{L}^{-1}$ ) and COD ( $484.6\pm6.6\text{ mg}\cdot\text{L}^{-1}$ ) in the supernatant after cultivation remained above the emission limit values (ELVs) (50 and  $150\text{ mg}\cdot\text{L}^{-1}$ , respectively), established by Portuguese legislation (Decree-Law No. 236/98). This regulation is used to assess the feasibility of discharging the wastewater into aquatic environments or soil after biomass harvesting and separation, indicating that the treated wastewater did not meet the requirements for direct discharge into the environment. At industrial scale, the recycling and reuse of culture supernatants as a cultivation medium is a common practice. The supernatant obtained after biomass harvesting still contained residual nutrients, highlighting its potential for water reuse through subsequent cultivation cycles and additional nutrient removal. This suggests that the treated effluent could be recirculated within the cultivation process, contributing to improved resource efficiency and process sustainability. Previous studies have already demonstrated that water reuse in microalgal cultivation systems is not only technically possible but also operationally feasible (Cunha et al., 2026). This strategy may enable process scale-up and near-complete nutrient removal, allowing treated wastewater to meet discharge regulations and be safely released or reused for agricultural irrigation (Lima e Silva et al., 2024). Therefore, the reuse of this industrial effluent may thus represent an environmentally friendly alternative for microalgal cultivation, contributing to water recycling and to the reduction of production costs. This approach may allow a significant reduction in the consumption of fresh water, which is particularly relevant for large-scale microalgal production (de Souza Silva et al., 2025).

Ammonium, which was present at  $18.1\text{ mg}\cdot\text{L}^{-1}$  (Table 1) during the initial elemental characterisation, decreased to  $0.2\pm0.0\text{ mg}\cdot\text{L}^{-1}$  (Table 4) immediately before microalgal cultivation. This reduction is likely associated with the sample thawing process and the addition of chlorine for wastewater disinfection, which may have promoted ammonia oxidation, leading either to its conversion into nitrogen gas ( $\text{N}_2$ ) under breakpoint chlorination conditions or to nitrate ( $\text{NO}_3^-$ ) formation (Brandt et al., 2017; Mugwili et al., 2023). This chemical transformation may also explain the relatively high nitrate concentrations observed in the wastewater, suggesting that part of the reduced ammonia was oxidised rather than removed from the system.

The biochemical composition of *C. amblystomatis* cultivated in BW+U was assessed to validate the nutritional potential of this biomass. The protein content of microalgae is typically reported to range between 30 and 70% of dry weight (DW) (Dolganyuk et al., 2020). In agreement with previous studies on *C. amblystomatis*, protein was the main

macronutrient present in the biomass, presenting values from  $48.22 \pm 0.43$  to  $73.45 \pm 1.91\%$  of DW (Correia et al., 2020; 2023). In the present work, no significant differences were observed in protein content between the optimised medium (OPT) and brewery wastewater supplemented with urea (BW+U) conditions, with values of  $59.86 \pm 1.88$  and  $57.52 \pm 3.76\%$  DW (Table 5), respectively, indicating that cultivation in BW+U does not impair protein accumulation when compared to optimised conditions. Regarding the lipid content in microalgae is generally reported to vary between 20 and 50% of DW (Dolganyuk et al., 2020), Lower values have been described for species commonly used in food and feed applications. For example, *Chlorella vulgaris*, widely used for nutritional purposes, typically presents lipid contents ranging from 5 to 9% DW (Maurício et al., 2023) while *Chlorococcum amblystomatis* reported from  $13.28 \pm 0.45$  to  $31.44 \pm 4.10\%$  of DW (Conde et al., 2021; Correia et al., 2020; 2023). In the present work, the total lipid content, from  $11.26 \pm 0.58$  to  $12.68 \pm 1.07\%$  obtained for BW+U and OPT, respectively, is in line with the previous described for the same species. The absence of significant differences between conditions further confirms that BW+U can sustain lipid production at levels comparable to those achieved in OPT medium. In contrast, carbohydrate content differed significantly, presenting a higher percentage ( $21.05 \pm 1.73\%$ ) in biomass cultivated in BW+U compared to OPT ( $16.81 \pm 1.16\%$ ). The higher carbohydrate content observed in wastewater-grown biomass compared to optimised medium can be attributed to nutrient imbalance, which can influence carbon partitioning and storage metabolism in microalgal cells, being considered a key mechanism of carbon partitioning under stress conditions. This might be advantageous, as polysaccharides are known to protect microalgae from environmental stress (Hanifzadeh et al., 2018; Maltsev et al., 2023). Ash content also differed significantly between conditions, with higher values observed in OPT ( $12.64 \pm 0.56\%$ ) compared to BW+U ( $9.64 \pm 1.36\%$ ). Wastewater-based media often present a more variable and less balanced mineral composition, which may limit the assimilation of certain inorganic elements. Additionally, the lower ash content in BW+U may be associated with the higher accumulation of organic compounds, such as carbohydrates, under nutrient stress conditions, leading to a relative decrease in mineral fraction (Chia et al., 2018).

Fatty acid profiles from both cultivation conditions were characterised by a predominance of  $\alpha$ -linolenic acid (ALA, FA  $18:3n - 3$ ), accounting for  $28.47 \pm 3.19\%$  of total esterified fatty acids (TEFA) in OPT and  $23.69 \pm 3.78\%$  in BW+U. Polyunsaturated fatty acids (PUFAs) represented  $58.30 \pm 5.34\%$  and  $57.58 \pm 5.20\%$  of TEFA in OPT and BW+U, respectively, confirming the high degree of lipid unsaturation in this strain. These findings are consistent with previous reports for the same microalgal strain, which also identified ALA as the dominant fatty acid and highlighted its high PUFA content (Correia et al., 2023). The elevated proportion of PUFAs, particularly  $\omega$ -3 fatty acids such as ALA, reinforces the potential of this microalga as a valuable source of nutritionally relevant lipids for food, feed, and biotechnological applications (Baker et al., 2016; Ahmmed et al., 2020; Gutierrez et al., 2025). No significant differences were observed in the overall fatty acid composition between the two cultivation conditions. This indicates that growth in brewery wastewater does not adversely affect lipid quality, preserving the biochemical profile of the biomass. Such stability is particularly advantageous for industrial applications, as it supports the use of low-cost and sustainable substrates without compromising the production of high-value lipid fractions (Markou et al., 2019; Ngo et al., 2022; Lima e Silva et al., 2024).

Carotenoid-derived compounds, including xanthophylls such as neoxanthin, act as key intermediates in abscisic acid (ABA) biosynthesis, a central phytohormone regulating seed germination and plant stress responses. In addition, neoxanthin contributes directly to photoprotection and the mitigation of oxidative stress, enhancing plant resilience under adverse environmental conditions (Jia et al., 2022; Mo et al., 2024). Consistent with this

functional relevance, pigment analysis revealed that neoxanthin was the only pigment showing variation between cultivation conditions, with higher content observed under optimised medium ( $1.32 \pm 0.27 \text{ mg} \cdot \text{g}^{-1}$ ).

Overall, pigment composition did not show substantial differences between the two cultivation conditions. This strain maintained high levels of lutein and  $\beta$ -carotene, ranging from  $4.11 \pm 0.26$  to  $4.28 \pm 0.59 \text{ mg} \cdot \text{g}^{-1}$  and  $4.03 \pm 1.04$  to  $4.23 \pm 0.76 \text{ mg} \cdot \text{g}^{-1}$ , respectively. These values are in line with those reported for well-known pigment-producing microalgae, further supporting the potential of this strain for carotenoid-based applications (Del Campo et al., 2007). Pigments have received increasing attention due to their antioxidant properties and broad applications in the food and cosmetic industries (Iwamoto et al., 2024; Varela et al., 2015). The growing demand for natural pigment sources supports the use of *C. amblystomatis* as a potential alternative. This microalga, even when cultivated in brewery wastewater, showed high contents of chlorophylls and lutein without the need for intentional biosynthesis induction, indicating its suitability for applications requiring natural, high-value bioactive compounds.

In general, microalgal polysaccharides are heteropolymers, i.e., they are composed of different monosaccharides present in varying proportions and linked by glycosidic bonds (Costa et al., 2021). However, their detailed composition and structure is species-specific. In the present study, arabinose was the predominant monosaccharide quantified after hydrolysis in both cultivation conditions, accounting for  $44.58 \pm 0.72 \text{ mol}\%$  in OPT and  $45.95 \pm 4.99 \text{ mol}\%$  in BW+U. Galactose was the second most abundant sugar, representing  $26.47 \pm 1.44 \text{ mol}\%$  in OPT and  $23.11 \text{ mol}\%$  in BW+U. The exopolysaccharides of Chlorophytes exhibit considerable heterogeneity among species. However, galactose is frequently reported as the predominant monosaccharide, often accompanied by glucose, while arabinose is also commonly detected, although generally at lower proportions (Laroche, 2022). In previous studies, arabinose was identified as the major monosaccharide in *Chlorococcum* sp., followed by galactose (Badary et al., 2024). To the best of the authors' knowledge, no studies have specifically described the detailed monosaccharide composition of *Chlorococcum amblystomatis*. Nevertheless, the monosaccharide profile observed in the present study is consistent with previous investigations on *Chlorococcum*, which reported heteropolysaccharides predominantly composed of arabinose and galactose. This monosaccharides profile is markedly different from that commonly reported for *Chlorella* species. For instance, Banskota et al., (2024) reported galactose and glucose as the main constituents in *Chlorella sorokiniana*, while arabinose was not detected. This defines a fundamentally distinct monosaccharide signature for *C. amblystomatis* and may confer added value in terms of novelty and functional diversification of microalgal biopolymers. Arabinose occurs in both D- and L-isoforms, with L-arabinose being commonly associated with plant and algal cell wall matrices (Kotake et al., 2016). Beyond its structural role, L-arabinose has been reported to exhibit functional bioactivities, including modulation of glucose metabolism and antioxidant-related effects, which may contribute to protection against hyperglycaemia (Newman et al., 2025). Overall, the dominance of arabinose clearly distinguishes *C. amblystomatis* from established *Chlorella* production strains and expands the known diversity of microalgal polysaccharide architectures. In contrast, the low glucose content suggests a limited presence of cellulose in the cell wall, supporting the idea of a branched arabinogalactan-like matrix rather than a cellulose-dominated architecture. This unique biochemical fingerprint reinforces its potential as a non-conventional and high-value platform for the development of functional polysaccharides with applications in several biotechnological sectors, including biodegradable films, thickeners, and bioactive compounds (e.g., antioxidant or immunomodulatory effects) (Khavari et al., 2021; Moreira et al., 2022). On the other hand, uronic acids confer an anionic nature to exopolysaccharides (EPS), a feature that enhances their affinity for metal ions and makes them promising candidates for bioremediation applications

(Naveed et al., 2019; Costa et al., 2021). The presence of uronic acids in the biomass of *C. amblystomatis*, with higher levels observed under brewery wastewater cultivation (BW+U,  $5.66 \pm 3.83$  mol%) compared to OPT ( $3.06 \pm 2.20$  mol%), suggests a potential role of this biomass in the removal of heavy metals from contaminated environments. Moreover, microalgal polysaccharides have also been associated with plant biostimulant activity and stress mitigation, further supporting the potential of *C. amblystomatis* biomass for downstream biotechnological applications (Prisa & Spagnuolo, 2023).

Despite some differences, the overall biochemical profile of the biomass grown with BW+U remained within the range for the biomass grown with OPT. The ability to maintain similar biochemical composition while using an industrial effluent as culture medium highlights the potential of this approach for reducing production costs and freshwater consumption. In addition, the reuse of brewery wastewater contributes to a more sustainable and circular production process, supporting the integration of microalgal cultivation into industrial wastewater management strategies (Ngo et al., 2022; Pinar & Rodríguez-Couto, 2025). However, despite this potential, regulatory constraints remain a significant limitation for the valorisation of biomass produced on waste-derived media. Current frameworks, particularly within the European Food Safety Authority and under regulations such as Regulation (EU) 2015/2283, impose strict safety and traceability requirements that may restrict the use of such biomass in food and feed applications, even when the waste originates from the food industry. This regulatory uncertainty represents a key barrier to the large-scale implementation of this otherwise promising and sustainable approach (Caporgno & Mathys, 2018).

Overall, these findings highlight the potential of *C. amblystomatis* cultivated in brewery wastewater as an effective and sustainable approach for wastewater valorisation, reinforcing its relevance within circular bioeconomy strategies. This biomass can be further explored in different application contexts, including agricultural uses, reinforcing its relevance within circular bioeconomy strategies.

## Conclusions

This study demonstrated that *C. amblystomatis* can be effectively cultivated in brewery wastewater supplemented with urea without compromising productivity, biochemical composition, or functional properties. The use of industrial effluents as culture media represents a sustainable and cost-effective strategy, enabling simultaneous wastewater valorisation and the production of functional biomass with applications in agriculture and environmental bioremediation. Importantly, the biomass produced under both cultivation conditions exhibited a valuable biochemical profile, characterised by high protein content, together with relevant levels of  $\omega$ -3 fatty acids, lutein and  $\beta$ -carotene, and structurally distinctive polysaccharides enriched in arabinose and galactose. These components further enhance its interest for several biotechnological applications, reinforcing the potential of this strain. In this context, wastewater-grown biomass provides a dual benefit by coupling resource recovery with environmental remediation. This integrated approach aligns strongly with circular bioeconomy principles, supporting the reuse of wastewater-derived biomass for pollution mitigation and contributing to more sustainable environment. Overall, this work confirms the feasibility of directly upcycling brewery wastewater into metabolically robust *Chlorococcum amblystomatis* biomass with intrinsic biotechnological value, establishing a solid foundation for its future application in downstream valorisation strategies.

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