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Mixotrophic cultivation of *Galdieria sulphuraria* for C-phycocyanin and protein production

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ABSTRACT

G. sulphuraria is a polyextremophilic microalga that can tolerate low pH, high temperature and high osmotic pressure. We cultivated *G. sulphuraria* ACUF 064 in chemostat at a biomass concentration of 134 to 243 $g \cdot m^{-2}$ aiming for maximal pigment content without compromising biomass productivity. Autotrophy was compared to 'oxygen balanced' mixotrophy with intracellular recirculation of oxygen and carbon dioxide. No differences were found in C-phycocyanin (*C-PC*) and protein content between autotrophic and mixotrophic cultures. In mixotrophy the biomass productivity and concentration were doubled compared to the photoautotrophic counterpart. In mixotrophy aeration was not needed and 89% of the substrate carbon was converted into biomass.

Mixotrophically grown biomass contained 10% w/w *C-PC* which, combined with its high areal biomass productivity (49 $g \cdot m^{-2} \cdot day^{-1}$), sums up as one of the highest *C-PC* areal productivities ever reported (5 $g \cdot m^{-2} \cdot day^{-1}$) under 24 h/24 h illumination. *C-PC* extracted from *G. sulphuraria* was more stable than the currently used *C-PC* extracts from *Spirulina*. No significant loss of color was observed down to a pH of 3 and up to a temperature of 55 °C. *G. sulphuraria* had a protein content of 62% w/w and compared favorably with FAO dietary recommendation of adults regarding amino acid composition. *G. sulphuraria* contains a high proportion of essential, sulfur amino acids compared to *Chlorella*, *Spirulina* and soybean protein.

Due to its attractive amino acid profile and high protein content, *G. sulphuraria* is a good candidate for food and feed applications to overcome sulfur amino acid deficiencies. In addition, oxygen balanced mixotrophy allows for efficient and productive cultivation of *G. sulphuraria* biomass.

1. Introduction

Galdieria sulphuraria is a polyextremophilic microalgae able to tolerate low pH (as low as 0.2) [1], high temperature (up to 57 °C) [2] and high osmotic pressure (up to 400 $g \cdot L^{-1}$ of sugar and 2–3 M of salt) [3]. Due to these exceptional traits, *G. sulphuraria* is often the only organism able to colonize acidic hot springs where it forms mats of deep blue-green color [4]. This peculiar color is due to the presence of blue phycobiliproteins C-phycocyanin (*C-PC*), allophycocyanin, and chlorophyll *a* [5]. Phycocyanins are used in diagnostic histochemistry and as colorants in cosmetics and foods. Phycocyanins have also been found to have antioxidant properties and may have potential as therapeutic agents [6].

When cultivated autotrophically, *G. sulphuraria* expresses a *C-PC* content of 10% w/w, similar to the *C-PC* content commonly reported in *Arthrospira platensis* [7–10], henceforth referred to with its commercial name *Spirulina*. Compared to the *C-PC* extracted from *Spirulina*, *C-PC* extracted from *Galdieria* has shown greater stability at low pH and at high temperature, increasing the possible industrial applications of this pigment [11,12].

Nowadays commercial production of *C-PC* is almost exclusively based on *Spirulina* cultivation and extraction. *Spirulina* extracts were approved for use in candy, chewing gum and other types of confection in the US in 2013 and 2014 [13]. In the EU *Spirulina* extracts were approved in 2013 as coloring food [14]. While *Spirulina* has been consumed for centuries [15] and its consumption is approved and

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considered safe worldwide, *G. sulphuraria* has no previous history of use in feed or foods. Recent studies indicated that *G. sulphuraria* could be safe as food ingredient or supplements [16,17], but *G. sulphuraria* still needs to be approved as novel food before it can be used as such.

G. sulphuraria is also considered as a promising source for protein [17]. The protein content of *G. sulphuraria* has been reported in several studies resulting in a range from as little as 22% to an impressive 72% of total biomass dry weight [9,18–20]. In addition to bulk protein content, protein quality in terms of amino acid composition is a core marker for nutritional value. Humans have a limited ability to (bio)synthesize amino acids, out of the 20 common amino acids, nine "essential" amino acids must be provided through food for adults [21]. Therefore, the amount of essential amino acids is key in determining the quality of a protein source. To the best of our knowledge, there have been no studies on the amino acid profile of *G. sulphuraria*. In this work we investigated the amino acid profile of *G. sulphuraria* in chemostat, both under autotrophy and a recently designed 'oxygen balanced' mixotrophic cultivation [22,23].

'Oxygen balanced' mixotrophy allows for operation of a closed photobioreactor without any gas exchange [22,23]. This is achieved by carefully adjusting substrate supply so that photosynthetic oxygen production is balanced with respiratory oxygen consumption. In turn, carbon dioxide released by substrate oxidation is recycled by the photosynthetic machinery, resulting in only a small loss of the organic carbon provided. Previously, we successfully applied this cultivation strategy to *G. sulphuraria* and due to the low pH, the reactor was operated for over two months without contamination [1]. *G. sulphuraria* proved to be photosensitive, which is the reason why optimization of the light regime was a key point for successful cultivation. In order to find the optimal specific light supply rate (q_{ph}, $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$) in our previous work, we cultivated *G. sulphuraria* autotrophically and mixotrophically in repeated batch [1]. After each dilution, we found evidence of photoinhibition due to the sudden change in q_{ph}. Photoinhibition was followed by a period of photo-acclimation, during which pigmentation of the culture was reduced and the signs of photoinhibition disappeared. The autotrophic culture reached maximum biomass productivity between biomass concentrations of 2 and 5 $g \cdot L^{-1}$. When biomass concentration surpassed this range, light limitation became more evident and biomass productivity decreased. The productivity of the mixotrophic culture progressively increased during each batch and in the third and last batch linear growth could be maintained until a biomass concentration of 9.7 $g \cdot L^{-1}$. Despite the high productivities that were obtained, it was concluded that ideally *G. sulphuraria* is cultivated under a constant light regime without stepwise reductions of biomass concentration as in repeated batch.

In this work we cultivated *G. sulphuraria* in chemostat, in which the specific light supply rate q_{ph} can be maintained constant to obtain stable biomass and pigment production. In order to reach this goal, we estimated a dilution rate based on our previous work [1], in which maximal pigment content could be achieved without negatively affecting biomass productivity. Once the steady state was reached, C-phycocyanin (*C-PC*) and protein content, as well as amino acid profile of the produced biomass were determined. The produced *C-PC* was tested for its acid- and thermostability. In addition, autotrophic and oxygen balanced mixotrophic cultures were compared with respect to biomass productivity.

2. Materials and methods

2.1. Strain, growth conditions and medium

Galdieria sulphuraria ACUF 064 (http://www.acuf.net) was kindly provided by Prof. A. Pollio (University of Naples, Italy) while *Arthrospira platensis* (Gomont) Geitler A1 was provided by Algreen B.V. (The Netherlands). Axenic stock cultures of *G. sulphuraria* were incubated in 250 mL flasks containing 100 mL of culture at 37 °C, 2% v/v CO_2, 120 rpm, and under a photon flux density (*PFD*) of 75 $\mu mol\ m^{-2} \cdot s^{-1}$. These cultures were used to inoculate the photobioreactor for the experiments described below. The medium used for flask and reactor cultivation contained the following components at the concentration given ($mol \cdot L^{-1}$): $12.2 \cdot 10^{-3}$ H_3PO_4, $80.0 \cdot 10^{-3}$ $(NH_4)_2SO_4$, $6.5 \cdot 10^{-3}$ $MgSO_4 \cdot 7H_2O$, $4.7 \cdot 10^{-4}$ $CaCl_2 \cdot 2H_2O$, $6.3 \cdot 10^{-4}$ FeNaEDTA, $0.2 \cdot 10^{-3}$ $Na_2EDTA \cdot 2H_2O$, $1.7 \cdot 10^{-3}$ NaCl, $8.1 \cdot 10^{-3}$ KCl, $8.0 \cdot 10^{-4}$ H_3BO_3, $8.1 \cdot 10^{-5}$ $MnCl_2 \cdot 4H_2O$, $8.2 \cdot 10^{-5}$ $ZnCl_2$, $3.2 \cdot 10^{-5}$ $CuSO_4 \cdot 5H_2O$, $1.7 \cdot 10^{-5}$ $Na_2MoO_4 \cdot 2H_2O$ and $1.7 \cdot 10^{-5}$ $CoCl_2 \cdot 6H_2O$. pH was adjusted to 1.8 with 6 $mL \cdot L^{-1}$ of 2 M H_2SO_4.

A. platensis cultures were incubated in 250 mL flasks containing 100 mL of Zarrouk medium [24] at pH 9.2, 25 °C, 2% v/v CO_2, 120 rpm and under a *PFD* of 75 $\mu mol\ m^{-2} \cdot s^{-1}$. The flasks were inoculated at an initial biomass concentration of 0.3 $g \cdot L^{-1}$ and harvested at the end of the linear phase. These cultures were used for the extraction of phycocyanins.

2.2. Photobioreactor setup and experiments

Autotrophic and mixotrophic cultures of *G. sulphuraria* were performed in a stirred-tank bioreactor with a volume of 3 L and an internal diameter of 0.13 m (Applikon, The Netherlands). During the experiments, the working volume (V_{PBR}) was kept at 2 L by maintaining the liquid height at 0.165 m. The resulting cylindrical illuminated surface (*IS*) was 0.067 m^2. The reactor was continuously stirred at 500 rpm and homogeneously illuminated over the cylindrical vessel surface with an average *PFD* of 511 ± 29 $\mu mol \cdot m^{-2} \cdot s^{-1}$, as described in detail in Abiusi et al. [22]. The temperature was maintained at 37 °C by a heat exchanger placed inside the reactor. Water evaporation was prevented with a condenser connected to a cryostat that fed water at 2 °C. The pH was controlled at 1.8 by automatic base addition (2 M NaOH). Dissolved oxygen (*DO*) was measured by a VisiFerm DO ECS 225 *DO* sensor (Hamilton, USA). The sensor was calibrated inside the reactor containing medium at aforesaid working temperature and pH. Dinitrogen gas (N_2) and air sparging were applied to obtain 0% and 100% *DO* reads, respectively. For autotrophic operation the reactor was sparged with air enriched with 2% v/v CO_2 at a flow rate of 1 $L \cdot min^{-1}$ using Smart TMF 5850S mass flow controllers (Brooks Instruments, USA).

The reactor was inoculated with an initial biomass concentration of 1.25 $g \cdot L^{-1}$. It was operated in batch until a biomass concentration (C_x) of 4 $g \cdot L^{-1}$ was reached after which the system was operated in autotrophic chemostat mode. Reactor volume was maintained constant by a level-probe based control system. A dilution rate (D) of 0.2 day^{-1} was chosen as optimal for *G. sulphuraria* ACUF 064 based on our previous study [1]:

$$D = \frac{r_{x,opt}}{C_{x,opt}} \quad (1)$$

where $C_{x,opt}$ (4.8 $g \cdot L^{-1}$) represents the maximal concentration within the range of linear growth during autotrophic repeated batch and $r_{x,opt}$ (0.97 $g \cdot L^{-1} \cdot day^{-1}$) the volumetric productivity achieved at that range. After steady state was reached under such autotrophic conditions, the harvest bottle was placed in ice-cooled water bath and the harvested biomass was daily collected and analyzed for 3 consecutive days. Dry weight and phycocyanin contents were measured, and amino acid composition was determined. Additional samples were taken aseptically from the reactor for measurements of photosystem II maximum quantum, absorption cross section and carbon and nitrogen content of the biomass.

After the autotrophic chemostat, the reactor was operated in batch for 1.5 days with constant glucose addition and sparging with 2% CO_2 enriched air. A 10% w/w glucose solution was added at a rate of 3.7 $g \cdot h^{-1}$. After this period of adaptation to glucose, aeration was switched off and the dissolved oxygen level (*DO*) was controlled at 90% air saturation by 10% w/w glucose solution addition (i.e. oxygen-balanced mixotrophy). Only when a C_x of 8 $g \cdot L^{-1}$ was reached, chemostat

operation was activated again. Also in the mixotrophic culture a dilution rate (D) of 0.2 day^{-1} was chosen as optimal. The D was calculated using Eq. (1) and a value of 8.6 $g{\cdot}L^{-1}$ and 1.72 $g{\cdot}L^{-1}{\cdot}day^{-1}$ were used for $C_{x,opt}$ and $r_{x,opt}$ respectively. After steady state was reached under oxygen-balanced mixotrophic conditions, culture was harvested daily for 4 days. Identical analyses as under the autotrophic reference condition were done and in addition glucose analysis from the reactor was performed several times per day.

2.3. Photobioreactor calculations

Volumetric biomass productivity r^V_x (r^V_x, $g{\cdot}L^{-1}{\cdot}day^{-1}$) was determined by multiplying the measured C_x in the harvest with the measured reactor dilution rate D. Areal biomass productivity (r^A_x, $g{\cdot}m^{-2}{\cdot}day^{-1}$) was calculated as follows:

$$r^A_x = \frac{r^V_x \cdot V_{PBR}}{IS} \tag{2}$$

where IS is the illuminated reactor area (m^2). In the autotrophic chemostat, r^V_x was used to calculate the autotrophic yield of biomass on photons ($Y_{x/ph}$, $g_x{\cdot}mol_{ph}{}^{-1}$) according to the formula:

$$Y_{x/ph} = \frac{r^V_x \cdot V_{PBR}}{PFD \cdot IS} \tag{3}$$

In the mixotrophic chemostat, first the volumetric substrate consumption rate (r_s, $g_s{\cdot}L^{-1}{\cdot}day^{-1}$) was calculated:

$$r_s = \frac{F_{glu} \cdot C_{sglu} - D \cdot V_{PBR} \cdot C_s}{V_{PBR}} \tag{4}$$

where F_{glu} ($L{\cdot}day^{-1}$) and C_{sglu} ($C\text{-}g_s{\cdot}L^{-1}$) stand for the glucose feeding rate and the carbon concentration in the solution. C_s ($C\text{-}g_s{\cdot}L^{-1}$) represents the carbon substrate concentration measured in the reactor. Then, the mixotrophic biomass yield on substrate ($Y_{x/s}$, $C\text{-}g_x{\cdot}g_s{}^{-1}$) was derived as follows:

$$Y_{x/s} = \frac{r^V_x \cdot C_{\%}}{r_s} \tag{5}$$

where $C_{\%}$ (% $w_C{\cdot}w_x{}^{-1}$) represents the carbon content in the harvested biomass. C-phycocyanin volumetric $r^V_{C\text{-}PC}$ ($r^V_{C\text{-}PC}$, $g{\cdot}L^{-1}{\cdot}day^{-1}$) and areal ($r^A_{C\text{-}PC}$, $g{\cdot}m^{-2}{\cdot}day^{-1}$) productivities were calculated by multiplying r^V_x and r^A_x by the phycocyanin content in the biomass (% $w_{C\text{-}PC}{\cdot}w_x{}^{-1}$).

3. Analytical methods

3.1. Photon flux density measurements

PFD was measured by means of a Li-Cor 190-SA 2π PAR quantum sensor (LI-COR Biosciences, USA). Incident light intensity on the reactor surface was determined at 12 points inside the empty reactor vessel, as explained in detail in Abiusi et al. [22].

3.2. Dry weight concentration

C_x was estimated by biomass dry weight determination. Aliquots of culture (1–5 mL) were diluted to 30 mL with demineralized water and filtered over pre-weighed Whatman GF/F glass microfiber filters (diameter of 55 mm, pore size 0.7 μm). The filters were washed with 30 mL of deionized water and dried at 100 °C for at least 3 h.

3.3. Average absorption cross section

Average absorption cross section (a_x, $m^2{\cdot}kg_x{}^{-1}$) in the PAR region (400–700 nm) of the spectrum was determined as meticulously described in de Mooij et al. [25]. In short, the absorbance was measured with a UV-VIS/double beam spectrophotometer (Shimadzu, Japan) equipped with an integrating sphere (ISR-2600) and using cuvettes with an optical path of 2 mm.

3.4. Photosystem II quantum yield

Biomass samples were diluted to an optical density at 750 nm between 0.3 and 0.8 and incubated in darkness and 35 °C for 20 min. The dark-adapted photosystem II maximum quantum yield of photochemistry (QY, F_v/F_m) was measured at 455 nm with an AquaPen-C AP-C 100 (Photon Systems Instruments, Czech Republic).

3.5. Assessment of contamination

Flask and reactor cultures were checked weekly for contamination by DNA staining of culture samples with SYBR Green I (Sigma-Aldrich, USA) and fluorescence microscopy with an EVOS FL auto microscope (Thermo Fisher Scientific, USA). Presence of fluorescent bacterial or fungal cells is easily detectable by difference in size and shape compared to algal fluorescent cells.

3.6. Glucose concentration, total organic carbon and total organic nitrogen determination

During the steady state, two 1-mL aliquots of culture were sampled daily from the reactor and centrifuged for 10 min at >20,000 RCF. The supernatant was used for estimation of glucose concentration with a YSI 2950 Biochemistry Analyzer (YSI Life Sciences, USA). The pellets were washed twice with deionized water following the aforesaid centrifugation procedure. Then, they were used for total carbon (TC, $g_C{\cdot}L^{-1}$) and total nitrogen (TN, $g_N{\cdot}L^{-1}$) determination using a TOC-L analyzer (Shimadzu, Japan). The biomass carbon ($C_{\%}$, % $w_C{\cdot}w_x{}^{-1}$) and nitrogen ($N_{\%}$, % $w_N{\cdot}w_x{}^{-1}$) content was calculated dividing the obtained TC and TN by the C_x of the same sample.

3.7. Extraction of phycocyanin and quantification

Phycocyanins from *G. sulphuraria* and *Spirulina* were quantitatively extracted by bead beating (Precellys 24, Bertin Technologies, France) 10 mg of lyophilized biomass. Cell were resuspended in 50 mM Na-acetate at pH 5.5 and exposed to 5 beating cycles of 60 s with 300 s breaks on ice between each cycle. Cell debris was removed through centrifugation at 10,000 rpm for 10 min and the supernatant was collected in fresh tubes. This extract is called crude extract. The C-phycocyanin (*C-PC*) and allo-phycocyanin contents were calculated measuring the absorbance at 620 nm and 652 and converting the measured absorbance to concentration using the Kursar and Alberte equation [26].

3.8. Temperature and pH stability of C-phycocyanin

The effects of temperature and pH on the stability of *C-PC* from *G. sulphuraria* and *Spirulina* were investigated on crude extracts. The crude extracts from cultures grown in auto- and mixotrophic conditions were divided into aliquots and diluted in 50 mM Na-acetate pH 5.5 buffer. This *C-PC* solution was incubated for 30 min in a water bath at 45 °C, 55 °C, 65 °C, and 75 °C. After the heat treatment, absorbance at 620 nm was measured and the residual absorbance at 620 nm ($C\text{-}PC_R$) was calculated as a percentage of the initial absorbance in samples kept at room temperature (20 °C). To determine the pH stability, 200 μL of *C-PC* crude extracts added with 800 μL 50 mM Na-acetate at different pH ranging from 1.2 to 5.5 were incubated for 30 min. The pH of each solution was measured. After the pH treatment, absorbance at 620 nm was measured and the residual absorbance at 620 nm ($C\text{-}PC_R$) was calculated as a percentage of the initial absorbance in samples at pH 5.5.

3.9. Amino acid composition

Biomass samples from *G. sulphuraria* obtained during autotrophic and mixotrophic steady states were freeze-dried in a Sublimator 2 × 3 × 3-5 (Zirbus Technology, Germany). The amino acid content of the freeze-dried biomass was then analyzed following the standardized method ISO 13903:2005 for animal feedstuffs [27] based on the procedure developed by Llames and Fontaine [28]. Tryptophan content was determined by the method ISO 13904:2016 for animal feedstuffs [29]. The analyses were performed in duplicate.

3.10. Statistical analyses and procedures

Significant differences between autotrophic and mixotrophic cultures were analyzed by unpaired *t*-test and two-tailed *P* value with a confidence level of 95% in GraphPad Prism 5.03 (GraphPad Prism Software, USA).

Propagation of errors for addition operations was calculated according to Eq. (6) to determine the error.

$$\Delta z = \sqrt{\Delta x^2 + \Delta y^2 + \ldots} \tag{6}$$

where Δx is the absolute error related to the value x, etc.

When comparing the mixotrophic and the autotrophic cultures each day at steady state was considered a replicate. Three and four days of steady state were maintained for the autotrophic (n = 3) and mixotrophic (n = 4) cultures respectively.

4. Results and discussion

4.1. High density chemostat cultivation of G. sulphuraria ACUF 064

We previously demonstrated that *G. sulphuraria* can be successfully cultivated without any gas exchange by making use of oxygen balanced mixotrophy [1]. However, our strain, as most *Galdieria* strains, turned out to be photosensitive, which is why optimization of the light regime was a key point for successful cultivation. In this work we cultivated *G. sulphuraria* in chemostat autotrophically and mixotrophically at biomass concentration higher than 4 $g{\cdot}L^{-1}$ aiming for maximum pigment content without affecting biomass productivity.

4.2. Biomass productivity

Chemostat production of *G. sulphuraria* biomass concentration higher than 4 $g{\cdot}L^{-1}$ proved to be a successful strategy for constant biomass production with a high pigment content under both autotrophic and mixotrophic conditions (Fig. 1, Table 1). High C-phycocyanin (*C-PC*) (Table 2) content and high PSII maximum quantum yield (*QY*, F_v/F_m) indicated that neither the autotrophic nor the mixotrophic culture were photo-inhibited. It must be noted that even under optimal conditions F_v/F_m of *Galdieria* is at most 0.5 which is intrinsically lower than green algae expressing a maximum F_v/F_m of 0.7 or more. In our previous repeated batch experiment high biomass productivity was obtained only under optimal specific light supply rate (q_{ph}) [1]. After each dilution the sudden change in q_{ph} caused photo-inhibition that strongly decreased biomass productivity in the 1–2 days following the dilution, reducing the overall performance of each batch. We confirmed that by adjusting the biomass concentration, thus reaching optimal light regime, *G. sulphuraria* can be continuously cultivated at high light intensity without signs of photo-inhibition. Therefore, while in our previous repeated batch experiment high biomass productivity was obtained only for a few days, in chemostat high biomass productivity can be maintained.

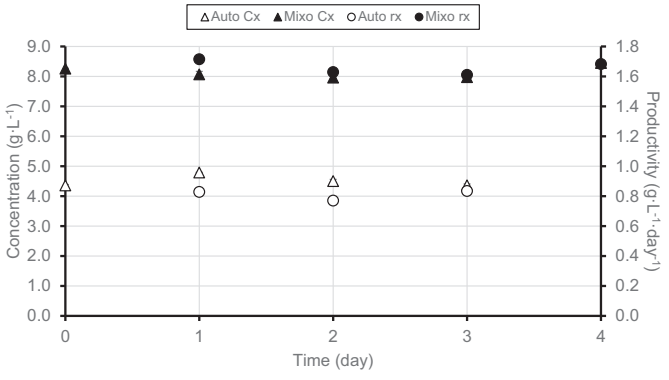

Fig. 1. Volumetric biomass production rate (r_x) (circles) and concentration (C_x) (triangles) of *G. sulphuraria* ACUF 064 grown autotrophically (white) and mixotrophically (black). Values expressed as averages ± standard deviation.

The biomass concentration of 8.1 $g{\cdot}L^{-1}$ obtained in the mixotrophic culture did not affect biomass areal productivity (49.3 $g{\cdot}m^{-2}{\cdot}day^{-1}$) (Table 2) that was not significantly different from the areal productivity obtained in repeated batch [1]. Such high areal biomass productivity (r^A_x, $g{\cdot}m^{-2}{\cdot}day^{-1}$) is 3 times to 7 times greater than previously reported in a 24 h/24 h illuminated culture of *G. sulphuraria*. The mixotrophic r^A_x was similar to one of the highest r^A_x reported in an autotrophic culture of *Spirulina* [8]. This maximal biomass productivity of *Spirulina* was obtained at a light intensity twice as high as used in this study and a thin layer photobioreactor was used. *Spirulina* has been the focus of extensive research for almost a century; therefore its biomass productivity is optimized and no significant increase in production has been reported during the last 20 years. Meanwhile, the genus *Galdieria* was discovered in 1981, but it wasn't until 2005 that a high cell density culture was reported [30]. We believe that further research on *Galdieria* will lead to a further improvement of biomass productivity.

In our mixotrophic experiment, volumetric biomass productivity (r^V_x, $g{\cdot}L^{-1}{\cdot}day^{-1}$) was 30 times lower than the highest heterotrophic r^V_x productivity reported in literature for *G. sulphuraria* [31]. The authors reported a maximum biomass yield on substrate of 0.52 g of biomass per gram of glucose ($g_x{\cdot}g_s^{-1}$) while under oxygen balanced mixotrophy we obtained 0.75 $g_x{\cdot}g_s^{-1}$. Heterotrophic high biomass yield on substrate can only be obtained through aerobic respiration requiring active aeration, while in our culture gas exchange was completely avoided. Our mixotrophic culture had a carbon content of 47.3% (Table 1) resulting in a $Y_{x/s}$ of 0.89 $C\text{-}g_x{\cdot}C\text{-}g_s^{-1}$. This means that in our culture 89% of the carbon in the substrate was transformed into biomass carbon and only 11% lost in the form of CO_2. This result is equivalent to the maximum $Y_{x/s}$ obtained in repeated batch (Table 1). While in our previous work $Y_{x/s}$ was not stable between batches, in the present work $Y_{x/s}$ was constant over the whole steady state.

In one of the few works in which *G. sulphuraria* was cultivated mixotrophically in chemostat, with aeration, a maximum biomass yield of 0.84 $g_x{\cdot}g_s^{-1}$ on substrate was obtained [32], representing a 12% increment to our current reported yield. For a fair comparison, the biomass yield on substrate should be estimated based on carbon content ($Y_{x/s}$, $C\text{-}g_x{\cdot}C\text{-}g_s^{-1}$). Such comparison was not possible with the works mentioned above [31,32]. Assuming a carbon content similar to our culture (47.3%) Sloth et al. [32] might have succeeded in converting 100% of the substrate (glucose) into biomass, obtaining a carbon balanced culture. Since the ratio between O_2 produced per mol of CO_2 consumed is always higher than 1 [22], a carbon balanced culture needs aeration to prevent O_2 accumulation while oxygen balanced mixotrophy operates without aeration.

Autotrophic biomass productivity and concentration were half of the mixotrophic culture values (Table 1). During autotrophic cultivation, the average biomass concentration (C_x) at steady state was 4.50 $g{\cdot}L^{-1}$ and the average volumetric productivity (r^V_x) was 0.81 $g{\cdot}L^{-1}{\cdot}day^{-1}$ (Table 1). In comparison, the average productivity obtained in the optimal range during the autotrophic repeated batches was 0.97 $g{\cdot}L^{-1}{\cdot}day^{-1}$ (Table 1), the highest biomass productivity ever reported in

Table 1
Overview of the offline measurements and process parameters of *Galdieria sulphuraria* ACUF 064 under autotrophic and oxygen-balanced mixotrophic conditions in chemostat (this study) and the best values determined in repeated batch (batches I and III under autotrophic and VI under mixotrophic conditions) [1]. Values expressed as averages ± standard deviation.

	Unit	Autotrophic (this study)	Mixotrophic (this study)	Autotrophic [1]	Mixotrophic [1]
C_x	$g{\cdot}L^{-1}$	4.5 ± 0.2^a	8.1 ± 0.2^b	4.9	9.7
D	day^{-1}	0.18 ± 0.00^a	0.21 ± 0.00^b	–	–
r_x	$g{\cdot}L^{-1}{\cdot}day^{-1}$	0.81 ± 0.03^a	1.66 ± 0.04^b	0.97	1.72
C-PC	$\%\ w_{C\text{-}PC}{\cdot}w_x^{-1}$	9.6 ± 0.8^a	10.1 ± 0.5^a	–	–
$C_{\%}$	$\%\ w_C{\cdot}w_x^{-1}$	47.5 ± 1.7^a	47.3 ± 2.2^a	46.6	47.6
$N_{\%}$	$\%\ w_N{\cdot}w_x^{-1}$	9.9 ± 0.0^a	9.7 ± 0.0^a	10.2	9.4
a_x	$m^2{\cdot}kg_x^{-1}$	231 ± 5^a	184 ± 45^b	181	132
QY	(F_v/F_m)	0.38 ± 0.06^a	0.44 ± 0.03^a	0.49	0.45
$Y_{x/s}$	$C\text{-}g_x{\cdot}C\text{-}g_s^{-1}$	–	0.89 ± 0.02	–	0.91
$Y_{x/ph}$	$g_x{\cdot}mol_{ph}^{-1}$	0.55 ± 0.02	–	0.65	–

Among the rows the same letter indicates no significant differences ($p > 0.05$).

Table 2
Comparison of biomass and *C-PC* volumetric- and areal productivities of this study and other values under 24 h/24 h illumination reported in literature.

Reference	Trophic mode	Strain	IS (m^2)	C^V_x ($g{\cdot}L^{-1}$)	C^A_x ($g{\cdot}m^{-2}$)	r^V_x ($g{\cdot}L^{-1}{\cdot}day^{-1}$)	r^A_x ($g{\cdot}m^{-2}{\cdot}day^{-1}$)	C-PC (% w/w)	$r^V_{C\text{-}PC}$ ($mg{\cdot}L^{-1}{\cdot}day^{-1}$)	$r^A_{C\text{-}PC}$ ($g{\cdot}m^{-2}{\cdot}day^{-1}$)
This study	Autotrophic	*G. sulphuraria* ACUF 064	0.067	4.5	134	0.81	24.1	9.6	78	2.3
This study	Mixotrophic	*G. sulphuraria* ACUF 064	0.067	8.1	243	1.66	49.3	10.1	168	5.0
[32]	Mixotrophic	*G. sulphuraria* 74G	0.084	0.8	24	0.50	15.0	2.81	14	0.4
[9]	Photoinduction	*G. sulphuraria* 74G	0.057	0.6–6.0	11–105	0.90	15.8	13.2	119	2.1
[10]	Photoinduction	*G. sulphuraria* 74G	0.029	0.9–5.5	31–188	0.38	13.1	12.3	46	1.6
[36]	Autotrophic	*G. sulphuraria* ACUF 064	–	–	10–225	–	7.2	3.2	–	0.2
[31]	Heterotrophic	*G. sulphuraria* 74G	–	51.6–83.1	–	50	–	1.6	861	–
[8]	Autotrophic	*A. platensis* M2 (*Spirulina*)	0.192	3.2	80	1.9	45.6	9.3	179	4.2

autotrophic culture of *G. sulphuraria*. This 20% reduction in r^V_x in the current experiment was unexpected, as we applied a similar light regime as in the optimal range of the batches. When looking at the C_x, 4.50 $g{\cdot}L^{-1}$ is on the upper limit of the linear growth range in batch [1]. Moreover, in the current work the absorption cross section (a_x, $g{\cdot}m^{-2}$) was 28% higher than the maximum value in repeated batch (Table 1) (see next section). High a_x decreases the light penetration within the culture accentuating possible light limitation. Despite the lower performance compared to our previous experiment, the obtained autotrophic areal productivity is still 1.5 times to 3.4 times higher than previously reported in *G. sulphuraria* (Table 2) and comparable to other commercially relevant microalgae such as *Tisochrysis lutea* [33], *Rhodomonas* sp. [34], *Nannochloropsis* sp. [35], indicating the potential of this strain for autotrophic biomass production.

4.3. Phycocyanin productivity and stability

Increasing biomass concentration proved to be a successful strategy to achieve high pigment productivity under both autotrophic and mixotrophic chemostat conditions with *G. sulphuraria* (Table 2). Pigment content can be monitored by calculating the fraction of light that is absorbed by the cells, the so called absorption cross section (a_x, $m^2{\cdot}g_x^{-1}$). We succeeded in increasing a_x by 28% and 39% in the autotrophic and mixotrophic cultures respectively, compared to our previous maximal values in repeated batch (Table 1). Moreover, a_x was constant over the whole autotrophic and mixotrophic chemostat (data not shown) while in repeated batch pigmentation temporarily decreased after each dilution [1]. In the mixotrophic culture a_x was 20% lower than in the autotrophic culture, confirming that the addition of an organic carbon source does affect a_x of *G. sulphuraria*. In contrast, no differences in a_x were found between autotrophic and oxygen balanced mixotrophic cultures of *C. sorokiniana* [22,23].

Despite the lower a_x, the mixotrophic culture had one of the highest C-phycocyanin (*C-PC*) content and *C-PC* productivity reported in this species (Table 2). No significant difference ($p > 0.05$) was found in *C-PC* content between autotrophic and mixotrophic cultures (Table 2) and both cultures had approximately 10% w/w of *C-PC* and 1.7% of allophycocyanin (data not shown). *C-PC* content of 10% w/w is the highest obtained in this strain [19,36]. Moreover, in the mixotrophic culture high *C-PC* combined with high biomass productivity lead to a *C-PC* areal productivity ($r^A_{C\text{-}PC}$, $g{\cdot}m^{-2}{\cdot}day^{-1}$) of 5 $g{\cdot}m^{-2}{\cdot}day^{-1}$, 2.4 times to 25 times higher than previously reported in 24 h/24 h illuminated culture of *G. sulphuraria* (Table 2). The mixotrophic $r^A_{C\text{-}PC}$ was even higher than reported in an autotrophic culture of *Spirulina* [8], which reached 4.2 $g{\cdot}m^{-2}{\cdot}day^{-1}$.

In our mixotrophic experiment *C-PC* volumetric productivity ($r^V_{C\text{-}PC}$, $g{\cdot}L^{-1}{\cdot}day^{-1}$) was 5 times lower than the highest heterotrophic *C-PC* productivity reported in literature for *G. sulphuraria* (Table 2). The superior heterotrophic *C-PC* productivity is due to its high biomass volumetric productivity (see previous section). Moreover, heterotrophic culture can be productive 24 h a day and does not suffer of seasonal variation on light intensity and photoperiod, making even greater the gap on biomass and *C-PC* productivity between the two cultivation strategies.

Despite the higher volumetric productivity compared to mixotrophic culture, the downside of heterotrophic pigment production is that the *C-PC* content is lower than 3% w/w [31,32]. The low *C-PC* content results in higher pigment extraction costs and limitations with the commercial application of this technology. Within the EU, the initial concentration of a pigment in the food of origin determines if the extract can be considered a "food extract with coloring properties" (>3% w/w) or a "natural food additive" (<3% w/w) [37]. Food extracts are considered "food ingredients" and are used in clean label products while natural additives require an "E" number. Due to the low *C-PC* concentration, *C-*

PC extracts from heterotrophic biomass fall into the additives category. On the contrary, *C-PC* extracts from our mixotrophic biomass would belong to the first category, not requiring any labelling.

We determined thermal- and acid-stability of the *C-PC* extract. No significant differences ($p > 0.5$) were found in *C-PC* stability between autotrophic and mixotrophic cultures (Figs. 2 and 3). *C-PC* produced in *G. sulphuraria* ACUF 064 proved to be stable at low pH (Fig. 1). The absorbance at 620 nm remained high down to pH 3. A further reduction of pH, from 3 to 1.9, led to a linear decrease in *C-PC* content, until it reached 39% of the reference (pH 5.5) absorbance. *C-PC* extracted from *Spirulina* was significantly less acid stable. The absorbance at 620 nm with pH 3.5 was only 40% of the absorbance at pH 5.5. Similar results were obtained by Carfagna et al. [11] in autotrophic and heterotrophic cultures of *G. phlegrea*. The authors reported similar *C-PC* stability in their autotrophic cultures with 80% of the color maintained from pH 4 to pH 3 followed by a rapid drop below pH 3.

Acid stability is an important trait in natural colorants. Most commercial beverages have a pH of less than 4 [38]. Acids are added to beverages and compose a flavor profile giving the beverage a distinctive taste. Acids provide a tartness and tangy taste that helps to balance the sweetness of sugar present in the beverage; they are key factors in the taste of the beverage. Therefore, while the *C-PC* extracted from *Spirulina* will lose its blue color in most beverages, the *C-PC* of *G. sulphuraria* can be used in almost any beverage increasing the market size of this pigment.

Thermostability is another important characteristic of a natural pigment. We incubated *G. sulphuraria* and *Spirulina* extracts for 30 min at temperatures ranging from 25 to 75 °C (Fig. 3). In *G. sulphuraria* the absorbance at 620 nm remained stable until 55 °C, maintaining 94% of its original color. Then when the temperature was elevated from 55 to 75 °C, *C-PC*'s absorbance decreased steadily, until at 75 °C it was 39% of the original. *Spirulina's C-PC* extracts started losing color already at 45 °C having lost 18% of initial absorbance. The absorbance linearly decreased until 75 °C, at which only 21% of initial absorbance was maintained. Our strain was more thermostable than the two *G. phlegrea* grown autotrophically by Carfagna et al. [11] but less stable than the *G. sulphuraria* strain described by Moon et al. [12]. The latter reported a thermostable *C-PC* that maintained 62% and 60% of its initial absorbance at 620 nm at 75 °C and 85 °C respectively.

The differences in *C-PC* stability between *G. sulphuraria* and *Spirulina* can be explained by looking at their amino acid sequences. Both the α and β subunits of the *C-PC* from *G. sulphuraria* differ in several residue positions from *C-PC* of *Spirulina* [12,39]. In the closely related *G. phlegrea,* residue mutations outside of conserved regions are responsible for an increased *C-PC* thermostability [40]. Possibly, the hot

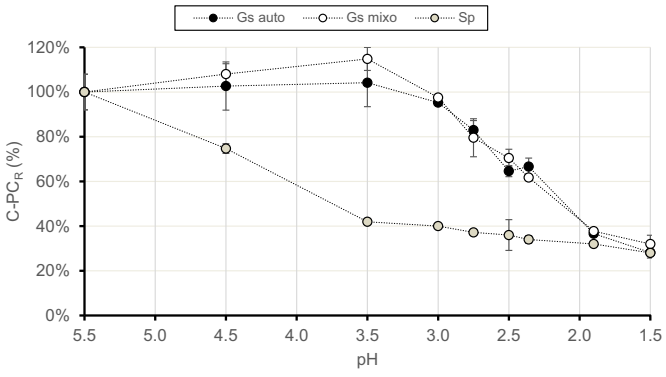

Fig. 2. Effect of pH on the residual absorbance at 620 nm ($C\text{-}PC_R$) in *G. sulphuraria* ACUF 064 cultures grown autotrophically (Gs auto) and mixotrophically (Gs mixo). An autotrophic culture of *A. platensis* (Sp) is used as comparison. The extracts were incubated at the indicated pH for 30 min. The absorbance at pH 5.5 represents 100%. Values expressed as averages ± standard deviation.

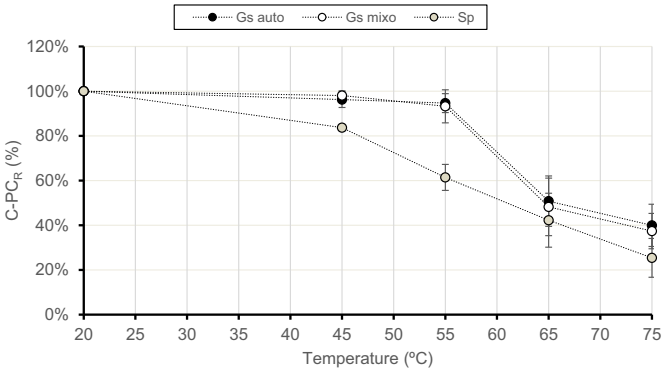

Fig. 3. Effect of temperatures (T) on the residual absorbance at 620 nm ($C\text{-}PC_R$) in *G. sulphuraria* ACUF 064 cultures grown autotrophically (Gs auto) and mixotrophically (Gs mixo). An autotrophic culture of *A. platensis* (Sp) is used as comparison. The extracts were incubated at the indicated T for 30 min. The absorbance at 20 °C represents 100%. Values expressed as averages ± standard deviation.

natural environment of *G. sulphuraria* creates a selective pressure to maintain similar mutations that is absent in *Spirulina*. In conclusion, mixotrophic cultivation of *G. sulphuraria* ACUF 064 is a promising strategy to produce C-phycocyanin (*C-PC*). Areal mixotrophic *C-PC* productivity was the highest ever reported in a phototrophic or mixotrophic culture of this species, and slightly higher than the highest productivity obtained in autotrophic culture of *Spirulina* [8]. Moreover, the *C-PC* extracted from *G. sulphuraria* showed superior acid- and thermo- stability compared to *C-PC* extracted in Spirulina. These traits may increase the commercial application of this pigment.

4.4. Amino acid content of G. sulphuraria ACUF 064

In order to address the knowledge gap existing in the amino acid composition of *G. sulphuraria*, in this work we analyzed the total amino acid content of *G. sulphuraria* ACUF 064 in steady state under autotrophic and mixotrophic metabolic regimes.

The results of the amino acid analysis are displayed in Table 3. For most amino acids, there were no significant differences in content between autotrophy and mixotrophy. The exceptions are aspartate, threonine, glycine, alanine and methionine, which were found in slightly larger quantities in the autotrophic biomass. The most abundant amino acids in both metabolic regimes were glutamate, aspartate and leucine, constituting nearly 9%, 6% and 5% of the total biomass dry weight, in that order. Furthermore, the least abundant amino acids were tryptophan, cysteine and histidine, which accounted for less than 1.1% of the total biomass dry weight each. The remaining amino acids were found in concentrations ranging from 2 to 4% of total biomass dry weight.

These results are in line with the relative amino acid frequency found in several microalgal species and seaweeds [41–44]. The acidic amino acids predominate, partially influenced by the concurrent detection of their amides by most analytical procedures. As elicitors of the umami taste, glutamate and aspartate are pivotal in the sensory perception of food [45]. Moreover, leucine has also been found to be the most abundant of the essential amino acids in *Chlorella*, *Spirulina* and *Chlamydomonas reinhardtii* [46]. Brown et al. [47] analyzed the amino acid content of 16 microalgae and showed that the content of sulfur amino acids, histidine and tryptophan are generally the lowest of all amino acids. However, similar relative frequency does not mean that the total amino acid content is equivalent among different species, as absolute amino acid quantities are subject to great variability, even within the same species, under different experimental conditions [48].

Interestingly, our analysis also found taurine in the biomass of *G. sulphuraria* (Table 3). Taurine is a sulfur-containing β-amino acid that does not form peptide bonds and is common in animal tissues. While

Table 3
Amino acid composition of steady state autotrophic and mixotrophic *G. sulphuraria* ACUF 064 (% w/w). Values expressed as averages ± standard deviation.

	Asp*	Thr	Ser	Glu*	Gly	Ala	Val	Ile	Leu	Tyr
Autotrophic	6.00 ± 0.02^{a}	3.71 ± 0.02^{a}	4.21 ± 0.06^{a}	9.25 ± 0.10^{a}	2.81 ± 0.00^{a}	4.09 ± 0.01^{a}	3.66 ± 0.00^{a}	3.62 ± 0.00^{a}	5.29 ± 0.07^{a}	4.14 ± 0.18^{a}
Mixotrophic	5.76 ± 0.02^{b}	3.60 ± 0.01^{b}	4.07 ± 0.03^{a}	9.14 ± 0.06^{a}	2.74 ± 0.01^{b}	3.89 ± 0.00^{b}	3.59 ± 0.05^{a}	3.51 ± 0.03^{a}	5.12 ± 0.00^{a}	4.10 ± 0.16^{a}
	Phe	His	Lys	Arg	Pro	Cys	Met	Trp	Tau	Total**
Autotrophic	2.95 ± 0.02^{a}	1.06 ± 0.02^{a}	3.89 ± 0.00^{a}	4.12 ± 0.06^{a}	2.83 ± 0.02^{a}	1.03 ± 0.02^{a}	1.59 ± 0.01^{a}	0.86 ± 0.00^{a}	0.48 ± 0.01^{a}	65.12 ± 0.24^{a}
Mixotrophic	2.89 ± 0.03^{a}	1.08 ± 0.04^{a}	3.88 ± 0.03^{a}	3.99 ± 0.03^{a}	2.73 ± 0.07^{a}	1.06 ± 0.02^{a}	1.51 ± 0.01^{b}	0.85 ± 0.01^{a}	0.37 ± 0.00^{b}	63.50 ± 0.21^{b}

Among the columns the same letter indicates no significant differences ($p > 0.05$).
* Aspartate and glutamate results include their amides, asparagine and glutamine.
** Total protein content excluding the free amino acid-like taurine.

plants and fungi contain scarce amounts of taurine, algae have been proposed as an alternative source of this compound for application in the animal feed industry [49]. We found significant differences in the content of taurine between autotrophy and mixotrophy, representing 0.48 and 0.37% of the total biomass dry weight, respectively. Taurine had been already identified in other rhodophytes [50] but never within the genus *Galdieria*. The role of this molecule in *G. sulphuraria* is unknown. Taurine contains sulfur, which is found in large quantities in the acidic hot springs where *G. sulphuraria* is commonly found. As such, it could play a role in sulfur metabolism. Tevatia et al. showed that sulfur supplementation increases the levels of intermediates in the synthesis pathway of taurine in other microalgae [51]. Additionally, in the same study it was showed that taurine participated in the osmoregulation of *Tetraselmis* sp. and *C. reinhardtii*. High salt concentrations are also a characteristic of the natural habitat of *G. sulphuraria*, so taurine could also have a function as osmolyte in this species.

4.5. Protein content of G. sulphuraria ACUF 064

We calculated the total protein content of the biomass by adding up quantities of the individual 20 common protein-forming amino acids (Table 3). This method could overestimate the amount of protein due to the inclusion of free amino acids within the cells. However, the potential overestimation does not affect the determination of the nutritional value of the biomass. The total protein content for the autotrophic biomass was 65.1% of the total biomass dry weight, whereas for the mixotrophic biomass it was 63.5%. This difference is derived from the aforementioned higher content of certain amino acids during autotrophy and was found to be significant. In literature, the reported protein contents of autotrophic *G. sulphuraria* show a great variability, ranging from 22% to 72% of total biomass dry weight [9,18–20]. From these studies, only Graziani et al. used *G. sulphuraria* ACUF 064. They analyzed the protein content under heterotrophy and autotrophy, obtaining protein content of 26.5% and 32.5% of the biomass dry weight, respectively. In comparison, our result from autotrophic culture is twofold larger.

The variability in *G. sulphuraria* protein content among different studies might be caused by differences in the physiological state of the culture. Different types of microalgal cell walls affect the solubilization of intracellular proteins in diverse manners thus influencing protein content determination [44]. *G. sulphuraria* has a rigid cell wall that contains an unusual large fraction of proteins, up to 55% [52]. As a consequence, if the cell wall is not properly broken, neither the proteins of other parts of the cell nor the proteins that constitute the cell wall will be accurately accounted for. Several class III peroxidases that are involved in cell wall hardening have been identified in *G. sulphuraria* [53]. Peroxidase activity was mainly detected during the stationary phase of cell growth. In contrast to batch processes, in chemostat this effect might be absent and consequently cell protein may be more accessible, explaining the differences between our study and Graziani et al. [19].

Another reason for the variability in *G. sulphuraria* protein content among different studies could be the different extraction methods employed [42]. In our study we determined the total protein both by adding up the individual amino acids and by the total nitrogen ($N_{\%}$) (Table 1) obtaining similar results. The method applied in the amino acid quantification [27] is based on strong HCl oxidation and hydrolysis at 120 °C followed by precise HPLC quantification. This method is successfully used for amino acid analyses in complex feed stuff mixtures and in plants that are very resistant to hydrolysis [54].

Apart from our study, all the other reported values of protein content in *G. sulphuraria* were determined by multiplying the total nitrogen ($N_{\%}$) content of the sample by a nitrogen-to-protein factor of 6.25. $N_{\%}$ was determined either by Kjeldahl [9,19,20], or a similar method [18], which are based on strong oxidation of organic nitrogen. Multiplying the $N_{\%}$ by a factor of 6.25 is generally thought to lead to an overestimation of the total amount of proteins in plant biomass, as a considerable fraction of the $N_{\%}$ is non-proteinic [55]. Moreover, the factor 6.25 is based on the assumption that the nitrogen content of protein is 16%, which is not correct for several protein sources. Species-specific factors have been published for several microalgae based on their amino acid composition [42,56]. Yet, it is a common practice to use 6.25 when a specific factor has not been determined for a certain species, as is the case for *G. sulphuraria*. Therefore, the reported results using this method can expected to overestimate the real protein content. In our study, we also determined nitrogen content (Table 1), resulting in 9.9% and 9.7% of the total biomass dry weight for autotrophy and mixotrophy, respectively. If we multiply those $N_{\%}$ by a factor of 6.25, we obtain 62% and 61% protein content for said trophic modes. On the one hand, these values are close to the sum of all the individual amino acids. On the other hand, the $N_{\%}$ based value is slightly lower than summing all amino acids while a higher value was expected. The combination of the unique amino acid profile and proportion of non-proteinic nitrogen of *G. sulphuraria* may result in an underestimation of protein when using 6.25 as a factor. Further insight is needed with respect to the small discrepancy between the resulting protein content following both approaches.

The highest protein content of *G. sulphuraria* reported in literature is 71.6% in autotrophy [18]. However, this protein content was corrected for ash content. We can undo this correction by multiplying their reported nitrogen content, 9.8%, by 6.25. The result then is 61%, which is the same as in our study. Wan et al. [9] reported a protein content of 58% under an autotrophic regime. All in all, the protein contents obtained in autotrophic and mixotrophic chemostat in our current study are among the highest reported in *G. sulphuraria*. A protein content of 60% is on the high side of values ever reported for all microalgae [57], highlighting the potential of *G. sulphuraria* as a protein source.

Heterotrophic metabolism could be the reason why lower protein contents are reported in certain studies. Studies that compared protein content under autotrophic and heterotrophic growth reported higher protein contents under autotrophy [9,19]. In fact, the highest protein content reported under heterotrophic metabolism is only 32% [20]. Among other factors, the reduction of phycocyanin levels in the heterotrophic cells [3] and their higher content in carbohydrates [19] could explain this observation.

4.6. Essential amino acid profile of G. sulphuraria ACUF 064

In order to assess the nutritional value of *G. sulphuraria*, the amino acid content not only needs to be analyzed quantitatively but also qualitatively. That is, focusing on the relative proportion of the essential amino acids in the proteins. For that reason, we compared the essential amino acid profile determined in this study with the FAO dietary requirements for adults [21] in Table 4. Moreover, we also included the essential amino acid profiles of the currently main industrial microalgae used in food applications, *Spirulina* and *Chlorella*, and of the main vegetable protein source worldwide, soybean [58,59]. We chose reported values in literature that correspond to commercially available ingredients for human nutrition. For the microalgae, the commercial products were constituted by the whole biomass, while in soybean they were protein isolates (>90% protein) and concentrates (>70% protein). The comparison includes the 9 essential amino acids for adults: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine but also two conditionally essential ones, cysteine and tyrosine. Cysteine is added together to methionine under the denomination of sulfur amino acids (SAAs) and tyrosine is added to phenylalanine under the denomination of aromatic amino acids (AAAs).

G. sulphuraria ACUF 064 compared favorably to the FAO requirements for every amino acid, in both autotrophic and mixotrophic biomass. Even if threonine and methionine showed a lower content in mixotrophy than in autotrophy, these small differences did not affect the nutritional value of the mixotrophic biomass. Histidine exhibited the lowest margin above the nutritional requirement, and thus can be considered the most limiting essential amino acid in *G. sulphuraria* proteins. In comparison, *Chlorella* is deficient in histidine and SAAs while the average lysine content is just at the limit. *Chlorella* SAAs content showed the largest difference with the FAO requirement and they are the limiting factor for this chlorophyte. *Spirulina* on average is also below the requirements for histidine and SAAs, although the limiting amino acid is histidine instead of SAAs. Tryptophan was not analyzed in the study that we used for comparison and thus was not taken into account in these two microalgae. Soybean protein contains a balanced amino acid profile, surpassing the requirement for every essential amino acid, but compares unfavorably with *G. sulphuraria*. *G. sulphuraria* contains higher contents of every essential amino acid with the exception of lysine, that is equivalent to soybean, and histidine, in which soybean is superior. Average soybean content of SAAs, 26 mg/g protein, is just above the requirement of 22 mg/g protein and hence SAAs are the most limiting essential amino acid in this protein source. In fact, there have been several efforts to increase SAAs content in soybean [60].

Besides the overall superior amino acid profile of *G. sulphuraria*, special attention must be given to SAAs content. *G. sulphuraria* contains a high proportion of SAAs compared with *Chlorella*, *Spirulina* and soybean protein. Methionine and cysteine cannot be synthesized de novo by animals and therefore their supply is dependent on the diet. Most plant biomasses contain scarce amounts of these sulfur compounds [61] and they have to be provided by other sources, mostly animal protein in the case of human nutrition and external SAAs supplementation or excess protein addition in the case of animal feed. Due to its remarkable amino acid profile and high protein content, *G. sulphuraria* is a good candidate to overcome SAAs deficiencies for food and feed applications. Nevertheless, amino acid composition and overall protein fraction are not the only characteristics that make a protein source suitable. Further research is needed to determine the digestibility and utilization of absorbed amino acids from *G. sulphuraria* biomass.

5. Conclusions

Chemostat production of *G. sulphuraria* ACUF 064 at biomass concentration higher than 4 $g \cdot L^{-1}$ proved to be a successful strategy for constant biomass production with high pigment content both under autotrophic and mixotrophic conditions. The biomass grown mixotrophically contained 10% w/w C-phycocyanin (*C-PC*), which combined with high areal biomass productivity led to the highest *C-PC* areal productivity reported under 24 h/24 h illumination in *Galdieria* and to a higher productivity than with autotrophic cultivation of *Spirulina*. The *C-PC* extracted from *G. sulphuraria* showed superior acid- and thermal stability compared to *C-PC* extracted in *Spirulina*. *G. sulphuraria* had over 60% w/w protein content and an essential amino acid profile that complied to the FAO dietary requirements for adults, in both autotrophic and mixotrophic biomass. Moreover *G. sulphuraria* contains a high proportion of sulfur amino acid compared with *Chlorella*, *Spirulina* and soybean protein. Due to its attractive amino acid profile and high protein content, *G. sulphuraria* is a good candidate to overcome sulfur amino acid deficiencies for food and feed applications.

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CRediT authorship contribution statement

Fabian Abiusi: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing-original draft, Writing review & editing. **Pedro Moñino Fernández:** Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing-original draft, Writing review & editing. **Stefano Canziani:** Investigation, Formal analysis, Data curation. **Marcel Janssen:** Supervision, Writing review & editing. **René H. Wijffels:** Funding acquisition, Supervision, Writing review & editing. **Maria Barbosa:** Funding acquisition, Supervision, Writing review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

Table 4
Essential amino acid comparison of autotrophic and mixotrophic *G. sulphuraria* ACUF 064, commercial *Chlorella* and *Spirulina* and FAO requirements (mg AA/g protein). Values expressed as averages ± standard deviation.

	His	Ile	Leu	Lys	Thr	Val	SAAs[b]	AAAs[c]	Trp	Reference
Requirement[a]	15	30	59	45	23	39	22	38	6	[21]
Autotrophic	16.3 ± 0.4	55.6 ± 0.0	81.1 ± 1.1	59.6 ± 0.0	56.9 ± 0.0	56.1 ± 0.1	40.2 ± 0.4	108.8 ± 2.4	13.2 ± 0.1	This study
Mixotrophic	17.0 ± 0.6	55.4 ± 0.5	80.9 ± 0.0	61.3 ± 0.5	56.8 ± 0.2	56.7 ± 0.8	40.6 ± 0.4	110.5 ± 2.0	13.4 ± 0.1	This study
Chlorella	10.4 ± 2.1	35.0 ± 5.4	83.4 ± 8.3	45.1 ± 7.4	29.5 ± 2.8	50.4 ± 5.0	12.7 ± 4.3	56.9 ± 3.7	n. d.[d]	[59]
Spirulina	10.4 ± 2.4	57.3 ± 5.1	92.2 ± 9.1	53.3 ± 6.0	34.1 ± 3.8	56.7 ± 5.3	16.9 ± 5.3	59.3 ± 13.6	n. d.[d]	[59]
Soybean	24.7 ± 0.2	46.4 ± 0.9	79.0 ± 1.9	62.3 ± 0.3	36.5 ± 0.2	49.4 ± 1.3	26.0 ± 1.1	88.4 ± 1.2	12.7 ± 0.2	[58]

[a] Indispensable amino acid requirement for adults.
[b] Sulfur-containing amino acids, namely cysteine and methionine.
[c] Aromatic amino acids, namely phenylalanine and tyrosine.
[d] Not determined.

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