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Amino acid profile and protein bioaccessibility of two *Galdieria sulphuraria* strains cultivated autotrophically and mixotrophically in pilot-scale photobioreactors

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ABSTRACT

Galdieria sulphuraria is considered one of the most promising microalgae for food applications. In this study, we compared two strains of *G. sulphuraria* cultivated autotrophically and mixotrophically over 35 days in pilot-scale photobioreactors under nonsterile conditions. The low pH (<1.9) used for cultivation successfully prevented microbial contamination. The two strains had similar autotrophic and mixotrophic biomass productivities, the latter being 2.3 times higher than autotrophic productivity. Comparing the two strains, *G. sulphuraria* SAG 108.79 and ACUF 064 had 51% and 64% (*w*/w) protein and 4% and 9% (w/w) C-phycocyanin content, respectively. Interestingly, *G. sulphuraria* SAG 108.79 showed a protein bioaccessibility of 62%, in line with other microalgal species, whereas *G. sulphuraria* ACUF 064 had a protein bioaccessibility of only 14%. No differences in the amino acid profile were found between the two strains or between trophic modes. Stable and well-balanced protein profiles are encouraging results for future applications of this species.

Industrial relevance: The main focus of this study was the production of single-cell proteins using two strains of the polyextremophilic microalgae *Galdieria sulphuraria*. The acidic cultivation condition was sufficient to guarantee axenic production in not sterile conditions, even in the presence of glucose. Both strains were rich in proteins with a similar amino acid profile rich in all of the essential amino acids. Interestingly, there was a 4.4-fold difference in protein bioaccessibility between the two strains. Simple production of axenic microalgal biomass rich in protein is an encouraging result for future food applications of this species.

1. Introduction

Galdieria sulphuraria is a polyextremophilic microalgae species that can tolerate pH values close to zero (Abiusi, Trompetter, Hoenink, Wijffels, & Janssen, 2021), temperatures up to 57 °C (Ott & Seckbach, 1994), and osmotic pressures up to 2–3 M (Schmidt, Wiebe, & Eriksen, 2005). These unique characteristics can be used to create a selective environment that prevents the proliferation of microbial contaminants, one of the main challenges of large-scale microalgae cultivation (Day, Gong, & Hu, 2017).

Most microalgae are obligate photoautotrophic organisms able to use only inorganic carbon and light as carbon and energy sources, respectively. However, most photoautotrophic cultivations have limited growth efficiency, as light penetration can be hindered by high cell density. Some microalgal species are able to utilize organic carbon as carbon and energy source. The organic carbon can be provided in absence of light, resulting in a heterotrophic metabolism, or in presence of light, resulting in a mixotrophic metabolism. Mixotrophic cultivation of microalgae has been proposed as a possible solution to overcome photoautotrophic limitation. When cultivated under mixotrophic conditions, *G. sulphuraria* doubled its biomass productivity compared to autotrophic conditions (Abiusi et al., 2022). Moreover, linear growth was observed at a high cell density (9.7 $g_x\,L^{-1}$) (Abiusi et al., 2021). The authors reported that acidic growth conditions (pH 1.6) prevented

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microbial contamination even after over 1 month of continuous operation (Abiusi et al., 2021; Abiusi, Moñino Fernández, et al., 2022). However, experiments were performed using a 2-L fermenter operated aseptically. Recently, Pleissner, Lindner, and Händel (2021) cultivated *G. sulphuraria* heterotrophically under nonsterile conditions in a 1-L fermenter without signs of contamination, providing evidence that low pH is sufficient to prevent unwanted microbial growth.

Galdieria sulphuraria is a promising source of the pigment blue C-phycocyanin (*C-PC*) and high-quality proteins. Illuminated cultures of *G. sulphuraria* have been reported to have *C-PC* and amino acid contents of 10% and 65% (w/w), respectively (Abiusi, Moñino Fernández, et al., 2022). Moreover, *C-PC* produced by *G. sulphuraria* had superior acid and thermo-stability compared to *C-PC* extracted from *Arthrospira platensis* (Abiusi, Moñino Fernández, et al., 2022; Böcker et al., 2019), which is the current commercial source of this pigment. *C-PC* can be utilized for several applications, such as natural colorants, antioxidant compounds, and fluorescent markers in the food, nutraceutical, and biomedical industries (Eriksen, 2008).

The amino acid profile of *G. sulphuraria* is reportedly ideal for human consumption (Abiusi, Moñino Fernández, et al., 2022). Indeed, 9 of the 20 common amino acids need to be introduced into our body through the diet, as we are not able to synthetize them. The amino acid profile of *G. sulphuraria* is rich in all of the essential amino acids, especially the two that contain sulphur (Abiusi, Moñino Fernández, et al., 2022). Sulphur-containing amino acids are generally scarce in most plant sources (Day, 2013); therefore, they are particularly relevant for persons consuming plant-based diets.

Bioaccessibility is as important as the quantity of the protein fraction. Generally, the bioaccessibility of intracellular nutrients from whole microalgae is limited by the indigestible cell wall (Canelli et al., 2020). To the best of our knowledge, only one study reported the protein bioaccessibility of a *G. sulphuraria* strain cultivated heterotrophically (Massa et al., 2019). When grown heterotrophically, *G. sulphuraria* typically contains approximately 20–40% (w/w) protein (Graziani et al., 2013; Massa et al., 2019; Pleissner et al., 2021), while autotrophic and mixotrophic cultivation of *G. sulphuraria* reportedly yields a higher protein content of up to 64–72% (w/w) (Abiusi, Moñino Fernández, et al., 2022; Cheng et al., 2019). Trophic mode might also impact protein bioaccessibility, as it was shown to affect the lipid bioaccessibility of high-pressure homogenized *Chlorella vulgaris* cells grown under mixotrophic and heterotrophic conditions (Canelli et al., 2022). To date, no study has compared the protein bioaccessibility of microalgae grown autotrophically and mixotrophically. Moreover, although over 100 *G. sulphuraria* strains have been described to date (www.acuf.net), no studies have been conducted to identify possible differences between strains in terms of protein and *C-PC* content, amino acid profile, and protein bioaccessibility.

In this work, we cultivated two strains of *G. sulphuraria*: *G. sulphuraria* SAG 108.79, which was isolated from a sulphur spring and reported as being rich in carbohydrates (Martinez-Garcia, Kormpa, & van der Maarel, 2017; Martinez-Garcia & van der Maarel, 2016), and *G. sulphuraria* ACUF 064, which was isolated from the ceiling of a Gill oven used for sulphur extraction and previously reported as being rich in protein (Abiusi, Moñino Fernández, et al., 2022). Both strains were cultivated in pilot-scale annular column photobioreactors (*PBRs*) under nonsterile conditions in both autotrophic and mixotrophic modes. Biomasses were collected in linear phase and compared in terms of protein and *C-PC* content, amino acid profile and protein bioaccessibility.

2. Materials and methods

2.1. Strains, growth conditions, and medium

In this study, we used two *Galdieria sulphuraria* strains: *G. sulphuraria* SAG 108.79, purchased from the algae culture collection of Göttingen University (SAG), and *G. sulphuraria* ACUF 064, donated by the Algal Collection of University Federico II of Naples (ACUF). Stock cultures were incubated in 250-mL flasks at 37 °C and 2% (*v*/v) CO_2. Both strains were cultivated in the medium described by Abiusi, Trompetter, Pollio, Wijffels, and Janssen (2022) at pH 1.7 ± 0.2, 37 °C, and under 24/24 h illumination with an average photon flux density of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. These cultures were used as inocula for the *PBR* experiments described below.

2.2. Cultivation system and operation

The two *G. sulphuraria* strains were cultivated in 17-L annular column *PBRs* (*AC-PBRs*). The reactors consisted of two Plexiglas cylinders of different external diameters (11 and 20 cm) placed one inside the other. The cylinders were 1 m high and 3 mm thick, forming an internal annular cultivation chamber with a thickness of 3.9 cm. The reactors were illuminated by six 18-W LED tubes 1.2 m long (T8 5000K, Intec light, Italy) positioned inside the inner cylinder. The experiments were conducted under 24/24 h illumination at an average photon flux density of 340 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured inside the inner cylinder in the empty reactors. The *AC-PBRs* were screened from ambient light and filled up to 0.9 m of their height, resulting in an internal illuminated surface of 0.314 m^2. The *PBRs* were housed in a greenhouse located in Wageningen (The Netherlands). The minimum temperature of the greenhouse was set to 15 °C.

The culture was mixed *via* aeration through a perforated tube positioned at the bottom of the culture chamber. The flow rate was set at 0.3 $L \cdot L^{-1} \cdot min^{-1}$, and air enriched with 2% (*v*/v) CO_2 and air alone was used in the autotrophic and mixotrophic cultures, respectively. The cultures were heated using the heat generated from the lights. Overheating of the culture was prevented by automatically switching on a fan to cool down the lamps when the culture temperature exceeded 38 °C for *G. sulphuraria* SAG 108.79 and 35 °C for *G. sulphuraria* ACUF 064. The initial pH was set to 1.7 using 1 M H_2SO_4.

Autotrophic and mixotrophic experiments were conducted in batches lasting 11 and 6 days, respectively. In the mixotrophic experiments, glucose was used as the carbon source. A 15% $w \cdot w^{-1}$ glucose solution was provided to the reactors *via* peristaltic pumps. The substrate supply rate (r_s, C-g glucose$\cdot L^{-1} \cdot day^{-1}$) was calculated according to Eq.1:

$$r_s = \frac{r_x^V \bullet \alpha}{Y_{x/s}} \quad (1)$$

where r_x^V is the autotrophic volumetric biomass productivity ($g_x \cdot L^{-1} \cdot day^{-1}$), α is the expected increase in r_x^V in the mixotrophic culture, and $Y_{x/s}$ ($g_x \cdot g_s^{-1}$) is the mixotrophic biomass yield on the substrate. Autotrophic r_x^V was determined for each strain in a batch experiment using CO_2 as the carbon source. Previous works (Abiusi et al., 2021; Abiusi, Moñino Fernández, et al., 2022) reported that mixotrophy doubled the autotrophic r_x^V. We arbitrarily decided to increase this value by 20%, resulting in an α of 2.4. We used a $Y_{x/s}$ of 0.75 $g_x \cdot g_s^{-1}$, the average of the previous mixotrophic growth of *G. sulphuraria* ACUF 064 (Abiusi et al., 2021; Abiusi, Moñino Fernández, et al., 2022). Cultures were adapted to autotrophic or mixotrophic conditions in the reactor for at least 1 week prior to starting the experiment. The cultures were then diluted with fresh medium to reach initial concentrations of approximately 1 and 2 $g_x \cdot L^{-1}$ in the autotrophic and mixotrophic experiments, respectively. Experiments were conducted in biological duplicates using two *AC-PBRs* operated in parallel. The reactors were operated under nonsterile conditions, meaning that the reactors, medium, and air were not sterilized, and samples were collected under non aseptic conditions.

2.3. Productivity calculations

The volumetric biomass productivity r_x^V was calculated as the linear regression of the increase in biomass concentration C_x ($g_x \cdot L^{-1}$) over

time. r_x^V was converted into areal biomass productivity (r_x^A, $g{\cdot}m^{-2}{\cdot}day^{-1}$) according to Abiusi et al. (2021). Biomass yield on light ($Y_{x/ph}$, $g_x{\cdot}mol_{ph}^{-1}$) was derived from autotrophic r_x^A according to Eq. 2:

$$Y_{x/ph} = \frac{r_x^A}{r_{ph}} \quad (2)$$

where r_{ph} is the photon supply rate ($mol_{ph}{\cdot}m^{-2}{\cdot}day^{-1}$).

2.4. Daily cultivation and biomass measurements

Daily measurements were conducted to assess culture growth, the photosystem II maximum quantum yield of photochemistry, pH, temperature, and the presence of microbial contaminants. Culture growth was monitored by measuring biomass dry weight (C_x, $g_x{\cdot}L^{-1}$), whereas the photosystem II maximum quantum yield of photochemistry (QY, $F_v{\cdot}F_m^{-1}$) was measured at 455 nm with an AquaPen-C AP-C 100 (Photon Systems Instruments, Czech Republic) following the procedure described in Abiusi et al. (2021). pH and temperature were measured offline 3 times each day (9:00, 13:00, and 17:00) throughout the entire experiment. Moreover, the temperature probes recorded minimum and maximum temperatures over 24 h. Glucose accumulation in the medium was measured daily using a bioanalyser (YSI 2700, YSI Life Sciences, USA). The presence of microbial contamination was measured qualitatively daily using an optical microscope (Laborlux S, Leica, Germany) and at the end of each batch using fluorescence microscopy (EVOS FL, Thermo Fisher Scientific, USA) after DNA staining with SYBR Green I (Sigma-Aldrich, USA).

2.5. Protein and amino acid measurements

Galdieria sulphuraria biomasses obtained during autotrophic and mixotrophic cultivation were centrifuged at 3600 ×*g* for 5 min (DL6M, Kaida, China). The pellet was resuspended in 6 L of demineralised water and centrifuged again using the same settings. The harvested algae paste was spread onto an aluminium tray, creating a 1-cm-thick layer of algae biomass. The tray was frozen at −20 °C and then freeze-dried. The total nitrogen content of the biomass was measured by dispersing approximately 10 mg of freeze-dried biomass in 15 mL demineralised water using a TOC-L connected to a TN module (Shimadzu Europe, Germany). The protein content was estimated by multiplying the nitrogen content by the protein using a nitrogen conversion factor of 6.25 (Sáez-Plaza, Michałowski, Navas, Asuero, & Wybraniec, 2013). The analyses were performed in triplicate. Amino acid composition was determined by following the standardized method described in ISO 13903.

2.6. Phycocyanin measurements

Freeze-dried biomasses were resuspended in 50 mM sodium acetate, disrupted by bead beating, and centrifuged, and *C-PC* in the supernatants was quantified by measuring the absorbance at 620 nm and 652 nm according to the protocol reported in Abiusi, Trompetter, et al. (2022). The analyses were performed in triplicate.

2.7. Protein bioaccessibility by in vitro digestion

An *in vitro* digestion model was used to measure protein bioaccessibility, following the harmonised protocol INFOGEST 2.0, as described in Canelli et al. (2020). After enzymatic digestion, the digestate was centrifuged (30 min, 10,000 ×*g*, 4 °C). The micellar phase (supernatant) and the pellet were separately frozen. A blank containing water (2 mL) instead of algal biomass was used to quantify the nitrogen coming from the digestion protocol.

The protein content of the full digesta and micellar phase was analysed by measuring the total nitrogen concentration. Protein bioaccessibility was defined as the protein concentration in the micellar phase (corrected by the protein in the micellar phase of the enzyme blank) divided by the protein concentration in the full digesta (corrected by the protein in the full digesta of the enzyme blank), expressed as a percentage (%) (Eq. 3):

$$Protein\ bioaccessibility\ (\%) = \frac{[Protein]_{micellar\ phase}}{[Protein]_{full\ digesta}} \text{ x } 100\% \quad (3)$$

3. Results and discussion

3.1. Absence of contamination in mixotrophic G. sulphuraria cultures

Contamination by unwanted microorganisms is a challenge in the developing microalgae industry. Several protist species can graze on microalgae, causing culture crush (Day et al., 2017). Preventing microbial contamination is even a larger challenge in the presence of an organic substrate in the medium, as bacteria and fungi can outcompete microalgae for substrate utilization (Unnithan, Unc, & Smith, 2014).

In this work, two *G. sulphuraria* strains were cultivated in pilot-scale *PBRs* (17 L) under nonsterile conditions. Each strain was grown first autotrophically and then mixotrophically for a total time of 35 days. Microscopic observation did not reveal the presence of microbial contaminants in any of the analysed cultures. This result indicates that pH < 1.9 is sufficient to prevent microbial contamination. Previous works involving heterotrophic cultures conducted under nonsterile conditions are consistent with this result (Pleissner et al., 2021; Russo et al., 2021). However, those studies were conducted in bench-scale *PBRs* (1−2 L), and each experiment lasted no longer than 12 days, making this study the first to report axenic cultivation of *G. sulphuraria* at the pilot scale over an extended period of 35 days.

3.2. Autotrophic cultivation of G. sulphuraria ACUF 064 and SAG 108.79

Galdieria sulphuraria ACUF 064 and *G. sulphuraria* SAG 108.79 were cultivated autotrophically at 340 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$. Both strains maintained linear growth (Eq. A1, A3) during the 11 days of autotrophic cultivation, reaching a final biomass concentration of 3.6 ± 0.1 $g_x{\bullet}L^{-1}$ (Fig. 1, Table 1). *Galdieria sulphuraria* ACUF 064 and SAG 108.79 exhibited similar photosystem II maximum quantum yields of photochemistry (*QY)* between 0.38 and 0.42 (Table 1), which is in the higher range of *G. sulphuraria* (Abiusi et al., 2021; Abiusi, Moñino Fernández, et al., 2022; Abiusi, Trompetter, et al., 2022) and indicates that our cultures were not photoinhibited. This finding confirms that it is possible to successfully cultivate *G. sulphuraria* at high light intensity. Maintaining a high biomass concentration (1–6 $g_x{\cdot}L^{-1}$) in the reactor allowed cultivation at high light intensity, as the specific light exposure of each single cell was limited by self-shading (Abiusi et al., 2021).

Volumetric biomass productivity (r_x^V) was in the low range of values previously reported (Table 1). However, a fair comparison of the autotrophic performance of different *PBR* designs needs to consider the reactor geometry and incident light intensity. For these reasons, we calculated the areal biomass productivity (r_x^A) and biomass yield on light ($Y_{x/ph}$) (Section 2.3). For both strains, the values of r_x^A and $Y_{x/ph}$ were in the high range of previously reported values but 48% and 24% lower than the highest reported respective values (Table 2). The superior autotrophic performance reported by Abiusi, Moñino Fernández, et al. (2022) was obtained in a better-controlled, 2-L stirred-tank *PBR*. Scaling up illuminated cultures of microalgae is known to negatively affect productivity and therefore $Y_{x/ph}$ (Barros et al., 2017). Aside from less efficient process control, the observed decrease in productivity could be attributed to contamination, biofilm formation and reduced turbulence. During the cultivation period, we did not observe contamination or biofilm formation. Lower turbulence and a less detailed temperature control (Table 1) could be the primary causes of the lower performance. Recently, Zanolla et al. (2022) cultivated *A. platensis* in 6-L *AC-PBRs*

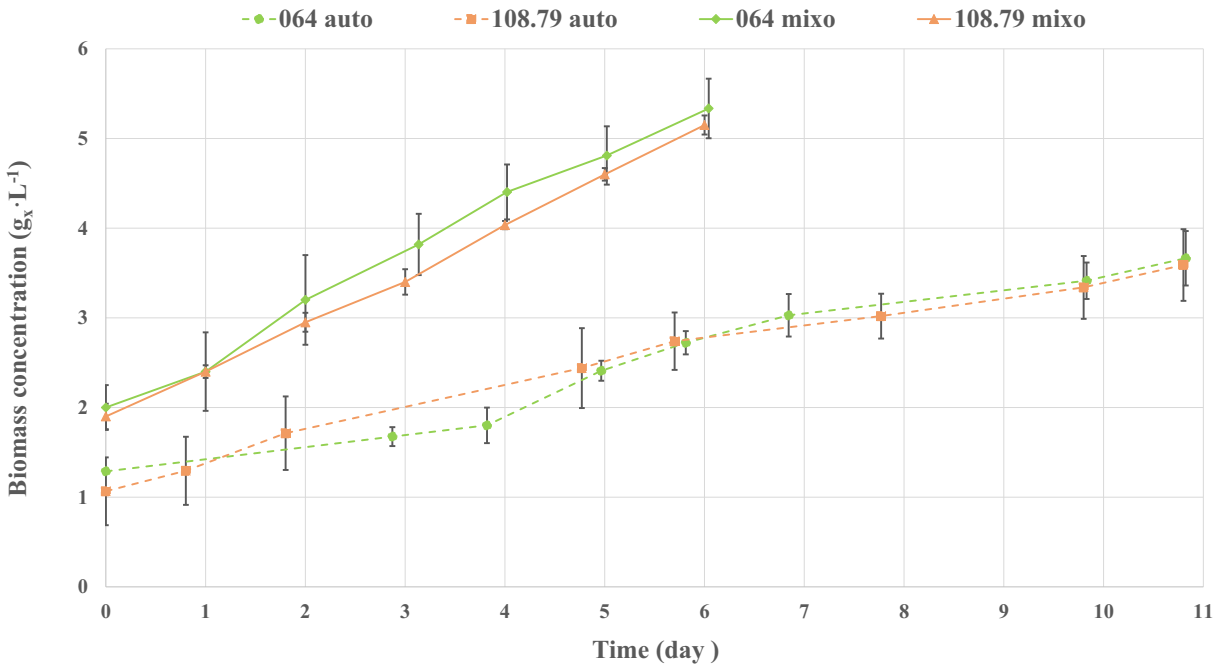

Fig. 1. Autotrophic (dotted lines) and mixotrophic (solid lines) batches of *G. sulphuraria* SAG 108.79 (■,▲; orange) and ACUF 064 (●,◆; green) cultivated in two 17-L annular column photobioreactors. Values are expressed as averages ± standard deviation of the two reactors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Overview of the offline measurements of *G. sulphuraria* SAG 108.79 and ACUF 064 cultivated autotrophically and mixotrophically in batch. Experiments were conducted in biological duplicates ($n = 2$) and are reported with the standard deviation of measurements.

	Units	108.79 auto	108.79 mixo	064 auto	064 mixo
pH (av)		1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
T (av)	°C	42 ± 3	43 ± 3	37 ± 5	38 ± 3
T max/ min	°C	44/35	42/ 32	41/25	40 /26
C_x (end)	$g \cdot L^{-1}$	3.6 ± 0.3	5.2 ± 0.1	3.7 ± 0.1	5.3 ± 0.3
r_x^V	$g_x \cdot L^{-1} \cdot day^{-1}$	0.23 ± 0.00	0.55 ± 0.00	0.2 ± 0.00	0.55 ± 0.00
r_x^A	$g_x \cdot m^{-2} \cdot day^{-1}$	12.3 ± 0.1	29.6 ± 0.3	12.4 ± 1.4	29.7 ± 0.1
r_s	$g_s \cdot L^{-1} \cdot day^{-1}$	–	0.82 ± 0.00	–	0.82 ± 0.00
QY	(F_v/fm)	0.38 ± 0.01	0.42 ± 0.00	0.38 ± 0.01	0.39 ± 0.04
$Y_{x/s}$	$g_x \cdot g_s^{-1}$	–	0.67 ± 0.01	–	0.67 ± 0.01
$Y_{x/ph}$	$g_x \cdot mol_{ph}^{-1}$	0.42 ± 0.03	–	0.42 ± 0.00	–

similar to the one used in this study, obtaining $Y_{x/pH}$ values comparable to that of our study and confirming that the obtained $Y_{x/ph}$ can be attributed to the type of reactor design and operation rather than the strain.

3.3. Mixotrophic cultivation of G. sulphuraria ACUF 064 and SAG 108.79

To the best of our knowledge, this is the first report of a scaling up of mixotrophic cultivation of *G. sulphuraria* under nonsterile conditions. Glucose was supplied at a constant rate, and the cultures were grown in *AC-PBRs* mixed with air without CO_2 enrichment.

Supplying CO_2 to microalgal cultures on an industrial scale can be challenging and might represent one of the major costs. Between 25% and 50% of the provided CO_2 is not taken up from the culture and thereby lost into the atmosphere (Acién, Fernández, Magán, & Molina, 2012; Doucha, Straka, & Lívanský, 2005). Even considering flue gas containing 10–15% (*v*/v) CO_2, the infrastructure associated with CO_2 capture and transport would represent a considerable cost and thus restrict the area suitable for microalgae cultivation (Chisti, 2013; Pate, Klise, & Wu, 2011). Suppling inorganic carbon with an acidophilic microalga is even more challenging, because at low pH, CO_2 in solution does not form either HCO_3^- or CO_3^{2-}, which are used by various microalgal species as soluble forms of inorganic carbon. The reduced total amount of inorganic carbon in the liquid phase negatively affects the gas–liquid mass transfer of CO_2. The first estimations of the autotrophic production cost of *G. sulphuraria* biomass highlighted CO_2 supply as a major cost (Abiusi, 2021).

In mixotrophy, glucose was supplied at a constant rate calculated to provide excess carbon to the culture (Section 2.2). Previous works conducted in laboratory-scale *PBRs* indicated that *G. sulphuraria* can be cultivated mixotrophically without supplying CO_2 (Abiusi, 2021; Abiusi, Trompetter, et al., 2022; Sloth, Wiebe, & Eriksen, 2006). Our study demonstrated that this process can be scaled up. To avoid possible carbon limitation, we chose a glucose supply rate that we expected would increase the autotrophic volumetric biomass production rate (r_x^V) by 2.4-fold (Section 2.2). The glucose concentration in the medium was below the detection level throughout the entire experiment. The mixotrophic r_x^V value was 2.3 times higher than the autotrophic r_x^V value (Table 1). This difference can be explained by the lower biomass yield on substrate ($Y_{x/s}$) observed in this study (0.67 $g_x \cdot g_s^{-1}$) (Table 1) compared to the $Y_{x/s}$ of 0.75 $g_x \cdot g_s^{-1}$ reported in previous studies (Abiusi, 2021; Abiusi, Moñino Fernández, et al., 2022) and used for calculating the substrate supply rate. However, in these previous studies, the substrate feeding rate was automatically regulated to obtain constant dissolved oxygen. This strategy doubled biomass productivity. A lower increase in productivity has been linked to more balanced mixotrophic growth, in which autotrophic and mixotrophic metabolism contribute equally to overall mixotrophic growth (Abiusi, Wijffels, & Janssen, 2020). Therefore, a larger fraction of CO_2 produced in heterotrophic metabolism can

Table 2
Comparison of biomass (auto) and mixotrophic (mixo) volumetric (r_x^V) and areal (r_x^A) biomass productivities, yield on light ($Y_{x/ph}$), C-phycocyanin content (*C-PC*) and areal productivity ($r_{C\text{-}PC}^A$) of this study and other values under 24 h/24 h illumination reported in the literature. Experiments were conducted in biological duplicates (n = 2) and are reported with the standard deviation of measurements. The table reports the photobiorector type (*PBR*) used for the comparison (AC = annular column; STR = stirred tank reactor; BC = bubbled column), photobiorector volume (*V*), illuminated surface (*IS*), and incident light intensity (I_o).

Reference	Trophic mode	PBR	Strain	*V* (L)	*IS* (m^2)	I_0 ($\mu molph \cdot m^{-2} \bullet s^{-1}$)	r_x^V ($g \cdot L^{-1} \cdot day^{-1}$)	r_x^A ($g \cdot m^{-2} \cdot day^{-1}$)	$Y_{x/ph}$ ($g_x \bullet mol_{ph}^{-1}$)	*C-PC* (% w/w)	$r_{C\text{-}PC}^A$ ($g \cdot m^{-2} \cdot day^{-1}$)
This study	Auto	AC	*GS* ACUF 064	17	0.314	340	0.24 ± 0.00	12.4 ± 1.4	042 ± 0.00	9.7 ± 1.4	1.2 ± 0.00
This study	Mixo	AC	*GS* ACUF 064	17	0.314	340	0.55 ± 0.00	29.7 ± 0.1	–	8.7 ± 0.3	2.6 ± 0.1
This study	Auto	AC	*GS* SAG 108.79	17	0.314	340	0.23 ± 0.00	12.3 ± 0.1	0.42 ± 0.00	4.2 ± 0.3	0.5 ± 0.0
This study	Mixo	AC	*GS* SAG 108.79	17	0.314	340	0.55 ± 0.00	29.6 ± 0.3	–	4.4 ± 0.0	1.3 ± 0.0
Abiusi, Trompetter, et al., 2022	Auto	STR	*GS* ACUF 064	2	0.067	514	0.81	24.1	0.55	9.6	2.3
Abiusi, Trompetter, et al., 2022	Mixo	STR	*GS* ACUF 064	2	0.067	514	1.66	49.3	–	10.1	5.0
Baer, Heining, Schwerna, Buchholz, & Hübner, 2016	Auto	BC	*GS* SAG 108.79	0.9	0.079	100	0.30	3.5	0.40	1.6	0.6
Graziani et al., 2013	Auto	BC	*GS* ACUF 064	4	0.181	150	0.18	3.9	0.30	0.8	
Zanolla et al., 2022	Auto	AC	*A. platensis F&M-C260*	6	0.178	700	0.77	25.9	0.43	11.2	2.9

be fixed in autotrophic metabolism, resulting in a higher biomass yield on the substrate ($Y_{x/s}$). However, despite $Y_{x/s}$ being 10% less than that in a previous mixotrophic study (Abiusi et al., 2021; Abiusi, Moñino Fernández, et al., 2022), it was still significantly higher than the 0.3–0.5 $g_x \cdot g_s^{-1}$ reported for heterotrophic cultures (Abiusi, Trompetter, et al., 2022; Massa et al., 2019; Rahman, Sarian, & van der Maarel, 2020).

The 2.3-fold increase in biomass productivity obtained under mixotrophic conditions in this study represents one of the most productive illuminated culture of *G. sulphuraria* to date (Table 2). Interestingly, the mixotrophic areal biomass productivity (r_x^A) was comparable to that obtained in culture of *A. platensis* in a similar *AC-PBR* but illuminated with double the amount of light used in this study (Zanolla et al., 2022) (Table 2).

Another import finding of the present study was that under mixotrophy, linear growth (Eq. A2, A4) was maintained throughout the experiment, reaching a final biomass concentration of 5.3 ± 0.1 $g_x \cdot L^{-1}$, a value 44% higher than that of the autotrophic culture (Fig. 1, Table 1). This result confirms the exceptional capacity of *G. sulphuraria* to maintain linear growth under mixotrophy at a low specific light supply rate (Abiusi et al., 2021).

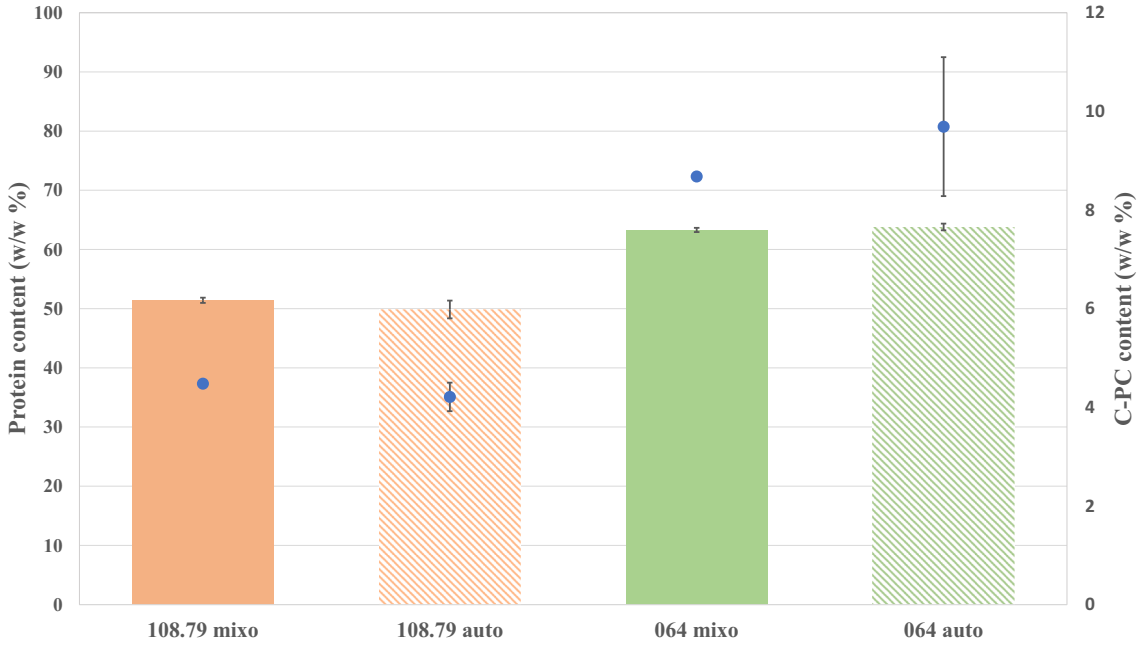

Fig. 2. Protein (bars) and C-phycocyanin (*C-PC*) contents (dots) (*w*/w, %) of biomasses of *G. sulphuraria* ACUF 064 (green) and SAG 108.79 (orange) cultivated in mixotrophic (solid) and autotrophic modes (diagonal stripes). The results are the average of triplicates ($n = 3$), and error bars represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Biomass concentration is an important parameter in microalgae cultivation, as it directly affects harvesting costs and represents one of the major contributors to production costs (Ruiz et al., 2016). Therefore, the economics and sustainability of the process are maximised when working at high biomass concentrations.

3.4. Protein and phycocyanin content

We compared the *C-PC* and protein contents of *G. sulphuraria* SAG 108.79 and ACUF 064 (Fig. 2). Both strains were grown under autotrophic and mixotrophic conditions. Both *C-PC* and protein contents were similar within the same strain, irrespective of the trophic mode. *G. sulphuraria* SAG 108.79 protein and *C-PC* content were on average between the two trophic modes 50.6 ± 1.1 *w*/w and 4.3 ± 0.2 (*w*/w), respectively. *G. sulphuraria* ACUF 064 had on average between the two trophic modes 26% (w/w) more protein than *G. sulphuraria* SAG 108.79 and approximately double of its *C-PC* content (Fig. 2). High biomass areal productivity combined with high *C-PC* content, make *G. sulphuraria* ACUF 064 a promising strain for *C-PC* production (Table 2). This is especially in mixotrophy where *G. sulphuraria* ACUF 064*C-PC* areal productivity ($r_{AC\text{-}PC}$) was among the highest ever reported (Table 2).

Previous studies reported high variability in the *C-PC* and protein content of *G. sulphuraria*, ranging from 0.8% to 13% and 33% to 72% of the total biomass dry weight for *C-PC* and proteins, respectively (Abiusi, Moñino Fernández, et al., 2022; Cheng et al., 2019; Graziani et al., 2013; Wan et al., 2016). Such great variability can be explained by the different cultivation conditions and strains used in those studies.

Galdieria sulphuraria ACUF 064 is the most studied strain for *C-PC* and protein production, and it has been reported to have great intraspecies variability in both *C-PC* and protein content (Abiusi, Moñino Fernández, et al., 2022; Carbone, Olivieri, Pollio, & Melkonian, 2020; Graziani et al., 2013). Cultivation conditions are key to maximising the production of these two compounds. In this study, the same cultivation conditions were applied for the two different *G. sulphuraria* strains; therefore, the differences in *C-PC* and protein content are strain specific. Concurrently to the submission of this work, Montenegro-Herrera, Vera-López Portillo, Hernández-Chávez, and Martinez (2022) compared four *G. sulphuraria* strains cultivated autotrophically under the same conditions. *C-PC* and proteins and content ranged from 0.2% to 4.7% and 39.3% to 46.9% (*w*/w) respectively, indicating significant strain specific differences. Interestingly, in their study one of the four strains tested was *G. sulphuraria* SAG 108.79, the same used in our study. In this strain, the authors reported a *C-PC* and protein content of 3.4% and 43.4% (w/w) respectively, 19% and 13% lower than the value here reported. Carfagna et al. (2018) cultivated two strains of *G. phlegrea* under the same autotrophic and heterotrophic conditions. The authors reported strain-specific differences in *C-PC* content and in thermal and acid stability. A possible explanation for these strain-specific characteristics relies on the evolutionary history of each strain, which made each strain fit to colonize a specific niche (Carfagna et al., 2018). Moreover, the genus *Galdieria* exhibits an unexpected level of genetic biodiversity (Ciniglia, Yoon, Pollio, Pinto, & Bhattacharya, 2004). The evolution of this genus is still under debate (Toplin, Norris, Lehr, McDermott, & Castenholz, 2008), however, and at least 5% of its protein-encoding genes were acquired by horizontal gene transfer (Schönknecht, Weber, & Lercher, 2014). We formulated a possible hypothesis to explain the differences between the two strains found in this study, which is described in Section 3.7.

3.5. Amino acid composition and profile

The amino acid contents of *G. sulphuraria* SAG 108.79 and *G. sulphuraria* ACUF 064 were analysed during linear growth under autotrophic and mixotrophic metabolic conditions. The relative abundance of each amino acid was calculated by dividing the content of each amino acid by the total amino acid content. The two strains displayed a comparable profile (Table 3). This shows that there is high intraspecies biological conservation of the amino acid profile in different *G. sulphuraria* strains. Moreover, these results indicate that trophic mode does not significantly affect the amino acid profile and total content, confirming the results presented in Fig. 2.

The absolute amino acid composition for the whole biomass is reported in the supplementary material (Table A1). Interestingly, in all the samples, the sum of all the individual amino acids was comparable to the protein content calculated by multiplying the biomass nitrogen content by the conversion factor 6.25 (Fig. 2). This method in other algal species has been shown to overestimate the protein content (Sáez-Plaza et al., 2013), and species-specific conversion factors have been published for several microalgae based on their amino acid profile (González López et al., 2010; Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004). The results of the present study indicate that use of the conversion factor 6.25 provides a good estimation of the protein content of *G. sulphuraria*, confirming the findings of Abiusi, Moñino Fernández, et al. (2022). The amino acid profile and content of *G. sulphuraria* ACUF 064 found in this study (Tables 3 and A1) are comparable to the profiles previously described by Abiusi, Moñino Fernández, et al. (2022). Such a high amino acid content and reproducible amino acid profile are desirable traits for future scale-up in production. The amino acid content and profile of four *G. sulphuraria* strains has been recently published by Montenegro-Herrera et al. (2022). Unfortunately, the methodology used by the authors did not allow to determine the content of tryptophan, asparagine, and glutamine. According to our data (Table A1), those three amino acids are abundant in *G. sulphuraria*, representing about one fourth of the total amino acid content. This might partially explain the lower total amino acid content measured by the authors in *G: sulphuraria* SAG 108.79. Interestingly, excluding the three not detected amino acids, the amino acid profile among the four strains was almost identical, confirming our findings (Table 3).From a nutritional perspective, it is important that the relative proportion of essential amino acids is well

Table 3
Amino acid (AA) profile expressed as mg AA per g of protein of *G. sulphuraria* SAG 108.79 and ACUF 064 cultivated autotrophically and mixotrophically in 17-L annular columns. Values are expressed as the average of duplicates (n = 2). The relative deviation of duplicates was <5% or as otherwise indicated.

	mg AA/g protein					
	This study				Abiusi, Trompetter, et al., 2022)	
	108.79 auto	108.79 mixo	064 auto	064 mixo	064 auto	064 mixo
Cysteine + cystine	14	13 [a]	13 [a]	15	16	17
Methionine	22	21 [b]	23	21	24	24
Tryptophan	14	12	12	12	13	13
Alanine	63	63	64	62	63	61
Aspartic acid	90	92	93	90	92	91
Arginine	61	66	64	61	63	63
Glutamic acid	140	137	140	144	142	144
Glycine	44	45	42	43	43	43
Histidine	16	16	15	15 [a]	16	17
Isoleucine	52	53	54	53	56	55
Leucine	80	82	81	79	81	81
Lysine	65	66	63	62	60	61
Phenylalanine	46	46	45	44	45	46
Proline	51	61	42[a]	45	43	43
Serine	65	61	66	67	65	64
Threonine	57	54	59	64[b]	57	57
Tyrosine	63	54	65	66	64	65
Valine	58	57	58	58	56	56

[a] Relative deviation between duplicates was between 5 and 10%.
[b] Relative deviation between duplicates was between 10 and 15%.

balanced and fulfils the FAO dietary requirements for adults (WHO, 2007). The amino acid profile of *G. sulphuraria* reported here corresponds to the profile previously described in Abiusi, Moñino Fernández, et al. (2022), which was shown to be consistent with the FAO dietary requirements. *G. sulphuraria* has a better amino acid profile than *Arthrospira* and *Chlorella*, the primary microalgae used in food applications, and soybean, the primary plant protein source worldwide (Abiusi, Moñino Fernández, et al., 2022). Finding that both strains have an equal high-quality amino acid profile is an encouraging result for future food applications of this species.

3.6. Protein bioaccessibility

A balanced amino acid profile and high protein content are not the only characteristics that make a biomass a good protein source for human consumption. The limited bioaccessibility of proteins from microalgae is a major challenge that needs to be addressed (Canelli et al., 2020).

This study assessed the protein bioaccessibility of *G. sulphuraria* SAG 108.79 and ACUF 064 biomasses grown under mixotrophic and autotrophic conditions (Fig. 3). The protein bioaccessibility of *G. sulphuraria* SAG 108.79 grown under mixotrophic and autotrophic conditions was 55.3% ± 1.8% and 69.3% ± 2.8%, respectively. *Galdieria sulphuraria* ACUF 064 showed a lower protein bioaccessibility of 16.0% ± 1.5% and 12.1% ± 1.0% under mixotrophic and autotrophic conditions, respectively. A large difference in protein bioaccessibility was found between the two strains, with *G. sulphuraria* SAG 108.79 having 4.4-fold higher protein bioaccessibility than *G. sulphuraria* ACUF 064.

The only previous study of the protein bioaccessibility of *G. sulphuraria* was conducted on strain SAG 107.79 cultivated heterotrophically and reported a protein digestibility of 63–79% depending on the medium used for cultivation (Massa et al., 2019). These results are in the same range of the bioaccessibility reported here for *G. sulphuraria* SAG 108.79. Microalgal protein bioaccessibility has been reported to be between 40% and 82%, with high variability recorded not only between species but also between strains (Canelli et al., 2020; Niccolai, Chini Zittelli, Rodolfi, Biondi, & Tredici, 2019). One of the main reasons behind such high variability is the composition of the recalcitrant cell wall, which varies widely between species, strains and even growth phases (Canelli et al., 2022, 2020). *G. sulphuraria* has a unique cell wall that contains low amounts of cellulose and up to 55% protein (Bailey & Staehelin, 1968; Oesterhelt, Vogelbein, Shrestha, Stanke, & Weber, 2008). In the human digestive tract, there are no enzymes able to digest cellulose, while there are several enzymes able to hydrolyse protein, therefore a high protein bioaccessibility in both strains was expected. The explanation for the different protein bioaccessibility likely involves the different cell wall biochemical compositions and morphologies, as the cell wall is the major hurdle for the bioaccessibility of microalgal intracellular compounds (see Section 3.7).

3.7. Hypothesis: Different traits for different locations

The two strains used in this study were characterised by similar productivity and amino acid profiles, but they showed major differences in *C-PC*, amino acid and protein content and protein bioaccessibility. Our hypothesis is that these differences are correlated with the niche colonized by the strain. *Galdieria sulphuraria* SAG 108.79 was isolated from the effluent of a sulfuric pond in Yellowstone National Park (US) that was exposed to direct sunlight and not subjected to changes in salinity. *Galdieria sulphuraria* SAG 108.79 might have a lower *C-PC* content because it evolved in an environment in which light is not the limiting growing factor. Moreover, its higher protein bioaccessibility might be explained by a potentially thinner cell wall, which would be easier to degrade by digestive enzymes. The lower *C-PC* content and a thinner cell wall (rich in proteins) might explain the lower protein content of this strain and should be the focus of future research.

In contrast, *G. sulphuraria* ACUF 064 was isolated in the ceiling of a Gill oven used for sulphur extraction in Sicily (Italy); it was not exposed to direct sunlight, but the environment was characterised by variable humidity, and therefore, the strain was subjected to periods of desiccation. The limited sunlight might be the reason for the higher expression of pigments (*e.g.*, *C-PC*) in *G. sulphuraria* ACUF 064. Moreover, to cope with the period of desiccation, this strain might have evolved a thicker cell wall, as thicker and more rigid cell walls have been

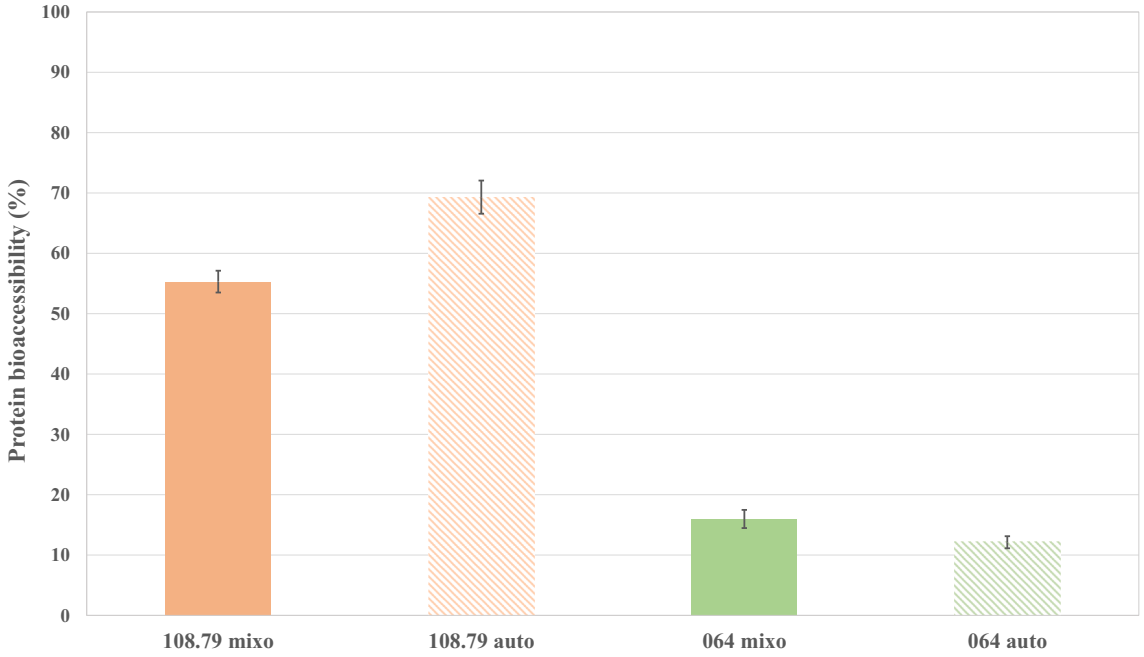

Fig. 3. Average protein bioaccessibility (%) of biomasses of *G. sulphuraria* ACUF 64 (green) and SAG 108.79 (orange) cultivated in mixotrophic (solid) and autotrophic (diagonal stripes) modes. The results are the average of triplicates (n = 3), and error bars represent the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated with increased tolerance to desiccation (Ciniglia et al., 2004). Such a thicker cell wall could explain the lower protein bioaccessibility. A thicker cell wall and higher *C-PC* content may also explain the higher protein content. Further studies are needed to confirm this hypothesis.

4. Conclusions

In the present study, *G. sulphuraria* ACUF 064 and SAG 108.79 were cultivated autotrophically and mixotrophically in pilot-scale *PBRs* under nonsterile conditions. No contaminants were observed in any of the cultures, indicating that pH < 1.9 was sufficient to prevent microbial contamination. The two strains displayed similar autotrophic and mixotrophic biomass productivity. In mixotrophy, glucose was used as the sole carbon source, and biomass productivity was 2.3 times higher than autotrophic productivity. The *C-PC* and protein contents were similar within the same strain, irrespective of the trophic mode. When comparing the two strains, the protein content of *G. sulphuraria* SAG 108.79 was 26% (*w*/w) lower than the protein content of *G. sulphuraria* ACUF 064, and the *C-PC* content was approximately half. The amino acid profile was well balanced and fulfilled the FAO dietary requirements for adults. No differences in amino acid profile were found between the two strains or between trophic modes. Stable and high-quality protein profiles are encouraging results for future food applications of this species. Interestingly, *G. sulphuraria* SAG 108.79 showed protein bioaccessibility in line with other microalgal species, whereas *G. sulphuraria* ACUF 064 had one of the lowest protein bioaccessibility values ever reported for an algal species. We hypothesised that such a difference in bioaccessibility might be due to differences in cell wall composition.

CRediT authorship contribution statement

Greta Canelli: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Fabian Abiusi:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Albert Vidal Garcia:** Methodology, Formal analysis, Writing – review & editing. **Stefano Canziani:** Resources, Writing – review & editing, Funding acquisition. **Alexander Mathys:** Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that Algreen B.V. supported the research conducted for this manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2023.103287.

References

Abiusi, F. (2021). *Oxygen balanced mixotrophy in microalgae*. Wageningen University. https://doi.org/10.18174/542064

Abiusi, F., Moñino Fernández, P., Canziani, S., Janssen, M., Wijffels, R. H., & Barbosa, M. (2022). Mixotrophic cultivation of *Galdieria sulphuraria* for C-phycocyanin and protein production. *Algal Research, 61*, Article 102603. https://doi.org/10.1016/j.algal.2021.102603

Abiusi, F., Trompetter, E., Hoenink, H., Wijffels, R. H., & Janssen, M. (2021). Autotrophic and mixotrophic biomass production of the acidophilic *Galdieria sulphuraria* ACUF 64. *Algal Research, 60*. https://doi.org/10.1016/j.algal.2021.102513

Abiusi, F., Trompetter, E., Pollio, A., Wijffels, R. H., & Janssen, M. (2022). Acid tolerant and acidophilic microalgae: An underexplored world of biotechnological opportunities. *Frontiers in Microbiology, 13*. https://doi.org/10.3389/fmicb.2022.820907

Abiusi, F., Wijffels, R. H., & Janssen, M. (2020). Doubling of microalgae productivity by oxygen balanced Mixotrophy. *ACS Sustainable Chemistry & Engineering, 8*. https://doi.org/10.1021/acssuschemeng.0c00990

Acién, F. G., Fernández, J. M., Magán, J. J., & Molina, E. (2012). Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnology Advances, 30*, 1344–1353. https://doi.org/10.1016/j.biotechadv.2012.02.005

Baer, S., Heining, M., Schwerna, P., Buchholz, R., & Hübner, H. (2016). Optimization of spectral light quality for growth and product formation in different microalgae using a continuous photobioreactor. *Algal Research, 14*, 109–115. https://doi.org/10.1016/j.algal.2016.01.011

Bailey, R. W., & Staehelin, L. A. (1968). The chemical composition of isolated cell walls of Cyanidium caldarium. *Microbiology, 54*, 269–276. https://doi.org/10.1099/00221287-54-2-269

Barros, A., Guerra, L. T., Simões, M., Santos, E., Fonseca, D., Silva, J., … Navalho, J. (2017). Mass balance analysis of carbon and nitrogen in industrial scale mixotrophic microalgae cultures. *Algal Research, 21*, 35–41. https://doi.org/10.1016/j.algal.2016.10.014

Böcker, L., Ortmann, S., Surber, J., Leeb, E., Reineke, K., & Mathys, A. (2019). Biphasic short time heat degradation of the blue microalgae protein phycocyanin from *Arthrospira platensis*. *Innovative Food Science and Emerging Technologies, 52*, 116–121. https://doi.org/10.1016/j.ifset.2018.11.007

Canelli, G., Tarnutzer, C., Carpine, R., Neutsch, L., Bolten, C. J., Dionisi, F., & Mathys, A. (2020). Biochemical and nutritional evaluation of *Chlorella* and *Auxenochlorella* biomasses relevant for food application. *Frontiers in Nutrition, 7*. https://doi.org/10.3389/fnut.2020.565996

Canelli, G., Tevere, S., Jaquenod, L., Dionisi, F., Rohfritsch, Z., Bolten, C. J., … Mathys, A. (2022). A novel strategy to simultaneously enhance bioaccessible lipids and antioxidants in hetero/mixotrophic *Chlorella vulgaris* as functional ingredient. *Bioresource Technology, 347*, Article 126744. https://doi.org/10.1016/j.biortech.2022.126744

Carbone, D. A., Olivieri, G., Pollio, A., & Melkonian, M. (2020). Biomass and phycobiliprotein production of *Galdieria sulphuraria*, immobilized on a twin-layer porous substrate photobioreactor. *Applied Microbiology and Biotechnology, 104*, 3109–3119. https://doi.org/10.1007/s00253-020-10383-8

Carfagna, S., Landi, V., Coraggio, F., Salbitani, G., Vona, V., Pinto, G., … Ciniglia, C. (2018). Different characteristics of C-phycocyanin (C-PC) in two strains of the extremophilic *Galdieria phlegrea*. *Algal Research, 31*, 406–412. https://doi.org/10.1016/j.algal.2018.02.030

Cheng, F., Mallick, K., Henkanatte Gedara, S. M., Jarvis, J. M., Schaub, T., Jena, U., … Brewer, C. E. (2019). Hydrothermal liquefaction of *Galdieria sulphuraria* grown on municipal wastewater. *Bioresource Technology, 292*, Article 121884. https://doi.org/10.1016/j.biortech.2019.121884

Chisti, Y. (2013). Constraints to commercialization of algal fuels. *Journal of Biotechnology, 167*, 201–214. https://doi.org/10.1016/j.jbiotec.2013.07.020

Ciniglia, C., Yoon, H. S., Pollio, A., Pinto, G., & Bhattacharya, D. (2004). Hidden biodiversity of the extremophilic Cyanidiales red algae. *Molecular Ecology, 13*, 1827–1838. https://doi.org/10.1111/j.1365-294X.2004.02180.x

Day, J. G., Gong, Y., & Hu, Q. (2017). Microzooplanktonic grazers – A potentially devastating threat to the commercial success of microalgal mass culture. *Algal Research, 27*, 356–365. https://doi.org/10.1016/j.algal.2017.08.024

Day, L. (2013). Proteins from land plants – Potential resources for human nutrition and food security. *Trends in Food Science and Technology, 32*, 25–42. https://doi.org/10.1016/j.tifs.2013.05.005

Doucha, J., Straka, F., & Lívanský, K. (2005). Utilization of flue gas for cultivation of microalgae (*Chlorella sp.*) in an outdoor open thin-layer photobioreactor. *Journal of Applied Phycology, 17*, 403–412. https://doi.org/10.1007/s10811-005-8701-7

Eriksen, N. T. (2008). Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. *Applied Microbiology and Biotechnology, 80*, 1–14. https://doi.org/10.1007/s00253-008-1542-y

González López, C. V., García, M. D. C. C., Fernández, F. G. A., Bustos, C. S., Chisti, Y., & Sevilla, J. M. F. (2010). Protein measurements of microalgal and cyanobacterial biomass. *Bioresource Technology, 101*, 7587–7591. https://doi.org/10.1016/j.biortech.2010.04.077

Graziani, G., Schiavo, S., Nicolai, M. A., Buono, S., Fogliano, V., Pinto, G., & Pollio, A. (2013). Microalgae as human food: Chemical and nutritional characteristics of the thermo-acidophilic microalga *Galdieria sulphuraria*. *Food & Function, 4*, 144–152.

Lourenço, S. O., Barbarino, E., Lavín, P. L., Lanfer Marquez, U. M., & Aidar, E. (2004). Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. *European Journal of Phycology, 39*, 17–32.

Martinez-Garcia, M., Kormpa, A., & van der Maarel, M. J. E. C. (2017). The glycogen of *Galdieria sulphuraria* as alternative to starch for the production of slowly digestible

and resistant glucose polymers. *Carbohydrate Polymers, 169*, 75–82. https://doi.org/10.1016/j.carbpol.2017.04.004

Martinez-Garcia, M., & van der Maarel, M. J. E. C. (2016). Floridoside production by the red microalga *Galdieria sulphuraria* under different conditions of growth and osmotic stress. *AMB Express, 6*, 71. https://doi.org/10.1186/s13568-016-0244-6

Massa, M., Buono, S., Langellotti, A. L., Martello, A., Russo, G. L., Troise, D. A., … Fogliano, V. (2019). Biochemical composition and in vitro digestibility of *Galdieria sulphuraria* grown on spent cherry-brine liquid. *New Biotechnology, 53*, 9–15. https://doi.org/10.1016/j.nbt.2019.06.003

Montenegro-Herrera, C. A., Vera-López Portillo, F., Hernández-Chávez, G. T., & Martinez, A. (2022). Single-cell protein production potential with the extremophilic red microalgae *Galdieria sulphuraria*: Growth and biochemical characterization. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-022-02733-y

Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Research, 42*. https://doi.org/10.1016/j.algal.2019.101617

Oesterhelt, C., Vogelbein, S., Shrestha, R. P., Stanke, M., & Weber, A. P. M. (2008). The genome of the thermoacidophilic red microalga *Galdieria sulphuraria* encodes a small family of secreted class III peroxidases that might be involved in cell wall modification. *Planta, 227*, 353–362.

Ott, F. D., & Seckbach, L. (1994). A review on the taxonomic position of the algal genus Cyanidium Geitler 1933 and its ecological cohorts *Galdieria* Merola in Merola et al. 1981 and Cyanidioschyzon De Luca, Taddei and Varano 1978. In L. Seckbach (Ed.), *Evolutionary pathways and enigmatic algae: Cyanidium Caldarium (Rhodophyta) and related cells* (pp. 113–132). Netherlands, Dordrecht: Springer.

Pate, R., Klise, G., & Wu, B. (2011). Resource demand implications for US algae biofuels production scale-up. *Applied Energy, 88*, 3377–3388. https://doi.org/10.1016/j.apenergy.2011.04.023

Pleissner, D., Lindner, A. V., & Händel, N. (2021). Heterotrophic cultivation of *Galdieria sulphuraria* under non-sterile conditions in digestate and hydrolyzed straw. *Bioresource Technology, 337*. https://doi.org/10.1016/j.biortech.2021.125477

Rahman, D. Y., Sarian, F. D., & van der Maarel, M. J. E. C. (2020). Biomass and phycocyanin content of heterotrophic *Galdieria sulphuraria* 074G under maltodextrin and granular starches–feeding conditions. *Journal of Applied Phycology, 32*, 51–57. https://doi.org/10.1007/s10811-019-01957-9

Ruiz, J., Olivieri, G., de Vree, J., Bosma, R., Willems, P., Reith, J. H., … Barbosa, M. J. (2016). Towards industrial products from microalgae. *Energy & Environmental Science, 9*, 3036–3043. https://doi.org/10.1039/c6ee01493c

Russo, G. L., Langellotti, A. L., Oliviero, M., Baselice, M., Sacchi, R., & Masi, P. (2021). Valorization of second cheese whey through cultivation of extremophile microalga Galdieria sulphuraria. *AIMS Environmental Science, 8*, 435–448. https://doi.org/10.3934/environsci.2021028

Sáez-Plaza, P., Michałowski, T., Navas, M. J., Asuero, A. G., & Wybraniec, S. (2013). An overview of the Kjeldahl method of nitrogen determination. Part I. early history, chemistry of the procedure, and titrimetric finish. *Critical Reviews in Analytical Chemistry, 43*, 178–223. https://doi.org/10.1080/10408347.2012.751786

Schmidt, R. A., Wiebe, M. G., & Eriksen, N. T. (2005). Heterotrophic high cell-density fed-batch cultures of the phycocyanin-producing red alga *Galdieria sulphuraria*. *Biotechnology and Bioengineering, 90*, 77–84. https://doi.org/10.1002/bit.20417

Schönknecht, G., Weber, A. P. M., & Lercher, M. J. (2014). Horizontal gene acquisitions by eukaryotes as drivers of adaptive evolution. *BioEssays, 36*, 9–20. https://doi.org/10.1002/bies.201300095

Sloth, J. K., Wiebe, M. G., & Eriksen, N. T. (2006). Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*. *Enzyme and Microbial Technology, 38*, 168–175. https://doi.org/10.1016/j.enzmictec.2005.05.010

Toplin, J. A., Norris, T. B., Lehr, C. R., McDermott, T. R., & Castenholz, R. W. (2008). Biogeographic and phylogenetic diversity of thermoacidophilic *cyanidiales* in Yellowstone National Park, Japan, and New Zealand. *Applied and Environmental Microbiology, 74*, 2822–2833. https://doi.org/10.1128/AEM.02741-07

Unnithan, V. V., Unc, A., & Smith, G. B. (2014). Mini-review: A priori considerations for bacteria–algae interactions in algal biofuel systems receiving municipal wastewaters. *Algal Research, 4*, 35–40. https://doi.org/10.1016/j.algal.2013.11.009

Wan, M., Wang, Z., Zhang, Z., Wang, J., Li, S., Yu, A., & Li, Y. (2016). A novel paradigm for the high-efficient production of phycocyanin from *Galdieria sulphuraria*. *Bioresource Technology, 218*, 272–278. https://doi.org/10.1016/j.biortech.2016.06.045

WHO, J. (2007). *Protein and amino acid requirements in human nutrition* (p. 1). Ser: World Health Organ. Tech. Rep.

Zanolla, V., Biondi, N., Niccolai, A., Abiusi, F., Adessi, A., Rodolfi, L., & Tredici, M. R. (2022). Protein, phycocyanin, and polysaccharide production by *Arthrospira platensis* grown with LED light in annular photobioreactors. *Journal of Applied Phycology*. https://doi.org/10.1007/s10811-022-02707-0