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Autotrophic and mixotrophic biomass production of the acidophilic *Galdieria sulphuraria* ACUF 64

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Abstract

Galdieria sulphuraria is an acidophilic microalga isolated in proximity of sulfuric ponds where pH is below 3 and most organisms cannot grow. We cultivated *G. sulphuraria* ACUF 64 free of contamination for over 2 months in a medium containing organic carbon at pH 1.7 with continuous, high intensity, lighting. We compared biomass productivity of chemostat and repeated batch cultivations. The optimal biomass density in autotrophic and mixotrophic cultures was identified. In autotrophy biomass productivity was 28.3 $g_x \cdot m^{-2} \cdot day^{-1}$, 1.8 to 7.7-fold higher than previously reported. Autotrophy was compared to 'oxygen balanced' mixotrophy where intracellular recirculation of O_2 and CO_2 take place. Aeration was not needed and 92% of the substrate carbon was converted into biomass. In mixotrophy biomass productivity was 1.8 times higher than autotrophic culture and linear growth was maintained at high biomass concentration (9.7 $g_x \cdot L^{-1}$). Light tolerance and high productivity in dense culture make our strain promising for mixotrophic outdoor cultivation.

1. Introduction

Microalgae are oxygenic photoautotrophic (hereafter referred to as autotrophic) microorganisms, that use light energy to convert CO_2 into organic matter and they release O_2 as a by-product. Compared to crops, microalgae can be cultivated under more controlled conditions and, as such, they can reach higher areal productivity [1]. In addition, microalgal production systems can be placed on non-arable lands, and allow for fertilizer use near 100% efficiency [2]. These unique qualities make microalgae a promising sustainable source of food and feed [3].

Some microalgal species are able to exploit light and organic carbons simultaneously as energy sources resulting in a mixotrophic metabolism. Mixotrophic cultivation can significantly increase biomass productivity [4,5]. We recently created a new cultivation strategy named 'oxygen balanced' mixotrophy [6]. In this strategy the dissolved oxygen concentration (*DO*) is maintained at a fixed set-point through continuous and automatic adjustment of the supply rate of a concentrated solution of the organic substrate. This strategy results in a balance between the oxygen produced by photosynthesis and oxygen consumed by respiration while at the same time carbon dioxide is recycled within the microalgal culture. Employing oxygen balanced mixotrophy avoids [6], or minimizes [7], gas-liquid exchange of oxygen and carbon dioxide. Under these conditions biomass productivity and biomass concentration was doubled and more than 90% of substrate carbon was incorporated within the biomass.

Contamination by bacteria and fungi is a notable challenge when microalgae are cultivated in a medium containing a source of organic carbon, because these microbes generally grow faster than microalgae. Cultivation of extremophilic microalgae has been proposed as a strategy to prevent microbial contamination [8]. These algae are able to grow in conditions defined as "extremes" such as very acidic or alkaline pH, unusually high or low temperatures, or high salinity which are all unfavorable to most other micro-organisms.

The microalgal genus *Galdieria* emerged as a promising extremophile [9]. Among the *Galdieria* genus, *G. sulphuraria* is the most studied species. *G. sulphuraria* is a polyextremophile that can tolerate low pH (1–4) [10], high temperature (up to 57 °C) [11] and high osmotic pressure (up to 400 $g \cdot L^{-1}$ of sugar and 2–3 M of salt) [12]. Due to these exceptional traits, *G. sulphuraria* is often the only organism able to colonize acidic hot springs where it forms mats of a deep blue-green color [13]. The peculiar color is due to the presence of the blue pigment phycocyanin and chlorophyll *a* [14]. In addition to phycocyanin, *G. sulphuraria* is rich in proteins [15], insoluble dietary fibers [16], and antioxidants [17]. Given its high nutritional value, *Galdieria* biomass is a potential feedstock for food [16].

Given its benthonic nature, *G. sulphuraria* has been considered

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extremely photosensitive with light inhibition occurring at intensities above 200 μmol·m^{-2}·s^{-1} [10,18]. Due to photosensitivity, most of the research on *G. sulphuraria* has focused on heterotrophic cultivation. *G. sulphuraria* has been successfully grown heterotrophically using 27 organic substrates [16,19] although most *G. sulphuraria* strains completely lose pigmentation when cultivated in the dark [16,19]. Previous studies also indicated that the presence of organic substrates in the light strongly reduced photosynthesis of *G. sulphuraria* [20,21]. [22] reported that if glucose is available *G. sulphuraria* prefers heterotrophy over autotrophy, repressing O_2 production and CO_2 fixation.

A screening performed by ACUF collection on 43 *G. sulphuraria* strains [16], identified the strain *G. sulphuraria* ACUF 64 as the most promising autotrophic strain. The aim of our work was to assess if the strain *G. sulphuraria* ACUF 64 could be cultivated under 'oxygen balanced' mixotrophy and therefore grow without any gas exchange in a closed photobioreactor (*PBR*). The strain was cultivated at pH 1.7 and we investigated the potential of such an acidic environment to prevent bacterial contamination. In addition, we studied both autotrophic and mixotrophic cultivation of *G. sulphuraria* ACUF 64 at a high light intensity to identify the cell concentration resulting in maximal biomass productivity. We explored the hypothesis that in *G. sulphuraria* ACUF 64 mixotrophic growth is the linear combination of heterotrophic and autotrophic growth.

2. Materials and methods

2.1. Organism, media and cultivation conditions

Galdieria sulphuraria ACUF 64 (http://www.acuf.net) was kindly donated by Prof A. Pollio (University of Naples, Italy). Axenic algal cultures were grown in Erlenmeyer flasks (250 mL) containing 100 mL of medium.The culture medium for flasks was composed of the following salts (in mol·L^{-1}): 2.2·10^{-3} KH_2PO_4, 20.0·10^{-3} $(NH_4)_2SO_4$, 1.6·10^{-3} $MgSO_4·7H_2O$, 0.1·10^{-3}·$CaCl_2$, 0.16·10^{-3} EDTA ferric sodium salt, 0.05·10^{-3} $Na_2EDTA·2H_2O$, 0.9·10^{-3} NaCl, 0.2·10^{-3}·H_3BO_3, 20.2·10^{-6} $MnCl_2·4H_2O$, 20.6·10^{-6} $ZnCl_2$, 8.0·10^{-6} $CuSO_4·5H_2O$, 4.1·10^{-6} $Na_2MoO_4·2H_2O$, 4.2·10^{-6}$CoCl_2·6H_2O$. The pH was adjusted to 1.7 ± 0.1 with about 18 mL·L^{-1} of 2.5 M H_2SO_4. By means of weekly dilution with fresh medium these cultures were kept in the linear growth phase. The flasks were placed in an incubator described by [6] at a temperature of 37 °C while stirring at 100 rpm. Incubator headspace was enriched with 4.5% v/v CO_2 and the flasks were illuminated from below at a photon flux density (*PFD*, μmol m^{-2} s^{-1}) of 300 ± 35 μmol m^{-2} s^{-1}. To ensure adequate supply of nutrients for a larger biomass production during photobioreactor experiments, the concentrations of the salts in the medium mentioned above were increased fourfold with exception of EDTA ferric sodium salt and $Na_2EDTA·2H_2O$, which were doubled, and NaCl, which was not increased.

2.2. Heterotrophic flask experiments

Dark batch experiments were used to determine the biomass yield on substrate ($Y^{het}_{x/s}$, C-mol$_x$·C-mol$_s^{-1}$). The heterotrophic experiments were conducted in flasks adding glucose monohydrate at 0.3C-mol$_s$·L^{-1} to the culture medium. The experiments were initiated by adding an inoculum that was acclimated to heterotrophic growth for at least two weeks. Cultures were maintained in exponential growth by diluting the culture with fresh medium every 3–5 days. Flasks were wrapped in aluminum foil and placed in darkness at 37 °C in an orbital shaker incubator (250 rpm). During the experiments, multiple samples per day were taken until glucose was depleted. The microalgae concentration was assessed by measuring optical density at 750 nm (OD_{750}). A 1 mL aliquot of sample was centrifuged to obtain a clear supernatant for the measurement of the glucose concentration. The dry weight (Cx, g$_x$·L^{-1}) was determined from the OD_{750} according to the following linear correlation:

$$C_x\ (g_x \cdot L^{-1}) = 0.55 \cdot OD_{750} + 0.06\ (R^2 = 1.00)$$

This correlation was obtained with biomass cultivated under heterotrophic conditions at the same C_x range used in the experiment. The validity of this correlation was confirmed at the end of each experiment by an additional measurement of dry weight and optical density. The biomass yield on organic substrate $Y^{het}_{x/s}$ (C-mol$_x$·C-mol$_s$) was determined by taking the ratio of the carbon-based biomass productivity over the substrate consumed according to Abiusi et al. [6]. The specific growth rate (μ, h^{-1}) in the logarithmic growth phase was calculated dividing the natural logarithm of the increase in the C_x over the period of exponential growth.

2.3. Photobioreactor setup and experiments

Galdieria sulphuraria ACUF 64 was grown in a stirred tank bioreactor of 3 L volume and 0.13 m diameter (Applikon, The Netherlands) according to [6]. Experiments were conducted with a working volume (V_{PBR}) of 2 L under continuous lighting provided by warm white LEDs. The LED lamps created a homogenous light field over the reactor surface with a cylindrical area of 0.068 m^2. The average *PFD* was 514 ± 17 μmol m^{-2} s^{-1}.

The bioreactor was fitted with a dissolved oxygen (*DO*) probe (VisiFerm DO ECS 225, Hamilton, US) calibrated according to Abiusi et al. [6]. The temperature was maintained at 37 °C and evaporation was prevented via a condenser cooled at 2 °C. The culture was stirred at 500 rpm in all the experiments. When aerated, 1 L·min^{-1} was provided to the reactor using Smart TMF 5850S mass flow controllers (Brooks Instruments, USA). This air flow was enriched with 2% v/v CO_2.

The pH was maintained at 1.7 through automatic base (2 M, NaOH) or acid (2 M H_2SO_4) addition. Bottles with acid and base, 2.2 M glucose solution, and harvest were placed on analytic balances. All sensors and controllers were connected to a data acquisition system that was interfaced via a PC by means of a LabView virtual instrument (National Instruments, USA). The complete setup was autoclaved for 60 min at 121 °C.

In one experiment the reactor was operated in chemostat while in another experiment it was operated in repeated batch. The chemostat experiment was performed at a dilution rate (D, day^{-1}) of 0.5 day^{-1}. The culture was first grown autotrophically for two weeks. For the 4 last days of this period the harvest bottle was placed in ice water. The harvested culture was collected daily. A 10 mL aliquot was used for dry weight determination. Other measurements (see Section 3.2) were taken multiple times per day directly from the reactor during these 4 days. After these first 14 days oxygen balanced mixotrophy was initiated at a *DO* of 90%. In this period the reactor was not aerated and a glucose solution was automatically supplied when the *DO* exceeded the set-point of 90%. Mixotrophic cultivation was maintained for 14 days and during the last 4 days samples were taken again according to the same procedures as described for the autotrophic experiment.

In another experiment the photobioreactor was operated in repeated batch mode. The experiment was started inoculating the reactor with an autotrophic culture at 0.4 g$_x$·L^{-1}. The microalgal culture in the reactor was diluted every 5–9 days for three times (batches I-III) for an overall cultivation period of 21 days. After the first 21 days, the glucose solution was supplied at a constant rate while maintaining gassing with CO_2 enriched air resulting in a mixotrophic culture. After 2 days we switched to oxygen balanced mixotrophy as we stopped gassing and switched to the automatic supply of glucose to maintain the DO at 90% air saturation. The culture was diluted every 6–8 days for three times (batches IV–VI) for an overall cultivation period of 23 days. During experiments, each batch samples were taken daily for different analyses.

2.4. Photobioreactor calculations

In chemostat the volumetric biomass production rate r_x (g$_x$·L^{-1}·day^{-1}) was determined taking the product of dilution rate D

(day^{-1}) and biomass concentration C_x ($g_x \cdot L^{-1}$). In repeated batch r_x was calculated from a linear regression of the increase of C_x over time. The biomass production rate was also expressed in carbon moles equivalent (r_c, C-$mol_x \cdot L^{-1} \cdot day^{-1}$) according to [6]. This r_c was to calculate the biomass yield on light $Y_{x/ph}$ (C-$mol_x \cdot mol_{ph}^{-1}$) for the autotrophic cultures:

$$Y_{x/ph} = \frac{r_{c,auto} \bullet V_{PBR}}{PFD \cdot A_{PBR}} \quad (1)$$

where A_{PBR} (m^2) represents the illuminated area of the photobioreactor.

The volumetric substrate consumption rate r_s (C-$mols \cdot L^{-1} \cdot day^{-1}$) in the mixotrophic experiment was determined assuming ideal mixing according to:

$$\frac{r_s = F_{glu} \bullet C_{s,glu} - D \bullet V_{PBR} \bullet C_s}{V_{PBR}} \quad (2)$$

where F_{glu} ($L \cdot day^{-1}$) represents the feeding rate of the glucose solution and $C_{s,glu}$ (C-$mol_s \cdot L^{-1}$) the concentration of this solution, while *Cs* is the glucose concentration measured in the reactor. The biomass yield on substrate under mixotrophic conditions $Y^{mixo}_{x/s}$ (C-$mol_x \cdot mol_s^{-1}$) was determined taking the ratio of r_c over r_s.

The specific light supply rate q_{ph} ($\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$) was calculated following the formula:

$$q_{ph} = \frac{PFD \bullet A_{PBR}}{C_x \cdot V_{PBR}} \quad (3)$$

3. Analytical methods

3.1. Photon flux density measurements

The photon flux density *PFD* was measured with a LI-COR 190-SA 2π PAR quantum sensor according to [6].

3.2. Culture sampling and off-line measurements

Samples were taken aseptically from the reactor culture several times per days. Aliquots of 1 mL were centrifuged at 20,000 RCF (10 min). When needed, the supernatant was immediately analyzed for glucose or phosphorus contents. Biomass pellets were washed twice with demineralized water and stored at −20 °C prior to analysis. Biomass carbon ($C_{\%}$, $w_c \cdot w_x^{-1}$) and nitrogen ($N_{\%}$, $w_c \cdot w_x^{-1}$) content were measured from the pellets using a TOC-L analyzer (Shimadzu, Japan) according to [6]. Dry weight concentration (C_x, $g_x \cdot L^{-1}$) was determined by filtering an aliquot of sample over pre-weighed glass microfiber filters as described in Abiusi et al. [6]. Optical density at 750 (OD_{750}) was measured in duplicate using on a spectrophotometer (DR6000, Hach-Lange, US). Average absorption cross section (a_x, $m^2 \cdot Kg^{-1}$) was measured in duplicate using on a UV-VIS/double beam spectrophotometer (Shimadzu UV-2600, Japan) equipped with integrating sphere (ISR-2600) according to Abiusi et al. [6]. The photosystem II maximum quantum yield of photochemistry (*QY* or Fv/Fm) was determined at 455 nm with an AquaPen-C (AP-C 100, Photon Systems Instruments, Czech Republic) according to [6]. Assessment of possible contaminations was carried out by DNA staining of culture samples with SYBR Green I (Sigma-Aldrich, US) and using fluorescence microscopy (EVOS FL auto, Thermo Fisher Scientific, US) according to Abiusi et al. [6].

3.3. Glucose and phosphorus determination

Glucose concentrations were determined by means of a YSI 2700 analyzer (YSI Life Sciences, Yellow Springs, OH, USA). Total phosphorus was quantified with a spectrophotometric phosphorus detection kit (LCK 349/350, Hach Lange, Germany).

3.4. Statistical analysis

Propagation of errors by summation and multiplication of individual measurements were calculated according to [6]. In the chemostat experiment each day of the steady state was considered as a replicate ($n = 4$). In the autotrophic repeated batch experiment, batch I and III were considered as replicates ($n = 2$) while in the mixotrophic experiment each batch was considered as a replicate ($n = 3$). In each experiment autotrophic and mixotrophic cultures were compared and significant differences were analyzed by one-way ANOVA ($P < 0.05$).

4. Results and discussion

4.1. Contamination of algal cultures at low pH

A major challenge of outdoor mixotrophic cultivation of microalgae is contamination by mainly bacteria and fungi [23]. Such contaminating microbes compete with the microalgae for the available organic carbon and they are likely to spoil the product. In the present work *G. sulphuraria* was cultivated at pH 1.7 ± 0.1 and fluorescence DNA staining did not show contaminations neither during the several weeks of heterotrophic cultivation in flasks, nor in our closed photobioreactor (*PBR*) for 33 days (chemostat), and for 42 days (repeated batch) (Fig. A1).

Prior studies [8] demonstrated that low pH dramatically reduced the initial bacterial population and resulted in complete removal of pathogens when *G. sulphuraria* was cultivated in unsterilized primary effluent at pH 2. However, *G. sulphuraria* has been reported to be prone to fungal contamination when grown mixotrophically in open biofilms [24]. In the current work, we employed a closed cultivation vessel and all the inputs and outputs used in our experiments were filter sterilized. This reactor configuration combined with the low pH was effective in preventing contaminations.

4.2. Heterotrophic experiments

Strict heterotrophic batch experiments were used to determine the biomass yield on substrate under heterotrophic conditions. *G. sulphuraria* ACUF 64 was cultivated without light in flasks with a medium containing glucose. The culture was allowed to adapt to heterotrophic growth conditions for two weeks. During this pre-cultivation period, the culture lost most of its pigments (Fig. A2). This was verified by measuring the absorption cross section spectrum (Fig. A3). The loss of pigmentation during heterotrophic cultivation of this strain has been previously reported [16].

Fig. 1 displays the experimental data on heterotrophic biomass production and substrate consumption. Providing the culture with 329 ± 10 C-$mmol_s \cdot L^{-1}$ of substrate, exponential growth was observed for 116 h at a specific growth rate (μ) of 0.74 ± 0.00 day^{-1}. The only published heterotrophic experiment performed with *G. sulphuraria* ACUF 64 [16] reported a μ of 1.0 day^{-1} which is in line with the most studied *G. sulphuraria* 74G [25]. The final biomass concentration was 195 ± 3 C-$mmol_x \cdot L^{-1}$ corresponding to $Y_{x/s}$ of 0.59 ± 0.02 (C-$mol_s \cdot C\text{-}mol_s^{-1}$). A previous study on heterotrophic growth of *G. sulphuraria* 74G in an aerobic fermenter [25], reported a biomass yield on substrate of 0.53–0.63C-$mol_s \cdot C\text{-}mol_s^{-1}$. Aerobic heterotrophic organisms have a maximal yield ($Y^{het}_{x/s}$) of 0.7 mol_x mol_s^{-1} which is bound by thermodynamic constraints [26]. Our results indicate that, despite the low pH, when grown heterotrophically *G. sulphuraria* can efficiently convert organic substrate into biomass but at a maximal growth rate which is 4 and 9 times lower than the most studied *Chlamydomonas* and *Chlorella* species, respectively [27].

4.3. Oxygen balanced mixotrophy in Galdieria sulphuraria ACUF 64

We previously demonstrated with *Chlorella sorokiniana* that a

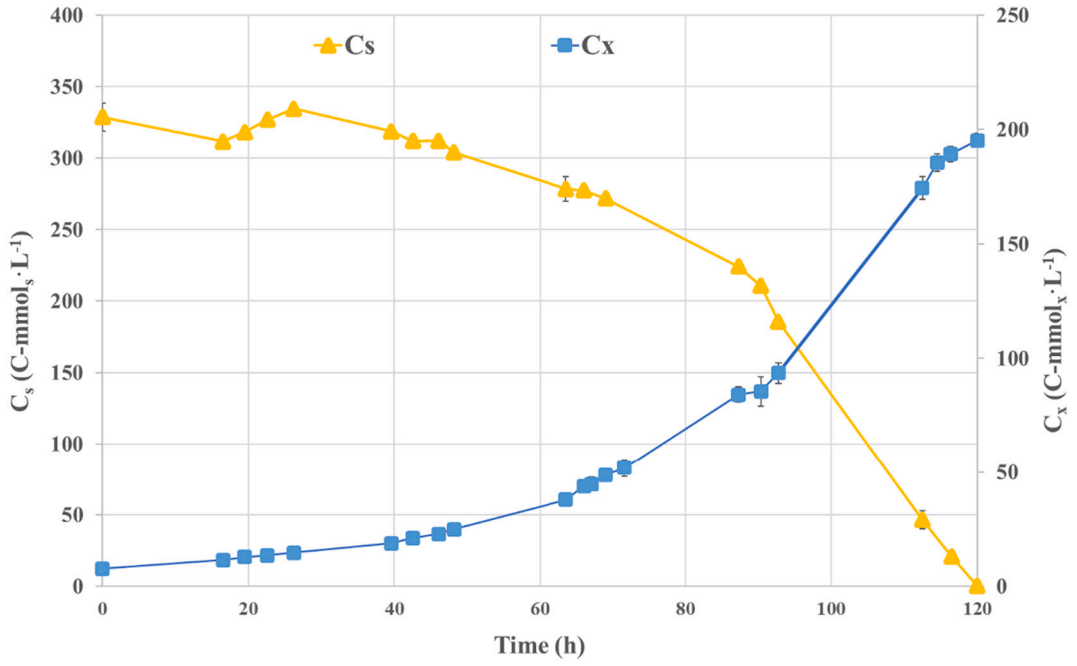

Fig. 1. Heterotrophic biomass production (squares) and substrate consumption (triangle) of *G. sulphuraria* ACUF 64 cultivated at pH 1.6 with 330 C-mmol·L^{-1} glucose.

mixotrophic culture can operate without any aeration [6] by coupling the substrate supply rate to the rate of photosynthesis. In the present study the same concept was applied to *Galdieria*, and we compared biomass productivity of chemostat and repeated batch cultivations. In both operating strategies the cultures were also grown under autotrophic conditions without the addition of organic carbon but with continuous gassing with CO_2 enriched air. These autotrophic cultures where used as reference to determine the biomass yield on photons ($Y_{x/ph}$).

4.4. Oxygen balanced mixotrophy in chemostat

Oxygen balanced mixotrophy was applied successfully to *G. sulphuraria* ACUF 64 and the *PBR* was operated without any gas exchange for 14 days. Under these conditions the biomass concentration and productivity were 3.6 times higher than under autotrophic reference conditions (Table 1). The results clearly indicate that, at least in our strain, the presence of an organic substrate does not inhibit oxygen production as was previously suggested [20,22]. In order to estimate the fraction of biomass produced autotrophically ($r_{c,auto}$) during mixotrophic growth, we subtracted the estimated fraction of the biomass heterotrophically produced ($r_{c,het}$) from the overall mixotrophic productivity ($r_{c,mixo}$). The estimated $r_{c,auto}$, and therefore the biomass yield on light ($Y_{x/ph}$), was not significantly ($P > 0.05$) different from the $r_{c,auto}$ of the autotrophic culture leading us to conclude that in *G. sulphuraria*, the overall mixotrophic productivity is the sum of the heterotrophic and autotrophic metabolisms (Table 1).

The pigmentation of the mixotrophic and autotrophic cultures were found to be different. The average absorption cross section (a_x) of the autotrophic culture was double the mixotrophic culture a_x (Table 1). A recent study on *G. phlegrea* found a lower chlorophyll *a* content in a mixotrophic culture compared to an autotrophic culture grown under otherwise similar conditions [28]. This indicates that the addition of organic substrate has an impact on pigmentation, which is not surprising considering the complete loss of pigments when *G. sulphuraria* was cultivated in darkness (see previous section).

In the chemostat culture of *G. sulphuraria* ACUF 64 cultivated in autotrophy without the addition of organic carbon, but with continuous gassing with CO_2 enriched air, the biomass yield on light ($Y_{x/ph}$) was 12.3 ± 0.6 C-mmol$_x$·mol$_{ph}$$^{-1}$, approximately 3.3 times lower than $Y_{x/ph}$ found in *C. sorokiniana* under similar experimental conditions [6]. The lower autotrophic performance can be partially explained by photoinhibition. *G. sulphuraria* is well known to be photosensitive with light inhibition occurring at intensities above 200 μmol·m^{-2}·s^{-1} [10,22]. Light inhibition was confirmed by measuring the dark-adapted quantum yield of PSII photochemistry (*QY*). The *QY* was 0.22 ± 0.01 in autotrophic culture, significantly ($P < 0.05$) lower than the value of 0.32 ± 0.02 measured in the mixotrophic culture, and both definitely lower than 0.72 generally reported in *C. sorokiniana* [6,29]. The low *QY* confirms the lower autotrophic performance of *G. sulphuraria* compared to *C. sorokiniana*. Moreover, the mixotrophic culture displayed a higher *QY* than the autotrophic culture, indicating a lower degree of photoinhibition. This higher *QY* is potentially explained by a 3.5 times lower specific light supply rate (q_{ph}) in the mixotrophic culture compared to the autotrophic culture (Table 1). This lower specific light supply rate is

Table 1
Overview of the off-line and *D* measurements on the chemostat cultivation of *G. sulphuraria* ACUF 64 under mixotrophic and autotrophic conditions. The data presented are the average of the last 4 consecutive days in chemostat ($n = 4$) and reported with the standard deviation of measurements.

	Unit	Autotrophic	Mixotrophic
C_x	g$_x$·L^{-1}	0.86 ± 0.05^a/7.4 ± 0.2*	3.06 ± 0.16^b
D	day^{-1}	0.51 ± 0.04^a	0.50 ± 0.01^a
$C_{\%}$	% w$_C$·w$_x$$^{-1}$	49.0 ± 1.1^a	49.1 ± 1.1^a
$N_{\%}$	% w$_N$·w$_x$$^{-1}$	9.8 ± 0.3^a	9.3 ± 0.1^b
r_x	g$_x$·L^{-1}·day^{-1}	0.45 ± 0.02^a	1.62 ± 0.07^b
$r_{c,mixo}$	C-mmol$_x$·L^{-1}·day^{-1}	n.a	66.0 ± 2.9
r_s	C-mmol$_s$·L^{-1}·day^{-1}	n.a	−82.6 ± 3.8
$Y^{mixo}_{x/s}$	C-mol$_x$·C-mol$_s$$^{-1}$	n.a	0.80 ± 0.04
$r_{c,het}$	mmol$_x$·L^{-1}·day^{-1}	n.a	48.7 ± 2.2
$r_{c,auto}$/$r_{c,auto}$	C-mmol$_x$·L^{-1}·day^{-1}	18.5 ± 1.0^a	17.3 ± 3.1^a
$Y_{x/ph}$	C-mol$_x$·C-mol$_{ph}$$^{-1}$	12.3 ± 0.6^a	11.5 ± 2.0^a
a_x	m^2·kg^{-1}	114 ± 6^a/180 ± 3*^{b}	62 ± 7^c
q_{ph}	μmol$_{ph}$·g$_x$$^{-1}$·s^{-1}	20.2 ± 1.8^a/2.3 ± 0.1*^{b}	5.7 ± 0.5^c
QY	Fm/Fv	0.22 ± 0.01^a/0.48 ± 0.02*^{b}	0.32 ± 0.02^c

*Values obtained in the flasks used as inoculum.
Along the rows, the same letter indicates no significant differences ($P > 0.05$).

related to the higher biomass concentration under mixotrophy.

In the autotrophic inoculum *QY* was 0.48 ± 0.02, indicating that not only the autotrophic culture in the *PBR* but probably even the mixotrophic culture was experiencing light stress. The inoculum was grown in batch at 300 μmol·m^{-2}·s^{-1} and at the time of *QY* and a_x measurements, just prior to inoculation, a biomass concentration (C_x) of 7.4 g_x·L^{-1} was reached (data not shown). Assuming the flaks used for the inoculum were illuminated solely from the bottom, the inoculum had a volumetric light supply rate similar to the one observed in the reactor (17 μmol·L^{-1}·s^{-1}). However, given the high C_x, the specific light supply rate (q_{ph}) was 2.3 μmol·$g_x$$^{-1}$·s^{-1}. Therefore, the low q_{ph} explains the higher a_x and *QY* measured in the inoculum compared to the autotrophic culture (Table 1).

Despite low $Y_{x/ph}$ and photoinhibition, the autotrophic culture reached a steady state which was maintained for more than a week. In a chemostat culture, once a steady state is obtained, the specific growth rate (μ) equals the dilution rate. The dilution rate applied was 0.50 day^{-1} which then results in a μ of 0.50 day^{-1}. A similar autotrophic μ has been reported in our strain earlier [16] and it is about double the autotrophic μ reported in other *Galdieria* strains [22,30] pointing towards potential of this strain in autotrophic biomass production. The observed effect of q_{ph} on photosynthetic performance strongly suggests that high C_x, and therefore low q_{ph}, is an effective strategy to minimize photoinhibition in *G. sulphuraria*. In this study such an optimization was done through repeated batch experiments where the specific light supply rate continuously decreases during the batch phase because of an increasing biomass concentration.

4.5. Oxygen balanced mixotrophy in repeated batch

The purpose of the repeated batch experiment was to identify the biomass concentration (C_x) that results in an optimal light regime maximizing biomass productivity of *G. sulphuraria* ACUF 64 under our experimental settings. The repeated batch approach also provided information on the effect of a sudden change of the specific light supply rate (q_{ph},) on the autotrophic and mixotrophic metabolism after culture dilution. Six consecutive batches were performed: three autotrophic batches (I, II, III), and three mixotrophic batches (IV, V, VI).

Referring to Fig. 2, it can be observed that in the autotrophic cultures linear growth was obtained between 2 and 5 g_x·L^{-1} and, at lower C_x the culture was photoinhibited while at higher C_x light limitation became evident and biomass productivity (r_x) decreased. In batches I and III the r_x was 0.97 ± 0.02 g_x·L^{-1}·day^{-1} during the linear phase and the biomass yield on light (*Yx/ph*) was 24.8 ± 1.3 $mmol_x$·$mol_{ph}$$^{-1}$. Those values are about double the values of the autotrophic culture operated in chemostat confirming that in our chemostat experiment the autotrophic culture was light inhibited. Biomass productivity and yield on light in *G. sulphuraria* were comparable to other commercially relevant microalgae such as *Isocrysis lutea* [31], *Rhodomonas* sp. [32], *Nannochloropsis* sp. [33], indicating the potential of this strain for autotrophic biomass production.

In three mixotrophic repeated batches (IV, V, VI) linear growth started at 2.8 g_x·L^{-1} and it was maintained until a biomass concentration (C_x) between 7.3 and 9.7 g_x·L^{-1} was reached (Fig. 2, Table 2). The upper biomass limit, at which linear growth rate was still maintained and optimal biomass productivity (r_x) observed, progressively increased during each batch (Fig. 2, Table 2). The same trend was observed for the mixotrophic biomass yield on substrate ($Y^{mixo}_{x/s}$), while the maximum absorption cross section area (a_x) at the end of each batch progressively decreased (Fig. 3). Together these four observations indicate adjustment of metabolism from autotrophy to mixotrophy over time, resulting in a culture that in the last batch (VI) was more acclimated to the presence of glucose. The three mixotrophic batches lasted 22 days in total. The fact that the cultures kept adapting to mixotrophy over such a long time was unexpected. A recent study on cellular changes that occur in *Galdieria phlegrea* during a switch from heterotrophic to mixotrophic cultivation suggested that 7–10 days were needed to fully recover the photosynthetic capacity lost during the heterotrophic growth [28]. In our experiment the culture was switched from autotrophy to mixotrophy. A possible explanation for the slow adaptation time observed in the mixotrophic repeated batch is that the sudden increase in the photon supply rate (q_{ph}) (Fig. 3) after each dilution affected cellular metabolism and temporarily destabilized the culture. A cessation of glucose uptake was observed after each dilution (Fig. 4) supporting this hypothesis, which will be discussed later.

Comparing mixotrophic and autotrophic cultures, we will focus mainly on mixotrophic batch VI, and on the average between autotrophic batches I and III. The autotrophic batch II was excluded from the

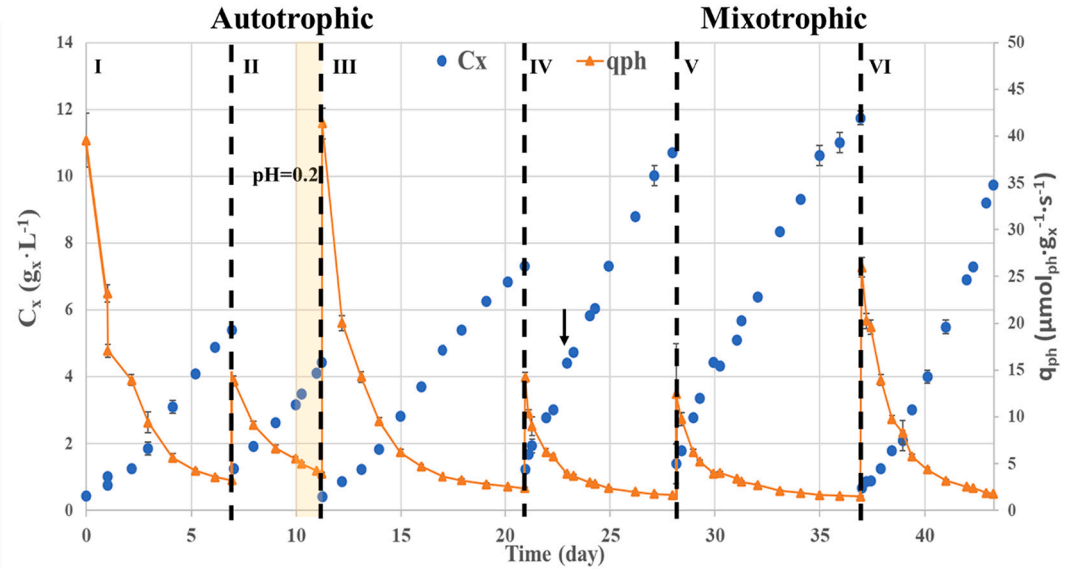

Fig. 2. Autotrophic (I–III) and mixotrophic (IV–VI) repeated batches. Dotted lines indicate the time of dilution. The black arrow indicates the end of aeration. Orange area indicates pH 0.2. In the graph are reported biomass concentration (C_x, blue dots) and specific light supply rate (q_{ph}, orange triangles). The data presented are the average technical duplicate ($n = 2$) and reported with the standard deviation of measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Overview of the off-line measurements on the repeated batch cultivation of *G. sulphuraria* ACUF 64 under mixotrophic and autotrophic conditions. The table reports the optimal range of C_x, q_{ph}, QY and the value of a_x at the end of this range in each repeated batch. The other data presented are the average of at least 4 consecutive measurements obtained in those optimal ranges and are reported with the standard deviation of measurements.

	Unit	Autotrophic			Mixotrophic		
		I	II	III	IV	V	VI
C_x	$g_x \cdot L^{-1}$	1.9–4.9	1.9–4.4	1.8–4.8	2.8–7.3	2.8–8.3	3.0–9.7
$C_{\%}$	% $w_C \cdot w_x^{-1}$	45.4 ± 1.4	46.1 ± 2.3	47.5 ± 1.0	48.3 ± 1.0	47.0 ± 2.8	47.6 ± 2.9
$N_{\%}$	% $w_N \cdot w_x^{-1}$	10.3 ± 0.3	10.4 ± 0.5	10.0 ± 0.2	9.8 ± 0.2	9.1 ± 0.5	9.4 ± 0.6
r_x	$g_x \cdot L^{-1} \cdot day^{-1}$	0.95 ± 0.05	0.76 ± 0.05	0.98 ± 0.05	1.50 ± 0.08	1.60 ± 0.08	1.72 ± 0.09
$r_{c,mixo}$	$C\text{-}mmol_x \cdot L^{-1} \cdot day^{-1}$	–	–	–	60.4 ± 0.2	62.8 ± 0.5	68.2 ± 0.5
r_s	$C\text{-}mmol_s \cdot L^{-1} \cdot day^{-1}$	–	–	–	84.2 ± 6.7	78.4 ± 3.9	74.5 ± 3.7
$Y^{mixo}_{x/s}$	$C\text{-}mol_x \cdot C\text{-}mol_s^{-1}$	–	–	–	0.72 ± 0.07	0.80 ± 0.06	0.92 ± 0.04
$r_{c,het}$	$mmol_x \cdot L^{-1} \cdot day^{-1}$	–	–	–	49.4 ± 3.0	46.0 ± 1.8	43.7 ± 1.7
$r_{c,auto}/r_{c,auto}$	$C\text{-}mmol_x \cdot L^{-1} \cdot day^{-1}$	35. 8 ± 2.1	29.0 ± 2.1	38.6 ± 2.1	11.0. ±0.6	16.8 ± 1.3	24.4 ± 1.9
$Y_{x/ph}$	$C\text{-}mmol_x \cdot mol_{ph}^{-1}$	23.8 ± 1.7	19.3 ± 1.6	25.6 ± 1.7	7.28 ± 0.5	11.14 ± 1.0	16.29 ± 1.4
a_x	$m^2 \cdot kg^{-1}$	177 ± 1	194 ± 2	184 ± 5	138 ± 0	125 ± 2	132 ± 11
q_{ph}	$\mu mol_{ph} \cdot g^{-1} \cdot s^{-1}$	9.4–3.6	9.1–3.9	9.5–3.6	6.3–2.4	6.3–2.1	5.8–1.8
QY	Fm/Fv	0.37–0.49	0.37–0.44	0.36–0.49	0.31–0.52	0.33–0.55	0.32–0.45

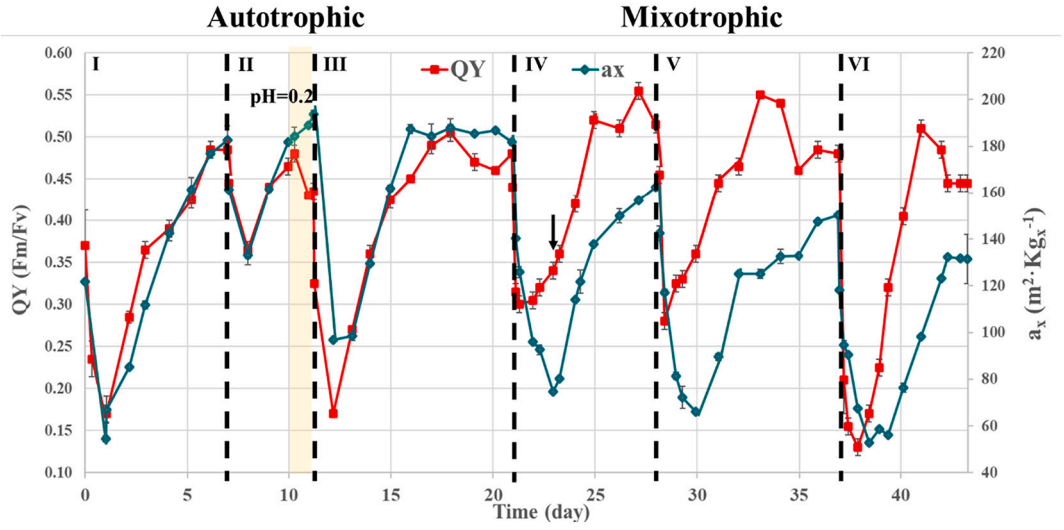

Fig. 3. Autotrophic (I–III) and mixotrophic (IV–VI) repeated batches. Dotted lines indicate the time of dilution. Black arrow indicates the end of aeration. Orange area indicates pH 0.2. In the graph are reported photosystem II maximum quantum yield (QY, Fv/Fm, red squares) and average absorption cross section (a_x, green diamonds). The data presented are the average technical duplicate ($n = 2$) and reported with the standard deviation of measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

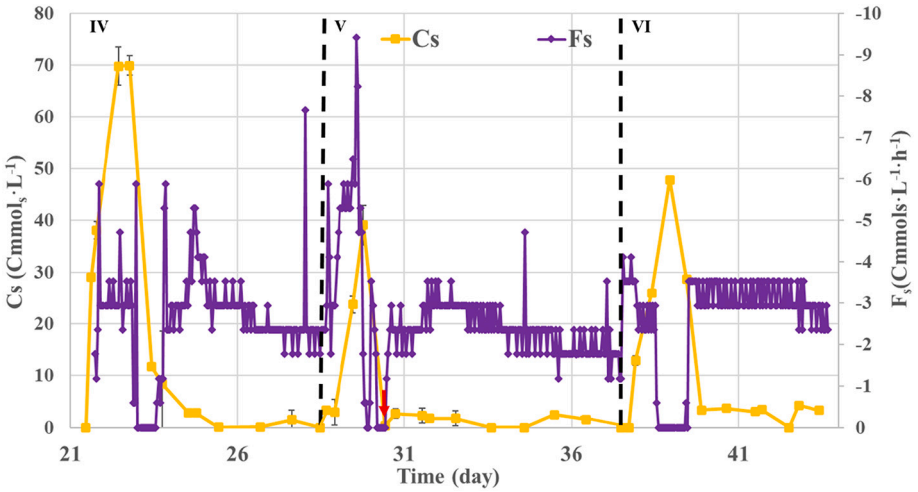

Fig. 4. Mixotrophic repeated batches. Reported in the graph are substrate concentration in the reactor (C_s, yellow squares) and substrate supply rate (F_s, violet diamonds). Dotted lines indicate the time of dilution. The red arrow indicates substrate depletion. C_s is presented as average technical triplicate ($n = 3$) and reported with the standard deviation of measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

comparison because the culture grew at pH 0.2 (see next section). In the mixotrophic culture (VI) biomass productivity (r_x) was 1.8-fold higher than in the autotrophic cultures (I and III) and optimal r_x was maintained at a biomass concentration (C_x) that was double the biomass concentration in the autotrophic culture. Moreover, it was possible to operate the mixotrophic culture without any gas-liquid transfer of oxygen or carbon dioxide for 20 days. The internal carbon dioxide recirculation led to a mixotrophic biomass yield on substrate of 0.92 ± 0.04 ($Y^{mixo}_{x/s}$, $C\text{-}mol_x \cdot C\text{-}mol_s^{-1}$) making the process close to carbon neutrality. These results are in line with our previous finding obtained with *Chlorella* [6,7] suggesting that 'oxygen balanced' mixotrophy can, be applied to other mixotrophic algae.

In the three mixotrophic repeated batches (IV, V, VI) the stoichiometry was not the sum of the autotrophic and heterotrophic metabolisms (Table 2). To calculate the fraction of biomass produced autotrophically ($r_{c,auto'}$), the estimated fraction of the biomass produced heterotrophically ($r_{c,het}$) was subtracted from the overall mixotrophic productivity ($r_{c,mixo}$).

This $r_{c,auto'}$, was 2.6, 2.3, and 1.5 fold lower than the autotrophic reference (batches I and III) respectively in batch IV, V and VI. It must be highlighted that in the estimation of the autotrophic fraction of the mixotrophic biomass productivity ($r_{c,auto'}$) we assumed that the heterotrophic biomass yield on substrate ($Y_{x/s}$), measured in the heterotrophic experiment, did not change in the presence of light. This calculation shows that the yield of biomass on light (autotrophy) or, possibly, the yield of biomass on glucose (heterotrophy) is affected by either the presence of glucose, or the presence of light. As a result, mixotrophy cannot be approached as the sum of the heterotrophic and the autotrophic metabolism, and there appears to be an interaction between these metabolic pathways. The negative interaction between the heterotrophic and the autotrophic metabolism decreased over time. However, even in batch VI the hypothetical autotrophic rate under mixotrophy was still 1.5 lower than the autotrophic rate without the addition of organic carbon.

Interaction between autotrophic and heterotrophic metabolisms was also noticed in the dissolved oxygen (*DO*) control by means of substrate addition (Fig. 5). After each dilution, the sudden increase in specific light supply rate (q_{ph}) partially inhibited glucose uptake and it was not possible to control the *DO*, which temporarily increased to 230% air saturation. Since the substrate was supplied to the culture without being consumed, substrate accumulated in the reactor. In order to deplete the accumulated substrate (C_s), we manually interrupted the glucose supply (*Fs*). The initial accumulation of substrate, followed by its depletion, provoked unstable *DO*, that lasted the first 2–4 days after dilution and resulted in *DO* fluctuations from 30 to 230%. After this initial phase, the *DO* was successfully maintained in the desired range by automatic substrate addition at an average feed rate *Fs* of −2.95 ± 0.59 $Cmmol_s \cdot L^{-1} \cdot h^{-1}$.

Previous studies indicated reduced photosynthetic performance, and even suppression of oxygen evolution, in mixotrophic cultures of *G. sulphuraria* supplemented with glucose [20,22]. According to our results, rather than glucose affecting photosynthesis, it was the sudden increase in q_{ph} that inhibited glucose uptake. This hypothesis is supported by measuring the dark-adapted quantum yield of PSII photochemistry (*QY*) (Fig. 3), that in mixotrophic cultures was equal to, or higher than the autotrophic cultures, indicating that photosynthesis was not negatively affected by glucose.

Another sign of interaction between autotrophic and heterotrophic metabolism was the reduction in pigmentation observed in the mixotrophic repeated batches (IV, V, VI) when compared to the autotrophic culture grown under the same conditions. In mixotrophic repeated batches, at the end of the linear growth phase, a_x was 132 ± 6 $m^2 \cdot Kg^{-1}$, 27% less than the autotrophic culture under the same condition. This decrease in pigmentation was much less severe than in the mixotrophic cultivated in chemostat where a_x decreased by half compared to the autotrophic culture grown under the same conditions (Table 1). The dramatic reduction in pigments observed in the mixotrophic culture grown in chemostat therefore is most likely related to the low C_x resulting in light inhibition instead of glucose inhibition of photosynthesis.

The repeated batch clearly indicated that the optimization of the light regime is a key point for successful cultivation of light sensitive *G. sulphuraria*. In the autotrophic repeated batch the optimal specific light supply rate (q_{ph}) was 3.6–9.5 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$ (Table 2). Under this light regime biomass production rate (r_x) was the double of the autotrophic culture in chemostat cultivated under the same incident light intensity but at 20.2 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$ (Tables 1, 2). This result clearly suggests that photoinhibition can be mitigated by finding the range of biomass concentration (C_x) which results in optimal q_{ph}. At an optimal q_{ph} *G. sulphuraria* can successfully grow even at a high incident light intensity.

The present work is the first report of an autotrophic *G. sulphuraria* culture grown in a photobioreactor (*PBR*) at incident light intensity above 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Reports on other trophic modes at this high

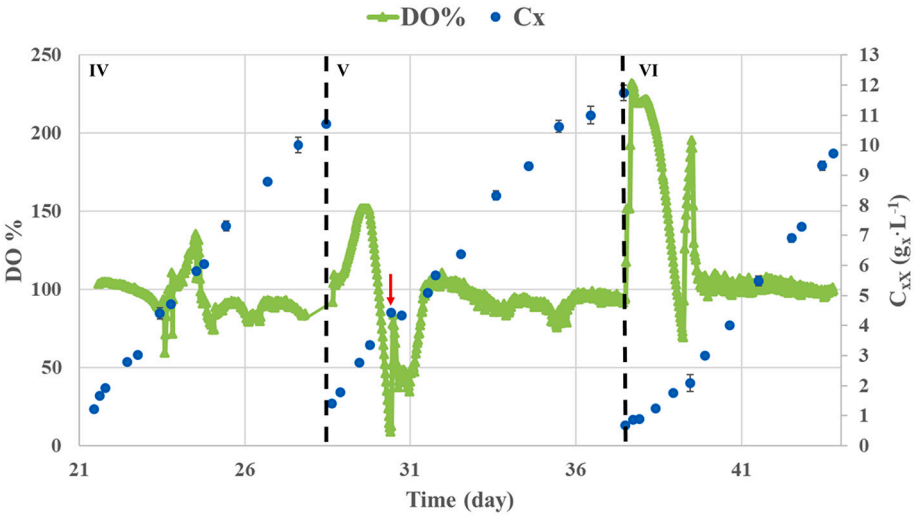

Fig. 5. Mixotrophic repeated batches. Dotted lines indicate the time of dilution. The red arrow indicates substrate depletion. In the graph is reported biomass concentration (C_x blue dots) and dissolved oxygen (*DO*, green triangles). C_x is presented as average technical duplicate ($n = 2$) and reported with the standard deviation of measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of final biomass concentration (C_x), biomass productivity, specific light supply rate (q_{ph}), volumetric (r_c) and areal (r_A) biomass productivity among this study and other reports. STR: stirred tank reactor; BC: bubbled column; ITL: immobilized twin layer; RWP: race way pond.

Strain	PBR	Operation mode	Trophic mode	Illuminated area (m^2)	I_0 ($\mu mol_{ph} \cdot m^{-2} \cdot s^{-1}$)	C_x ($g_x \cdot L^{-1}$)	q_{ph} ($\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$)	r_x ($g_x \cdot L^{-1} \cdot day^{-1}$)	r_A ($g_x \cdot m^{-2} \cdot day^{-1}$)	Reference
G. sulphuraria ACUF 64	STR	Chemostat	Autotrophic	0.068	514	0.86	20.2	0.45	13.3	This study
G. sulphuraria ACUF 64	STR	Chemostat	Mixotrophic	0.068	514	3.07	5.7	1.62	47.8	This study
G. sulphuraria ACUF 64	STR	Repeated Batch	Autotrophic	0.068	514	1.9–4.9	9.5–3.6	0.97	28.6	This study
G. sulphuraria ACUF 64	STR	Repeated Batch	Mixotrophic	0.068	514	3.0–9.7	5.8–1.8	1.72	50.7	This study
G. sulphuraria ACUF 64	BC	Batch	Autotrophic	0.190	150	0.4–5.7	17.8–1.3	0.18	3.7	[16]
G. sulphuraria ACUF 64	ITL	Batch	Autotrophic	n.a.	50	10–107[a]	5.0–0.5	n.a.	5.2	[24]
G. sulphuraria ACUF 64	ITL	Batch	Autotrophic	n.a.	100	10–185[a]	10–0.5	n.a.	6.8	[24]
G. sulphuraria ACUF 64	ITL	Batch	Autotrophic	n.a.	200	10–195[a]	20–1.0	n.a.	10.4	[24]
G. sulphuraria 74G	STR	Chemostat	Mixotrophic	0.071	125	0.78	5.2	0.49	14.5	[10]
G. sulphuraria 74G	STR	Chemostat	Mixotrophic	0.071	175	0.84	7.1	0.53	15.6	[10]
G. sulphuraria 74G	STR	Chemostat	Mixotrophic	0.071	395	0.85[b]	9.0	0	0	[10]
G. sulphuraria 74G	BC	Batch	Photo-induction	0.057	250	0.6–6.0	23.8–2.4	0.77	13.0	[34]
G. sulphuraria 74G	BC	Batch	Photo-induction	0.029	1092	0.9–5.5	35.3–5.8	0.38	13.0	[35]
G. sulphuraria 74G	RWP	Batch	Photo-induction	1.35	641[c]	0.7–2.4	8.5–2.5	0.13	10.0	[35]

[a] Biomass concentration (C_x) expressed as $g \cdot m^{-2}$.
[b] Initial biomass concentration. It was not possible to obtain a steady state and the culture was washed out of the reactor.
[c] The culture was grown outdoor with at a light intensity of 30 $MJ \cdot m^{-2} \cdot day^{-1}$. This value was converted into average PFD.

light intensity are scarce (Table 3). Sloth et al. [10] reported that exposure of a mixotrophic *G. sulphuraria* 74G culture to an incident light intensity of 395 $\mu mol \cdot m^{-2} \cdot s^{-1}$ leads to photoinhibition. The culture was operated in chemostat at a dilution rate of 0.63 day^{-1} and the photoinhibition prevented it from reaching a steady state. The specific growth rate reported by Sloth et al. was 0.49 day^{-1} and it was calculated as the sum of the wash out rate and the dilution rate. We estimated the initial q_{ph} of their culture to have been 9.0 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$, which is on the higher side of the range we found in our autotrophic culture. Moreover, in the study of Sloth et al. the culture was washed out at a rate of 0.14 day^{-1} which led to a rapid increase of q_{ph} causing photoinhibition.

With the intent of avoiding photoinhibition, Wan et al. [34] proposed a two-phase cultivation strategy where *G. sulphuraria* 74G is firstly grown heterotrophically for biomass production. During heterotrophic growth pigmentation is lost. In the second phase, this heterotrophic culture is used as an inoculum for an autotrophic phase (photoinduction) in *PBRs* for phycocyanin accumulation. Photoinduction has been recently scaled up outdoors in *PBRs* at light intensity reaching up to 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at solar noon [35]. During photoinduction, cultures started without pigmentation (heterotrophic inoculum) needed up to 14 days to fully regain their pigmentation. The initial low pigmentation improved light tolerance, in fact using this strategy the authors cultivated *G. sulphuraria* in bubble columns at an initial q_{ph} of 35.3–47.6 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$. Moreover the authors demonstrated that the initial biomass concentration (C_x), and therefore the initial q_{ph}, is crucial to successfully cultivate *G. sulphuraria* at high light intensity.

In order to compare our work with other *PBR* designs, we converted the volumetric biomass concentration (C_x) and productivity (r_x) into areal biomass concentration (C_A, $g \cdot m^{-2}$) and areal productivity (r_A, $g_x \cdot m^{-2} \cdot day^{-1}$). This was done using a correction factor α (m):

$$\alpha = \frac{V_{PBR}}{A_{PBR}} \quad (4)$$

where V_{PBR} (m^3) represents the volume of the photobioreactor. Volumetric biomass concentration (C_x) and productivity (r_x) can be converted in their areal equivalent multiplying C_x and r_x for the correction factor α.

In our study the autotrophic areal biomass production rate (r_A) obtained in repeated batch was 28.6 $g_x \cdot m^{-2} \cdot day^{-1}$. This areal productivity was 1.8 to 7.7 times higher than previously reported (Table 3). Moreover, the mixotrophic r_A was 1.8 and 1.7 times higher than the autotrophic r_A in the repeated batch (VI) and in chemostat respectively, making the present study the highest r_A ever obtained in a photosynthetic culture of *G. sulphuraria*.

G. sulphuraria ACUF 64 displayed an outstanding capacity to maintain linear growth at low specific light supply rate (q_{ph}) (Fig. 2, Table 2). In the mixotrophic batch (VI) linear growth was maintained at 1.8 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$, corresponding to 9.7 $g_x \cdot L^{-1}$ or 340 $g_x \cdot m^{-2}$. In the autotrophic culture linear growth was maintained until 3.6 $\mu mol_{ph} \cdot g_x^{-1} \cdot s^{-1}$, corresponding to 4.8 $g_x \cdot L^{-1}$ or 170 $g_x \cdot m^{-2}$, values half of those obtained in the mixotrophic culture. Linear growth at high areal biomass densities has recently been reported by [24]. The authors cultivated *G. sulphuraria* ACUF 64 autotrophically in a biofilm at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ obtaining linear growth until 195 $g_x \cdot m^{-2}$. Despite the higher density, r_a was 2.8 times lower than in our autotrophic culture in repeated batch. The ability of *G. sulphuraria* to efficiently perform photosynthesis in dense culture will lead to a significant reduction in downstream processing costs, making this a promising candidate for large scale cultivation.

4.6. Biomass productivity at pH 0.2

Most *G. sulphuraria* strains have been isolated from highly acidic hot springs where the pH is close to zero [13]. Although optimal pH for *G. sulphuraria* is reported to be between pH 1 and 4 [10,16,22,36] only two of those studies [12,18] investigated pH below 1. During

autotrophic batch II at day 9.9, the pH suddenly dropped from 1.8 to 0.2 and we stopped the automatic pH control (Fig. A4). After a few hours, we confirmed pH to be 0.2 by taking several samples from the reactor and measuring the pH with an external probe. The culture was maintained at pH 0.2 for 1.3 days (Fig. 2, orange area). The pH in our medium was buffered by $H_3PO_4 \rightleftharpoons H_2PO_4^- + H+$ ($pKa_1 = 2.14$) and $H_2SO_4 + H_2O \rightleftharpoons H^+ + HSO^-_4$ ($pK_{a1} = 1.92$). Since H_2SO_4 was used as titrant we excluded sulfur depletion and we analyzed phosphorus (P) content in the culture. The measurements indicated that P was completely depleted at the point in time where the pH dropped. This finding was unexpected, because even considering a high biomass P content of 1.5% w_P/w_x, the P concentration in the medium should have been sufficient to sustain the growth up to 18 $g{\cdot}L^{-1}$ of biomass, while in batch II only 4 $g{\cdot}L^{-1}$ of biomass was produced. We used the software MINTEQ 3.1 (https://vminteq.lwr.kth.se) to estimate possible salt precipitation and the calculations allowed us to reject any risk of precipitation. Therefore, the only possible explanation was an error in the medium preparation. For this reason, after batch II the medium was changed and H_3PO_4 was chosen as P source. This change allowed a reduction from 88 to 14 $mL{\cdot}L^{-1}$ of the addition of 2.5 M H_2SO_4 to set the pH at 1.6. In the new medium pH was constant for the remainder of the experiment without the addition of any titrant.

Despite being an unintentional event, this is the first report of *G. sulphuraria* grown in a *PBR* at pH 0.2. In previous studies no growth [12] or 30% reduction in μ grow has been reported [18] when *G. sulphuraria* was grown below pH 0.5. Nevertheless, pH optima and tolerance are strain specific and it might be that our *G. sulphuraria* strain is more tolerant to low pH than others. Surprisingly, in our study during the 2 days of growth at pH 0.2 r_x was not significantly different ($P > 0.05$) from the value found in batch I and III (Table 2). Further studies, where pH 0.2 will be maintained for a longer time and without P starvation are needed to confirm this finding. Tolerance to extremely low pH might further decrease the risk of contamination.

5. Conclusions

In the present study *G. sulphuraria* ACUF 64 was cultivated at high incident light intensity autotrophically and under oxygen balanced mixotrophy. The autotrophic biomass productivity surpassed by far all other ever reported in literature. The reactor operated without any gassing and biomass productivity and concentration were almost double the autotrophic culture grown under similar conditions. All of these characteristics make *G. sulphuraria* ACUF 64 a promising candidate for outdoor cultivation.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

CRediT authorship contribution statement

Fabian Abiusi: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing-original draft, Writing review & editing. **Egbert Trompetter**: Investigation, Formal analysis, Data curation, Writing-original draft. **Hugo Hoenink**: Investigation, Formal analysis, Data curation. **Rene H. Wijffels**: Funding acquisition, Supervision, Writing review & editing. **Marcel Janssen**: Data curation, Supervision, Writing review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102513.

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