Title: PRODUCTIVITY AND SELECTIVE ACCUMULATION OF CAROTENOIDS OF THE NOVEL EXTREMOPHILE MICROALGA *Chlamydomonas acidophila* GROWN WITH DIFFERENT CARBON SOURCES IN BATCH SYSTEMS

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Abstract

Cultivation of extremophilic microorganisms has gained interest due to their ability to accumulate high value compounds. *Chlamydomonas acidophila* is an acidophilic green microalga isolated by our group from Tinto River, an acidic river that flows down from the mining area in Huelva, Spain. That microalga accumulates high concentrations of lutein, a very well known antioxidant. The aim of this study was to assess the use of different carbon sources (CO_2, glucose, glycerol, starch, urea and glycine) for the growth and carotenoid production of *C. acidophila*. Our results revealed that the utilization of different carbon sources also resulted in different biomass productivities, urea being as efficient as CO_2 when used as the only carbon source (~ 20 $g/m^2 \cdot d$). Mixotrophic growth on glucose is also efficient in terms of biomass production (~ 14 $g/m^2 \cdot d$). Regarding carotenoid accumulation, mixotrophic growth on urea even results in higher carotenoid productivities (mainly lutein, probably via α-carotene) than those obtained with photoautotrophic cultures (70% vs 65% of relative abundance for lutein, respectively). The accumulated lutein concentrations of *C. acidophila* reported in this work (about 10 g/kg dry weight, produced in batch systems) are among the largest ones produced with a microalga and ever published. Glycerol and glycine seem to enhance β-carotene biosynthesis, and if glycine is used as carbon source, zeaxanthin becomes the most accumulated carotenoid in the microalga. Strategies for production of lutein and zeaxanthin are suggested from the obtained results.

Keywords: Extremophile microalgae; mixotrophic growth; energy source; carotenoid

Introduction

Cultivation of extremophilic microorganisms has gained interest due to their ability to accumulate and produce high value compounds, namely metabolites, enzymes, surfactants, etc [1]. Microalgae can also be found among extremophile microorganisms that grow at e.g. extreme pH, salinity or temperature environments [2]. Besides, microalgae have been widely recognized as producers of value compounds, carotenoids among them [3]. The acidic drainages from the mining area in Huelva (Spain) constitute an extreme environment where many extremophile microorganisms grow based on sulphur and iron oxidation to obtain metabolic energy. From that low pH environment, our group isolated the acidophilic microalga *Chlamydomonas acidophila*. The extremely oxidant conditions of such an environment [4] suggest that the microorganisms growing in the river should express different antioxidant mechanisms to defend themselves from oxidative stress. For that reason, our group studied the biotechnological value of *C. acidophila* for antioxidants production.

C. acidophila accumulates high concentrations of lutein, a very well known antioxidant also accumulated by other "common" microalgae [5-6], which has recently gained interest for the treatment of oxidative diseases like macular degeneration [7-8]. Lutein is a xanthophyll, an oxygenated carotenoid. Carotenoids are produced through carotenogenesis pathway, which starts from basic precursors of terpenoids, including piruvate or acetyl-CoA [9]. In particular, xanthophylls are synthesised from very basic C5-isoprenoid compounds which first elongate to phytoene (C40) [10-11] and are further processed to lycopene and cyclic carotenoids including α,β,γ-carotenes (C40) [12]. Carotene oxidation leads to lutein. Some of the organic carbon sources selected to grow *C. acidophila* cultures are closely related to carotenoid biosynthesis. Both acetate and glycerol are precursors of acetyl-CoA biosynthesis. Glycine, a combined source of

carbon and nitrogen, was chosen due to its possible role for zeaxanthin production. Previous research at our laboratory [6] showed that in *C. acidophila* an active xanthophyll cycle seems to operate which would produce zeaxanthin in photoautotrophic cultures exposed to high light conditions. In the xanthophyll cycle, zeaxanthin is produced from violaxanthin by the action of violaxanthin de-epoxidase enzyme activity [13-14], which depends on the presence of reduced glutathion [15]. Glycine is required to synthesise glutathion and that could expectedly be used as a successful tool for large production of zeaxanthin.

On the other hand, urea, the other combined C-N source chosen, has also been tested to assess biomass and carotenoid productivity of that extremophile microalga. Urea has been successfully used as nitrogen source in *Spirulina platensis* cultivation, obtaining similar productivities to those obtained with nitrate as nitrogen source [16-17]. Besides, the search for cheaper nitrogen sources like urea [18] or ammonium salts [19] is particularly attractive from the economic point of view.

The aim of the present work was to determine whether mixotrophic cultivation could be used for carotenoid production enhancement in *C. acidophila* and for conducting accumulation of different carotenoids depending on the organic carbon source added into the culture medium. This work is the first published report about the carotenoid accumulation of mixotrophically grown acidophilic green microalgae ever.

Materials and Methods

Microorganism and culture conditions

C. acidophila, the algal material used in this study, was isolated from the acidic water of the Tinto River, which runs through a mining area in Huelva (Spain). In this river, the pH ranges during the year between 1.7 and 3.1. An axenic culture of the alga was obtained by streaking it on basal agar medium at pH 2.5. Isolated colonies were transferred from the agar medium to the liquid medium; both with the same composition. To prepare agar medium, 300 mL of 7% agar and 700 mL of modified K9 medium [20] were autoclaved separately and mixed before use *C. acidophila* was maintained by periodic transfers in sterile modified K9 medium adjusted at pH 2.5 with concentrated H_2SO_4. During the runs, pH of cultures was monitored and adjusted at 2.5 with diluted NaOH or KOH.

Control cultures were bubbled with air containing 5% (v/v) CO_2, whereas the other cultures were incubated in absence of carbon dioxide but in presence of different carbon sources (glucose, glycerol, starch, glycine and urea). In those cases where CO_2 were not supplied to the cultures, it was necessary to put a carbon dioxide trap with KOH 5M buffer for removing it from the air mix. All cultures were grown at 25 ºC in 1 liter batch reactors, and continuously illuminated with fluorescent lamps (Philips TLD, 30W, 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the surface of the flasks). The irradiance was measured with a photoradiometer Delta OHM (mod. HD9021).

Specific growth rate calculations

The specific growth rates of cultures incubated under different carbon sources were calculated from the linear portion of the biomass time-course evolution using the following expression:

$$\mu = \text{Ln } (C/Co)/t$$

where μ is the specific growth rate; Co, the initial biomass concentration; C the biomass concentration at any time (t).

Dry weight measurements

To measure the dry weight, 5 mL of each culture were taken and the liquid medium was removed by means of a vacuum pump using a cellulose acetate filter (weighed before using it) with 0.45 μm pore size from Sartorius (Goettingen, Germany) to separate the cells from the medium. The cells were then washed with demineralized water before drying the filters. The filters with the cells were dried and stored at 70 ºC. The filters with the dried cells were weighed after 24 h.

Oxygen evolution

The biological activity used to test cell viability was photosynthetic activity. For photosynthetic activity determinations, 1 mL of cellular microalgae suspensions was placed into a Clark-type electrode (Hansatech, UK) to measure O_2-evolution. Chlorophyll concentrations used for these determinations were below 15 $\mu g \cdot ml^{-1}$ so that self-shading was minimized. Measurements were made at 25 ºC under saturating, but non-photoinhibitory, white light (600 $\mu E \cdot m^{-2} \cdot s^{-1}$) emitted by Philips lamps, or in darkness (endogenous respiration).

Analytical determinations

Aliquots (1 mL) of the cultures were spinned down for 10 min at 5000 rpm. The supernatant was discarded and the obtained pellet was placed for 1 min in boiling water. After that the pellet was resuspended in the proper volume of absolute methanol to

extract pigments completely. The suspension was shaken vigorously for 1 min and centrifuged for 10 min at 5000 rpm. Chlorophyll and total carotenoid concentrations were determined spectrophotometrically in the supernatant, using the equations proposed in [21] or by HPLC analysis (see below). Protein content was determined following the Bradford method [22]. The cell concentration was determined by measuring the optical density of the culture at 680 nm in a spectrophotometer Ultrospec 3100 pro UV/Visible manufactured by Biochrom Ltd.

HPLC analysis of carotenoids

Separation and chromatographic analysis of pigments was performed in a Merck Hitachi HPLC equipped with a UV–Vis detector as described in [23], using a RP-18 column and a flow rate of 1 $mL \cdot min^{-1}$. The mobile phase consisted on ethyl acetate (solvent A) and acetonitrile/water (9:1 v/v) (solvent B), and the gradient programme applied was: 0–16 min, 0–60% A and 100-40% B; 16–30 min, 60% A and 40% B; 30–35 min, 100% A. Pigments detection was carried out at 450 nm, and their identification and quantification was achieved by injecting known amounts of pigment standards supplied by DHI-Water and Environment (Denmark).

Cell counting

The number of cells was determined by microscopy Olympus CX41 in a Neubauer chamber.

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

Results and Discussion

Growth kinetics with different carbon sources

In this part of the work, the efficiency of different energy sources (carbon sources) on the growth of *C. acidophila* was investigated. The use of glucose and starch for mixotrophic growth to drive biomass production of microalgae is quite extended [24–27]. Regarding other carbon sources, glycerol and acetate can also play the role of natural precursors for terpenoid biosynthesis [25,28,29]. Based on that, growth on either glycerol or acetate could in theory enhance carotenoid accumulation in *Chlamydomonas acidophila*. However, heterotrophic growth should be less suitable for massive carotenoid production as these are accessory photosynthetic pigments for whose massive synthesis light uses to be required [30]. As a consequence, in our experiments the growth (even on acetate, photoheterotrophic conditions) was always done by illuminating the cultures, that way the microalga synthesises carotenoids while growing [25,31]. Glycine and urea were tested as a combined form of carbon and nitrogen. Its expected role in carotenoid biosynthesis is discussed later. Besides, the alternative use of urea as combined source of nitrogen and carbon has been shown to enhance *Spirulina platensis* growth [32].

Batch experiments were run to study time-course evolution of biomass concentration (C) of *C. acidophila* grown with those carbon sources above referred. Several cultures of *Chlamydomonas acidophila* were grown in standard culture medium, each one of them supplemented with only one of the following carbon sources: glucose, starch, glycine and urea – as combined C and N sources –, glycerol, acetate and CO_2 (5% v/v in air, control cultures). Results are shown in Figure 1.

The lag phase takes from 30 to 50 hours for all of the cultures, whatever the carbon source used. Lag phase duration seems to be not dependent on either type of

199 carbon source or its concentration in the culture medium, although in those cultures
200 grown on urea lag phase seems to be shorter. When compared to growth rates obtained
201 from batch experiments with common microalgae [33], the exponential growth of *C.*
202 *acidophila* was found to be a bit slower. After lag phase cultures were allowed to grow
203 during sufficient time in order to calculate growth rates accurately.

204 As shown in Figure 1, in all cultures the end of the exponential growth phase
205 was reached within the period 150–250 h, whatever the carbon source used. No
206 additional amounts of organic carbon sources were added along the growth as all
207 cultures were run as batch. The lowest concentration of each organic nutrient added to
208 the medium was 5 mM, except for starch (0.5 $g \cdot L^{-1}$). From previous experiments
209 (results not shown), that starch concentration added to the cultures was high enough to
210 allow the cultures to fully complete the growth. This is in good agreement with other
211 author results [34,35], who report from 3 to 5 mM glucose to be carbon saturation
212 conditions for microalgal growth in batch cultures. Starch was the only exception to that
213 empiric rule, since concentrations higher than 2 $g \cdot L^{-1}$ rapidly inhibited microalgal
214 growth. This is in good agreement with [25] for mixotrophic growth of the microalga
215 *Phaeodactylum tricornutum*. Starch, a common chemical form of carbon reservoir in
216 nature and a suitable raw material for the growth of microorganisms, could be used to
217 sustain the growth of *C. acidophila* during about 100 h at a growth rate that was almost
218 as fast as that obtained with CO_2 as the only carbon source. Addition of more starch
219 could sustain microalgal growth for longer (results not shown). Therefore, starch could
220 also be a suitable carbon source for growing *C. acidophila*.

221 Some significant differences in growth rates of *C. acidophila* were observed for
222 the different energy sources and concentrations. By using the data in Figure 1, growth
223 rates of *C. acidophila* for the best nutrient concentration of each one of the organic

carbon sources assayed were calculated from the linear portion of each one of the growth curves. The results are shown in Figure 2, in which each one of the calculated growth rates is expressed as a percentage of the maximum growth rate obtained for those cultures grown on CO_2 (100% ≅ 0.5 d^{-1}).

Best results -the highest growth rate and maximum dry weight- (~ 0.5 d^{-1} and ~ 6.0 $g \cdot L^{-1}$, respectively) were obtained by cultivating *C. acidophila* with CO_2 (5% v/v in air) or 0.01M urea as the only carbon source (Figure 2). Indeed, the time-course growth evolution obtained on 10 mM urea was actually the same that one of control cultures grown with 5% CO_2 in air (v/v), although in this case the lag phase almost did not take place. Glucose was the only organic carbon source with which cultures reached similar biomass concentrations to those obtained by using CO_2 or urea as carbon source (Figure 2). However, *C. acidophila* growth on glucose only takes place as long as glucose concentration remains below 0.01M. Higher glucose concentrations easily induce bacterial growth in the culture medium with the subsequent growth inhibition of *C. acidophila*.

C. acidophila was also able to grow in the presence of other organic carbon sources including starch, glycerol and glycine, the last being a combined source of carbon and nitrogen. As observed in Figure 2, the amount of produced biomass with glucose and urea was significantly higher than that obtained with any other of the organic carbon sources mentioned above, by at least a factor of about 4. Among the less productive organic carbon sources (starch, glycerol and glycine), starch led to the fastest growth (Figure 2), though final biomass concentration was similar to that obtained in the presence of glycerol and glycine.

The growth of *C. acidophila* on 5 mM either glycerol or glycine developed much slower (by a factor of 2, Figure 1) than that on 5 mM glucose. Growth on higher

concentrations of either glycerol or glycine even led to slower growth due to bacterial growth that competes for the carbon source. This is shown in terms of growth kinetics (Figure 1). Acetate was also tested as carbon source but no growth was observed.

Glucose and specially urea were found to be the carbon sources that did enable *C. acidophila* to grow as fast as on CO_2 (control cultures). Indeed, urea was the only carbon source with which culture growth rate almost equalled that of cultures grown on glucose. Growth rate of *C. acidophila* grown on starch accounted for 60% of that obtained with CO_2, whereas in the presence of either glycerol, glycine or acetate the growth rate was less than 50% of that obtained with CO_2. Results of dry weight collected from each best nutrient concentration culture are also shown in Figure 2.

Biomass productivity for each culture was calculated and the results shown in Table 1. Productivity clearly differs depending on the carbon source the growth was based on, and acetate was the only organic carbon source with which no net growth was detected, therefore productivity being negligible. Those cultures grown on glucose, urea, starch, glycerol and glycine developed along the same growth time, but final dry weight (Figure 2), biomass productivity and maximum number of cells (Table 1) decreased where a carbon source other than urea or glucose was used, as an evidence for minor affinity of *C. acidophila* for those substrates.

The biological state of the cultures growing on the different carbon sources was also assessed and followed through photosynthetic activity. Results of averaged O_2 production rates when cultures were in mid exponential growth phase are included in Table 1. Cultures grown on acetate did not yield any measurable photosynthetic activity, in good agreement with the growth results. The rest of cultures, grown on each nutrient best concentration, showed oxygen production rates within the range of expected values for viable cultures (control cultures grown on CO_2).

From our results, urea seems to be a suitable and economic alternative to other conventional carbon (mainly CO_2) and nitrogen sources (usually nitrate) that keeps *Chlamydomonas acidophila* growth at even higher levels than those obtained with carbon dioxide (5% v/v in air). Therefore, only urea and, to some minor extend, glucose could speed *C. acidophila* growth up to those values obtained with CO_2 as carbon source. In addition, *C. acidophila* showed low capacity to make use of glycerol, glycine and, specially, acetate. The low pH of the culture medium (2.5) modifies the chemical form of glycine and acetate which become totally protoned. That way, both molecules has less polar character which might make substrate uptake through cell membrane more difficult.

Pigment production

Total carotenoids

In this paper we also made an approach to carotenoids accumulation by *C. acidophila* during its growth based on different carbon sources. Best concentration of each organic carbon source (glucose, urea, starch, glycerol, glycine and acetate), obtained from previous growth experiments, was used for growing *C. acidophila* and investigating both quantity and type of the accumulated carotenoids. During growth, samples were taken at different times for pigment analysis by HPLC.

Figure 3 shows maximum chlorophyll and carotenoid content of *C. acidophila* in each one of the cultures grown on the different carbon sources. Each value represents the percentage with respect to the maximum concentration found in cultures grown on glycerol. Maximum chlorophyll and total carotenoid content per biomass unit were found in cultures grown on glycerol (100% = 33.6 and 7.4 $mg \cdot g^{-1}$ dry weight for chlorophyll and carotenoids, respectively).As observed, chlorophyll content remained

similar for all of the carbon sources tested. The lowest chlorophyll content was found in cultures grown on glycine (77%). However, significant differences in the content of carotenoids of the different cultures were observed, depending on the carbon source used. The lowest carotenoid content was found in algal samples of those cultures grown on urea, CO_2 and glucose (77%, 71% and 66% respectively, with respect to that maximum of cultures grown on glycerol). These carbon sources induced the fastest growth of *C. acidophila*.

On the opposite, the highest carotenoid content was found in those cultures grown on glycerol and acetate (100%), which also showed the highest carotenoid to chlorophyll ratio and led to the lowest growth. These results are in good agreement with those published in [25] for *Phaeodactylum tricornutum*. They showed maximum pigment production for cultures grown on glycerol or glycine, and the opposite effect for cultures grown on glucose or urea. Regarding the use of acetate, those cultures grown on acetate oddly showed no growth, meaning the carbon source was hardly consumed and catabolized. Coherently, the high content of carotenoids per biomass unit in cultures grown on acetate could partly be due to the stress produced by nutrient limitation. In that situation cell metabolism uses to partly divert carbon flows to lipid biosynthesis including carotenoids, as described by other authors for several microalgae strains [36-37]. In particular, lack of carbon has been reported to increase phycocyanin content in the acidophilic red microalga *Galdieria* [27], one of the few examples of mixotrophic growth reported for acidophilic microalgae. Obviously, such that production of carotenoids per biomass unit in cultures grown on acetate can not be sustained in time as there is almost no carbon source (acetate) uptake and, therefore, culture finally becomes non-viable.

The high content of carotenoids per biomass unit in viable *C. acidophila* cultures grown on glycerol should be the consequence of enhanced carotenoid biosynthesis produced by that organic carbon source. In terms of total volume productivity of chlorophyll and carotenoids (mg carotenoids per mL), best results were found in those cultures grown on glucose, urea and CO_2, as expected; because of their higher biomass concentration, in these cultures the total amount of chlorophyll and carotenoids (per culture volume unit) was found to be about 5-6 fold that amount of those cultures grown on glycerol (Table 2). Therefore, it seems to be clear that carotenoid enrichment based on glycerol should always be a second step in the carotenoid production process, just after biomass production on whatever CO_2, urea or glucose.

Specific carotenoids

To investigate whether the use of different reduced carbon sources would address carbon flows to the production of specific carotenoids in different ways, chromatographic separation of the main carotenoids produced by *C. acidophila* grown under the different carbon sources was performed by HPLC. A typical chromatogram from photoautotrophically grown *C. acidophila* extracts is shown in Figure 4. Lutein appears as the major carotenoid, and β-carotene, zeaxanthin and violaxanthin are also accumulated significantly.

Figure 5 shows the maximum content of each one of the main carotenoids accumulated by *C. acidophila* grown in culture media with the different carbon sources. The carotenoid contents are given as mg per g of dry biomass. Two different trends in carotenoid content evolution are observed depending on the carbon source added into the culture media. In those cultures incubated in the presence of whatever CO_2, glucose, urea, starch or glycerol, the maximum carotenoid content corresponded to lutein and β-

carotene. In particular, very similar contents of these carotenoids were found in those cultures incubated in the presence of CO_2, starch, urea and glucose, about 9–10 $mg \cdot g^{-1}$ of lutein and 1.5–4 $mg \cdot g^{-1}$ of β-carotene, respectively. Thus, in all of the cultures grown with CO_2, glucose, urea, starch or glycerol, zeaxanthin and violaxanthin contents remained below 2 $mg \cdot g^{-1}$.

On the contrary and surprisingly, β-carotene was the most abundant carotenoid in cultures grown on glycerol (12 $mg \cdot g^{-1}$, 2-fold that lutein content), and zeaxanthin was the major carotenoid in cultures grown on glycine (7.5 $mg \cdot g^{-1}$, 2-fold that lutein content). When *C. acidophila* was incubated with glycine as carbon source, both zeaxanthin and violaxanthin content increased surprisingly up to about that level of lutein and β-carotene of *C. acidophila* photoautotrophically grown, whereas lutein and β-carotene content decreased down to 3.7 and 2.2 $mg \cdot g^{-1}$. As shown in Table 3, lutein plus β-carotene to zeaxanthin plus violaxanthin (xanthophyll cycle pigments) ratio ranges from 9 to 13 in *C. acidophila* cultures grown with CO_2, glucose, starch and glycerol, whereas it is 0.6 and 4.5 in cultures grown on glycine and urea, respectively.

The results obtained from cultures grown on glycine seem to evidence zeaxanthin accumulation to occur inversely to lutein accumulation. As explained, among all the xanthophylls, zeaxanthin is the only one that accumulates exclusively under excess light [38]. Furthermore, lutein, the most abundant xanthophyll in the thylakoid membrane, has been shown to be not essential for light harvesting and photoprotection functions when zeaxanthin is present [39]. However, *C. acidophila* accumulates large concentrations of lutein that remain constant under high light conditions [6]. The oxidative conditions of the natural habitat of *C. acidophila*, metal ions and high light, might be behind these adaptation mechanisms. Besides, the

accumulated lutein concentrations of *C. acidophila* reported in this work (about 10 $g \cdot kg^{-1}$ dry weight) are among the largest produced with a microalga and published ever [40]. This is just an example for reinforcing the idea (widely supported by many algal biotechnologists) that nature is a *huge larder* for searching microalgae with certain biological activities, if that search takes places in a suitable ecological niche which meets specific environmental conditions as to induce in the microalga the desirable metabolic response, e.g. lipid synthesis and carotenoid accumulation [5,41].

The high zeaxanthin and violaxanthin content obtained in cultures grown on glycine deserves special attention. The obtained results would be in good agreement with an increased de-epoxidase activity of the xanthophyll biosynthetic pathway in *Chlamydomonas acidophila* under high light (via lycopene, [39]), which forms zeaxanthin from violaxanthin de-epoxidation. However, it is under relatively low light conditions (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) that zeaxanthin accumulates whereas lutein decreases, in the presence of glycine as carbon source instead of CO_2. This is an unexpected result as zeaxanthin was expected to accumulate only in cells exposed to oxidative conditions (e.g. high light intensity).

Zeaxanthin accumulation does not occur for light harvesting functions. Therefore, that accumulation in glycine cultures should anyhow be explained in terms of how metabolic carbon flows are addressed depending on the carbon source added. In that sense and bearing in mind that there is surely no an unique explanation to that, glycine supplies carbon for glutathione biosynthesis, whose reduced form is required for the action of violaxanthin de-epoxidase activity [42]. Therefore and first, glycine is indeed being use as a precursor for reduced ascorbate formation that is further required for zeaxanthin biosyntesis, which could partly explain the higher content of that xanthophyll pigment per biomass unit (Figure 5). Second and inferred from results

(Figures 1 and 2), *C. acidophila* growth on glycine becomes slower; in such these conditions, a higher zeaxanthin accumulation occurs and it seems to be consistent with the cell need for additional antioxidant activity in order to diminish the oxidative stress produced by the apparent carbon limitation for basic metabolic activities [27]. And third and more interestingly, according to that biosynthesis pathway for carotenoids in microalgae [43], zeaxanthin and violaxanthin are directly formed from lycopene via β-carotene; lycopene can also diverts to lutein formation via α-carotene. Thus, zeaxanthin and violaxanthin biosynthesis from β-carotene would be enhanced in *C. acidophila* grown on glycine while lutein synthesis from α-carotene would become significantly lowered. Growth on glycerol also seems to enhance that pathway via β-carotene (high β-carotene content, low lutein content, compared to photoautotrophic growth), but it does not finally progress towards ketocarotenoids formation.

From our results, we conclude that the use of different carbon sources to grow *C. acidophila* leads to different biomass productivities, urea becoming as efficient as CO_2 as sole carbon source. Mixotrophic growth on glucose is also efficient in terms of biomass production, but a bit less than urea. The use of different organic carbon sources also results in different both quantitative and qualitative profile of accumulated carotenoids. Particularly, mixotrophic growth on urea can even yield higher carotenoids productivities (mainly lutein, probably via α-carotene) than those obtained from photoautotrophic growth. The accumulated lutein concentrations of *C. acidophila* reported in this work (about 10 $g \cdot kg^{-1}$ dry weight) are among the largest produced with a microalga and published ever. Glycerol and glycine seems to enhance β-carotene biosynthesis pathway. If glycine is used as carbon source, that pathway evolves to yield high zeaxanthin content. These results also suggest that two pathways (via α-carotene

and β-carotene) might be operating for carotenoid biosíntesis in acidophile green microalgae. Finally, the results suggest strategies for production of lutein by continuous cultivation of *C. acidophila* on urea or CO_2, and for production of zeaxanthin by semi-continuous processes with *C. acidophila*, in a first step by growing the cultures photoautotrophically or mixotrophically with urea for biomass production and, as a second step, by adding only glycine as carbon source for zeaxanthin accumulation.

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616 **Table 1.** Maximum cell number, averaged photosynthetic activity per unit of
617 chlorophyll and biomass productivity for the best concentration of each carbon source
618 assayed

Carbon source	**CO_2**	**Glucose**	**Starch**	**Glycerol**	**Glycine**	**Acetate**	**Urea**
Best nutrient concentration	5% (v/v) in air	0.005M	2 $g \cdot L^{-1}$	0.005M	0.01M	0.05M	0.01M
Maximum cell number ($n \times 10^8$)	5.4 ± 0.3	4.2 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	0.50 ± 0.02	0.10 ± 0.01	4.9 ± 0.2
Photosynthetic activity ($\mu mol\ O_2 \cdot h^{-1} \cdot mg^{-1}$ Chl)	40 ± 2	35 ± 2	61 ± 3	33 ± 2	34 ± 2	5.0 ± 0.3	26 ± 1
Biomass productivity ($g \cdot m^{-2} \cdot d^{-1}$)	20 ± 1	13.9 ± 0.7	3.3 ± 0.2	2.7 ± 0.1	2.1 ± 0.1	1.0 ± 0.1	20 ± 1

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623 **Table 2.** Maximum chlorophyll content and maximum carotenoids content on culture

624 volume for the best concentration of each carbon source assayed

Carbon source	**CO_2**	**Glucose**	**Starch**	**Glycerol**	**Glycine**	**Acetate**	**Urea**
Best nutrient concentration	5% (v/v) in air	0.005M	2 g·L^{-1}	0.005M	0.01M	0.05M	0.01M
Chlorophyll (mg·L^{-1})	185.9 ± 9.3	144.5 ± 7.2	29.3 ± 1.5	26.9 ± 1.3	15.6 ± 0.8	4.8 ± 0.2	160.2 ± 8.0
Carotenoids (mg·L^{-1})	31.6 ± 1.6	22.5 ± 1.1	6.2 ± 0.3	5.9 ± 0.3	3.5 ± 0.2	1.1 ± 0.1	28.6 ± 1.4
Total pigments (mg·L^{-1})	217.5 ± 10.9	167.0 ± 8.3	35.5 ± 1.8	32.8 ± 1.6	19.1 ± 1.0	5.9 ± 0.3	188.8 ± 9.4

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Table 3. Maximum pigment content ($mg \cdot g^{-1}$ of dry weight), main carotenoids content (relative abundance in dry biomass) and (lutein+β-carotene) to xanthophylls cycle pigments ratio (zeaxanthin+violaxanthin), for the best concentration of each carbon source assayed.

Carbon source	*Lut (%)*	*β-car (%)*	*Zea (%)*	*Vio (%)*	*(Lut+β-car)/ (Zea+Vio)*	*Maximum pigment content*
CO_2	65.2	24.8	5.7	4.3	9.1	14.1 ± 0.7
Glucose	66.9	24.2	4.8	4.1	10.3	13.0 ± 0.6
Starch	66.3	25.9	4.8	3	11.8	16.6 ± 0.8
Glycerol	32.7	60.3	5	2	13.2	19.9 ± 0.9
Glycine	23.7	14.1	47.4	14.8	0.6	15.6 ± 0.8
Urea	70.6	11.2	12.1	6.1	4.5	12.4 ± 0.6

Figures

Figure 1

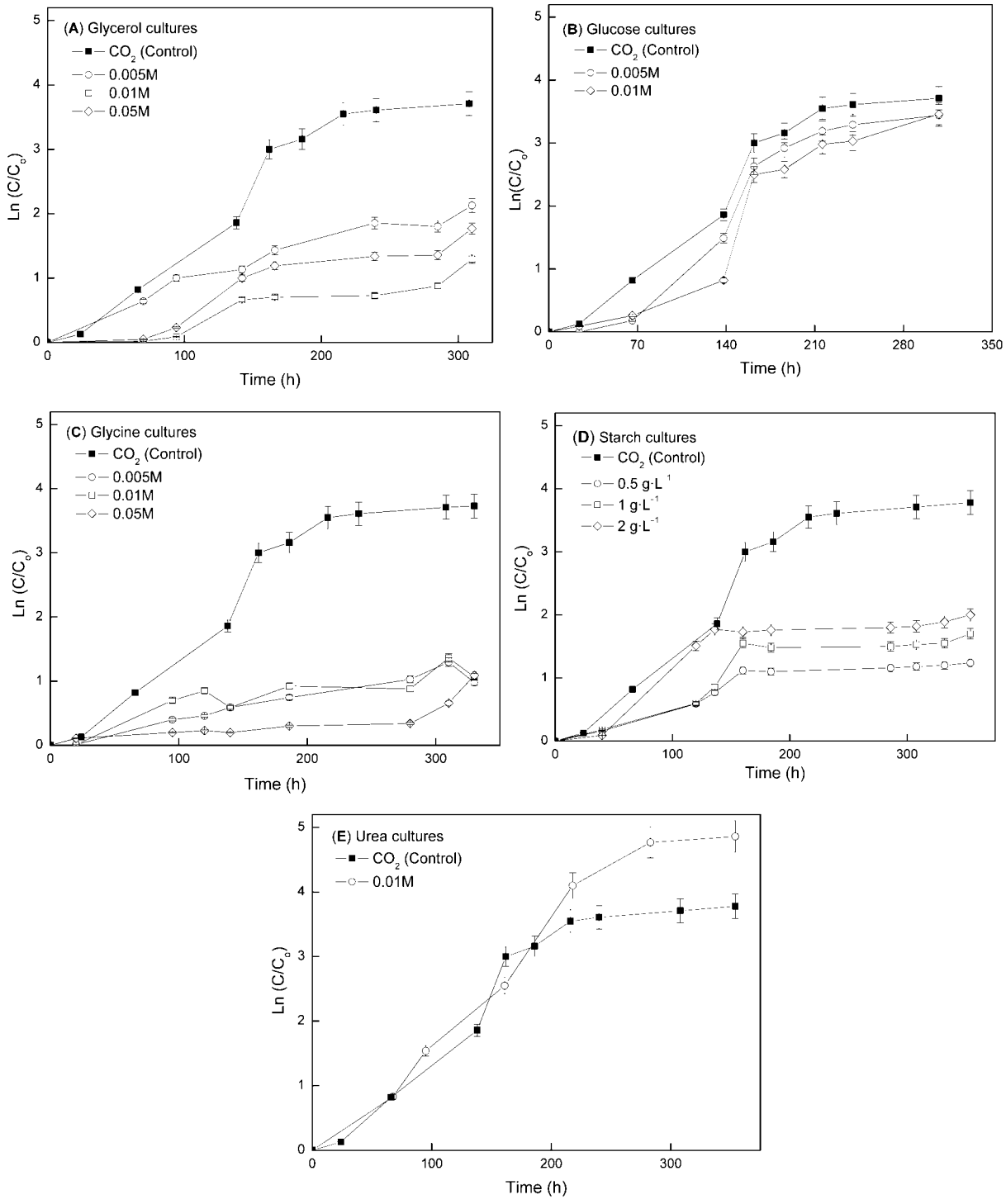

Figure 1. Time-course biomass evolution of *C. acidophila* cultures grown on different carbon sources. Several aliquots of *C. acidophila* cultures were prepared from a standard culture and each one was supplemented with one of the following assayed carbon sources: glucose, starch, glycine, urea, glycerol, acetate and CO_2 (bubbled in air, 5% v/v). These latter are included into each graph as reference to compare.

Figure 2

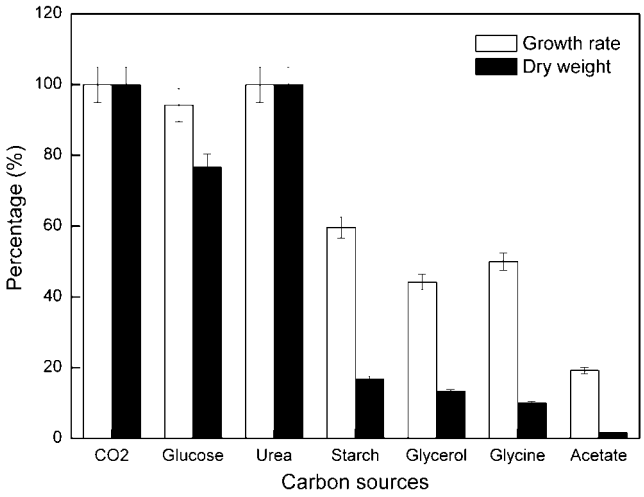

Figure 2. Specific growth rates and maximum dry weight of *C. acidophila* for the best nutrient concentration of each one of the organic carbon sources. Calculated growth rates are expressed as a percentage of the maximum growth rate obtained for those cultures grown on CO_2 (100% = 0.5 d^{-1}). Maximum dry weight values are expressed as a percentage of the maximum dry weight obtained for those cultures grown on CO_2 (100% = 6 $g \cdot L^{-1}$).

Figure 3

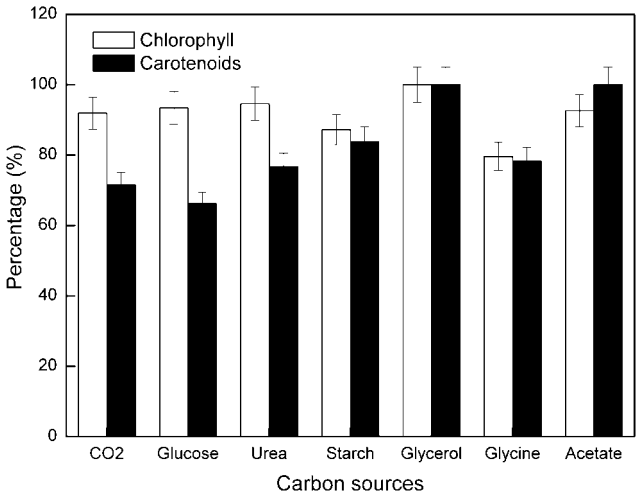

Figure 3. Maximum chlorophyll and carotenoid content of *C. acidophila* of each one of the cultures grown on different organic carbon sources. Each value represents the percentage with respect to the maximum content found in cultures grown on glycerol (100% = 33.6 and 7.4 $mg \cdot g^{-1}$ dry weight for chlorophyll and carotenoids, respectively).

Figure 4

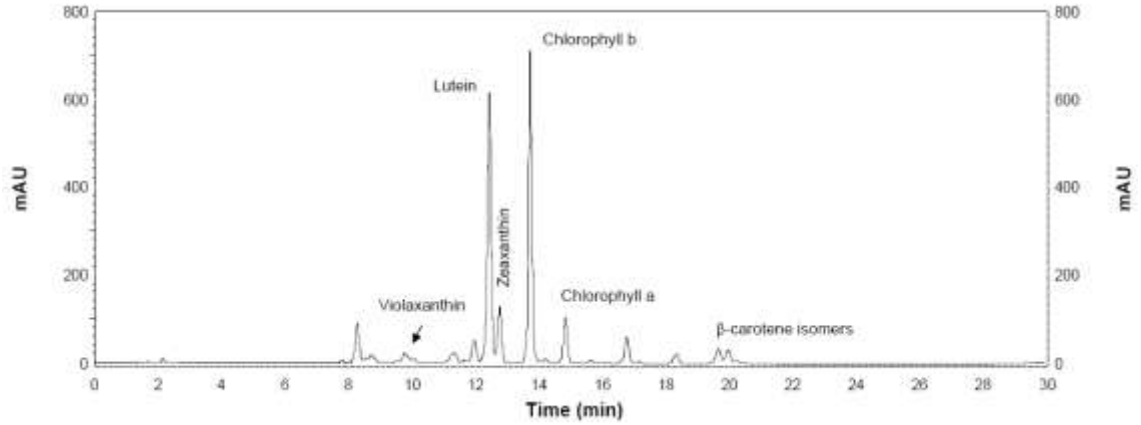

Figure 4. Typical chromatogram from photoautotrophically grown *C. acidophila* extracts. Peaks for major carotenoids are indicated.

Figure 5

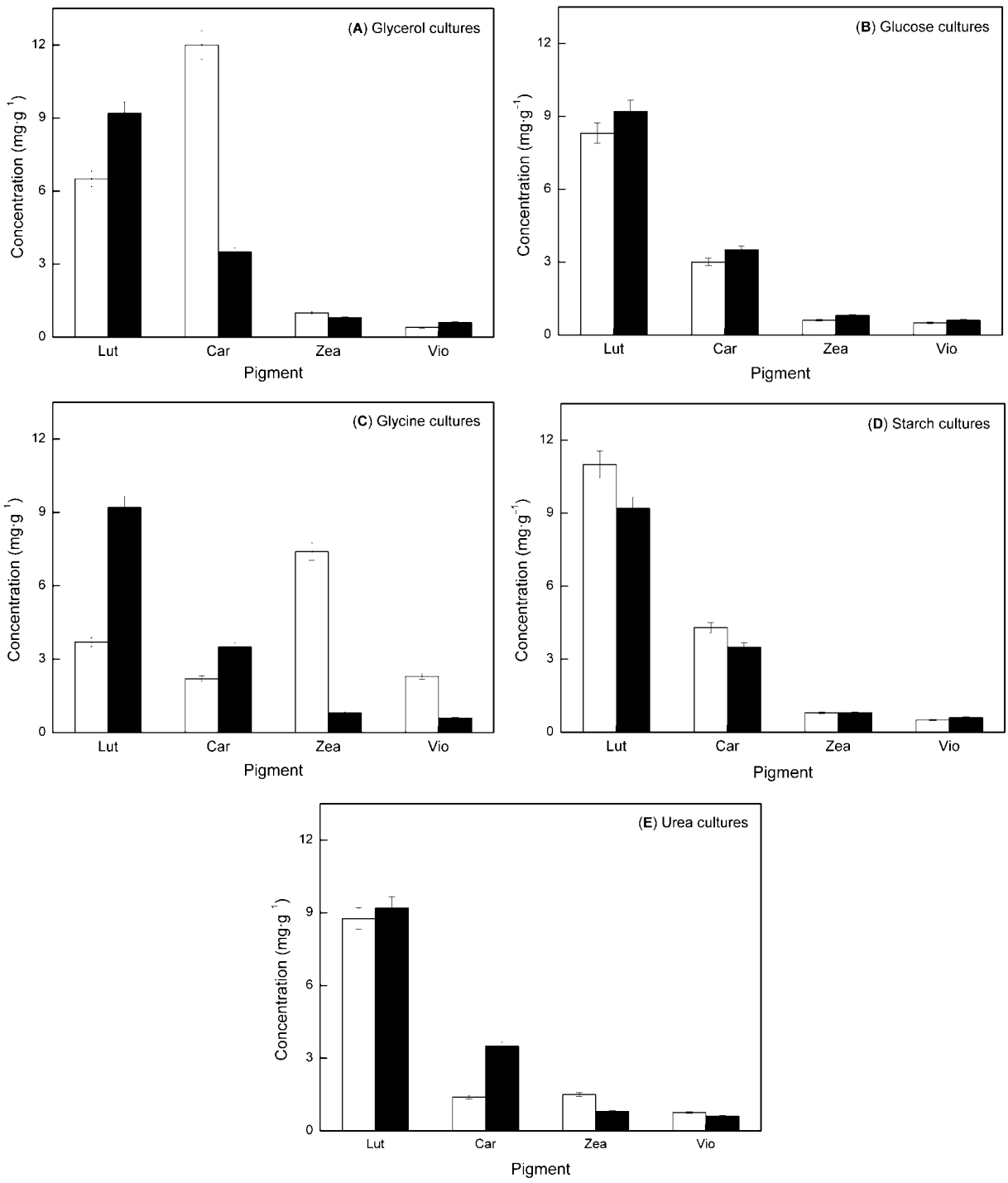

Figure 5. Maximum content of each one of the main carotenoids accumulated by *C. acidophila* grown in culture media with the different carbon sources. Carotenoid contents are given as mg per g of dry biomass. Black bars included into each graph

represent control cultures. Lut, lutein; car, β-carotene; zea, zeaxanthin; vio, violaxanthin.