EFFECT OF ABIOTIC STRESS ON THE PRODUCTION OF LUTEIN AND β-CAROTENE BY *Chlamydomonas acidophila*

Authors:

Inés Garbayo[(1)], María Cuaresma[(1)], Carlos Vílchez[(1)] and José M. Vega[(2)*]

Addresses

[(1)]Departamento de Química y Ciencia de los Materiales "Profesor J.C. Vílchez Martín", Facultad de Ciencias Experimentales, Universidad de Huelva, 21017 Huelva, Spain.

[(2)]Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Química, Universidad de Sevilla, c/o Prof. García González, Sevilla 41071, Spain.

[*]**Autor for correspondence**

Prof. José M. Vega

Departamento de Bioquímica Vegetal y Biología Molecular

Facultad de Química. c/ Prof. García González, nº 1. Sevilla, 41071. Spain

Tel.: 34.954.557142

Fax.: 34.954.626853

e-mail: jmvega@us.es

Abstract

Chlamydomonas acidophila growing autotrophically with continuous PAR light (160 $\mu E.m^{-2}.s^{-1}$) and 30 ºC may accumulate carotenoids which increase in response to abiotic stress, like high light intensity, UV-A radiation and temperature fluctuation. At 240 $\mu E.m^{-2}.s^{-1}$ the alga contains 57.5 ± 1.6 $mg.l^{-1}$ of total carotenoids after 20 days of growing, which does not significantly change by an irradiance of 1000 $\mu E.m^{-2}.s^{-1}$. Lutein (20 ± 0.5 $mg.l^{-1}$) and β-carotene (8.3 ± 0.2 $mg.l^{-1}$) production were particularly high in *C. acidophila*, while zeaxanthine (0.2 ± 0.1 $mg.l^{-1}$) was low. Enhanced production of these carotenoids was also observed in cultures illuminated with PAR light (160 $\mu E.m^{-2}.s^{-1}$) supplemented with moderate UV-A radiation (10 $\mu E.m^{-2}.s^{-1}$). Optimum algae growth takes place at 40 ºC, like the maximum amount of intracellular lutein and β-carotene. On the other hand, the presence of iron in the culture medium, in a range between 5-35 mM, significantly decreased the cell viability and the intracellular content of carotenoids, however cupper, at 1-5 mM, appears to increase the synthesis of β-carotene. The alga can growth under mixotrophic conditions, with glucose or acetate, 10 mM, as carbon source, but such conditions did not improved the intracellular content of carotenoids.

Keywords: extremophiles, *Chlamydomonas acidophila*, environmental stress, carotenoids, lutein, β-carotene

Introduction

Microalgal production of high added value products, particularly carotenoids for human health and nutrition, are gaining relevance during the last years, but its broad industrial application still requires studies to improve the methods in order to be economically competitive in the market [1-3]. Carotenoids accumulation by microalgae depends on both nutritional and environmental conditions and it can be stimulated by high luminical intensity [1], type of light [4] or limiting nutrients, particularly P or N [5,6]. Besides to be secondary pigments for PAR light absorption for photosynthetic activity, carotenoids protect the algae against oxidative stress, generally associated to the high light and/or UV-A radiation [4]. Particularly interesting is lutein because its presence in the human eye where it protects the macula against oxidation and, in general against the age-related macular deseases [7]. Due to these important benefits, lutein is recommended as dietary supplement for humans [8].

Extremophiles microorganisms, like *Dunaliella bardawill,* are frequently involved in carotenoids photoproduction and they rise new possibilities of biotechnological applications [9,10]. Massive accumulation of β-carotene in *D. salina* is triggered by environmental stresses such as intense irradiance, high salinity, nutrient starvation and extreme temperatures [11,12]. Ben-Amotz, [13] found in *D. bardawil* that decreasing the culture temperature from 30ºC to 10ºC caused a four-fold increase in the 9-cis/all-trans β-carotene ratio (0.5 to 2.0), with no significant changes in the other cell pigments

Our purpose is to study the possibilities of *Chlamydomonas acidophila* as carotene source, by changing the environmental conditions of growth.

Materials and Methods

Microorganism and culture conditions

Chlamydomonas acidophila was isolated from water of Tinto river (Huelva, Spain). The natural environment of the alga was pH 2.5 and high contamination by heavy metals, specially iron, magnesium and aluminium, as more as sulfate and nitrate. The alga was cleaned in Petri dishes with agar medium, at the indicated acidic pH, and from the agar medium to liquid medium. Unless otherwise indicated, cultures were grown at 30 ºC, bubbled with air containing 5 % (v/v) CO_2, as unique carbon source, and continuously illuminated with white fluorescent lamps (Philips TLD, 30 W, 160 $\mu Em^{-2}.s^{-1}$, at the surface of the flask). Under these standard conditions the generation time for *C. acidophila* was about 60 h [14]. The growth rate was determined by measuring the chlorophyll content in 1 ml of culture at different times. Biomass production was usually determined by the dry weight contained in 300 ml of alga culture, at the end of each experiment.

The irradiance was measured with a photoradiometer Delta OHM (mod. HD9021).

Analytical determinations

Pigments were measured spectrophotometrically using aliquots (1 ml) of the cultures which cells were spinned down for 10 min at 5000 rpm and the obtained pellet was treated with boiled water during 1 min. Then 4 ml of pure methanol was added and the resulted suspension shaked vigorously for 1 min and centrifuged during 10 min at 5000 rpm. Chlorophyll and total carotenoid concentrations were determined in the supernatant, using the equations proposed by Wellburn [15].

Carotenoids were separated and characterized by HPLC (Merck Hitachi, equipped with a UV-V detector) analysis as described by Young [16]. Pigments detection was performed at 450 nm and quantified using standards supply by DHI-Water and Environment (Denmark).

Statistic

Unless otherwise indicated, the presented data are the means of three independent experiments. The standard deviations use to be lower than 3 %.

Results and Discusion

Light intensity and nutritional conditions induce carotenogenesis in extremophiles, as an answer to the associated oxidative stress [1]. We study the best conditions to improve the quantity and quality of produced carotenoids by *C. acidophila*.

Effect of light on carotenoids accumulation by C. acidophila

Cultures of *C. acidophila* were irradiated with PAR light of 160, 240 and 1000 $\mu E.m^{-2}.s^{-1}$ and total intracellular chlorophyll and carotenoids were determined. Fig. 1 shows that high light intensity inhibits the alga growth, while an optimal carotenoids accumulation of 57.5 ± 1.6 $mg.l^{-1}$ was observed in the culture after 20 days of growing at 240 $\mu E.m^{-2}.s^{-1}$, which suppose 15 g/kg of dry weight. In all cases β-carotene biosynthesis is higher than in the control, thus indicating a probably stimulation of enzymes involved in carotenogenesis. Similarly, light induces stimulation of the phytoene synthase and phytoene desaturase in *Chlamydomonas reinhardtii* [17]. An obligate photoautotroph, *Spirulina platensis*, was also reported to disply increased

carotenoid levels under strong illumination [18] and in most cases, light causes a quantitative improvement in carotenoid content in microorganisms [1], however, the microalga *Phaeodactilum tricornutum* was reported to show a five-fold improvement in cellular carotenoid content in response to a decrease in light intensity [19].

In order to determine the type of carotenoid accumulated by *C. acidophila*, we analyze the above mencioned cultures and the obtained results are shown in Table 1. Lutein is present as high as 20.2 $mg.l^{-1}$ which suppose 34.7 % of total carotenoids, β-carotene (8.3 $mg.l^{-1}$), violaxanthin (3.2 $mg.l^{-1}$) and zeaxanthin (0.2 $mg.l^{-1}$). These carotenoids suppose a 55.6 % of the total carotenoids fraction, as estimated by HPLC. These data show that *C. acidophila* is very adequate for the biotechnological production of lutein (10.13 $g.kg^{-1}$ of dry weight) as compared with other microalgae previously studied, like *Muriellopsis* sp, 5.7 [20], *Dunaliella salina,* 5.5 [21] and *Chlorococcum*, 2.0 $g.kg^{-1}$ [22]. The increase in the PAR light up to 1000 $\mu E.m^{-2}.s^{-1}$ does not has any effect on lutein and/or β-carotene intracellular concentration in *C. acidophila*, which is different to other microalgae [4].

Carotenogenesis rate was stimulated when PAR light was supplemented with UV light [23], however UV radiation has been proved to have both positive or negative effects on the viability of the microalgal culture [24]. *C. acidophila* improves the viability when PAR light of 160 was supplemented with UV-A light of 10 $\mu E.m^{-2}.s^{-1}$. This effect paralells with the intracellular carotenoids accumulation (Fig. 2), as well as lutein and β-carotene (Fig. 3). UV lights higher than 10 $\mu E.m^{-2}.s^{-1}$ produces a significant inhibition of both alga growth and carotenoids accumulation (Fig. 2). UV radiation has been probed to have both positive and negative effect on the viability of the microalgae cultures [24]. *Dunaliella bardawill* accumulates carotenoids because the oxidative stress generated by UV radiation [4].

Effect of temperature on C. acidophila *growth and carotene accumulation*

Temperature is consider the main factor controlling growth rate in the commercially important microalgae *Dunaliella* sp. and the fresh water green alga *Haematococcus pluvialis*, and the parallel with carotene production [1]. We studied the viability of *C. acidophila* in the temperature range 25-50 ºC, and the best results either for growth and carotenoid production were obtained at 40 ºC (Fig. 4), while 50 ºC was letal for the alga. Growth-limiting conditions, such as pH value and increasing temperature were found to stimulate carotenogenesis in *Muriellopsis* sp. [25]. In addition temperature was found to be more effective than irradiance in changing the qualitative and quantitative carotenoid composition in several species of *Dunaliella salina* [26]. Production of lutein and β-carotene were also optima at 40 ºC in *C. acidophila* (Fig. 5).

Effect of nutritional conditions on carotene production by C. acidophila

The alga can be grown under mixotrophic conditions with glucose or acetate as carbon source, and the intracellular carotene content of alga was 13.3 and 11.2 g/kg, respectively, which did not improve the carotene production under phototrophic conditions (15 g/kg). Similar situation was observed with other potential carbon source for the alga, like acetate, sugars or amino acids (data not shown).

Effect of heavy metals on viability and carotene production by C. acidophila

Addition of ferric iron to the alga culture medium, between 5 – 35 mM final concentration, produces a strong inhibition of the algal growth and thus on the carotenoids production (data not shown). *Haematococcus pluvialis* improved

astaxanthin production when cultured in growth medium supplemented with ferrous ion, probably because the high amount of hydroxyl radical generated by the Fenton reaction [27]. On the other hand cupper addition up to 4 mM final concentration increases carotenoids production after 3 days of alga growth (Fig. 6). In this contest only cobalt, at low concentration was reported to stimulate carotenogenesis in the cyanobacyerium *Spirulina platensis* [1].

Acknowledgements

This work has been supported by the Ministerio de Educación y Cultura (Proyecto AGL2006-12741) and PAI (Plan Andaluz de Investigación, Junta de Andalucía, Grupo CVI 214).

References

[1] Bhosale P (2004) Environmental and cultural stimulants in the production of carotenoids from microorganisms. Appl. Microbiol. Biotechnol. 63: 351-361.

[2] Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. 65: 635-648.

[3] Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J. Biosc. Bioeng. 101; 87-96

[4] Janhke LS (1999) Massive carotenoid accumulation in *Dunaliella bardawil* induced by ultraviolet-A radiation. J Photochem Photobiol B 48: 68-74.

[5] Cerón MC, Sánchez A, Fernández JM, Molina E, García F (2005) Mixotrophic growth of the microalga *Phaeodactylum tricornutum*. Influence of diferent nitrogen and organic carbon source on productivity and biomass composition. Proc. Biochem. 40: 297-305.

[6] Tittel J, Bissinger V, Gaedke U, Kamjunke N (2005) Inorganic carbon limitation and mixotrophic growth in *Chlamydomonas* from an acidic mining lake. Protist 156: 63-75.

[7] Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA, Willett W (1994) Dietary

201 carotenoids, vitamins A, C and E, and advanced age-related macular degeneration. J.
202 Am. Med. Assoc. 272: 1413-1420.
203 [8] Bartlett H, Eperjesi F (2004) An ideal ocular nutritional supplement? Ophtal.
204 Physiol. Opt. 24: 339-349.
205 [9] Fujiwara S (2002) Extremophiles: developments of their special functions and
206 potential resources. J. Biosci. Bioeng. 94: 518-525.
207 [10] Podar M, Reysenbach AL (2006) New opportunities revealed by biotechnological
208 explorations of extremophiles. Curr Opp Biotechnol 17: 250-255.
209 [11] Ben-Amotz A, Avron M (1983) Accumulation of metabolites by halotolerant algae
210 and its industrial potential. Annu. Rev. Microbiol. 37: 95-119.
211 [12] Borowitzka MA, Borowitzka LJ, Kessly D (1990) Effects of salinity increase on
212 carotenoid accumulation in the green alga *Dunaliella salina*. J. Appl. Physiol. 2: 111-
213 119.
214 [13] Ben-Amotz A. (1996) Effect of low temperature on the stereoisomer composition
215 of β-carotene in the halotolerant alga *Dunaliella bardawil* (Chlorophyta). J. Phycol. 32:
216 272-275.
217 [14] Cuaresma M, Garbayo I, Vega JM, Vílchez C (2006) Growth and photosynthetic
218 utilization of inorganic carbon of the microalga *Chlamydomonas acidophila* isolated
219 from Tinto river. Enz. Microbial Technol. 40: 158-162.
220 [15] Wellburn AR (1994) The spectral detrmination of chlorophyll a and b, as well as
221 total carotenoids, using various solvents with spectrophotometers of different
222 resolutions. J Plant Physiol. 144: 307-313.
223 [16] Young A, Orset S, Tsavalos A. (1997) Methods for carotenoids analysis. In:
224 Pessarakli M ed.. Handbook of Photosynthesis. pp. 597-622. Marcel & Dekker. New
225 York.

226 [17] Bohne F, Linden H (2002) Regulation of carotenoid biosynthesis genes in response
227 to light in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta 1579: 26-34.
228 [18] Liu HI (1984) Effects of temperature and light intensity on growth rate,
229 physiological and biochemical characteristics of *Spirulina platensis*. Zhonghua Nongye
230 Yanjiu 33: 276-291.
231 [19] Sánchez Mirón A, Ceron García MC, García Camacho F, Molina Grima E, Chisti
232 Y (2002) Growth and biochemical characterization of microalgal biomasa produced in
233 bubble column and airlift photobioreactors: studies in fed-bach cultura. Enz. Microbial
234 Technol. 31: 1015-1023.
235 [20] Del Campo JA, Moreno J, Rodríguez H, Vargas MA, Rivas J and Guerrero MG
236 (2000) Carotenoid content of chlorophycean microalgae: factors determining lutein
237 accumulation in *Muriellopsis* sp. (Chlorophyta) J. Biotechnol. 76: 51-59.
238 [21] García-González M, Moreno J, Manzano JC, Florencio FJ, Guerrero MG (2005)
239 Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed
240 tubular photobioreactor. J. Biotechnol. 115: 81-90.
241 [22] Yuan J-P, Chen F, Liu X, Li X-Z (2002) Carotenoid composition in the green
242 microalga *Chlorococcum*. Food Chemistry 76: 319-325.
243 [23] Salguero A, León R, Mariotti A, De la Morena B, Vega JM, Vílchez C (2005) UV-
244 A mediated induction of carotenoid accumulation in *Dunaliella bardawil* with retention
245 of cell viability. Appl. Microbiol. Biotechnol. 66: 506-511.
246 [24] Horwitz BA (1994) Properties and transduction chains of the UV and blue light
247 photoreceptors. In: Kendrick KE, Kronenberg GHM (eds.). Photomorphogenesis in
248 plants. Kluwer, Drordencht. pp. 327-350.

[25] Del Campo JA, Rodríguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2001) Lutein production by *Muriellopsis* sp in an outdoor tubular photobioreactor. J. Biotechnol. 85: 289-295.

[26] Gómez PI, González MA (2005) The effect of temperature and irradiance on the growth and caratenogenic capacity of seven strains of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions. Biol. Res. 38: 151-162.

[27] Tjanjono AE, Hayama T, Terada Y, Nishio N, Nagai S (1994) Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. Biotechnol. Lett. 16: 133-138.

TABLES

Table 1

Influence of light intensity on the carotenoids accumulation by *C. acidophila*

Carotenoids (mg.l^{-1})	PAR Intensity ($\mu E.m^{-2}.s^{-1}$)		
	160	*240*	*1000*
Lutein	11.0 ± 0.2	20.2 ± 0.5	15.3 ± 0.2
β-carotene	6.0 ± 0.1	8.3 ± 0.2	4.9 ± 0.1
Violaxanthin	1.5 ± 0.01	3.2 ± 0.1	1.5 ± 0.01
Zeaxanthin	0.1 ± 0.01	0.2 ± 0.01	0.7 ± 0.01

Cultures growing autotrophically, under standard conditions and logarithmic phase, were illuminated with PAR light, at the indicated intensity. After 9 days of growing, the intracellular carotenoids were determined, using aliquots of the corresponding culture, as indicated in Methods section. Given values are mean ± S.D. from three independent experiments.

Figure captions

Figure 1. HPLC analysis of carotenoids from *C. acidophila* extracts. Peak assignment is as follows: (1) violaxanthin; (2) lutein; (3) zeaxanthin; (4) chlorophyll b; (5) chlorophyll a; (6) β-carotene.

Figure 1. Influence of PAR light intensity on cells viability and carotenoids accumulation by *C. acidophila*, under different PAR light intensity.

Standard autotrophic cultures were continuously illuminated with PAR light of 160 (◆); 240 (■); or 1000 (▲) $\mu E.m^{-2}.s^{-1}$. At the indicated times the intracellular content of total carotenoids (A) and chlorophyll (B) were determined in aliquots of the corresponding culture.

Figure 2. Influence of UV-A radiation on the cell viability and total carotenoids accumulation by *C. acidophila*.

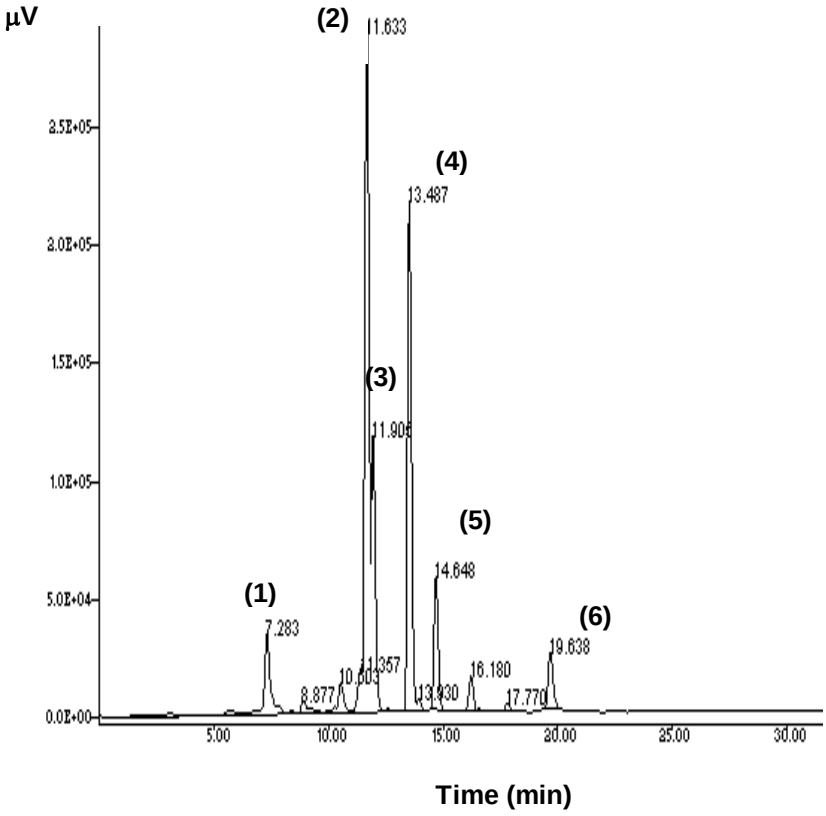
Standard autotrophic cultures were continuously illuminated with PAR light of 160 (◆) $\mu E.m^{-2}.s^{-1}$, supplemented with UV-A light of 5 (□), 10 (■), 25 (▲) or 40 (X) $\mu E.m^{-2}.s^{-1}$. At the indicated times the intracellular content of total carotenoids (A) and chlorophyll (B) were determined in aliquots of the corresponding culture.

Figure 3. Influence of UV-A radiation on lutein and β-carotene accumulation by *C. acidophila*.

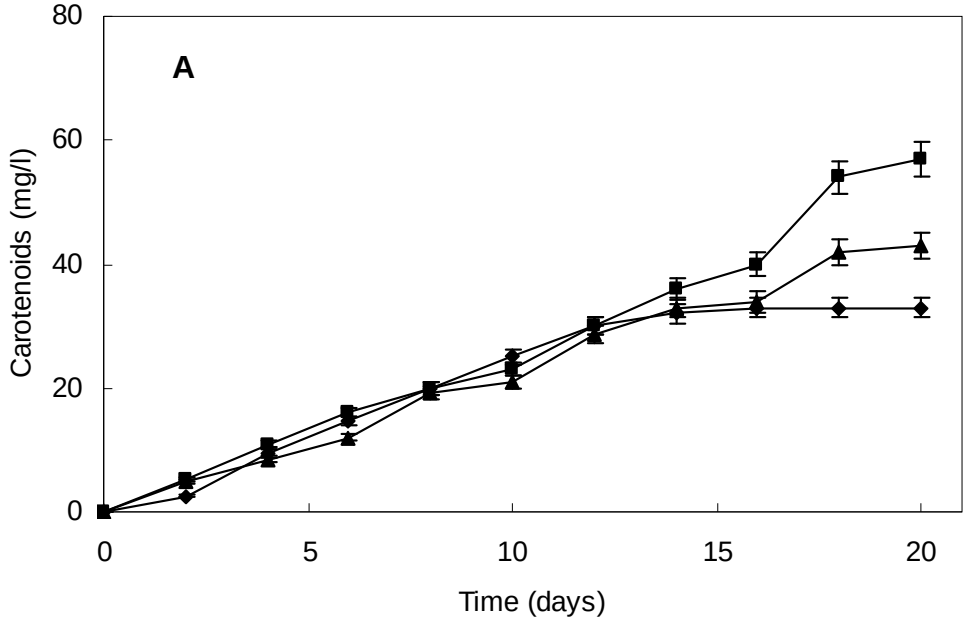
Standard autotrophic cultures were continuously illuminated with PAR light of 160 (◆) $\mu E.m^{-2}.s^{-1}$, supplemented with UV-A light of 10 (■), 25 (▲) or 40 (X) $\mu E.m^{-2}.s^{-1}$. At the

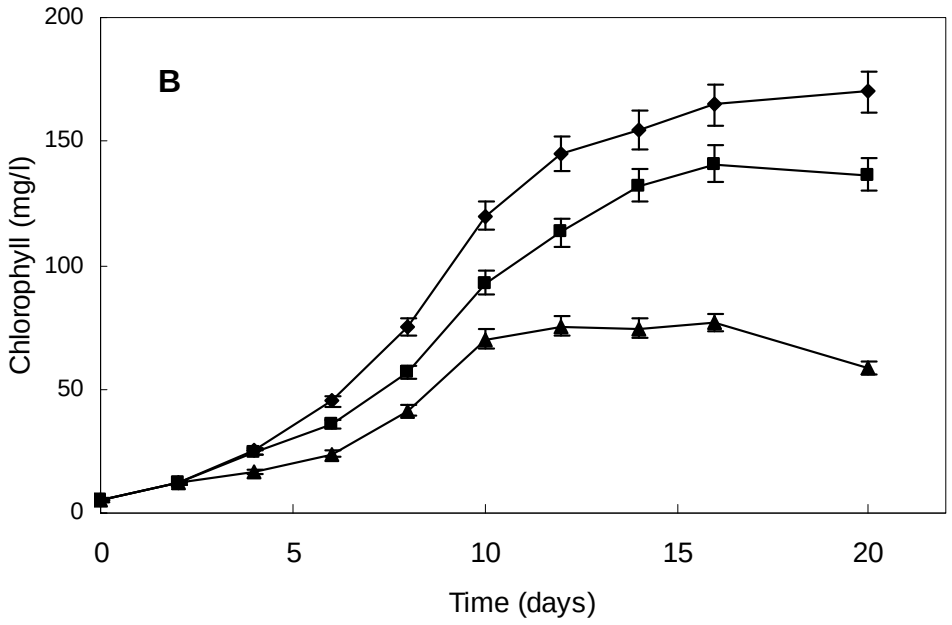
303 indicated times the intracellular content of β-carotene (A) and lutein (B) were
304 determined in aliquots of the corresponding culture.
305
306 **Figure 4. Influence of temperature on the cell viability and total carotenoids**
307 **accumulation by *C. acidophila*.**
308 Standard autotrophic cultures, at the beginning of the logarithmic phase of growth, were
309 continuously illuminated with PAR light of 160 $\mu E.m^{-2}.s^{-1}$, and grown at the following
310 temperatures: 25º C (◆); 30 ºC (■); 40 ºC (X) or 50º C (○). At the indicated times the
311 intracellular content of total carotenoids (A) and chlorophyll (B) were determined in
312 aliquots of the corresponding culture.
313
314 **Figure 5. Influence of temperature on lutein and β-carotene accumulation by *C.***
315 ***acidophila*.**
316 Standard autotrophic cultures, at the beginning of the logarithmic phase of growth, were
317 continuously illuminated with PAR light of 160 $\mu E.m^{-2}.s^{-1}$, and grown at the following
318 temperatures: 25º C (◆); 30 ºC (■); 35 ºC (▲) or 40º C (X). At the indicated times the
319 intracellular content of β-carotene (A) and lutein (B) were determined in aliquots of the
320 corresponding culture.
321
322 **Figure 6. Influence of different Cu^{2+} concentrations on lutein and β-carotene**
323 **content of *C. acidophila*.**
324 Standard autotrophic cultures, at the logarithmic phase of growth, were supplemented
325 with the indicated amounts of Cu^{2+}. After 72 h of growing the intracellular content of
326 lutein (□) and β-carotene (■) was determined by HPLC (A % of β-carotene and lutein
327 by ml of culture respect to standard culture content) and (B % of β-carotene and lutein

by number of cells respect to standard culture content) in aliquots of the corresponding culture.



334



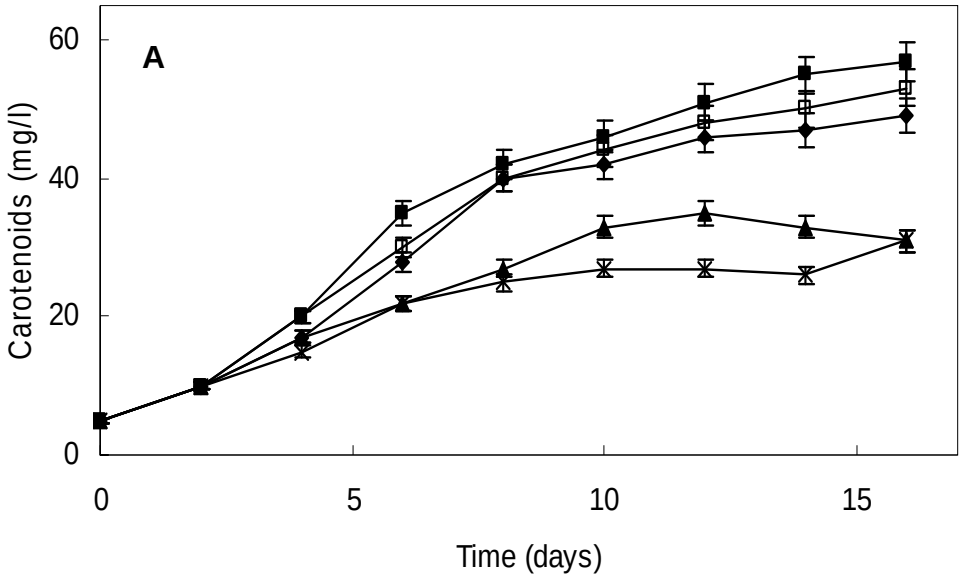


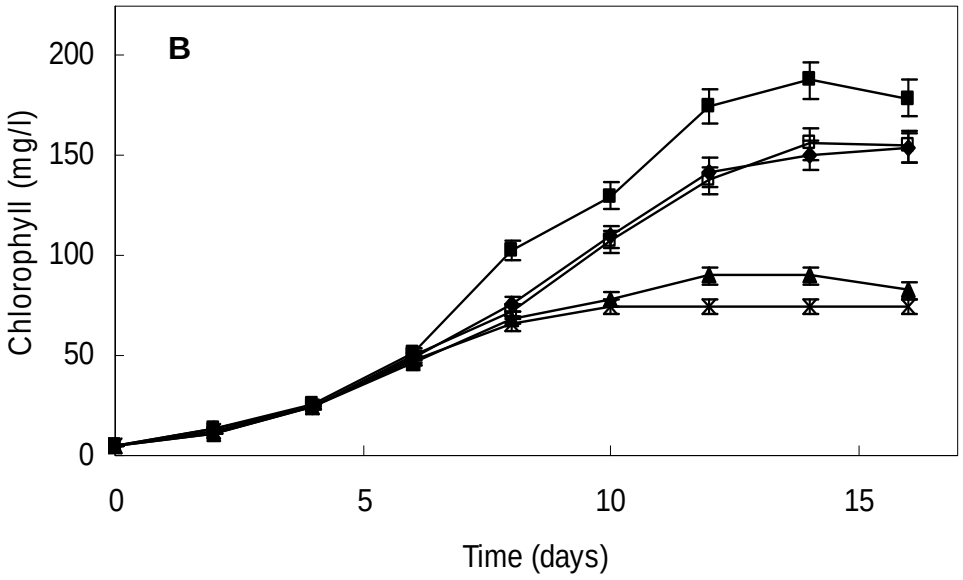
335 **Figure 1**

336

337

338



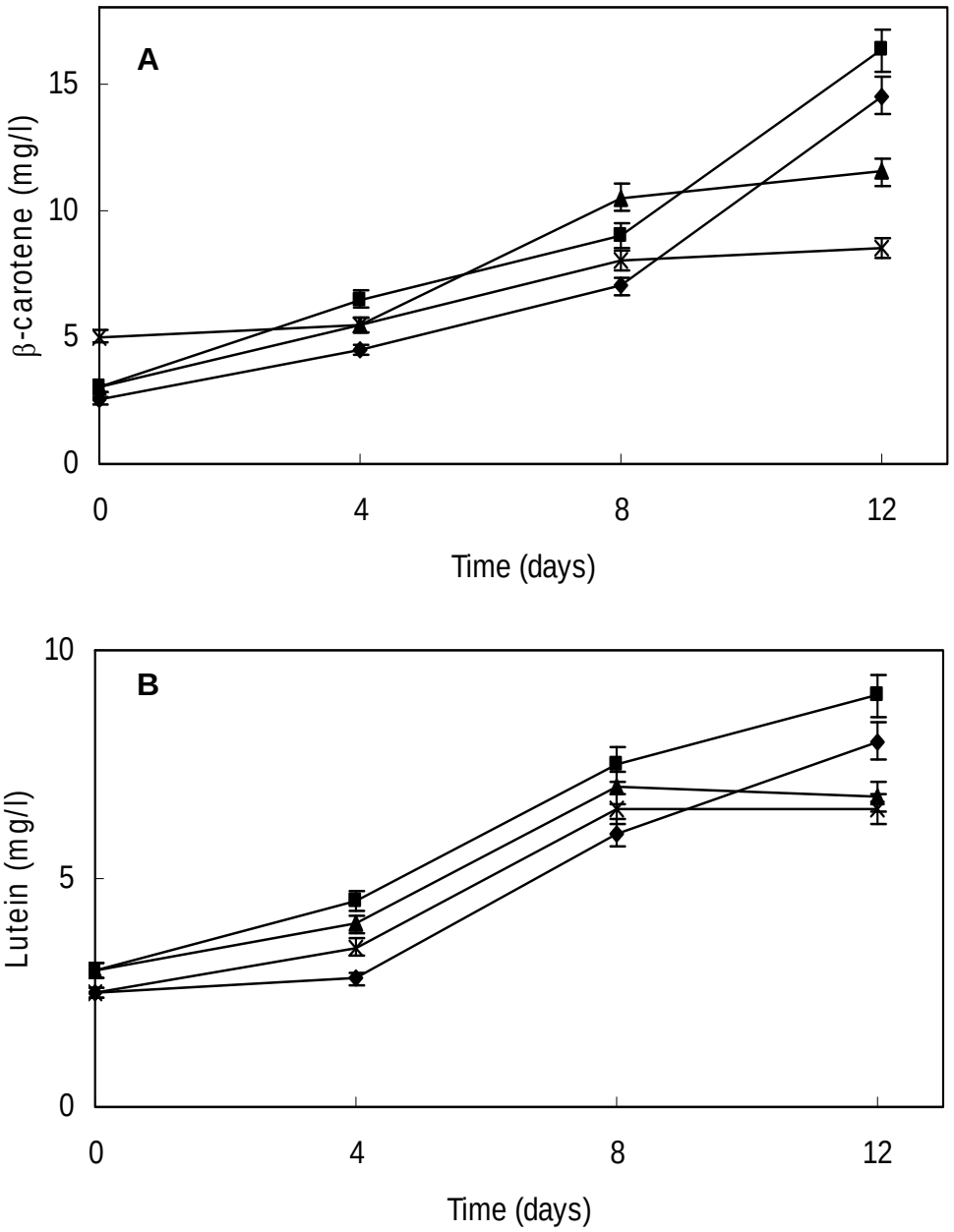


339 **Figure 2**

340

341

342

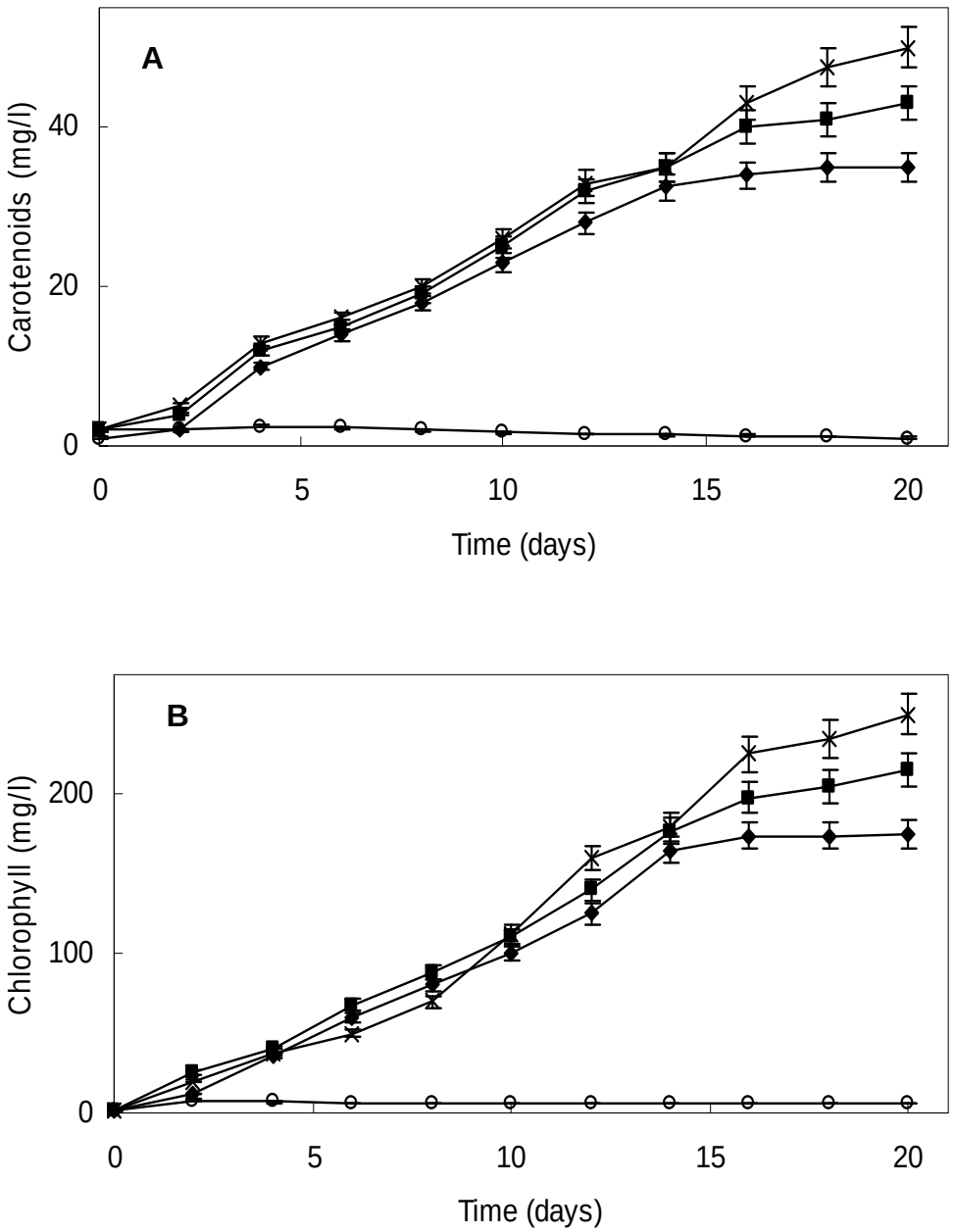


343 **Figure 3**

344

345

346

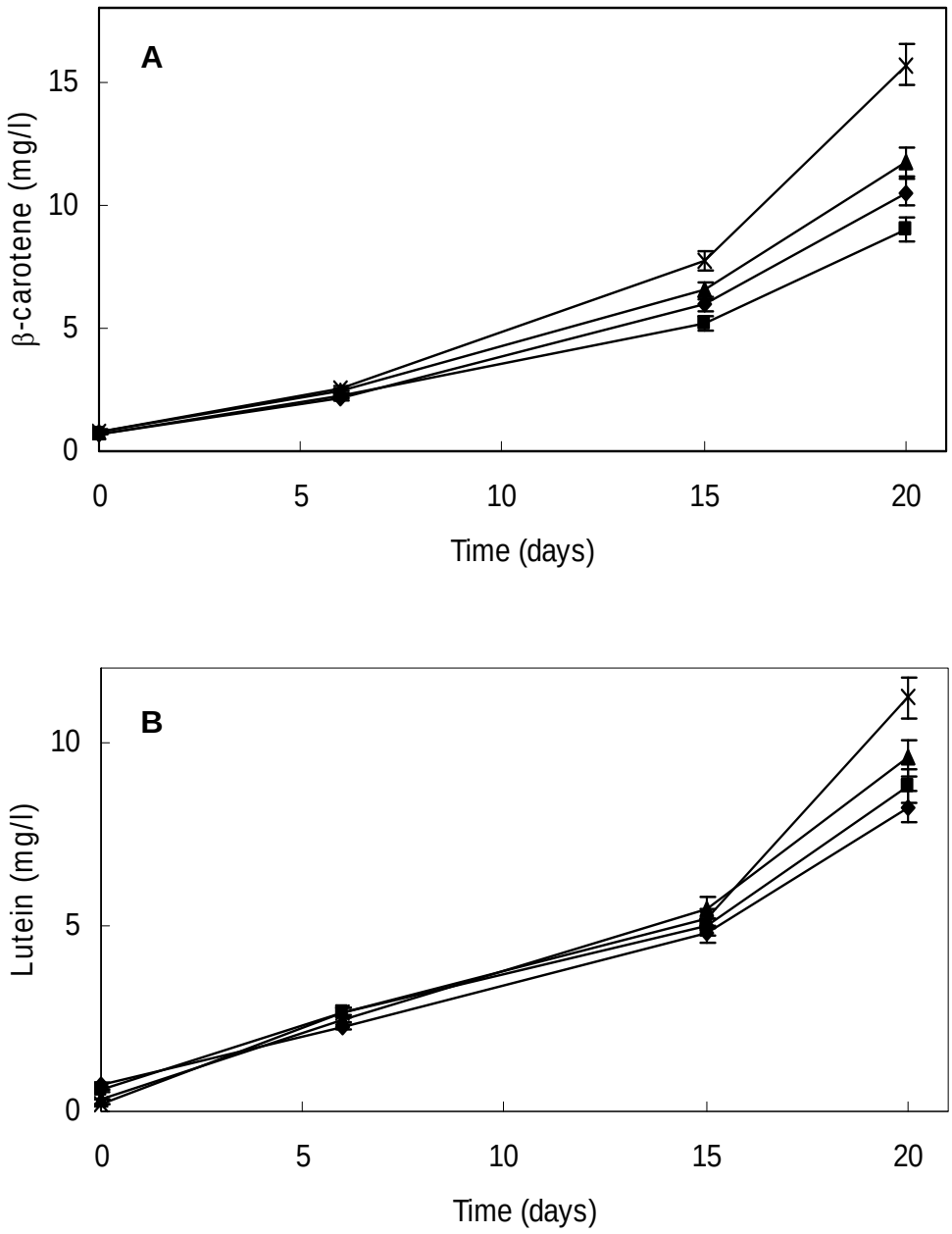


347 **Figure 4**

348

349

350

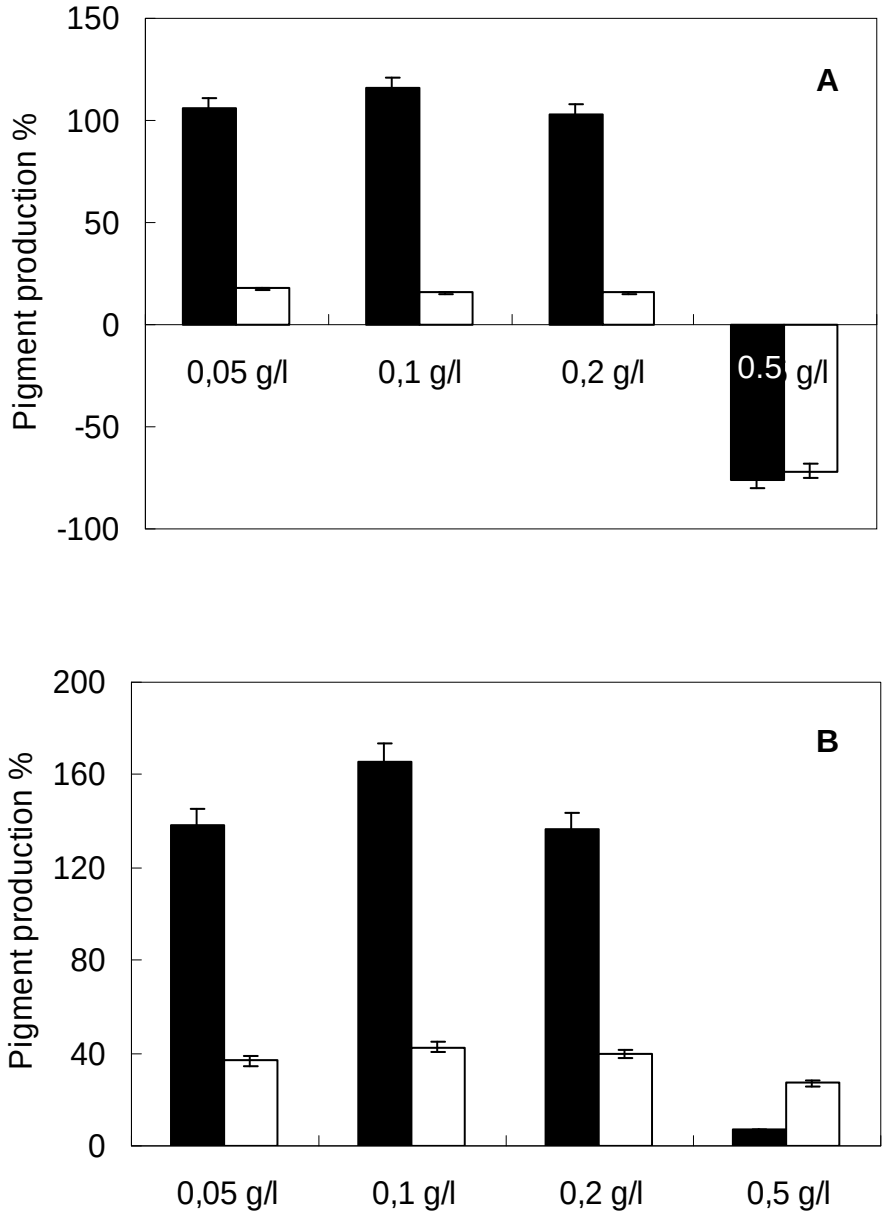


351 **Figure 5**

352

353

354



355

356 **Figure 6**

357