Gayana Bot. 66(1): 58-70, 2009 ISSN 0016-5301

GROWTH AND TOTAL CAROTENOID CONTENT IN FOUR CHILEAN STRAINS OF *HAEMATOCOCCUS PLUVIALIS* FLOTOW, UNDER LABORATORY CONDITIONS

CRECIMIENTO Y CONTENIDO TOTAL DE CAROTENOIDES EN CUATRO CEPAS CHILENAS DE HAEMATOCOCCUS PLUVIALIS *FLOTOW, BAJO CONDICIONES DE LABORATORIO*

Mariela A. González, Ana Silvia Cifuentes & Patricia I. Gómez

Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas. Universidad de Concepción, Casilla 160-C, Concepción. Chile.
mgonzale@udec.cl

ABSTRACT

Haematococcus pluvialis is a unicellular green microalga cultivated on a large scale as a natural source of astaxanthin, a ketocarotenoid of high commercial value due to its colorant and antioxidant properties. The aim of this study was to compare growth and total carotenoids among four strains of *H. pluvialis* isolated from different microhabitats in two geographical locations in Chile. Growth was carried out under autotrophic and mixotrophic conditions and under two photon flux densities (PFD) (20 and 85 µmol $m^{-2}s^{-1}$). Total carotenoid content was estimated on day 45. Most of the strains grew better under the autotrophic conditions; the highest exponential growth rates were exhibited at the high PFD in all the strains (ranging from 0.73 to 1.20 div day^{-1}) with the exception of CCM-UDEC 023 which showed the same exponential growth rate with either PFD (0.73 div day^{-1}). However, the cell density estimated on day 14 was higher at the low PFD in CCM-UDEC 022 and CCM-UDEC 023 (4.4 x 10^5 and 8.9 x 10^4 cells ml^{-1}, respectively). The mixotrophic condition slightly improved the growth rate of the strain CCM-UDEC 021 at the high PFD (from 1.04 div day^{-1} at autotrophic condition to 1.25 and 1.14 div day^{-1} at 2mM and 10mM sodium acetate, respectively). The algal dry weight estimated on day 14 was not significantly correlated with the cell density, but it was, to a certain extent, related to the proportion and size of the different cell types quantified on that day. The strain with the highest dry weight at the end of the experimental period and under all the assayed conditions was CCM-UDEC 023. It was also the strain that produced the highest total carotenoid content under both autotrophic (5.6 mg l^{-1} and 95.6 pg $cell^{-1}$ at the high PFD) and mixotrophic (with 2mM acetate) conditions (10.8 mg l^{-1} and 297 pg $cell^{-1}$ at the high PFD). A high intraspecific variability in the physiological attributes analyzed existed among the strains under study. Even though the mixotrophic condition assayed did not substantially improve the growth rate of any strain, it did improve the total carotenoid yield in the strain CCM-UDEC 023.

KEYWORDS: Batch culture, *Haematococcus pluvialis*, intraspecific physiological differences.

RESUMEN

Haematococcus pluvialis es una microalga unicelular verde cultivada masivamente como fuente natural de astaxantina, un cetocarotenoide de alto valor comercial debido a sus propiedades como colorante y antioxidante. El objetivo de este estudio fue comparar el crecimiento y el contenido de carotenoides totales en 4 cepas de *H. pluvialis* aisladas de diferentes microhabitats de dos regiones geográficas de Chile. El crecimiento se llevó a cabo en condiciones autotróficas y mixotróficas a dos densidades de flujo fotónico (DFF) (20 y 85 µmol $m^{-2}s^{-1}$). El contenido de carotenoides totales se estimó el día 45. La mayoría de las cepas crecieron mejor en condiciones autotróficas; las más altas tasas de crecimiento exponencial se presentaron a la mayor DFF en todas las cepas (fluctuó entre 0,73 y 1,20 div $día^{-1}$) a excepción de la cepa CCM-UDEC 023 la cual mostró la misma tasa de crecimiento exponencial a cualquier DFF (0,73 div $día^{-1}$). Sin embargo, la densidad celular estimada el día 14 fue mayor a la menor DFF en CCM-UDEC 022 y CCM-UDEC 023 (4,4 x 10^5 y 8,9 x 10^4 céls ml^{-1}, respectivamente). La condición mixotrófica mejoró levemente la tasa de crecimiento de la cepa CCM-UDEC 021 a la mayor DFF (de 1,04 div $día^{-1}$ en condición autotrófica a 1,25 y 1,14 div $día^{-1}$ a 2mM y 10mM de acetato de sodio, respectivamente). El peso seco algal estimado el día 14 no mostró correlación significativa

con la densidad celular, pero sí, en un cierto sentido, con la proporción y el tamaño de los diferentes tipos celulares a ese día. La cepa que presentó el mayor peso seco al final del periodo experimental y bajo todas las condiciones ensayadas fue CCM-UDEC 023. Esta fue también la cepa que produjo la cantidad de carotenoides totales más alta, tanto en condiciones autotróficas (5,6 mg l^{-1} y 95,6 pg $cél^{-1}$ a la mayor DFF) como mixotróficas (con 2mM acetato) (10,8 mg l^{-1} y 297 pg $cél^{-1}$ a la mayor DFF). Las cepas en estudio presentaron una alta variabilidad intraespecífica en los atributos fisiológicos analizados. Aún cuando las condiciones mixotróficas ensayadas no aumentaron sustancialmente las tasas de crecimiento de alguna de las cepas (CCM-UDEC 020, CCM-UDEC 022), esta condición sí mejoró la producción de carotenoides totales en la cepa CCM-UDEC 023.

Palabras clave: Cultivo estático, *Haematococcus pluvialis*, diferencias fisiológicas intraespecíficas.

INTRODUCTION

Haematococcus pluvialis Flotow is a green microalga which is considered one of the richest biological sources of astaxanthin (Boussiba *et al*. 1999, Borowitzka 1999). The species inhabits small natural or artificial ephemeral freshwater bodies which are formed by granite or other material such as marble, cement or plastic and periodically filled with rain or tap water. *H. pluvialis* has a complex life cycle (Elliott 1934) which includes flagellated cells, nonmotile cells ("palmella" stage) containing a variable number of cells and the typical resting cell or cyst. The size of all these cell types is extremely variable during the life cycle.

Although considerable information exists regarding the factors that promote growth (i.e. Kobayashi *et al*. 1991, 1993, Kakizono *et al*. 1992, Barbera *et al*. 1993, Zlotnik *et al*. 1993, Fan *et al*.1994, Lee & Ding 1995, Harker *et al*. 1996, Gong & Chen 1997, Triphathi *et al*. 1999, Hagen *et al*. 2001, Orosa *et al*. 2001, García-Malea *et al*. 2005, 2006, Jeon *et al*. 2006) and astaxanthin accumulation (review in Boussiba 2000, Domínguez-Bocanegra *et al*. 2004, García-Malea *et al*. 2005, Orosa *et al*. 2005) in *H. pluvialis*, most of the studies have looked at single strains mainly from culture collections (i.e., UTEX, NIES, CCAP, ATCC, SAG, NIVA) and examined under a wide range of culture conditions.

The aim of this study was to compare growth rates and total carotenoid content among four strains recently isolated from different microhabitats in the Bío-Bío and Los Lagos Regions of Chile, grown under two sodium acetate concentrations and two irradiances, in batch mode. The frequencies of the different cell types in the culture during growth and in the stationary growth phase were also compared. The results will show if physiological differences exist among the strains and if some of the mixotrophic conditions under low light improve the growth of any of the strains when compared to growth under the autotrophic conditions.

MATERIALS AND METHODS

Algal strains and culture conditions

The four strains of *H. pluvialis* studied ([1] CCM-UDEC 020, CCM-UDEC 021, CCM-UDEC 022 and CCM-UDEC 023) were isolated by single-cell isolation by micropipette and/or streaking cells across agar plates (Hoshaw & Rosowski 1973) from field samples obtained in the Bío-Bío and Los Lagos Regions of Chile. CCM-UDEC 020 was taken from a small, granitic bird bath in the backyard of a house in Puerto Varas (41°29'S; 73° 00'W). CCM-UDEC 021 and CCM-UDEC 022 were collected in the cemetery in the city of Concepción (36°51'S; 72°59'W): CCM-UDEC 021 was isolated from a reddish liquid contained in a metal flower pot and CCM-UDEC 022 was taken from a dry red cyst layer on the side of an ornamental marble pot. The strain CCM-UDEC 023 was taken in liquid state from a small water fountain in the campus of the Universidad de Concepción.

After isolation, unialgal cultures were maintained in tubes with Bristol's medium (Starr & Zeikus 1993), at a photon flux density (PFD) of 15-20 µmol m^{-2} s^{-1} (measured by a Model LI-250 Quantum/Radiometer/Photometer Light Meter manufactured by LI-COR Inc. U.S.A.) and at a temperature of 22 ± 1 ºC.

For the experimental trials, the strains were grown in Bristol's medium under autotrophic and mixotrophic conditions (Bristol's with addition of 2 and 10 mM of sodium acetate) in 500 ml flasks with

[1] CCM-UDEC = Colección de Cultivos de Microalgas de la Universidad de Concepción, Chile.

200 ml of culture medium, under two different continuous PFD: 20 and 85 µmol m^{-2} s^{-1} (supplied by cool fluorescent daylight lamps), at 22 ± 1 ºC, without aeration and agitated manually twice a day. Exponentially growing cells from stock cultures were used as inocula, and the initial cell density was of 1,000 cells ml^{-1}.

Growth and relative number of cell types

Cell growth was monitored over a 14-day period by cell counting using 1 ml Utermöhl chambers and a Zeiss inverted microscope. Growth rate k (divisions per day) was calculated using the formula: k= (3,322/ t_2- t_1) x log N_2/N_1 (t= time, N=number of cells. Subscripts denote values at different times) (Guillard 1973). A maximal growth rate, k_{max}, during the exponential phase of growth was calculated. The algal dry weight and the relative number (%) of motile cells and cysts were determined on day 14 as in Cifuentes *et al*. (2003).

Total carotenoid analysis

Total carotenoids and cell density were both estimated on day 45. The number of motile cells and cysts were also recorded on that day. Raw extracts of pigments were obtained by grinding the algal cell pellet, utilizing either a manual tissue homogenizer or a mortar with liquid nitrogen. Then, the pigments were extracted with 90% acetone and left overnight at 4 ºC in darkness. The extracts were centrifuged and the carotenoid content was calculated from the extracts´ absorbance at 480 nm (UV-VIS Spectrophotometer, Shimadzu, Tokyo), according to Strickland and Parsons (1972).

Statistical analysis

All of the experiments were carried out in triplicate. A three-way analysis of variance (ANOVA) with the strain, irradiance and sodium acetate concentration as independent variables was applied to cell biomass (number of cells ml^{-1} and cell dry weight) and total carotenoids. The Tukey test was used for multiple comparisons. Differences were considered to be significant at a probability of 5% (P= 0.05). Correlation analysis was also carried out between cell density and cell dry weight at day 14 using Pearson's correlation coefficient. Statistical analyses were performed with the SigmaStat software program, version 2.03.

RESULTS

Growth rates

Maximal growth rates fluctuated between 0.57 div day^{-1} and 1.25 div day^{-1}. The CCM-UDEC 021 and CCM-UDEC 022 strains exhibited the highest maximal growth rates of 1.25 and 1.14 div day^{-1} at the high PFD (85µmol $m^{-2}s^{-1}$), under mixotrophic (with 2mM acetate) and autotrophic conditions, respectively (Table I a and I b).

Under autotrophic conditions, the maximum growth rate of all strains was higher at the high light (85 µmol $m^{-2}s^{-1}$), except for CCM-UDEC 023, which exhibited growth during the first six days that was very similar at either PFD (Fig. 1d). The mixotrophic condition slightly increased the growth rate of the CCM-UDEC 021 strain at both acetate concentrations and light conditions and the CCM-UDEC 023 strain at 2mM acetate and low light. On the other hand, the CCM-UDEC 020 and CCM-UDEC 022 strains grew better in the autotrophic mode.

Cell density (N° cells ml^{-1}) and dry weight (mg L^{-1})

At day 14 the values of biomass measured both as cell density and as dry weight were significantly different among all the strains (P= 0.05). The acetate concentration significantly influenced both parameters, whereas PFD affected only growth estimates of cell density. The three-way ANOVA analysis indicated that the strain, PFD and acetate concentration interacted significantly. The interaction between strain identity and PFD was dependent on the acetate concentration in the medium. For both parameters of growth (cell density and dry weight), the strain identity was the most important factor in determining the magnitude of the variation reported on day 14 ([2] F = 53.282 and F= 51.936 for cell density and dry weight, respectively), while acetate was the most important factor determining the cell density on day 45 (F= 137.872).

At day 14, the highest cell density was attained by the CCM-UDEC 022 strain (4.4 x 10^5 cells ml^{-1}) under autotrophic conditions at the low PFD. The CCM-UDEC 023 strain, on the other hand, showed the highest cell density (1.2 to 1.3 x 10^5 cells ml^{-1}) under mixotrophic conditions (20mM acetate) at either PFD.

[2] F= F test statistic value.

The CCM-UDEC 023 strain exhibited higher cell dry weights than the other strains in most of the experimental conditions, with maximum values under both mixotrophic conditions assayed (383 and 305 mg l^{-1} at 2mM and 10mM acetate and under the low and the high PFD, respectively) (see Table I b and I c). On the contrary, CCM-UDEC 022 showed the highest cell dry weight under the autotrophic conditions (227 mg) at the high PFD. No significant correlation (Pearson's coefficient= -0.189; P>0.05) was found between the cell density and the respective cell dry weight when compared among all the strains.

In autotrophic conditions, cell densities estimated on day 45 indicated that most of the strains continued growing after day 14 (Table I), with maximal cell densities of 4.7×10^5 and 3.5×10^5 cells ml^{-1} in CCM-UDEC 023 and CCM-UDEC 021, respectively. In mixotrophic conditions, on the contrary, the cell densities achieved on day 14 increased slightly in some strains at 2mM acetate, but were barely maintained or, as occurred in most strains, decreased substantially by day 45 at 20mM acetate and at the high PFD (see Table I).

Relative number of motile cells and cysts in the culture

Although the data analyzed include the four strains, the results presented in Figure 2 summarize the results of the two strains presenting the greatest contrast: CCM-UDEC 022 and CCM-UDEC 023. At day 14, the cultures of most of the strains showed a predominance of motile cells, while CCM-UDEC 023 formed cysts very early under almost all experimental conditions (Fig. 2a). At day 45, motile cells had decreased in most of the strains, with a predominance of cysts principally under mixotrophic conditions. The clearest exception occurred in the CCM-UDEC 022 strain, in which the proportion of motile cells was mostly higher than 80% (except at 10mM acetate and low light) at day 45 (see Fig. 2b).

The statistical analysis applied to these results (three-way ANOVA) showed significant differences in the proportions of the two cell types among all the strains, both on day 14 and on day 45, and under all experimental conditions (P= 0.05). Even though strain, PFD and acetate concentration significantly influenced this proportion on day 14, strain was the most important factor determining this variation (F= 248.176 and F=237.626 for motile cells and cysts, respectively). At day 45, on the contrary, acetate concentration was the single factor significantly affecting the proportion of these cell types (F=185.264 and F=170.783 for motile cells and cysts, respectively).

Total carotenoid accumulation

When compared with the other strains, CCM-UDEC 023 was the most carotenogenic strain under all the conditions, except at 10 mM acetate. Under this condition, the amount of carotenoids accumulated by all the strains were very low. The maximum amount of total carotenoids per volume in CCM-UDEC 023 occurred under autotrophic conditions (3.3 and 5.6 mg l^{-1}) and at 2mM acetate (10.1 and 10.8 mg l^{-1}), at the two PFD, respectively (Table I).

The three-way ANOVA analysis revealed significant differences in the total amount of carotenoids per volume accumulated on day 45 among all the strains, under all the culture conditions (P= 0.05). The acetate concentration (F=602.804) and the PFD (F=203.404) significantly influenced this parameter. The three factors –strain, PFD and acetate concentration– interacted significantly, and the effect of the interaction between strain identity and PFD was dependent on the acetate concentration.

When carotenoid accumulation was expressed on a cell basis, the amount accumulated by CCM-UDEC 023 at 2mM acetate was much greater (217 pg $cell^{-1}$ and 296 pg $cell^{-1}$ at 20 and 85 µmol m^{-2} s^{-1}, respectively) than the amount accumulated by the other strains (8.5 to 62.6 pg $cell^{-1}$). The two-way ANOVA analysis, with PDF and strain as variable factors, revealed that strain was the main factor in determining carotenoid accumulation on a cell basis (F=68.459), and that there were significant differences (P=0.001) in the amount of carotenoid accumulation per cell between the strain CCM-UDEC 023 and the other three strains.

TABLE I. Summary of some physiological parameters of four strains of *Haematococcus pluvialis* (CCM-UDEC 020 from Puerto Varas, CCM-UDEC 021 and CCM-UDEC 022 from the Concepción Cemetery and CCM-UDEC 023 from the campus of the Universidad de Concepción), cultivated autotrophically (Table I a) and mixotrophically [2mM (Table I b) and 10 mM (Table I c) of sodium acetate] at two different PFD (20 and 85 µmol m^{-2} s^{-1}) and at an initial cell density of 10^3 cells ml^{-1}. The values are the mean of three replications and the variation (±) is the standard deviation; "t" denotes time and "d" denotes days.

TABLA I. Resumen de algunos parámetros fisiológicos de cuatro cepas de *Haematococcus pluvialis* (CCM-UDEC 020 de Puerto Varas, CCM-UDEC 021 y CCM-UDEC 022 del Cementerio de Concepción y CCM-UDEC 023 del Campo de la Universidad de Concepción), cultivadas autotrófica (Tabla I a) y mixotróficamente [2mM (Tabla I b) y 10 mM (Tabla I c) de acetato de sodio], ambas a dos DFF (20 and 85 µmol m^{-2} s^{-1}) y a una densidad celular inicial de 10^3 céls ml^{-1}. Los valores son el promedio de tres réplicas y la variación (±) corresponde a la desviación estándar; "t" y "d" indican tiempo y días.

(TABLE I a)

	Without acetate-20 µmol m^{-2} s^{-1}				Without acetate-85 µmol m^{-2} s^{-1}			
Parameters	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023
$N_{(t=14d)}$ cells ml^{-1}	$6.3x10^4$± $4.2x10^3$	$7.3x10^4$± $1.0x10^4$	$4.4x10^5$± $5.5x10^4$	$8.9x10^4$± $3.3x10^4$	$7.1x10^4$± $3.2x10^4$	$2.8x10^5$± $9.2x10^4$	$1.9x10^5$± $1.6x10^4$	$2.2x10^4$± $3.2x10^3$
k_{max} (div day^{-1})	0.75	0.98	0.97	0.73	0.85	1.04	1.20	0.73
Algal dry weight (mg l^{-1}) (t=14 d)	82 ± 4.36	194 ± 3.51	81± 43	214 ± 23	87 ± 23.79	73 ± 7.57	227 ± 46.36	143 ± 13.50
$N_{(t=45d)}$ cells ml^{-1}	$3.1x10^5$ ± $5.3x10^4$	$2.3x10^5$ ± $8.1x10^4$	$1.6x10^5$ ± $1.2x10^4$	$4.7x10^5$ ± $4.4x10^4$	$3.5x10^5$ ± $2.6x10^4$	$1.5x10^5$ ± $2.1x10^4$	$2.2x10^5$ ± $1.0x10^4$	$5.8 x 10^4$ ± $2.5x10^3$
Total Car (mg l^{-1}) (t=45 d)	0.98 ± 0.072	2.56 ± 0.55	0.93 ± 0.034	3.29 ± 0.31	1.14 ± 0.02	2.93 ± 0.17	3.25 ± 0.24	5.58 ± 0.36
Total Car (pg $cell^{-1}$) (t=45 d)	3.2 ± 0.5	12.6 ± 6.5	5.72 ± 0.54	7.0 ± 0.17	3.3 ± 0.21	19.3 ± 1.9	15.0 ± 1.4	96.0 ± 3.2

(Table I b)

	2 mM acetate-20 µmol m^{-2} s^{-1}				2 mM acetate-85 µmol m^{-2} s^{-1}			
Parameters	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023
$N_{(t=14d)}$ (cells ml^{-1})	$1.1x10^5 \pm 2.5x10^4$	$2.0x10^5 \pm 6x10^4$	$2.0x10^5 \pm 1.7x10^4$	$5.3x10^4 \pm 1.4x10^4$	$8.2x10^4 \pm 8.5x10^3$	$1.1x10^5 \pm 1.7x10^4$	$1.2x10^5 \pm 1.6x10^4$	$2.2x10^4 \pm 1.6x10^3$
k_{max} (div day^{-1})	0.68	1.02	0.92	0.90	0.73	1.25	0.79	0.74
Algal dry weight (mg l^{-1}) (t=14 d)	210 ± 20.88	239 ± 53.00	63 ± 33.12	383 ± 103.44	161 ±21.19	208 ± 24.24	115 ± 10.06	245 ± 25.10
$N_{(t=45d)}$ (cells ml-1)	$2.0x10^5 \pm 3.5x10^4$	$1.9x10^4 \pm 5.8x10^3$	$2.8x10^5 \pm 1.2x10^4$	$4.9x10^4 \pm 1.3x10^4$	$1.7x10^5 \pm 4.7x10^4$	$9.2x10^4 \pm 1.7x10^4$	$2.0x10^5 \pm 2.1x10^4$	$3.7x10^4 \pm 6.5x10^3$
Total Car (mg l^{-1}) (t=45d)	4.77± 0.67	4.2± 0.29	2.37± 0.20	10.10± 0.78	1.54± 0.78	5.52± 1.27	3.90± 0.29	10.86± 1.10
Total Car (pg $cell^{-1}$) (t =45d)	24.3 ± 7.3	24.7 ± 10.7	8.5 ± 1.1	217.0 ± 70	9.8 ± 5.7	62.6 ± 24.6	21.0 ± 3.7	297.0 ± 61

(TABLE I c)

	10 mM acetate-20 µmol m^{-2} s^{-1}				10 mM acetate-85 µmol m^{-2} s^{-1}			
Parameters	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023	CCM-UDEC 020	CCM-UDEC 021	CCM-UDEC 022	CCM-UDEC 023
$N_{(t=14d)}$ (cells ml^{-1})	$8.6x10^4 \pm 2.6x10^3$	$1.1x10^5 \pm 4.6x10^3$	$4.0x10^4 \pm 1.8x10^4$	$1.3x10^5 \pm 3.4x10^4$	$5.7x10^4 \pm 1.2x10^4$	$1.3x10^5 \pm 2.3x10^4$	$2.7x10^4 \pm 3.3x10^3$	$1.2x10^5 \pm 2.6x10^4$
k_{max} (div day^{-1})	0.62	1.02	0.57	0.74	0.68	1.14	0.72	0.73
Algal dry weight (mg l^{-1}) (t=14d)	136 ± 94.87	95 ± 16	43 ± 19.07	291 ± 26.35	194 ± 66.42	87 ± 12.16	35 ± 11.37	305 ± 71.84
$N_{(t=45d)}$ (cells ml^{-1})	$8.4x10^4 \pm 6.6x10^3$	$2.7x10^4 \pm 2.6x10^3$	$5.9x10^4 \pm 2.0x10^3$	$2.4x10^5 \pm 2.1x10^4$	$1.4x10^5 \pm 3.6x10^4$	$7.0x10^3 \pm 1.4x10^3$	$2.6x10^4 \pm 3.5x10^3$	$7.1 \times 10^4 \pm 1000$
Total Car (mg l^{-1}) (t=45d)	1.0 ± 0.14	0.11± 0.01	0.584 ± 0.01	0.71± 0.04	1.5 ± 0.23	0.06 ± 0.017	0.2 ± 0.026	0.946 ± 0.06
Total Car (pg $cell^{-1}$) (t=45d)	11.9 ± 0.75	4.3 ± 0.16	9.9 ± 0.6	2.9 ± 0.33	11.0 ±1.1	9.0 ± 4.2	7.9 ± 3.0	13.3 ± 0.7

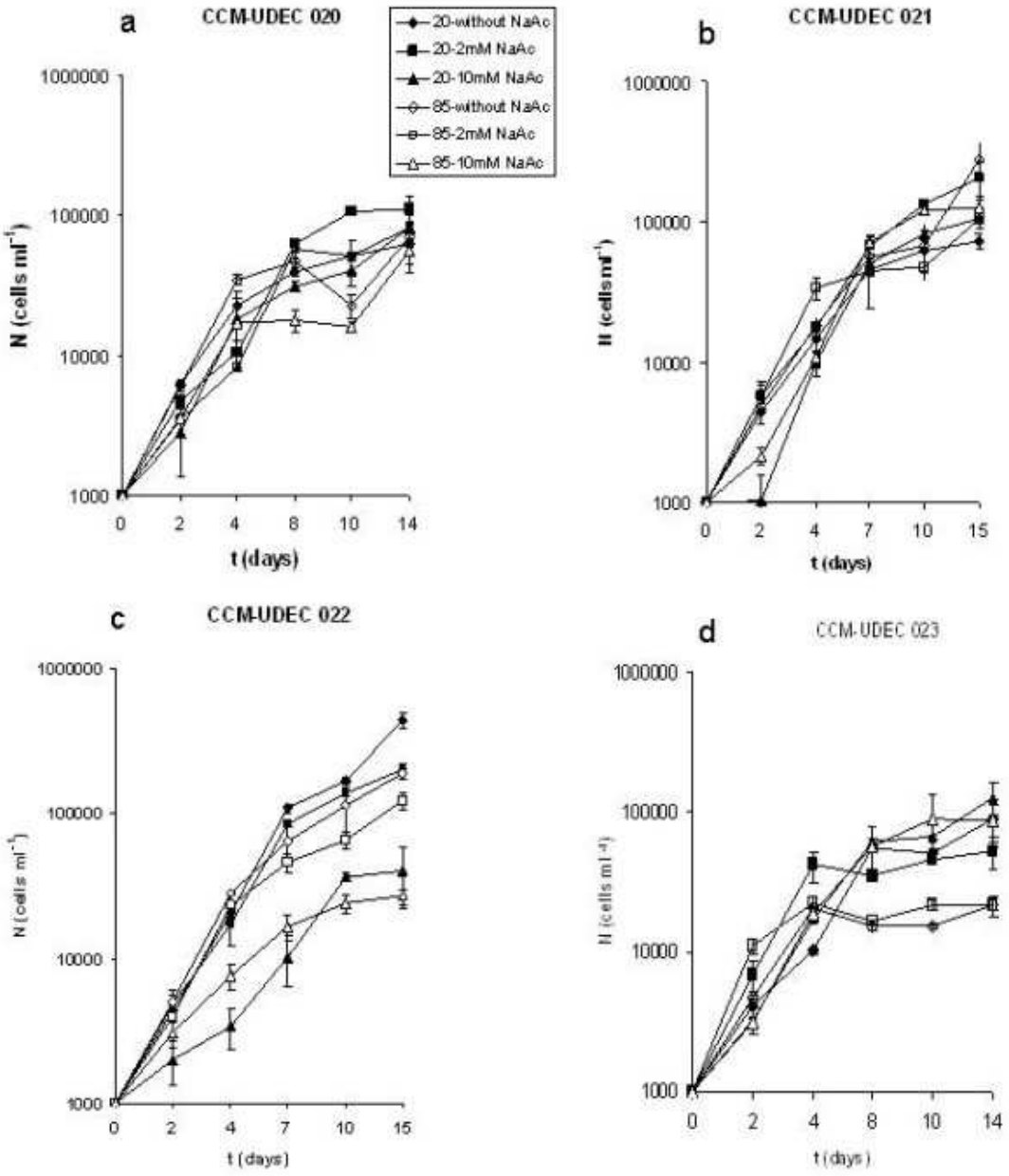

Figure 1. Comparison of growth (indicated by cell density) of four strains of *Haematococcus pluvialis* in Bristol´s medium under different concentrations of sodium acetate (NaAc) (2mM and 10mM) and photon flux densities (20 and 80 µmol $m^{-2}s^{-1}$). (a) CCM-UDEC 020 strain, (b) CCM-UDEC 021 strain, (c) CCM-UDEC 022 strain and (d) CCM-UDEC 023 strain.

Figura 1. Comparación del crecimiento (como densidad celular) de cuatro cepas de *Haematococcus pluvialis* en medio Bristol bajo diferentes condiciones de acetato de sodio (NaAc) (2mM y 10 mM) y de densidades de flujo fotónico (20 y 80 µmol $m^{-2}s^{-1}$). (a) cepa CCM-UDEC 020, (b) cepa CCM-UDEC 021, (c) cepa CCM-UDEC 022 y (d) cepa CCM-UDEC 023.

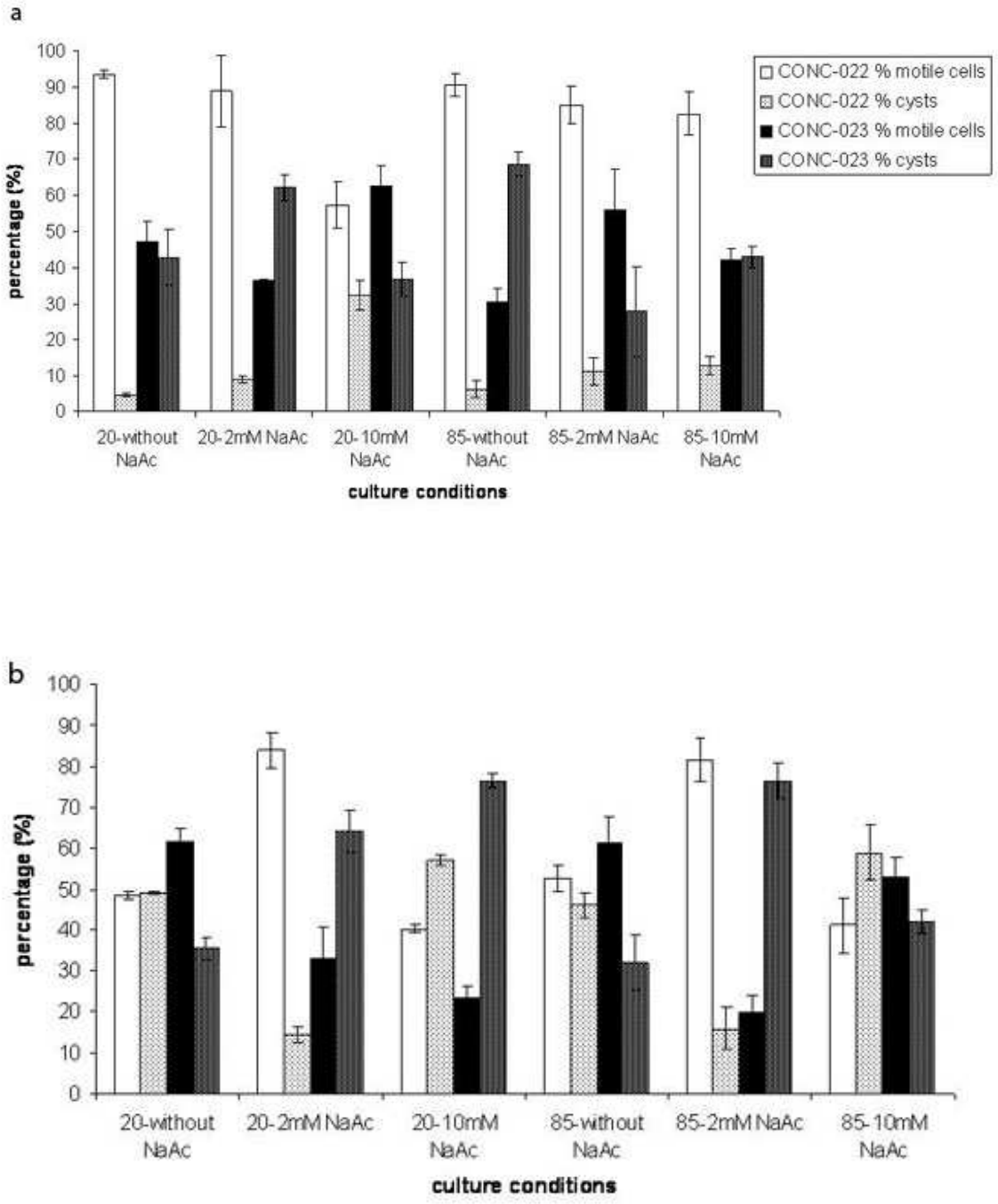

FIGURE 2. Relative number (%) of motile cells and cysts in the CCM-UDEC 022 and CCM-UDEC 023 strains, in cultures grown under different sodium acetate (NaAc) concentrations (2 mM and 10mM) and photon flux densities (20 and 80 µmol m^{-2} s^{-1}). (a) on day 14 and (b) on day 45.

FIGURA 2. Número relativo (%) de células móviles y cistos en las cepas CCM-UDEC 022 y CCM-UDEC 023, en cultivos que crecieron con diferentes concentraciones de acetato de sodio (NaAc) (2 mM y 10mM) y a dos densidades de flujo fotónico (20 y 80 µmol m^{-2} s^{-1}). (a) el día 14 y (b) el día 45.

DISCUSSION

The variability of the responses obtained from the different strains of *H. pluvialis* cultured under the same conditions are evidence of the difficulty of establishing effective experimental conditions for promoting growth and total carotenoid accumulation which are valid for all the strains. Growth patterns differed even among those strains taken from the same location (CCM-UDEC 021 and CCM-UDEC 022), similar to findings for strains of *Dunaliella salina* Teod. (Gómez & González 2005). Like *Dunaliella salina*, *H. pluvialis* inhabits temporary isolated pools with extreme environmental conditions that vary within a wide range. The strains must adapt to these conditions with great celerity.

In contrast to *Dunaliella salina*, *Haematococcus pluvialis* exhibits a more complex life cycle. A predominance of green motile cells in the culture is a sign that the alga is still growing (i.e., the number of cells is increasing). A higher proportion of cysts than motile cells in the cultures is on the other hand an indicator of aging and/or unfavorable environmental conditions for the cells. Although the formation of cysts has been related to the cessation of cell growth (Borowitzka *et al*. 1991, Boussiba & Vonshak 1991, Kobayashi *et al*. 1991), in old cultures (i.e., two months old or more) it has been found that the cysts can grow either by increasing their cell size or by internal cell division, giving rise to new non-motile cells.

The strains that exhibited the most varied results were CCM-UDEC 022 and CCM-UDEC 023. In fact, CCM-UDEC 022 showed the highest division rates (0.97 div $day^{-1)}$ and the highest cell densities (4.4 x 10^5 cells ml^{-1}) under autotrophic conditions on day 14; both parameters were much higher than the normal range found in the literature for the species (0.5-0.7 div day^{-1} and 1.5-2.5 x 10^5 cell ml^{-1}, Hagen *et al*. 1993, 2001, Lee & Ding 1994, 1995, Harker *et al*. 1996, Orosa *et al*. 2001). The CCM-UDEC 023 strain, in contrast, exhibited lower division rates than CCM-UDEC 022 (ca. 0.73 div day^{-1}) under all conditions, reaching a maximum cell density of 1.2 to 1.3 x 10^5 cells ml^{-1} under mixotrophic conditions (10 mM acetate) and independent of the irradiance.

The acetate concentrations assayed in the present study (2 and 10 mM), in relation to improving growth in motile cells, could be considered low in comparison to the concentrations utilized by other authors for other strains [i.e., 12 mM in ATCC 30453 (Barbera *et al*. 1993), 20 mM in UTEX 16 (Gong & Chen 1997) and 30.5 mM in CCAP 34/7 (Orosa *et al*. 2001)] with the same objective. Preliminary growth assays carried out in our laboratory with a wide range of acetate concentrations (2-60 mM) showed that acetate concentrations higher than 10 mM did not support good growth of motile cells in the four studied strains (unpublished data); on the contrary, they triggered the settling of cells and a slow growth of nonmotile cells ("palmella stage"). These findings agree with the studies done by Orosa *et al*. (2005) and Jeon *et al*. (2006) which found that the effect of acetate on growth depends on the acetate concentration. Even though the growth rates estimated in the CCM-UDEC 023 and CCM-UDEC 021 strains were a little higher when they were grown with 2 mM acetate than under autotrophic conditions (Table I), the biomass estimated as cell dry weight showed a marked increment in CCM-UDEC 023 under both mixotrophic conditions. In general, the strains attained higher cell dry weight in media with low concentrations of acetate (2mM) irrespective of the PFD, with the exception of CCM-UDEC 022. This latter strain achieved its maximum growth rate and growth (cell number and dry weight) under autotrophic conditions.

When the relative abundance of the different cell types during growth in the culture was examined, a significant difference also appeared between CCM-UDEC 022 and CCM-UDEC 023. While CCM-UDEC 022 exhibited a predominance of motile cells (81-93%) on day 14 under most of the experimental conditions, the CCM-UDEC 023 strain showed a similar proportion of cysts and motile cells (Fig. 2a). According to these results, the CCM-UDEC strain 023 fits well with other *H. pluvialis* strains studied in batch system; that is, an exponential growth period of 5 to 8 days with predominance of motile cells, followed by a linear phase with decrease of motile cells and an increment of "palmella" stages and cysts (Lee & Ding 1994, Moya *et al*. 1997). Many studies on *H. pluvialis* have been focused on trying to find culture conditions that lengthen the active growing phase (first stage, according to Kobayashi *et al*. 1991, Olaizola 2000), in order to improve the astaxanthin yield per dry weight at the end of the inductive phase. The CCM-UDEC 022 strain fulfilled the first requirement, i.e. an exponential growth period of 14 days, with predominance of motile cells (ca. 93%), reaching a high cell density (4.4 x 10^5 cells ml^{-1}) when growing under autotrophic conditions at low light. Moreover, on day 45, this strain still

exhibited a higher proportion of motile cells than cysts in 2mM acetate, both at the low and high PFD (ca. 83% and 81%, respectively) (Fig. 2b).This is a promising and uncommon characteristic of old *H. pluvialis* cultures (Borowitzka *et al.* 1991, Kobayashi *et al*. 1992, Lee & Ding 1994), which makes this strain very interesting from a biotechnological point of view, as long as this high cell density is coupled with high astaxanthin production once stress mechanisms are applied.

Many researchers utilize cell density to estimate growth in *H. pluvialis* (Borowitzka *et al*. 1991, Kobayashi *et al*. 1991, Lee & Ding 1994, Fábregas *et al*. 1998, 2000, 2001, Domínguez-Bocanegra *et al*. 2004, Orosa *et al*. 2005). In this study, growth was estimated as cell density and as dry weight, but correlations between both parameters were not found under any experimental condition on day 14. This is probably due to the complex life cycle of the species, where cell division and cell enlargement simultaneously occur in the culture. For example, on day 14, a dry weight of 305 mg l^{-1} in CCM-UDEC 023 (at 10mM acetate and at the high PFD) was in fact more closely related to the high proportion of cysts (43%) than to the culture cell density (1.2 x 10^5 cells ml^{-1}), since the same cell density was exhibited by the CCM-UDEC 022 strain (at 2mM acetate and high PFD), with a dry weight of 115 mgl^{-1} and a low proportion of cysts (6%) (Table I and Fig. 2a). Cell density is a good estimator of biomass when motile cells are predominant in the culture, but when batch growth experiments are being planned, care should be taken to choose the adequate parameter for estimating and comparing cell growth among strains.

The levels of carotenoids (mg l^{-1}) quantified on day 45 also showed considerable inter-strain variation. While in most strains the total amount of carotenoids accumulated was influenced significantly by both PFD and acetate, in CCM-UDEC 021 this parameter was only affected by the acetate concentration.

The amount of carotenoids on a per-cell basis, estimated in almost all the strains and culture conditions, may be considered low, if the full culturing period is taken into account (45 days). The only exception occurred in CCM-UDEC 023, where levels of carotenoids of 200-300 pg $cell^{-1}$ were registered. Thus, strain and growth condition can result in as much as a 30-fold difference in carotenoid/cell at 45 days. In addition, these values are in line with those reported by Harker *et al*. (1996) and Triphathi *et al*. (1999). The former found that the greatest increase in the pigment content of the cells occurred after day 40 and accumulated at a constant rate until day 90 and preferably in nonmotile cells.

Most of the large-scale cultures of *H. pluvialis* around the world are carried out under autotrophic growth conditions (Olaizola & Huntley 2003). However, finding strains that can achieve satisfactory growth and carotenoid accumulation in a reliable mixotrophic system would improve the outlook for commercial cultivation at higher latitudes, where integral of the natural radiation is low. The CCM-UDEC 023 strain seems to fulfill these requirements but needs additional study.

The physiological variability found among the four strains of *H. pluvialis* studied have provided a strong motivation for continuing to seek new strains from the field, based on the conviction that the simple selection of strains from nature would be the easiest way to produce genetic improvement in this species.

ACKNOWLEDGMENTS

This study was supported by a grant to M.A. González from Dirección de Investigación, Universidad de Concepción (DIUC N° 202.111.3.0-1.3).

REFERENCES

Barbera, E., X. Tomas, M.J. Moya, A. Ibáñez & M.B. Molins. 1993. Significance Tests in the Study of the Specific Growth Rate of *Haematococcus lacustris*: Influence of carbon Source and Light Intensity. Journal of Fermentation and Biotechnology 76: 403-405.

Borowitzka, M.A., J.M. Huisman & A.Osborn. 1991. Culture of astaxanthin –producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. Journal of Applied Phycology 3: 95-304.

Borowitzka, M.A. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of Biotechnology 70: 313-321.

Boussiba, S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiology Plantarum 108: 111-117.

Boussiba, S. & A. Vonshak. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiology 32: 1077-1082.

Boussiba, S., B. Wang, J.P. Yuan, A. Zarka & F. Chen. 1999. Changes in pigment profile in the green alga

Haematococcus pluvialis exposed to environmental stresses. Biotechnology Letters 21: 601-604.

Cifuentes, A.S., M.A. González, S. Vargas, M. Hoeneisen & N. González. 2003. Optimization of biomasa, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions. Biological Research 36: 343-357.

Domínguez-Bocanegra, A.R., I.G. Legarreta, F.M. Jeronimo & A.T. Campocosio. 2004. Influence of environmental and nutritional factors in the production of astaxanthin from *Haematococcus pluvialis*. Bioresource Technology 92: 209-214.

Elliott, A.M. 1934. Morphology and life history of *Haematococcus pluvialis*. Archiv für Protistenkunden 82: 250-272.

Fábregas. J., A. Domínguez, D. García-Alvarez, T. Lamela & A.Otero. 1998. Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*. Biotechnology Letters 20: 623-626.

Fábregas, J., A. Domínguez, M. Regueiro, A. Maseda & A. Otero. 2000. Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. Applied Microbiology and Biotechnology 53: 530-535.

Fábregas, J., A. Otero, A. Maseda & A. Domínguez. 2001. Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis*. Journal of Biotechnology 89: 65-71.

Fan, L., A. Vonshak & S. Boussiba. 1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). Journal of Phycology 30: 829-833.

García-Malea, M.C., C. Brindley, E. Del Río, F.G. Acién, J. M. Fernández & E. Molina. 2005. Modeling of growth and accumulation of carotenoids in *Haematococcus pluvialis* as a function of irradiance and nutrients suply. Biochemical Engineering Journal 26: 107-114.

García-Malea, M.C., F.G. Acién, J.M. Fernández, M.C. Cerón & E. Molina. 2006. Continuous production of green cells of *Haematococcus pluvialis*: Modeling of the irradiance effect. Enzyme and Microbial Technology 38: 981-989.

Gómez, P.I. & M.A. González. 2005. The effect of temperature and irradiance on the growth and carotenogenic capacity of seven strains of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions. Biological Research 38(2-3): 151-162.

Gong, X. & F. Chen. 1997. Optimization of culture medium for growth of *Haematococcus pluvialis*. Journal of Applied Phycology 9: 437-444.

Guillard, R.R.L. 1973. Division rates. In: J.R. Stein (ed.), Handbook of Phycological Methods. Culture Methods and Growth Measurements, pp. 289-311. London, Cambridge University Press.

Hagen, C., W. Braune & F.Greulich. 1993. Functional aspects of secondary carotenoids in *Haematococcus lacustris* [Girod] Rostafinski (Volvocales). IV. Protection from photodynamic damage. Journal of Photochemical and Photobiology 20: 153-160.

Hagen, C., K. Grunewald, M. Xylander & E. Rothe. 2001. Effect of cultivation parameters on growth and pigment byosynthesis in flagellated cells of *Haematococcus pluvialis*. Journal of Applied Phycology 13: 79-87.

Harker, M., A.J. Tsavalos & A.J.Young. 1996. Factors responsible for astaxanthin formation in the chlorophyte *Haematococcus pluvialis*. Bioresources Technology 5: 207-214.

Hoshaw, R.W. & J.R. Rosowski. 1973. Methods for microscopic algae. In: J. Stein (ed.), Handbook of Phycological Methods. Culture Methods and Growth Measurements, pp. 53-68. Cambridge University Press.

Jeon, Y.Ch., Ch.W. Cho & Y.S.Yun. 2006. Combined effects of Light intensity and acetate concentration on the growth of unicellular microalga *Haematococcus pluvialis*. Enzyme and Microbial Technology 39: 490-495.

Kakizono, T., M. Kobayashi & S. Nagai. 1992. Effect of C/N on encysment accompanied with astaxanthin formation in a green alga *Haematococcus pluvialis*. Journal of Fermentation and Bioengineering 74: 403-405.

Kobayashi, M., T. Kakizono & S. Nagai. 1991. Astaxanthin production by a Green Alga, *Haematococcus pluviaeis* Accompanied with Morphological Changes in Acetate Media. Journal of Fermentation and Bioengineering 71: 335-339.

Kobayashi, M., T. Kakizono, N. Nishio & S. Nagai. 1992. Effects of light intensity, light quality and illumination cycle on astaxanthin formation in a green alga *Haematococcus pluvialis*. Journal of Fermentation and Bioengineering 74: 61-63.

Kobayashi, M., T. Kakizono & S. Nagai. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Applied Environmental Microbiology 59: 867-873.

Lee, Y.K. & S.Y. Ding. 1994. Cell cycle and accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). Journal of Phycology 30: 445-449.

Lee, Y.K. & S.Y. Ding. 1995. Effect of dissolved oxygen partial pressure on the accumulation of astaxanthin in chemostat cultures of *Haematococcus lacustris* (Chlorophyta). Journal of Phycology 31: 922-924.

Moya, M.J., M.L. Sánchez-Guardamino, A. Vilavella & E. Barbera. 1997. Growth of *Haematococcus lacustris*: A contribution to kinetic modelling. Journal of Chemical Technology and Biotechnology 68: 303-309.

Olaizola, M. 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25000-liter outdoor photobioreactors. Journal of Applied Phycology 12: 499-506.

Olaizola, M. & M. E. Huntley 2003. Recent advances in commercial production of astaxanthin from

microalgae. In: M. Fingerman & R. Nagabhushanam (eds.), Biomaterials and Bioprocessing, Science Publishers. 143-164.

Orosa, M., D. Franqueira, A. Cid & J. Abalde. 2001. Carotenoid accumulation in *Haematococcus pluvialis* in mixotrophic growth. Biotechnology Letters 23: 373-378.

Orosa, M., D. Franqueira, A. Cid & J. Abalde. 2005. Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*. Bioresource Technology 96: 373-378.

Starr, R.C. & J.A. Zeikus. 1993. UTEX-The culture collection of algae at the University of Texas at Austin. Journal of Phycology 29: 1-106.

Strickland, J.D.H. & T.R. Parsons. 1972. A manual of seawater analysis. Bulletin of Fisheries Research Bd Can 125: 1-310.

Triphathi, U., R. Sarada, R.S. Ramachandra & G.A. Ravishankar . 1999. Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. Bioresource Technology 68: 197-199.

Zlotnik, I., A. Sukenik & Z. Dubinsky. 1993. Physiological and photosynthetic changes during the formation of red aplanospore in the chlorophyte *Haematococcus pluvialis*. Journal of Phycology 29: 463-469.

Recibido: 18.12.08
Aceptado: 20.03.09