

# Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*

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## Abstract

The green microalga *Haematococcus pluvialis* was cultured with different concentrations of NaNO<sub>3</sub> to determine the effect on cell growth and astaxanthin accumulation. The optimum nitrate concentration to obtain astaxanthin and to avoid the cessation of cell division was 0.15 g/l NaNO<sub>3</sub>. The ratio chlorophyll *a*/total carotenoids proved a good physiological indicator of nitrogen deficiency in the cell. The effect of different carbon sources, malonate and acetate, on astaxanthin accumulation was also studied; up to 13 times more carotenoids per cell were accumulated in cultures with malonate than in cultures without this compound.

The pigment analysis was performed by a new low toxicity HPLC method capable of separating chlorophylls *a* and *b*, carotenes and xanthophylls in a short-period of time,

using low volumes of solvents and with an economical price. With this method even echinenone was separated, which had been unsuccessful by any other method.

### **Keywords**

Astaxanthin; Canthaxanthin; Echinenone; *Haematococcus pluvialis*; Secondary carotenoids; Pigments; HPLC analysis

## **1. INTRODUCTION**

The fresh-water green unicellular alga *Haematococcus pluvialis* (Chlorophyceae) has been exhaustively studied due to its ability to accumulate the orange-red pigment astaxanthin (3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione) and other related carotenoids and their esters (Grung et al., 1992; Yuan and Chen, 1998). These carotenoids are the main source of the red or yellow colour of the fins, skin and flesh of the wild rainbow trout as well as other kinds of salmonid fish (Marusich and Bauerfeind, 1981).

Animals cannot synthesise astaxanthin *de novo* therefore it must be added in their diets to obtain the desired flesh coloration of trout and salmon for better consumer acceptance. In the last few years the microalga *H. pluvialis* has been considered as a possible natural source of this pigment (Sommer et al., 1991).

Astaxanthin accumulation by this microalga is related to the formation of the palmella and aplanospore stages of the life cycle of the microalga (Elliot, 1934), usually induced by stress conditions.

Different studies have been carried out on the nutrient and culture condition requirements of *Haematococcus*. Massive accumulation of astaxanthin is obtained under nitrogen starvation, high light intensity and with agents which prevent cell division without impairing the ability of the alga to assimilate carbon ( Boussiba and Vonshak, 1991; Harker et al., 1996; Kobayashi et al., 1997; Fábregas et al., 1998). However, the main problem of nitrogen starvation is the growth rate reduction. The effect of nitrate concentration on *Haematococcus* growth rate and carotenoid production throughout the culture was assayed in order to determine the optimum conditions for mass culture and astaxanthin production.

Previous studies on the nutrition of *H. pluvialis* have shown that acetate appears to be an important carbon source, enhancing both growth and carotenogenesis (Borowitzka et al., 1991; Kobayashi et al., 1993). However, there is too little information about the effect of acetate concentration on accumulation of secondary carotenoids in *Haematococcus*.

In the present study, carotenoid formation was studied under different acetate concentrations and also using another carbon source (malonate), in order to find the optimal concentrations for mass culture and maximum production of astaxanthin and other carotenoids of biotechnological interest.

Due to the great interest in knowing the carotenoid composition during culture and in the different stages of the life cycle of *H. pluvialis*, a new high performance liquid chromatography (HPLC) system was developed for the separation and identification of the pigments in a single chromatographic run.

## 2. METHODS

*H. pluvialis* (strain 34/7) was obtained from the CCAP (FBA Ambleside Cumbria, United Kingdom). Microalgal cells were cultured in modified Bold's Basal Medium supplemented with ALGAL-1 trace elements solution (Fabregas et al., 1984). To determine the suitable nitrate concentration to obtain the best growth and ketocarotenoids production a range of nitrate concentrations was tested: 0, 0.15, 0.25, 0.5, 0.75 and 1 g/l NaNO<sub>3</sub>. For subsequent experiments, a NaNO<sub>3</sub> concentration of 0.15 g/l was chosen. To test the effect of acetate concentration on cell growth and pigment synthesis, sodium acetate was added in the culture medium at concentrations of 0%, 0.25%, 0.5%, 1% and 2% (w/v), adjusting the pH to 7.0. In the other carbon source experiment, sodium acetate was replaced with sodium malonate at the same concentrations and culture conditions.

Cultures were carried out in aerated mini-reactors containing 400 ml of medium and maintained at  $18 \pm 1$  °C and  $68.25 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , with a dark:light cycle of 12:12 h. All cultures were carried out in triplicate.

Algal densities were determined by daily counting triplicate samples in a haematocytometer.

For pigment spectrophotometric analysis, 10 ml samples were centrifuged at 3000g for 15 min. Cell pigments were extracted with dimethyl sulfoxide (DMSO), preheated to 55 °C and vortexed for 30 s (Sedmak et al., 1990). The extracts were centrifuged again and chlorophylls *a* and *b* and total carotenoids were spectrophotometrically determined by recording the absorbance at 665, 649 and 480 nm, respectively, and using the equations of Wellburn (1994).

Carotenoids from the different experiments were qualitatively and quantitatively analysed. The pigment extracts in acetone were separated on a Hewlett Packard HPLC equipped with a photodiode array detector. A reversed-phase 250 × 4 mm Hypersil C<sub>18</sub>(5 µm) column (Hewlett Packard) was used.

The elution gradient was run as follows with eluent A (water), eluent B (methanol), eluent C (acetone): 0 min 9% A, 76% B, 15% C; 9 min 5% A, 45% B, 50% C; 15 min 4% A, 38% B, 58% C; 17 min 3% A, 27% B, 70% C; 22 min 3% A, 27% B, 70% C; 25 min 100% C; 26 min 100% C.

The flow rate was 1 ml min<sup>-1</sup>. The detection wavelengths for integration were 444 and 476 nm.

β-carotene (Sigma) and astaxanthin, canthaxanthin and echinenone (F. Hoffman. La Roche Ltd.) were used as standards to calculate the concentrations of the other carotenoids.

Data are given as mean values ± standard error of the means.

### 3. RESULTS

#### 3.1. Effect of nitrate concentration

*Haematococcus* cultures with different NaNO<sub>3</sub> concentrations showed no differences in growth until the eleventh day, when the cultures with 0.15 g/l NaNO<sub>3</sub> had a decrease in growth rate. Cultures under nitrogen-free conditions did not grow (Fig. 1).

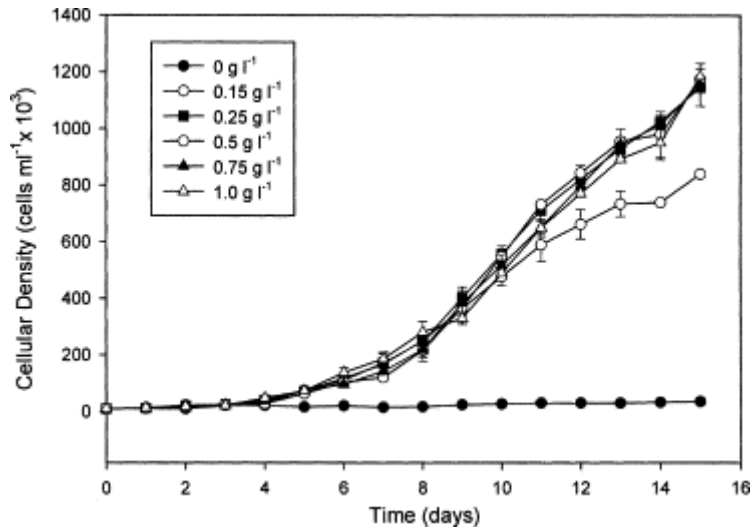


Fig. 1. Growth of *H. pluvialis* in cultures with different nitrate concentrations.

The ratio between chlorophyll *a* and total carotenoids was about 4 when nitrate was present in the culture medium. However, this ratio dropped quickly under nitrate deficiency (Fig. 2). In nitrogen-free cultures this drop was observed on the first day of culture and in cultures with 0.15 and 0.25 g/l NaNO<sub>3</sub>, the drop occurred after the ninth and eleventh day, respectively. The decrease of this relation showed a nitrogen deficiency in the cultures before growth had stopped.

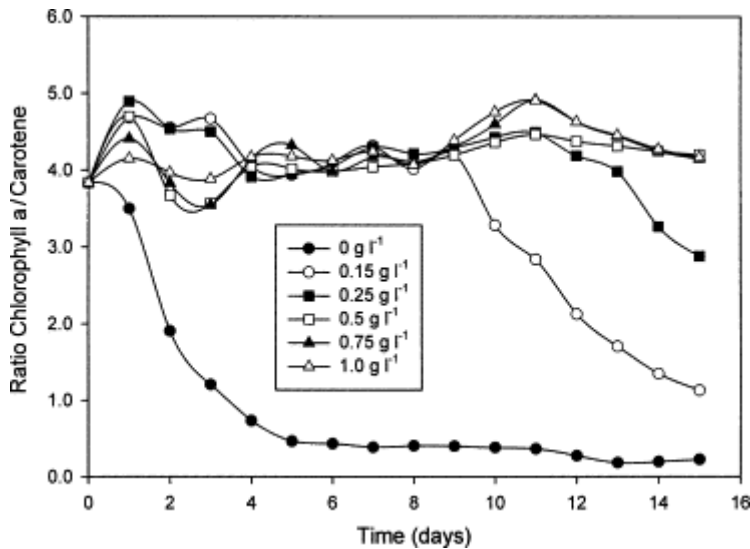


Fig. 2. Ratio chlorophyll *a*/total carotenoids in cultures of *H. pluvialis* with different concentrations of NaNO<sub>3</sub>.

While NaNO<sub>3</sub> remained available for the cultures, chlorophylls and total carotenoid concentrations remained at constant levels until there was N-deficiency; beyond this point, a rapid chlorophyll decline and carotenoid increase occurred throughout the further development of these cultures. Therefore, the total carotenoid

content of cells reached a maximum value in cultures under nitrogen deficiency while the chlorophylls reached a minimum value.

The HPLC analysis at the last day of culture (Table 1) showed that the ketocarotenoids were only detected in cultures with 0, 0.15 and 0.25 g/l NaNO<sub>3</sub>, corresponding to the cultures with a chlorophyll *a*/total carotenoids ratio below 4 ( Fig. 2). The secondary carotenoids were mainly synthesised as astaxanthin esters, which amount was higher than 80% of the total carotenoids (Table 1). Canthaxanthin and echinenone were also detected but at lower concentrations. Lutein, the most abundant carotenoid in green cells of *H. pluvialis* was drastically reduced in red cultures under conditions of nitrogen deficiency.

The HPLC method used resolves, in a short time one-step separation, a wide variety of carotenoids, including the separation of echinenone from the astaxanthin esters peaks which could not be obtained with other methods.

Table 1. Quantitative carotenoid composition (in pg cell<sup>-1</sup>), obtained by HPLC analysis, of *H. pluvialis* cells at the stationary phase in cultures with different concentrations of nitrate (g l<sup>-1</sup>), acetate and malonate (% w/v)

	Astaxanthin free	Astaxanthin ester	Cantaxanthin	Echinenone	β-Carotene	Other carotene
<i>Nitrate</i>						
0	0.40 ± 0.00	24.11 ± 0.38	0.62 ± 0.04	0.50 ± 0.01	0.53 ± 0.00	3.08 ± 0.07
0.15	0.11 ± 0.00	5.41 ± 0.46	0.14 ± 0.00	0.13 ± 0.00	0.65 ± 0.18	3.30 ± 0.04
0.25	0.19 ± 0.00	0.40 ± 0.01	0.07 ± 0.00	0.19 ± 0.00	0.69 ± 0.01	4.92 ± 0.04
0.5	n.d.	n.d.	n.d.	n.d.	1.16 ± 0.00	5.28 ± 0.00
0.75	n.d.	n.d.	n.d.	n.d.	1.03 ± 0.00	5.48 ± 0.00
1	n.d.	n.d.	n.d.	n.d.	1.06 ± 0.01	5.09 ± 0.01
<i>Acetate</i>						
0	0.38 ± 0.00	1.48 ± 0.00	0.20 ± 0.00	0.25 ± 0.00	2.25 ± 0.01	10.88 ± 0.01
0.25	0.33 ± 0.00	17.59 ± 0.73	0.24 ± 0.00	0.64 ± 0.02	1.52 ± 0.00	3.35 ± 0.91
0.5	0.43 ± 0.01	28.92 ± 0.00	0.36 ± 0.00	1.19 ± 0.02	2.34 ± 0.14	2.96 ± 0.09
1	0.30 ± 0.01	30.61 ± 0.03	0.27 ± 0.00	0.82 ± 0.00	1.78 ± 0.01	1.55 ± 0.01
2	0.80 ± 0.01	48.50 ± 0.00	0.31 ± 0.00	0.90 ± 0.01	2.57 ± 0.02	2.46 ± 0.00
<i>Malonate</i>						
0	0.24 ± 0.00	0.59 ± 0.00	0.08 ± 0.00	0.10 ± 0.00	0.90 ± 0.00	4.65 ± 0.00
0.25	0.16 ± 0.00	8.62 ± 0.11	0.12 ± 0.00	0.25 ± 0.02	0.80 ± 0.00	2.02 ± 0.17
0.5	0.21 ± 0.00	9.99 ± 0.23	0.13 ± 0.00	0.32 ± 0.01	0.93 ± 0.01	1.74 ± 0.35
1	0.13 ± 0.00	11.55 ± 0.01	0.12 ± 0.00	0.31 ± 0.00	0.71 ± 0.00	0.76 ± 0.01
2	1.31 ± 0.02	73.16 ± 0.51	0.52 ± 0.06	1.00 ± 0.04	3.18 ± 0.41	4.85 ± 0.26

When the ratio chlorophyll *a*/total carotenoids was about 4, corresponding to cultures with 0.5, 0.75 and 1 g/l NaNO<sub>3</sub> (Fig. 2), the pigment profile showed the typical pattern of major carotenoids of green algae,  $\beta$ -carotene, lutein, violaxanthin and neoxanthin.

### 3.2. Acetate effect

Growth rates of *H. pluvialis* were enhanced by the addition of 0.25% (w/v) acetate with respect to control cultures without this compound, but a concentration of acetate higher than 0.5% caused growth inhibition ( Table 2). With supplementation of acetate to the cultures, *Haematococcus* was induced to form cyst cells, which was closely associated with a concomitant increase in the astaxanthin content per cell ( Fig. 3).

Table 2. Growth rates of *H. pluvialis* cultured with different concentrations of nitrate, acetate and malonate

Nitrate (g l <sup>-1</sup> )	$\mu$ (d <sup>-1</sup> )	Acetate (%)	$\mu$ (d <sup>-1</sup> )	Malonate (%)	$\mu$ (d <sup>-1</sup> )
0	0.12 $\pm$ 0.020	0	0.51 $\pm$ 0.046	0	0.44 $\pm$ 0.013
0.15	0.45 $\pm$ 0.005	0.25	0.64 $\pm$ 0.008	0.25	0.56 $\pm$ 0.008
0.25	0.47 $\pm$ 0.002	0.5	0.49 $\pm$ 0.010	0.5	0.43 $\pm$ 0.030
0.5	0.48 $\pm$ 0.001	1	0.28 $\pm$ 0.018	1	0.08 $\pm$ 0.015
0.75	0.50 $\pm$ 0.010	2	-0.03 $\pm$ 0.022	2	-0.07 $\pm$ 0.055
1	0.46 $\pm$ 0.037				

In control cultures, there were few variations in the cellular concentration of total carotenoids throughout the culture, but with increasing amounts of acetate there was an increase in the accumulation of total carotenoids. (Fig. 3). The addition of acetate to the medium had a strong effect on the cellular accumulation of carotenoids at the end of the stationary phase (Fig. 4). Acetate also affected the amount of chlorophyll present. Maximum chlorophyll content per cell was detected in cultures without acetate in the medium (Fig. 4).

Maximum total carotenoid content per volume of culture was obtained in cultures with low acetate concentrations (0.25%, 0.5% and 1% (w/v)) (Fig. 5). An increase in acetate concentration in the medium produced lower cell and carotenoid yield because growth was inhibited. Total carotenoid concentration (mg l<sup>-1</sup>) was almost double in cultures with 0.25% and 0.5% (w/v) acetate compared with controls. Acetate enhanced the accumulation of total cellular carotenoids, with values up to three times higher than

in autotrophic control cultures. However, the major accumulation occurred in cultures with 2% acetate, which presented a strong growth inhibition accompanied by cell encystment.

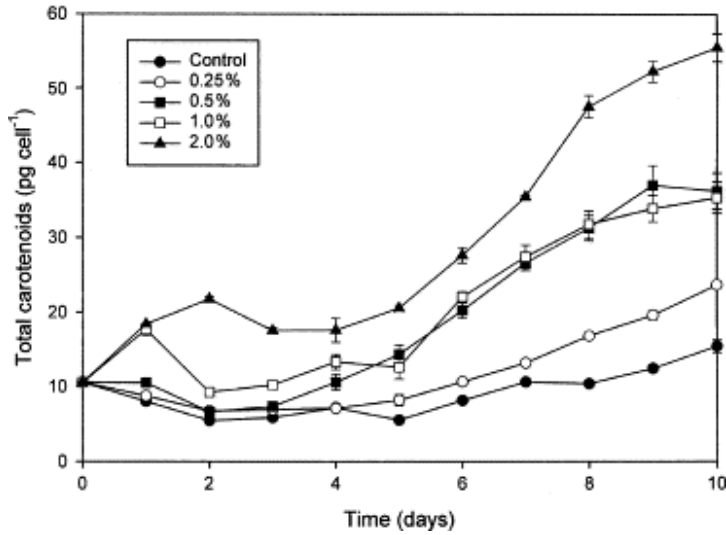


Fig. 3. Total cellular carotenoids in cultures of *H. pluvialis* with different concentrations of acetate.

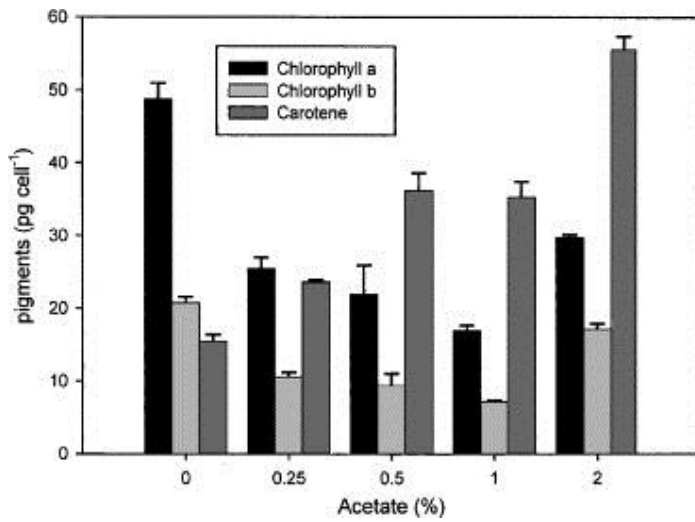


Fig. 4. Pigment content at the stationary phase in cells of *H. pluvialis* cultured with different concentrations of acetate.

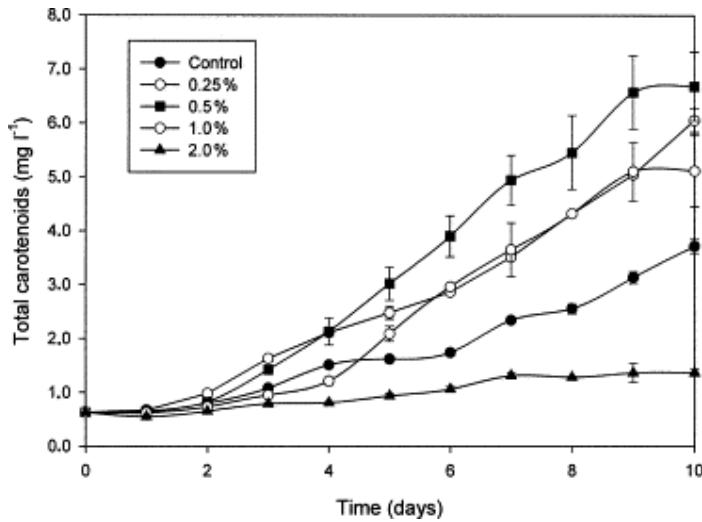


Fig. 5. Total carotenoids (expressed as mg l<sup>-1</sup>) in cultures of *H. pluvialis* cultured with different concentrations of acetate.



The HPLC analysis showed that acetate mainly induced the accumulation of astaxanthin esters (almost 90% of total carotenoids), as well as a strong primary carotenoids reduction (Table 2); the lutein amount per cell was more than five times lower in cultures with 2% acetate than in control cultures.

### 3.3. Malonate effect

Over the range of concentrations tested, malonate enhanced the growth of *H. pluvialis* only at a concentration of 0.25% (w/v); higher malonate concentrations caused a slight or total growth inhibition (Table 2). However, an increase in cell size was microscopically observed under high malonate concentrations.

The total carotenoid content per cell increased in malonate-stressed algae compared with controls (Fig. 6). Maximum cellular carotenoid content was obtained at the higher malonate concentration assayed, in cultures with 2% (w/v) malonate. In these cultures up to thirteen times more carotenoids per cell were accumulated than in cultures without this compound, but cell density was lower because cell division was totally inhibited in these cultures.

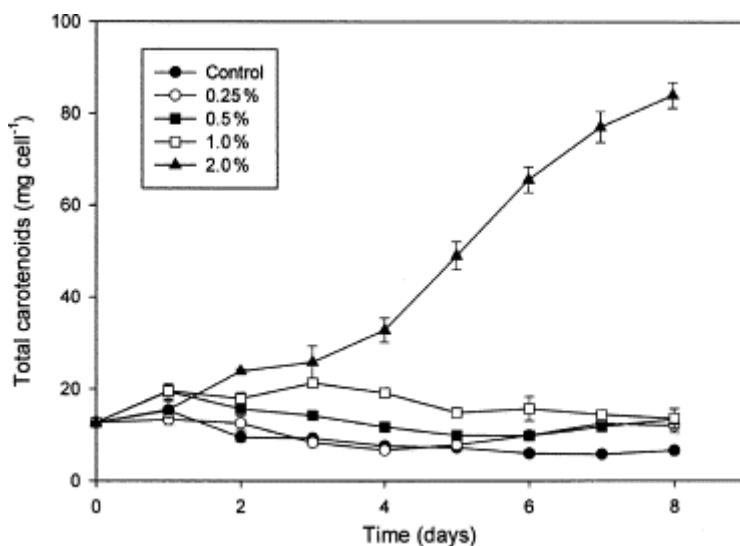


Fig. 6. Total carotenoids (expressed as  $\text{pg cell}^{-1}$ ) in cultures of *H. pluvialis* with different concentrations of malonate.

Both chlorophylls and total carotenoids per litre of culture reached the maximum value with 0.25% (w/v) malonate, because higher concentrations ( $\geq 0.5\%$  w/v) produced a decrease in biomass production with respect to control cultures.

The HPLC analysis of the cultures with malonate (Table 1) showed that the major compounds accumulated were the astaxanthin esters, as occurred in cultures with

acetate, and that the cellular content of primary carotenoids was less than in cultures without this carbon source.

#### 4. DISCUSSION

Nitrate concentration played a very important role in the cell division rate and in the accumulation of secondary carotenoids of *H. pluvialis* (Boussiba and Vonshak, 1991). This suggests that the synthesis of astaxanthin requires nitrogen, and most likely reflects the need for continuous synthesis of protein in order to support the massive accumulation of the pigment. However, the source of this nitrogen could not be in the culture media, but possibly in a nitrogen intracellular store as RuBisCo, since it has been reported that RuBisCo supports cell survival, and even growth for several hours under nitrogen starvation (García-Ferris et al., 1996).

Nitrogen starvation is an effective way to enhance astaxanthin accumulation in *Haematococcus* (Table 1), but cell density is low due to the cessation of cell division (Fig. 1). One solution to this problem could be the use of a low nitrate concentration, so that in a few days the nitrate present in the medium would be exhausted, but allowing to obtain the highest cell density. The optimum nitrate concentration to avoid this problem seems to be 0.15 g/l.

The ratio chlorophyll *a*/total carotenoids could be a good indicator of the physiological state of the culture. In favourable growth conditions this value was about 4; this value indicated nutrient replete conditions (Fig. 2); however, a value below 3 implied limitation of growth by reduced nutrient supply or another kind of stress, diminishing this value quickly when nutrient supply was reduced or another kind of stress occurred. Thus, it is possible to quickly check the N status of the cell, and this is very important since N availability is probably the main factor affecting astaxanthin accumulation.

As observed previously by Droop (1954) and Borowitzka et al. (1991), acetate appeared to be an important carbon source, enhancing both growth and carotenogenesis at small quantities (Table 1 and Table 2; Fig. 3 and Fig. 4). However, the effect of acetate was concentration-dependent, higher concentrations inhibiting growth but

markedly increasing astaxanthin content per cell (Table 1). Acetate addition in excess may generate a relative shortage of nitrogen inducing cyst formation and astaxanthin accumulation triggered by a high carbon/nitrogen (C/N) ratio (Kakizono et al., 1992). The algal cells seem to reduce their nitrogen uptake and begin to use the cellular nitrogen as in typical N-deficiency, although nitrogen exists in the culture medium.

The same effect has been observed in cultures with malonate. The effect is faster, cells accumulating more astaxanthin in less time than in cultures without this compound, or than in cultures with acetate. Even at the lower malonate concentrations assayed, the amount of astaxanthin per cell is at least twice higher than in control cultures (Table 1). Malonate is toxic for cells at high concentrations, however, at low concentrations it may enhance growth as a carbon source.

In spite of the growth inhibition at higher acetate or malonate concentrations, it is important to note the huge effect over the stimulation of astaxanthin synthesis and accumulation per cell that was more than thirteen times higher than in cultures without these compounds (Table 1). A similar effect was observed in *Phaffia rhodozyma*, where the addition of another carotenoid pathway precursor (mevalonic acid) had a strong effect on the accumulation of carotenoids (Calo et al., 1995).

## 5. CONCLUSION

The high efficiency, sensitivity and reliability of this HPLC method, as well as the short run time, low toxicity of the solvents used and low price, makes it ideal for its application for on-line analysis of secondary carotenoids accumulated in green algae at different stages of the life cycle.

The results obtained in the present study support the idea that low concentrations of acetate or malonate must be added to the growth medium to increase cell yield and carotenogenesis. The ratio chlorophyll *a*/total carotenoids is a good indicator of the nutrient state of the culture and it announces the beginning of astaxanthin accumulation before it could be detected by HPLC.

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