[36] Enhancement and Determination of Astaxanthin Accumulation in Green Alga *Haematococcus pluvialis*

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Introduction

The ketocarotenoid astaxanthin (3,3′-diketo-4,4′-dihydroxy-β-carotene) was first described in aquatic crustaceans as an oxidized form of β-carotene, which gives the carapace of these animals its pinkish color.[1] It was later found that this pigment is very common in certain species of fish and birds, in which it plays an important role in coloration during the mating season.[1] Astaxanthin is also found in algae such as *Chlamydomonas nivalis* and *Haematococcus pluvialis,*[2] *Euglena rubida,*[3] and *Acetabularia mediterranea.*[4] There has been a growing interest in the use of this pigment as a colorant for egg yolk in the poultry industry and in aquaculture, where it is used as a feed supplement in the production of salmon and shrimp. In addition, the carotenoids are lipophilic oxygen quenchers with potential anticancer activities, and it has been shown that this carotenoid possesses a higher antioxidant activity than β-carotene.[5]

Little research has been done on the conditions favoring accumulation of this ketocarotenoid by the unicellular alga *H. pluvialis* and what has been published is contradictory.[6] It has been suggested that nitrogen deficiency and high light intensity cause massive accumulation of this red pigment in *H. pluvialis.*[2,7,8] Other hypotheses concerning astaxanthin production in *H. pluvialis* argue either that it is favored by agents that prevent cell division without impairing the ability of the alga to assimilate carbon[9] or that the carbon–nitrogen balance in the medium determines the degree of carotene formation.[10] The present study was aimed at defining conditions favoring astaxanthin accumulation in *H. pluvialis.*

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Growth Conditions and Measurements

Haematococcus pluvialis Flotow (Chlorophyceae, Volvocales) is obtained from the culture collection of algae at the University of Göttingen, Germany.

The algae are cultivated in a modified BG_{11} medium,[11] which contains $NaNO_3$ (1.5 g/liter) in 500-ml sterilized columns placed in a transparent Plexiglas circulating water bath maintained between 25 and 28°. Light is supplied at a photon flux density of 85 to 120 μmol/(m^2·sec). Continuous aeration is provided by bubbling air containing 1.5% CO_2. Under these conditions, the pH is maintained between 6.8 and 7.5 and the algae remain green until they enter the stationary phase. For growth measurements, four parameters are used[12]: (1) cell number is determined with a Thomas blood cell counter; (2) chlorophyll is extracted with dimethyl sulfoxide (DMSO), the absorbance of the extracts is determined at 672 nm, and chlorophyll content is calculated with an $E^{1\%}_{1cm}$ of 898 according to Seely *et al.*[13]; (3) protein is determined according to Lowry *et al.*[14]; and (4) dry weight is measured by heating sample filtrated on a preweighed filter paper at 70° overnight.

Extraction and Measurement of Astaxanthin

The following procedures are used: cells are harvested by centrifugation (3500 rpm, 5 min), the pellet is resuspended in a solution of 5% (w/v) KOH in 30% (v/v) methanol and heated in a 70° water bath for 5 min to destroy the chlorophyll. The mixture is centrifuged and the supernatant is discarded. The remaining pellet is extracted with DMSO after adding 5 drops of acetic acid, and homogenized to recover the astaxanthin. The mixture is then heated for 5 min at 70°. This last step is repeated if necessary until the cell debris is totally white. The absorbance of the combined extracts is determined at 492 nm, and the amount of the pigment is calculated according to Davies[15] ($E^{1\%}_{1cm}$ 2220). All the processes should be conducted in darkness if possible.

The method described above is a simple and rapid way for ketocarotenoid determination. When the cells are green (Fig. 1a and b), the result is

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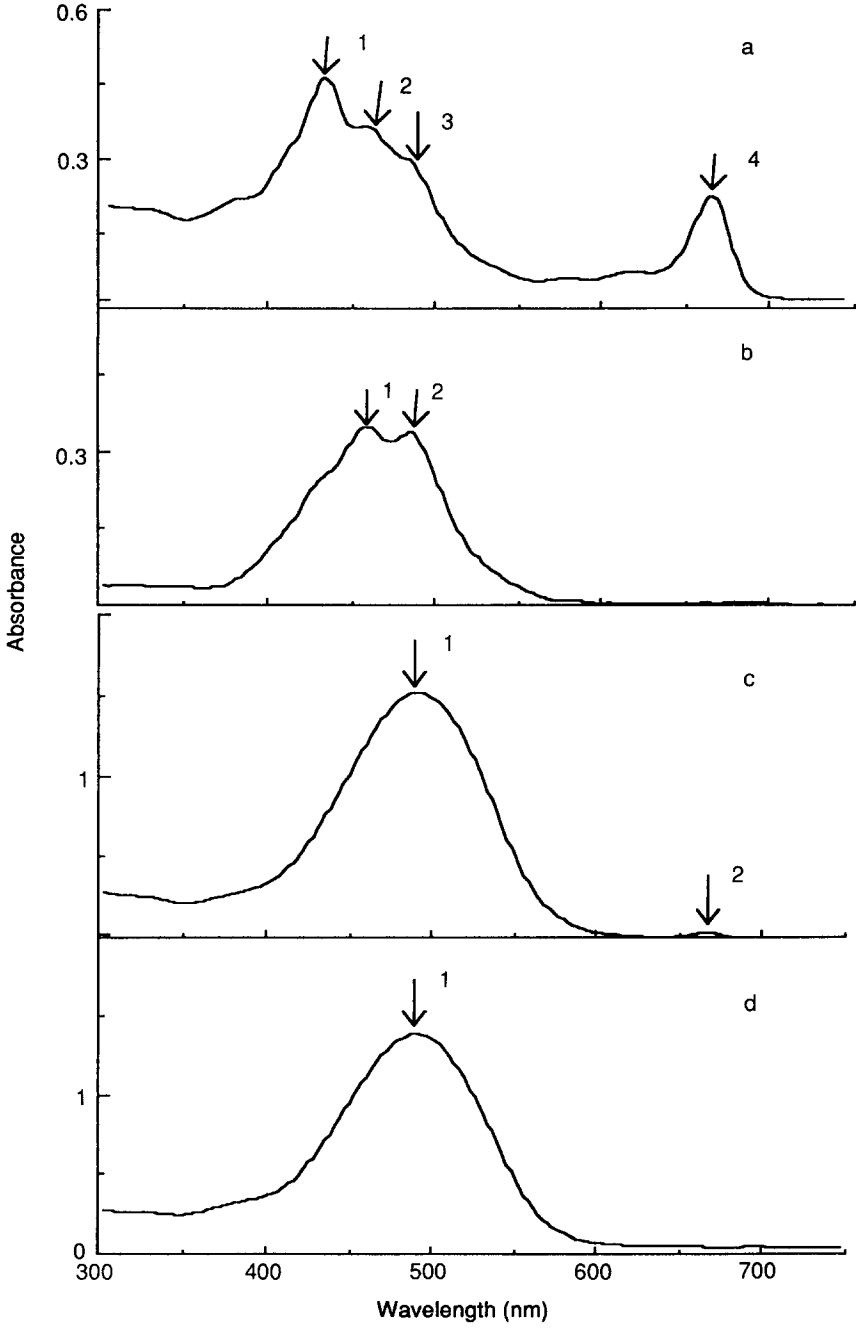

FIG. 1. Absorbance of DMSO extraction of *H. pluvialis*. (a) Chlorophyll (arrows 1 and 4) and total carotenoids (arrows 2 and 3) in green cells. (b) Astaxanthin (arrow 2) and other carotenoids (arrow 1) in green cells. (c) Chlorophyll (arrow 2) and astaxanthin (arrow 1) in red cells. (d) Astaxanthin (arrow 1) in red cells.

the amount of total ketocarotenoids instead of astaxanthin; however, after the cells become red, astaxanthin is the dominant form because of the conversion of intermediates to that pigment (Fig. 1c and d).

Conditions Inducing Astaxanthin Accumulation

Light and Nitrogen

At optimal light intensity for growth [85 μmol/(m^2·sec), L1] the astaxanthin content of logarithmically growing cells remained almost constant. When cultures were exposed to high light intensity [170 μmol/(m^2·sec), L2], a massive accumulation of astaxanthin was observed and the pigment reached a value of 65 pg/cell. Under the same light conditions but a lower nitrogen concentration (0.15 g/liter of $NaNO_3$, N2) this level was somewhat lower, 52 pg/cell (Table I). In high light intensity, the onset and rate of accumulation of astaxanthin was dependent on nitrogen concentration: in high nitrogen the massive accumulation started only on the fifth day, while in lower nitrogen the accumulation started on the second day and increased at a much faster rate.

TABLE I
ENVIRONMENTAL CONDITIONS INDUCING ASTAXANTHIN ACCUMULATION

Treatments[a]	Astaxanthin content (pg/cell)	
	Start	After 4 days
L1 and N1	2.31	4.5
L2 and N1	2.47	65
L2 and N2	1.89	52
L1 and NP	2.34	20.13
L2 and NP	2.25	58.7
L1 and NS1	1.75	11.29
L1 and NS2	2.73	10.27
L1 and SS	8.75	47.2
L1 and vinblastine	2.18	14.84

[a] L1, 85 μmol/(m^2 · sec); L2, 175 μmol/(m^2 · sec); N1, 1.5 g/liter $NaNO_3$; N2, 0.15 g/liter $NaNO_3$; SS, 0.8% NaCl: NP, no phosphate addition; NS1, without $MgSO_4$; NS2, $MgCl_2$ substituted for $MgSO_4$.

Phosphate Starvation

The influence of phosphate supply in combination with nitrogen on astaxanthin accumulation was studied. Logarithmic cells of *H. pluvialis* grown under optimal conditions are harvested and resuspended in a phosphate-free medium containing either high (N1) or low (N2) concentrations of nitrogen under high light intensity (L2). Phosphate-deprived cells exposed to the high nitrogen concentration do not divide, whereas those grown under the low level of nitrogen grow for 2 days and then stop. Astaxanthin accumulation follows the opposite pattern, being maximal in the phosphate-deprived culture containing a high nitrogen level. It should be noted that the rate and extent of astaxanthin accumulation per cell in phosphate-deprived cells is also dependent on light intensity: under 170 μmol/($m^2 \cdot$ sec) astaxanthin content is 58.7 pg/cell after 4 days, while it was 20.13 pg/cell under 85 μmol/($m^2 \cdot$ sec) (Table I).

Salt Stress

Exposing the logarithmic cells to salt stress by the addition of 0.8% (w/v) NaCl (SS) to the growth medium under optimal light intensity (L1) causes complete cessation of growth. Growth arrest is accompanied by a massive accumulation of astaxanthin, which reaches 47.2 pg/cell after 4 days (Table I).

Sulfate Starvation

Logarithmically growing cells are sulfur starved by suspending them in medium either without $MgSO_4$ (NS1), or in which $MgCl_2$ is substituted for $MgSO_4$ at a concentration of 0.06 g/liter (NS2). Under these conditions, astaxanthin is observed to reach 11.29 and 10.27 pg/cell, respectively, after 4 days (Table I). When $MgSO_4$ is substituted by Na_2SO_4 at a concentration of 0.043 g/liter, astaxanthin contents remained as constant as that of the control (2.68 and 2.53 pg/cell, respectively).

Cell Division Inhibitor

To evaluate the interaction between cell division and astaxanthin accumulation further, the effect of vinblastine was studied. Cell division in cultures grown under the L1/N1 regime and exposed to 2 or 5 mg/ml of vinblastine is completely inhibited. This inhibition is followed by an increase in the accumulation of astaxanthin in the resting cells (Table I).

In conclusion, astaxanthin accumulation is induced whenever a disturbance in cell division is imposed, and nitrogen is required for this process. Of the five conditions described above, phosphate starvation under high light intensity induced astaxanthin accumulation with the fastest rate and largest cell content. In all cases, the amount of astaxanthin did not exceed 3% of the dry weight.

[37] Simultaneous Quantitation and Separation of Carotenoids and Retinol in Human Milk by High-Performance Liquid Chromatography

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Introduction

The importance of carotenoids in human health has been known for decades.[1] Carotenoids have received the attention of researchers in various fields due to the role carotenoids play as precursors of retinol, as antioxidants, and as effectors of immune function. The effects of β-carotene on the immune system is of particular importance to infants, especially those in less developed countries, as these infants rely almost entirely on breast milk as a source of nutrients. Further, it is now clear that breast milk provides significant immunoprotection to the infant. However, there is essentially nothing known about the quantity or identity of the individual carotenoids in mature breast milk. Previous studies measuring carotenoids in mature breast milk have reported values for total carotene, being unable to quantify individual carotenoids due to the less sensitive and precise methods utilized.[2-6] In a study of carotenoid content of colostrum, Patton *et al.*[7] reported a method for separating and quantifying carotenoids by high-performance liquid chromatography (HPLC). Due to the significantly lower levels of lipid and higher levels of carotenoids in colostrum compared with mature milk, this method has not been directly applicable

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