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The Biosynthetic Pathway of Astaxanthin in a Green Alga *Haematococcus pluvialis* as Indicated by Inhibition with Diphenylamine

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The effect of diphenylamine on astaxanthin biosynthesis in *Haematococcus pluvialis* was studied. Cultures induced to produce astaxanthin accumulated β-carotene in the presence of the inhibitor. It was found that 30 μM diphenylamine specifically inhibits the biosynthesis of astaxanthin at the step of conversion of β-carotene to echinenone and canthaxanthin. The results imply that these two compounds are genuine intermediates in the pathway of astaxanthin biosynthesis in *H. pluvialis*.

Key words: Astaxanthin — Diphenylamine — *Haematococcus pluvialis* — Ketocarotenoid-biosynthesis.

The ketocarotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a red pigment common in many marine animals contributing to the pinkish color of their flesh. Only few animals can synthesize it de novo from other carotenoids and most of them acquire it in their food. In the plant kingdom astaxanthin occurs mainly in some species of cyanobacteria, lichens and algae. It is found rarely also in petals of higher plants (Goodwin 1980).

The biflagellate green alga *Haematococcus pluvialis* Flotow has recently received much attention due to its capability to accumulate large amounts of astaxanthin. Under unfavorable growth conditions, or following different types of environmental stresses, cells of this alga form cysts and accumulate massive amounts of astaxanthin in their cytoplasm to the extent that their color changes to red (Goodwin and Jamikorn 1954, Borowitzka et al. 1991, Boussiba and Vonshak 1991, Kobayashi et al. 1991, 1992, Yong and Lee 1991, Boussiba et al. 1992, Fan et al. 1994). It is possible to induce astaxanthin accumulation in cultures of *H. pluvialis* by depletion of phosphate or nitrogen, by increasing salt in the growth medium or increasing light intensity (Boussiba and Vonshak 1991, Fan et al. 1994). Some of the physiological aspects of astaxanthin accumulation in *H. pluvialis* were investigated by several researchers, but the exact pathway by which astaxanthin is synthesized has not yet been elucidated. It is accepted that astaxanthin is formed by hydroxylation reactions of β-carotene (Donkin 1976). In algae it is synthesized as a (*3S,3'S*)-stereoisomer (Andrewes et al. 1974), whereas the yeast *Phaffia rhodozyma* produces the (*3R,3'R*)-stereoisomer (Andrewes et al. 1976). Recently, Yokoyama and Miki (1995) reported the biosynthetic pathway of astaxanthin in a bacterium *Agrobacterium aurautiacum*, in which astaxanthin was formed from β-carotene through either canthaxanthin or zeaxanthin depending on the culture conditions.

It was therefore postulated that in the algae, the C-3 hydroxy groups are introduced first, to give the normal stereoisomer (*3R,3'R*)-zeaxanthin, into which the C-4-keto groups are then introduced (Britton 1988). This is in contrast to the case of *P. rhodozyma*, where a keto group is introduced first to give echinenone, which is converted to 3-hydroxy echinenone and adonirubin (Andrewes et al. 1976). The presence of echinenone and canthaxanthin in two spcies of *Haematococcus* (Donkin 1976, Grung et al. 1992) implied that in algae these carotenoids are intermediates in astaxanthin synthesis.

In this work we report that diphenylamine inhibits astaxanthin synthesis in *H. pluvialis*. By using diphenylamine we demonstrate that (*3S,3'S*) astaxanthin is synthe-

Abbreviations: DPA, diphenylamine; TLC, thin layer chromatography.

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sized in *H. pluvialis* from β-carotene via echinenone and canthaxanthin.

Materials and Methods

Haematococcus pluvialis Flotow was obtained from the culture collection of the University of Göttingen. Medium composition and optimal growth conditions were as previously described (Boussiba and Vonshak 1991, Boussiba et al. 1992, Fan et al. 1994). Diphenylamine stock solution (30 mM) was prepared by dissolving pure crystalline diphenylamine (BDH Chemicals Ltd.) in 70% basic (pH 9.0) ethanol (Paerl 1984). Astaxanthin content and growth parameters (chlorophyll, dry weight and cell number) were measured as described previously (Boussiba and Vonshak 1991, Fan et al. 1994). Cells in the logarithmic growth phase were harvested by centrifugation (1,160 × *g*, 2 min) for measurements of photosynthetic activity and respiration as described by Vonshak et al. (1988).

For pigment extraction, cells of *H. pluvialis* were harvested and freeze-desiccated. 0.1 g of dried cells were ground to powder and extracted several times with acetone (total volume 15 ml) until almost colorless. The extract was filtered through a Whatmann 0.2 mm pore size filter, and the filtrate was analyzed by HPLC. Efficiency of pigment extraction in this procedure is estimated as 95%.

Chromatography was carried out by HPLC on a reversed-phase C_{18} column, Spherisorb ODS2 (5 mm particle size). Samples were injected into a 100 μl loop, and mobile phases were pumped by a Merck-Hitachi L-6200A high pressure pump, at a flow rate of 1 ml min^{-1}. Peaks were detected at 450 nm by a Merck-Hitachi diode-array detector L-4500. The solvent system included acetonitrile : H_2O (9 : 1)—solvent A, and 100% ethylacetate—solvent B. The pigments were separated by a step gradient between solvents A and B for 32 min as follows: 0–10 min 100 : 0 (solvent A : solvent B), 10–15 min 40 : 60; 15–22 min 24 : 76; 22–32 0 : 100. Areas under the peaks were integrated by the Merck-Hitachi HPLC software and quantified against peaks of known quantities of standard pigments: chlorophyll *a*, chlorophyll *b*, β-carotene, lutein, violaxanthin, neoxanthin, zeaxanthin, echinenone, canthaxanthin and astaxanthin. The latter three carotenoids were kindly provided by Dr. A. Young of the Liverpool John Moores University.

Thin layer chromatography (TLC) of pigments on silica gel was carried out to determine the astaxanthin esters, using hexane : acetone (7 : 3) as a running solvent.

Results

Effect of diphenylamine on growth—Utilization of inhibitors was found to be very helpful in elucidating the biosynthetic pathway of carotenoid. In this work diphenylamine was used to study the biosynthetic pathway of astaxanthin. In order to avoid any non-specific effect of the inhibitor a rather low concentration of diphenylamine (30 μM) was used. To determine whether this concentration of the inhibitor has any deleterious effect on the growth of *H. pluvialis*, cells were grown under optimal growth condition in the presence or the absence of the diphenylamine. Growth, as expressed in cell number, chlorophyll content, dry weight and the rates of oxygen evolution and uptake (Table 1), as well as the carotenoid profile (Fig. 1A, B), showed no significant differences between two treatments, indicating that diphenylamine did not cause any toxic-effect at the concentration tested.

Effect of diphenylamine on carotenogenesis—Phosphate starvation is very effective for astaxanthin induction in *H. pluvialis* (Boussiba and Vonshak 1991). When cells at the logarithmic growth phase were incubated in a phosphate-deficient BG-11 medium, they gradually changed their color from green to red (Fig. 2A, C), due to accumulation of astaxanthin. Separation of the pigments by thin layer chromatography (TLC) indicated that most of the astaxanthin accumulated in the form of mono-ester (50%) and di-ester (45%) and very little is free astaxanthin as reported previously (Grung et al. 1992). The carotenoid composition of cells of *H. pluvialis* following induction of astaxanthin synthesis is given in Fig. 1C and Table 2.

Addition of 30 μM diphenylamine when phosphate was omitted from the culture resulted in yellow cells

Table 1 Effect of DPA on growth of *H. pluvialis*

Time (h)	Cell number (× 10^8 cells $liter^{-1}$)		Chlorophyll (mg $liter^{-1}$)		Dry weight (g $liter^{-1}$)		O_2 evolution (μmol O_2 (mg Chl)$^{-1}$ h^{-1})		O_2 uptake (μmol O_2 (mg Chl)$^{-1}$ h^{-1})	
	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA	−DPA	+DPA
0	3.3	3.3	2.67	2.67	0.17	0.17	211	205	29	33
96	17.3	17.5	17.0	18.3	0.96	0.88	209	202	32	35

Conditions for the growth are as described in Materials and Methods. +DPA = 30 μM DPA; −DPA = control. Data represent means of three replicates.

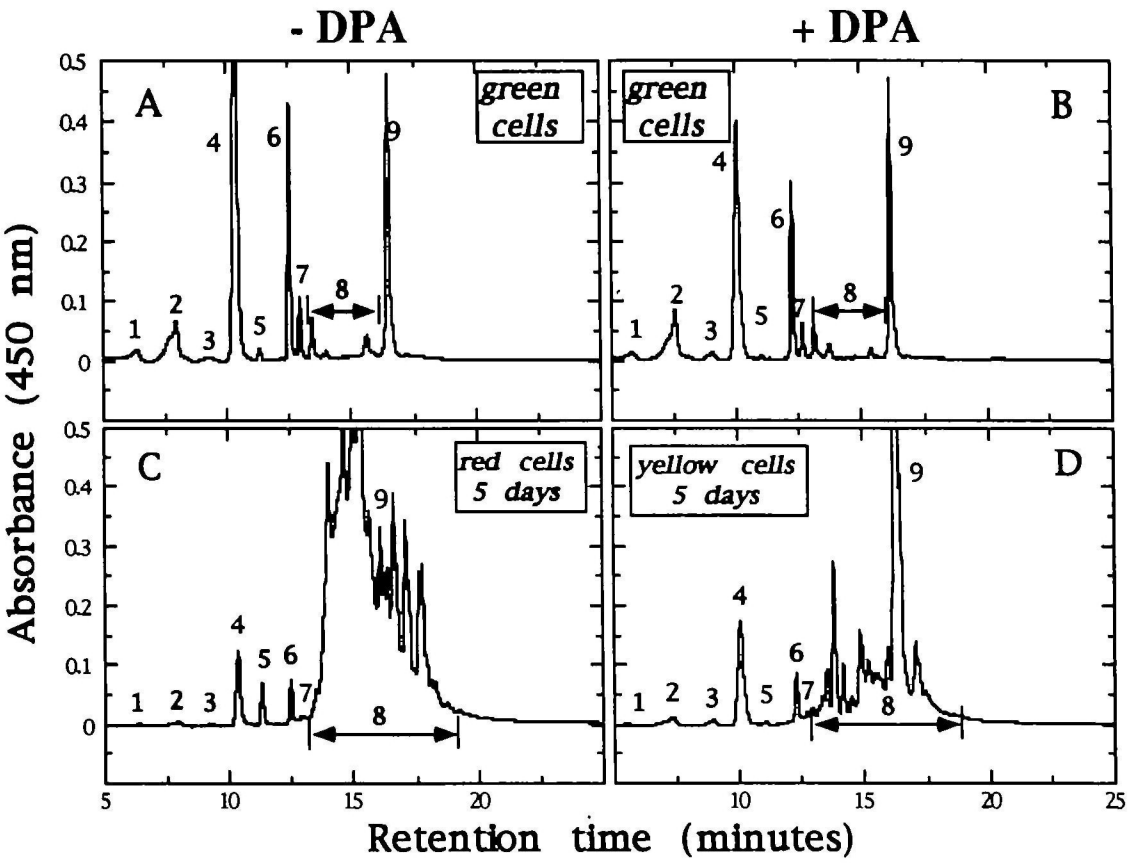

Fig. 1 Total pigments profile analyzed by HPLC. Green cells grown without (A) or with (B) diphenylamine; cells were induced to synthesize astaxanthin by phosphate starvation in the absence (C) or presence (D) of diphenylamine. Peaks were identified by typical retention time and absorption spectra compared with their respective standard compounds: (1) neoxanthin; (2) violaxanthin; (3) Antheraxanthin; (4) lutein; (5) canthaxanthin; (6) chlorophyll *b*; (7) chlorophyll *a*; (8) astaxanthin and its esters; (9) β-carotene. Echinenone could not be separated from the astaxanthin peaks.

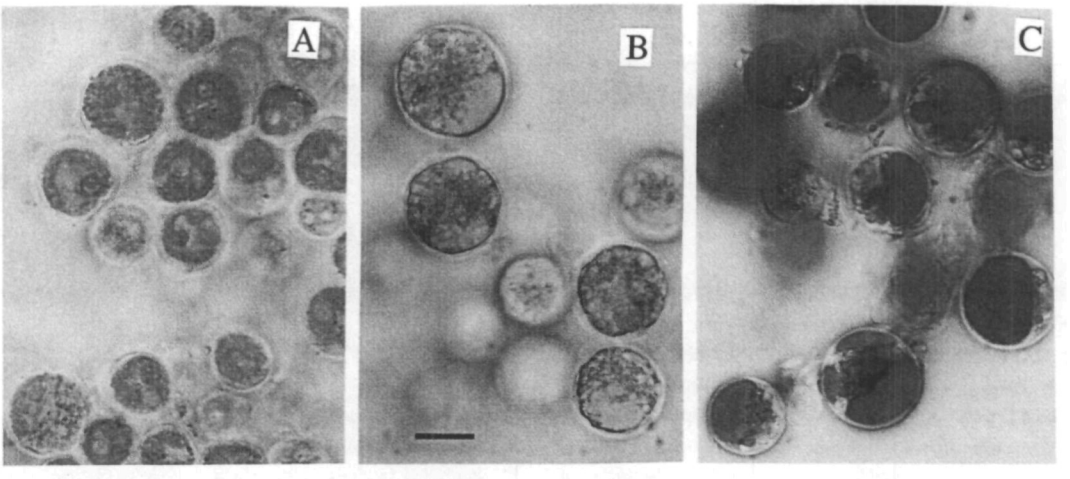

Fig. 2 *H. pluvialis* cells grown under optimal conditions (A) and under phosphate starvation for 12 days with (B) or without (C) 30 μM diphenylamine treatment. The bar represents 10 μm.

Table 2 Effect of diphenylamine on carotenoids profile of *Haematococcus* under different stress conditions

		Carotenoid content [mg (g dry weight)$^{-1}$]				
		Violaxanthin	Lutein	β-Carotene	Canthaxanthin	Astaxanthin
Non inductive conditions	No DPA	1.2±0.2	17.3±2.0	9.1±1.1	<0.5	<0.5
	+DPA	1.1±0.2	8.4±1.3	8.2±0.9	<0.5	<0.5
Inductive conditions	3 days −DPA	0.9±0.18	6.3±0.7	<0.5	2.3±0.14	19.1±0.6
	3 days +DPA	0.7±0.17	8.1±0.68	17.3±1.9	<0.5	1.5±0.3
	5 days −DPA	<0.5	4.6±0.6	<0.5	2.5±0.2	27.2±2.2
	5 days +DPA	<0.5	5.4±0.63	22.3±2.6	<0.5	1.8±0.2
	14 days −DPA	<0.5	3.7±0.33	<0.5	2.1±0.13	32.1±2.2
	14 days +DPA	<0.5	4.8±0.5	31.3±1.7	<0.5	<0.5
	washed from DPA[a]	<0.5	3.7±0.3	19.4±1.6	1.3±0.11	17.4±1.4

[a] Cells grown with diphenylamine under inductive conditions for 5 days were washed twice and re-suspended in phosphate free BG-11 and incubated at the same growth conditions for further 9 days.

(Fig. 2B), indicating possible inhibition of astaxanthin accumulation. Analysis by HPLC of pigment composition in the cells at different times after induction of astaxanthin biosynthesis revealed that the yellow pigment accumulated in diphenylamine-treated cells was β-carotene (Fig. 1D and Table 2). A drastic decrease in the concentration of canthaxanthin was observed in the diphenylamine-treated cells (Table 2). But when diphenylamine was removed by washing at the day 5, canthaxanthin re-appeared and astaxanthin was accumulated at the day 14, accompanied by the decrease of β-carotene (Table 2). Echinenone and adonirubin could not be separated from astaxanthin esters by our elution solvent in HPLC, but they were clearly identified by silica gel TLC. The R_f values of β-carotene, echinenone, canthaxanthin, adonirubin and free astaxanthin were 1.00, 0.90, 0.65, 0.46 and 0.36, respectively. Zeaxanthin was not detected by HPLC or TLC in all the treatments.

These results indicate that under phosphate starvation accumulation of astaxanthin is inhibited by diphenylamine, leading to accumulation of β-carotene. Furthermore, it is implied that echinenone and canthaxanthin, not zeaxanthin, are intermediates in the conversion of β-carotene to astaxanthin in *H. pluvialis*.

Discussion

Inhibition of carotenogenesis by diphenylamine was found in various organisms, mainly in fungi, to block the desaturation of phytoene (reviewed by Goodwin 1980). Cohen-Bazire and Stanier (1958) described a similar accumulation of phytoene in a photosynthetic bacteria *Rhodospirillum rubrum* by using 70 μM diphenylamine. Sassu (1972) however reported that diphenylamine at low concentration (10 μM) caused an accumulation of β-carotene and at high concentration (50 μM) resulted in an accumulation of phytoene in *Dictyococcus cinnabarinus*. Recently, Vorst et al. (1994) showed that the accumulation of β-carotene in *Dunaliella bardawil* was arrested by 40.6 μM diphenylamine. Our results demonstrate that in *H. pluvialis* 30 μM diphenylamine inhibits the conversion of β-carotene to keto-carotenoids. Since no deleterious effects of diphenylamine were detected on growth, metabolism and carotenoid composition of *H. pluvialis* grown under optimal conditions (Table 1, Fig. 1A, B), we conclude that this inhibitor at the concentration of 30 μM only affects astaxanthin biosynthesis in this alga under stress conditions. Thus, we have identified another inhibitory site of diphenylamine on carotenogenesis. The different results obtained from *D. bardawil* by Vorst et al. (1994) may be due to the difference between species in sensitivities towards the inhibitor. In fact, we did observe an accumulation of phytoene in *H. pluvialis* treated with 60 or 90 μM diphenyl-

Table 3 Effect of diphenylamine on phytoene accumulation in *Haematococcus pluvialis*

Treatment[a]	A_{288}/A_{458}[b]	A_{288}	A_{458}
0 time	0.24	0.14	0.58
After 7 days Control	0.25	0.68	2.74
With DPA addition 30 μM	0.32	0.62	1.95
60 μM	0.57	0.43	0.75
90 μM	0.76	0.31	0.41

[a] Cells were treated with various concentration of DPA under inductive condition for 7 days.

[b] A_{288}/A_{458} indicates the relative accumulation of phytoene. The maximum absorbance of phytoene (A_{288}) and β-carotene (A_{458}) in dimethylsulfoxide

amine (Table 3). Since at this stage it is unclear whether or not diphenylamine interacts directly with the oxygenase, we do not understand the concentration-dependent dual inhibitory effects on carotenogenesis by diphenylamine.

The biosynthesis of ketocarotenoids has been studied in several systems. In the yeast *Phaffia rhodozyma* it was proposed (Andrewes et al. 1976) that the (*3R,3'R*) astaxanthin is produced from β-carotene via echinenone, hydroxyechinenone and adonirubin (=phoenicoxanthin). The identification of small but significant amounts of echinenone and canthaxanthin in *Haematococcus lacustris* (Donkin 1976) and *H. pluvialis* (Grung et al. 1992) has led to the assumption that in algae the pathway of (*3S,3'S*) astaxanthin biosynthesis is different since it occurs via canthaxanthin. We have shown that diphenylamine inhibition of astaxanthin biosynthesis is associated with β-carotene accumulation and decreace of canthaxanthin. Our results support the canthaxanthin biosynthetic pathway by providing evidence that the C-4 keto groups are introduced first to β-carotene. These two steps might be carried out by the same enzyme, thus additions of the two keto-groups will be both sensitive to diphenylamine. This latter speculation is based upon the detection of a sharp decrease in the concentration of canthaxanthin (Table 2) that occurs in diphenylamine-treated cells, while β-carotene is the only carotenoid that accumulates. The proposed pathway of astaxanthin biosynthesis is depicted in Fig. 3.

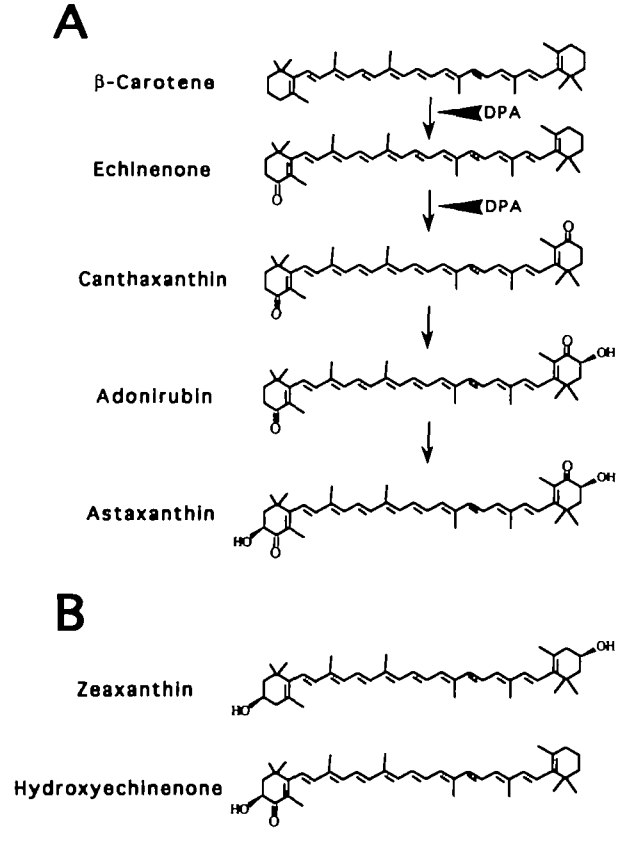

Fig. 3 Proposed pathway for astaxanthin biosynthesis in *H. pluvialis* (A); related carotenoids, which do not take part in this pathway, are given in (B).

In *Phaffia rhodozyma* the addition of a C-4 keto group also precedes the introduction of the C-3 hydroxy group and, therefore, it is clear that the type of stereoisomer that is produced is determined not by the sequence of these reactions, but by the mechanism of the enzymatic reactions. It is apparent that different enzymes carry out these reactions in yeast and algae. Recent studies of enzymes in the carotenoid biosynthesis pathway revealed that each one of them operates in a symmetric manner on each half of the carotenoid molecule (reviewed by Sandmann 1994, Armstrong 1994). Since in *P. rhodozyma* the echinenone is converted to hydroxyechinenone and no canthaxanthin is found, it is possible that the two consecutive reactions in *P. rhodozyma* are carried out by the same enzyme, in which case a single enzyme in *P. rhodozyma* is responsible for (*3R,3'R*) astaxanthin production from β-carotene. This hypothesis is corroborated by a recent study of mutations in astaxanthin synthesis in *P. rhodozyma*, where no mutation could be recovered in any of the intermediate steps in the oxidation β-carotene to astaxanthin (Girard et al. 1994). In contrast, two enzymes are expected to be involved in the synthesis of (*3S,3'S*) astaxanthin in *H. pluvialis*.

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References

Andrewes, A.G., Borch, G., Liaaen-Jensen, S. and Snatzke, G. (1974) Animal carotenoids. IX. On the absolute configuration of astaxanthin and actinioerythrin. *Acta Chem. Scand.* B28: 730–736.

Andrewes, A.G., Phaff, H.J. and Starr, M.P. (1976) Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. *Phytochemistry* 15: 1003–1007.

Armstrong, G.A. (1994) Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. *J. Bacteriol.* 176: 4795–4802.

Borowitzka, M.A., Huisman, J.M. and Osborn, A. (1991) Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. I. Effects of nutrients on growth and cell type. *J. Appl. Phycol.* 3: 295–304.

Boussiba, S., Fan, L. and Vonshak, A. (1992) Enhancement and determination of astaxanthin accumulation in green alga

Haematococcus pluvialis. Methods Enzymol. 213: 386–391.

Boussiba, S. and Vonshak, A. (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis. Plant Cell Physiol.* 32: 1077–1082.

Britton, G. (1988) Biosynthesis of Carotenoids. *In* Plant Pigments. Edited by Goodwin, T.W. pp. 133–182. Academic Press, London.

Cohen-Bazire, G. and Stanier, R.Y. (1958) Inhibition of Carotenoid Synthesis in Photosynthetic Bacteria. *Nature* 181: 250–252.

Donkin, P. (1976) Ketocarotenoid biosynthesis by *Haematococcus pluvialis. Phytochemistry* 15: 711–718.

Droop, M.R. (1954) Conditions governing Haematochrome formation and loss in the alga *Haematococcus pluvialis* Flotow. *Arch. Mikrobiol.* 20: 391–397.

Fan, L., Vonshak, A. and Boussiba, S. (1994) Effect of temperature and irradiance on Growth of *Haematococcus pluvialis (Chlorophyceae). J. Phycol.* 30: 829–833.

Girard, P., Falconnier, B., Bricout, J. and Vladescu, B. (1994) β-Carotene producing mutants of *Phaffia rhodozyma. Appl. Microbiol. Biotechnol.* 41: 183–191.

Goodwin, T.W. (1980) *The Biochemistry of Carotenoids.* Vol. 1, Plants. Chapman and Hall, New York.

Goodwin, T.W. and Jamikorn, M. (1954) Studies in carotenogenesis. 11. Carotenoid synthesis in the alga *Haematococcus pluvialis. Biochem. J.* 57: 376–381.

Grung, M., D'Souza, FM-L., Borowitzka, M. and Liaaen-Jensen, S. (1992) Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (*3S,3'S*)-astaxanthin esters. *J. Appl. Phycol.* 4: 165–171.

Kobayashi, M., Kakizono, T. and Nagai, S. (1991) Astaxanthin production by a green alga *Haematococcus pluvialis* accompanied with morphological changes in acetate media. *J. Ferment. Bioengin.* 71: 335–339.

Kobayashi, M., Kakizono, T., Yamaguchi, K., Nishio, N. and Nagai, S. (1992) Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. *J. Ferment. Bioengin.* 74: 17–20.

Paerl, H.W. (1984) Cyanobcterial carotenoids: their roles in maintaining optimal photosynthetic production among aquatic bloom forming genera. *Oecologia* (Berlin) 61: 143–149.

Sandmann, G. (1994) Carotenoid biosynthesis in microorganisms and plants. *Eur. J. Biochem.* 223: 7–24.

Sassu, O.G. (1972) Effect of diphenylamine on carotenoid synthesis in *Dictyococcus cinnabarinus. Phytochemistry* 11: 3195–3198.

Vonshak, A., Guy, R., Poplowsky, R. and Ohad, I. (1988) Photoinhibition and its recovery in two different strains of *Spirulina. Plant Cell Physiol.* 29: 721–726.

Vorst, P., Baard, R.L., Mur, L.R., Korthals, H.J. and van den Ende, H. (1994) Effect of growth arrest on carotene accumulation and photosynthesis in *Dunaliella. Microbiology* 140: 1411–1417.

Yokoyama, A. and Miki, W. (1995) Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum. FEMS Microbiol. Lett.* 128: 139–144.

Yong, Y.Y.R. and Lee, Y.K. (1991) A possible role of photoprotection for secondary carotenoids in the cytoplasm of the green alga *Haematococcus lacustris (Chlorophyta). Phycologia* 30: 257–261.

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