Secondary Carotenoid Accumulation in *Haematococcus* (Chlorophyceae): Biosynthesis, Regulation, and Biotechnology

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Abstract Unicellular green algae of the genus *Haematococcus* have been studied extensively as model organisms for secondary carotenoid accumulation. Upon environmental stress, such as strong irradiance or nitrogen deficiency, unicellular green algae of the genus *Haematococcus* accumulate secondary carotenoids in vesicles in the cytosol. Because secondary carotenoid accumulation occurs only upon specific environmental stimuli, there is speculation about the regulation of the biosynthetic pathway specific for secondary carotenogenesis. Because the carotenoid biosynthesis pathway is located both in the chloroplast and the cytosol, communication between both cellular compartments must be considered. Recently, the induction and regulation of astaxanthin biosynthesis in microalgae received considerable attention because of the increasing use of this secondary carotenoid as a source of pigmentation for fish aquaculture, as a component in cancer prevention, and as a free-radical quencher. This review summarizes the biosynthesis and regulation of the pathway, as well as the biotechnology of astaxanthin production in *Haematococcus*.

Key words: *Haematococcus*, astaxanthin, biosynthesis, regulation, biotechnology

Carotenoids, 40-carbon isoprenoids, are integral and essential components of the photosynthetic membranes in all plants. Essential carotenoids that function within the photosynthetic machinery are commonly referred to as primary carotenoids. In contrast, secondary carotenoids are defined functionally as those carotenoids that are not required for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. The unicellular green alga *Haematococcus pluvialis* is distinguished from other organisms in that it

accumulates large amounts of carotenoids in lipid vesicles outside the plastid [6]. This has given rise to speculation about the possible existence of a biosynthetic pathway specific for secondary carotenoids that occurs in the cytoplasm. This is supported by the existence of two different IPP isomerases in *H. pluvialis* [74]. However, no pathway specific to the biosynthesis of secondary carotenoid (SC) of *H. pluvialis* was found at the level of the enzyme phytoene desaturase in the cytosol [33]. Therefore, it was hypothesized that carotenoids are transported from the site of biosynthesis (chloroplast) to the site of accumulation (cytoplasmic lipid vesicles). Upon environmental stress, such as high salt concentrations, high irradiance, or nitrogen deficiency, SCs such as β-carotene or the ketocarotenoid astaxanthin accumulate in the lipid vesicles in the chloroplast stroma or in the cytosol of various unicellular green algae (carotenogenesis, Fig. 2) [5, 30]. Astaxanthin, the end product of the biosynthesis pathway and its immediate precursors, echinenone and canthaxanthin, can be found in stressed cells. Proposed functions of SC in *H. pluvialis* include action as a sunscreen [37], protection from photooxidative damage [36, 75, 80]), and protection against photoinhibition [76]. SCs in microalgae have been reviewed previously [54]. This review focuses on and adds more information about the biosynthesis, regulation, and metabolic engineering of SCs, based on updated results.

General Biology

The green microalga *Haematococcus pluvialis* Flotov (Chlorophyceae) is a unicellular biflagellate living in fresh water. Under optimal growth conditions, cells are spherical to ellipsoid and enclosed by a cell wall. Figure 1 shows that the flagellated green cells transform to red cyst cells in response to environmental stress conditions. These cells, also called aplanospores, develop their reddish color from astaxanthin accumulation. The aplanospores are

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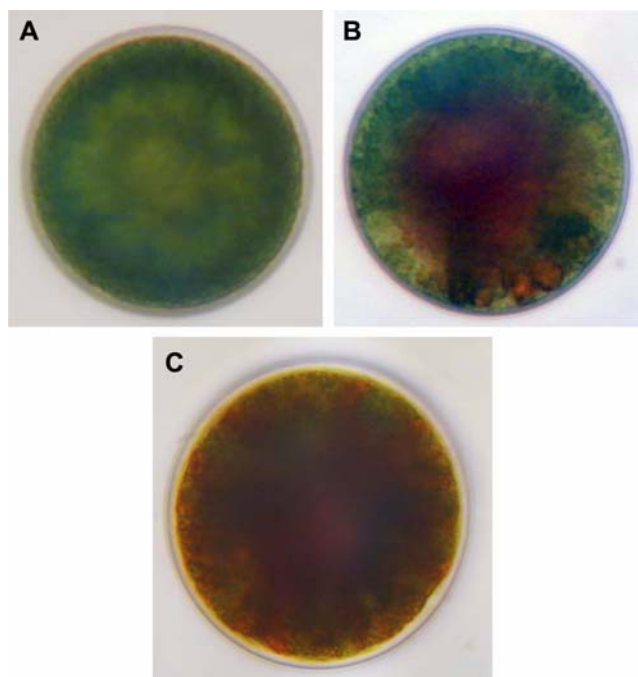E-mail: jpolle@brooklyn.cuny.edu

Fig. 1. Photographs of cells of *Haematoccus pluvialis* in different stages of astaxanthin accumulation: **A**. Green cell; **B**. Cell in the process of astaxanthin accumulation in the cytosol; and **C**. Orange cell.

considered to be the resting stage of the algae. Several morphological features accompany the transformation of vegetative cells into aplanospores. The cell wall becomes thicker, immobilizing the flagellae, which eventually are shed. The volume of the algae increases (Fig. 1), sometimes creating giant red aplanospores with a diameter of over 40–50 μm. This is ten times the diameter of the vegetative cell. However, giant aplanospores are usually not predominant; most of the red cysts have much smaller diameters. Under nonstressed conditions after maturation, the cysts germinate, releasing mostly flagellated cells and leaving behind the typical cell wall. Reproduction is usually by cell division throughout the vegetative stage. Enlarged cysts containing many new cells may be observed [45]. The duration of the growth cycle of *H. pluvialis* varies according to nutritional and environmental conditions, but it is usually between less than 10 days and several weeks.

The process of accumulation of SCs in *H. pluvialis* and *H. lacustris* is not necessarily associated with the formation of aplanospores [5, 17, 19, 31, 34, 38, 47]. Some authors report that pigment production is not exclusive to aplanospores, but may also be seen in growing flagellates [31, 32].

Biosynthesis of Secondary Carotenoids
The chloroplasts of green algae are very similar to the chloroplasts of higher plants with respect to pigment composition, photosynthetic performance, biochemistry, and
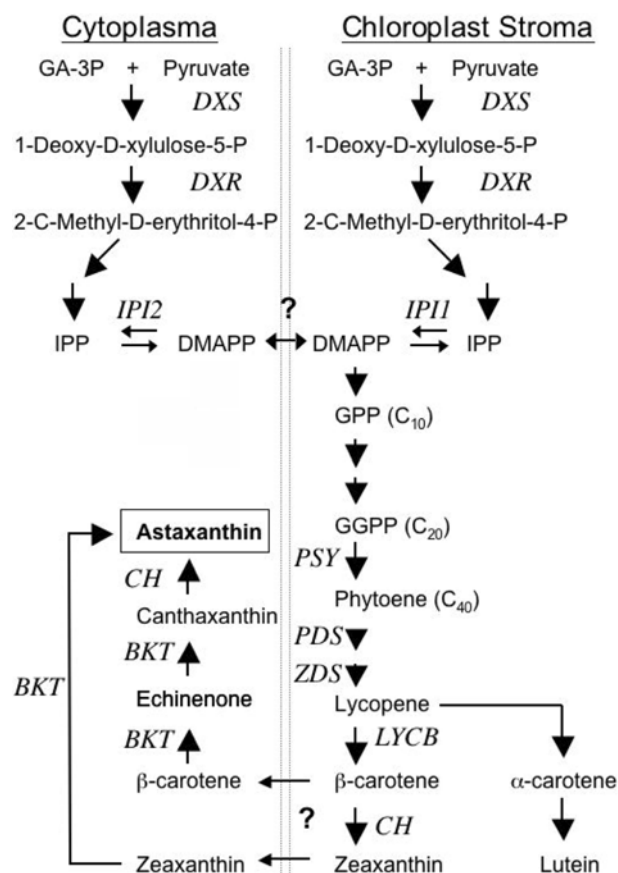

Fig. 2. Proposed pathway of secondary carotenoids production in *Haematococcus pluvialis*.
Enzymes are designated as DXS (Deoxy Xylulose-5-P Synthase), DXR (Deoxy Xylulose-5-P Reductase), IPI (Isopentenyl Pyrophosphate Isomerase), LCYB (Lycopene β-Cyclase), PSY (Pytoene synthase), PDS (Phytoene Desaturase), ZDS (ζ-Carotene Desaturase), CH (β-Carotene Hydroxylase), BKT (β-Carotene ketolase).

physiology. The biosynthesis pathway of secondary carotenoids in *H. pluvialis* is elucidated using inhibitor studies and the C-labeling technique [35, 38, 51]. Figure 2 schematically depicts the key steps in the carotenoid biosynthesis pathway. The key building block of carotenoids is isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) (Fig. 2) [32, 50]. The enzyme isopentenyl pyrophosphate isomerase (IPI) carries out this reversible isomerization reaction. Two *Haematococcus* cDNAs for IPP isomerase genes have been identified [74, 75]. This is similar to higher plants in which two distinct cDNAs for IPP isomerase have been identified [70]. Two separate and biochemically different IPP biosynthesis pathways exist in higher plants [51]. Isopentenyl pyrophosphate is produced in the cytosol through the acetate/mevalonate pathway and in the chloroplast through the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway. Genes for the enzymes participating in the biosynthetic pathway of plastidal DOXP are localized in the nucleus and the gene products

are imported to the chloroplast [50]. In contrast to higher plants, there is no evidence for activity of the cytosolic acetate/mevalonate biosynthetic pathway of isoprenoids in unicellular green algae belonging to the class chlorophyceae [50, 69]. Thus, it is proposed that these algae produce their isoprenoids only through the DOXP pathway that operates in both the cytosol and plastids [16, 69].

Another important enzyme of the carotenoid biosynthesis pathway is phytoene synthase (PSY) (Fig. 2, right). It catalyzes the first committed step in carotenoid biosynthesis by condensing two 20-carbon geranylgeranyl pyrophosphate (GGPP) molecules to form the 40-carbon molecule phytoene, the precursor molecule for all other carotenoids. The amino acid sequence of the PSY contains domains that are highly conserved among bacteria, cyanobacteria, algae, and higher plants. This emphasizes its importance within the carotenoid biosynthetic pathway [40]. PSY shares amino acid sequence similarity with GGPP synthase and other prenyl-transferases. Although only one gene for PSY in *Haematococcus* has been discovered so far, two genes exist for PSY in some higher plants. *Psy-1* encodes a fruit- and flower-specific isoform and *Psy-2* encodes an isoform that predominates in green tissues [2, 23, 27]. PSY is a rate-limiting enzyme of carotenoid biosynthesis in ripening tomato fruits [8, 24], in canola (*Brassica napus*) seeds [71], and in marigold flowers [58]. This rate-limiting feature makes PSY suitable as a key regulator of carotenogenesis.

Two structurally and functionally similar enzymes, phytoene desaturase PDS and ζ-carotene desaturase (ZDS), convert phytoene to lycopene via ζ-carotene. These FAD-containing enzymes each catalyze two symmetric dehydrogenation reactions that require plastoquinone [55, 59] and a plastid terminal oxidase as electron acceptors [10]. When coexpressed in *E. coli*, PDS and ZDS from *Arabidopsis* convert phytoene to 7,9,7',9'-tetra-*cis*-lycopene (poly-*cis* lycopene, also called pro-lycopene), whereas the bacterial phytoene desaturase (CRTI) produces the all-*trans* lycopene [4]. The mechanism of carotenoid isomerization is unknown. It is predicted, however, that a gene product at the *tangerine* locus of the tomato that codes for a carotenoid isomerase is involved in this process [42]. In fruits of the recessive mutant *tangerine*, lycopene is replaced by poly-*cis* lycopene.

Carotenoids in the photosynthetic apparatus and secondary carotenoids are bicyclic compounds. Therefore, cyclization of lycopene to α-carotene and β-carotene is an important branch point in carotenoid biosynthesis (Fig. 2). In contrast to some recent reports for higher plants, *Haematococcus* genes for ZDS and the carotenoid isomerase are not known. Cyclization of lycopene to β-carotene involves lycopene β-cyclases (LYCB) carrying out two cyclization reactions, thus introducing two β-rings. In contrast, generation of α-carotene involves two enzymes, the lycopene ε-cyclases and the lycopene β-cyclases. Since only β-carotene

or its derivatives are accumulated upon environmental stress, the amount and/or activity of the lycopene β-cyclase is crucial for carotenogenesis. A special lycopene β-cyclase is upregulated in tomatoes upon fruit ripening [65], suggesting the importance of the lycopene β-cyclase in secondary carotenoid accumulation.

As shown in Fig. 2, β-carotene is the precursor of astaxanthin that accumulates in lipid globules in the cytosol. The enzymes carrying out the necessary oxygenation reactions in the alga *Haematococcus* are β-carotene hydroxylase (crtO) and β-carotene oxygenase (crtB; the β-carotene ketolase [BKT] is synonymous). Because one of the desaturase enzymes introducing double bonds into phytoene was found only within the chloroplast, it was proposed that β-carotene is transported from the chloroplast to the cytosol where the oxygenation reactions of β-carotene take place [6, 32, 40].

In summary, several genes for the carotenoid biosynthesis pathway in the alga *Haematococcus* are known, but questions remain. For example, the presence of more than one gene for the PSY is not confirmed. Furthermore, no genes for or the carotenoid isomerase have been reported so far.

Regulation of Carotenogenesis

Although many nuclear-encoded genes for enzymes of the carotenoid biosynthetic pathway have been identified in several species including algae [13, 72, 79, 83], not much is known about regulation of carotenogenesis *in vivo*. Nevertheless, expression studies of several genes involved in carotenoid biosynthesis indicate upregulation
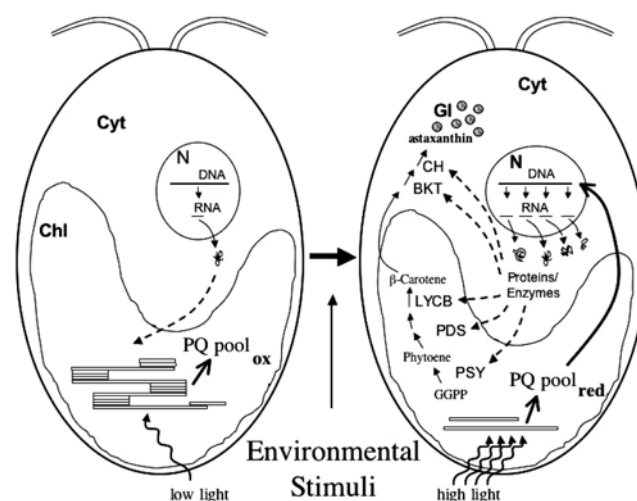

Fig. 3. Regulation of astaxanthin overaccumulation in algae of the genus *Haematococcus*.
The left panel shows a cell that does not overaccumulate astaxanthin when under nonstressed growth conditions. Upon environmental stress, cells overaccumulate astaxanthin through a mechanism that involves the induced expression of all genes of the carotenoid biosynthesis pathway studied so far. One hypothesis is that the redox state of the plastoquinone pool in the thylakoid membrane is involved in regulation of gene expression.

Table 1. Comparison of the regulation of all known enzymes of the carotenoid biosynthesis pathway of the alga *H. pluvialis*. +, upregulation; o, no change of mRNA or protein level; na, not known.

Enzyme	Level of expression after exposure to environmental stress	
	mRNA	protein
Isopentenyl pyrophosphate isomerase 1	+	o
Isopentenyl pyrophosphate isomerase 2	+	+
Phytoene synthase	+	na
Phytoene desaturase	+	+
Lycopene β-cyclase	+	o
β-Carotene hydroxylase	+	na
β-Carotene oxygenase	+	+

of genes participating in carotenoid biosynthesis in the alga *Haematococcus* at either the mRNA level, the protein level, or both [33, 72, 74]. The regulation of carotenogenesis is summarized in Fig. 3 and Table 1. In general, for *Haematococcus*, all investigated genes that code for enzymes involved in carotenoid biosynthesis were shown to be upregulated at the mRNA level in response to environmental stress conditions. Concerning the biosynthesis of precursors of the carotenoid biosynthesis pathway, Sun *et al.* [74] showed that both isopentenyl pyrophosphate isomerase (IPI) genes are upregulated at the mRNA level (Table 2). However, only the IPI2 found in the cytosol is also upregulated at the protein level. This differential expression suggests strict regulation at different levels (transcriptional/ translational/post-translational) of the initial steps in the biosynthesis of precursors for carotenoid biosynthesis. The PSY is the enzyme that catalyzes the entry-step into the carotenoid biosynthesis pathway. As mentioned above, this feature makes it a target for possible regulation of metabolic flux into the pathway. As expected, the mRNA of the phytoene synthase is upregulated in response to various stress conditions [72, 64]. The addition of sodium acetate, Fe^{2+}, and growth under high light leads to a strong induction of steady-state mRNA levels for the PSY gene

Table 2. Environmental factors affecting astaxanthin overaccumulation in the alga *Haematococcus*. Examples of recent studies are listed.

Environmental factor	Stimulus	Reference
Light	Increased irradiance	[35, 61]
Salt (NaCl)	High concentration	[66, 67]
Nutrients:		
Phosphate	Deficiency	[7]
Nitrogen	Deficiency	[7, 35, 61, 82]
$CaNO_3$	Presence	[67]
Acetate	Presence	[47, 60, 61, 67]
Malonate	Presence	[60, 61]

[72]. These conditions are reported to bring about the highest astaxanthin production. Unfortunately, nothing is known about the regulation of the PSY at the protein level. However, a promoter sequence for the PSY gene has recently been deposited in GenBank (Accession #DQ152009). For the first time, this will allow the investigation of gene regulation including promoter studies.

It is reported that the enzyme phytoene desaturase (PDS) is induced at the mRNA level and at the protein level in response to stress conditions that lead to SC biosynthesis [33]. Grünewald *et al.* [33] report that PDS is found exclusively in the chloroplast. This suggests that the carotenoid precursors of astaxanthin are made exclusively in the chloroplast. Because astaxanthin accumulates in vesicles in the cytoplasm, the export of carotenoids from chloroplast to cytoplasm must occur.

Because lycopene β-cyclase (LYCB) controls the metabolic flux towards zeaxanthin, a precursor of astaxanthin, the expression of LYCB was examined during the induction of astaxanthin biosynthesis [52, 73, 74]. As mentioned above, higher plants have LYCB genes that are differentially regulated. However, only one gene has been found for LYCB in *Haematococcus*. Although the level of mRNA increased upon exposure to environmental stress conditions, the protein level of the LYCB remained constant [73, 74]. This raises the question of whether more of the LYCB enzyme present in nonstressed cells becomes active in response to the exposure of cells to stress conditions.

Once the precursor β-carotene is made, two slightly different pathways for generation of astaxanthin are postulated. In both pathways, the enzymes β-carotene ketolase (CRTO), also called β-carotene oxygenase (BKT), and carotene hydroxylase (CH) are required. CRTO/BKT is upregulated at the mRNA level as well as at the protein level [32, 33, 74]. Grünewald *et al.* [32] showed that the β-carotene oxygenase is located in the chloroplast and in the cytosolic vesicles. Moreover, CRTO activity is demonstrated *in vitro* in the lipid vesicles, indicating that CRTO acts not only in the chloroplast, but also in the cytosol. For CH, only upregulation at the mRNA level has been demonstrated so far [52, 72, 73].

In many plant species, the total xanthophyll content increases under strong light and the ratio between lutein (L) and the xanthophyll-cycle components [zeaxanthin, antheraxanthin, and violaxanthin (Z+A+V)] decreases. Conversely, the ratio L:(Z+A+V) increases in low light. For example, a five-fold increase in the ratio between levels of *Lcy-b* and *Lcy-e* mRNAs in both *Arabidopsis* and tomato leaves was observed when plants were shifted from low light to high light [40]. This result suggests that the xanthophyll composition of the light-harvesting complexes can be modulated by excitation pressure. This hypothesis was confirmed in a recent study demonstrating that regulation of gene expression upon exposure to stress conditions is

linked to the redox state of the plastoquinone pool [72]. It was shown that blocking the photosynthetic electron transport chain at the level of plastoquinone reduction will either completely prevent or diminish the light-stress response of upregulation in gene expression.

It has previously been reported that reactive oxygen species (ROS)-generating compounds such as Fe^{2+}, methyl viologen, and methylene blue result in increased astaxanthin accumulation. This leads to the hypothesis that the stress response in *H. pluvialis* may be mediated by ROS [6, 21, 47]. However, results from Steinbrenner and Linden [73] suggested that ROS generators are not involved in the transcriptional regulation of PYS and carotenoid hydroxylase. In corroboration with this finding, previous reports showed that the effect of Fe^{2+} on astaxanthin accumulation is independent of *de novo* protein biosynthesis and it is suggested that there is a function of ROS at the post-translational level [47].

Only a few kinetic analysis of enzymes that are related to the biosynthesis of astaxanthin in *H. pluvilais* have been reported [14, 25, 26]. In these reports, astaxanthin synthesis requires oxygen, NADPH, and Fe^{2+} It was proposed that a cytochrome P450-dependent enzyme might be involved in the transformation of β-carotene to astaxanthin. The hydroxylase activity appears to be cytochrome P450-dependent. When hydroxylase activity is inhibited by ellipticine, which is a specific inhibitor of cytochrome P450 enzyme activity, only canthaxanthin accumulates [68]. The BKT enzymes are not so defined in their substrate preference, responding readily to fluctuations of substrate levels. However, the enzymes are strictly oxygen-requiring; and a cofactor mixture of 2-oxoglutarate, ascorbic acid, and Fe^{2+} is beneficial to activity.

Recently, in addition to studies of the enzymes of the carotenoid biosynthetic pathway, genomic [18] and proteomic [41, 78] studies were undertaken to investigate global gene and protein expression under stress conditions that lead to astaxanthin accumulation in *H. pluvialis*. Such global analysis of the process of carotenogenesis resulted in identification of numerous up- or downregulated novel genes and gene products not known from other organisms. It is possible that some of these gene products are involved in regulation of the stress response mechanisms in *H. pluvialis*.

Overall, the summary of results concerning enzyme functioning in the carotenoid biosynthetic pathway, shown in Table 2, demonstrates that all genes investigated so far are upregulated at the level of mRNA in response to stress. However, there exist at least two types of regulation at the translational level. These two types can be distinguished as (1) No change in the protein level of an enzyme (*e.g.*, lycopene β-cyclase) and (2) upregulation at the translational level (example, phytoene desaturase). A comparison of expression studies shows a lack of general study of the activity of enzymes involved in the carotenoid biosynthetic

pathway. In the future, differential regulation at the mRNA or protein level needs to be complemented with studies of enzymatic activities during induction of SC accumulation.

Metabolic Engineering of Astaxanthin Biosynthesis and Its Production

There is growing interest worldwide in manipulating carotenoid biosynthesis in plants. All of the carotenoid species that contain a β-ring can be converted to retinol, and therefore, precursors of vitamin A. Although this is the major value of carotenoids in human nutrition, additional health benefits are attributed to the antioxidant activity of carotenoids *in vivo* [28, 29, 44, 56, 62].

Industrial applications of carotenoids include their use as colorants for human food, feed additives to enhance the pigmentation of fish, eggs, cosmetics, and pharmaceutical products. As natural pigments, carotenoids furnish attractive colors to fruits, vegetables, and ornamental flowers. Their composition in these crops has enormous economic value. Specifically, astaxanthin provides a characteristic pink color to salmonids, trout, and shrimp. Cloning of most of the astaxanthin biosynthesis genes in *Haematococcus* has now opened the door to genetically manipulating this pathway not only in algae, but also in other organisms. Production of natural astaxanthin by genetically engineered microorganisms has been reported [57]. The highest yield achieved in *E. coli* is 1.4 mg/g dry weight (0.14%) [77]. Other carotenoids, such as lycopene, which is synthesized in *E. coli*, reached a concentration of 0.5% of dry weight [1]. Mutants of the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) accumulate (3*R*, 3*R*) astaxanthin up to 0.5% of the dry weight [11, 45].

Since the supply of the common precursors, IPP, DMAPP, GPP, FPP, or GGPP are limited in the bacterium *E. coli*, a host strain that increases the supply of GGPP gave the highest astaxanthin yield of 1.25 mg/g dry weight [77]. This is 50-fold higher than that of previous reports [9]. Tobacco plants that expressed *CRTO* in a regulated manner accumulated a high concentration of ketocarotenoids, including astaxanthin, in the chromoplasts of the nectary tissue in their flowers. This changed the flower color from yellow to red [53]. The concentration of the red ketocarotenoids in the nectary reached 0.2 mg/g fresh weight, which corresponds to ~2 mg/g dry weight (0.2%). However, petals of many plants contain considerably higher concentrations of carotenoids. Currently, the natural carotenoids lutein and zeaxanthin are commercially produced by extraction of *Tagetes* (marigold) flowers. The molecular mechanisms responsible for accumulation of carotenoids in various *Tagetes* cultivars are under investigation [58]. Converting flower carotenoids in *Tagetes* to astaxanthin by metabolic engineering could provide an abundant source of this pigment. However, in contrast to other organisms, cells of the alga *H. pluvialis* can naturally accumulate (3*S*, 3'*S*)

astaxanthin up to 4–5% of their dry weight [7, 81]. The reason that plants and algae accumulate carotenoids at concentrations that are 10- to 50-fold higher than other microorganisms is that they possess special mechanisms for storing large amounts of carotenoids inside lipid vesicles located in the cytoplasm or in lipid globules within plastids. Unfortunately, bacteria and yeast are not able to sequester the carotenoids in large amounts. High intracellular concentrations of carotenoids can damage the membranes of these cells. The property of plants and algae to sequester large amounts of carotenoids should be taken into account during development of the biotechnological production of carotenoids.

As expected, the astaxanthin molecule that is synthesized artificially in the nectary occurs in the "natural" molecular configuration of the stereoisomer 3*S*,3'*S*. This feature distinguishes the astaxanthin produced in plants from the synthetic pigment, which is obtained as a mixture of molecules where 75% have different chiralities than the natural (3*S*,3'*S*) astaxanthin [53]. The biological significance of the chirality of astaxanthin is not known. In the nectaries of transgenic plants, canthaxanthin and adonirubin are more abundant than zeaxanthin and adonixanthin, suggesting that the β-carotene ketolase (CRTO) preferentially uses β-carotene as a substrate and is more active than the hydroxylase. Trace amounts of ketocarotenoids found in the leaves of transgenic plants can be explained by the relatively low expression of *CRTO*, driven by the tomato PDS promoter, as indicated from the GUS assay. Unavailability of proper substrates for the β-carotene ketolase in chloroplasts and a lack of the ketocarotenoid-accumulating mechanism, i.e. binding proteins and lipid globules, could also affect astaxanthin formation in plant cells.

Cultivation of *H. pluvialis* and Enhancement of Astaxanthin Production

Microalgae convert solar energy efficiently [64]. Many attempts have been made to cultivate them in simple and inexpensive systems, such as shallow open ponds. Despite these ideal features, phototrophic single-species cultivation of microalgae has met only limited success, such as with the algae *D. salina* and *Spirulina*. This is because selective conditions for their growth exist. In contrast, there is no selective environment known that would allow selective cultivation of *H. pluvialis*. Nevertheless, *H. pluvialis* is grown commercially by various companies to generate astaxanthin. In order to grow *H. pluvialis* competitively on a grand scale in outdoor cultures, a large amount of inoculum must be produced under sterile conditions. The generation of the required amounts of inoculum is a major bottleneck for production of astaxanthin by the alga *H. pluvialis*. In order to improve productivity, various factors must be considered. Listed below are some of these factors with recent examples from the literature:

1. Strain improvement [11, 12]
2. Development of better growth media and induction conditions [19, Table 2]
3. Improvement of cultivation parameters including bioreactor design [15, 19, 35, 39, 49, 60]

Light is another problem in the phototrophic cultivation of microalgae. In dense mass cultures, algae are acclimated to low light conditions. As a consequence, the productivity of algal cultures is low. Tubular bioreactors, which overcome some problems, have not been commercially employed for the cultivation of *H. pluvialis*. More recently, an attempt has been made to grow *Haematococcus* autotrophically in a 30-l air-lift photo-bioreactor [38]. If only algal growth is considered, the production of a substantial biomass (10^5 cells/ml) requires no less than 3 weeks, with the unavoidable problems of contamination. Microalgae that utilize organic carbon substrates as their sole carbon and energy source may be used for heterotrophic growth. However, heterotrophic media may invite rapid bacterial contamination, which again may be overcome only by rigorous aseptic operations. *H. pluvialis* can be grown heterotrophically in a variety of media, most of which contain low concentrations of acetate and asparagine [48]. However, the specific growth rate of the flagellate under heterotrophic (dark) conditions is considerably lower than under autotrophic (light) conditions (0.22 day^{-1} compared with 0.32 day^{-1}). On the other hand, photoheterotrophic conditions, *i.e.*, media supplied with organic substrates in the presence of light, give the best results with a specific growth rate of 0.58 day^{-1}. Under the latter conditions, a cell concentration of up to 8×10^5/ml could be obtained in less than 7 days of batch cultivation in conical flasks [48]. The outcome of the complete cycle for carotenogenic microalgae is greatly dependent on algal concentrations and the time required to reach maximal cell density. Aplanospore formation under conditions that favor carotenogenesis, but not algal growth, may yield high astaxanthin values per cell, but very low yields on a volume basis. Among the various environmental conditions that affect the rate of carotenoid production, temperature and light are the most critical factors in both autotrophic and mixotrophic systems. Most authors use temperatures in the range of 20–25°C for astaxanthin formation. According to Fan *et al.* [22], increased temperatures lead to increased volumes of the cultivated cells. Light may be supplied continuously or with dark periods of 10±14 h. Irradiation levels, as measured at the surface of the culture, vary between 20 and 430 μmol photon $m^{-2} s^{-1}$. Fluorescent lights usually provide the indoor light source. Increased irradiation levels generally yield higher carotenoid concentrations [22]. The fact that *Haematococcus* has both a vegetative and an aplanospore phase, in which most of the astaxanthin is formed, has led many researchers to adopt a two-stage approach in the cultivation of *Haematococcus* for carotenoid production. While low photon flux densities

are provided during the first vegetative/green phase in the photoheterotrophic system, much higher irradiance levels (up to ten times higher) are supplied to induce aplanospore formation and astaxanthin biosynthesis [22, 31].

Induction of Ketocarotenoids Biosynthesis Yields

As shown in Table 2, many publications deal with the problem of induction of astaxanthin biosynthesis. From the biotechnological point of view, this issue is of great importance because artificial induction may cause the duration of the fermentation cycle to be significantly reduced. Therefore, many attempts have been made to better understand the conditions that regulate the transformation of green flagellated cells into aplanospores. From an earlier work, it was surmised that this transformation is a result of the slowing down of cell division or depletion of certain food ingredients or both [84]. For example, cells taken from the logarithmic phase and exposed to conditions of low nitrogen or phosphate concentrations induce carotenogenesis. However, some nitrogen content seems to be required for astaxanthin accumulation. It was assumed that the reduction in the rate of cell division was considered to be a prerequisite for cyst formation and astaxanthin overaccumulation. Nevertheless, it was recently demonstrated that the production of secondary carotenoids is not necessarily a process associated with the formation of resting cells [31].

Other systems have also been employed for the induction of cyst formation. Stress imposed by the exposure of cells to sodium chloride (up to 0.8%) was found to lead to encystment and astaxanthin accumulation [5]. Independently, Kobayashi *et al.* [50] showed that various chlorides (0.1% NaCl, $MgCl_2$) induced aplanospore formation and carotenogenesis under dark heterotrophic conditions. Light-independent induction yielded much lower carotene values compared with those achieved under illumination. However, it should be emphasized that salt induction also leads to a considerable reduction in cell density [5, 38].

In order to ensure accelerated carotenoid production, Kobayashi *et al.* [47] used culture supplementation. After 4 days of growth, sodium acetate and ferrous sulfate were added at 45 mM and 450 μM, respectively, producing good yields within 5 days. The reported yields of astaxanthin were 100 pg/cell and less. In fermentation liquids, usually less than 10 mg/l was obtained after 5–8 days of growth. These data, together with the fact that usually less than 1% algal dry material/l is obtained, must be kept in mind during any feasibility studies for commercial uses. Obviously, in order to secure high yields in the carotenogenic phase, a high density of vegetative growth is required. When the acetate/asparagine-containing medium developed by Kobayashi and colleagues [46] was used, cell densities of 10^5/ml and above (1.0–1.5 g/l dry weight) were obtained within 6–8 days of cultivation.

Overall, secondary carotenoid formation by *H. pluvialis* can be influenced by nitrogen limitation, strong light intensity, pH, salinity, and organic nutrients such as acetate (Table 2). In general, almost any factor that causes cessation of growth can have an effect on secondary carotenoid accumulation [5, 6, 38, 45, 60, 61, 63]. However, more

Table 3. Some major producers of natural astaxanthin from *Haematococcus* and their merchandise brand name (NA, not applicable).

Company	Trademark	Use	Web site
Mera Pharmaceuticals (former Aquasearch), U.S.A.	Astafactor[TM]	Antioxidant	http://www.merapharm.com, http://www.astafactor.com,
Cyanotech Corp., U.S.A.	BioAstin[R], NatuRose[R]	Antioxidant, neutraceutical, aquaculture	http://www.cyanotech.com, http://www.bioastin.com
Nutrex Hawaii Inc., U.S.A.	BioAstin[R]		http://www.nutrex-hawaii.com
BioReal, Inc. (former MicroGia and subsidiary of Fuji Chemical Industry Co., Ltd.), U.S.A.	NA	Neutraceutical, aquaculture, pharmaceutical	http://www.bioreal.com
Valensa International, U.S.A.[a]	Zanthin[R]	Antioxidant	http://www.usnutra.com/
AstaReal (subsidiary of Bioreal, Inc.), Sweden	AstaCarox	NA	http://www.astareal.com
SUBITEC GmbH, Germany	NA	NA	http://www.subitec-gmbh.de
Algatechnologies Ltd., Israel[b]	NA	Animal feed, neutraceutical	NA
Natural Astaxanthin Co. Ltd. (PA-Shun Group), China	NaturAsta[TM]		http://www.pa-shun.com/xiaqingsu/astaxanthin.htm
Parry Neutraceuticals (Murugappa Group), India	OleoResin	Neutraceutical	http://www.parrynutraceuticals.com

[a]Not a primary producer.
[b]Exclusive production for the Japanese market.

knowledge of the intracellular triggering factors at the molecular level that lead to high secondary carotenoid accumulation in different microalgae is required for developing strategies towards optimization of SC production.

Commercial Production of "Natural" Astaxanthin Using *Haematococcus* Algae

Until a few years ago, astaxanthin was used only in aquaculture. More recently, however, because of its antioxidant activity and potential role in human health, astaxanthin has received increasing attention. This attention and new research findings have prompted companies to grow *Haematococcus* in mass cultures for production of natural astaxanthin. Table 3 lists several companies that are currently engaged in or plan to launch cultivation of *Haematococcus*. All the companies are marketing the positive health effects and powerful biological antioxidant activity of astaxanthin when it is included in the human diet. Several companies protect their formulas containing astaxanthin and its esters with patents or trademarks (Table 3). A recent review by Guerin *et al.* [34] dealt with the variety of uses of astaxanthin. Some companies dedicate specific Web sites (Table 3) to their products, providing information about the proven benefits of astaxanthin. The Web sites offer information about the structure of astaxanthin, differences between natural and synthetic astaxanthin, action of astaxanthin as an antioxidant, and safety for human consumption.

An increasingly important issue for marketing astaxanthin produced by *Haematococcus* is the emphasis on the "natural" process. Any product containing astaxanthin obtained from *Haematococcus* algae grown in outdoor open ponds could be labeled as a "natural" product. This will promote sales, especially when it is advertised as a neutraceutical. According to Mera Pharmaceuticals, Inc. and Cyanotech, Inc., the estimated market for synthetic astaxanthin in salmon and trout aquaculture is about $185–200 million. Although the potential market for natural astaxanthin is large, it is a cutting edge market and any increase in competition that results in lower prices could ruin existing companies. In this context, it is interesting to note that to date, no or only limited commercial astaxanthin production has been reported from China. However, with growing interest in algal mass cultivation and increasing knowledge transfer, it is expected that *Haematococcus* will soon be used for astaxanthin production in China.

Acknowledgments

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