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Xanthophylls in Microalgae: From Biosynthesis to Biotechnological Mass Production and Application

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Abstract Xanthophylls are oxygenated carotenoids that serve a variety of functions in photosynthetic organisms and are essential for survival of the organism. Within the last decade, major advances have been made in the elucidation of the molecular genetics and biochemistry of the xanthophyll biosynthesis pathway. Microalgae, yeast, or other microorganisms produce some of the xanthophylls that are being commercially used due to their own color and antioxidant properties. Currently, only a few microalgae are being considered or already being exploited for the production of high-value xanthophylls. However, new developments in molecular biology have important implications for the commercialization of microalgae, and make the genetic manipulation of the xanthophyll content of microalgae more attractive for biotechnological purposes. Accordingly, the current review summarizes the general properties of xanthophylls in microalgae and the recent developments in the biotechnological production of xanthophylls.

Key words: Xanthophylls, microalgae, photoprotection, biotechnology, commercialization

Carotenoids are among the most common pigments in nature [3, 17]. Most naturally occurring carotenoids are hydrophobic tetraterpenoids that contain a C_{40} methyl-branched hydrocarbon backbone. The polyene chains of carotenoids, consisting of conjugated double bonds, are responsible for the pigmentation of carotenoids and their ability to absorb photons in visible wavelengths. Carotenoids are synthesized by all photosynthetic organisms as well as by many non-photosynthetic bacteria and fungi. There are two main classes of naturally occurring carotenoids: carotenes, which are hydrocarbons that are either linear or cyclized at one or both ends of the molecule (such as β-carotene, α-carotene); and xanthophylls, which are oxygenated derivatives of carotenes. All xanthophylls produced by higher plants, for example violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein, are also synthesized by green algae. However, in contrast to land plants, specific green algae possess additional xanthophylls such as loroxanthin [5], astaxanthin [35], and canthaxanthin [35]. In addition, diatoxanthin, diadinoxanthin, and fucoxanthin are produced in brown algae or diatoms [55, 56].

In general, a distinction can be made between primary and secondary carotenoids. Primary xanthophylls are here defined as xanthophylls that are structural and functional components of the photosynthetic apparatus of the cell and therefore essential for cellular survival. Secondary xanthophylls are defined as xanthophylls that microalgae produce in large quantities only after having been exposed to specific environmental stimuli (carotenogenesis). Although many microalgae are known to produce various levels of xanthophylls [13], at present, only algae, such as *Haematococcus pluvialis* that accumulate secondary xanthophylls in large quantities, are commercially grown on a large scale. One determining factor is that the cutting edge market of microalgal product development dictates that the organism of choice produces as much of the high-valuable xanthophylls as possible. This constraint also explains why, so far, only a few microalgae are used for the production of high-value xanthophylls.

Biosynthesis of Xanthophylls and Enzymatic Reactions

In the past 10 years, the biochemistry of carotenogenesis and especially the cloning of carotenogenic genes have made considerable progress. Availability of these genes was a great help in the characterization of enzymes in the xanthophyll pathways and their regulation. In return, biochemical insights facilitated the cloning of novel carotenogenic genes. A summary of the chemical structures and xanthophyll biosynthetic pathways of microalgae is

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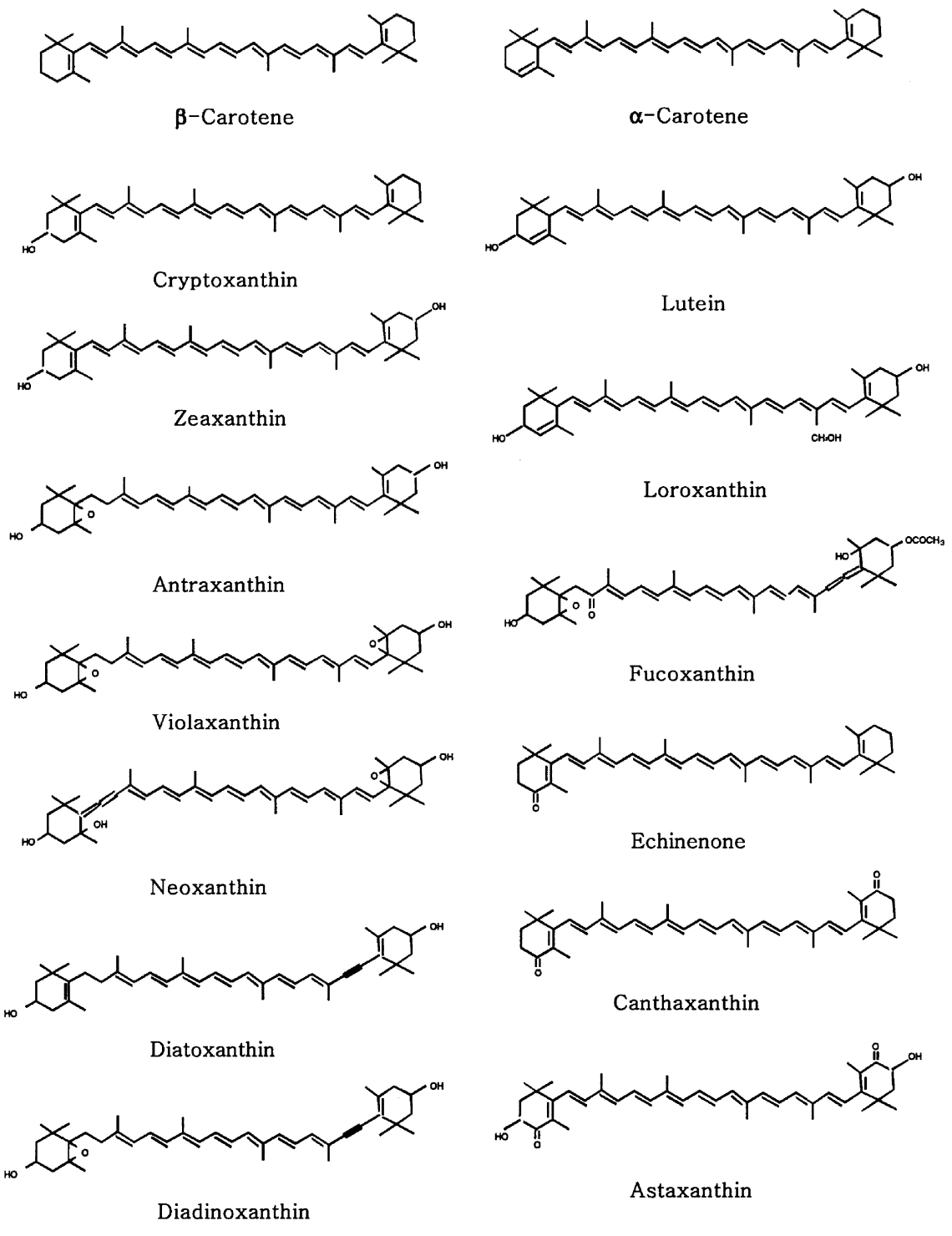

Fig. 1. Chemical structures of xanthophylls and carotenes found in microalgae.

shown in Fig. 1 and Fig. 2. In addition, Table 1 specifies known genes and enzymes involved in xanthophyll biosynthesis. Lycopene is cyclized on both ends by the enzyme lycopene β-cyclase to form β-carotene. The two beta rings of β-carotene are subjected to identical hydroxylation reactions to yield zeaxanthin, which in turn is epoxidated once to form antheraxanthin and twice to form violaxanthin. Neoxanthin is derived from violaxanthin by an additional rearrangement [72]. Higher plants and green algae have additional carotenoids, α-carotene derivatives (β,ε-carotenoids),

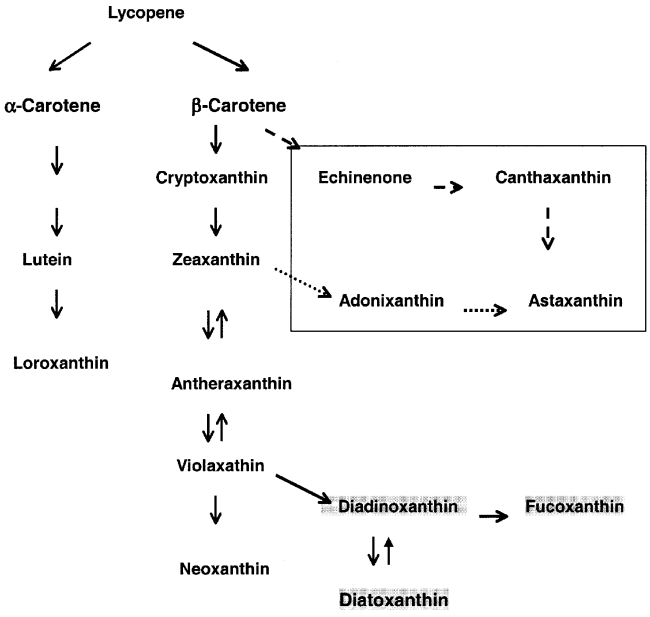

Fig. 2. Schematic diagram of pathway of xanthophyll biosynthesis in microalgae.
The pathway, connected by a solid arrow, shows the primary xanthophyll biosynthetic pathway in green algae. The secondary astaxanthin biosynthetic pathway is also presented in the box, where there are two possible ways of astaxanthin biosynthesis. The proposed pathway for xanthophyll synthesis in diatom is also included with gray font letters.

which are derived from lycopene by the action of two structurally related enzymes, lycopene β-cyclase and lycopene ε-cyclase. Hydroxylation of the β-ring and ε-ring of α-carotene forms lutein [69, 70]. During xanthophyll formation, the carotenoid structures are modified such that the end product pigments are often species-specific (Fig. 2). Although the biosynthesis of the secondary xanthophyll astaxanthin has not been fully elucidated, it is widely assumed that the early part of carotenoid biosynthesis follows a similar pathway to that found in other green algae [59]. Also the presence of echinenone (one keto group) and canthaxanthin (two keto groups) indicates that astaxanthin synthesis requires keto carotenoids prior to the hydroxylation of β-carotene [59, 80]. Therefore, the conversion of β-carotene into astaxanthin in *H. pluvialis* is carried out by the two enzymes β-carotene ketolase and carotenoid hydroxylase. Besides green algae, several groups of algae, such as diatoms, dinophytes, and haptophytes, are known to contain more diverse xanthophyll pigments, including diatoxanthin, diadinoxanthin, and fucoxanthin. Recently, a biosynthetic pathway has been proposed (Fig. 2, designated by gray letter font). Detailed studies on diatoms have also revealed that violaxanthin is an intermediate in the biosynthesis of diadinoxanthin, and this in turn can serve as a precursor of fucoxanthin [56].

Localization of Xanthophylls in Microalgae

Xanthophylls are relatively hydrophobic molecules. Therefore, they are typically associated with membranes and/or non-covalently bound to specific proteins. In general, primary carotenoids are localized in the thylakoid membrane, while secondary carotenoids are found in lipid vesicles either in the plastid stroma or the cytosol. Most xanthophylls that are found in cyanobacteria and oxygenic photosynthetic bacteria are associated with chlorophyll (Chl)-binding polypeptides of the photosynthetic apparatus [34]. Among non-photosynthetic bacteria and, to a lesser extent, among photosynthetic bacteria and cyanobacteria, xanthophylls and their glycosides can be found in cytoplasmic and cell wall membranes where they are thought to influence membrane fluidity [3].

In the majority of green algae, most carotenes and xanthophylls are synthesized within plastids and only

Table 1. Enzymes and genes that contribute to the biosynthesis of various xanthophylls found in algae. Through a number of different combinations of successive reactions a variety of xanthophylls can be produced.

Genes	Enzymes	Function	References
Formation of alpha and/or beta carotene from lycopene			
lcyE, crtL-e	Lycopene ε-Cyclase	Introduction of ε-rings	9, 79
lcyB, crtL-b	Lycopene β-Cyclase	Introduction of β-rings	18, 82
Formation of cyclic xanthophyll from carotene			
chyE	ε-ring Hydroxylase	Hydroxylation of ε-rings	79,
chyB, crtZ (crtr-b)	β-ring Hydroxylase	Hydroxylation of β-rings	3, 35, 58
Introduction of a keto-group in the 4 position of β-rings			
crtO	β-C-4 Oxygenase	Introduction of a keto-	40,
crtW, bkt	β-Carotene Ketolase	group in the 4 position of β-rings	3, 35, 62, 82
Epoxidation and deepoxidation of β-rings of carotene			
zep	Zeaxanthin Epoxidase	Epoxidation of β-rings	45, 65
vde	Violaxanthin Deepoxidase	Deepoxidation of β-rings	65

* crt designation for genes that encode carotenoid biosynthesis enzymes has now been adopted by many researchers in this field.

accumulate within plastids. By contrast, in some green algae, secondary xanthophylls such as astaxanthin in *Haematococcus* accumulate in the cytoplasm. This accumulation of xanthophylls in the cytosol raises the possibility that there is an extra-plastidic site of carotenoid biosynthesis in *Haematococcus*. Alternatively, xanthophylls synthesized in the chloroplast may be exported and accumulate in the cytoplasm [71, 83]. In summary, it is important to note that xanthophylls can be found in all cellular compartments.

Biological Function of Xanthophylls in Microalgae

The function of xanthophylls in photosynthetic microalgae is manifold. Xanthophylls can function as accessory light-harvesting pigments, structural entities within the light-harvesting complexes (LHC), molecules required for the protection of photosynthetic organisms from the potentially toxic effects of light, and antioxidants in membranes to mitigate against lipid peroxidation [5].

In almost all photosynthetic eukaryotes, the majority of xanthophylls are bound with chlorophyll (Chl) molecules to proteins in the integral membrane, LHCs [32, 33, 44]. The LHCs absorb and transfer excitation energy to the photosynthetic reaction centers to drive electron transport: photosynthetic reactions convert light energy into chemical energy that is used to fix atmospheric CO_2 into sugars. It is generally accepted that xanthophylls serve as essential structural components in the light-harvesting complexes of plants, including microalgae [28, 50]. Xanthophylls also function as accessory light-harvesting pigments by absorbing photons and transferring them to Chl molecules [53, 73].

Another paramount function of xanthophylls in all photosynthetic organisms, including cyanobacteria, is to provide photooxidative protection. The importance of carotenoids in photoprotection is evident from the phenotypes of organisms that cannot synthesize carotenoids, either as a consequence of mutations or treatment with herbicides (e.g., norflurazon) that block carotenoid biosynthesis [64, 85]. There are several mechanisms by which xanthophylls function to protect plants against photodamage. For example, in one proposed mechanism, zeaxanthin protects the membrane directly against lipid peroxidation by reactive radicals that have been created as toxic byproducts during photosynthetic reactions. By yet another mechanism, specific xanthophylls are involved in the deexcitation of singlet Chl (^{1}Chl) that accumulates in the LHC under conditions of excessive illumination [23, 24, 25, 30, 35, 41]. This deexcitation, measured as non-photochemical quenching of Chl fluorescence (NPQ), depends on a large trans-thylakoid proton gradient that is established in excessive light. In general, the development of NPQ correlates with the synthesis of zeaxanthin and antheraxanthin from violaxanthin via the xanthophyll cycle [22, 24, 31, 32]. When photosynthetic irradiance is greater than that required for the saturation of photosynthesis in the chloroplasts of plants and green alga, a reversible violaxanthin deepoxidation reaction occurs to form antheraxanthin and subsequently zeaxanthin, resulting in the accumulation of zeaxanthin in the chloroplast thylakoids. The enzyme that catalyzes this reaction, violaxanthin deepoxidase, is localized in the lumen of the chloroplast thylakoids [38]. When the absorbed irradiance is lower than that required for saturation of photosynthesis, zeaxanthin is converted back to violaxanthin by the enzyme zeaxanthin epoxidase [37], with the monoepoxidide antheraxanthin being an intermediate in this reversible oxidation-reduction process. Genetic studies with *Arabidopsis thalina* [74, 75], *Nicotiana plubaginifolia* [60], and the green alga *Chlamydomonas reinhardtii* [65] revealed the presence of a single gene coding for the zeaxanthin epoxidase enzyme. Thus, a single gene product is apparently responsible for both the biosynthesis of violaxanthin during growth and development and the epoxidation reaction, leading to the return of zeaxanthin via antheraxanthin to violaxanthin following recovery after irradiance stress. Mutants with lesions in the zeaxanthin epoxidase gene are consequently deficient not only in antheraxanthin and violaxanthin, but also fail to synthesize neoxanthin [8, 45, 65]. In addition, these mutants accumulate, even when grown under nonstressed conditions, large amounts of zeaxanthin that are almost equivalent to those levels of violaxanthin found in the wild-type. It was shown recently that under moderate light intensities, the photosynthetic efficiency was unaffected in mature mutant plants lacking antheraxanthin, violaxanthin, and neoxanthin [42, 45, 72, 83]. Analogous mutations affecting zeaxanthin production exist in green algae, *Scenedemus obliquus* [9], *Chlamydomonas reinhardtii* [65], and *Dunaliella salina* [45]. Also, in mutants of these microalgae, no significant differences in the photosynthetic efficiency and photosynthetic capacity have been observed under moderate growth conditions.

Other xanthophylls of Chl *a/c*-containing algae, e.g., fucoxanthin (Fig. 1) in diatoms and brown algae, or peridinin in dinophytes, are present in the LHCs of those algae, thereby playing the same role as lutein and violaxanthin in the light harvesting complexes of higher plants and green algae [82]. In those algal groups, the xanthophyll cycle is replaced by a xanthophyll-cycle alternating diadinoxanthin with diatoxanthin [55, 56, 81, 86]. This cycle comprises a single deepoxidation step, because only one of the ionon rings of diadinoxanthin carries an epoxide group (Fig. 1 and 2). Epoxidation of the second ionon ring by the respective xanthophyll-cycle epoxidase does not occur. As is the case with zeaxanthin in green algae, the formation of diatoxanthin correlates with a higher ability for nonradiative relaxation of singlet chlorophyll (Chl) [4, 21, 66] and is assumed to act by the same mechanisms as the cycle in green algae [66].

Application and Biotechnological Production of Xanthophylls from Microalgae

Currently, the xanthophylls lutein and its stereoisomer zeaxanthin as well as astaxanthin are used as neutraceuticals against macular degeneration. Lutein and zeaxanthin are known to play a critical function in maintaining a normal visual function. These polar compounds are the predominant carotenoids in the macula, while other nonpolar carotenoids, including β-carotene and lycopene (the principal circulating carotenoids) [49] are absent [11, 39, 87]. High concentrations of lutein and zeaxanthin are responsible for the yellowish color of this region in the retina, designated as the macula lutea or "yellow spot." The function of the macular pigments explains why zeaxanthin and lutein, as opposed to β-carotene or any of the other carotenoids, are presented in the macular. The most striking characteristic of macular pigments is their ability to absorb and attenuate blue light striking the retina. One functional benefit of xanthophyll presence is that it reduces chromatic aberration in the eyes [52, 76].

Lutein is also an important xanthophyll that creates the pigmentation in fish and poultry, plus it is used for the coloration of drugs and cosmetics. Sales of lutein as a feed additive in the United States amount to about $150 million per year [20]. Regulations on the use of synthetic dyes in the food industry are very stringent. As such, this has stimulated research and development of the production and use of carotenoids from microalgae as food additives. Carotenoids have also been proposed as effective preventive agents for a variety of human diseases. Lutein, for example, has been claimed to display cancer-preventing properties [54, 72, 92]. In addition, intake of lutein has been strongly correlated with a decreased risk of cataracts and age-related retinal degeneration [43, 72, 75, 78]. Currently, the chlorophycean microalgae *Muriellopsis* sp., which has a high lutein content (up to 35 mg l^{-1} culture under specific culture conditions), as well as high growth rate and standing cell density [19], is being exploited for the production of lutein.

In general, the zeaxanthin content of microalgae is regulated by light irradiance, therefore, nonstressed photosynthetic organisms do not contain much zeaxanthin. However, recently, a zeaxanthin-overproducing mutant strain *zea1* generated from *Dunaliella salina* [45, 46] has been considered for commercial exploitation. This mutant strain has a defect in the zeaxanthin-epoxidation step. Thus, the *zea1* mutant lacks neoxanthin, violaxanthin, and antheraxanthin, but constitutively accumulates zeaxanthin in the thylakoid membrane even under normal growth conditions. Under normal growth conditions (low-light), the mutant strain has a 15-fold higher zeaxanthin content on a per cell basis than the wild-type. Previous efforts to generate zeaxanthin overproducing *E. coli* strains using metabolic engineering [1] have resulted in the production of 1.6 mg zeaxanthin/g dry weight, however, this value is just one third of that produced by the *zea1* strain of the photosynthetic microalgae *Dunaliella salina* (6 mg zeaxanthin/g dry weight) [45]. Also, trials for the increased production of zeaxanthin using the photosynthetic bacteria *Synecocystis* sp. resulted in a 2.5-fold increase in zeaxanthin accumulation in the mutant strain [51].

Astaxanthin is ubiquitous in nature, especially in the marine environment, and is probably best known for eliciting the pinkish-red hue in the flesh of salmonoids, shrimp, lobsters, and crayfish. Because these animals are unable to synthesize astaxanthin *de novo*, carotenoid pigments must be supplied through their diet. In the marine environment, astaxanthin is biosynthesized in the food chain by microalgae or phytoplankton, as the primary production level. Microalgae are consumed by zooplankton, insects, or crustaceans that accumulate astaxanthin and which, in turn, are ingested by larger animals that will then take on a pinkish-red color [29, 47]. One typical example of a xanthophyll-producing unicellular microalga is *Haematococcus pluvialis*, well known for its massive accumulation of ketocarotenoids, mainly astaxanthin and its acylesters, in response to various stress conditions, *e.g.* nutrient deprivation or high irradiation [15, 48, 68]. Different functions of astaxanthin in *Haematococcus pluvialis*, such as acting as a sunshade [36], protecting from photodynamic damage, or minimizing the oxidation of storage lipids [82], have all been proposed. There is growing commercial interest in the biotechnological production of astaxanthin, due to its antioxidative properties and the increasing amounts needed as a supplement in the aquaculture of salmonoids and other seafood [57]. *Haematococcus pluvialis* is one of the preferred microorganisms for this purpose, because it accumulates astaxanthin up to 4% of its dry mass [15]. Recently, the expression of a *Haematococcus pluvialis* β-C-4-oxygenase in *Synechococcus* PCC7942 [40] has been attempted to explore the possibilities of producing the valuable ketocarotenoid astaxanthin in organisms in which this pigment is not normally made. The gene encoding β-C-4-oxygenase (*crtO*), converting β-carotene to canthaxanthin, was cloned from the green alga *Haematococcus pluvialis*. The β-C-4-oxygenase gene was then transferred to the cyanobacterium *Synechococcus* PCC7942, which contains a β-carotene hydroxylase gene and normally accumulates β-carotene and zeaxanthin. The new genetically engineered cyanobacterium produced astaxanthin as well as other ketocarotenoids. These results confirm that *crtO* can function in cyanobacteria in conjunction with the intrinsic carotenoid enzymes to produce astaxanthin. Specifically, this finding indicates that the enzyme β-carotene hydroxylase, which normally converts β-carotene to zeaxanthin, can also function in the biosynthesis of astaxanthin. Besides this transgenic approach to increase production of astaxanthin, and in addition to the already

commercially exploited microalga *Haematococcus pluvialis*, *Chlorococcum* sp. strain MA-1 has been developed for culture systems to produce astaxanthin and other ketoxanthophylls [91]. Other microalgae are also under investigation for their potential in the commercial application of astaxanthin production [67].

Factors Determining Xanthophyll Production by Microalgae and Microalgal Cultivation

The most important feature of microalgae is, of course, their photosynthetic ability, which makes them promising organisms for photoautotrophic cultivation on simple mineral media for various biotechnological purposes. Since microalgae convert solar energy efficiently, many attempts have been made to cultivate them in simple systems, such as shallow open ponds, race-way ponds, or large round open ponds [2, 7, 8]. In spite of the many attractive features of microalgae, phototropic single-species cultivation of microalgae has so far had only limited success. Severe contamination by bacteria or protozoa has made large-scale commercial propagation possible only if suitable selective environments can be assured. Thus, currently, only a few microalgae are being commercially used. *Dunaliella salina*, a halo-tolerant alga, is being cultivated in open ponds under high saline conditions, while another microalga, *Sprirulina platensis*, is successfully cultivated in highly alkaline (pH-9.2) waters. Until now, no selective environment has become available for *Haematococcus pluvialis*. Therefore, photobioreactor systems, including tubular bioreactors, are being employed to produce astaxanthin [57].

Among the various environmental conditions that affect the rate of xanthophyll production, temperature and light are the most critical factors in microalgal cultivation. In addition, nutrition, salinity, and pH are also important factors to be considered to induce the production of xanthophylls. Irradiance appears to induce increased levels of astaxanthin in *Haematococcus* or zeaxanthin in *Dunaliella* and *Muriellopsis* sp. [8, 10, 20, 27, 48, 68, 77]. The zeaxanthin concentration in high-light grown cultures (2,000 μmol photon m^{-2} s^{-1}) of *Dunaliella* is increased 10 times [46] compared to that in low-light (100 μmol photon m^{-2} s^{-1}) grown cultures. Astaxanthin and canthaxanthin accumulation in *Muriellopsis* sp. exhibits a similar pattern, being maximal at the highest irradiance [19].

The effect of temperature on astaxanthin accumulation in *Haematococcus* has also been reported. It has been suggested that endogenously generated active oxygen is responsible for stimulation of carotenogenesis by high temperature in *Haematococcus* [13, 84]. Also, the level of lutein per cell in *Muriellopsis* sp. increases about six-fold, when the temperature is raised from 28 to 33°C. Nonetheless, the maximal lutein concentration in the culture (about 30 mg l^{-1}) was obtained at 28°C, since the maximum standing cell density was considerably higher at this temperature.

Another factor determining the amount of carotenoids and initiation of secondary carotenoid accumulation in microalgae is the nitrogen availability [13, 14, 16, 19]. Nitrogen limitation results in the initiation of carotenogenesis and increases the level of astaxanthin in *Haematococcus* and other microalgae [15]. However, it is also important to distinguish whether the xanthophylls that are being investigated are primary (photosynthetic) or secondary xanthophylls. For example, nitrogen limitation resulted in decreased levels of the primary xanthophyll lutein in cultures of *Muriellopsis* sp., yet the lutein concentration in this culture was doubled when the nitrate in the medium was increased, remaining practically constant at higher nitrate concentrations. In contrast, the nature of the nitrogen source ($NaNO_3$, NH_4Cl, or NH_4NO_3) did not influence the lutein level in *Muriellopsis* sp. [19]. As such, the data show that high nitrogen concentrations favor the accumulation of lutein in *Muriellopsis* sp., which may reflect a need for the continued synthesis of the light-harvesting protein and its structural xanthophyll lutein under optimal growth conditions.

Enhanced carotenoid accumulation at extreme pH values has also been reported for *Chlamydomonas zofingiensis* and *Dunaliella salina* [8, 12]. The maximum lutein level in the culture (32.5 mg l^{-1}) was obtained when the cells were grown at pH 6.5, while the levels of lutein decreased markedly at higher and lower pH values. However, when the data were alternatively expressed on a per cell basis, the maximum levels of lutein were obtained at pH 6 and 9, being five to seven-fold higher than those at pH 6.5. This is a significant finding, because it demonstrates clearly the importance of the maximum cell density of mass cultures, when considering the commercial application of a microalga.

Salinity is a major factor inducing carotenogenesis in some algae, such as *Haematococcus* and *Dunaliella* [6, 13, 14, 16]. However, the lutein level in *Muriellopsis* sp. per cell was virtually constant at the different NaCl concentrations assayed [19]. Moreover, salinity does not seem to influence lutein accumulation in other algae. From this comparison of the effect of salinity on the xanthophyll concentration in various microalgae, it would seem that changes in salinity only affect the accumulation of secondary rather than the level of primary carotenoids.

Molecular Biology and Genetic Manipulation of Carotenoid Biosynthesis Pathway

The recent genetic elucidation of bacterial and plant carotenoid biosynthetic pathways leading to the accumulation of zeaxanthin, canthaxanthin, and astaxanthin may offer interesting alternatives for their *in vivo* production [61, 62, 63, 88]. For example, blue-green algae can be readily transformed with autonomously replicating plasmids, while endogenous genes can be disrupted by homologous

recombination. A number of commercial possibilities have been proposed for recombinant blue green algae [51]. In a recent report, *Synecocystis* sp. strain PCC 6830 was used as a transformation host to overproduce zeaxanthin *in vivo*. Furthermore, the system developed in that study allowed for gene replacement without the introduction of antibiotic resistance cassettes in the final overexpressing strains. The absence of cassettes containing genes that confer antibiotic resistance in such strains is a positive feature highlighting the increasing desire of the biotechnology industry to avoid spreading antibiotic resistant cassettes, thereby respecting the concerns of consumers and environmentalists.

Advances in eukaryotic algal recombinant techniques have recently been extensively reviewed [80]. The unicellular green alga *Chlamydomonas reinhardtii* has developed into a sophisticated molecular system that has made important contributions to the understanding of photosynthetic and other cellular processes. Although recombinant *Chlamydomonas* does not at this time have direct commercial applications, the molecular and genetic technology developed for *Chlamydomonas* has provided direction for the development of transformation techniques using other algae. Recently developed transformation techniques for *Chlorella* [18] and diatoms [2, 26, 90] have potential use in direct commercial applications. At the very least, recombinant techniques in economically valuable algae can provide heterotrophy [89], thereby increasing the cell mass per liter and enhancing the economic value of algae. However, at this point, algal strains generated through new recombinant techniques are not being used commercially on a large scale, because products from genetically modified organisms are not accepted on the European and American markets.

Conclusions and Prospects

Xanthophylls serve a variety of functions in photosynthetic organisms and are essential for the survival and the ecological success of these organisms in their environment. Despite their roles and great abundance, information on the biosynthesis of xanthophylls is still incomplete. However, major progress has recently been achieved through the development of new molecular tools that facilitate the partial dissection of the biosynthetic pathway of xanthophylls in microalgae. To date, the majority of carotenoids used industrially are chemically synthesized (astaxanthin, β-carotene, etc). However, as more complex xanthophylls reveal their pharmaceutical value, mass culturing in photobioreactors or fermentors using natural or genetically modified microalgae may become indispensable for xanthophyll production. In this regard, the genes encoding enzymes that can produce many species-specific xanthophylls still need investigating. Further development of transformation techniques in microalgae promises to increase the cellular xanthophyll content, which, depending on consumer acceptance, may become commercially attractive. In this regard, it has to be stressed that the major factor for the commercial application of new recombinant technologies is the public acceptance of products generated with genetically modified microalgae. If this constraint of consumer opposition can be overcome by safer and better molecular and genetic methods for algal systems, significant contributions could be realized in the near future.

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REFERENCES

1. Albrecht, M., N. Misawa, and G. Sandmann. 1999. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechnol. Lett.* **21:** 791–795.
2. Apt, K. E. and P. G. Kroth-Pacific. 1996. Stable nuclear transformation of diatom *Phaeodactylum tricornutum*. *Mol. Gen. Genet.* **252:** 572–579.
3. Armstrong, G. A. 1997. Genetics of eubacterial carotenoid biosynthesis: A colorful tale. *Annu. Rev. Microbiol.* **51:** 629–659.
4. Arsalane, W., B. Rousseau, and J.-C. Duval. 1994. Influence of the pool size of the xanthophyll cycle on the effects of light stress in a diatom-competition between photoprotection and photoinhibition. *Photochem. Photobiol.* **60:** 237–243.
5. Baroli, I. and K. K. Niyogi. 2000. Molecular genetics of xanthophylls-dependent photoprotection in green algae and plants. *Phil. Trans. R. Soc. Lond. B.* **355:** 1385–1394.
6. Ben-Amotz, A., A. Katz, and M. Avron. 1982. Accumulation of β-carotene in halotolerant algae: Purification and characterization of β-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *J. Phycol.* **18:** 529–537.
7. Ben-Amotz, A. and M. Avron. 1990. The biotechnology of cultivating the halotolerant alga *Dunaliella*. *Trends Biotechnol.* **8:** 121–126.
8. Ben-Amotz, A., A. Shaish, and M. Avron. 1989. Mode of action of the massively accumulated β-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. *Plant Physiol.* **91:** 1040–1043.
9. Bishop, N. I., Y. Urbig, and H. Senger. 1995. Complete separation of the β,ε and ββ-carotenoid biosynthesis by a unique mutation of the lycopene cyclase in the green alga, *Senedesmus obliquus*. *FEBS Lett.* **367:** 158–162.
10. Bishop, N. I., B. Bulga, and H. Senger. 1998. Photosynthetic capacity and quantum requirement of three secondary mutants of *Scenedesmus obliquus* with deletions in carotenoid biosynthesis. *Bot. Acta* **111:** 231–235.
11. Bone, R. A., J. T. Landrum, L. Fernandez, and S. L. Tarsis. 1988. Analysis of the macular pigment by HPLC: Retinal

distribution and age study. *Invest. Ophthalmol. Vis. Sci.* **29:** 843–849.
12. Borowitzka, M. A. 1988. Vitamins and fine chemicals from microalgae, pp. 153–196. *In* Borowitzka, M. A. and L. J. Borowitzka (eds.), *Micro-algal Biotechnology.* Cambridge University Press, Cambridge, U.K.
13. Borowitzka, M. A. 1992. Comparing carotenogenesis in *Dunaliella* and *Haematococcus*: Implcations for commercial strategies, pp. 301–310. *In* Villa, T. G. and J. Abalde (eds.), *Profiles on Biotechnology.* Servicio de Publicaciones, Universidad de Santiago, Santiago de Compostela, Spain.
14. Borowitzka, M. A., J. M. Huisman, and A. Osborn. 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.
15. Boussiba, S. 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: Cellular physiology and stress response. *Physiol. Plant.* **108:** 111–117.
16. Boussiba, S. and A. Vonshak. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol.* **32:** 1077–1082.
17. Daun, H. 1988. The chemistry of carotenoids and their importance in food. *Clin. Nutr.* **7:** 97–100.
18. Dawson, H. N., R. Burlingame, and A.C. Cannons. 1997. Stable transformation of *Chlorella*: Rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. *Curr. Microbiol.* **35:** 356–362.
19. Del Campo, J. A., J. Moreno, H. Rodríguez, M. A. Vargas, J. Rivas, and M. G. Guerrero. 2000. Carotenoid content of chlorophycean microalgae. Factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). *J. Biotechnol.* **76:** 51–59.
20. Del campo, J. A., H. Rodríguez, J. Moreno, M. A. Vargas, J. Rivas, and M. G. Guerrero. 2001. Lutein production by *Muriellopsis* sp. in an outdoor tubular photobioreactor. *J. Biotechnol.* **85:** 289–295.
21. Demers, S., S. Roy, R. Gagnon, and C. Vignault. 1991. Rapid light-induced changes in cell fluorescence and in xanthophyll-cycle pigments of *Alexandrium excavatum* (Dinophyceae) and *Thalassiosira pseudonana* (Bacillariophyceae) - A photoprotection. *Mar. Ecol. Prog. Ser.* **76:** 185–193.
22. Demmig, B., K. A. Winter, A. Krüger, and F. C. Czygan. 1987. Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. *Plant Physiol.* **84:** 218–224.
23. Demmig-Adams, B., A. M. Gilmore, and W. W. Adams. 1996. Carotenoids 3: *In vivo* function of carotenoids in higher plants. *FASEB. J.* **10:** 403–412.
24. Demmig-Adams, B. 1990. Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochim. Biophys. Acta* **1020:** 1–24.
25. Demmig-Adams, B. and W. W. Adams III. 1992. Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43:** 599–626.
26. Dunahay, T. G., E. E. Jarvis, and P. G. Rossler. 1995. Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. *J. Phycol.* **31:** 1004–1012.
27. Fan, L., A. Vonshak, and S. Boussiba. 1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). *J. Phycol.* **30:** 829–833.
28. Formaggio, E., G. Cinque, and R. Bassi. 2001. Functional architecture of the major light-harvesting complex from higher plants. *J. Mol. Biol.* **314:** 1157–1166.
29. Foss, P. 1987. Natural occurrence of enantiomeric and meso astaxanthin in crustaceans including zooplankton. *Comp. Biochem. Physiol.* **86:** 313–314.
30. Gilmore, A. M. 1997. Mechanistic aspects of xanthophyll cycle dependent photoprotection in higher plant chloroplasts and leaves. *Physiol. Plant.* **99:** 197–209.
31. Gilmore, A. M. and H. Y. Yamamoto. 1993. Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching: Evidence that antheraxanthin explains zeaxanthin-independent quenching. *Photosynth. Res.* **35:** 67–78.
32. Goss, R., K. Böhme, and C. Wilhelm. 1998. The xanthophyll cycle of *Mantoniella squamata* converts violaxanthin into antheraxanthin but not to zeaxanthin: consequences for the mechanism of enhanced non-photochemical energy dissipation. *Planta* **205:** 613–621.
33. Green, B. R. and D. G. Durnford. 1996. The chlorophyll-carotenoid proteins of oxygenic photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47:** 685–714.
34. Grossman, A. R., D. Bhaya, K. E. Apt, and D. M. Kehoe. 1995. Light-harvesting complexes in oxygenic photosynthesis: Diversity, control, and evolution. *Annu. Rev. Genet.* **29:** 231–288.
35. Grünewald, K., J. Hirschberg, and C. Hagen. 2001. Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. *J. Biol. Chem.* **276:** 6023–6029.
36. Hagen, C., W. Braune, K. Vogel, and P. P. Häder. 1993. Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Giröd) Rostafinski (Volvocales). V. Influences on photomovement. *Plant Cell Environ.* **16:** 991–995.
37. Hager, A. 1980. The reversible, light-induced conversions of xanthophylls in the chloroplast, pp. 57–79. *In* Czygan, F. C. (ed). *Pigments in Plants*. Stuttgart, Fischer.
38. Hager, A. and K. Holocher. 1994. Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. *Planta* **192:** 581–589.
39. Handelman, G. J., E. A. Dratz, C. C. Reay, and F. J. G. M. van Kuijk. 1988. Carotenoids in the human macula and whole retina. *Invest. Ophthalmol. Vis. Sci.* **29:** 850–855.
40. Harker, M. and J. Hirschberg. 1997. Biosynthesis of ketocarotenoids in transgenic cyanobacteria expressing the algal gene for β-C-4-oxygenase, *crtO*. *FEBS Lett.* **404:** 129–134.
41. Horton, P., A. V. Ruban, and R. G. Walters. 1996. Regulation of light harvesting in green plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47:** 655–684.

42. Hurry, V., J. M. Andersson, W. S. Chow, and C. B. Osmond. 1997. Accumulation of zeaxanthin in abscicic acid-deficient mutants of *Arabidopsis* does not affect chlorophyll quenching or sensitivity to photoinhibition *in vivo*. *Plant Physiol*. **113:** 639–648.
43. Jacques, P. F., L. T. Chylack, R. B. McGandy, and S. C. Hartz. 1988. Antioxidant status in persons with and without senile cataract. *Arch. Ophthalmol*. **106:** 337–340.
44. Jansson, S. 1994. The light-harvesting chlorophyll a/b-binding proteins. *Biochim. Biophys. Acta* **1184:** 1–19.
45. Jin, E. S., B. Feth, and A. Melis. 2003. A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. *Biotech. Bioeng.* **81:** 115–124.
46. Jin, E. S., J. W. E. Polle, and A. Melis. 2001. Involvement of zeaxanthin and of the Cbr protein in the repair of photosystem-II from photoinhibition in the green alga *Dunaliella salina. Biochim. Biophys. Acta* **1506:** 244–259.
47. Kitahara, T. 1984. Carotenoids in the Pacific salmon during the marine period. *Comp. Biochem. Physiol.* **78:** 859–862.
48. Kobayashi, M., T. Kakizono, N. Nishio, and S. Nagai. 1992. Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in a green alga, *Haematococcus pluvialis. J. Ferment. Bioeng.* **74:** 61–63.
49. Krinsky, N. I., M. D. Russett, G. J. Handelman, and D. M. Snodderly. 1990. Structural and geometric isomers of carotenoids in human plasma. *J. Nutr.* **120:** 1654–1662.
50. Kuehlbrandt, W., D. N. Wang, and Y. Fujiyoshi. 1994. Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367:** 614–621.
51. Lagarde, D., L. Beuf, and W. Vermaas. 2000. Increased production of zeaxanthin and other pigments by application of genetic engineering techniques to *Synechocystis* sp. strain PCC 6803. *Appl. Environ. Microbiol.* **66:** 64–72.
52. Landrum, J. T. and R. Bone. 2001. Lutein, zeaxanthin and the macular pigment. *Arch. Biochem. Biophys.* **385:** 28–40.
53. Lawlor, D. W. 2001. Accessory light-harvesting pigments, pp. 43–47. *In*: Photosynthesis 3rd Ed., BIOS Scientific Publishers Limited, Springer-Verlag New York Inc., NY, U.S.A.
54. Le Marchand, L., J. H. Hankin, L. N. Kolonel, G. R. Beecher, L. R. Wilkens, and L. P. Zhao. 1993. Intake of specific carotenoids and lung cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **2:** 183–187.
55. Lohr, M. and C. Wilhelm. 1999. Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle. *Proc. Natl. Acad. Sci. USA* **96:** 8784–8789.
56. Lohr, M. and C. Wilhelm. 2001. Xanthophyll synthesis in diatoms: Quantifications of putative intermediate and comparison of pigment conversion kinetics with rate constants derive form a model. *Planta* **212:** 382–391.
57. Lorenz, R. T. and G. R. Cysewski. 2000. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **18:** 160–167.
58. Lotan, T. and J. Hirschberg. 1995. Cloning and expression in *E. coli* of the gene encoding β-C-4-oxygenase that converts β-carotene to the keto carotenoids cantaxanthin in *Haematococcus pluvilais*. *FEBS Lett.* **364:** 125–128.
59. Margalith, P. Z. 1999. Production of keto carotenoids by microalgae. *Appl. Microbiol. Biotechnol*. **51:** 431–438.
60. Marin, E., L. Nussaume, A. Quesada, M. Gonneau, B. Sotta, P. Hugueney, A. Frey, and A. Marion-Poll. 1996. Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in absicic acid biosynthesis and corresponding to ABA locus of *Arabidopsis thaliana. EMBO J.* **15:** 2331–2342.
61. Misawa, N. and H. Shimada. 1998. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeast. *J. Biotechnol.* **59:** 169–181.
62. Misawa, N., S. Kajiwara, K. Kondo, A. Yokoyama, and Y. Satomi. 1995. Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon β-carotene by a single gene. *Biochem. Biophys. Res. Commun.* **209:** 867–876.
63. Misawa, N., Y. Satomi, K. Kondo, A. Yokoyama, and S. Kajiwara. 1995. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J. Bacteriol.* **177:** 6575–6584.
64. Niyogi, K. K., O. Björkman, and A. R. Grossman. 1997. The roles of specific xanthophylls in photoprotection. *Proc. Natl. Acad. Sci. USA* **94:** 14162–14167.
65. Niyogi, K. K., O. Björkman, and A. R. Grossman. 1997. *Chlamydomonas* xanthophyll cycle mutants identified by video imaging of chlorophyll fluorescence quenching. *Plant Cell* **9:** 1369–1380.
66. Olaizola, M., J. LaRoche, Z. Kolber, and P. G. Falkowski. 1994. Nonphotochemical fluorescence quenching and the diadinoxanthin cycle in a marine diatom. *Photosynth. Res.* **41:** 357–370.
67. Orosa, M., J. F. Valero, C. Herrero, and J. Abalde. 2001. Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under N-starvation and high light conditions. *Biotechnol. Lett.* **23:** 1079–1085.
68. Park, E. K. and C. G. Lee. 2001. Astaxanthin production by *Haematococcus pluvialis* under various light intensity and wavelengths. *J. Microbiol. Biotechnol.* **11:** 1024–1030.
69. Pogson, B. J., K. A. McDonald, M. Truong, G. Britton, and D. DellaPenna. 1996. *Arabidopsis* carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. *Plant Cell* **8:** 1627–1639.
70. Pogson, B. J., K. K. Niyogi, O. Björkman, and D. DellaPenna. 1998. Altered xanthophyll compositions adversely affect chlorophyll accumulation and non-photochemical quenching in *Arabidopsis* mutants. *Proc. Natl. Acad. Sci. USA* **95:** 13324–13329.
71. Rabbani, S., P. Beyer, J. von Lintig, P. Hugueney, and H. Kleinig. 1998. Induced β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil. Plant Physiol.* **116:** 1239–1248.
72. Richmond, A. 1990. Large scale microalgal culture and applications, pp. 269–330. *In* Round, M. and S. Chapman

(eds.). *Progress in Phycological Research* 7. Biopress, Bristol.
73. Rissler, M. H. and B. J. Pogson. 2001. Antisense inhibition of the beta carotene hydroxylase enzyme in *Arabidopsis* and the implication for carotenoids accumulation, photoprotection and antenna assembly. *Photosynth. Res.* **67:** 127–137.
74. Rock, C. D. and J. A. D. Zeevaart. 1991. The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy carotenoid biosynthesis. *Proc. Natl. Acad. Sci. USA* **88:** 7496–7499.
75. Rock, C. L., R. A. Jacob, and P. E. Bowen. 1994. Update on the biological characteristics of the antioxidant micronutrients: Vitamin C, vitamin E and the carotenoids. *J. Am. Diet. Assoc.* **96:** 693–702.
76. Seddon, J. M., U. A. Ajani, R. D. Sperduto, R. Hiller, N. Blair, T. C. Burton, M. D. Farber, E. S. Gragoudas, J. Haller, D. T. Miller, L. A. Yannuzzi, and W. Willett. 1994. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *J. Am. Med. Assoc.* **272:** 1413–1420.
77. Shaish, A., M. Avron, U. Pick, and A. Amotz. 1993. Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil? Planta* **190:** 363–368.
78. Snodderly, M. D. 1995. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* **62:** 1448–1461.
79. Steinbrenner, J. and H. Linden. 2001. Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. *Plant Physiol.* **125:** 810–817.
80. Stevens, D. R. and S. Purton. 1997. Genetic engineering of eukaryotic algae: Progress and prospects. *J. Phycol*. **33:** 263–270.
81. Stransky, H. and A. Hager. 1970. The carotenoid pattern and the occurrence of the light-induced xanthophyll cycle in various classes of algae. VI. Chemosystematic study. *Arch. Mikrobiol*. **73:** 315–323.
82. Sun, Z., F. X. Jr. Cunningham, and E. Gantt. 1998. Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. *Proc. Natl. Acad. Sci. USA* **95:** 11482–11488.
83. Tardy, F. and M. Havaux. 1996. Photosynthesis, chlorophyll fluorescence, light-harvesting system and photoinhibition resistance of a zeaxanthin-accumulating mutant of *Arabidopsis thaliana*. *J. Photochem. Photobiol.* **34:** 87–94.
84. Tjahjono, A. E., Y. Hayama, T. Kakizono, Y. Terada, N. Nishio, and S. Nagai. 1994. Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. *Biotech. Lett.* **16:** 133–138.
85. Vandana, P. and L. C. Rai. 2002. Interactive effect of UV-B and pesticides on photosynthesis and nitrogen fixation of *Anabaena doliolum*. *J. Microbiol. Biotechnol*. **12:** 423–430.
86. Wilhelm, C. 1990. The biochemistry and physiology of light-harvesting processes in chlorophyll *b* containing and chlorophyll *c* containing algae. *Plant Physiol. Biochem.* **28:** 293–306.
87. Yeum, K. J., A. Taylor, G. Tang, and R. M. Russell. 1995. Measurement of carotenoids, retinoids, and tocopherols in human lenses. *Invest. Ophthalmol. Vis. Sci.* **36:** 2756–2761.
88. Yokoyama, A., Y. Shizuri, and N. Misawa. 1998. Production of new carotenoids, astaxanthin glucosides, by *E. coli.* transformations carrying carotenoid biosynthetic genes. *Tetrahedron Lett*. **39:** 3709–3712.
89. Zaslavskaia, L. A., J. C. Lippmeier, C. Shih, D. Ehrhardt, A. R. Grossman, and K. E. Apt. 2001. Trophic conversion of an obligate photoautotrophic organism through engineering. *Science* **292:** 2073–2075.
90. Zaslavskaia, L. A., J. C. Lippmeier, P. G. Kroth, A. R. Grossman, and K. E. Apt. 2000. Transformation of the diatom *Phaeodactylum tricornutum* (*Bacillariophyceae*) with a variety of selectable marker and reporter genes. *J. Phycol*. **36:** 379–386.
91. Zhang, D. H and Y. K. Lee. 2001. Twostep process for ketocarotenoid production by a green alga, *Chlorococcum* sp. strain MA-1. *Appl. Microbiol. Biotechnol*. **55:** 537–540.
92. Ziegler, R. G., E. A. Colavito, P. Hartge, M. J. McAdams, J. B. Schoenberg, T. J. Mason, and J. F. Fraumeni Jr. 1996. Importance of α-carotene, β-carotene and other phytochemicals in the etiology of lung cancer. *J. Natl. Cancer Inst*. **88:** 612–615.