Microbial Carotenoids

Eric A. Johnson and William A. Schroeder
University of Wisconsin, Departments of Food Microbiology and Toxicology, 1925 Willow Drive, and Bacteriology, 1550 Linden Drive, Madison, Wisconsin 53706, USA

Carotenoids occur universally in photosynthetic organisms but sporadically in nonphotosynthetic bacteria and eukaryotes. The primordial carotenogenic organisms were cyanobacteria and eubacteria that carried out anoxygenic photosynthesis. The phylogeny of carotenogenic organisms is evaluated to describe groups of organism which could serve as sources of carotenoids. Terrestrial plants, green algae, and red algae acquired stable endosymbionts (probably cyanobacteria) and have a predictable complement of carotenoids compared to prokaryotes, other algae, and higher fungi which have a more diverse array of pigments. Although carotenoids are not synthesized by animals, they are becoming known for their important role in protecting against damage by singlet oxygen and preventing chronic diseases in humans. The growth of aquaculture during the past decade as well as the biological roles of carotenoids in human disease will increase the demand for carotenoids. Microbial synthesis offers a promising method for production of carotenoids.

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List of Symbols and Abbreviations

ψ (psi)	Acyclic
β (beta)	Cyclohexene
ε (epsilon)	Cyclohexene
λ (lambda)	Lambda (wavelength)
CHEF	Contour-clamped Homogenous Electric Field electrophoresis
C/N	Carbon to nitrogen ratio
DCW	Dry cell weight
EMS	Ethyl methane sulfonate
ER	Endoplasmic reticulum
E/Z	*cis/trans* Stereoisomers
FACS	Fluorescence activated cell sorting
HDCO	3-Hydroxy-3′,4′-didehydro-β,ψ-caroten-4-one
HMG-CoA-reductase	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
IUPAC	International Union of Pure and Applied Chemistry
NAD(P)	β-Nicotinamide Adenine Dinucleotide (Phosphate)
NTG	*N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine
PG	*n*-Propyl gallate
RFLP	Restriction Fragment Length Polymorphism
SHAM	Salicylhydroxamic acid
$^1O_2$	Singlet oxygen
μ	Specific growth rate
Yx/s	Yield of cells on substrate

1 Introduction

Although carotenoids occur widely in nature, they are by no means unified in biological organisms. Carotenoids show much diversity in natural distribution, structure, and function. The primary functions of carotenoids in photosynthetic organisms are as light harvesting accessory pigments and as protectants against porphyrin-catalyzed photooxidations [1–3]. Carotenoids have the specific and catalytic ability to quench singlet oxygen ($^1O_2$) generated from metabolism or by the reaction of triplet-state porphyrins with oxygen. Even in nonphotosynthetic organisms, a universal role of carotenoids is to sequester toxic oxygen species from metabolism or the environment, most specifically $^1O_2$. The diversity of carotenoids has evolved in relation to functional requirements, but they have apparently been deposited in animals by availability in the food chain. Like hormones and sterols, they initially occurred by necessity in some organisms, but apparently in others by gratuity.

Carotenoids were initially synthesized by anoxygenic phototrophic bacteria and oxygenic photosynthetic prokaryotes (cyanobacteria) [4–6]. In eukaryotes, the capacity to synthesize carotenoids was acquired by symbiosis with bacteria or with eukaryotic chloroplasts [7–9]. In terrestrial plants, and red and green algae, stable symbioses were established with cyanobacteria or related prochlorophytes. The origin of carotenoids in non-red and non-green algae as well as the fungi appears polyphyletic and possibly evolved independently and repeatedly in different taxonomic groups [7, 8, 10]. Carotenoids are not synthesized by animals although carotenes can be altered in some animals by oxidation or isomerization and by conversion to vitamin A and retinoids.

Since many animals of agricultural importance contain carotenoids and require them as nutrients, these pigments are industrially significant as components in animal feeds. Carotenoids are required as feed supplements in the poultry industry and in aquaculture of fishes and crustaceans. Besides providing nutrition and possibly disease resistance, carotenoids give brilliant pigmentation and aesthetic value to crustacea, animals, birds, and their ova. Recent studies have suggested that carotenoids have health benefits in humans and animals by preventing or delaying some chronic diseases including cancer, arteriosclerosis, cataracts, and other maladies. These developments have contributed greatly to the industrial and medical interest in carotenoids. Traditionally, carotenoids for agriculture and food uses were obtained by extraction of natural plant sources (e.g. marigold flowers or corn) and by chemical synthesis. During the past ten years, much interest and effort has been devoted to designating and developing microbial sources for industrially important carotenoids. Microbial synthesis could provide a significant proportion of the pigments used in terrestrial agriculture and marine aquaculture, and potentially as human nutrients or "nutraceuticals".

2 Nomenclature and Structure

Carotenoids are isoprenoids containing a characteristic polyene chain of conjugated double bonds. The two general groups of pigments are the hydrocarbons (carotenes) and oxygenated derivatives (xanthophylls). Carotenoids are formed from two geranylgeranyl precursors by head to head condensation which gives a symmetrical acyclic $C_{40}H_{56}$ basic structure (Fig. 1) [11]. The basic molecule is arranged such that the two central methyl groups are in a 1,6 position. About 600 natural carotenoids are derived from the basic molecule by various enzymatic chemical processes. About 370 of the naturally occurring carotenoids have chiral centers, and in some cases both the *R* and *S* configurations have been isolated. In addition, *cis/trans* (*E/Z*) -isomerism occurs widely in carotenoids. Normally carotenoids exist in nature as the *Z* form, but several exceptions exist [11].

Rules for nomenclature of carotenoids have been recommended by IUPAC [12, 13]. Trivial names will be used in this review; the semisystematic names have been tabulated [14]. It should be kept in mind that several of the carotenoids cited in this review were identified by absorbance and chromatographic characteristics alone. The structure of these pigments should be confirmed (including the stereochemistry) by high resolution MS, NMR and CD spectroscopy.

3 Optical Properties of Carotenoids

The region of the carotenoid molecule related to light absorption is the polyene chain consisting of a series of conjugated double bonds. The wavelength of maximum absorption is directly related to the number of conjugated double

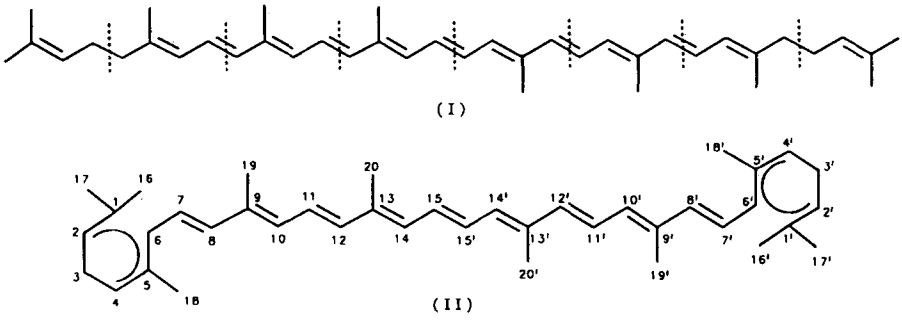

Fig. 1. I: Basic acyclic $C_{40}H_{56}$ carotene structure from which all carotenoids are derived; II: Structure and numbering of carotene stem molecule

bonds. The absorption maximum can be estimated from the equation λ_{max}(nm) = 300.5 + 65.5 × n where n is the number of conjugated double bonds present. Each additional double bond increases the λ_{max} by 7–35 nm (5–9 nm if the double bond is located in a ring). This is referred to as a bathochromic shift. The addition of carbonyls conjugated to the polyene chain has two effects on optical properties. The first is to increase the λ_{max} by ~28 nm for a first carbonyl in the polyene chain. Additional carbonyls either in the polyene chain or in a ring structure will increase the λ_{max} by 1–9 nm. Normally, the polyene chain has a characteristic absorbance spectrum with three peaks. This characteristic shape is referred to as having high persistence. The introduction of the carbonyl results in loss of persistence and loss in fine structure. This yields a rounded, symmetrical peak of absorption. Other chemical modifications of the carotenoid structure, with the exception of epoxides, have little effect on absorbance characteristics, since they do not sterically interfere with the polyene chain. Epoxides decrease the λ_{max} by 10–20 nm (a hypsochromic shift). The 9 terminal carbons of each end of microbial xanthophylls are usually arranged in one of 3 configurations. In the acyclic ψ configuration, steric hindrance is minimized, yielding a longer wavelength chromophore. When the end group is cyclized to a β group, steric hindrance is increased, shortening the λ_{max}. In the third common end group, the ε configuration, the terminal double bond is displaced one position relative to the β end group double bond. This bond is no longer conjugated to the chromophore and therefore gives a hypsochromic shift.

4 Biological Properties of Carotenoids

4.1 Evolutionary Origins of Carotenoids

Advances in microbial phylogeny, particularly by 16s rRNA sequencing [15, 16] and by analysis of conserved gene and protein sequences [7, 17, 18] have improved our understanding of the evolutionary origins of prokaryotes and eukaryotes. Molecular comparisons have demonstrated three primary taxa of life on Earth, the Eubacteria, Archaebacteria, and Eukaryota (Protista) [9, 15] [Fig. 2]. Of the three groupings, carotenoids are known to be produced in eubacteria including all phototrophic bacteria and sporadically in heterotrophic bacteria, but by only one group of archaebacteria, the halobacteria. Carotenoids are produced universally in photosynthetic algae and plants, and by some fungi. They are not found in strictly anaerobic eubacteria and archaebacteria nor are they synthesized by animals.

Xanthophylls, like sterols [19], probably evolved in relation to the appearance of oxygen. The evolution of life consisted of two main periods: (I) the age of bacteria 3500–1700 million years ago and (II) the age of eukaryotes from 1700 million years ago to the present [20]. Carotenes and xanthophylls found in

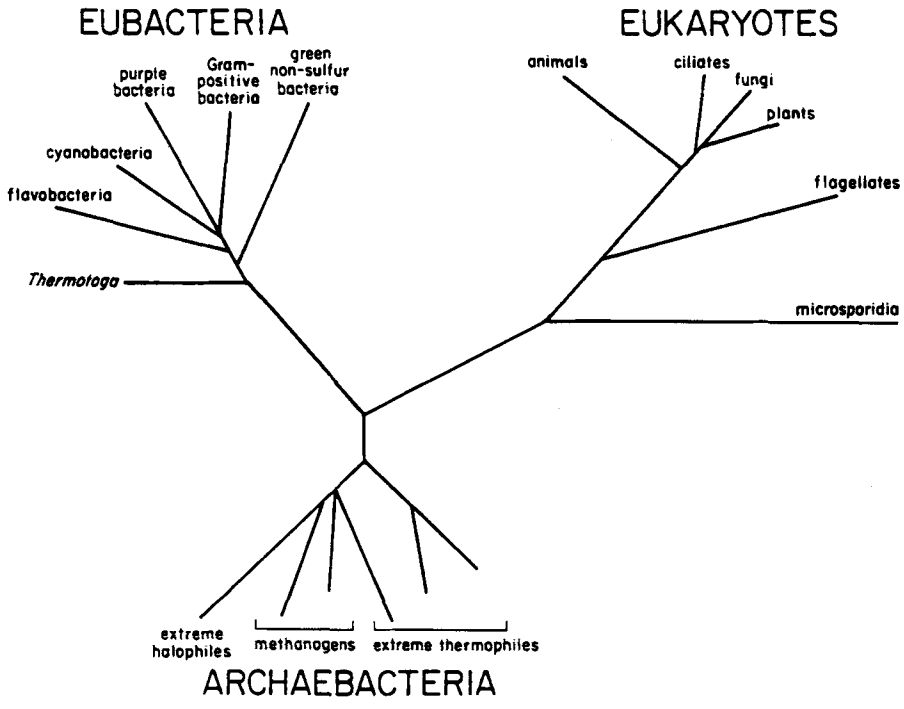

Fig. 2. Universal phylogenetic tree determined from rRNA sequences [from Ref. 16]

anoxygenic photosynthetic bacteria probably evolved in the bacterial epoch and were adapted as accessory pigments in anoxygenic photosynthesis. From 16s rRNA-based phylogeny, 12 major groups of evolutionary lineages of bacteria diverged from each other at essentially the same time, which corresponded to the onset of photosynthesis [5, 21]. Carotenoid biosynthesis probably occurred initially in anoxygenic photosynthetic bacteria. These organisms, which include the photosynthetic purple bacteria, green bacteria, and heliobacteria, contain bacteriochlorophylls and group-specific aliphatic and monocyclic carotenoids as the primary light harvesting pigments [22–24]. Ancient rock sediments formed about 3.5 billion years ago in Australia and South Africa showed evidence of at least four types of filamentous prokaryotes living in stromatolites in shallow lakes [25, 26]. The organisms resemble filamentous mat-forming contemporary cyanobacteria and anoxygenic photosynthetic bacteria [5, 25]. The ancestral carotenogenic cell may have resembled the present day *Heliobacterium chlorum* (gram-positive line) [27], which contains the simple alicyclic carotene neurosporene as its primary carotenoid. This organism is strictly anaerobic and has the simplest known porphyrin-based photosynthetic reaction center [28]. The high O_2-sensitivity of this bacterium is related to its low content of neurosporene [27] which is a relatively poor quencher of $^1O_2$. As the

Table 1. Carotenoid groups and characteristic features from anoxygenic phototropic bacteria Adapted from [23, 30]

Group	Cartenoid groups	Major pigments	Structural characteristics
1	Normal spirilloxanthin	Neurosperene, lycopene, rhodopin, spirilloxanthin	Aliphatic, methoxyl groups
2	Alternative spirilloxanthin	Chloroxanthin, spheroidene, speheroidene, spirilloxanthin	Aliphatic, methjoxyl groups keto group
3	Okenone series	Okenone	Aromatic, keto and methoxyl groups
4	Rhodopinal series (related to group 1)	Lycopene, lycopenal, lycopenol, rhodopin, rhodopinal, spirilloxanthin	Aliphatic, Aldehyde (C_{20}), methoxyl and hydroxyl groups
5	Chlorobactene	Chlorobactene, isorenieratene, β-carotene, γ-carotene	Aromatic and cyclohexane rings
6	Chloroflexus	Lycopene, γ-carotene, echinenone, β-carotene, carotenoid glycosides	Cyclohexene ring, keto groups, glycosyl groups.

anoxygenic phototrophic bacteria evolved, there was an accompanying diversification in the complement of carotenoids (Table 1). Cell suspensions of these organisms are striking, showing purple-violet, purple, red, orange-brown, yellowish-brown, and orange-green colors [29]. The colors are due to carotenoids produced by the various groups reflecting quite different pathways of biosynthesis in the later stages (Table 1) [30]. Many of the pigments contain oxygen functions (especially ketone groups at the C_4 and C_{20} positions), which is interesting since it is believed that xanthophylls commonly derive from reaction of molecular oxygen by mixed-function oxidase (e.g. flavoenzymes or cytochrome P-450) enzyme systems, and oxygen groups would not be expected to be involved in biosynthesis in strictly anaerobic environments.

The differences in carotenoid structures in the anoxygenic photosynthetic bacteria is probably necessary for the optimum exploitation of the spectra of light that filters through microbial assemblages or mats [5, 24]. The carotenoids in the bacteria are located in chromatophores or "grana" in the extensive lamellar, tubular or vesicular membrane systems continuous with the cytoplasmic membrane. The purple nonsulfur bacteria are the most diverse group metabolically, in internal membrane structure, and in carotenoid composition. Most representatives grow under microaerophilic to aerobic conditions in the dark as chemoorganotrophs, and under these conditions the synthesis of photosynthetic pigments including carotenoids is repressed. The green sulfur bacteria, which contain chlorobactene or isorenieratene, are strictly anaerobic and obligately phototrophic [31]. Of the anoxygenic phototrophs, *Chloroflexus* is unusual in its pigment content producing β-carotene and hydroxy-γ-β-carotene glucoside anaerobically and echinenone and 4-keto-γ-carotene glucoside (myxobactone) in aerobic conditions [32]. *Thermomicrobium roseum* is a green nonsulfur bacterium originally isolated from the effluent of a hot spring in Yellowstone National Park [33] which also has an unusual complement of

carotenoids. The primary carotenoids in *T. roseum* appear to be torulene and 3,4-dehydrolycopene [33]. The carotenoids found in the anoxygenic groups are listed in Table 1.

An extremely important event in the evolution of carotenoids was the development of photosystem II, which allowed the utilization of H_2O as an electron donor in photosynthesis and release oxygen as a product. The geological record particularly the changes in iron-banded layers indicates that substantial quantities of oxygen were present in the atmosphere about 1.7 billion years ago [34]. Oxygenic photosynthesis occurred in bacteria similar to present day cyanobacteria and prochlorophytes [6, 18]. In cyanobacteria, the major light-harvesting pigments are chlorophyll a and phycobiloproteins. The carotenoids in the cyanobacteria are structurally different than the pigments found in the anoxygenic phototrophic bacteria. Cyanobacteria form cyclic ketocarotenoids including echinenone, canthaxanthin, and in some species caloxanthin and nostoxanthin. They also synthesize glycosylated xanthophylls including myxoxanthophyll and oscillaxanthin. The carotenoids apparently are not integrally involved in light harvesting but may provide protection against photooxidation. The second order of oxygenic photosynthetic bacteria are the Prochlorales. This group has an unusual pigment content; it contains chlorophylls a and b but lacks phycobiloproteins as light harvesting pigments [35, 36]. The prochlorophytes contain chlorophylls a and b, β-carotene, zeaxanthin, cryptoxanthin, echinenone, and mutatochrome as the primary light harvesting pigments [22, 37, 38]. The pigment composition of the prochlorophytes appears to more closely resemble the pigment composition of green algae and higher plants except for a lack of lutein and has been considered as the evolutionary ancestor of chloroplasts [8, 35]. A novel prochlorophyte was reported to contain chlorophyll a and b, and the carotenoids α-carotene and zeaxanthin (typical plant pigments) but they lack β-carotene, lutein and prasinoxanthin [39]. The presence of α-carotene was puzzling because procaryotes are not known to synthesize ε rings, but its presence suggests a relation to plant and green algal chloroplasts. The prochlorphytes have been proposed as being a descendant of ancestral chlorophytes, the free-living ancestor of chloroplasts, or a deviant cyanophyte derivative [40].

The evolution of oxygenic photosynthesis about 3 to 2.5 billion years ago [6] had enormous consequences for biochemical evolution: O_2 began to accumulate and the atmosphere changed from reducing to oxidizing. As O_2 gradually accumulated, the fossil record suggests that there was an enormous burst in the rate of evolution leading to the formation of eukaryotes with organelles and eventually to multicellular plants and animals [18]. Terrestrial plants and probably the red algae (Rhodophyta) evolved their photosynthetic capability through endosymbiosis with bacteria resembling present day cyanobacteria [9, 41–45]. The common ancestor of cyanobacteria and purple bacteria was probably an O_2-evolving photoautoroph, related to the purple bacteria, which had earlier lost its capacity to use water as an electron donor [6, 46].

In certain lineages of eukaryotes, including some orders of algae and fungi, multiple endosymbiotic events with cyanobacteria or with eukaryotic chloroplasts expanded organelle diversity [47]. The nonphotosynthetic carotenogenic bacteria probably evolved from photosynthetic bacteria by loss of photosynthesis [6, 16, 21].

The gradual appearance of oxygen probably had tremendous consequences for the diversification of carotenoid structure. Like sterols, which primarily evolved in parallel with the appearance of oxygen [6], the diversity of carotenoids undoubtedly expanded and adapted to special functions. The reaction of carotenoids with oxygen-dependent enzyme systems has created diversity in structure. During the transition from anaerobic to an O_2-containing environment on earth, the carotenoids could have played a role as a rudimentary antioxidant system for coping with reactive oxygen species.

4.2 Carotenoid Structures and Phylogeny

About 600 carotenoids structures are now known to occur naturally [11]. There is considerable similarity in the carotenes of various bacteria, plants, and protists suggesting a universal early pathway (at least up to phytoene or neurosporene), but divergence occurred in later steps of carotene synthesis and many variations exist in the structure of xanthophylls (Table 2). The diversity of carotenoid structures is greatest in the algae and bacteria, less diverse in fungi, and least variable in terrestrial plants. The diversity of photopigment systems is of ecological significance for the harvesting of light energy in mat ecosystems in the algae and prokaryotes [48, 49] and probably for inactivating toxic oxygen species in heterotrophic species.

4.2.1 Bacteria

The distribution of carotenoids has evolved in several groups of prokaryotes. This distribution is reviewed in light of recent advances in our understanding of a natural phylogeny [16, 18] and the need to identify potential new sources of industrially important carotenoids.

Within the two kingdoms of bacteria, only the halobacteria in the archaebacteria are known to contain carotenoids, whereas many species of eubacteria produce carotenoids. The bright red color of cell suspensions of halobacteria e.g. (*Halobacterium*, and other halotolerant species of archaebacteria) is due to carotenoids. C_{40} carotenoids are present including β-carotene, but the C_{50} bacterioruberins are the primary pigments. These are alicyclic pigments with 2 to 4 hydroxyl groups. Carotenes in *Halobacterium* include the usual series of intermediates, except that the pathway differs from the one found in plants by the formation of *trans*-isomers instead of the *cis*-isomers [50]. Carotenoids

Table 2. Representative carotenoids reported in bacteria, plants, algae and fungi, Adapted and expanded from [73, 87, 88])

Phylogenetic group	Representative Carotenoids
A. Eubacteria (classified according to Ref. 5)	
Purple phototrophic bacteria (see Table 1)	Anaerobic: Neurosporene, rhodopin, lycopene, spirilloxanthin Aerobic: spheroidene, hydroxy-spheroidene, okenone
Green sulfur bacteria	
Chlorobium	Chlkorobactene (green strains) β-isorenieratene, isorenieratene
Green nonsulfur bacteria	
Chloroflexus	β-Carotene, γ-carotene
Herpetosiphon	Myxobacton fatty esters
Cyanobacteria	
Synechococcus	β-Carotene, echinenone, 3-hydroxyechinenone, zeaxanthin, cryptoxanthin, caloxanthin, nostoxanthin. Glycosides myxoxanthophyll, and oscillaxanthin also occur.
Procholorophytes	β-Carotene, zeaxanthin
Purple nonphotorophic bacteria	
Myxococcus	Myxobacton and glycosides, 4-keto torulene
Stigmatella	Myxobacton and acyl esters
Sorangium	γ-Carotene, carotenoid glucoside acyl esters
Chondromyces	γ-Carotene, carotenoid glucoside acyl esters
Bradyrhizobium	2,3,2′3′-Ditrans-tetrahydroxy-β-carotene and 2,3,2′3′-ditrans-tetrahydorxy-β-caroten-4-one
Pseudomonas	Rhodoxanthin, nostoxanthin, decaprenoxanthin
Xanthomonas	Brominated arylpolyenoic esters
Spirchetes	
Spirochaeta	1′,2′-Dihydro-1′-hydroxytorulene 4-keto-1′,2′-dihydro-1′-hydroxytorulene
Bacteroides-Flavobacterium-Cytophagas	
Flavobacterium	Zeaxanthin, astaxanthin (?). Also C_{45} and C_{50} carotenoids.
Flexibacter	Flexirubin, Chloroflexirubin, flexixanthin, deoxyflexixanthin
Cytophaga	Zeaxanthin, flexirubins, chloroflexirubins, flexixanthin, deoxyflexixanthin
Saprospira	Saproxanthin
Deinococcus-Thermus	
Deinococcus	β-Carotene, keto-carotenoids
Thermus	α-Carotene (?), γ-carotene, unidentified polar carotenoids
Gram-positive bacteria-high GC	
Streptomyces	Leporotene and hydroxy derivatives
Nocardia	Lycopene, γ-carotene, 4-keto-γ-carotene, carotenoid glycosides.
Corynebacterium	C_{45} and C_{50} carotenoids, glycoside of decaprenoxanthin
Brevibacterium	Canthaxnthin, β-carotene, astaxanthin
Mycobacterium	Isorenieratene (aromatic carotene), β-carotene, carotenoid glycosides
Micrococcus	Sarcinaxanthin, canthaxanthin, echinenone
Gram-positive bacteria-high GC	
Bacillus (alkalophilic)	β-Carotene, C_{50} carotenoids
Staphylococcus	C_{30} apocarotenoids
Sarcina	Lycopene, β-carotene, zeaxanthin, C_{50} carotenoids
Enterococcus	C_{30} carotenoids

Table 2. (*continued*)

Phylogenetic group	Representative Carotenoids
B. Archaebacteria	
Extreme halophiles-*Halobacterium*	β-Carotene, C_{50} xanthophylls-derivatives of acyclic bacterioruberin. Possess retinal protein complex, bacterorhodopsin
Hyperthermophiles-Sulfolobus	
C. Protista	
Chlorophyta (green alage)	β-Carotene, lutein, violaxanthin, neoxanthin, zeaxanthin, loroxanthin, siphonoxanthin, astaxanthin
Euglenophyta (euglenids)	Diadnoxanthin, diatoxanthin, heteroxanthin, eutreptiellanone (oxabicycloheptane carotenoid), siphonein, neoxanthin.
Phaeophyta (brown algae)	Fucoxanthin, violoaxanthin, acyl-fucoxanthins
Pyrrophyta (dinoflagellates)	Peridinin (C_{37}), diadinoxanthin, acyloxyfucoxanthins, gyroxanthins
Chrysophyta (golden-brown algae, diatoms)	Fucoxanthin, other acetylenic carotenoids
Rhodophyta (red algae)	α-Carotene, β-carotene, lutein, zeaxanthin
Cryptophyta	Alloxanthin, crocoxanthin, monadoxanthin
D. Higher plants	
Chloroplasts	α-Carotene, β-carotene, lutein, β-cryptoxanthin, Violaxanthin, neoxanthin
Chromoplasts (species specific)	Lycopene, β-carotene, β-cryptoxanthin, zeaxanthin, apocarotenoids, 5,6-epoxy- and 5,8-epoxycarotenoids, capsanthin
E. Fungi	
Myxomycota	
Myxomycetes (slime molds)	γ-Carotene, β-carotene, 3,4-didehydrolycopene, neurosporaxanthin
Eumycota	
Mastigomycotina	
Chytridiomycetes	γ-Carotene, lycopene, β-carotene
Oomycetes	
Zygomycotina	
Zygomycetes	β-Carotene
Ascomycotina	
Hemiascomycetes	None known
Plectomycetes	γ-Carotene, β-carotene, lycopene
Pyrenomycetes	γ-Carotene, β-carotene, lycopene, torulene, lycoxanthin, neurosporaxanthin
Discomycetes	β-Carotene, γ-carotene, 3,4-didehydrolycopene, torulene, phillipsiaxanthin, aleuriaxnthin, plectaniaxanthin
Loculoascomycetes	None known?
Basidiomycotina	
Hymenomycetes	γ-Carotene, β-carotene, cryptoxanthin, canthaxanthin, ataxanthin (?)
Gasteromycetes	γ-Carotene, β-carotene
Teliomycetes	β-Zeacarotene, γ-carotene, β-carotene, torulene, cryoptoxanthin
Basidiomycetous yeasts	β-Carotene, torulene, torularhodin
	β-carotene, HDCO, echinenone, astaxanthin
Deuteromycotina	γ-Carotene, β-carotene, torulene

Many of the designated carotenoids were identified based on chromatographic and visual absorbance; it would be valuable to confirm the structures by modern spectroscopic methods

protect the halophilic cells against damage by bright light [51]. The halobacteria also have evolved energy-producing bacteriorhodpsin [52], which contains retinal derived from carotenoids as a prosthetic group. Bacteriorhodopsin can occupy as much as 50% of the protein of the cell membrane.

The eubacteria possess a rich variety of carotenoids and the pigments occur in specific phylogenetic groups. From the sequences of nucleotides of fragments of 16s rRNA, 12 distinct eubacterial groups have been delineated [16, 53] [Fig. 3]. These include: (1) cyanobacteria, (2) green sulfur bacteria, (3) multicellular filamentous green nonsulfur bacteria (*Chloroflexus*) and chemotrophic related bacteria, (4) phototrophic purple bacteria, (5) planctomyces, (6) spirochetes, (7) flavobacteria-*Bacteroides*, (8) chlamydiae, (9) deinococci-*Thermus*, (10) Gram-positive bacteria, (11) *Thermotoga*, and (12) *Aquifex* [16]. Of the 12 groups, carotenoids occur in the photosynthetic bacteria (groups 1–4), spirochetes, flavobacteria-cytophages, deinococci, and gram-positive lineages. The carotenoids of the phototrophic bacteria (in groups 1–4) were described in the previous section. Carotenoids occur sporadically in the nonphotosynthetic bacteria. They are rarely found in strict anaerobes except for the phototrophic bacteria.

Carotenogenic purple bacteria include pseudomonads and myxobacteria [54, 55]. Although carotenoids are not common in rhizobia, the species *Bradyrhizobium* contains hydroxy-derivatives of nostoxanthin [56]. The same pig-

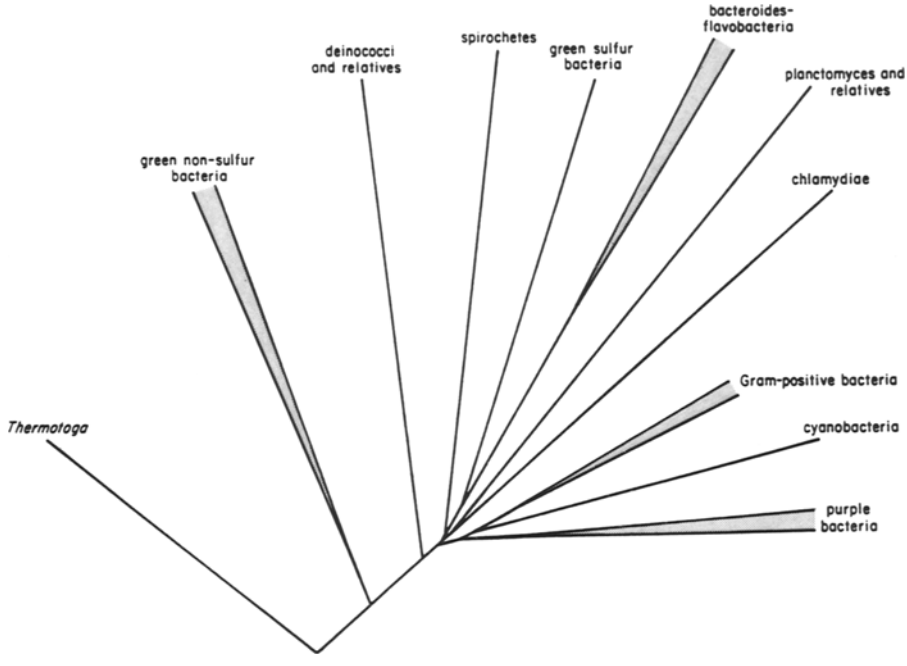

Fig. 3. Eubacterial phylogenetic tree from comparison of 16s rRNA sequences [from Ref. 16]

ment also occurs in *Pseudomonas rhodos* [57]. Carotenoids of other pseudomonads are rather poorly characterized. A pigment similar to 3,5,3′-trihydroxy-5,6-dihydro-β-carotene was reported in *P. echinoides*, and mutants that accumulated lycopene, γ-carotene, or phytoene were obtained [54]. Nostoxanthin was identified as the primary carotenoid in several *Pseudomonas* species [58]. The obligate methylotrophic bacteria, which utilize methane and methanol as sole carbon source, are pink colored apparently due to the presence of a ketocarotenoid associated with the outer membrane [59]. The aquatic organism *Azospirillum* produces unidentified carotenoids under aerobic conditions [60]. The parasite *Bdellovibrio* also produces an unidentifed carotenoid [61]. Carotenoids are relatively uncommon in the family Enterobacteriaceae which includes many human pathogens. However, *Erwinia* is a yellow colored plant-associated enteric bacterial genus whose species contain β-carotene, β-cryptoxanthin, and zeaxanthin and glycosides as its major carotenoids [62]. *Xanthomonas* species produce unique carotenoids containing an aryl polyene acid chain with bromine covalently attached [63]. One interesting group, isolated recently and included in the genera *Roseobacter* and *Erythrobacter*, possesses unidentified carotenoids whose synthesis is stimulated by light [64].

Carotenoids are common in spirochetes which produce the torulene derivatives 4-keto-1′, 2′-dihydro-1′-hydroxytorulene and 1′, 2′-dihydro-1′-hydroxytorulene. The flavobacteria lineage is an important source of carotenoids. β-Carotene and zeaxanthin have been isolated from flavobacteria. In addition, the C_{45} and C_{50} pigments nonaprenoxanthin and 11′, 12′-didehydrononaprenoxanthin, an apocarotenoid, have been isolated from *Flavobacterium dehydrogenans* [54]. Phytoene in *F. dehydrogenans* is all *trans*, in contrast to the *cis*-isomer found in most bacteria [65]. Other *Flavobacterium* spp. produce carotenoids and a recent isolate from Yellowstone Park and a marine isolate from Japan apparently produce high levels of astaxanthin which is glycosylated and possibly secreted [66].

The gliding bacteria (Cytophagales) produce several carotenoids which occur in the cytoplasm, while the novel flexirubin pigments are bound to the membrane [67]. The carotenoids include zeaxanthin and the monocyclic ketocarotenoids, flexixanthin and deoxyflexixanthin [67]. The flexirubins, which have been valuable in chemosystematics, have a chromophore of omega-phenyloctaenic acid which is connected via an ester linkage to a resorcinol derivative. The basic chemical structure can be modified by the introduction of methyl and chlorine on the omega-phenyl ring. The carotenoids in *Myxococcus fulvus* increased in the presence of light and in late logarithmic phase of growth to reach levels of 0.06% of the dry weight [68]. The carotenoids are mainly located in the cytoplasmic membrane, comprising 0.14% of its weight. With the exception of *Nannocystis exedens*, the main pigments are carotenoid glycosides with a fatty acid attached to the sugar. Myxobacton, the primary pigment in many myxobacteria, is characteristic for this taxonomic subgroup [69]. Other carotenids include γ-carotene, lycopene, and 4-ketotorulene. The only known role of carotenoids in myxobacteria is to provide protection against photooxi-

dation [69]. The aquatic gliding bacterium *Saprospira* Sp. shows colored colonies of shades of yellow, orange, pink and red [70]. The xanthophyll saproxanthin (a modified 3,1′-dihydroxy-γ-carotene) [71] is the major carotenoid. Pigmentation in *Saprospira thermalis* is stimulated by light, by suboptimal concentrations of cobalamin or tyrosine, and by subinhibitory concentrations of thiamine or sodium thiosulfate [70].

Carotenoids are found in the gram-positive lineage, which is separated into two groups according to high or low GC content of genomic DNA [16]. Carotenoids occur frequently in the high GC gram-positive genera. The high GC carotenogenic organisms include carotenogenic actinomycetes, micrococci, nocardias, corynebacteria, and mycobacteria. In the actinomycete lineage, the mycobacteria produce β-carotene, glycosidic carotenoids (phleioxanthophyll) as well as the aromatic carotenoid isorenieratene. *Streptomyces mediolani* also produces isorenieratene and 3 and 3′ hydroxyderivatives of the aromatic pigment. Mycobacteria have been reported to produce industrially significant xanthophylls including astaxanthin [72]. Among the corynebacteria, C_{45} and C_{50} pigments and glycosides are present [73]. *Nocardia* sp. contains γ-carotene, 4-keto-γ-carotene, and β-carotene [54], while *Nocardia kirovani* produces xanthophyll glycoside esters. Carotenoid pigments are common in *Micrococcus* sp. The primary pigments are the C_{50} carotenoids sarcinaxanthin [74] and its mono- and di-glycosides [75, 76]. *Micrococcus roseus* contains β-carotene, echinenone, and canthaxanthin [54]. *Brevibacterium* sp. strain KY-4313 produces canthaxanthin as its primary carotenoid [77]. Strain 103 was reported to produce astaxanthin [78].

Of the low GC gram-positive genera, *Staphylococcus aureus* produces C_{30} apocarotenoids [79]. These include the C_{30} analogues of phytoene, phytofluene, 7, 8, 11, 12-tetrahyrolycopene, and neurosporene. The primary pigment is 4,4′ diaponeurospren-4-oic acid [80]. *Enterococcus faecium* produces several C_{30} carotenoids similar to those in the staphylococci. The major carotenoid in 15-cis-4-4′-diapophytoene [81] and also present are the xanthophylls diaponeurosporen-4-al, 4,4′-diapolycopenal-4-al, and the glucoside ester of 4,4′-diaponeurosporene [82]. An alkalophilic *Bacillus* sp. contains β-carotene and a C_{50} carotenoid tentatively identified as tetra-anhydrobacterioruberin [83]. Other bacilli apparently also produce carotenoids but they have not been characterized. The motile, marine bacterium *Planococcus* is yellow-orange is color due to carotenoid pigments which consist of unidentified carotenes and xanthophylls [84]. The pigment levels increase as the cells age and as the salt level in the medium increases.

Deinococcus is an unusual genus of gram-positive bacteria that is highly resistant to ultraviolet radiation and heat which may be related to the presence of carotenoids. The structures of the carotenoids have not been adequately characterized but appear to include β-carotene as well as xanthophylls [85]. Sixty percent of the membrane lipids of the related species, *Thermus aquaticus*, a gram-negative thermophile, were reported to consist of carotenoids [86]. Carotenes appear in *Thermus* but the structures apparently have not been reported [86].

Overall, the bacteria produce several unusual pigments including C_{30}, C_{45}, and C_{50} pigments, as well as esters and acyl derivatives. They generally lack typical plant and algal carotenoids such as α-carotene, lutein, and epoxides.

4.2.2 Algae (Protista)

The composition and distribution of carotenoids in the algae has recently been extensively reviewed [87–89] and we will only briefly consider the evolution of the carotenoids in the algae. Pigments characteristic of the various algal classes are listed in Table 2.

The algae (Protista) are not a natural assemblage of organisms but rather represent a highly diverse group of protists [90]. The diversity dwarfs that of the Plantae, Fungi, and Animalia combined [18]. Each major algal class can be distinguished in part by the properties of the chloroplasts, especially by the membrane structure and composition of photopigments [8, 10, 87]. The eukaryotic algae produce unique xanthophylls which are often class-specific [88, 89, 91]. The pigments are usually associated with chlorophylls within plastids but in overproducers extraplastidic carotenoids which do not function as accessory pigments also occur. Extraplastidic carotenoids accumulate in the commercially important green algae *Haematococcus* and *Dunaliella.*

Carotenoids in algae originated from endosymbionts acquired during evolution [92]. The different classes of algae acquired chloroplasts from symbiotic prokaryotes or symbiotic eukaryotic algae. The red algae acquired chloroplasts from symbiotic organisms resembling cyanobacteria [93]. The origins of chloroplasts in other phyla of algae probably evolved from endosymbiotic eukaryotic algae [90, 92]. Dinoflagellates probably acquired chloroplasts by endosymbiososis with a photosynthetic prokaryote. Dinoflagellate chloroplasts typically contain peridinin as the characteristic carotenoid. However, some dinoflagelates also contains unusual carotenoids: *Gyrodinium aureolum* lacks peridinin but contain 19′-hexanoyloxyfucoxanthin as the main carotenoid [94], suggesting possible multiple endosybiotic relationships during its evolution. The promiscuous retention and disposal of plastids by algae and protozoa [92] has probably contributed to the unpredictable array of carotenoids in protists. The sporadic occurrence, loss, and reappearance argues against the use of carotenoids in chemotaxonomy for designating major phyla, a conclusion also supported by other authors [87, 94, 95]. However, carotenoids could be used as tracers to determine the sources of endosymbionts [96]. Since several genes and protein products are involved in carotenoid biosynthesis, Liaaen-Jensen and colleagues have suggested that descriptions of the pathway reactions would be superior to identification of carotenoids for phylogenetic distinctions [87, 97].

The Chlorophyceae is the class of algae of primary industrial interest. They generally accumulate carotenoids similar to those of higher plants, namely α-carotene, β-carotene, lutein, violoxanthin, and neoxanthin but some species also accumulate astaxanthin. The red appearance of some green algae is due to the appearance of extraplastidic carotenoids (secondary carotenoids), often in

quite high amounts. Secondary carotenoids usually occur under nutritional (nitrogen or phosphate) or environmental stress (temperature, oxidative, salt, light) conditions in algae living in extreme conditions. Patches of "red snow" are caused by cryophilic chlorophytes, which accumulate red pigments in oil droplets [98] or in the cell membrane. Only a few species in the Chlamydomonadaceae are red-pigmented in the vegetative state [99]. The majority are snow algae or are found in locations subjected to high light intensities. Astaxanthin occurs as a secondary carotenoid in *Chlamydomonas* [100] and *Haematococcus lacustris* [101], and may function as a photoprotectant or "sunshade" [102, 103]. Similarly, massive accumulation of β-carotene in *Dunaliella* has been postulated to protect the alga against excess irradiation [104].

4.2.3 Plants

Terrestrial plants have a limited and uniform complement of pigments consisting of α-and β-carotenes and the corresponding xanthophylls with the hydroxyl groups in the 3 and 3′ positions (zeaxanthin and lutein). β-carotene and lutein are the main components. Also occurring in higher plants are the 5,6-epoxides of β-carotene and lutein, violaxanthin and neoxanthin. Other minor carotenoids present are β-cryptoxanthin and antheraxanthin [88, 89]. Carotenoids in plants are located in chloroplasts/chromoplasts where they are noncovalently bound in protein complexes. Some ketocarotenoids such as rhodoxanthin in gymnosperms and cycads, are not present in chloroplasts but in extraplastidic oil droplets [89].

Chloroplasts in terrestrial plants were acquired by a stable endsymbiosis of cyanobacteria [105–107], but during evolution considerable modification of the chloroplast genome has occurred through transfer of genes to the host nucleus and the loss of nonessential genes from the chloroplast. The relatively simple complement of plant carotenoids suggests that a monophyletic stable symbiosis was established that performed several important functions for the plant and became indispensable. This is in contrast to the polyphyletic acquisition and loss of chloroplasts in certain groups of protists [92].

The monophyletic origin of chloroplasts in plants is supported by the identification of two groups of genes that are clustered in chloroplast genomes [108]. DNA sequencing of the chloroplast genome of the red alga *Porphyra purpurea* demonstrated 125 genes, of which 58 (~46%) were not found on the chloroplast genomes of land plants. *crtE* (encoding prephytoene pyrophosphate dehydrogenase) was present in the red algal chloroplast genome but not in the chloroplast of land plants [108]. In land plants, apparently this gene has been transferred to the nucleus. *crtE* is also incoded in cyanelle DNA in the flagellated alga *Cyanophora paradoxa* [109]. In plants, all chloroplast regulatory genes including those for carotenoid synthesis appear to be encoded in the nucleus [108].

4.2.4 Fungi

Recent phylogenetic studies have led to the surprising conclusion that fungi and animals share an evolutionary history which is independent from plants [110, 111]. Sequences of 25 proteins and comparisons of small subunit ribosomal RNA sequences indicated that animals and fungi are a monophyletic group while plants constitute an independent evolutionary lineage [110]. The results imply that animals and fungi share many similarities at the molecular and cellular levels and that yeast or other fungi may be the model system of choice for understanding metabolic processes related to carotenoids in eukaryotes [110].

Carotenoids occur only sporadically in fungi. Of more than 330 species, about 200 species of carotenogenic fungi have been described [89]. The lower fungi and ascomycetes produce mostly carotenes, especially γ-carotene and β-carotene (Table 2). However, some of the brilliantly colored Discomycetes and Pyrenomycetes synthesize carotenoids of increased complexity including phillipsiaxanthin, aleuriaxanthin, and plectaniaxanthin [96]. The richness of the carotenoid diversity increases in the most advanced fungal phylum, the basidiomycetes.

Goodwin [89] proposed several generalizations from his analysis of fungal carotenoids. Among those pertinent to this review are: (1) many fungi produce only carotenes, including the Phycomycetes, Chytridiales, and Blastocladiales; (2) torulene appears in red yeasts and in certain ascomycetes but rarely in Basidiomycetes and never in Phycomycetes; (3) no carotenoids with α-ionone rings (e.g. α-carotene) have yet been isolated, and (4), no xanthophylls characteristic of the green tissues of higher plants and algae (e.g. cryptoxanthin), 5,6-epoxides (except as possible degradation products), or allenes (e.g. neoxanthin) have been demonstrated in fungi. Characteristic fungal xanthophylls are carboxylic acid apocarotenoids such as torularhodin; somewhat similar xanthophylls are found in some fruits (e.g. lycoxanthin) and algae (loroxanthin). Ketocarotenoids including echinenone, canthaxanthin, and astaxanthin are also occasionally found in fungi [89]. Carotenoids typically found in the four subdivisions of fungi (Zygomycotina, Ascomycotina, Basidomycotina, and Deuteromycotina) are listed in Table 2.

Carotenoids are relatively rare in yeasts confined only to genera of heterobasidiomycetes [112]. They unfortunately do not occur naturally in common industrial yeasts such as *Saccharomyces cerevisiae*, *Kluyveromyces*, and *Candida utilis*. Carotenoids characteristic of the red heterobasidomycetous yeasts are torulene and the carboxylic acid torularhodin, which appear *Rhodotorula*, its perfect stage *Rhodosporidium*, in *Sporobolomyces*, *Sporidiobolus* and in some species of *Cryptococcus*. Plectaniaxanthin, a pigment normally present in Discomycetes, was detected in the imperfect yeast *Cryptococcus laurentii* [113]. The presence of carotenoids is used as a criterion in yeast classification [112]. This appears reasonable since carotenoid synthesis involves several genes and is

expressed in only a limited group of yeasts although it can be an unstable property.

The discovery of *Phaffia* in the 1960's [114], the description in 1976 of its unusual physiological characteristics compared to other pigmented yeasts [114, 115], and the elucidation of the structure of its pigments [116, 117] has added important diversity to yeast carotenoids. *Phaffia* produces astaxanthin, 3-hydroxy-3′,4′-didehydro-β,ψ-caroten-4-one (HDCO), torulene and β- and γ-carotene as its primary pigments. The levels of pigments in wild isolates are low but they have been increased greatly by strain development in industry. *Phaffia* accumulates the 3*R* and 3*R*′ configurational isomers which are the opposite configuration found in *Haematococcus*:

Certain fungi have the ability to accumulate cartenoids to quite high levels. For example, *Phycomyces* can accumulate up to 25 mg of β-carotene per g of mycelium when cultivated on agar plates and subjected to genetic activation and external agents [118]. A high capacity to synthesize carotenoids is evident another fungi as well, e.g. cells of some jelly fungi (*Dacrymyces*) are packed with oil globules containing carotenes. Carotenoids in fungi probably accumulate in lipid globules and in membranes, and may provide antioxidant protection.

The evolutionary origins of carotenoids in fungi are not known. The choanoflagellates or perhaps the true slime molds may be the closest protistan ancestor to the fungi [119]. However, like the algae, it is probable that the source of endosymbionts in fungi is polyphyletic. There is strong homology between the bacterial and fungal genes encoding enzymes early in the isoprenoid and carotenoid pathways [120], but these genes may be universally congruent in all carotenogenic organisms. Fungi may have acquired the ability to produce carotenoids by endoymbiosis of bacteria, algal (e.g. Rhodophyta or Chlorophyta) chloroplasts, or by horizontal gene transfer from bacteria. Intimate pairing in lichens may also have encouraged symbiotic transfer of carotenogenic genes. The finding of phillipsiaxanthin and plectaniaxanthin in fungi, which share structural characteristics with photosynthetic bacteria, support the polyphyletic nature of fungal carotenoids. If fungi acquired plastids as a source of carotenoids, it is possible that a morphologic or genetic study would reveal some relics of this plant and algal organelle in the carotenogenic fungi. Alternatively, the findings that fungi and animals are sister groups indicates a common ancestor which may have been an unidentified protist. Suggestions for the ancestor have included choanoflagellates [119, 121] or certain slime molds. The choanoflagellates lack carotenoids [122] whereas many slime molds are pigmented. The slime mold *Lycogola epidendeon* in the class Myxomycetes produces γ- and β-carotenes, 3,4-didehydrolycopene, torulene, and neurosporaxanthin [113].

The lichens, which are symbiotic associations between fungi (often ascomycetes) and green algae or cyanobacteria, contain a wide diversity of carotenoids. This is expected considering the polyphyletic origins of carotenoids in algae and fungi. Extensive studies of carotenoid composition in lichens have been carried out by Czeczuga and his colleagues in Poland and recently summarized [123].

The carotenoid composition of lichens depends on their exposure to light. Both typical algal and fungal carotenoids appear in lichens. Lutein, epoxides, α-carotene, zeaxanthin and other typical plant and algal carotenoids have been reported. Fungal carotenoids including torulene are also present. Several carotenoids of industrial interest occur in lichens including β-carotene, zeaxanthin, canthaxanthin, lutein, and astaxanthin. Most of the carotenoids in lichens are present in low concentrations ($\leq 100\ \mu g\ g^{-1}$). Nonetheless the lichens could be valuable as biological material for isolation of new genetic sources of carotenoids and also may be of interest because of possible genetic and physiologic interchange between the symbiotic pairing of fungi and algae.

5 Biological Functions of Carotenoids

Interest in carotenoids has increased considerably during the past ten years, due in part to the growing evidence of benefits to human health and also to the growth of certain areas of agriculture, especially aquaculture and the poultry industry. In the field of human benefits of carotenoids, several proposed attributes and protective roles in chronic disease are speculative, but clinical trials should critically evaluate the benefits during the next few years. As roles in human disease prevention become known, the commercial demand for carotenoids should increase.

5.1 Roles of Carotenoids in Preventing Degenerative Diseases in Humans

The possibility that carotenoids have roles in preventing cancer and other degenerative diseases has generated considerable interest and activity within the carotenoid field. The primary degenerative diseases associated with aging are cancer, cardiovascular disease, immune-system decline, brain dysfunction, arthritis, and cataracts [124]. Epidemiological investigations have shown that consumption of green and yellow vegetables is associated with reduced incidence of some cancers [125, 126]. In fact, good dietary consumption of fruits and vegetables approximately halves the likelihood of developing cancer [124]. Research has supported the theory that life expectancy is related to oxidative damage of DNA, proteins, and lipids [124, 127 128] and that natural antioxidants including carotenoids among other substances are important in the prevention of these oxidations.

An important factor in longevity appears to be the basal metabolic rate [124] since metabolism produces oxidative by-products which can cause extensive damage to cellular components. The primary oxidants generated in metabolism

are superoxide (O_2-, hydrogen peroxide (H_2O_2), hydroxyl radical, lipid epoxides, hydroperoxides, lipid alkoxyl and hydroperoxides, and singlet oxygen $^1O_2$. All of these are products of normal metabolism and millions of each species are produced each day [124]. Singlet oxygen is formed from transfer of energy from light, the respiratory burst of neutrophils, lipid peroxidation, or other dark reactions [129–131]. Singlet oxygen has benefits as well as detriments: it acts as a meditator of the antibacterial action of leukocytes [132] but its reactivity with human tissues can cause damage and it probably contributes to acute and chronic health disorders [133–138]. Antioxidant defenses against these damaging agents include ascorbate, tocopherol, and carotenoids [124]. One of the first classes of compounds demonstrated to effectively quench $^1O_2$ was the carotenoids [139]. Carotenoids physically quench $^1O_2$, generating ground state oxygen and a carotenoid triplet which returns to its ground state with release of heat [140]. Carotenoids vary in their quenching capacity; $^1O_2$ quenching capacity generally increases as the number of conjugated double bonds in the carotenoid increases, but quenching also varies with chain structure, functional groups, *cis/trans* isomerism and the technique used for determination [141, 142, 143].

β-Carotene has been proposed as an important dietary anticarcinogen, and evidence has been obtained that β-carotene prevents cancers caused by chemicals and viruses [144–146]. However, several other carotenoids occur in plant foodstuffs including α-carotene, γ-carotene, lycopene, lutein, neoxanthin, violaxanthin, and others. Murakoshi et al. [147] presented evidence that α-carotene was more effective than β-carotene in preventing lung and skin carcinogenesis in mice. Recently, it was shown that astaxanthin but not canthaxanthin effectively prevented urinary bladder carcinogenesis [148]. Since astaxanthin is not a precursor to vitamin A in animals, the action of astaxanthin may have been related to suppression of cell proliferation. Astaxanthin was shown in vitro to enhance antibody production to sheep red blood cells in normal mice [149, 150]. This immunomodulating activity could reduce the chance of developing autoimmunity and malignancies by enhancing T-helper functions and promoting specific

Table 3. Functions and uses of carotenoids in humans–known and proposed [153, 154]

Quenching of singlet oxygen
Termination of lipid peroxidation
Cancer prevention
Immunomodulation
Gap junction cell-cell communications
Modulation of lipoxygenase activity
Cardiovascular disease prevention
Cataract prevention
Photosensitivity prevention
Vitamin A precursor-vision, cell differentiation
Membrane modulators, fluidity control
Modulators of membrane enzymes
Precursors of hormones, vitamins, e.g. retinoids for human development

antibody responses [149,150]. Astaxanthin is an excellent quencher of $^1O_2$ compared to several other carotenoids tested, which may contribute to its anticarcinogenic activity. Damage to ocular tissue occurs by radiant energy in the 295–450 nm range [151], and blue light and near-UV radiation damage is often increased by natural photosensitizers in cells (i.e riboflavin). Of the mechanisms by which radiant energy damages ocular tissues, the involvement of $^1O_2$ has been best documented [151,152]. It is possible that ocular tissue, damage could be lessened by carotenoids. Another example is the formation of oxidized low-density plasma lipoprotein (LDL), which contributes to cardiovascular disease. Carotenoids prevented oxidation of human LDL [153]. Other biological functions of carotenoids in humans have been proposed and are being investigated in animals and in human clinical trials (Table 3) [154,155].

5.2 Functions of Carotenoids of Microorganisms, Plants, and Animals

Carotenoids and their derivatives are known to have several functions in photosynthetic bacteria and eukaryotes. Besides their universal role in photosynthetic organisms as light-harvesting pigments and as photoprotectants (quenching of triplet-state prophyrins and $^1O_2$) in photosynthetic membranes [1–3], they have several additional biological functions. In corn coleoptiles, zeaxanthin has been proposed as the receptor for blue light-induced phototropism [156]. Carotenoids are a source of hormones and stress response in plants: the growth regulator abscisic acid is formed in water stressed plants by cleavage of carotenoids [157]. In algae, carotenoids are cleaved to retinoids, which combine with opsin to form the photoreceptor for phototaxis [158].

The well-established function of carotenoids in animals is as vitamin A precursors [159] and as a source of retinoids crucial for vertebrate development [160]. In addition, they probably have a role in quenching singlet oxygen and terminating lipid peroxidation [161]. This activity possibly prevents mutations in the ova of marine fish and shellfish which may be a reason the eggs of many of these species are so highly pigmented. Many of the functions proposed above for humans may apply to lower animals as well.

Carotenoids in oceanic animals probably have their origin in phytoplankton at the ocean surface. Still, deep sea animals distant from the surface waters often have substantial concentrations of carotenoids obtained through the food chain. The function of carotenoids is probably for reproduction, osmotic tolerance, or other purposes, and not for photoprotection since these animals are not exposed to bright light. In contrast, hydrothermal vent animals generally lack carotenoid pigmentation, which is consistent with the nutritional input of these animals coming from carbon fixed within the vent area and not deriving from photosynthetically fixed carbon at the ocean surface. The exceptions are the vent decapod crustaceans [162]. Although the exoskeletons of deep-vent shrimp and crabs are nearly colorless, their eggs and hepatopancreas are colored, though

less carotenoid is present than in benthic crustacea [162]. The carotenoids present in the vent brachyuran crab *Bythograea thermhydron* include astaxanthin (80%), β-carotene (10%) and the remainder echinenone, canthaxanthin and presumably carotenes. Interestingly, α-carotene was not detected suggesting that the carotenoids were not of algal origin. The restricted composition of pigments suggests that the pigments may be synthesized by bacteria in the thermal vent environment which are then ingested. The vent habitat could offer a new location for isolation of novel organisms producing astaxanthin and other pigments.

Carotenoids have several roles in fungi. They are the precursors to mating hormones (trisporic acids) in the Mucorales. They may also be involved in development and reproduction in *Neurospora* since biosynthesis of carotenoids is photoregulated in the mycelium but not in the conidia. The most general function is to protect against $^1O_2$ and phytoalexin/oxygen mediated killing of fungi in the phyllosphere. Carotenoids can stabilize membranes and lessen damage by microbiocidal medium chain fatty acids common on the leaf surface. Cross-linked carotenoids in pollens and in the zygospores of ascopspores of some fungi provide extraordinary resistance to chemical and environmental stresses [163]. They may also have antimicrobial properties in certain ecological niches: the pepper carotenoid capsanthin inhibits growth and aflatoxin production by *Aspergillus* [164].

Carotenoids can stabilize lipid membranes by decreasing water permeability and increasing rigidity [165–167]. Carotenoids decreased membrane fluidity and increased resistance to oleic acid killing in *Staphylococcus aureus* [168]. This decrease in membrane fluidity could also contribute to heat and radiation damage. The resistance provided to organisms by carotenoids may have ecological significance allowing organisms to colonize certain environments, for example staphylococcal invasion of wounds, myocbacteria in AIDS, and colonization of leaf surfaces by pigmented heterobasidiomycetous yeasts.

6 Biosynthesis of Carotenoids

Excellent recent reviews of the biosynthesis of isoprenoids to cholesterol and carotenoids have been published [169–171], and only some new developments are presented here, particularly as they pertain to microbial synthesis of industrially important carotenoids.

A crucial aspect of the regulation of biosynthesis of carotenoids in microorganisms is the location of synthesis, the spatial separation of carotenoid biosynthesis from sterol and lipid synthesis (which also use acetyl-CoA as a substrate), and the deposition and transport of isoprenoids throughout the cell. The rate limiting enzyme in the isoprenoid biosynthetic pathway in *Saccharomyces cerevisiae* and in mammalian cells is 3-hydroxy-3-methylglutaryl-coenzyme

A reductase (NADPH) (i.e. HMG-CoA reductase). In contrast to mammalian cells which have a single reductase, the yeast has two functional genes encoding the enzyme with limited homology to HMG-CoA reductase from hamster cells [172]. Plants also contain multiple reductase genes which are differentially regulated. *Arabidopsis thaliana* contains two differentially expressed reductase genes, whose product is inserted into the endoplasmic reticulum-derived microsomal membranes [173]. One of the yeast reductases (Hmg1p) resides in the perinuclear ER, while the other is present in peripheral ER membrane [174]. The different cellular locations may allow specific compartmentalization of mevalonate synthesis. One of the two isozymes (Hmg2p) is rapidly degraded in response to feedback signals from the isoprenoid pathway [175]. Decreased mevalonate pathway flux reduces the rate of degradation of Hmg2p, and one signal is a nonsterol intermediate prior to squalene. Overexpression (10-fold) of Hmg1p causes the proliferation of the membrane in which the reductase resides, forming perinuclear stacked structures termed karmellae and thus increased compartmentalization of the enzymes [176]. Selective protein degradation in the ER is controlled partly by redox potential [177], which could be influenced by respiratory activity of the cells or cyanide-insensitive oxygen uptake by cytochrome P-450s which are abundant in the ER.

Sterol synthesis in *S. cerevisiae* is differentially regulated and partitioned in different compartments [178]. The HMGR isozymes directed essentially equal quantities of carbon to the biosynthesis of sterols when heme was available despite a large (67-fold) difference in specific activity of the isozymes. Palmitoleic acid (16:1) acted as a positive regulator and ergosterol and oleic acid (18:1) as inhibitors but the regulation pattern differed for each isozyme. Casey et al. [178] concluded that separate compartmentalized isoprenoid pathways exist in *S. cerevisiae*. Carotene and sterol pathways in *Phycomyces* were reported to be independently regulated and physically separated in different subcellular compartments [179]. The reactions of carotene biosynthesis in *Phycomyces* and zeaxanthin synthesis in a *Flavobacterium* sp. were also tightly channeled in the cell because of the presence of enzyme aggregates [180–183]. Enzyme complexes have also been observed for the reactions in early isoprenoid synthesis leading to phytoene [184]. Therefore, cellular topography, compartmentation, and the presence of enzyme aggregates adds a hierarchy of complexity to carotenoid biosynthesis, and conclusions from in vitro 'solubilized' enzymes on carotenoid biosynthesis will have to be made cautiously.

By analogy with other isoprenoid systems, carotenes probably originate in the smooth ER and then are transported within the cytoplasm in lipid vesicles or in complexes with lipoproteins. Transmission electron micrographs of sections of *Phaffia rhodzyma* and comparison to confocal autofluorescent images indicates that carotenoids are deposited in lipid globules, which in wild-type yeasts are mainly associated with the lipid membrane and are especially prevalent in the growing tip of buds. In hyperproducing mutants the fluorescence was more widely distributed throughout the cell. Xanthophylls may also be biosynthesized in the ER since cytochrome P-450 and cyanide-insensitive oxygen uptake is

abundant in this organelle. Alternatively, carotenes could be formed in the ER, and deposited in lipid globules or ER vesicles, and transported to other membraneous regions or remain in the cytosol depending on the strain of yeast and its rate of production. Once in the membranous region, the carotenes may then react enzymatically with oxygen metabolites to form the characteristic xanthophylls depending on the species. Secondary carotenoids (astaxanthin and other ketocarotenoids) in *Haematococcus* first accumulated in lipid bodies surrounding the nucleus [185], and were suggested to function as a physicochemical barrier protecting the genome from free-radical damage [186]. The smooth ER in yeasts is not only associated with the nuclear region but also may be contiguous with the cytoplasmic membrane and growing tips of the bud. Membrane-bound, small, ER derived vesicles at the growing tips of the bud have been observed in *S. cerevisiae* and in the heterobasidiomycetous yeasts *Rhodosporidium* sp. and *Sporidiobolus salmonicolor* [187]. The quantity of ER in yeast cells varies greatly, and is related to growth rate and oxygen availability [188], conditions which also affect carotenoid synthesis.

The ontogeny of lipid bodies in plants and *Neurospora crassa* was examined by electron microscopy during massive lipid accumulation [189]. The lipid bodies as well as the outer membrane of plastids of fat-storing cells were associated with ER-like cytoplasmic membrane structures, suggesting that both plastids and ER may be involved in the synthesis of lipid bodies. Chromoplast development in fruits and flowers of plants results during differentiation of plastids and is often associated with accumulation of large quantities of carotenoids [190]. Carotene bodies contribute to the morphology of the mature chromoplast. Depending on the type of chromoplast, carotene molecules crystallize, dissolve in lipid globules, or become tightly bound to protein molecules. More than 60% of the plastid membrane protein participate in carotene binding or synthesis. The ontogeny of the chromoplasts appears to offer an ideal system for investigating the biosynthesis of carotenoids.

It is likely that carotenoids are formed and deposited in the cell in the proximity of enzyme systems that generate toxic forms of oxygen such as mitochondria or microbodies. Microbodies (peroxisomes, glyoxosomes, glyoxiperoxisomes) are frequently associated with strands of endoplasmic reticulum [191]. Carotenoid biosynthesis and modification in eukaryotic cells may occur in proximity to peroxisomes, since these organelles are involved in oxidative reactions and generate and consume H_2O_2. Similar to the mitochondrion, the peroxisome is a major site of oxygen utilization, and may represent the vestige of an ancient organelle that carried out all of the oxygen metabolism in procaryotic or primitive eucaryotic cells. The protein composition of peroxisomes is limited and the total peroxisomal protein consists of up to 40% catalase. Peroxisomes are responsible for the degradation of alcohol to acetaldehyde and for the degradation of fatty acids to acetyl-CoA producing H_2O_2 in the process. The acetyl-CoA generated from fatty acid breakdown can be transported to the mitochondria for utilization in the citric acid cycle, or it could be used for biosynthetic reactions such as isoprenoid biosynthesis. The meta-

bolic roles of peroxisomes have been investigated in green leaves. At high light intensities and in hypoxic conditions, chloroplasts generate the 2-carbon compound glycolic acid, which exits the chloroplasts and enters into the peroxisomes. Glycolic acid is oxidized forming hydrogen peroxide and glyoxylate, and glyoxylate can then be converted to the amino acid glycine for protein synthesis or transported to mitochondria for further metabolism. Oxidized glycolic acid can also condense with two molecules of acetyl-CoA from fat degradation forming isocitric acid, and degraded to succinate and glyoxylate, thus perpetuating the cycle. Populations of peroxisomes and mitochondria increased in population during leaf senescence and were associated with the transition of leaf peroxisomes into glyoxysomes [192]. Aging and senescence in fungi is associated with increased generation of reactive oxygen metabolites and differentiated cells have a relatively more prooxidizing or less reducing intracellular environment [193].

The involvement of peroxisomes in isoprenoid biosynthesis has been demonstrated in mammalian cells. Mevalonate kinase has been localized to rat liver peroxisomes [194]. Isoprenoid biosynthesis or squalene has been demonstrated in mammalian cells [195]. The physiological importance of peroxisomal isoprenoid formation to overall polyisoprenoid synthesis is not yet established. The synthesis in peroxisome could contribute to compartmental regulation of isoprenoid synthesis.

7 Genetics of Carotenoids

The genetics of carotenoid biosynthesis in bacteria, plants, and fungi has been recently reviewed [169, 170] and only selected areas are discussed. Genes for carotenoid biosynthesis, i.e. the *crt* genes, are clustered on the chromosome in many eubacteria such as *Rhodobacter capsulatus* [196], *Rhodobacter sphaeroides* [197], *Erwinia herbicola* [198, 199], *Erwinia uredovora* [200], and *Mycobacterium aurum* [202]. The structures are unlinked to the regulatory genes for carotenogenesis in *Myxococcus xanthus* [201]. A carotenogenic gene cluster containing the *crtB* gene is on a large plasmid in the gram-negative thermophile *Thermus thermophilus* [203]. Gantotti and Beer [204] reported that pigmentation and thiamine prototrophy in *Erwinia herbicola* was controlled by a large plasmid. The genes controlling conversion of lycopene into xanthophylls were not linked to the gene cluster encoding carotene biosynthesis in *M. aurum* [202]. These reports indicate that differences exist in the genomic locations and expression of *crt* genes in certain nonphotosynthetic and photosynthetic bacteria. The carotenoid biosynthetic genes in purple nonsulfur bacteria are present in superoperons, in which transcription is coupled to genes encoding bacteriochlorophyll [205]. This arrangement enables rapid expression and assembly of the photosynthetic apparatus when oxygen concentrations become limiting.

The *crt* genes encoding carotenoid biosynthesis in *E. herbicola*, *E. uredovora* and *R. capsulatus* have been expressed in *E. coli* [200, 206, 207]. The *crt* genes of *R. sphaeroides* were expressed in nonphotosynthetic bacteria including *Paracoccus denitrificans*, *Agrobacterium tumefaciens*, and *Azotomonas insolita* [208], which is interesting from an evolutionary point of view because it indicates that nonphotosynthetic bacteria could acquire the ability to synthesize carotenoids from photosynthetics through a symbiotic relation or by genetic exchange of the carotenogenic genes.

Among carotenogenic organisms, the *crt* and regulatory genes in the purple nonsulphur photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* and in the chemoorganotrophs *Erwinia herbicola* and *Erwinia uredovora* have been studied most extensively. *Rhodobacter* synthesizes acyclic carotenes and methoxy carotenoids while *Erwinia* forms cyclic and hydroxylated pigments including β-carotene and zeaxanthin and glycosides of zeaxanthin. Eight carotenogenic genes of *R. capsulatus* are organized in 4 operons: *crtA*, *crtIBK*, *crtDE* and *crtEF*. The ninth gene, *crtJ*, lies on a fragment separated by 12 kb. Many of the *R. capsulatus* gene products have been identified. *crtE* encodes phytoene synthetase, *crtB* and *crtJ* code for prephytoene pyrophosphate synthetase, while *crtI* has been found to code for phytoene dehydrogenase. However, in *Thermus thermophilus*, *crtB* was found to code for phytoene synthase. By introducing *crtB* on a multicopy plasmid into a hyperproducing mutant, carotenogenesis was increased 20 fold. This suggests that phytoene synthase is a rate limiting step for carotenogenesis in this organism [209]

In *Erwinia* the carotenogenic genes coding for the first three enzymes in the pathway (prephytoene synthase, phytoene synthase and phytoene dehydrogenase) share a common nomenclature with *Rhodobacter* (*crtB*, *crtE* and *crtI*). The remaining three genes, however, coding for lycopene cyclase, β-carotene hydroxylase and zeaxanthin glycosylase have different nomenclature in the two *Erwinia* species. In *E. herbicola* they are *crtZ*, *crtH* and *crtG* respectively, while in *E. uredovora* they are *crtY*, *crtZ* and *crtX*. Although the GC contents of *Erwinia* and *R. capsulatus* are different (52.6–57.7% in *E. herbicola*, 53–54.5% in *E. uredovora* and 65.5–66.8% in *R. capsulatus*), and the organisms are not closely related by several other properties, the sequences of the putative proteins encoded by *crtI*, *crtB* and *crtE* all share homology [210]. *crtB* and *crtE* gene products also have similar domains among various prenyltransferases involved in isoprenoid biosynthesis [210]. In *Rhodobacter*, *Erwinia*, and *Neurospora*, a single gene encodes a gene product (CrtI/A1–1) which performs multiple dehydrogenations to neurosporene or even lycopene, while cyanobacteria, green algae, and higher plants presumably require two gene products [211]. The carotenoid dehydrogenases of *R. capsulatus* and *E. herbicola* are homologous (26 to 76%) and contain an evolutionarily conserved FAD/NAD(P) and carotene binding regions, while the dehydrogenase from *Neurospora crassa* is more distantly related. Phytoene dehydrogenase in tomato, a single polypeptide catalyzing the conversion of phytoene to ξ-carotene, is transcriptionally

regulated during fruit ripening [212]. The deduced amino acid sequence of the tomato desaturase shows high homology to phytoene desaturases of cyanobacteria and algae, but was unrelated to those from purple bacteria and fungi. Thus, two evolutionarily unrelated lineages of phytoene desaturases appear to exist in nature.

The genetics of fungal carotenoids have mostly been investigated in *Phycomyces* and *Neurospora crassa*. Carotenoid pigments are induced by blue light in *N. crassa*, and carotenogenesis is also induced developmentally in the dark during formation of macroconidia [213]. Colorless (albino) mutants are common in *N. crassa*, in which *al*-1 mutants accumulate phytoene [214]. Four genes of *N. crassa* encode enzymes for carotenoid biosynthesis, *al-1*, *al-2*, *al-3*, and the yellow-1 locus [214]. Mutations in any of the three albino genes gives white mycelia and conidia. The *al-1*, *al-2*, and *al-3* genes encoding phytoene dehydrogenase, phytoene synthase, and geranylgeranyl pyrophosphate synthetase have been cloned and sequenced [215–217]. All three genes are photoinduced by blue light. Phytoene synthase activity is membrane associated in *Neurospora* [218]. The phytoene dehydrogenase deduced amino acid sequence has homology to those of *Rhodobacter* and *Glycine max* (soybean). Bacterial and eukaryotic phytoene synthases and prephytoene pyrophosphate synthase proteins share homology [219], supporting the hypothesis of structural relatedness of very early carotenoid biosynthetic enzymes (phytoene synthases and prephytoene pyrophosphate) in phylogenetically diverse organisms, while two distinct lineages of phytoene desaturases apparently have evolved. Further divergence is expected for later enzymes, but the genes and enzymes catalyzing the formation of xanthophylls in different organisms remain to be isolated and characterized.

Carotenoid expression in various bacteria and fungi is controlled by several mechanisms including blue light, oxygen, conidial development, and growth stage. In purple nonsulfur bacteria (*Rhodobacter*), the "superoperons" harboring *bch* and *crt* genes, appear to be required for optimal transition of cells from aerobic respiration to anaerobic photosynthesis. Genes encoding the superoperons are repressed by a mechanism involving oxygen. In low oxygen conditions, the expression of the photosynthetic apparatus is regulated by light intensity [220, 221]. Little is known of the mechanism governing the oxygen-mediated expression of the *crt* operons [222]. A gene (*ppsR*) was isolated from the photosynthetic gene cluster of *R. sphaeroides* that represses Crt protein levels [222]. Inactivation of the *ppsR* gene causes overproduction (5-fold) of carotenoid pigments and bacteriochlophylls, but these mutants are unstable [222]. The deduced amino acid sequence of the protein product of *ppsR* is homologous to the CrtJ protein of *R. capsulatus*, and also to the C-terminal region of various other regulatory proteins, which have similar structure as response regulators of two-component regulatory systems. While ppsR appears to be a transcriptional repressor, the mechanism by which oxygen affects expression is not known. Oxygen or a derivative could react with transcriptional regulators directly leading to an oxidized inactive state, or oxygen could be sensed at the cell membrane by a redox active two component system.

Other bacteria apparently regulate carotenogenesis by other mechanisms. In *Myxococcus xanthus*, carotenoids accumulate after the transition from exponential to stationary phase and their formation is absolutely dependent on light [224]. Expression of carotenoids in stationary phase is due to exhaustion of the carbon source [224], a condition that also stimulates the cell's response to oxidative stress [225]. Hodgson and Murillo [224] have proposed the interesting hypothesis that $^1O_2$ is involved directly in activation of the *carORS* operon in *M. xanthus*. Carotenoid formation in *M. xanthus* seems to depend on protoporphyrin IX as a photosensitizer [226], which has an absorption maximum of 410 nm. In a model in which expression is regulated by $^1O_2$, blue light excites protoporphyrin IX in the membrane to the triple-state, which in turn reacts with O_2 to form $^1O_2$. Reaction of the membrane bound CarR causes release of CarQ and expression of genes encoding carotenogenic enzymes as well as CarR. The carotenoids in turn quench $^1O_2$ and shut down expression.

The genetics of β-carotene hyperproduction in *Phycomyces* has been investigated in depth by Cerda-Olmedo and his coworkers. Carotene and sterol synthesis in *Phycomyces* are physically separated in different compartments and the biosynthetic reactions of carotene biosynthesis are channeled because of an enzyme aggregate [227]. β-Carotene production in *Phycomyces* is dramatically affected by culture conditions, the strain, chemical stimulators, illumination, sexual activity, and endogenous genetic changes. Mutations in the genes *carS*, *carD*, and *carF* dramatically increased carotenoid levels. In contrast, carotene levels were decreased by mutations in *carA* and *carC*. Sexual interactions between heterothallic Mucorales gave heterokaryons and increased the carotene content. Physical contact for sexual stimulation was not necessary, which led to the isolation of diffusible trisporic acids that are produced from β-carotene. A third impressive component of carotene overproduction in *Phycomyces* is chemical activators. Several chemicals added to the medium increase the β-carotene content [228]. One group of chemical activators such as retinol and β-ionone are related in structure to β-carotene, while another group are phenols. When sex is combined with *carS* and *carF* mutations, the carotene content increased from 100 to 25000 $\mu g\, g^{-1}$ mycelium [118]. End-product regulation by β-carotene is mediated by the *carS* gene product. Mutations in *carS* result in massive accumulation of the intermediates lycopene and phytoene whereas others were white because the *carS* product stops carotene synthesis with very low levels of β-carotene [229]. The *carS* gene product also combines with the main regulator *carA* product pA, a complex which represses expression of the carotenogenic genes. pA activity is affected by light and sexual activity. The genes of the isoprenoid pathway in *Phycomyces* are also investigated at the sequence level [230].

Despite the growing commercial importance of *Phaffia rhodozyma*, very little is known of its genetics. In part, this was due to the inability to find sexual activity. Quite recently Golubev [231] reported mother-daughter cell conjugation on polyol-containing media. This resulted in holobasidia formation with terminal basidiospores. No discharge of basidiospores, nor mycelium formation were reported. This telomorphic stage was given the name *Xanthophyllomyces*

dendrorhous. Golubev [231] suggested that *Phaffia* had been described much earlier by Ludwig [232, 233] and named *Rhodomyces dendrorhous*.

By using Contour-clamped Homogeneous Electrical Field (CHEF) gel electrophoresis of 6 strains of *P. rhodozyma*, it has been shown that heterogeneity exists in chromosome size [234]. While most chromosomes have a size of 1000–2000 kb, some strains were found to have an additional chromosome of 3000 kb. Most, but not all strains contained an additional chromosome of 250–350 kb. The total chromosomal number was 7 in two of 6 strains studied and 6 in the remaining 4 strains. When the chromosomes in the CHEF gels were transferred to nitrocellulose and probed with the *S. cerevisiae ura3* and *S. pombe ura4* genes the *ura3* sequence (but not the *ura4* sequence) hybridized to *Phaffia* genomic DNA. The total DNA content of *P. rhodozyma* has been estimated by flow cytometry to be $\sim 4\times$ the content of haploid *S. cerevisiae* [235].

Although certain classes of auxotrophic and other mutations in essential genes have been difficult to obtain in *Phaffia* in some laboratories, color mutants occur commonly. Girard et al. [236] generated a series of color mutants from a wild-type strain by EMS and UV mutagenesis. The color of the mutants ranged from white to intense red. Twenty eight color mutants were recovered and carotenoid analysis showed three main classes: white (lacking carotenoids or accumulating phytoene), yellow mutants that accumulated β-carotene, and astaxanthin-hyperproducers. Interestingly, in several β-carotene and phytoene mutants, the levels of these pigments occurred at much higher levels than in the wild-type. This suggests that astaxanthin or another xanthophyll may regulate the pathway by feedback inhibition. Similar results have been observed in our laboratory in experiments where astaxanthin was decreased by chemical treatment [237]. According to the carotenoid analysis by Girard et al. [233], no mutants were obtained that accumulated carotenes at quantities higher than β-carotene. Nor were mutants obtained that accumulated xanthophylls in the Andrewe's sequence between β-carotene and astaxanthin, except for two mutants that produced mostly echinenone and hydroxechinenone and one strain that accumulated an unknown xanthophyll (presumably 3-hydroxy-3′,4′-didehydro-β-ψ-caroten-4-one [HDCO]). Only two of the isolated mutants produced higher levels of total cartenoids than the wild-type and both of these grew poorly compared to the parent. One yellow mutant produced nearly 2 mg of β-carotene g^{-1} when yeast extract/peptone medium was supplemented with 3% glycerol.

8 Industrial Aspects of Microbial Carotenoids

8.1 Industrially Important Carotenoids

Although more than 600 carotenoids have been identified from various sources, only about 40 are ingested by humans, and only a handful are used industrially

for food colors, animal feeding, pharmaceuticals and cosmetics [238, 239]. The primary colorants used in foods and feeds are presented in Table 4.

Most of the carotenoids used in the food, feed, nutritional supplement, cosmetic, and pharmaceutical industries, and for other industrial purposes are produced by total chemical synthesis. β-Carotene has been produced commercially since 1954 [11]. Six synthetic carotenoids or apo-derivatives have become important commercially [11]: β-apocarotenal, β-apo-8′-carotenoic acid ethyl ester, citranaxanthin (5′,6′-dihydro-5′-apo-18′-nor-β-carotene-6′-one), β-carotene, canthaxanthin, and racemic astaxanthin (Fig. 4). According to Pfander [11], approximate current market prices for stabilized dispersible powders containing 5–10% active carotenoid are \$600 kg^{-1} for β-carotene, \$900 for β-apo-8′-carotenoids, \$1300 for cantaxanthin, and \$2500 for astaxanthin. Total sales for synthetic carotenoids was estimated to be about \$300 million and may be more than \$500 million by 1997 [11]. The world market estimates for synthetic and biological sources of astaxanthin, canthaxanthin, and β-carotene are presented in Table 5 [240]. It is nearly impossible to estimate the market for natural extracts since many of the companies are privately held and in development. Of course, the market and demand for carotenoids may change if medical benefits are demonstrated in clinical trials.

Table 4. Carotenoid sources used as colorants in foods and in feeds

A. Natural extracts	
Compound	Major Carotenoids
Annatto (*Bixa orellano*)	Bixin, norbixin
Carrot oil (*Daucus carota*)	α-Carotene, β-carotene
Orange peels	Lutein esters
Palm oil	Carotenes, lutein
Paprika (*Capsicum annuum*)	Capsanthin, β-carotene, cryptoxanthin, capsorubin
Tomato (*Lycopersicon esculentum*) extracts	Lycopene, β-carotene
Saffron (*Crocus sativus*)	Crocin, β-carotene, zeaxanthin
Yellow corn (*Zea mays*)	Lutein, zeaxanthin, cryptoxanthin, carotenes
Alfalfa meal	Lutein, zeaxanthin, cryptoxanthin, violaxanthin, neoxanthin
Marigold extracts (*Tagetes erecta*)	Lutein (ester), β-carotene
B. Culture products	
Phaffia rhodozyma	Astaxanthin, HDCO, β-carotene
Blakeslea trispora	β-Carotene
Dunaliella salina	β-Carotene
Haematococcus sp.	Astaxanthin
C. Synthetic carotenoids	
β-Carotene	
β-Apo-8′-carotenal	
β-Apo-8′-carotenoic esters	
Canthaxanthin	
Citranaxanthin	
Astaxanthin	

Presently, carotenoids for industrial purposes are nearly exclusively obtained by chemical synthesis or by extraction of plant materials, but these carotenoids also occur naturally in species of microalgae, bacteria, and fungi. Much interest has been devoted to microbial synthesis of carotenoids, and several patents have been filed in recent years (Table 6). Microbial carotenoid products from *Dunaliella*, *Haemotococcus*, and *Phaffia rhodozyma* are now being commercialized.

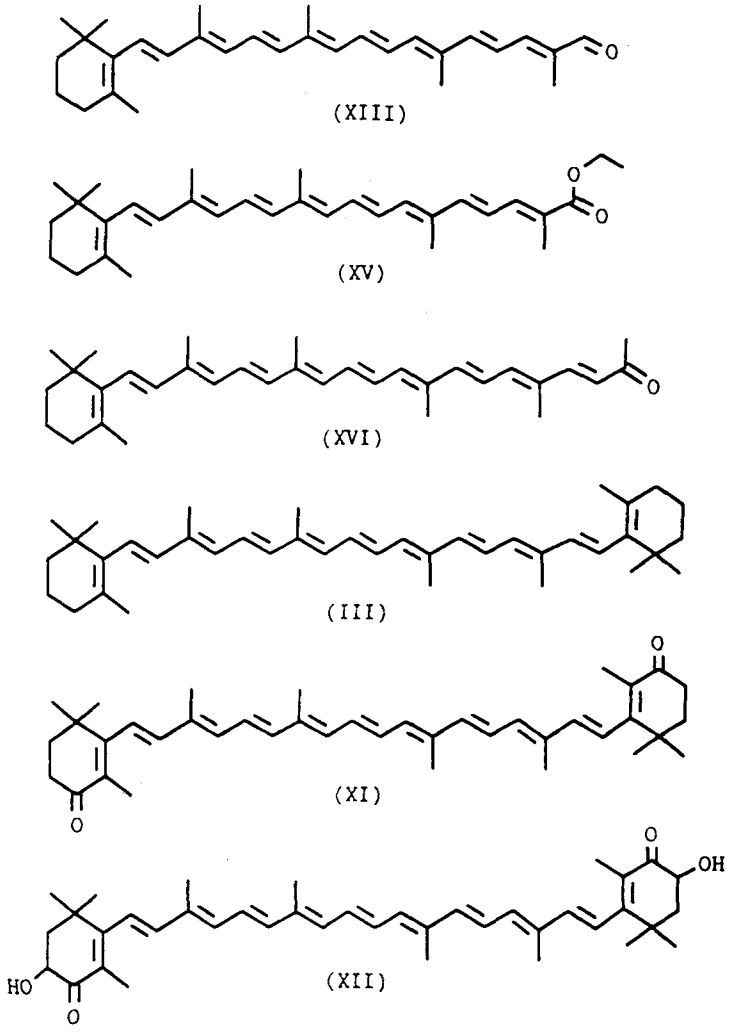

Fig. 4. Structures of six synthetic industrially important carotenoids. **XIII:** β-apo-8'-carotenal, (C_{30}); **XV:** β-apo-8'-carotenoic acid ethyl ester, (C_{32}); **XVI:** citranaxanthin, (C_{30}); **III:** β-carotene, (C_{40}); **XI:** canthaxanthin, (C_{40}); **XII:** racemic astaxanthin, (C_{40}). From Ref. [11]

Table 5. Estimated world market for synthetic and biological carotenoids (1992–2000) [235]

	1992 $ million		1996 $ million		2000 $ million	
	Synth.	Bio.	Synth.	Bio.	Synth.	Bio.
β-carotene						
Food	50	10	75	35	110	60
Nutritional/ Cosmetic	45	15	95	45	140	130
Astaxanthin/ canthaxanthin	120	10	210	40	305	150

Table 6. World patents concenring microbial synthesis of carotenoids

Subject	Patenting Company/Individual	World Patent #
1) *P. rhodozyma* protoplast fusions	1) Lion Co. (2)	9290687, 9290686
	2) Phillips Petroleum Co.	9015322
2) Novel *P. rhodozyma* strains	1) Lion Co.	9554017
	2) Gist-Brocades NV	9520648
	3) Enzymatix Ltd.	8637888
	4) Unilever Plc.	8954610
3) *P. rhodozyma* fermentation conditions	1) Phillips Petroleum Co.	8815125
	2) Osaka Chem. Alloy KK	8493807
	3) Biocolours I/S	7673631
4) Astaxanthin feeding	1) Kyowa Hakko Co. Ltd.	9711774
	2) Sanraku Ocean	3650795
	3) Aquatic Diet Techno.	3141032
5) *P. rhodozyma* mutagenesis protocols	1) Villadsen, I.S.	9324671
	2) Pillips Petroleum Co.	8712855
	3) Igene Biotechnology Inc. (2)	8569501, 8196510
6) Astaxanthin extraction procedures	1) Phillips Petroleum Co.	9551253
	2) Ki Kasei KK	9127959
	3) Mikalsen, E.; Mikalsen, G.	8306420
7) Algal astaxanthin production	1) Dainippon Ink Chem. KK	8641955
	2) Commiss. Energie Atomique	7820438
	3) Chlorella Kogyo KK	7993977
	4) Higashimaru Shoyu KK	9443386
	5) Univ. Ben-Gurion Negev Res. & Dev.	9729356
8) Zeaxanthin production Novel organisms	1) Applied Food Biotechnology Inc.	8598054
	2) Kaiyo Biotech. Kenkyusho KK (3)	9742075, 9742974 9418382
	3) Zeagen Inc.	9418382
9) Zeaxanthin production Genetic engineering	1) Amoco Co.	8777393
	2) Kirin Beer KK	8335212

Microbial carotenoids have attracted much interest in recent years for several reasons. One of the primary reasons is the growth of aquaculture [241, 242], currently the fastest growing sector of agriculture. The farming of salmon and shrimp increased greatly during the 1980s. Abount 260000 metric tons of salmon and 700000 metric tons of shrimp were farmed in 1991 in over 40

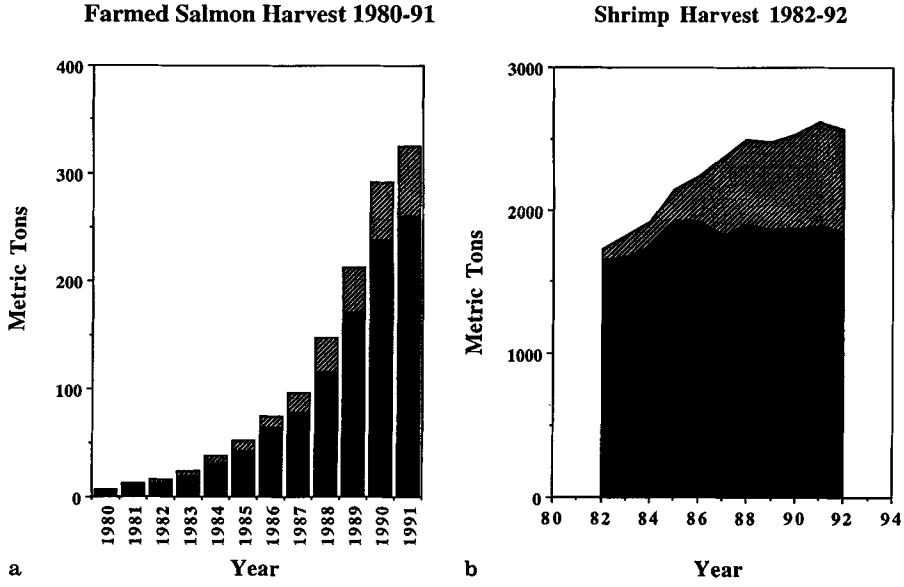

Fig. 5. Worldwide farmed salmon and shrimp crops in thousands of metric tons. **a** (salmon): *solid bars* represent Atlantic salmon, *hatched bars* represent Pacific salmon; **b** (shrimp): *solid area* represents wild-catch, *hatched area* represents farmed shrimp

countries (Fig. 5). Ten years ago, farmed salmon and shrimp supplied 3–5% of the world consumption, but this has expanded to 25–30%. Aquaculture is currently about a $20 billion per year industry which is expected to grow to $40 billion per year by the year 2000. Astaxanthin is an important source of pigmentation in farm-raised salmonids and crustaceans. Carotenoids also are the source of vitamin A in animal diets. Carotenes, particularly β-carotene, have traditionally served this role in animal and human nutrition but other carotenoids including the xanthophylls, zeaxanthin and astaxanthin can be converted to vitamin A in crustaceans and some other animals. Certainly, a primary contributing factor to the increased interest in natural carotenoids is the current trend of avoiding food additives and synthetic chemicals in foods. The structures of carotenoids under investigation for industrial production are shown in Fig. 6.

8.2 Biological Processes for Production of Carotenoids

8.2.1 Lutein

Lutein and lutein extracts are important carotenoid sources of pigmentation of broilers and hens' eggs [239, 243], and sales of pigmenters in the United States was estimated to about $150 million per year [244]. Lutein is also one of the

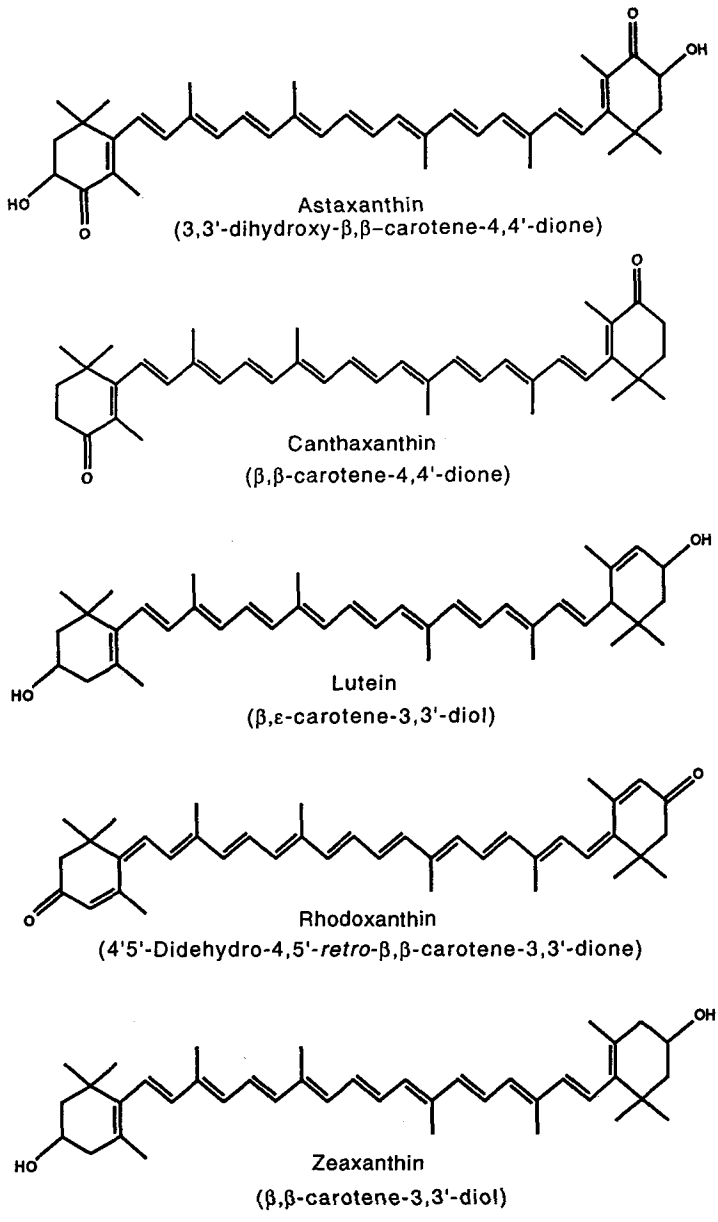

Fig. 6. Structures of some carotenoids under investigation for synthesis by microbial processes

most common carotenoids in freshwater fish, giving pond-reared trout a yellow coloration [245]. For effective egg yolk pigmentation a base pigment of lutein is required. Ketocarotenoids also enhance the color [246]. Generally, a combination of lutein and zeaxanthin is used to give the desired color intensity [246].

With improvements in the efficiency of conversion of feed to body weight the amount of carotenoid ingested in the standard diet containing alfalfa meal or corn meal was often insufficient to provide proper pigmentation [247]. Carotenes are poorly deposited in egg yolks which is reflected in the carotenoid composition of 70% lutein and 30% zeaxanthin [246]. Dehydrated marigold was more effective than meal; the composition of carotenoids in the meal was 64% lutein, 31% antheraxanthin, and 3.5% α-cryptoxanthin. Saponification of the esters to free lutein improved deposition by about 30% [246].

Lutein in alfalfa meal, corn gluten meal, and marigold flowers [248] and concentrates are available commercially for poultry pigmentation. Another commercial source of lutein is marigold petals (*Tagetes erecta*), which are dehydrated, ground and marketed for coloration [249]. Microbial processes for lutein production have been investigated. Patents exist for lutein production in the microalgae *Chlorella*, *Chlorococcum*, *Chlamydomonas* and *Spongiococcum* [250]. *Spongiococcum excentrum* was commercialized for use in chicken feed [250] but it is no longer available. F. Hoffmann-La Roche has patented the production of lutein using *Chlorella pyrenoidosa* cultured at 35 °C, however this was never commercialized [244, 251]. Industrial production of lutein by the blue green alga *Spongiococcum excentrum* by Grain Processing Co. (Muscatine Iowa) is marked as A-Zanth [246]. In this process, *S. excentrum* is grown in a 9 day culture at 28 °C on glucose, corn steep liquor, urea and mineral salts with glucose being added in a feed batch process. This process was reported to yield about 294 mg of lutein per liter of culture.

8.2.2 Zeaxanthin

Zeaxanthin is produced by plants, various algae, cyanobacteria, *Mycobacterium*, *Xanthobacter*, *Erwinia*, and *Flavobacterium*. *Flavobacterium* appear to be among the best sources and several patents for production of zeaxanthin have been filed [252]. *Flavobacterium* cultures generate 10–40 mg of 3*R*, 3′*R* zeaxanthin per liter. Supplementation of the media with isoprenoid precursors can increase titers to 335 $mg\,l^{-1}$ [249]. High liters of zeaxanthin (15 $mg\,g^{-1}$) have been obtained in *Flavobacterium* in laboratory batch cultures [253].

Processes for zeaxanthin production have also been developed using the cloned genes for zeaxanthin biosynthesis from *Erwinia* [254]. The clustered genes from *E. herbicola* were initially cloned into *E. coli*, which conferred a yellow color to the organism. Extensive alterations of the genes were necessary for expression in *Saccharomyces cerevisiae*. Gene reconstruction increased the activity of GGPP synthase for 6.35 to 23.4 $nmol\,min^{-1}$ by deleting portions of the gene. In order for the lycopene cyclase gene to be expressed in *S. cerevisiae*, the original GTG (methionine) start codon had to be changed to ATG. The carotenogenic genes were integrated into the chromosome of *S. cerevisiae*. The gene encoding phytoene synthase was first fused to the phosphglycerate kinase promoter of *S. cerevisiae*, then transformed into the yeast using an integration

vector. It was reported that several % zeaxanthin was obtained, and the carotenoids appeared to concentrate in lipid globules in the yeast.

8.2.3 Rhodoxanthin

Rhodoxanthin is distributed widely in leaves, fish and bird feathers [244]. In bacteria it has been detected in a methanol utilizing bacterium, *Pseudomonas extorquens* [255]. The pigment level in *P. extorquens* was found to be growth related, increasing 4-fold during the stationary phase. Nutrient conditions were also found to affect pigmentation. Ethanol and Mg^{2+} inhibited rhodoxanthin biosynthesis. Limitations of Mg^{2+} increased the production 2.3-fold over cultures with excess Mg^{2+} [255]. Use of glycerol and ammonium nitrate as carbon and nitrogen sources gave the highest productivity [255]. Surprisingly, high O_2 supply inhibited pigmentation, and pigment production in an O_2 limited culture was 1.9-fold higher than the yield in excess O_2 [255].

8.2.4 Canthaxanthin

Canthaxanthin was originally isolated from the mushroom *Cantharellus cinnabarinus* and from yam tubers (*Dioscornea*) [246, 256]. Canthaxanthin is currently approved as a food additive in the U.S. and 35 other countries [246]. In addition to other keto-carotenoids such as astaxanthin and echinenone, canthaxanthin is produced in certain green algae (*Haematococcus*, *Chlorella*, *Chalamydomonas*, *Scenedesmus*, and *Ankistrodesmus*), usually in response to stress and nutrient deficiency [257]. Three microbial sources have primarily been studied for commercial canthaxanthin production. *Rhodococcus maris* grown on hydrocarbons had a poor yield [250]. The green alga *Dictyococcus cinnabarinus* under nitrogen deficient conditions produced canthaxanthin, but also with a low yield and slow growth [250]. The most promising source of canthaxanthin appears to be a *Brevibacterium* sp. isolated from oil fields [250]. This process has been optimized for *Brevibacterium* strain KY-4313 by using a high carbon/nitrogen ratio. This involves a two phase process utilizing nitrogen starvation in the second phase, and supplementation of the medium with hydrocarbons and higher alcohols [77]. Since genetic techniques were not available for this organism, strain improvement has relied on random mutagenesis followed by visual screening [250]. Mutagenesis has yielded carotenoid hyperproducing mutants giving 490 $\mu g\, g^{-1}$ cell and 4200 $mg\, l^{-1}$ compared to 370 $\mu g\, g^{-1}$ and 3100 $mg\, l^{-1}$ in the wild type; echinenone levels also increased. A medium considered for industrial application was a hydrocarbon supplemented medium (pH 7.0) containing 2–4% octadecane, malt extract (0.1%), Tween 40 (0.01%) vitamin B_{12} and a high C/N ratio. This formulation yielded canthaxanthin levels of 600 $\mu g\, g^{-1}$ cell, 1–2 mg l^{-1} canthaxanthin, and a cell density of 3.5 $g\, l^{-1}$. Mg^{2+} depleted media and substitution of yeast extract for

malt extract improved these results, strong aeration was slightly inhibitory. The feeding of carotenoid biosynthesis intermediates or addition of alcohols did not affect pigmentation [77]. In hydrocarbon supplemented media the main limit to growth was the accumulation of toxic carboxylic acids [77]. This led to a production strategy of periodic renewal of the aqueous phase to remove toxic metabolites and periodic octadecane supplementation, which gave a two-fold increase in cell mass as well as increased pigmentation [77]. Because of the difficulties in using hydrocarbon supplemented media, the canthaxanthin synthesis was attempted in a rich medium (Brain Heart Infusion; BHI), which gave increased cell mass but decreased pigmentation [77]. Supplementation of BHI with higher alcohols (propanol, isopropanol or retinol) increased pigmentation. The best results were obtained using 10–20 g of propanol l^{-1}, while retinol as well as vegetable oils gave increased β-carotene levels at the expense of canthaxanthin and decreased cell mass [77]. Dicarboxylic acids (fumaric, maleic and malic acid) stimulated pigmentation. The optimum formulation contained fumaric acid (5%), beet molasses (4%) and sucrose ester (1%). This yielded 9.3 mg of canthaxanthin and 12.6 g of cell mass per 1 [77].

8.2.5 β-carotene

Syntheis of β-carotene in mucoral fungi and green algae has been thoroughly reviewed [258–260]. Industrial methods for β-carotene have been developed using the mucoral fungus *Blakeslea trispora.* Detailed investigations of media composition and culture conditions resulted in a process potentially competitive with chemical synthesis. The US Department of Agriculture process [260] yielded about 17 mg of β-carotene per g mycelium or about 1 g l^{-1}. The process has been improved and yields about 30 mg β-carotene per g mycelium and about 3 g l^{-1} [252]. The medium composition for the improved process using mated cultures of *B. trispora* had certain interesting components which may stimulate production. During the early exponential phase thiamine, $MnSO_4$, soya oil, cotton-seed oil, kerosene, and isoniazid were added. After 48 h, β-ionone, kerosene, and glucose were added and glucose continually until the end of the 185 h process.

Phycomyces also can accumulate very high levels of β-carotene (~30 mg g^{-1}) in lipid globules in the mycelium [256]. Production of β-carotene has only been studied in laboratory conditions, usually on solid agar media, but efforts are underway to develop a submerged culture process.

The superior process for microbial production of β-carotene appears to be algal culture using the green, biflagellate green alga, *Dunaliella.* Currently there are at least four commercial operations in Australia, Israel, and the United States. *Dunaliella* powder and extracted β-carotene have been marketed since 1985 [258]. β-Carotene accumulates in oil globules in the interthykaloid spaces of the chloroplast when *Dunaliella* is cultured with intense illumination, a stress temperature, high salinity, and a deficiency in nitrogen or sulfur. In inducing

conditions β-carotene reaches levels of up to 14% of the dry weight, while under noninducing conditions the content is about 0.3%. High β-carotene producing mutants have been obtained by selecting for the ability of β-carotene to protect the alga from killing in blue light [261]. Algal β-carotene cannot compete economically with synthesized product, but it has found a niche in "Natural" markets.

8.2.6 Astaxanthin

Astaxanthin is the primary pigment in salmonids and shrimps, which must be supplied in their diet. Currently, most of the astaxanthin is supplied through chemical synthesis. However, astaxanthin is a complex molecule and the synthesis can be a difficult one. Synthetic astaxanthin sells for about \$2000 kg^{-1}. Because of the potential market in aquaculture, there has been considerable industrial interest in developing competitive biological sources of astaxanthin.

Currently, biological astaxanthin is not approved as a feed additive in the U.S., forcing the industry to use canthaxanthin. However, it has been shown that canthaxanthin fades faster upon cooking and will fade in fish if feeding is stopped [245]. Consequently, higher canthaxanthin levels are recommended in feeds. For salmonids, ≥ 20 μg astaxanthin per g feed is required compared to ≥ 40 μg canthaxanthin per g over a two to four month period. It is likely that the Food and Drug Administration will approve astaxanthin in the near future for certain foods and feeds.

Salmon and shrimp differ in their capacity to metabolize carotenoids. Most crustacea deposit carotenoids in the eyes, blood, eggs and carapace [250]. Astaxanthin is the most common carotenoid found, either free, esterified to short chain fatty acids, or noncovalently linked to protein. β-Carotene, lutein, canthaxanthin and echinenone are also often present. Generally crustacea are non-selective and absorb xanthophylls and carotenes equally well. Brine shrimp can convert β-carotene to echinenone and to canthaxanthin but hydroxy carotenoids are not detected [262]. However, when hydroxycarotenoids were provided in the diet to a crustacean (*Daphnia*), they were converted to astaxanthin [250]. In contrast to crustacea, fish are very limited in their ability to metabolically transform carotenoids. Generally, chemical modifications are limited to the esterification of xanthophylls. Esterified compounds usually are deposited in the skin, while the free form is generally found in the flesh, liver, ovary and digestive organs [263].

Astaxanthin has been identified in several microorganisms including the basidiomyceteous fungus *Peniophora* [54], the heterobasidomycetous yeast *Phaffia rhodozyma* [116], the hydrocarbon-utilizing bacteria *Mycobacterium lacticola* and *Brevibacterium* [245], and in various green algae including *Haematococcus* sp, *Neochloris wimmeri* and *Chlamydomonas nivalis* [264]. The higher plant *Adonis aestivalis* species produces astaxanthin in its flower petals, which

can be dried and ground without major loss of pigment [265]. Commercialization of *Peniophora* has not been pursued and pigment production by *Mycobacterium* and *Brevibacterium* isolates were poor ($\sim 30\ \mu g\ g^{-1}$ and $3\ g\ l^{-1}$, respectively) [245].

The primary biological sources of astaxanthin currently being considered for industrial production are the green alga *Haematococcus pluvialis* and related species, the heterobasidiomyceteous yeast *Phaffia rhodozyma*, and crustacean extracts. With the large quantities of crustacean waste generated each year, much effort has gone into using this as a carotenoid source in fish aquaculture. Generally, the feed requires 20–30% by weight of crustacean waste meal. The major problem in using waste meals is the high level of ash and the low energy content of the shells [266]. Krill oil contains relatively high astaxanthin levels ($727–1080\ \mu g\ g^{-1}$), while krill meal contains only $15–200\ \mu g\ g^{-1}$ and frozen krill $15–77\ \mu g\ g^{-1}$. In krill, most of the astaxanthin is present as the diester which apparently is not absorbed as efficiently as the free form [266].

Haematococcus pluvialis is a unicellular, motile, green freshwater alga of the class Chlorophyceae. Under favorable growth conditions, *Haematococcus* exists as a single celled biflagellate swimmer capable of photosynthetic autotropic growth [264]. In unfavorable growth conditions cells lose motility and form cysts. This encystment is accompanied by the synthesis and deposition in lipid globules of large quantities of astaxanthin and other carotenoids. Formation begins in the perinuclear region and proceeds outward in the cytoplasm. Astaxanthin can account for up to 5% of the cell mass [15].

During the encystment process, the relative quantities of individual carotenoids change considerably, going from 75–80% lutein and 10–20% β-carotene to over 80% astaxanthin. However, this increase in astaxanthin is not due to conversion of these other carotenoids to astaxanthin [267]. The environmental and nutritional factors which trigger encystment and carotenogenesis have been investigated in *H. pluvialis*. Nitrogen starvation occurs in the same period as encystment although the two processes were reported not to be physiologically linked [268]. Encystment is also triggered by the addition of NaCl (up to 1%) [268]. Astaxanthin accumulates when cell division is inhibited, and phosphate but not nitrogen starvation and high light intensities also lead to accumulation of $\leq 3\%$ astaxanthin [269]. A study of nutritional requirements has shown that KNO_3 ($0.5\ g\ l^{-1}$) is the best nitrogen source for pigmentation, and intermediate levels of phosphate ($0.001–0.2\ g\ l^{-1}$) are optimum. Encystment is triggered by exhaustion of nitrogen and increasing phosphate levels [268]. Iron (Fe^{2+}) stimulates carotenogenesis, but only in the presence of acetate [270]. It was suggested that Fe^{2+} promotes the Fenton reaction which generates hydroxyl radicals. Exposing the cells to active oxygen species O_2^-, $OH\cdot$, $^1O_2$ and H_2O_2 in the absence of Fe^{2+} induces the production of astaxanthin [270], which is a potent quencher of active oxygen species. Carotenoid levels are increased by the feeding of Fe^{2+} and acetate following the cessation of growth in a two stage process [271]. Furthermore, it was found that activation by Fe^{2+} or H_2O_2 is proportional to light intensity. In the absence of light, no activation by Fe^{2+} or

H_2O_2 was observed. Light activation occurred more than 18 hours after acetate addition [272]. Technologies have also been investigated to maximize cell growth and density prior to encystment, as well as achieving the maximum levels of astaxanthin after encystment. Lwoff and Lwoff [273] first reported in 1930 that addition of acetate to mineral peptone media enhanced carotenogenesis. Goodwin and Jamikorn [267], however, suggest that acetate is responsible for increased growth but not increased pigment. More recently, Borowitzka et al. [268] demonstrated that acetate increases pigmentation in addition to growth. Although light is not strictly required for pigmentation, illumination gives 7-fold higher pigmentation and two-fold higher growth [274]. Thiamine was also found to stimulate growth.

The astaxanthin biosynthetic pathway has been investigated in *H. pluvialis*. During encystment, astaxanthin synthesis is not synthesized from pre-existing lutein and β-carotene pools [267]. Radiolabeling studies showed that exogeneous sources of carbon are a major source of ketocarotenoids and that there is no evidence for the conversion of β-carotene to astaxanthin [275], although echinenone and canthaxanthin were proposed as astaxanthin precursors [275]. This was supported by the increased demand for carbon in cells during encystment than during vegetative growth. Feeding cells during encystment with carotenoid precursors much as mevalonic acid, pyruvate, malonate or dimethylacrylate increased pigmentation [275].

Microbio Resources Inc. of San Diego, California, has developed a commercial process for the production of astaxanthin from *Haematococcus pluvialis*, marketed as Algaxan Red. *H. pluvialis* is grown in modified Bolds basal medium (pH 7.3). Scale up of the cultures involves a series of 10-fold increments, each stage requiring 5–7 d. The final production stage is in 4500 m^3 ponds with encystment being induced by nitrogen starvation or NaCl addition when a cell density of $3–6 \times 10^5$ cells per ml is obtained. Because production occurs in open ponds, contamination is a major obstacle. Contamination generally occurs by wild algae or protozoan predators. In the latter case, predation can eliminate up to 90% of *H. pluvialis* within 72 h.

Following harvesting of the cells, astaxanthin must be liberated from the cysts and protected from oxidation. This is accomplished by grinding the dried cysts to a diameter of $< 10\ \mu m$ at $-170\ °C$ in the presence of antioxidants such as butylated hydroxytoluene or ethoxyquin [264]. The powder, actually a mixture of 1% free astaxanthin (in the 3*S*, 3′*S* configuration), 76% astaxanthin monoester, 7% astaxanthin diester, 1% β-carotene, 7% lutein and 2% violaxanthin [276], is recommended for use at 25 to 100 μg astaxanthin per g salmon feed. Although costs vary, it is sold at \$20 kg^{-1} for a 1% astaxanthin product. This is competitive with pure synthetic astaxanthin sold at \$2000 kg^{-1} [264].

Another microbial source of astaxanthin is the yeast *Phaffia rhodozyma*. The natural habitat of the orange-red heterobasidiomyceteous yeast *P. rhodozyma* is slime fluxes of deciduous trees including birch, beech, alder, aspen, and dogwood in mountainous regions of Japan, the Pacific Northwest, and Russia [114]. Soon after its initial isolation in 1967, the yeast was recognized as representing a new genus "Rhodozyma". Later Golubev et al. [277] isolated 67

strains from birch fluxes near Moscow. The yeast was formally described as *Phaffia rhodozyma* in 1976 in recognition of the lifelong contributions of Herman J. Phaff to the biology of yeasts [115]. Characteristic properties of *Phaffia* including its repetitive budding from a single cellular site, presence of coenzyme Q-10, xylose in the cell wall, a positive diazonium blue B staining reaction, characteristic 18s rRNA sequences and production of carotenoid pigments. These characters indicate its affinity in the heterobasidiomycetes in the Cryptococcaceae [278–280]. Further evidence linking *Phaffia* to the Cryptococcacacae is the presence of killer activity. Double-stranded RNA (dsRNA) particles of molecular sizes ranging from 4.3 to 0.75 kilobase pairs were isolated. Strains containing these dsRNA were found to kill strains without dsRNA [281]. Gueho et al. [279] suggested that based on its 26S and 18S rRNA partial sequences *Phaffia* evolved from a fungus having a primitive dolipore such as in *Cystofilobasidium capitatum*. In exception to this proposal, Prillinger et al. [282] suggested that *Phaffia* was related to the Tremellaceae (*Tremella, Trimorphomyces*) which are Hymenomycetes that have complex septa. This is interesting in light of the fact that Tremellales form fruit-bodies that are brightly colored, and the basidiospores germinate by yeast-like budding and in culture the fungus grows in a yeast-like manner [283]. The Tremellaceae grow on stumps of various tress. *Tremella foliaceae* was shown [284] to be closely related to *Filobasidiella neoformans*, which is the perfect stage of the yeast *Cryptococcus neoformans*. The Dacrymycetales, closely related to the Trenellacea, include the jelly fungi whose cells can become packed with oil globules containing carotenes [283]. This taxonomic information suggests that *Phaffia* may have evolved from the Tremellaceae in the higher basidiomycetes and that it may be useful to investigate Tremellales as a new source of astaxanthin.

The carotenoid intermediates found in *P. rhodozyma* are quite different from those found in *H. pluvialis*. *H. pluvialis* contains β-carotene, lutein, violaxanthin, and astaxanthin, which has the 3*S*, 3′*S* stereoconfiguration [273]. In contrast, *P. rhodozyma* produces the 3*R*, 3′*R* configuration of astaxanthin [117] and contains several ketocarotenoids including echinenone, phoenicoxanthin, canthaxanthin, and HDCO [116]. The difference in configuration is intriguing and suggests distinct enzyme mechanisms or differences in the biosynthetic pathways. Bjerking suggested [285] that addition of oxygen at the C-4 position before oxygen addition at the C-3 position results in formation of the 3*R* astaxanthin configurational isomer while oxygen addition at the C-3 position before C-4 oxygen addition gives the 3*S* isomer.

The wild isolates of *P. rhodozyma* contain relatively low quantities of carotenoids ($\leq$ 500 μg total carotenoids per g yeast), of which 40 to 95% of the overall mixture is astaxanthin [116]. To develop an industrial process for astaxanthin production, it was necessary to develop strains of *P. rhodozyma* with a minimum astaxanthin content of 3000 $\mu g\, g^{-1}$. A number of genetic methods have been utilized to attain this goal. Mutagens have been utilized in *P. rhodozyma* strain development, with ethylmethyl sulfonate (EMS) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) being the most effective [286]. The first few rounds of random mutagenesis and visual screening yields mutants with

significantly elevated pigment levels and carotenoid concentrations of 1500 $\mu g\, g^{-1}$ are relatively easily obtained [286]. However later rounds of mutagenesis give smaller pigment incremental increases (0–10%). Beyond pigment levels of $\sim 1200\ \mu g\, g^{-1}$ it is very difficult to detect higher pigment levels by visual inspection possibly due to oxygen transfer limitations on plates [231,287]. Strain instability and the tendency of mutants to produce a lesser proportion of astaxanthin in the total complement of carotenoids is also a significant problem. This has led our laboratory and others to investigate positive genetic selection systems for carotenoid hyperproducing strains. Carotenoid synthesis in several carotenogenic fungi is subject to end product inhibition (e.g. β-carotene in *Phycomyces*) and it seemed feasible that analogs to carotenoids could be used to select analog resistant mutants with altered pathway regulation. Chun et al. [288] suggested that β-carotene feedback inhibits carotenoid synthesis in *P. rhodozyma* in a manner similar to *Phycomyces*. Lewis et al. [287] used the β-carotene analog β-ionone as an inhibitor and was able to isolate hyperproducing strains in *P. rhodozyma*. β-Ionone (10^{-4} M) decreased pigmentation 5-fold and killed ~20% of wild-type cells whereas the hyperproducing strains were resistant to 10^{-3} M β-ionone. This difference in viability was exploited as a selection system in conjunction with NTG mutagenesis to produce mutants with 1000 $\mu g\, g^{-1}$ pigmentation after 2 rounds. However, while β-ionone was effective under these conditions, it was concluded that β-ionone was effective only in initial mutation cycles [289]. Schroeder and Johnson [290] have demonstrated that the degradation of astaxanthin in live cells by tert-butylhydroperoxide resulted in an ~5-fold increase in β-carotene levels. The accumulation of β-carotene with decreased intracellular astaxanthin suggests that astaxanthin may be involved in feedback regulation. Astaxanthin analogs may be more effective than β-carotene analogs in selections for hyperproducing strains.

A second strategy for positive selections was to exploit the antioxidant activity of carotenoids. Chang [291] initially demonstrated hyperproducing cell lines to be more sensitive to H_2O_2. However, Schroeder and Johnson [292] showed an age dependence in which young hyperproducing cultures were more resistant to H_2O_2 than wild-type cultures. Use of this as a selection gave slightly increased pigmentation after repeated challenges [292]. A second oxidant which is specifically detoxified by carotenoids is $^1O_2$ [293]. Singlet oxygen is an excited electronic state of O_2 which when it decays to its ground state causes the peroxidation of lipids, cellular membrane damage, and damage to DNA. Singlet oxygen is relatively long-lived, especially in lipid environments, and its diffusion radius in cells has been estimated as 100 Å [294]. The lipid peroxidative activity of $^1O_2$ is particularly effective because of the increased lifetime of $^1O_2$ in membranes compared to the aqueous phase (25–100 μs vs 4 μs) [295]. Fatty acid analysis of *P. rhodozyma* showed that it is rich in fatty acids which are particularly sensitive to the damaging effect of $^1O_2$. Linoleic and oleic acids, which are quite sensitive, are the two most prevalent fatty acids in *P. rhodozyma* (Table 7). Carotenoids are well recognized as being able to detoxify $^1O_2$ catalytically,

Table 7. Comparison of the composition of the *Phaffia* products from Red Star (Universal Foods, Milwaukee, Wisconsin, USA and Natupink Gist-Brocades. Delft, The Netherlands)

Values reported as Red Star/Natupink

Bulk composition

Astaxanthin (ppm)	Protein (%)	Lipid (%)	Ash (%)	Mositure (%
3000/2500	22/32	23/28	3/5	5/5

Amino Acids (%)	Fatty Acids (%)	Vitamins (ppm)	Minerals (ppm)
Glu: 7.9/9.7	Linoleic (18:2n6)	Niacin	Calcium
Asp: 6.3/6.7	39.65/36.5	1520/ND	248/1900
Arg: 6.3/6.7	Oleic (18:1n9)	Riboflavin	Sodium
Ala: 5.6/4.8	32.43/33.2	100/ND	2770/4000
Leu: 5.1/5.0	Palmitic (16:0)	Vitamin D_2^*	Zinc
Lys: 4.7/4.8	13.18/12.7	85/ND	14/50
Thr: 3.9/3.9	Stearic (18:0)	Biotin	Phosphate
Ser: 3.7/4.6	5.62/9.8	1/ND	16200/15000
Val: 3.7/4.1	Linolenic (18:3n3)	Thiamine	Copper
Pro: 3.6/3.4	1.26/1.5	14/ND	ND/15
Gly: 3.6/3.9	Heptadecanoic (17:0)	Pyridoxine	Manganese
Ile: 2.9/4.2	1.24/1.2	12/ND	ND/11
Phe: 2.8/2.2	Behenic (22:0)	Pantothenate	Magnesium
Tyr: 1.9/2.2	0.76/1.1	34/ND	1489/700
His: 1.7/1.6	Eicosanoic (20:0)	Folate	Potassium
Trp: 0.7/ND	0.75/ND	6/ND	4135/10500
	Palmitoleic (16:1n7)		Iron
	0.4/ND		31/200

*IU/100g
ND: not determined

and a single β-carotene molecule can detoxify 250–1000 molecules of $^1O_2$ [295]. Carotenoids differ in their capacity to detoxify $^1O_2$, and lycopene and astaxanthin are more effective than β-carotene [296, 297]. Consequently, mutants of *P. rhodozyma* with increased astaxanthin would be expected to be more resistant to damage by $^1O_2$. When mixed cultures of wild-type and hyperproducing strains were cultured together and exposed to light activated $^1O_2$ generators, a monoculture of the hyperproducing line was selected [298]. Singlet oxygen can also be generated in the cell by chemical reactions such as the spontaneous dismutation of O_2^- or by the reaction of H_2O_2 with O_2^- (Haber-Weiss reaction) [299]. This suggests that protection of cells by carotenoids agianst H_2O_2 or O_2^- may be through a $^1O_2$ intermediate. These results indicate that resistance to $^1O_2$ may provide a basis for a positive genetic selection for astaxanthin and also that the function of carotenoids in *P. rhodozyma* is to protect against $^1O_2$ in its natural environment.

Because *P. rhodozyma* is imperfect and pedogamic sexuality has only been recently demonstrated, it has not been possible to perform conventional crosses and genetic analyses. Recombinant DNA technology has been attempted with *Phaffia* but not published. One of the most powerful methods of obtaining

recombinant strains in basidiomycetes is the development of transformation protocols [300]. Genetic transformations have been developed for several basidiomycetes including the yeasts *Cryptococcus neoformans* [301] and *Rhodosporidium toruloides* [[302]. van Ooyen [303] has reported a transformation system for *P. rhodozyma*. An actin sequence which hybridized with *Phaffia* genomic DNA was used to isolate a DNA fragment containing a *Phaffia* promoter. A marker gene encoding phleomycin resistance was fused with this promoter and used to transform *P. rhodozyma*. The development of this system should enable the expression of heterologous proteins in *Phaffia*, including enzymes catalyzing carotenoid biosynthesis.

Protoplast fusion has also been used for strain development of *P. rhodozyma* [288]. Previously it had been reported that the generation of auxotrophic mutants was difficult, possibly indicating polyploidy or aneuploidy in *P. rhodozyma* [304]. Chun et al. [288] were able to isolate strains with auxotrophic markers for tryptophan, leucine, methionine and arginine and suggested that *P. rhodozyma* was haploid. Using these auxotrophs, 30 hybrids were generated, three of which had significantly higher astaxanthin levels than the parents (2000 compared to 1600 $\mu g\, g^{-1}$). The hybrids were found to have 2-3-fold more DNA than the parents, were uninucleate indicating nuclear fusion, and were stable (segregation frequency $< 2.1 \times 10^{-4}$ reversion). Prevatt [305] also reported generating *P. rhodozyma* fusion strains which produced 4500–8600 mg of astaxanthin l^{-1} of culture broth. They fused early stationary phase spheroplasts for 15 min using polyethylene glycol as a stabilizer.

Adrio et al. [306] found the isolation of auxotrophic mutants more difficult. They were unable to isolate any auxotrophs from 90000 EMS- generated or 50000 NTG-generated mutants. Enrichment for auxotrophs by nystatin yielded mutants at a frequency of 1×10^{-3} after NTG mutagenesis. Nystatin gave 150-350-fold selection. The largest class of auxotrophs required adenine. It was possible to isolate *Ura3* mutants on 5-fluoroorotic acid (5-FOA) plates and then to complement the mutation using a plasmid containing the *Ura3* gene [306]. It has also been the experience of our laboratory that auxotrophs are diffucult to isolate in *P. rhodozyma*. This is puzzling since color mutants (white, yellow, red) occur frequently. Aneuploid strains have been isolated in *Phaffia* which express color phenotypes [307]. Aneuploidy in a polyploid strain could explain the difficulty in obtaining some classes of mutants.

As new strains of *P. rhodozyma* are developed it will become increasingly important to demonstrate the genetic uniqueness of the strains as well as confirming that strains used in production do not spontaneously revert during mass culture. Fingerprinting of DNA by restriction fragment length polymorphisms (RFLPs) is a common laboratory technique, and a modification of this strategy was used for typing *P. rhodozyma* [308]. The method used randomly amplified polymorphic DNAs (RAPD) generated with a single oligonucleotide primer containing an arbitrary sequence. Using the PCR template, random regions were amplified from the genomic DNA. Between 3 and 5 major DNA fragments were amplified, which varied in size from 0.7 to 2 kilobase pairs. The

banding pattern on using contour-clamped homogeneous electric field (CHEF) electrophoresis differed according to the strains. The analysis suggested that extensive mutations had occurred in the hyperproducing strains. This method could be of use for determining the gentic relatedness and uniqueness of strains.

Varga et al. [309] studied relatedness among 6 wild-type *Phaffia* strains. Relatedness was quantified using a combination of RFLP, RAPD, isoenzyme patterns and electrophoretic karyotyping. It was determined that although *Phaffia* was indeed distinct from other Crytococcaceae, there was considerable heterogeneity among strains. While isoenzyme profiles were the most stably maintained, RAPD analysis was the most sensitive to interstrain differences, even more sensitive than electrophoretic karyotyping.

Fluorescence Activated Cell Sorting (FACS) has been used as a strategy to isolate mutants that overproduce autofluorescent cellular components. This strategy was demonstrated to be successful in isolating algal cells based on chlorophylll autofluorescence [310] and anthocyanin producing mutants of *Aralia cordata* [311]. An et al. [312] investigated this novel screening method for carotenoid hyperproducers based on the autofluorescence of carotenoids. When excited by laser light of 488 nm, carotenoids fluoresce at a characteristic wavelength. Conditions were developed in which autofluorescence was due to cellular carotenoids. Using FACS, it was possible to individually evaluate and sort up to 4500 cells per second for increased fluorescence and consequently increased carotenoid content. When a mixed culture of a wild-type and carotenoid hyperproducer was sorted out by FACS, a 9- to 147-fold enrichment of hyperproducer was attained. NTG mutagenesis followed by cell sorting yielded a 10000-fold improved efficiency for isolating hyperproducing mutants in a population comprised mostly of wild-type cells [312]. This method could be used to isolate highly fluorescent cells if the optimum sorting windows can be devised and the viability of hyperproducing strains can be maintained.

Confocal fluorescence microscopy and transmission electron microscopy were used to study the subcellular distribution of carotenoids in *P. rhodozyma* [313]. Carotenoids were localized in the external membrane regions and in lipid globules but were absent from the mitochondria. Using fluorescence as an indication of carotenoid content in individual cells, an estimate was made of the maximum cellular content of carotenoids in *P. rhodozyma.* Log fluorescence as a function of carotenoid content gave a linear relationship, and the most highly fluorescent cells contained approximately 15000 μg astaxanthin g^{-1}, significantly above a commercialization target of 3000 to 5000 $\mu g\, g^{-1}$ [59]. However, since several carotenoids fluoresce, particularly phytoene and phytofluene, the content of astaxanthin is an unknown proportion of the total.

A linear reaction network mathematical model of astaxanthin production has been developed [314]. The model supported the hypothesis that the current yield of astaxanthin by high producing strains (3000 to 5000 $\mu g\, g^{-1}$) is much less than is theoretically possible. If no constraint is applied to the formation or consumption of ATP, and certain other assumptions are made regarding the chemical species in the astaxanthin pathway, then the maximum production of

astaxanthin from glucose is (in moles):

$$12 \text{ glucose} + 4\,O_2 + 14 \text{ NADPH} + 48 \text{ NAD}^+ = 1 \text{ astaxanthin} + 32\,CO_2 + 14 \text{ NADP}^+ + 48 \text{ NADH} + 12\,H_2O + 34\,H^+ \quad (1)$$

The overall reaction is the linear combination of the constituent reactions with assumed steady state rates. The rates are considered relative, for instance, if the reaction catalyzed by the C_{40} dehydrogenase proceeds at a specific rate, then the reaction catalyzed by HMG-CoA reductase must proceed at ≥ 8 times that specific rate. It is clear that a large quantity of reduced NADH is produced in the reaction which must be balanced. If the cells used part of the glucose as an electron sink, and the coefficient of oxygen is not greater than 4, then the net reaction maximizing astaxanthin production would be:

$$3.83 \text{ mol glucose} + 0.954 \text{ mol ammonia} + 4 \text{ mol } O_2 = 0.213 \text{ mol astaxanthin} + 0.954 \text{ mol biomass } (C_8H_{18}O_4N) + 6.82 \text{ mol } CO_2 + 12.67 \text{ ml } H_2O. \quad (2)$$

This pathway model suggests that the ratio of astaxanthin to biomass could be 2 to 9, tremendously higher than the ratio of 1 to 3300 obtained in wild-type cells. Furthermore, this model shows that 21 moles of O_2 are required per mol astaxanthin produced, and that 1.75 mol O_2 are required per mol glucose consumed for astaxanthin production to maintain redox balance compared to 1.53 O_2 consumed per mol glucose for aerobic biomass synthesis. This model infers that O_2 availability is a major bottleneck in astaxanthin fermentations, and that as O_2 becomes limiting more glucose is diverted to biomass at the expense of astaxanthin formation. It may be possible to reduce the oxygen limitation by providing the cells with an alternate electron acceptor to oxygen in order to oxidize reduced species generated in the astaxanthin pathway. If an alternative oxidant was used, then the cell would require only 1/3 molecule of oxygen per glucose for astaxanthin synthesis, greatly reducing the oxygen transfer problem. The model also infers that hyperproducing mutants with high growth rates may not be easily attained since biomass production is favored by conditions that discourage carotenogenesis [314]. This conclusion is born out by experimental evidence; many, if not all of the hyperproducing strains of *P. rhodozyma* grow more slowly than their parents.

The modeling analysis suggests that nutrient control and feeding during fermentation are critical for optimal synthesis of astaxanthin and biomass. Initial studies by Johnson and Lewis [315] showed than in wild-type strains as batch cultures entered stationary phase (30–40 h) xanthophyll levels increased over 2-fold and continued to increase up to 128 h. Meyer and DuPreez [316] reported that in batch culture pigment formation slowed after exhaustion of the carbon source. During exponential phase total pigment was produced at a rate of 28.8 $\mu g\,g^{-1}\,h^{-1}$ which slowed to 11.1 $\mu g\,g^{-1}\,h^{-1}$ during stationary phase.

Pigment levels on a dry weight basis increased because of the decrease in dry cell mass that occurs in *Phaffia* cultures during stationary phase. The pH of the medium only slightly affected final cell mass over the range 3.8–7.5. The growth rate was fastest at pH 5.8 and maximum astaxanthin was produced at pH 5.0. pH 4.5 was suggested to be optimal in fermentors. Buffers in this pH region were studied, lactate being unsatisfactory because it was metabolized. The best buffer was potassium hydrogen phthalate. At lower pHs (3.5) the carotene intermediate β-zeacarotene accumulated as shown earlier by Johnson and Lewis [315] indicating that cyclization in the carotenogenic pathway could be altered by stressful conditions. A primary limitation in the yeast astaxanthin culture is low temperature range for growth. Temperature affected growth and astaxanthin formation and the highest specific growth rate ($0.12\,h^{-1}$) was at 22 °C. Bleaching of the cells occurred at higher temperatures. A slightly lower temperature of 15–20 °C was recommended for astaxanthin production [317]. Polulyakh et al. [318] reported that growth of *P. rhodozyma* at 30 °C induced the production of torulene and torularhodin. Other laboratories have not reported that *Phaffia* can grow above 27 °C, and it is possible that their culture was contaminated with *Rhodotorula*. *Rhodotorula* has been found to produce predominantly carotenes at lower temperatures and synthesize torulene and torularhodin as the temperature is raised [316].

The carbon source and its feeding during growth has a pronounced effect on astaxanthin production by *P. rhodozyma*. Glucose at high concentrations inhibited carotenogenesis as well as the maximum specific growth rate (μ_{max}) [316]. Consequently, fed batch cultures are usually carried out, which avoids repression of carotenogenesis early in the process. Disaccharides, such as sucrose, maltose and particularly cellobiose were best for pigmentation while sucrose and glucose gave the best growth. Good pigmentation on cellobiose, which is probably slowly hydrolyzed compared to sucrose, suggests that astaxanthin biosynthesis may be regulated by catobolite repression. High concentrations of glucose ($50\,g\,l^{-1}$) increased carotenes and decreased astaxanthin [315]. Prevatt [319] suggested that astaxanthin levels and foaming increased with carbon limitation. Conversely, high levels of sugar in the medium, particularly in lag or early exponential phase, caused decreased astaxanthin levels and the production of alcohols and aldehydes. A 12-fold increase in β-carotene levels was found at high sugar (5%) levels [315]. Similarly, it was reported [320] that β-carotene and β-zeacarotene were the main pigments in cells cultured in media containing 100 g glucose l^{-1}. The high levels of carotenes and formation of ethanol and glycerol in media containing high sugar was proposed to be caused by limitations in O_2 uptake. A careful study of sucrose utilization and astaxanthin biosynthesis was carried out in batch culture [316]. It was reported that the uptake of the sucrose monomers was rate-limiting for growth. Sucrose was hydrolysed by invertase to its constituent monomers within 8 h. However, the resulting glucose was not completely utilized until 29 h and fructose uptake was delayed by the presence of glucose. Ethanol ($17\,g\,l^{-1}$) and glycerol ($2\,g\,l^{-1}$) were detected as metabolic products at high sugar concentrations. These results

emphasize that it is critical to control availability of sugar during *Phaffia* processes.

Nitrogen sources also affect growth and pigmentation in *P. rhodozyma.* Peptone was reported to be the best single source of nitrogen for pigmentation [317]. However, a mixture (1: 1: 1) of yeast extract, beef extract, and KNO_3 at a nitrogen concentration of $5\,g\,l^{-1}$ protein gave the best pigmentation. The positive effect of KNO_3 is puzzling since *Phaffia* cannot use nitrate as an N source. The use of yeast extract as a nitrogen source increased carotenoid levels [315, 316]. In another study, two-fold higher astaxanthin content was obtained with yeast extract [62]. Culturing cells under N limitation decreased the astaxanthin content of *P. rhodozyma* [320]. This is surprising since a high C/N ratio generally increases lipid biosynthesis in fungi. However, it is possible that the common substrate acetyl-CoA is diverted from carotenoid biosynthesis to lipid formation. The highest astaxanthin levels in batch cultures were observed when no residual ammonia remained in batch cultures. An et al. [286] observed that nitrogen was assimilated more slowly in hyperproducing mutants of *P. rhodozyma,* possibly indicating that astaxanthin biosynthesis might be subject to nitrogen regulation.

The synthesis of astaxanthin by *P. rhodozyma* was investigated in continuous culture [316]. The optimum conditions for pigment synthesis was carbon limitation. The total carotenoid and astaxanthin levels decreased with increasing dilution rate, again supporting that carbon limitation is very important for pigment synthesis. Even at the lowest dilution rate investigated ($0.043\,h^{-1}$), the carotenoid content was about half that obtained in batch cultures with the strain. An increase in the glucose concentration in the chemostat decreased μ_{max}, $Y_{x/s}$, total pigmentation and astaxanthin. An attempt to determine the maintenance energy coefficient was not possible since carotenogenesis is energy consuming, and varies with the dilution rate. The investigators determined the yield ($Y_{x/s}$) of *P. rhodozyma* (mutant J4-3) on various substrates (g cells per g substrate) : N, 17.7–19.62; PO_4^{2-}, 87.2; K, 65.8; Mg, 1040; Ca 2687 [316]. The yield on N was at the upper range for yeasts, suggesting that nitrogen utilization is related to pigment accumulation. It would be of value to determine the yield on N for wild type yeasts as well as hyperproducers. Continuous culture appears to be good approach to determine the effect of limiting nutrients (e.g. P, N, Mg) on astaxanthin synthesis but it is limited by the low dilution rates and poor pigment synthesis. Because astaxanthin production is highest at low dilution rates and is repressed under conditions that promote growth, continuous cultivation would be sensible only on an industrial scale, with an integrated process layout [316].

Oxygen is a critical nutrient affecting astaxanthin synthesis. At aeration rates of $<30\,mmol\,O_2\,l^{-1}\,h^{-1}$ β-carotene was the primary carotenoid in *P. rhodozyma* [319]. The combination of 4% glucose and $5\,mmol\,O_2\,l^{-1}\,h^{-1}$ aeration gave pigment levels of only $30\,\mu g\,g^{-1}$ [315]. Our laboratory has noted that *P. rhodozyma* switches from cyanide-sensitive to cyanide-insensitive respiration on entry into stationary phase and that carotenogenesis also increases after this transition. Cyanide-insensitive respiration has been associated with generation

of O_2^- [321]. These data support that oxygen species such as O_{2^-} or $^1O_2$ are involved in carotenoid regulation in *P. rhodozyma.*

Although carotenoid synthesis is many fungi is stimulated by light, illumination was initially reported to have no effect on pigmentation [315]. Later studies by An and Johnson [322] using higher light intensities showed that light depressed both growth and pigment formation with blue light having the strongest effect. Light also increased β-zeacarotene levels, indicating cellular stress. Carotenoid hyperproducing mutants were less apt to become bleached by illumination. The combination of the respiratory inhibitor antimycin and light strongly increased pigment levels in *P. rhodozyma.* Light also restored growth in cells inhibited by antimycin after a 12–24 h lag which suggested that light might induce or feed an alternate respiratory system that facilities NADPH oxidation and continued growth. Salicylhydroxamic acid (SHAM) and propyl gallate (PG), which are inhitiors of antimycin-insensitive respiration in fungi, blocked induction of growth in antimycin by light. These data supported that an antimycin-insensitive oxidase is present in *P. rhodozyma* which affects carotenoid production. It is intriguing that partially purified alternative oxidase (ubiquinol oxidase) preparations from plants contain the presence of carotenoids and the oxidase [323] suggesting they may be physically associated with carotenoids in the cell.

Exposure of cells to antimycin for extended periods gave rise to hyperproducing colonies which were more sensitive to antimycin, and had altered respiratory function [286]. Surprisingly, antimycin-sensitive mutants that produced higher levels of pigment showed increased sensitivity to photokilling, particularly by blue light. It is likely that photoreactive molecules other than carotenoids such as flavins or porphyrins are more associated with light sensitization in *P. rhodozyma,* as has been postulated for other microorganisms [324]. The data also imply that the endogenous sensitizers, flavins or porphyrins, are involved in regulating carotenoid biosynthesis. A yellow mutant, yan-1, which produces β-carotene under normal culture conditions, formed xanthophylls when treated with antimycin + light. These data suggest that induction of an alternative oxidase system, possibly a flavin oxygenase or cytochrome P-450, allows growth in antimycin-inhibited yeasts and also stimulates carotenoid biosynthesis. Preliminary work in our laboratory has shown that in the presence of the potent cytochrome P-450 inhibitors, metyrapone and piperonyl butoxide, *P. rhodozyma* grows well but is nonpigmented. These data indicate a role for cytochrome P-450 in carotenogenesis. Multiple plant cytochrome P-450s are well recognized in plants which are involved in hydroxylations of isoprenoids [325].

In industrial scale productions, costs could be reduced by the use of non-refined carbon sources. Beet and cane molasses have been investigated for production of *P. rhodozyma.* Haard [326] grew *P. rhodozyma* on 7–10% molasses and showed 2–3 times higher astaxanthin higher astaxanthin production than on simple sugars. With 10% molasses, astaxanthin production was 3 times that of a corresponding amount of glucose and 2 times a blend of sugars mimicking the composition of molasses. Biomass production on molasses was also higher than on the corresponding sugar blend, suggesting that non-sugar

nutrients in molasses support growth. Similarly, when grape juice was used as a growth substrate, higher growth was obtained than on the corresponding sugar mixture [320].

Crude and inexpensive sources of carotenoid precursors could be used to enhance yield of astaxanthin. Alfalfa residual juice (ARJ), expected to have carotenoid precursors was studied as a growth substrate for *P. rhodozyma.* However, ARJ levels above 1.25% decreased pigmentation and at 3.7% completely repressed carotenogenesis [327]. Growth was not significantly affected by ARJ. Saponin, a terpenoid-glycoside, was found to be responsible for inhibition of carotenoid formation [328]. Carotenoid depression was reversed by polysaturated fatty acids and sterols, but not by the addition of media components.

Attempts have been made to increase production of carotenoids in *P. rhodozyma* and other fungi by feeding of terpenoids and other chemicals such as phenols. Although β-ionone stimulates carotenogenesis in *Phycomyces blakesleeanus* [329] and *Blakeslea trispora* [330], it inhibited astaxanthin formation in *P. rhodozyma.* β-Ionone caused an accumulation of β-carotene and a depletion of xanthophylls. Feeding of the monoterpene α-pinene to a mutant of *P. rhodozyma* increased the total pigment content from 1652 to 2201 $\mu g\, g^{-1}$, but there was a sharp decrease in growth rate at the effective concentration [331]. Other monoterpenes were too toxic to be used as supplements. Meyer and du Preez [332] investigated whether acetic acid would increase pigmentation with the assumption that it could serve as a precursor to acetyl-CoA. However, low concentrations of acetic acid ($2\ g l^{-1}$) decreased the growth rate and astaxanthin production by *P. rhodozyma* on glucose. Titrating the culture with acetic acid to maintain pH control increased the biomass and astaxanthin. In *Phycomyces,* a variety of compounds are known to stimulate synthesis (mostly terpenoids and phenols) and recently an unidentified fungal metabolite produced by an *Apsergillus* sp was stimulatory [333]. Potential stimulants that work independently or synergistically would be worthy of investigation for industrial *P. rhodozyma* production.

Considerable progress has been made by several bio-industry companies in the development of the yeast *Phaffia rhodozyma* as a source of pigmentation and other nutrients for aquaculture. In Milwaukee, Wisconsin, Universal Bioventures has commercialized a yeast product that producers at least 4000 $\mu g\, g^{-1}$ of astaxanthin in reactor culture. Other companies also have developed processes for production of astaxanthin by yeast. Most of the strains with enhanced pigmentation have been obtained primarily by the use of chemical mutagenesis and visual screening coupled with the use of inhibitors of the isoprenoid pathoway. However, unwanted effects are often associated with these methods including genetic instability in overproducing strains, lack of reproducibility in industrial fermentations, and decreased growth of mutants compared to the wild-type.

There are presently several constraints in developing a commercial astaxanthin process utilizing *P. rhodozyma* including: (a) a scarcity of basic genetic

methods for understanding carotenogenesis and construction of stable hyperproducing strains, (b) a lack of understanding of cellular metabolism in relation to astaxanthin synthesis, (c) complexities in bioprocess control and monitoring, (d) lack of technologies to detect and isolate astaxanthin hyperproducing mutants, (e) and the intracellular nature of astaxanthin. Progress is being made in the development of basic genetic methods and in optimization of process conditions. The isolation and characterization of the genes and enzymes responsible for xanthophyll formation in *P. rhodozyma* would be a significant advance but this information is not yet available. For strain development, it is possible that automated technologies could be optimized for detection of and isolation of carotenogenic mutants including FACS possibly combined with positive genetic selections [334] and physiological measurements [335]. Confocal redox and fluorescence imaging of single cells [312, 336], and digital imaging spectroscopy of cells and colonies may be valuable for detecting pigment mutants [337, 338]. A bottleneck in obtaining the highest production of astaxanthin is its intracellular compartmentalization. An understanding of its site of synthesis, compartmentalization, and transport throughout the cell could possibly lead to new technologies such as the potential use of permeabilized cells for end product removal and accumulation in an extractive phase [339].

9 Future Prospects

The production of carotenoids by microbial synthesis represents a classic example of competition between biological and chemical processes. It is unlikely that biosynthesis will be a significant source of the simpler carotenoids that are produced by highly advanced chemical syntheses. Microorganisms have potential for industrial production of carotenoids with complex structures including many xanthophylls that exist in nature as configurational isomers. The phylogeneic distribution of carotenoids in microorganisms confirms that specific groups have evolved the ability to synthesize carotenoids of medical and industrial interest. This analysis also indicates that certain ecological niches possessing carotenogenic organisms are unexplored and potentially valuable organisms remain to be isolated.

Of the various functions of carotenoids, a unifying mechanism is the ability of this class of compounds to quench $^1O_2$ and possibly other reactive forms of oxygen. Knowledge of the biological roles of carotenoids in humans and lower life is advancing at a rapid rate which should provide an impetus for increased intensity of research. It is refreshing to realize that besides providing aesthetic beauty and photoprotection in nature, carotenoids may be quite important in increasing the health and longevity of humans and animals by preventing chronic diseases.

Appendix

Trivial Name	Systematic Name
Aleuriaxanthin	(2*R*)-1′,16′-Didehydro-1′,2′-dihyro-β,ψ-caroten-2′-ol
Alloxanthin	(3*R*,3′*R*)-7,8,7′,8′-Tetradehydro-β,β-carotene-3,3′-diol
Antheraxanthin	5,6-Epoxy-5,6-dihydro-β,β-carotene-3,3′-diol
β-Apo-8′-carotenoic acid	8′-Apo-β-caroten-8′-oic acid
Astaxanthin	3,3′-Dihydroxy-β,β-carotene-4,4′-dione
Bacterioruberin	(2*S*,2′*S*)-2,2′-Bis(3-hydroxy-3-mehylbutyl)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol
Bixin	Methyl hydrogen 9′-cis-6,6′-diapocarotene-6,6′-dioate
Caloxanthin	(2*R*,3*R*,3′*R*)-β,β-Carotene-2,3,3′-triol
Canthaxanthin	β,β-Carotene-4,4′-dione
Capsanthin	(3*R*,3′*S*,5′*R*)-3,3′-Dihydorxy-β,κ-caroten-6′-one
Capsorubin	(3*S*,5*R*,3′*S*,5′*R*)-3,3′-Dihydroxy-κ,κ-carotene-6,6′-dione
α-Carotene	(6′*R*)-β,ε-Carotene
β-Carotene	β,β-Carotene
γ-Carotene	β,ψ-Carotene
ξ-Carotene	7,8,7′,8′-Tetrahydro-ψ,ψ-carotene
Chlorobactene	ϕ,ψ-Carotene
Chloroxanthin	1,2,7′,8′-Tetrhydro-ψ,ψ-caroten-1-ol
Citranaxanthin	5′,6′-Dihydro-5′-apo-18′-or-β-caroten-6′-one
Crocin	Digentibiosyl-8,8′-diapocarotene-8,8′-dioate
Crocoxanhin	(3*R*,6′*R*)-7,8-Didehydro-β,ε-caroten-3-ol
Cryptoxanthin	(3*R*)-β,β-Caroten-3-ol
Decaprenoxanthin	(2*R*,6*R*,2′*R*,6′*R*)-2′-Bis-(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene
Deoxyflexixanthin	1′-Hydroxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one
Diadinoxanthin	(3*S*,5′*R*,6*S*,3′*R*)-5,6-Epoxy-7′,8′-didehydro-5,6-dihydro-β,β-carotene-3,3′-diol
4′,4′-Diaponeurosporene	7,8-Dihydro-4,4′-diapocarotene
4,4′-Diaponeurospren-4-oic acid	7′,8′-Dihydro-4,4′-diapocaroten-4-oic acid
4,4′-Diapophytoene	7,8,11,12,7′,8′11′,12′-Octahydro-4,4′-diapocarotene
Diatoxanthin	(3*R*,3′*R*)-7,8-Didehydro-β,β-carotene-3,3′-diol
Echinenone	β,β-Carotene-4-one
Eutreptiellanone	(3*S*,5*R*,6*S*)-3,6-Epoxy-3′,4′,7′8′-tetradehydro-5,6-dihydro-β,β-carotene-4-one
Flexixanthin	(3*S*)-3,1′-Dihydro-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one
Fucoxanthin	(3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*R*)-5,6-Epoxy-3,3′,5′-trihydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-caroten-8-one-3′-acetate
Heteroxanthin	(3*S*,5*S*,6*S*,3′*R*)-7′,8′-Didehydro-5,6-dihydro-β,β-carotene-3,5,6,3′-tetrol
19′-Hexanoyloxyfucoxanthin	(3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*)-5,6-Epoxy-3,3′,5′,19′-tetrahydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-carotene-8-oe 3′-acetate 19′-hexanoate
Hydroxyspheroidenone	1′-Methoxy-3′,4′-didehydro-1,2,7,8,1′,2′-hexahydro-ψ,ψ-caroten-1-ol
Isorenieratene	ϕ,ϕ-Carotene
β-Isorenieratene	β,ϕ-Carotene
Leoprotene	see Isorenieratene
Loroxanthin	(3*R*,3′*R*,6′*R*)-β,ε-carotene-3,19,3′-triol
Lutein	(3*R*,3′*R*,6′*R*)-β,ε-Carotene-3,3′-diol
Lycopenal	13-*cis*-ψ,ψ-Carotene-20-al
Lycopene	ψ,ψ-Carotene
Lycopenol	ψ,ψ-Carotene-16-ol
Lycoxanthin	see Lycopenol
Mutachrome	5,8-Epoxy-5,8-dihydro-β,β-carotene

Appendix (*continued*)

Monadoxanthin	7,8-Didehydro-β,ε-carotene-3,3′-diol
Myxobactone	1′-Glucosyloxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-4-one
Myxoxanthophyll	2′-(β-L-Rhamnopyranosyloxy)-3,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-3,1′:diol
Neoxanthin	(3*S*,5*R*,6*R*,3′*S*,5′*R*,6′*S*)-5′,6′-Epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol
Neurosporene	7,8-Dihydro-ψ,ψ-carotene
Neurosporoxanthin	4′-Apo-β-carotene-4′-oic acid
Nonaprenoxanthin	2-(4-Hydroxy-3-methyl-2-butenyl)-7′,8′,11′,12′-tetrahydro-ε,ψ-carotene
Norbixin	6,6′-Diapocarotene-6,6′-dioic acid
Nostoxanthin	(2*R*,3*R*,2′*R*,3′*R*)-β,β-Carotene-2,3,2′,3′-tetrol
Okenone	1-Methoxy-1′,2′-dihydro-ϰ,ψ-caroten-4′-one
Oscillaxanthin	2*R*,2′*R*)-2,2′-Bis(β-L-rhamnopyransyloxy)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol
Peridinin	5′,6′-Epoxy-3,5,3′-trihydroxy-6,7-didehydro-5,6,5′,6′-tetrahydro-10,11,20-trinor-β,β-caroten-19′,11′-olide-3-acetate
Phillipsiaxanthin	1,1′-Dihydrxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-2,2′-dione
Phleixanthophyll	(2′*S*)-1′-(β-D-Glucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-2′-ol
Phytoene	15-*cis*-7,8,11,12,7′,8′,11′,12′-Octohydro-ψ,ψ-carotene
Phytofluene	15-*cis*–7,8,11,12,7′,8′-Hexahydro-ψ,ψ-carotene
Plectaniaxanthin	(2′*R*)-3′,4′-Didehydro-1′,2′-dihydro-β,ψ-carotene-1′,2′-diol
Prasinoxanthin	(3′*R*,6′*R*)-3,6,3′-Trihydroxy-7,8-dihydro-γ,ε-caroten-8-one
Prephyoene pyrophosphate	7,8,11,12,13,14,15,7′,8′,11′,12′,15′-Dodecahydro-13,15′: 14,15′-bicyclo-15,15′-seco-ψ,ψ-carotene-15-ol 15 pyrophosphate
Rhodopin	1,2-Dihydro-ψ,ψ-carotene-1-ol
Rhodopinal	13-*cis*-1-Hydroxy-1,2-dihydro-ψ,ψ-caroten-20-al
Rhodoxanthin	4′,5′-Didehydro-4,5′-retro-β,β-carotene-3,3′-dione
Saproxanthin	3′,4′-Didehydro-1′,2′-dihydro-β,ψ-carotene-3,1′-diol
Sarcinaxanthin	2′-(4-Hydroxy-3-methyl-2′-butenyl)-2-(3-methyl-2-butenyl)-ε,ε-caroten-18-ol
Siphonaxanthin	3,19,3′-Trihydroxy-7,8-dihydro-β,ε-carotene-8-one
Siphonein	(3*R*,3′*R*,6′*R*)-3,19,3′-T rihydroxy-7,8-dihydro-β,ε-carotene-8-one-19-laurate
Spheroidene	1-Methoxy-3,4,-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-carotene
Spirilloxanthin	1,1′-Dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene
Torularhodin	3′,4′-Didehydro-β,ψ,-carotene-16′-oic acid
Torulene	3′,4′-Didehydro-β,ψ-carotene
Violaxanthin	(3*S*,5*R*,6*S*,3′*S*,5′*R*6′*S*)-5,6,5′,6′-Diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene
Zeaxanthin	(3*R*,3′*R*)-β,β-Carotene-3,3′-diol

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