Carotenoids
Volume 5: Nutrition and Health

Chapter 5

Microbial and Microalgal Carotenoids as Colourants and Supplements

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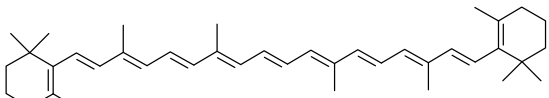
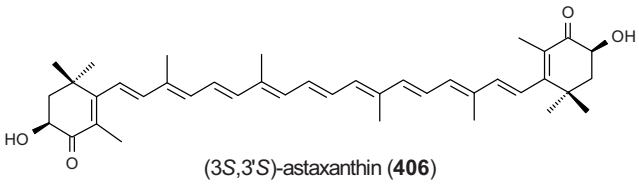
A. Introduction

General aspects of the production and use of carotenoids as colourants and supplements were discussed in *Chapter 4*. For several decades, these carotenoids have been produced commercially by chemical synthesis or as plant extracts or oleoresins, *e.g.* of tomato and red pepper. Some unicellular green algae, under appropriate conditions, become red due to the accumulation of high concentrations of 'secondary' carotenoids. Two examples, *Dunaliella* spp. and *Haematococcus pluvialis*, are cultured extensively as sources of β-carotene (**3**) and (3*S*,3′*S*)-astaxanthin (**406**), respectively.



β-carotene (**3**)



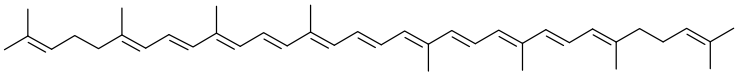
(3*S*,3′*S*)-astaxanthin (**406**)

Non-photosynthetic microorganisms, *i.e*. bacteria, yeasts and moulds, may also be strongly pigmented by carotenoids, so commercial production by these organisms is an attractive prospect. Penetration into the food industry by fermentation-derived ingredients is increasing year after year, examples being thickening or gelling agents (xanthan, curdlan, gellan), flavour enhancers (yeast hydrolysate, monosodium glutamate), flavour compounds (γ-decalactone, diacetyl, methyl ketones), and acidulants (lactic acid, citric acid). Fermentation processes for pigment production on a commercial scale were developed later but some are now in use in the food industry, such as production of β-carotene from the fungus *Blakeslea trispora*, in Europe, and the non-carotenoid heterocyclic pigments from *Monascus*, in Asia [1-3]. Efforts have been made to reduce the production costs so that pigments produced by fermentation can be competitive with synthetic pigments or with those extracted from natural sources. There is scope for innovations to improve the economics of carotenoid production by isolating new microorganisms, creating better ones, or improving the processes.

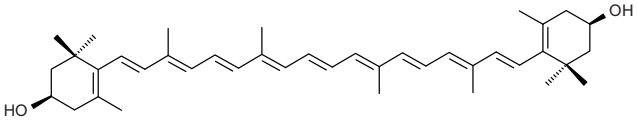
The microbial carotenoid products may be used as colour additives for food and feed, and are now under consideration for use as health supplements.

B. Carotenoid Production by Microorganisms and Microalgae

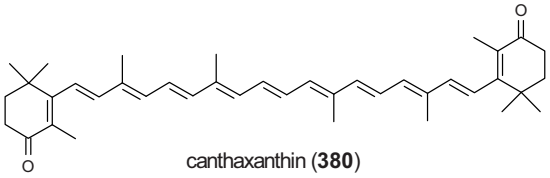
Commercial processes are already in operation or under development for the production of carotenoids by microalgae, moulds, yeasts and bacteria. The production of β-carotene by microorganisms, as well as by chemical synthesis or from plant extracts, is well developed, and the microbial production of several other carotenoids, notably lycopene (**31**), astaxanthin (**404-406**), zeaxanthin (**119**) and canthaxanthin (**380**), is also of interest. There is no microbial source that can compete with marigold flowers as a source of lutein (**133**).



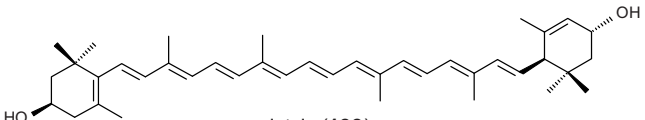
lycopene (**31**)



zeaxanthin (**119**)



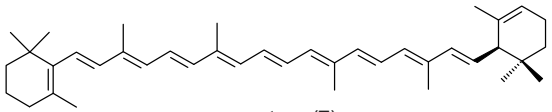
canthaxanthin (**380**)



lutein (**133**)

1. β-Carotene

β-Carotene is produced on a large scale by chemical synthesis, and also from plant sources such as red palm oil, in addition to production by fermentation and from microalgae. The various preparations differ in the composition of geometrical isomers and in the presence of α-carotene (**7**) and other carotenoids, particularly biosynthetic intermediates (Table 1).



α-carotene (**7**)

Table 1. Percentage composition of 'β-carotene' from various sources.

Source	(all-*E*)-β-carotene	(*Z*)-β-carotene	α-carotene	others
Fungus *(Blakeslea)*	94	3.5	0	2.5
Chemical synthesis	98	2	0	0
Alga *(Dunaliella)*	67.4	32.6	0	0
Palm oil	34	27	30	9

a) *Dunaliella* species

Although the cyanobacterium (blue-green alga) *Spirulina* is able to accumulate β-carotene at up to 0.8-1.0 % w/w, *Dunaliella* species (*D. salina* and *D. bardawil*) produce the highest yield of β-carotene among the algae.

Dunaliella is a halotolerant, unicellular, motile green alga belonging to the family Chlorophyceae [4]. It is devoid of a rigid cell wall and contains a single, large, cup-shaped chloroplast which contains the characteristic carotenoid complement of green algae, similar to that of higher plant chloroplasts. In response to stress conditions such as high light intensity [5], it accumulates a massive amount of β-carotene [6]. The alga can yield valuable products, notably glycerol and β-carotene, and is also a rich source of protein that has good utilization value, and of essential fatty acids. *Dunaliella* biomass has GRAS (Generally Recognized As Safe) status and can be used directly as food or feed. As a supplement, *Dunaliella* has been

reported to exhibit various biological effects, such as antihypertensive, bronchodilator, analgesic, muscle relaxant, and anti-oedema activity [7].

Dunaliella grows in high salt concentration (1.5 ± 0.1 M NaCl), and requires bicarbonate as a source of carbon, and other nutrients such as nitrate, sulphate and phosphate. The initial photosynthetic (vegetative) growth phase requires 12-14 days in nitrate-rich medium. The subsequent carotenogenesis phase requires nitrate depletion and maintenance of salinity. This technology is best suited for coastal areas where sea water is rich in salt and other nutrients. For carotenogenesis, nutrient, salt or light stress is essential; generally the vegetative phase requires 5-10 klux whereas the light should be around 25-30 klux for β-carotene accumulation.

Dunaliella salina can be cultivated easily and quickly compared to plants and, under ideal conditions, can produce a very high quantity of β-carotene compared to other sources (3-5%, w/w on a dry mass basis, 400 mg per square metre of cultivation area) [8]. At high light intensity, there can be a large proportion of *Z* isomers, with up to 50% of (9*Z*)-β-carotene. Cells are harvested by flocculation followed by filtration; the product can be directly utilized as feed or in food formulations, or it can be extracted for pigments.

For various food formulations and applications the carotene can be extracted either in edible oils or food grade organic solvents. Most of the pharmaceutical formulations are made with either olive oil or soybean oil. In the natural extracts, β-carotene is generally accompanied by small amounts of the residual chloroplast carotenoids and is marketed under the title 'Carotenoids Mix'.

The major sites of commercial production are Australia, China, India, Israel, Japan and the U.S., with smaller-scale production in several other countries with suitable environmental conditions. *Dunaliella* β-carotene is widely distributed today in many different markets under three different categories, namely β-carotene extracts; *Dunaliella* powder for human use; dried *Dunaliella* for feed use. Extracted purified β-carotene, sold mostly in vegetable oil in bulk concentrations from 1% to 20%, is used to colour various food products or, in soft gel capsules, for use as a supplement, usually 5 mg β-carotene per capsule.

b) *Blakeslea trispora*

Blakeslea trispora is a commensal mould associated with tropical plants. The fungus exists in (+) and (–) mating types; the (+) type synthesizes trisporic acid, which is both a metabolite of β-carotene and a hormonal stimulator of its biosynthesis. On mating the two types in a specific ratio, the (–) type is stimulated by trisporic acid to synthesize large amounts of β-carotene.

The production process proceeds essentially in two stages. Glucose and corn steep liquor can be used as carbon and nitrogen sources. Whey, a byproduct of cheese manufacture, has also been considered [9], with strains adapted to metabolize lactose. In the initial fermentation process, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β-carotene. In the

second stage, the recovery process, the biomass is isolated and converted into a form suitable for isolating the β-carotene, which is extracted with ethyl acetate, suitably purified and concentrated, and the β-carotene is crystallized [10]. The final product is either used as crystalline β-carotene (purity >96%) or is formulated as a 30% suspension of micronized crystals in vegetable oil. The production process is subject to Good Manufacturing Practices (GMP) procedures, and adequate control of hygiene and raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification and the final crystalline product also complies with the JECFA (**J**oint FAO/WHO **E**xpert **C**ommittee on **F**ood **A**dditives) and E.U. specifications as set out in Directive 95/45/EC for colouring materials in food.

The first β-carotene product from *B. trispora* was launched in 1995. The mould has shown no pathogenicity or toxicity, in standard pathogenicity tests in mice, by analysis of extracts of several fermentation mashes for fungal toxins, and by enzyme immunoassays of the final product, the β-carotene crystals, for four mycotoxins. HPLC analysis, stability tests and microbiological tests showed that the β-carotene obtained by co-fermentation of *Blakeslea trispora* complies with the E.C. specification for β-carotene (E 160 aii), listed in Directive 95/45/EC, including the proportions of *Z* and *E* isomers, and is free of mycotoxins or other toxic metabolites and free of genotoxic activity. In a 28-day feeding study in rats with the β-carotene manufactured in the E.U. no adverse findings were noted at a dose of 5% in the diet, the highest dose level used. The E.U. Scientific Committee considered that "β-carotene produced by co-fermentation of *Blakeslea trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a colouring agent for foodstuffs" [11].

There are now other industrial productions of β-carotene from *B. trispora* in Russia, Ukraine, and Spain [12]. The process has been developed to yield up to 30 mg of β-carotene per g dry mass or about 3 g per litre of culture. *Blakeslea trispora* is now also used for the production of lycopene (Section **B**.2.a).

c) *Phycomyces blakesleeanus*

Another mould, *Phycomyces blakesleeanus*, is also a potential source of various chemicals including β-carotene [13]. The carotene content of the wild type grown under standard conditions is modest, about 0.05 mg per g dry mass, but some mutants accumulate up to 10 mg/g [14]. As with *Blakeslea trispora*, sexual stimulation of carotene biosynthesis is essential, and can increase yields to 35 mg/g [15]. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *Blakeslea trispora* is more appropriate for production in usual fermentors [16].

d) *Mucor circinelloides*

Mucor circinelloides wild type is yellow because it accumulates β-carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction by blue light [17], are similar to those in *Phycomyces* and *Mucor* [18]. *M. circinelloides* is a dimorphic fungus that grows either as yeast cells or in a mycelium form, and research is now focused on yeast-like mutants that could be useful in a biotechnological production [12].

2. Lycopene

Lycopene is produced on a large scale by chemical synthesis, and from tomato extracts, in addition to production by fermentation. As with β-carotene, the various preparations differ in the composition of geometrical isomers (Table 2).

Table 2. Percentage of geometrical isomers in 'lycopene' from various sources.

Source	**(all-*E*)**	**(5Z)**	**(9Z)**	**(13Z)**	**Others**
Chemical synthesis	>70	<25	<1	<1	<3
Tomato	94-96	3-5	0-1	1	<1
Blakeslea trispora	≥ 90		(mixed *Z* isomers) 1-5		

Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β-carotene. In principle, therefore, blocking the cyclization reaction and the cyclase enzyme by mutation or inhibition will lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

a) *Blakeslea trispora*

A commercial process for lycopene (**31**) production by *Blakeslea trispora* is now established. Imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase [19]. The product, predominantly (all-*E*)-lycopene, is formulated into a 20% or 5% suspension in sunflower oil, together with α-tocopherol at 1% of the lycopene level. Also available is an α-tocopherol-containing 10% or 20% lycopene cold-water-dispersible (CWD) product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements. The proposed level of use for lycopene in food supplements is 20 mg per day.

Approval for the use of lycopene from *B. trispora* was sought under regulation (EC) No 258/97 of the European Parliament and the Council concerning novel foods and novel food ingredients [20]. The European Food Safety Authority was also asked to evaluate this product for use as a food colour. The conclusions were that the lycopene from *B. trispora* is considered to be nutritionally equivalent to lycopene in a natural diet, but further safety trials are necessary. Whilst the toxicity data on lycopene from *B. trispora* and on lycopene from

tomatoes do not give indications for concern, nevertheless these data are limited and do not allow an ADI to be established. The main concern is that the proposed use levels of lycopene from *B. trispora* as a food ingredient may result in a substantial increase in the daily intake of lycopene compared to the intakes solely from natural dietary sources. The use of lycopene as a health supplement was not considered.

b) *Fusarium sporotrichioides*

The fungus *Fusarium sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn-fibre material, the 'leftovers' of making ethanol [21]. By use of sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids *via* carotenoid biosynthesis genes introduced from the bacterium *Erwinia uredovora*. Cultures in laboratory flasks produced 0.5 mg lycopene per g dry mass within six days and improvements are predicted [22].

3. Astaxanthin

The application of astaxanthin in aquaculture feed to impart the desired colour to fish and crustaceans is described in *Volume 4, Chapter 12*. There are also reports of beneficial actions of astaxanthin for human health, so its use in supplements is of interest. The biotechnological production of astaxanthin from microalgae, yeasts and bacteria is the subject of intensive investigation, though synthetic astaxanthin remains the market leader.

a) *Haematococcus pluvialis*

Haematococcus pluvialis is a green alga known for its ability to accumulate (3*S*,3′*S*)-astaxanthin (**406**), up to 0.2 to 2.0% (on a dry mass basis). The alga can grow both under autotrophic and heterotrophic conditions. Astaxanthin from *Haematococcus* is under consideration for US Food and Drug Administration clearance and several European countries have approved its marketing as a dietary supplement ingredient for human consumption.

The production of astaxanthin by *Haematococcus pluvialis* is attractive [23], but has fewer advantages than the *Dunaliella* β-carotene process. *H. pluvialis* is a freshwater alga so open-air culture leads to contamination by undesirable species. Outdoor cultivation of *Haematococcus* is a challenge and requires curtailment of contamination and control of environmental conditions such as light and temperature. This organism grows at 20-28°C, below 15 klux light intensity and at pH 6.8-7.4, so contamination by bacteria, fungi and protozoa, is a serious problem. Under high light intensity the cell growth is significantly affected. Recently, however, processes involving completely closed photobioreactors with artificial light or a combination of closed photobioreactors and open culture ponds are being used for *Haematococcus* cultivation [24].

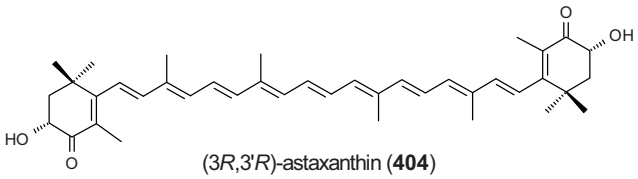
Unlike *Dunaliella*, *Haematococcus* changes from a motile, flagellated cell to a non-motile, thick-walled aplanospore during the growth cycle [25,26]; the astaxanthin is contained in the aplanospore. This means that the physical properties (density, settling rate, cell fragility) and nutrient requirements of the cells change during the culture process, and this alters the optimum conditions for growth and carotenoid accumulation during the growth cycle [27]. The content of astaxanthin in the aplanospores is about 1-2% of dry mass but their thick wall requires physical breakage before the astaxanthin can either be extracted or be available to organisms consuming the alga [28].

The development of a commercially viable algal astaxanthin process requires the development of an effective closed culture system and the selection (either from Nature or by mutagenesis) of strains of *Haematococcus* with higher astaxanthin content and an ability to tolerate higher temperatures than the wild strains. Successful commercial production is now operating in India, Japan and the U.S.

Astaxanthin is recognized by U.S. FDA under title 21 Part 73 (under List of Colour Additives Exempted from Certification) Subpart A - Foods (Sec.73.35 Astaxanthin). Formulations containing astaxanthin are: soft gelatin capsules containing 100 mg equivalent of total carotenoids; skin-care cream containing astaxanthin as one of the ingredients; food and feed formulations for shrimp and fish.

b) *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*)

Among the few astaxanthin-producing microorganisms, *Xanthophyllomyces dendrorhous* is one of the best candidates for commercial production of astaxanthin [29] though, in this case, the product is the (3*R*,3′*R*)-isomer (**404**).



(3*R*,3′*R*)-astaxanthin (**404**)

The effects of different nutrients on *Xanthophyllomyces dendrorhous* have generally been studied in media containing complex sources of nutrients such as peptone, malt and yeast extracts. By-products from agriculture were also tested, such as molasses [30], enzymic wood hydrolysates [31], corn wet-milling co-products [32], bagasse or raw sugarcane juice [33], date juice [34] and grape juice [35]. However, in order to elucidate the nature of nutritional effects as far as possible, chemically defined or synthetic media have been used [36-38]. In one major study [38], the optimal conditions stimulating the highest astaxanthin production were found to be: temperature 19.7°C; carbon concentration 11.25 g/L; pH 6.0; inoculum 5%; nitrogen concentration 0.5 g/L. Under these conditions the astaxanthin content was 8.1 mg/L.

Fermentation strategy also has an impact on growth and carotenoid production of *Xanthophyllomyces dendrorhous* [39], as shown by studies with fed-batch cultures (*e.g*. limiting substrate is fed without diluting the culture) [40] or pH-stat cultures (*i.e*. a system in which the feed is provided depending on the pH) [41]. The highest biomass obtained was 17.4 g/L. Another starting point in optimization experiments is the generation of mutants [42], but metabolic engineering of the astaxanthin biosynthetic pathway is now attractive [43].

A major drawback in the use of *Xanthophyllomyces dendrorhous* is that, for efficient intestinal absorption of the pigment, disruption of the cell wall of the yeast biomass is required before addition to an animal diet. Several chemical, physical, autolytic, and enzymic methods for cell-wall disruption have been described, inluding a two-stage batch fermentation technique [44]. The first stage was for 'red yeast' cultivation. The second stage was the mixed fermentation of the yeast and *Bacillus circulans*, a bacterium with a high cell-wall lytic activity.

The case of *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) is peculiar; hundreds of scientific papers and patents deal with astaxanthin production by this yeast [45,46] but the process has not yet become economically efficient. New patents are filed almost each year, with improved astaxanthin yield; yields up to 3mg/g dry matter have been achieved [47].

c) *Agrobacterium aurantiacum* and other bacteria

Astaxanthin is one of ten carotenoids present in *Agrobacterium aurantiacum* [48]. The biosynthetic pathway, the influence of growth conditions on carotenoid production and the occurrence of astaxanthin glucoside have been described [49,50], but commercial processes have not yet been developed.

Numerous screenings have been conducted in the search for new bacterial sources of astaxanthin, and positive targets were isolated such as *Paracoccus carotinifaciens* [51] and a *Halobacterium* species [52]. The latter is particularly interesting because: (i) the extreme NaCl concentrations (about 20%) used in the growth medium prevent contamination with other organisms so no particular care has to be taken with sterilization; (ii) NaCl concentrations under 15% induce bacterial lysis, so that no special cell breakage technique is necessary, and pigments may be extracted directly with sunflower oil instead of organic solvents. This would eliminate possible toxicity problems due to trace amounts of acetone or hexane and facilitate pigment assimilation by animals. No commercial processes have yet been developed, however.

4. Zeaxanthin

Zeaxanthin (**119**) can be used, for example, as an additive in feeds for poultry to intensify the yellow colour of the skin or to accentuate the colour of the yolk of their eggs [53]. It is also suitable for use as a colourant, for example in the cosmetics and food industries, and as a health supplement in relation to the maintenance of eye health (see *Chapter 15*).

In the mid-1960s, several marine bacteria that produce zeaxanthin were isolated. Cultures of a *Flavobacterium* sp. (ATCC 21588, classified under the accepted taxonomic standards of that time) [54] in a defined nutrient medium containing glucose or sucrose as carbon source, were able to produce up to 190 mg of zeaxanthin per litre, with a concentration of 16 mg/g dried cell mass. One species currently under investigation in many studies [55-57] is *Sphingobacterium* (formerly *Flavobacterium*) *multivorum* (ATCC 55238). This was recently shown to utilize the deoxyxylulose phosphate or methylerythritol phosphate pathway [58,59]. A strain was constructed for over-production of zeaxanthin in industrial quantities [60].

Another zeaxanthin-producing '*Flavobacterium*' was recently reclassified as a *Paracoccus* species, *P. zeaxanthinifaciens* [61]; earlier findings that isoprenoid biosynthesis occurs exclusively *via* the mevalonate pathway were confirmed [62-65]. A second strain, isolated in a mat from an atoll of French Polynesia, produces also exopolysaccharides [66]. Another member of the Sphingobacteraceae, *Nubsella zeaxanthinfaciens*, was isolated recently from fresh water [67].

Chemical synthesis remains the method of choice for production of zeaxanthin, however.

5. Canthaxanthin

Canthaxanthin (**380**) has been used in aquafeed for many years to impart the desired flesh colour to farmed salmonid fish, especially trout (*Volume 4*, *Chapter 12*). Because extreme overdosage with canthaxanthin can lead to the deposition of minute crystals in the human eye (*Chapter 15*), canthaxanthin is not likely to be accepted as a health supplement and there is some pressure to limit its use in aquafeeds.

Some bacteria have potential for commercial canthaxanthin production. A strain of a *Bradyrhizobium* sp. was described as a canthaxanthin producer [68] and the carotenoid gene cluster was fully sequenced [69]. A second organism under scrutiny for canthaxanthin production is the extreme halophile *Haloferax alexandrinus*, a member of the family Halobacteriaceae (Archaea). Most members of the Halobacteriaceae are red due to the presence of C_{50}-carotenoids [70]. Some species, however, have been reported to produce C_{40}-carotenoids, including ketocarotenoids, as minor components. Recently, the biotechnological potential of these members of the Archaea has increased because of their unique features, which facilitate many industrial procedures. For example, no sterilization is required, because of the extremely high NaCl concentration used in the growth medium (contamination by other organisms is avoided). In addition, no cell-disrupting devices are required, as cells lyse spontaneously in fresh water [71]. A 1-litre-scale cultivation of the cells in flask cultures (6 days) under non-aseptic conditions produced 3 g dry mass, containing 6 mg total carotenoid and 2 mg canthaxanthin [72]. Further experiments in a batch fermenter also demonstrated increases in the biomass concentration and carotenoid production.

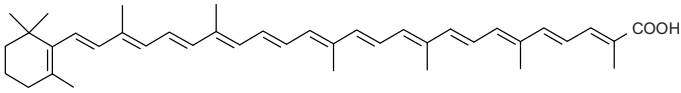
A third example is *Gordonia jacobea* (CECT 5282), a Gram-positive, catalase negative, G+C 61% bacterium which was isolated in routine air sampling during screening for

microorganisms that produce pink colonies [73], with canthaxanthin as the main pigment [74]. The low carotenoid content (0.2 mg/g dry mass) does not support an industrial application but, after several rounds of mutations, a hyper-pigmented mutant (MV-26) was isolated which accumulated six times more canthaxanthin than the wild-type strain and, by varying the culture medium, canthaxanthin concentrations between 1 and 13.4 mg/L were achieved. Mutants of this species have potential advantages from the industrial point of view: (i) the optimal temperature for growth and carotenogenesis, 30°C, is usual in fermentors; (ii) glucose, an inexpensive carbon source, gives optimal growth and pigmentation; and (iii) >90% of the total pigments can be extracted directly with ethanol, a non-toxic solvent allowed for human and animal feed [75].

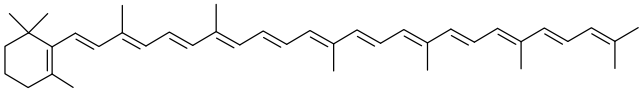
6. Torulene and torularhodin

Yeasts of the genus *Rhodotorula* synthesize carotenoids, mainly torularhodin (**428**) and torulene (**11**) accompanied by very small amounts of β-carotene. Most of the research has focused on the species *Rhodotorula glutinis* [76], though other species such as *R. gracilis*, *R. rubra* [77], and *R. graminis* [78] have been studied. These yeasts have potential as feed products rather than as health supplements.

Optimization studies [79,80] have mainly resulted in an increased yield of torulene and torularhodin, which are of minor interest, though some did succeed in increasing the β-carotene content up to about 70 mg/L.



torularhodin (**428**)



torulene (**11**)

C. Prospects for Carotenoid Production by Genetically Modified Microorganisms

1. *Escherichia coli* and other hosts

Metabolic engineering is defined as the use of recombinant DNA techniques for the deliberate modification of metabolic networks in living cells to produce desirable chemicals with

superior yield and productivity. The traditional assumption was that the most productive hosts would be microbes that naturally synthesize the desired chemicals, but microorganisms that have the ability to produce precursors of the desired chemicals with superior yield and productivity are also considered as suitable hosts [81].

As a starting point a large number (>200) of genes and gene clusters coding for the enzymes of carotenoid biosynthesis have been isolated from various carotenogenic microorganisms, and the functions of the genes have been elucidated (*Volume 3, Chapter 3*).

In bacteria such as *Escherichia coli*, which cannot synthesize carotenoids naturally, carotenoid biosynthesis *de novo* has been achieved by the introduction of carotenogenic genes. *E. coli* does possess the ability to synthesize other isoprenoid compounds such as dolichols (sugar carrier lipids) and the respiratory quinones. It is thus feasible to direct the carbon flux for the biosynthesis of these isoprenoid compounds partially to the pathway for carotenoid production by the introduction of the carotenogenic genes. For example, plasmids carrying *crt* genes for the synthesis of lycopene, β-carotene and zeaxanthin have been constructed and expressed in *E. coli*. Transformants accumulated lycopene, β-carotene, and zeaxanthin, at 0.2-1.3 mg/g dry mass, in the stationary phase. With a few exceptions, such as the zeaxanthin C(5,6) epoxidase gene, almost all cloned carotenoid biosynthetic genes are functionally expressed in *E. coli*. The use of shot-gun library clones constructed with *E. coli* chromosomal DNA [82] has revealed that genes not directly involved in the carotenoid biosynthesis pathway are important, such as *appY*, which encodes transcriptional regulators related to anaerobic energy metabolism and can increase the lycopene production to 4.7 mg/g dry cell mass.

A most important challenge for biotechnology is to identify rate-limiting steps or to eliminate regulatory mechanisms in order to enhance further the production of valuable carotenoids [83]. Sufficient amounts of endogenous precursors (*i.e.* substrates for the reactions involved) must be available; by control of the pyruvate/glyceraldehyde 3-phosphate ratio, a yield of 25 mg lycopene/g dry mass has been reported [84]. A balanced system of carotenogenic enzymes should be expressed, to enable efficient conversion of precursors without the formation of pools of intermediate metabolites. The correct plasmid combination is important to minimize the accumulation of intermediates and to increase the yield of the end product. Finally, the host organism should exhibit an active central terpenoid pathway and possess a high storage capacity for carotenoids [85].

As well as *E. coli*, the edible yeasts *Candida utilis* [86] and *Saccharomyces cerevisiae* [87] acquire the ability to produce carotenoids when the required carotenogenic genes are introduced.

2. Directed evolution and combinatorial biosynthesis

Directed evolution involves the use of rapid molecular manipulations to mutate the target DNA fragment, followed by a selection or screening process to isolate desirable mutants. By

various directed evolution protocols, several enzymes have been improved or optimized for a specific condition. Directed evolution was applied to geranylgeranyl diphosphate (GGDP) synthase (a rate-controlling enzyme) from *Archaeoglobus fulgidus* to enhance the production of carotenoids in metabolically engineered *E. coli* [88]. The production of lycopene was increased by about 2-fold.

A second example deals with the membrane-associated phytoene synthase which appears to be the major point of control over product diversity. By engineering the phytoene synthase to accept longer diphosphate substrates, variants were produced that can make previously unknown C_{35}-, C_{45}- and C_{50}-carotenoid backbones from the appropriate isoprenyl diphosphate precursors [89,90]. Once a carotenoid backbone structure is created, downstream enzymes, either natural or engineered, such as desaturases, cyclases, hydroxylases, and cleavage enzymes, can accept the new substrate, and whole series of novel C_{35}-, C_{45}- and C_{50}-carotenoid analogues can be produced.

A different approach is to combine available biosynthetic genes [91] and evolve new enzyme functions through random mutagenesis, recombination (DNA-shuffling) and selection. Prerequisites for this approach are that the enzymes from different species can function cooperatively in a heterologous host and display enough promiscuity regarding the structure of their substrates. The success of functional colour complementation in transgenic *E. coli* for cloning a number of carotenoid biosynthesis genes demonstrates that enzymes from phylogenetically distant species can assemble into a functional membrane-bound multi-enzyme complex through which carotenoid biosynthesis presumably takes place [92].

A related strategy [93] which can be used to produce novel carotenoids is to combine carotenogenic genes from different bacteria that alone normally produce different end products and to express them in a simple *E. coli* host that carries the biosynthetic machinery for phytoene production [94].

Much is now technically feasible, but there are still many problems to be overcome, especially in relation to control of the end product so that the desired target carotenoid is produced rather than a complex mixture, and to the ability of the host organism to accumulate the carotenoid in high concentration.

D. Concluding Comments

Nature is rich in colour, and carotenoid-producing microorganisms (fungi, yeasts, bacteria) are quite common.

The success of any pigment product manufactured by fermentation depends upon its acceptability in the market place, regulatory approval, and the size of the capital investment required to bring the product to market. A few years ago, doubts were expressed about the successful commercialization of carotenoids produced by fermentation because of the high capital investment needed for fermentation facilities and the extensive and lengthy toxicity

studies required by regulatory agencies. Also, public perception of GM organisms is an important factor in the acceptance of biotechnology-derived products.

Now, however, some carotenoids produced by fermentation are on the market. This, and the successful marketing of algal-derived or vegetable-extracted carotenoids, both as food colours and as nutritional supplements, reflects the importance of 'niche markets' in which consumers are willing to pay a premium for 'all-natural ingredients'. Carotenoids play an exceptional role in the fast-growing 'over-the-counter medicine' and 'nutraceutical' sector.

Among carotenoids under investigation for colouring or for biological properties, only a small number of the 700 or so carotenoids listed in the '*Carotenoids Handbook'* are currently available from natural extracts or by chemical synthesis [95]. With imagination, biotechnology could be a solution for providing additional pigments for the market.

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