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MINI-REVIEW

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Production of ketocarotenoids by microalgae

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Abstract Among the highly valued ketocarotenoids employed for food coloration, astaxanthin is probably the most important. This carotenoid may be produced biotechnologically by a number of microorganisms, and the most promising seems to be the freshwater flagellate *Haematococcus pluvialis* (Chlorophyceae), which accumulate astaxanthin in their aplanospores. Many physiological aspects of the transition of the flagellate into aplanospores have been described. Mixotrophic cultivation and suitable irradiance may result in fairly good yields (up to 40 mg/l; 43 mg/g cell dry weight) within a reasonable time, under laboratory conditions. In order to compete with synthetic astaxanthin, suitable scaling-up is required. However, large-scale production in open ponds has proved unsatisfactory because of severe contamination problems. A selective medium might overcome this difficulty. Further research for the development of suitable strains is thus warranted.

Introduction

Among the ketocarotenoids found in higher plants, algae, fungi or bacteria, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) are the most important from the biotechnological view point (Fig. 1). In addition to their well-known effect as food colorants (mainly in aquaculture), nutraceutical importance has been ascribed to these carotenoids following nutritional studies indicating various positive actions, such as free-radical scavenging, immunomodulation and cancer prevention. Many studies imply greater antioxidant activity of these ketocarotenoids in comparison with their non-oxygenated analogues (Miki 1991; Krinsky 1993; Jyonouchi et al. 1995).

Dunaliella spp. are the best-known microalgae employed widely for the production of β-carotene, while *Haematococcus pluvialis* (*lacustris*) and the heterobasidious yeast *Phaffia rhodozyma* are the only microbial systems so far offering potential commercial interest for the production of such ketocarotenoids, because of their greater concentration in their respective biomass when cultivated by conventional procedures for a reasonable time. This minireview will concentrate on the more important data available for the use of microalgae as potential ketocarotenoid producers. Ketocarotenoids in yeast have been reviewed extensively by Johnson and An (1991).

General biology

The green microalga, *Haematococcus pluvialis*, Flotov, Volvocales, Chlorophyceae, is a unicellular freshwater biflagellate (zoospore). Under optimal conditions the cells are spherical to ellipsoid and enclosed by a cell wall, which is separated from the protoplast by a region filled with a watery jelly and traversed by cytoplasmic threads (Santos and Mesquita 1984).

The nature of the flagellate cell wall has not been determined but it probably contains no cellulose (Triki et al. 1997). In accordance with environmental conditions, the flagellated cells cease to be motile and gradually transform into cyst cells – the aplanospores, developing a distinct red color by astaxanthin accumulation. This phase is considered the resting stage of the algae. After maturation, the cysts germinate releasing mostly flagellated cells, leaving behind the typical cell wall. Reproduction is usually by cell division throughout the vegetative stage. Enlarged cysts containing many new cells may be observed (Kobayashi et al. 1997b).

The transformation of the vegetative cells into the aplanospore is accompanied by several morphological

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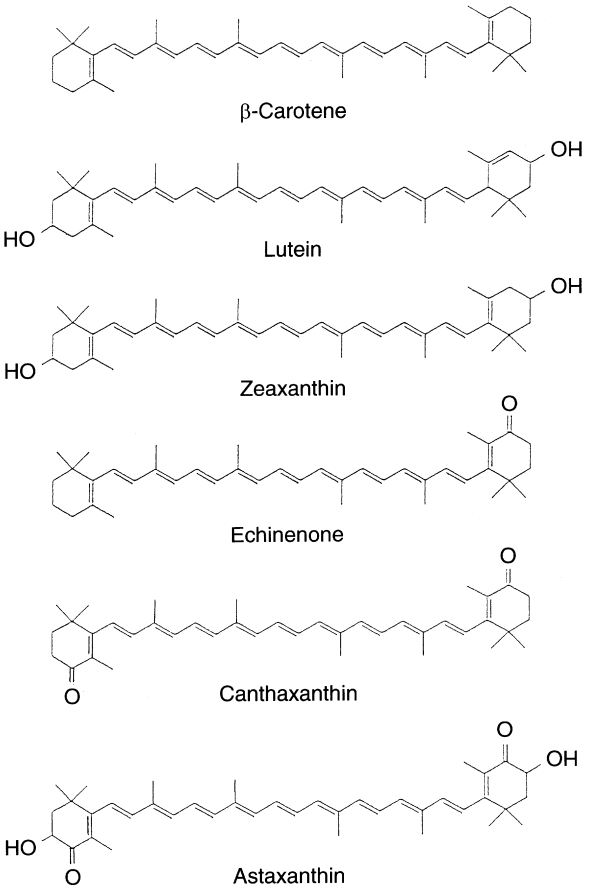

Fig. 1 Structures of the biotechnologically most important carotenoids

features. The cell wall becomes considerably thicker, immobilizing the flagellae which eventually are shed. The volume of the algae increases dramatically, sometimes creating giant red aplanospores with a diameter of over 40–50 μm, i.e. ten times the diameter of the vegetative cell. However, giant aplanospores are usually not predominant, most of the red cysts having much smaller diameters. The duration of the growth cycle of *H. pluvialis* may vary according to nutritional and environmental conditions, but is usually between less than 10 days and several weeks. Some authors report that pigment production is not exclusive to aplanospores but may also be demonstrated in growing flagellates (Lee and Ding 1994; Chaumont and Thepenier 1995; Grünewald et al. 1997).

Physiology

Carotenoids are intracellular products and are usually located in membranes of mitochondria, chloroplasts or endoplasmic reticulum. In *Dunaliella bardawil*, β-carotene is located as plastoglobuli in the matrix of the chloroplast. This is not the case with astaxanthin-producing organisms like *P. rhodozyma* or *H. pluvialis*, where the carotenoids are located in cytoplasmic lipid globules (Lang 1968; Johnson and An 1991). Such extraplastidic carotenoids are also referred to as secondary carotenoids (Grung et al. 1992). While *Phaffia* and *Dunaliella* produce their respective carotenoids during all stages of growth, *H. pluvialis* synthesizes ketocarotenoids, depending on environmental conditions, predominantly during the formation of the resting cell (aplanospore). This is also accompanied by active adaptation of the photosynthetic apparatus and a decline in photosynthetic activity (Hagen et al. 1993; Zlotnik et al. 1993). The accumulation of secondary carotenoids generally starts after the cessation of growth and usually occurs in the perinuclear cytoplasm at the center of the cell, giving the cell a distinct red color. This usually requires light induction (Yong and Lee 1991). Spreading of extrachloroplastic carotenoids takes place in the periphery of the cell during strong irradiation, leading to increased shading of the chloroplast (Hagen et al. 1994). However, Fan et al. (1998) doubt the photoprotective actin of astaxanthin. They suggest that astaxanthin formation is the result of the photoprotective process rather than the productive agent. If illumination is discontinued the pigments move back towards the center of the cell (Yong and Lee 1991).

Cultivation

The most important feature of algae is, of course, their photosynthetic ability, which makes them promising organisms for autotrophic cultivation on simple mineral media for various biotechnological purposes. Since microalgae are very efficient converters of solar energy (Pulz and Scheibenbogen 1998), many attempts have been made to cultivate them in simple systems, such as shallow open ponds. In spite of such attractive features, phototrophic single-species cultivation of microalgae has met only limited success. Severe contamination by bacteria or protozoa has made such propagation possible only if suitable selective environments can be assured. Thus *Dunaliella salina*, a halotolerant alga, is being cultivated commercially in open ponds with highly saline brines. *Spirulina platensis*, as a source of single-cell protein, is successfully cultivated in highly alkaline (pH > 9.2) waters. Fast-growing microalgae e.g. *Chlorella* spp. can be also grown in open ponds (Borowitzka and Borowitzka 1989). Such systems demand comparatively low investment for construction and maintenance and yield a biomass of about 10 g m^{-2} day^{-1}. No selective environment has become available for *H. pluvialis*. Light penetration (which is inversely proportional to cell concentration) is another problem in the phototrophic cultivation of microalgae. Also tubular bioreactors, which overcome some of these problems, have so far not been employed commercially for the cultivation of *H. pluvialis* (Chaumont 1993; Chen 1996).

More recently an attempt has been made to grow *Haematococcus* autotrophically in a 30-l air-lift photobioreactor (Harker et al. 1996). If we only consider algal growth at this stage, the production of a substantial biomass (10^5 cells/ml) requires no less than 3 weeks, with the unavoidable problems of contamination.

Microalgae that utilize organic carbon substrates as their sole carbon and energy source may be employed for heterotrophic growth. Usually very low concentrations of organic compounds are employed to prevent growth inhibition. This may be circumvented by using a suitable fed-batch system. Heterotrophic media may invite rapid bacterial contamination, which again may be overcome only by rigorous aseptic operations.

H. pluvialis can be grown heterotrophically in a variety of media mostly containing low concentrations of acetate and asparagine (e.g. Kobayashi et al. 1991). However, the specific growth rate of the flagellate under heterotrophic (dark) conditions was considerably lower than under autotrophic (light) conditions (0.22 day^{-1} compared to 0.32 day^{-1}). On the other hand, mixotrophic conditions, i.e. media supplied with organic substrates in the presence of light, gave the best results with a specific growth rate of 0.58 day^{-1}. Under the latter conditions a cell concentration of up to 8×10^5/ml could be obtained in less than 7 days of batch cultivation in conical flasks (Kobayashi et al. 1992).

The outcome of the complete cycle for carotenogenic microalgae is greatly dependent on algal concentrations and the time required to reach these levels. Aplanospore formation under conditions that favor carotenogenesis but not algal growth may yield high astaxanthin values per cell but very low yields on a volume basis.

Among the various environmental conditions that affect the rate of carotenoid production, temperature and light are the most critical factors in both autotrophic and mixotrophic systems. Most authors employ temperatures in the range of 20–25 °C for astaxanthin formation (see below). According to Fan et al. (1994), increased temperatures lead to increased volumes of the cultivated cells.

Light may be supplied continuously or with dark periods of 10–14 h. Irradiation levels, as measured at the surface of the culture, vary between 20 and 430 μmol quanta m^{-2} s^{-1}, usually provided from fluorescent light sources. Higher irradiation levels usually yield higher carotenoid concentrations (Fan et al. 1994; Pulz and Scheibenbogen 1998).

The fact that *Haematococcus* has both a vegetative and an aplanospore phase, where most of the astaxanthin is formed, has led many authors to adopt a two-stage approach in the cultivation of *Haematococcus* for carotenoid production. While light is provided at lower fluence rates during the vegetative/green phase in the mixotrophic system, much higher irradiation levels, up to ten times, are supplied during aplanospore formation and astaxanthin biosynthesis (Fan et al. 1994; Grünewald et al. 1997). The same reasoning was applied by Tjahjono et al. (1994a), who obtained much better results by raising the cultivation temperature during carotenogenesis.

Induction of ketocarotenoids biosynthesis–yields

Many publications deal with the problem of induction of astaxanthin biosynthesis. This is of great importance from the biotechnological view point, since artificial induction may cause the duration of the fermentation cycle to be significantly reduced. First, attempts were made to understand the conditions that regulate the normal transformation of flagellates into the colored aplanospores. From earlier work it may be surmised that this transformation is a result of the slowing down of cell division and/or depletion of certain food ingredients (Czygan 1970; Zlotnik et al. 1993).

Exposure of logarithmic-phase cells to growth in nitrogen, or phosphate starvation induced carotenogenesis, although some nitrogen content seems to be required for astaxanthin accumulation. In any case, the slowing down of cell division was considered a prerequisite for cyst formation. Furthermore, the addition of vinblastin, a specific inhibitor of cell division, led to massive astaxanthin accumulation (Boussiba and Vonshak 1991). On the other hand, the production of secondary carotenoids is not necessarily a process associated with the formation of resting cells (Lee and Ding 1994).

Other systems have also been employed for the induction of cyst formation. Stress imposed by sodium chloride (up to 0.8%) was also found to lead to encystment and astaxanthin accumulation (Boussiba and Vonshak 1991). In a recent paper (Kobayashi et al. 1997c) various chlorides (0.1% NaCl, $MgCl_2$) were shown to induce aplanospore formation and carotenogenesis under heterotrophic conditions in the dark (!) although light-independent induction yielded much lower carotene values. However, it should be emphasized that salt induction also leads to a considerable reduction in cell density (Boussiba and Vonshak 1991; Harker et al. 1996).

In order to ensure accelerated carotenoid production, Kobayashi et al. (1993) employ culture supplementation. After 4 days of growth, sodium acetate and ferrous sulfate were added at 45 mM and 450 μM respectively, producing good yields within 5 days.

Yields of astaxanthin may be expressed as pg/cell, mg/l and mg/g dry weight. Authors use different production units, which cannot always be compared. A summary of different production values is given in Table 1. It may be seen that, in most cases, values of 100 pg/cell and less were obtained. In fermentation liquids, usually less than 10 mg/l was obtained after 5–8 days of growth. These data, together with the fact that usually less than 1% algal dry material/l is obtained, must be kept in mind in any feasibility studies for commercial uses.

Obviously, in order to secure high yields in the carotenogenic phase, a high density of vegetative growth is

Table 1 Astaxanthin yields obtained by various authors

Author	Yield			Remarks
	(pg/cell)	(mg/l)	(mg/g dry weight)	
Lee and Soh 1991	–	–	40–43	Chemostat
Yong and Lee 1991	–	7–8	–	
Boussiba and Vonshak 1991	60–100	–	17–19	
Kobayashi et al. 1992	–	7.5	10–15	
Grung et al. 1992	–	–	7	
Kobayashi et al. 1993	40–50	–	–	
Zlotnik et al. 1993	77			30 days
Tjahjono et al. 1994	200–250	–	–	Elevated temperature
Lee and Ding 1995	30.6	3.9	7.3	
Fan et al. 1995	–	–	32	
Chaumont and Thepenier 1995	–	–	13.8	Tubular photobioreactor
Harker et al. 1996	–	27.5–40	–	Photobioreactor, 80 days
Chumpolkulwong et al. 1997	100	–	–	Compactin mutants
Kobayashi et al. 1997c	30	9	–	Light independence
Grünewald et al. 1997	40–70	–	–	In flagellates

required. When the acetate/asparagine-containing medium developed by Kobayashi and coworkers (1991) was employed, cell densities of 10^5/ml and above (1.0–1.5 g/l dry weight) could be obtained within 6–8 days of cultivation.

Chemical composition

The chemical constituents of the carotenoid complex formed during *Haematococcus* growth and encystment have been examined by a number of investigators. In green vegetative cells, β-carotene, lutein, violaxanthin and neoxanthin were found, in contrast to aplanospores where over 90% of the carotenoids were astaxanthin and astaxanthin mono and diesters. The bulk of the carotenoids were, however, astaxanthin esters, confirming earlier results (Grung et al. 1992). It should be borne in mind that astaxanthin esters are less well utilized in aquaculture than are the free carotenoids (Storebakken et al. 1987). *P. rhodozyma* apparently produces unesterified astaxanthin (Andrewes and Starr 1976).

Astaxanthin may appear in various isomeric configurations. *Haematococcus* produces predominantly the 3*S*,3′*S* isomer while *Phaffia* synthesizes 3*R*,3′*R*, the latter apparently being less well absorbed by fish in aquaculture (Bjerkeng et al. 1997).

Biosynthesis

Although the biosynthesis of ketocarotenoids in *Haematococcus* has not been fully elucidated, it is widely assumed that the early part of carotenogenesis, i.e. the formation of phytoene from mevalonate and the desaturation process up to β-carotene, follows a similar pathway to that found in other oxygenic carotenogenic organisms (Armstrong and Hearst 1996). According to Donkin (1976) and Grung et al. (1992) the presence of small amounts of echinenone (one keto group) and canthaxanthin (two keto groups) indicates a pathway from β-carotene to astaxanthin involving the above ketocarotenoids prior to hydroxylation at the 3 and 3′ carbons (Fig. 2). This has been further corroborated by Orset et al. (1995) and Fan et al. (1995), who employed low concentrations of diphenylamine (30 μM) to inhibit canthaxanthin and astaxanthin formation with the accumulation of β-carotene. The biosynthesis of astaxanthin in *Phaffia* is apparently somewhat different, since echinenone is converted to hydroxyechinenone without canthaxanthin. Hydroxyechinenone is transformed to phoenicoxanthin (diketo-monohydroxy-β-carotene) yielding the 3*R*, 3′*R* isomer of astaxanthin (Andrewes et al. 1976). However, in a more recent paper it was claimed that zeaxanthin (β-carotene-3,3′diol) may also have served as an intermediate when a membrane-bound enzyme fraction of *Heamotococcus* was incubated with β-carotene in the presence of NADPH and O_2, yielding astaxanthin with high conversion yields (Chumpolkulwong et al. 1997a).

Enhancement of astaxanthin biosynthesis

Little has been done so far to stimulate astaxanthin production by the addition of specific compounds or precursors of carotenogenesis. Similar attempts at stimulating β-carotene synthesis in the fungus *Blakeslea trispora* yielded some interesting results (Cerda-Olmedo and Hütterman 1986). Abscisic acid, best known for its function as a stress hormone in the adaptation to drought in higher plants, was examined for its effect on the morphogenesis and carotenogenesis in *Haematococcus*. Vegetative cells cultivated on agar plates, but not in liquid culture, with the addition of exogenous abscisic acid 0.1 mM), transform into red aplanospores at a much faster rate than the control, but

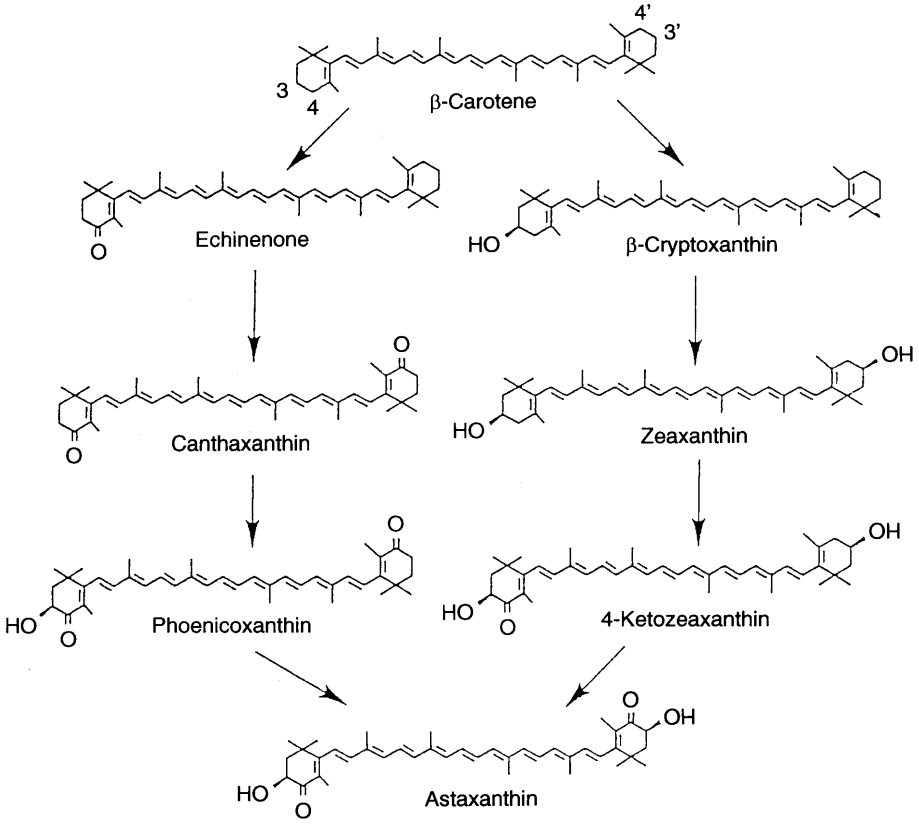

Fig. 2 Suggested routes for astaxanthin biosynthesis. *Left* preferred route in *Haematococcus pluvialis* (adapted from Fraser et al. 1997)

did not affect carotenogenesis when applied to cyst cells (Kobayashi et al. 1997a).

Strain improvement aiming to optimize astaxanthin production on an industrial scale has not received much attention. Gametogenesis and sexual hybridization is a possibility, but looks very difficult to perform. Mutants made resistant to inhibitors to carotenoid biosynthesis (e.g. norflurazon, fluoridone) have been used for hybridization by protoplast fusion, yielding cells with higher ploidy and higher carotenoid content (Tjahjono et al. 1994b). A different approach was employed by the group of Chumpolkulwong et al. (1997b) who showed that mutants of *Haematococcus* made resistant to compactin, a competitive inhibitor of hydroxymethylglutaryl-CoA reductase, a key regulatory enzyme of isoprenoid biosynthesis, accumulated 1.4–2.0 times more astaxanthin than did the wild type.

Molecular biology and genetic manipulation

At this juncture some information on the molecular background of cyst formation and carotenogenesis would be useful. In what way does the aplanospore differ from its green vegetative precursor besides having carotenoid pigments? The well-known decline in photosynthetic activity (Zlotnik et al. 1993) was studied by Tan et al. (1995) who found that, in red cultures of *Haematococcus*, there was a diminished photosynthetic activity because the level of cytochrome *f* was reduced to less than 1% that of the green cell. This would greatly impair the linear electron flow from photosystem PSII to PSI. The decline in cytochrome *f* and other essential components of photosynthesis must somehow be related to the stress imposed by unfavorable environmental conditions that prevent new synthesis and repair of the sensitive components of photosynthesis.

How does the environmental stress impose its effect on these sensitive systems? Studies on carotenogenesis in general, and particularly in *Haematococcus*, seem to indicate that the major environmental stress is related to an oxidative reaction induced by various oxygen radicals. Since photooxidation products formed under illumination, that lead to carotenogenesis in fungi, may be replaced by hydrogen peroxide in the dark, and since Fe^{2+} has been shown to enhance algal carotenogenesis in an acetate medium – the oxidative stress was probably carried out by oxygen radicals. Fe^{2+} is known to function as an ($OH^{\cdot}$) generator (Fenton reaction). Other active oxygen species ($^1O_2$, O_2^-, H_2O_2 and peroxy radicals) have also been shown to enhance carotenogenesis in the absence of Fe^{2+}. Scavengers of hydroxy radicals, e.g. KI, will annul the Fe enhancement of carotenogenesis. Interestingly, transcriptional (actinomycin) or translational (cycloheximide) inhibitors added to the vegetative growth phase prior to carotene induction by $Fe(OAc)_2$ will arrest encystment and prevent enhancement. However, after induction of encystment and

carotenogenesis by acetate, Fe enhancement was no longer blocked by the addition of these inhibitors. Evidently the activation of carotenoid biosynthesis is regulated by post-translational oxidation stress. This would be of special interest in the case of carotenoids that undergo oxygenation and hydroxylation. It can be postulated that active oxygen species may be involved in the structural modification of carotenogenic enzymes, in the regeneration of NAD(P) as an acceptor of hydrogen during dehydrogenation reactions, or by direct oxidation of the carotene molecule (Kobayashi et al. 1993). The activation of carotenoid synthesis as a result of reactive free oxygen radicals may, of course, also indicate that carotenoids have a role in the resistance of algal cells to high photooxidative stress by quenching radical reactions, in addition to the shading of chloroplasts by extrachloroplastidic secondary carotenoids (Hagen et al. 1993).

Studies on the molecular biology of genes and enzymes concerned with astaxanthin biosynthesis have revealed a number of interesting facts. Various carotenoids, including β-carotene and ketocarotenoids, may be found in a number of eubacteria (e.g. *Flavobacterium*, *Brevibacterium*, *Erwinia*) as well as in several archebacteria (the halophilic *Halobacterium*). The *Erwinia uredovora* carotenogenic genes have been introduced and expressed in the colorless *Escherichia coli* as well as in other non-carotenogenic bacteria (Misawa et al. 1990, 1991). More recently Kajiwara and coworkers (1995) and Lotan and Hirschberg (1995) employed this *E. coli* transformant to clone the *H. pluvialis bkt* gene, encoding β-carotene ketolase, thus producing an *E. coli* strain able to express and synthesize canthaxanthin. Furthermore, the *E. uredovora crtZ* gene was shown to encode β-carotene hydroxylase, which catalyzes the formation of zeaxanthin (β-carotene 3,3′-diol). Thus, in the presence of the *bkt* and *crtZ* genes, astaxanthin production could be demonstrated. Although no yields are given, it is quite possible that suitable genetic manipulations may result in improved biosynthetic activities of astaxanthin production (Kajiwara et al. 1995). In any case, this is the first time that information on the genes concerned with the later steps of astaxanthin biosynthesis has become available.

Applications and feasibility

In spite of their potential uses as antioxidants and chemo-preventive agents against carcinogens, current applications of ketocarotenoids are limited to fish farming. Astaxanthin is the predominant carotenoid in wild Atlantic salmon and rainbow trout as well as in various crustacean species. Salmonids preferentially deposit more polar carotenoids, i.e. astaxanthin rather than canthaxanthin or β-carotene, in their flesh (Schiedt et al. 1985). The natural pigmentation of fish is derived from plankton. There appears to be limited metabolism of ketocarotenoids in fish, the pigment being deposited in the free form mainly in the flesh, while the esterified compounds are deposited in the skin. Crustacean species are probably non-selective, absorbing a variety of carotenoids, with and without oxygenation. Salmonid flesh may contain various amounts of astaxanthin, usually 4 mg/kg and above appearing to endow satisfactory color. In order to obtain similar pigmentation, 20–50 mg/kg astaxanthin (FDA limits 80 mg/kg, see FDA 1997) is recommended in aquaculture feeds. The fast fading of canthaxanthin during cooking necessitates higher concentrations (50–100 mg/kg) in the feed (Torrissen et al. 1989).

Since the volume of salmon and trout farming is on the increase, the demand for carotenoid pigmentation will undoubtedly continue to grow. Ketocarotenoids in tonne quantities may be expected to be on the market in the coming years. Synthetic astaxanthin widely used by fish farmers sells at U.S. $ 2000/kg. Such feed supplementation may constitute 10%–20% of the feed cost (Torrissen et al. 1989). Although natural astaxanthin from microbial sources has been shown to be suitable after some pretreatment (US patent, 426002, 1989), competitiveness with synthetic astaxanthin would occur only if suitable scaling up of algal growth and encystment may be demonstrated. The very cheap method of growing *H. pluvialis* in open ponds will in all likelihood not materialize because of contamination problems.

Conclusion

The production of ketocarotenoids that show qualities superior to those of other carotenoids as antioxidants and possibly also as anticancer agents, as well as being suitable for fish pigmentation, has become an important area of research. We have dealt mainly with *H. pluvialis* as an astaxanthin producer. *P. rhodozyma* is also a good source of astaxanthin even though its absolute configuration is somewhat less desirable. The cultivation of *P. rhodozyma* is quite simple but yields obtained by various strains are usually less than 500 μg/g dry weight of cells. Many attempt have been made to employ various mutagenic procedures to produce superstrains. Yields of 4000–5000 μg/g and over have been claimed, but lower cell biomass and instability have not allowed wide commercialization (Johnson and Schroeder 1995).

H. pluvialis has so far gained the upper hand in the competition for the best astaxanthin-producing organism. Current yields of 40–45 mg/g dry weight are still not sufficient to compete with chemical synthesis. However, the great advantage of microbial astaxanthin as a nutriceutical will undoubtedly contribute to its commercial promotion. The mixotrophic mode of growth in closed systems should be the basis for further developments and advanced fermentation technology, possibly by well-regulated alterations between light-independent mass cultivation and light-dependent carotenogenesis in the algal life cycle.

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