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MINI-REVIEW

Biotechnological production of astaxanthin with *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*

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Abstract The oxygenated β-carotene derivative astaxanthin exhibits outstanding colouring, antioxidative and health-promoting properties and is mainly found in the marine environment. To satisfy the growing demand for this ketocarotenoid in the feed, food and cosmetics industries, there are strong efforts to develop economically viable bioprocesses alternative to the current chemical synthesis. However, up to now, natural astaxanthin from *Haematococcus pluvialis*, *Phaffia rhodozyma* or *Paracoccus carotinifaciens* has not been cost competitive with chemically synthesized astaxanthin, thus only serving niche applications. This review illuminates recent advances made in elucidating astaxanthin biosynthesis in *P. rhodozyma.* It intensely focuses on strategies to increase astaxanthin titers in the heterobasidiomycetous yeast by genetic engineering of the astaxanthin pathway, random mutagenesis and optimization of fermentation processes. This review emphasizes the potential of *P. rhodozyma* for the biotechnological production of astaxanthin in comparison to other natural sources such as the microalga *H. pluvialis*, other fungi and transgenic plants and to chemical synthesis.

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Introduction

The carotenoid astaxanthin is a 3,3′-dihydroxylated and 4,4′-diketolated derivative of β-carotene (3,3′-dihydroxy-β,β′-carotene-4,4′-dione). It mainly occurs in the marine environment, where it is ingested by seafoods such as salmon, trout, shrimp and lobster, subsequently leading to the prominent colour of the flesh and shell, respectively (Lorenz and Cysewski 2000). In general, animals do not synthesize carotenoids de novo. Astaxanthin found in marine animals is either a result of the direct accumulation from zooplankton, which on its part ingests phytoplankton or microalgae biosynthesizing the ketocarotenoid de novo (Kitahara 1984; Foss et al. 1987) or the consequence of the metabolic conversion of other carotenoids absorbed (Matsuno 2001). In nature particularly, the microalga *Haematococcus pluvialis* accumulates remarkably high levels of astaxanthin (Guerin et al. 2003). Due to its intense red colour, the pigment of *H. pluvialis* was first mentioned as "haematochrome" until it was later identified as astaxanthin (Tischer 1944; Hazen 1899). Beyond that, the red-pigmented heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous*, also known as *Phaffia rhodozyma*, was also found to abundantly de novo biosynthesize astaxanthin (Andrewes et al. 1976; Golubev 1995). *P. rhodozyma* was named after its discoverer Herman J. Phaff, who isolated this species from trees in the mountain areas of Japan and Alaska (Phaff et al. 1972).

Based upon not just its applications in colouring but also its antioxidative and health-promoting properties

(Guerin et al. 2003), astaxanthin has a significant and growing global commercial market (BCC Research 2008). A large majority of this commercial supply (about 97%) has to date been from synthetic astaxanthin (Frucht and Kanon 2005). Due to technical and marketing reasons, carotenoids produced by biotechnology has not to date been able to develop into serious competition for synthetic carotenes. Consequently, many promising leads in astaxanthin have disappeared with the market development of fermentation-derived carotenoids in general being largely disappointing (BCC Research 2009). For the biotechnological route, *H. pluvialis* and *P. rhodozyma* are the most promising systems for the commercial biotechnological production of astaxanthin since they accumulate the highest levels in nature (Bhosale and Bernstein 2005; Guerin et al. 2003). Astaxanthin derived from the bacterium *Paracoccus carotinifaciens* is an alternative that can also be found in the market (European Food Safety Authority 2010; Nakajima et al. 2008).

The purpose of this paper is to review the state-of-the-art of science and technology in one of these biotechnological alternatives, the use of *P. rhodozyma*, for the commercial supply of astaxanthin. Options and limitations of biotechnological astaxanthin production in other carotenogenic microorganisms such as *Rhodotorula* or *H. pluvialis* have been discussed and compared to *Phaffia* before (Bhosale and Bernstein 2005; Domínguez-Bocanegra et al. 2007; Frengova and Beshkova 2009; Lorenz and Cysewski 2000; Margalith 1999). The potential of genetic modification of the carotenoid pathway to increase astaxanthin yields in fungi and *Phaffia* has been reviewed previously (Sandmann and Misawa 2002; Visser et al. 2003). A recent publication gives a survey on astaxanthin production by *Phaffia* with its emphasis on strain selection, choices of substrates and fermentation conditions for optimized astaxanthin yields (Rodriguez-Saiz et al. 2010). In contrast, a major part of the present review is dedicated to the molecular mechanisms of astaxanthin biosynthesis and on concepts and progress made in astaxanthin pathway engineering. It integrates basic genetic, biochemical and bioengineering insights and proposes future strategies for improved astaxanthin production with *P. rhodozyma*.

Astaxanthin: properties, application, economic role

Like most of the carotenoids, astaxanthin belongs to the group of lipophilic tetraterpenes, which are assembled of eight isoprene units (C_5). The symmetric carbon backbone is derived from the acyclic lycopene, the structural precursor of carotenoids, where 40 carbon atoms are arranged in a chain of conjugated double bonds. Many carotenoids have two terminal β-ionone rings. In the astaxanthin molecule, each ionone ring carries a characteristic 3-hydroxyl (OH) and a 4-keto (C=O) moiety. Due to the two chiral centres at carbon 3 and 3′ in each ionone ring, astaxanthin can exist in three different stereoisomers: (3*S*,3′*S*), (3*R*,3′*R*) (enantiomers) and (3*R*,3′*S*) (meso form) (Fig. 1). The (3*S*,3′*S*) isomer is the most prevalent form in nature, where it is mainly found in *H. pluvialis* and wild salmon (Turujman et al. 1997). Remarkably, *P. rhodozyma* biosynthesizes astaxanthin in its (3*R*,3′*R*) configuration, which is so far the only known natural source for this stereoisomer (Andrewes and Starr 1976). Astaxanthin is formed as different geometrical isomers with *cis* and *trans* double bonds in the polyene chain. In *P. rhodozyma*, mainly the all-*trans* isomer exists, but the 9- and 13-*cis* isomers can be found in substantial amounts as well. These isomeric forms slightly differ in their optical spectrum. Both *cis* isomers show an absorbance maximum at 465 nm and all-*trans* astaxanthin at 470 nm (Visser et al. 2005). Being a xanthophyll, astaxanthin exerts chemical and physiological properties typically attributed to carotenoids. Hence, it is highly lipophilic and shows an intense red colour based on the light absorbance of its polyene system. However, the 3-hydroxyl and 4-keto groups on both terminal ionone rings cause some unique attributes assigned to astaxanthin only, namely, it is more polar than other carotenoids and has a higher antioxidant activity. In general, both 3-hydroxy moieties can be conjugated to proteins or esterified with fatty acids to a mono- or diester in order to increase the solubility and stability of free astaxanthin in the cell (Grung

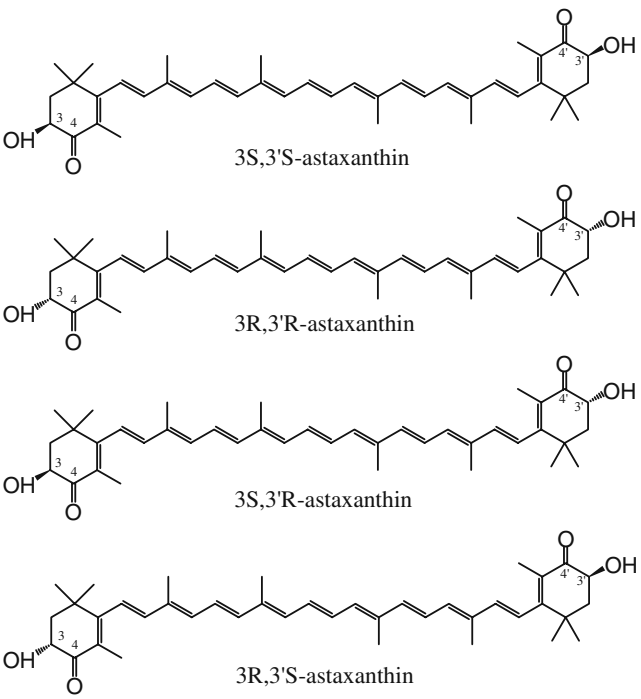

Fig. 1 Configurational isomers of astaxanthin

et al. 1992; Lorenz and Cysewski 2000). However, astaxanthin is present in a free and unmodified form in *P. rhodozyma* (Visser et al. 2005).

From an economic viewpoint, astaxanthin is the second most important carotenoid with a global market size of $219 million in 2007 (29% of total carotenoid sales) and is estimated to reach $253 million by 2015, a compound annual growth rate of 1.8% (BCC Research 2008). By far, the economically most significant area of application for astaxanthin is its use as a feed additive in aquaculture for appropriate pigmentation, growth and reproduction of primarily salmon and trout (Lorenz and Cysewski 2000; Higuera-Ciapara et al. 2006). Since these fishes have no access to their natural prey, they lack astaxanthin and would lose their natural flesh pigmentation if the carotenoid was not replaced in the feed. To satisfy the consumer's desire for a consistent product with familiar colouration, the salmon farming industry invests about 15% of the total production cost in this pigment (Mann et al. 2000). Astaxanthin is also utilized as a feed additive in the diet of chicken and quails to standardize the colour of flesh and egg yolks (Bhosale and Bernstein 2005). More and more health-promoting properties of astaxanthin have been revealed, which open up new possibilities for a potential application as a nutraceutical and as a constitutional ingredient for cosmetics. Several beneficial effects on health are due to astaxanthin's remarkably high antioxidative activity, which has been proven to be up to tenfold stronger than β-carotene and 100-fold higher than α-tocopherol (Miki 1991). As already extensively reviewed, astaxanthin exhibits anti-inflammatory, antibacterial, immunomodulatory, photoprotective, neuroprotective, anticancerogenic and antiatherogenic properties and therefore has a notably high potential for an application for medical purposes (Guerin et al. 2003; Higuera-Ciapara et al. 2006; Hussein et al. 2006). Accounting for only a few percentage points of the total astaxanthin market, the nutraceutical market for astaxanthin is currently very small but likely to show a greater growth rate in the coming years compared to the aquaculture and feed industries.

Chemical synthesis is currently acknowledged as the lowest-cost production process of astaxanthin and will very likely remain the main source of this compound for the predominantly cost-driven animal feed and aquaculture market in the near future. Synthetic astaxanthin has a market price of around $2,000 per kilogram while the natural product is sold for more than $7,000 per kilogram. The considerable price difference, in combination with the different target market dynamics, might be the reason for the about 97% market share of the chemically produced astaxanthin (Frucht and Kanon 2005). For a biotechnological production process of natural astaxanthin to directly compete only on market price with synthetic astaxanthin, significant technological advances are necessary. In the year 1999, it was estimated that a *P. rhodozyma* production strain should be able to produce astaxanthin preferably in excess of 4,000 μg/g of cell dry weight and to reach cell densities of more than 60 gl^{-1} in a large volume above 1,500 l to result in an economically viable commercial-scale fermentation process (Jacobson et al. 1999). With respect to the chemical synthesis as today's benchmark, these data do certainly not justify the operation of an industrial bioprocess anymore, whereas any process delivering natural astaxanthin can have niche market advantages that at least to some extent may counteract a higher sales price.

Sources of astaxanthin: chemical vs. natural

There are several strategies for the chemical synthesis of astaxanthin. One of the well-established procedures involves a Wittig reaction of two equivalents of a C_{15}-phosphonium salt with a central C_{10}-dialdehyde (Widmer et al. 1981). A $C_{10}+C_{20}+C_{10}$ synthesis via dienolether condensation was described by Rüttimann (1999). Astaxanthin produced by the hydroxylation of canthaxanthin was patented by Bernhard et al. (1984) and the isomerization of a lutein extracted from marigold to zeaxanthin and the subsequent oxidation to astaxanthin was described in the patent literature (Schloemer and Davis 2001). Current commercial astaxanthin from synthesis consists of a racemic mixture of (3*S*,3′*S*) (3*RS*,3′*RS*) and (3*R*,3′*R*) isomers. (3*S*,3′*S*)-Astaxanthin could be synthesized by using (4S)-4-hydroxy-3-methoxy-2,6,6-trimethyl-cyclohex-2-enon as building block, which can be obtained by enzymatic conversion from 2-methoxy-3,5,5-trimethylcyclohex-2-en-1,4-dion (Breuer et al. 2008).

Astaxanthin is produced by many microalgae, but the freshwater green alga *H. pluvialis* is the dominant species for commercial astaxanthin production. *H. pluvialis* contains 1.5% to 3% of (3*S*,3′*S*)-astaxanthin by dry weight, mainly as monoesters (Lorenz and Cysewski 2000). The alga is usually grown in a two-stage batch process. The first stage (green stage) is a growth phase with sufficient nutrient supply, controlled pH and temperature and low irradiation. In the second stage, the reddening stage, the cells are stressed by exposure to high irradiation (sun light), nutrient deprivation (mainly nitrogen and phosphate) and high temperature and/or salt concentration. These conditions induce haematocyst formation and astaxanthin production. Several cultivation systems are used ranging from open ponds and tubular or hemispherical photobioreactors to indoor photobioreactors with artificial light. The biomass is harvested by settling and subsequent centrifugation and processed by drying and milling and an optional supercrit-

ical CO_2 or oil extraction. Steinbrenner and Sandmann (2006) developed a genetic transformation system for *H. pluvialis* which enables the metabolic engineering of this organism to improve its astaxanthin synthesis capacity.

A number of bacteria are described to produce astaxanthin, mostly of the genus *Paracoccus* (Harker et al. 1998; Lee and Kim 2006; Tsubokura et al. 1999; Yokoyama et al. 1994). A detailed comparison of many *Paracoccus* species revealed that two of the astaxanthin-producing isolates, *Paracoccus marcusii* (Harker et al. 1998) and *Paracoccus carotinifaciens* (Tsubokura et al. 1999), are indistinguishable (Berry et al. 2003).

Plants are the source of a vast variety of carotenoids. However, astaxanthin is only found in a few species, particularly in the petals of *Adonis annua* (Seybold and Goodwin 1959) and other *Adonis* species (Cunningham and Gantt 2005). Plant breeding of *Adonis palaestina* resulted in increased flower size and enhanced astaxanthin content in dried petals from 7.9 to 17.9 mg/g (Rayton et al. 2006). Other researchers aimed at producing astaxanthin with transgenic plants. *Tagetes*, which is a commercial source of lutein, was engineered to produce astaxanthin by introducing a ketolase gene (Sauer et al. 2008a, 2008b), but the astaxanthin content in the best plant was only 27% of the total carotenoids. Hasunuma et al. (2008) describe the biosynthesis of astaxanthin in the leaves of transgenic tobacco expressing β-carotene ketolase (CrtW) and β-carotene hydroxylase (CrtZ) from *Brevundimonas* sp. strain SD212 in chloroplasts. The astaxanthin content reached more than 5 $mg\,g^{-1}$ dry weight. In transgenic carrots expressing the β-carotene ketolase gene from *H. pluvialis*, up to 70% of the carotenoids were converted to ketocarotenoids, 38% of which was astaxanthin amounting to up to about 90 $\mu g\,g^{-1}$ fresh root (Jayaraj et al. 2008). Astaxanthin production in transgenic potatoes was reported by two groups. Gerjets and Sandmann (2006) transformed the transgenic potato line Baltica 47#18 with the ketolase gene *crtO* from *Synechocystis* but only 0.7–1.8% of the total carotenoids was astaxanthin (max. 0.7 $\mu g\,g^{-1}$ tuber dry weight). Morris et al. (2006) transformed two potato species with the ketolase gene *bkt1* from *H. pluvialis*. The best result was obtained with a transgenic line of *Solanum phureja* cultivar Mayan Gold, which produced about 14 $\mu g\,g^{-1}$ tuber dry weight astaxanthin corresponding to almost 46% of total carotenoids.

On 26 March 2008, Cyanotech Corporation, who at the time claimed to have the largest production capacity of natural astaxanthin in the industry, announced that it would discontinue marketing its *H. pluvialis*-derived natural astaxanthin product NatuRose® for the animal feed market as a result of being unable to effectively compete in this market with synthetic astaxanthin. However, on May 27, 2010, Naturxan, Columbia, MD, USA, a joint venture between Archer Daniels Midland Company and Igene Biotechnology, Inc., announced that it has completed an expansion of its production and distribution capabilities to provide worldwide availability of Aquasta®, a naturally sourced astaxanthin made from *P. rhodozyma* for the aquaculture market (Naturxan 2010). These mixed developments in suppliers to the aquaculture market may indicate that the current biotechnological processes have a higher production cost than the synthetic astaxanthin. It is possible, however, that being able to charge a premium price for a product that can be marketed as natural allows for a profitable business.

In contrast, the emerging market of astaxanthin as a nutraceutical, mainly in the human supplement field, is currently dominated not by chemically synthesized astaxanthin but by product derived from *H. pluvialis.* The main reasons for this may be that *H. pluvialis* produces only (S, S')-astaxanthin, which is preferred for human applications, and that the main producers of synthetic astaxanthin are currently not very active in the nutraceutical market with this product. Currently, both *P. carotinifaciens* and *P. rhodozyma* production of astaxanthin for the nutraceutical market is small but may eventually be able to compete with *H. pluvialis*.

Astaxanthin biosynthesis in *P. rhodozyma*

P. rhodozyma was first isolated from its natural habitat, the wounds of birch trees in colder regions, from Phaff in the late 1960s. Later, it was taxonomically classified as the new genus *Phaffia* represented by a single species, *P. rhodozyma* (Phaff et al. 1972; Miller et al. 1976). Since Golubev (1995) discovered the sexual stage of *P. rhodozyma* in 1995, the sexual form was designated *X. dendrorhous* and the asexual state *P. rhodozyma* henceforth. He assessed that *P. rhodozyma* fulfils several criteria, which allow an allocation to the phylum of basidiomycota: its cell wall ultrastructure, the mode of bud formation, the presence of urease activity, the Q-10 ubiquinone system, the ability to synthesize starch-like compounds and pigments. The main pigments are xanthophylls, where astaxanthin represents the majority with 83–87% of total carotenoids, followed by phoenicoxanthin (5–7%), 3-hydroxyechinenone (3–4%), echinenone (2–4%) and β-carotene (2–2.5%) (Andrewes et al. 1976). They serve as antioxidants and quench reactive oxygen species to protect *P. rhodozyma* from damage caused by oxidative stress (Schroeder and Johnson 1993, 1995a).

The carotenoid pathway of *P. rhodozyma* is well established. All genes involved in phytoene synthesis, phytoene desaturation, cyclization and formation of 3-hydroxy and 4-keto groups have been cloned. Only three genes cover the whole specific carotenoid pathway from

phytoene synthesis to the end products. In addition, most genes of the mevalonate pathway and the following reactions which are responsible for the synthesis of prenyl pyrophospates including geranylgeranyl pyrophosphate synthase are also available (Hoshino et al. 1999). The major end product of carotenogenesis in *P. rhodozyma* is astaxanthin. However, a second product is 3-HO-4-keto-torulene (HKT) (Vázquez and Santos 1998), which is formed instead of astaxanthin as a by-product.

In *P. rhodozyma*, carotenoids arise via the mevalonate pathway, where three acetyl–CoA molecules are condensed forming mevalonate, which is then transformed to isopentenyl pyrophosphate (IPP, C_5), the general precursor of all isoprenoids (Fig. 2). By condensation with further IPP molecules, geranylgeranyl pyrophosphate (GGPP, C_{20}) is generated. Two GGPP molecules are condensated to yield the first carotenoid, which is the colourless phytoene. Subsequent dehydrogenation steps and one cyclization step at either end lead to β-carotene formation, from which astaxanthin derives (Andrewes et al. 1976). The conversion of GGPP to β-carotene is initiated by the product of the fusion gene *crtYB* (Verdoes et al. 1999a). Functional heterologous complementation demonstrated that it encodes for a bifunctional protein involved in both the synthesis of phytoene from GGPP and the cyclization of lycopene to β-carotene. Subsequently, phytoene is stepwise dehydrogenated by the insertion of double bonds into its polyene system. These desaturation reactions are catalyzed by the *crtI* gene product, a single enzyme, which was functionally identified by heterologous complementation in *Escherichia coli*, exhibiting a phytoene-accumulating background (Verdoes et al. 1999b). By this means, the enzyme catalyzes both the formation of lycopene, the precursor of β-carotene and the synthesis of 3,4-dehydrolycopene (G. Sandmann, unpublished results), which is subsequently cyclized to torulene, the precursor of HKT.

Typically, carotenogenic reactions are catalyzed by di-iron hydroxylases and ketolases in photosynthetic bacteria (Martín et al. 2008). According to these bacterial systems, it was initially hypothesized that the enzymatic conversion of

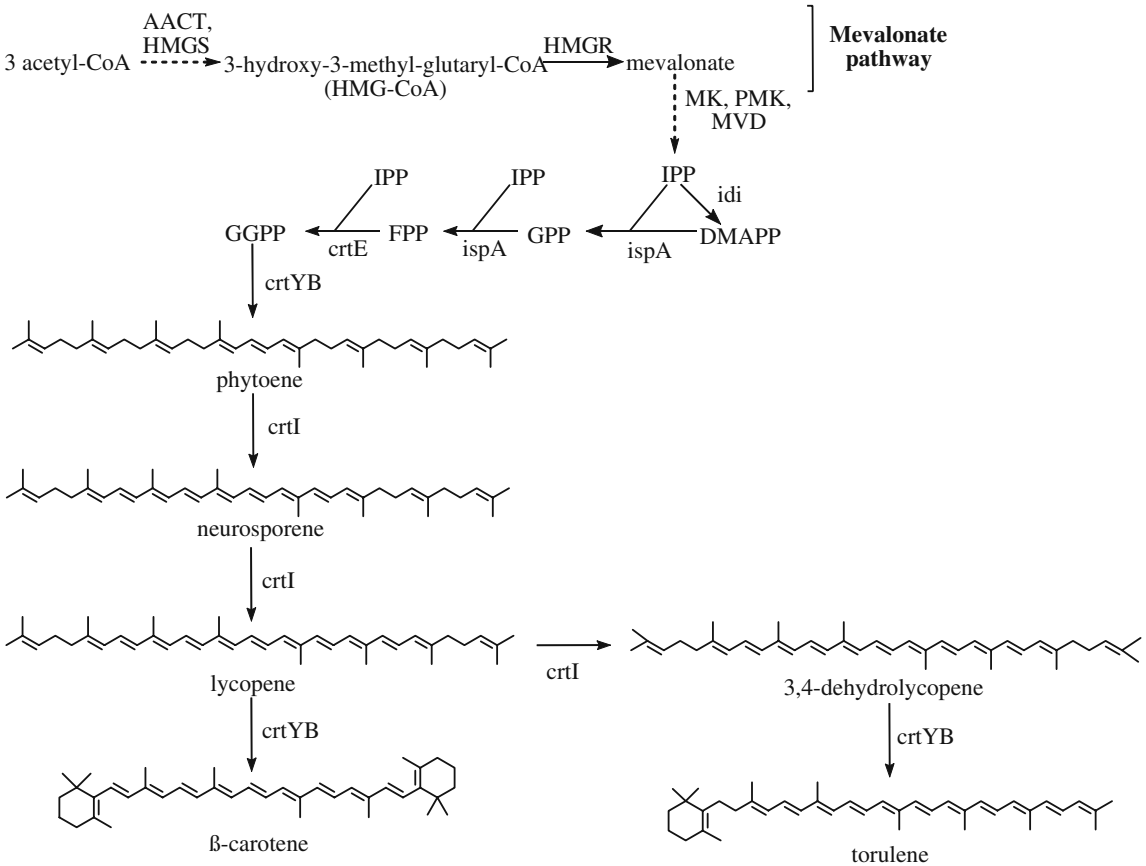

Fig. 2 Biosynthetic pathway of β-carotene in *P. rhodozyma* (Andrewes et al. 1976). Acetoacetyl–CoA thiolase (*AACT*), HMG–CoA synthase (*HMGS*), HMG–CoA reductase (*HMGR*), mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), mevalonate pyrophosphate decarboxylase (*MVD*), IPP isomerase (*idi*), prenyltransferase (*ispA*), FPP synthase (*ispA*), GGPP synthase (*crtE*), phytoene synthase (*crtYB*), phytoene desaturase (*crtI*), neurosporene desaturase (*crtI*), lycopene cyclase (*crtYB*), isopentenyl pyrophosphate (*IPP*), dimethylallyl pyrophosphate (*DMAPP*), geranyl pyrophosphate (*GPP*), farnesyl pyrophosphate (*FPP*), geranylgeranyl pyrophosphate (*GGPP*)

β-carotene to astaxanthin is carried out by a hydroxylase (crtZ) and a ketolase (crtW), but the genes encoding for these putative enzymes have never been isolated and identified in *P. rhodozyma*. However, astaxanthin formation in *P. rhodozyma* was shown to be different from that in other microorganisms since treatment with the cytochrome P450 monooxygenase inhibitor aminobenzotriazole resulted in β-carotene accumulation and astaxanthin stagnation (Ojima et al. 2006).

The gene of a cytochrome P450 monooxygenase of the 3A subfamily was cloned from β-carotene-accumulating *P. rhodozyma* mutants (Álvarez et al. 2006; Hoshino et al. 2000; Ojima et al. 2006). Its assignment as an astaxanthin synthase gene (*asy*) is based on the following results (Ojima et al. 2006): transformation of *asy* in PR-1-104, a β-carotene-accumulating *P. rhodozyma* mutant strain (Girard et al. 1994), successfully restored astaxanthin synthesis indicating that Asy is involved in astaxanthin biosynthesis. The possibility that *asy* encodes a specific β-carotene ketolase was ruled out by the transformation of PR-1-104 with a foreign ketolase gene since only canthaxanthin and no astaxanthin was formed. Furthermore, Asy is not a specific hydroxylase since the transformation of PR-1-104 with a foreign β-carotene hydroxylase yielded zeaxanthin instead of astaxanthin. These two foreign enzymes did not complement a putative hydroxylase or ketolase in PR-1-104 assumed to cooperate with Asy. A coherent conclusion from these finding is that Asy catalyzes the insertion of a 3-hydroxyl as well as a 4-keto group in both ionone rings of β-carotene. This multistep conversion of β-carotene to astaxanthin by this single enzyme is shown in Fig. 3. Since torulene resembles β-carotene at one end of the molecule, it can be assumed that it is converted to HKT by Asy as well.

Since cytochrome P450 enzymes usually require an electron-donating system (van den Brink et al. 1998), research focused on the identification of a corresponding system for Asy. Alcaíno et al. (2008) isolated a cytochrome P450 reductase (CrtR) from *P. rhodozyma* and circumstantiated its essential role in astaxanthin biosynthesis, supporting Asy which cooperates very poorly with P450 reductases of other origins (Ukibe et al. 2009). It should be pointed out that the designation CrtR is already assigned to a cyanobacterial β-carotene hydroxylase based on the *crt* nomenclature of bacterial carotenoid biosynthesis genes (Masamoto et al. 1998). Hence, we recommend designating this reductase Asr (astaxanthin synthase reductase) in

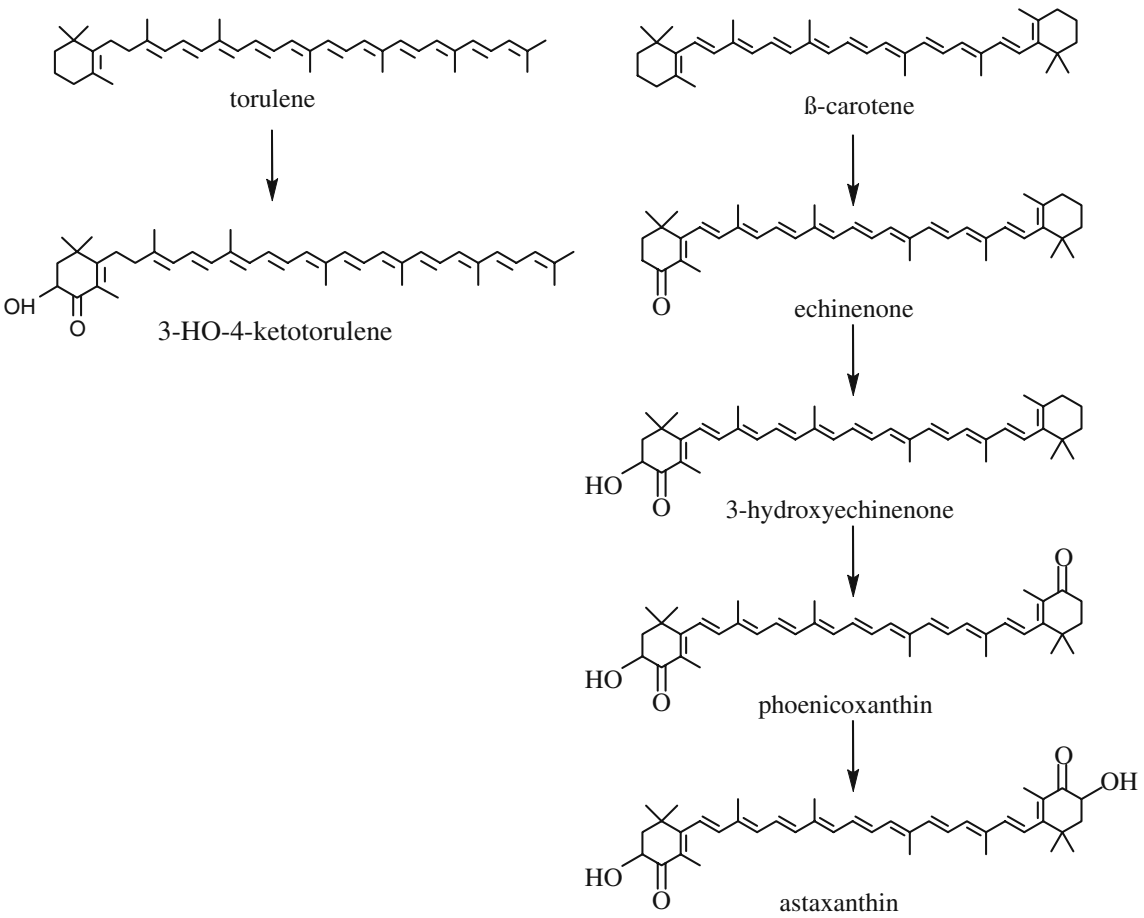

Fig. 3 Pathways for the conversion of β-carotene to astaxanthin by Asy in *P. rhodozyma* (Andrewes et al. 1976; Ojima et al. 2006)

accordance to the three-letter abbreviations of carotenogenic genes from eukaryotes. In analogy, the astaxanthin synthase gene should be assigned as *asy* (Ojima et al. 2006) and not as *crtS* (Álvarez et al. 2006) since *crtS* has already been used for another gene (Kato et al. 1995).

Attempts for the functional heterologous expression of *asy* turned out to be difficult. Due to incomplete reaction steps, intermediates of the Asy-catalyzed reaction may accumulate. After cloning *asy* in *E. coli*, only canthaxanthin was synthesized (Ojima et al. 2006), and in *Mucor circinelloides* β-cryptoxanthin and zeaxanthin were detectable (Álvarez et al. 2006). The reason might be the dependence of Asy on its specific reductase since the co-expression of *asy* and *asr* in non-carotenogenic *Saccharomyces cerevisiae* with an engineered β-carotene pathway successfully established astaxanthin synthesis, although at a low level (Ukibe et al. 2009). This experiment is a definite direct proof that Asy, in combination with its reductase, is a β-carotene-converting astaxanthin synthase catalyzing a ketolation and a hydroxylation step at each ionone ring. A model for its catalytic substrate recognition has been presented (Ojima et al. 2006). Results obtained by the heterologous expression of *asy* without its native reductase should therefore not be over-interpreted, e.g. in terms that Asy may exhibit hydroxylase activity only (Martín et al. 2008). However, to show its full activity, Asy might be dependent on further cellular components beyond its reductase, e.g. an additional electron carrier protein which cannot be found adequately in heterologous hosts. A definite certainty about the underlying reaction mechanism of Asy might be gained by systematic in vitro studies with functionally expressed and purified Asy and Asr.

Cultivation media and bioprocess engineering

P. rhodozyma has a wide biochemical ability for the assimilation and metabolization of mono-, di- and polysaccharides, organic acids and alcohols. It also shows the prompt utilization of simple nitrogen sources such as ammonium, nitrate, urea or amino acids as well as complex mixtures such as yeast, beef or malt extract, peptone, soytone or tryptone (Fang and Cheng 1993; Meyer et al. 1993; Palágyi et al. 2001; Sun et al. 2004; Yamane et al. 1997a). Hence, a useful measure to lower production costs is the use of reproducible industrial waste streams like those from sugar manufacturing processes or the corn wet milling industry. On the other hand, low-cost media may contain unknown inhibitors of carotenogenesis, rendering them unsuitable for a production process (Okagbue and Lewis 1984). In most cases, the medium has to be supplemented with essential nutritional components and may also include inducers or precursors of carotenogenesis (Calo et al. 1995). Examples of raw materials used as C and N sources are given in Table 1. Certain compounds or effects known to cause stress, such as trisporic acids (de la Fuente et al. 2005), plant extracts and monoterpenes (Meyer et al. 1994; Kim et al. 2007), fungal extracts (Echavarri-Erasun and Johnson 2004; Wang et al. 2006), copper limitation (Flores-Cotera and Sánchez 2001) or low light intensities (de la Fuente et al. 2005, 2010; Jacobson et al. 1999; Meyer et al. 1993; Vázquez 2001), have been described to have an advantageous impact on astaxanthin production (see "Elucidation and metabolic engineering of astaxanthin biosynthetic pathways in *P. rhodozyma* and use of other fungi").

Both nutritional (carbon or nitrogen sources, ions, vitamins) and physical (temperature, pH, oxygen supply, light) factors influence cell growth and carotenogenesis. The heterogeneity of medium compositions and process yields mentioned in the literature (cf. Table 1) may be explained by the diversity of natural isolates of *P. rhodozyma* or astaxanthin-hyperproducing mutant strains used. These strains contain randomly inserted mutations whose exact nature, number or locations remain unknown in most cases, which thus hinders comparability. This diversity may be the reason for controversial reports whether carotenoid production in *Phaffia* is growth-associated (Acheampong and Martin 1995; Johnson and Lewis 1979; Kim et al. 2003; Meyer and du Preez 1994b; Reynders et al. 1996) or continues after cessation of cell growth (de la Fuente et al. 2005; Hu et al. 2005, 2007; Jacobson et al. 1999; Johnson and Schroeder 1996; Kusdiyantini et al. 1998; Liu and Wu 2007a; Parajo et al. 1998; Zheng et al. 2006). The complex interaction of nutritional and physical factors in a multiparameter space seems to be different for every (mutant) strain, emphasizing the need for statistical methods such as response surface methodology (Florêncio et al. 1998; Kesava et al. 1998; Parajo et al. 1998; Ramírez et al. 2001; Vázquez and Martin 1998), statistical experiment design (Liu and Wu 2007a; Kim et al. 2005; Ni et al. 2007), genetic algorithm (de la Fuente et al. 2005; Peng and Li 2008) or the use of continuous fermentations (Meyer and du Preez 1994b; Acheampong and Martin 1995) for the optimization of process parameters. However, a few general statements about the influence of nutritional and physical factors on cell growth and astaxanthin production can be made.

P. rhodozyma is a moderately psychrophilic yeast with a temperature range of growth from 0 to 27 °C (Miller et al. 1976). Depending on the (mutant) strain used, the optimal temperature for maximal astaxanthin production and cell growth usually lies between 18 and 22 °C. Optimal pH values for astaxanthin production and cell growth usually range between 5 and 6. The optimal temperature or pH for cell growth and astaxanthin production does not necessarily have to be the same (Fang and Cheng 1993). Hence,

Table 1 Summary of bioprocesses using carotenoid-hyperproducing mutants of *P. rhodozyma*

					Astaxanthin yields					
Strain	Carbon source	Nitrogen source	Feed	Feeding method	Cell mass (gl^{-1}) (total process time (h))	Fermentation volume (l)	$Y_{P/X}$ ($\mu g g^{-1}$)	c_{Axn} ($mg l^{-1}$)	STY ($\mu g l^{-1} h^{-1}$)	Reference
ATCC 24228[a]	Enzymatic eucalyptus wood hydrolyzates	KNO_3		Batch	10 (74)	–	160[b]	1.6	22[b]	(Cruz and Parajo 1998)
NRRL Y-17268[a]	Hemicellulosic hydrolyzates of *Eucalyptus globulus*	KNO_3 Peptone		Batch	23.2 (162)	1	448[b]	10.4	79	(Parajo et al. 1998)
7B12	Glucose	$(NH_4)_2SO_4$		Batch	7.71 (54)	3.5	1,000	7.71	143[b]	(Ni et al. 2007)
ZJUT46	Glucose	$(NH_4)_2SO_4$ YE KNO_3		Batch (pH shift)	15.7 (132)	20	1,740	27.05	200	(Hu et al. 2006)
PR 190	Glycerol	YE Peptone		Batch	24.9 (168)	1.5	1,800	33.7	200	(Kusdiyantini et al. 1998)
2A2N	Molasses	Urea		Batch	36 (200)	100	1,100[c]	40[c]	200[b,c]	(An et al. 2001)
Mutant of AS 2.1557	Glucose	YE		Batch	17.25[b] (64)	10	830[b]	14.32	224[b]	(Wang and Yu 2009)
N9	White grape juice	Peptone ME YE		Batch	27.24 (120)	1	1,060	29	242[b]	(Meyer and du Preez 1994a)
ZJUT003	Glucose	$(NH_4)_2SO_4$ YE KNO_3		Batch	21.33 (156) 20.58 (156)	35 10,000	2,770 2,570	58.77 52.32	380 330	(Zheng et al. 2006)
E5042	Glucose	$(NH_4)_2SO_4$ YE KNO_3		Batch	30.7 (136)	35	2,510	77.1[b]	567[b]	(Liu et al. 2008)
ZJUT46	Glucose	$(NH_4)_2SO_4$ YE KNO_3	Glucose	Batch Pulse-feeding	15.8 (132) 16.34 (132)	20 20	1,390 2,090	21.89 33.91	166 257[b]	(Hu et al. 2005)
ZJUT46	Glucose	$(NH_4)_2SO_4$ YE KNO_3	Glucose Glucose	Batch Pulse-feeding Pulse-feeding (pH shift)	16.7 (132) 17.7 (132) 17.42 (132)	1,500 1,500 1,500	– 2,046[b] 2,266[b]	32.48[b] 36.21 39.47	248[b] 270 300	(Hu et al. 2007)
HT-5FO1C	Glucose	YE Urea	Urea YE Glucose	Pulse-feeding	40 (86)	2	1,100[c]	44[b,c]	511[b,c]	(An et al. 1996)
VKPM Y2976	Glucose	YE CSF PM CSS	Glucose Ethanol Duroquinone	Exponential	84 (212) 90 (184)	10 600	5,061[b] 4,444[b]	425 400	2,005[b] 2,174[b]	(de la Fuente et al. 2005)
2A2N	CSH	CSL	CSH CSL Urea KH_2PO_4	Constant	32 (96)	3	1,638[b,c]	52.4[c]	546[b,c]	(Kesava et al. 1998)

P-5-6	Glucose	$(NH_4)_2SO_4$ CSL YE HY	Glucose NH_4OH KH_2PO_4	pH-stat	15 (96)	2.5	350[c]	5.25[b,c]	55[b,c]	(Chan and Ho 1999)
ATCC 24202[a]	Glucose	$(NH_4)_2SO_4$ YE	Glucose Ethanol	pH-stat DO-stat	30 (140)	1.8	720	20.6	147[b]	(Yamane et al. 1997a)
N9	White grape juice	Peptone ME YE	Acetic acid	pH-stat	8.43 (120)	1	1,430	18	150[b]	(Meyer and du Preez 1993)
25-2	Yuca medium		Date juice YE Urea	pH-stat	39 (96)	1.2–1.6	611[b]	23.81	248	(Ramírez et al. 2006)
ENM5	Glucose	$(NH_4)_2SO_4$ YE CSL	Glucose CSL	Exponential (Monod)	29.2 (120)	1	918[b]	26.8	223	(Liu and Wu 2008)
J4-3	Glucose	$(NH_4)_2SO_4$	Glucose KH_2PO_4 $MgSO_4$	Exponential	70 (90)	12	720	40	560	(Cannizzaro et al. 2003)
UBV-AX3 UBV-AX2	Glucose Dextrose corn	$(NH_4)_2SO_4$ YE	Glucose	Exponential	86 (115) 109 (136)	14 1,500	6,660 5,321	573[b] 580[b]	4,983[b] 4,265[b]	(Jacobson et al. 1999)
UBV-AX1	Syrup				78 (157)	40,000	5,060	395[b]	2,516[b]	
P-5-6	Glucose	$(NH_4)_2SO_4$ CSL	Glucose	Constant Exponential	16.2 (96) 14.7 (92)	2.5	356[c] 412[c]	5.80[c] 6.04[c]	60[b,c] 66[b,c]	(Ho et al. 1999)
		YE		DO-stat	17.4 (92)		307[c]	5.33[c]	58[b,c]	
		Soy Peptone		pH-stat	16.3 (100)		341[c]	5.56[c]	56[b,c]	
ATCC 24202[a]	PH	YE	PH YE	Batch Continuous	4 (200) 4.6 (200)	1.5	1,090 700	4.4[b] 3.2[b]	22[b] 16[b]	(Acheampong and Martin 1995)
ATCC 24202[a]	SCJ	Urea	SCJ	Pulse-feeding Continuous	18.75 (96) 19.35 (96)	1.3	303.34 383.73	5.69 7.44	59 78	(Moriel et al. 2005)
ATCC 24202[a]	PH	YE Peptone	PH YE Peptone	Continuous	4.95 (44)	1.3	544	2.69[b]	61	(Vázquez and Martin 1998)
Aj-6-1	Glucose	YE	Glucose YE Ethanol	–	37.15 (97)	1.4	1,228[b]	45.62	470	(Kim et al. 2003)
VKPM Y2476	Glucose		Glucose	–	88 (216) 86 (228)	10 800	4,773[b] 4,070[b]	420 350	1,944[b] 1,535[b]	(de la Fuente et al. 2010)

c_{Axn} astaxanthin concentration per litre of fermentation volume, *CSF* cottonseed flour, *CSH* corn starch hydrolyzate, *CSL* corn steep liquor, *CSS* corn steep solid, *HY* hydrolyzed yeast, *ME* malt extract, *PH* peat hydrolyzate, *PM* pharmamedia (flour made from the embryo of cottonseed), *SCJ* sugarcane juice, *STY* space time yield, *YE* yeast extract, $Y_{P/X}$ astaxanthin yield per gram of cell dry weight

[a] Wild-type strain

[b] Values calculated from given data

[c] Data refers to total carotenoid content

shifting the pH (An et al. 2001; Hu et al. 2006, 2007) and/or temperature (de la Fuente et al. 2005) in the course of the process can be a powerful and simple tool to enhance astaxanthin yield during the stationary phase of growth. Both cultivation temperature and medium pH have a strong influence on intracellular astaxanthin content, carotenoid composition and/or cell growth kinetics, regardless if mutant or wild-type strains are used (Johnson and Lewis 1979; Liu et al. 2008; Ramírez et al. 2001).

Oxygen plays a pivotal role in astaxanthin biosynthesis. Experimental data such as the finding that concentration of accumulated astaxanthin correlated with oxygen transfer rate (Liu et al. 2006; Wang and Yu 2009; Liu and Wu 2006) or reports about inadequate oxygen supply leading to an accumulation of β-carotene hence indicating decreased efficiency of β-carotene oxidation (Johnson and Lewis 1979; Ramírez et al. 2006; Wang and Yu 2009) underline the necessity of an adequate oxygen supply. A critical dissolved oxygen concentration was found at 10–20% air saturation. Dissolved oxygen concentrations below these values lead to limitations in cell growth and carotenoid formation (Wang and Yu 2009; Liu et al. 2006). Since astaxanthin production is enhanced by respiration (sufficient oxygen supply) and repressed by fermentation (inadequate oxygen concentration and ethanol production) (Meyer and du Preez 1994a; Ramírez et al. 2006; Yamane et al. 1997b) and growth is inhibited by oxygen oversupply (An et al. 2001), an assessment of individual oxygen consumption and transfer rates of a respective strain and equipment used (Liu et al. 2006; Wang and Yu 2009) seems reasonable. In addition to changes of agitation and air flow rate, the oxygen transfer towards the cells can be improved by adding biocompatible organic solvents with high oxygen solubility as oxygen vectors to the medium (Liu and Wu 2006).

Batch fermentation processes seem inappropriate for astaxanthin production with *P. rhodozyma* since a high initial glucose concentration leads to metabolic pathway overflow and accumulation of ethanol, acetic acid or other metabolic products in the medium (Reynders et al. 1997; Liu and Wu 2008). Both ethanol and acetic acid exhibit positive effects on intracellular astaxanthin concentration (Gu et al. 1997; Yamane et al. 1997a; Meyer and du Preez 1993; Kim et al. 2003) but also result in inhibition of cell growth (Johnson and An 1991; Liu and Wu 2007b, 2008; Liu et al. 2008). Despite the similarities of the central carbon metabolism of *P. rhodozyma* to those of filamentous fungi rather than to those of yeast as indicated by metabolic flux analysis (Cannizzaro et al. 2004; Dong et al. 2006), the Crabtree effect known from *S. cerevisiae* applies to *P. rhodozyma* as well (Reynders et al. 1996, 1997). While being undesirable during exponential cell growth, high carbon source concentrations support carotenoid accumulation in the later phases of the process (Johnson and An 1991; Meyer and du Preez 1994a). This antagonism can be remedied by the application of a fed-batch process in order to obtain maximal productivity through both high cell dry weight and high intracellular astaxanthin concentrations. Employing an appropriate process control, a typical state-of-the-art industrial *Phaffia* fermentation process is divided into two phases: a cell growth phase and a maturation phase. By providing a low C/N ratio (ratio of carbon source to nitrogen source concentration), the cells initially show rapid growth, which gradually slows down as they approach high cell concentrations. During this phase, astaxanthin formation is slower than the growth rate of the cells. By switching to a high C/N ratio as the cells approach the stationary phase of growth, astaxanthin formation ultimately exceeds the cell growth rate (de la Fuente et al. 2005; Hoshino et al. 2008; Jacobson et al. 1999). Informative studies discussing the biochemical reasons behind this phenomenon have been published (Flores-Cotera et al. 2001; Yamane et al. 1997b).

The feed control used to reach maximal process productivity depends on the (mutant) strain and medium used and can be established by several means like an exponential feed based on Monod models, pH-stat control, DO-stat control or pulse-feeding after the determination of carbon source concentration (cf. Table 1). As an alternative to the fed-batch process, semi-continuous (Liu and Wu 2007b) and continuous processes can be considered. Assessing the data presented in Table 1, pulse-feeding and exponential feeds seem to be the feeding methods creating the highest space–time yields. An additional tool to increase astaxanthin productivity during maturation phase by the means of carbon source feed control is the addition of a slowly metabolized carbon source such as glycerol (de la Fuente et al. 2005; Jacobson et al. 1999), ethanol (Yamane et al. 1997a; Gu et al. 1997) or acetic acid (Kim et al. 2003; Meyer and du Preez 1993) after the depletion of the initially metabolized carbon source.

The highest space–time yield reported so far of almost 5 $mg\,l^{-1}\,h^{-1}$ and $Y_{P/X}$ of over 6.6 $mg\,g^{-1}$ cell dry weight we reached in a 14-l reaction volume (Jacobson et al. 1999). In situ product removal could further enhance astaxanthin productivity since a feedback control mechanism by astaxanthin (Schroeder and Johnson 1995a) or β-carotene (Ducrey Santopietro and Kula 1998) on carotenoid biosynthesis was assumed. However, the rigid cell wall structure of *P. rhodozyma* (Miller et al. 1976) and the lipophilic nature and size of carotenoids would pose an obstacle to such a process feature.

Classical strain development

The high cost of production is the still the limiting factor for the use of *P. rhodozyma* for natural astaxanthin production (Ramírez et al. 2001). Typically, wild-type *P. rhodozyma* synthesizes astaxanthin in concentrations of

about 200 to 400 $\mu g g^{-1}$ dry weight (Calo et al. 1995; Girard et al. 1994). These amounts are below the level which merits industrial production. Hence, the astaxanthin concentration of natural isolates is one of the measures that have to be improved to develop an industrial process with higher cost efficiency (Johnson and An 1991). As a straightforward solution, random mutagenesis by chemical or physical treatment can be applied to effectively create astaxanthin-hyperproducing mutants. Therefore, several attempts have been made to mutagenize *P. rhodozyma* and select for dark-red pigmented cells (An et al. 1989; Fang and Cheng 1993; Meyer et al. 1993). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was proven to be an effective chemical mutagen creating stable astaxanthin-hyperproducing mutants of *P. rhodozyma* (An et al. 1989) with considerable improvements of astaxanthin content (Meyer et al. 1993), while UV light did not seem to be appropriate to create stable mutants (An et al. 1989). However, low-energy ion beam implantation (Liu et al. 2008) or low-dose gamma irradiation (Sun et al. 2004) was described as an alternative physical method for the generation of stable mutants with increased astaxanthin concentration. The altered colouration of colonies can be used as a screening method for astaxanthin-hyperproducing mutants. For a more accurate measurement of increased intracellular astaxanthin concentrations, advanced technical methods like flow cytometry facilitate screening (An et al. 1991). Other methods for mutant creation combined with facilitated visual selection involve the use of respiration inhibitors (An et al. 1989; Gu et al. 1997), inhibitors of steroid (Ducrey Santopietro and Kula 1998) or carotenoid biosynthesis (Chumpolkulwong et al. 1997; Lewis et al. 1990; Liu et al. 2008; Meyer et al. 1993) or compounds that induce the formation of oxygen radicals (Schroeder and Johnson 1995a). Finally, astaxanthin concentrations of more than 1,000 $\mu g g^{-1}$ dry weight have been obtained. However, a drawback often encountered with random mutagenesis is the decrease in biomass yields, growth rates and process productivity (An et al. 1989; Jacobson et al. 1999; Johnson and An 1991; Meyer et al. 1993).

An alternative approach to mutagenesis is the construction of carotenoid-hyperproducing hybrids by protoplast fusion. This was followed by Chun et al. (1992) and resulted in 1.6 mg total carotenoid per gram of dry weight. However, any information on astaxanthin content was missing.

Elucidation and metabolic engineering of astaxanthin biosynthetic pathways in *P. rhodozyma* and use of other fungi

The elucidation of the carotenoid biosynthetic pathways in *P. rhodozyma* and the development of genetic tools for this yeast render targeted metabolic engineering a promising method to augment astaxanthin yields. In general, genetic modification may increase the flow of precursors into a specific pathway, mediate an efficient conversion of intermediates into the end product and divert a pathway at a branch point into the direction of the desired product. According to this, an improved astaxanthin yield in *P. rhodozyma* was obtained upon feeding precursors of carotenogenesis such as mevalonate (Calo et al. 1995), glutamate (de la Fuente et al. 2005) or citrate (Flores-Cotera et al. 2001) as a result of increased metabolic fluxes to carotenoid-producing pathways. This result confirms that the precursor supply for carotenogenesis is limited. On the genetic level, the expression of required enzymes can be modified by directed pathway engineering to overcome crucial bottlenecks in astaxanthin synthesis as outlined in Fig. 4. In other fungi with a manipulated carotenoid pathway, such as *Candida utilis* (Shimada et al. 1998) and *S. cerevisiae* (Verwaal et al. 2007), the limitation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase and GGPP synthase has been overcome by the over-expression of both genes. Typically, the key enzymes of a pathway are controlling the entire metabolite influx. Phytoene synthase might be such a key enzyme for carotenoid synthesis, regulating the carbon influx, and thereby controls overall carotenoid synthesis as shown for many organisms including the fungus *Neurospora crassa* (Schmidhauser et al. 1994). In *P. rhodozyma*, phytoene desaturase converts phytoene in several steps not only to lycopene (Verdoes et al. 1999b) but by introducing an additional double bond to 3,4-didehydrolycopene as well, which can be further converted by cyclization to HKT (Vázquez and Santos 1998). The branch point for the alternative synthesis of HKT and astaxanthin is lycopene. As shown by inhibitor studies (G. Sandmann, unpublished results), the lycopene cyclase and the desaturase compete

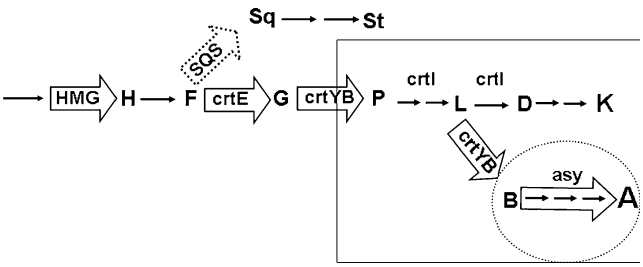

Fig. 4 Limiting steps in the terpenoid pathway through prenyl pyrophosphates to carotenoids in *P. rhodozyma*. *Open arrows* indicate enzymes as targets for engineering by over-expression in order to increase astaxanthin biosynthesis; *dashed arrow* indicates an enzyme target for down-regulation. The specific carotenoid pathway is *boxed*. *Sq*, squalene; *St*, sterols; *H*, hydroxymethyl glutaryl–CoA; *F*, farnesyl diphosphate; *G*, geranylgeranyl diphosphatel; *P*, phytoene; *L*, lycopene; *D*, 3,4-didehydrolycopene; *K*, 3-HO-4-ketotorulene; *B*, β-carotene; *A*, astaxanthin

for lycopene as a substrate. The relative activities of both enzymes versus each other determine the formation of astaxanthin. Thus, an increase of lycopene cyclase activity will shift the carotenoid pathway in the direction of astaxanthin. In addition to astaxanthin, several monoketolated precursors such as echinenone, 3-hydroxyechinenone and 3-hydroxycanthaxanthin accumulate (Fig. 3) and can add up to about 50% of the bicyclic ketocarotenoids (Visser et al. 2005). Since they all are intermediates and potential substrates of astaxanthin synthase (Ojima et al. 2006), increased levels of this enzyme could convert them all to astaxanthin.

In fungi, the dominating terpenoids are sterols. Therefore, apart from intra-pathway manipulation and inter-pathway manipulation, directing metabolite from sterols to carotenoids should be possible. The feasibility of this approach has been demonstrated for carotenoid synthesis in *C. utilis* (Shimada et al. 1998). Disruption of one of the squalene synthase genes in this yeast in combination with overexpression of the HMG–CoA reductase gene led to an almost twofold increase of lycopene synthesis. In contrast to the astaxanthin biosynthesis steps, the regulation of this pathway is poorly understood. One important function of astaxanthin in *P. rhodozyma* is quenching singlet oxygen and oxygen radicals (Schroeder and Johnson 1995a). Obviously, there is an up-regulation of astaxanthin synthesis on demand, i.e. when those oxygen species are generated (Schroeder and Johnson 1995b). Hence, generators of excited oxygen species like duroquinone have been applied to increase astaxanthin productivity in industrial-size processes (de la Fuente et al. 2005). High-intensity light inhibits growth and decreases the carotenoid content (An and Johnson 1990). However, under low intensities, light exerts a positive regulatory effect on carotenoid synthesis and affects the carotenoid composition in *P. rhodozyma* (de la Fuente et al. 2005, 2010; Jacobson et al. 1999; Meyer et al. 1993; Vázquez 2001).

Unless the regulatory mechanisms are better known, a direct genetic manipulation to up-regulate astaxanthin biosynthesis is not possible. However, random mutagenesis may hit a regulatory target resulting in increased astaxanthin formation. A promising astaxanthin-producing strain might be generated by mutagenesis and selection with subsequent pathway engineering at all the metabolic steps mentioned above.

For *P. rhodozyma* transformation, a vector was constructed based on pUC18 which allows plasmid propagation in *E. coli* containing a selection marker for transformed *P. rhodozyma* strains (Wery et al. 1997). This may be the *nptII* gene or *hph* gene conferring resistance of transformed *Phaffia* to geneticin (G418) or hygromycin, respectively. In each case, the resistance genes were flanked by the promoter and terminator of the *Phaffia* glyceraldehyde-3-phosphate dehydrogenase gene. For multiple-copy integration into the *P. rhodozyma* genome, this transformation vector was extended with a section of the ribosomal DNA sequences. Integration of the transformation vector is achieved after linearization by a single-cutting restriction enzyme before transformation. Integration of over 50 copies can be expected (Wery et al. 1997). Other available promoter and terminator sequences are from the *P. rhodozyma* NADP-dependent glutamate dehydrogenase gene (Rodriguez-Saiz et al. 2009). Transformation of *P. rhodozyma* can be carried out in two different ways. Electroporation was first established (Wery et al. 1998) and is very useful for routine transformations. However, biolistic transformation with gold particles is advantageous for library screening due to the high transformation efficiency of up to 4,000 transformants per microgram of DNA compared to the electroporation efficiency close to 1,000 transformants per microgram of DNA (Ojima et al. 2006).

An extensive protocol for plasmid construction with a gene related to carotenogenesis, its transformation into *P. rhodozyma* and transformant analysis have been presented as an example for engineering of the carotenoid pathway (Visser et al. 2005).

The carotenoid pathway of *P. rhodozyma* has been modified successfully at different steps. Introduction of extra copies of the *crtYB* fusion gene resulted in transformants with higher total carotenoid content due to the additional phytoene synthase activity either when a wild-type or a β-carotene-producing mutant was used (Verdoes et al. 2003). Interestingly, enhanced overall carotenoid synthesis did not increase the formation of the astaxanthin end product. Instead, β-carotene and ketocarotenoid intermediates accumulated, indicating that the Asy reaction is the next-appearing limiting step for astaxanthin synthesis in cells with enhanced flux into carotenoid synthesis. A second effect observed in the *crtYB* transformants was a change of the carotenoid composition with a strong decrease of HKT, the alternative product of *P. rhodozyma* carotenoid biosynthesis (Verdoes et al. 2003). This can be attributed to the lycopene cyclase activity of the *crtYB* gene product. As expected, higher activities of this enzyme directed the branching pathway at lycopene much stronger into β-carotene (Fig. 4). The opposite was observed when *crtI* was over-expressed. Torulene and HKT formation was higher because of higher desaturase activity. The increased ratio of phytoene desaturase versus lycopene cyclase is responsible for a much stronger lycopene conversion towards 3,4-didehydrolycopene, the direct precursor of torulene.

In *E. coli*, a bacterium which utilizes the methyl-erythritol phosphate (MEP) pathway for synthesis of the

prenyl building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), IPP isomerase encoded by *idi* is a limiting enzyme for carotenoid synthesis. Expression of the *idi* gene from *P. rhodozyma* in this bacterium increased the carotenoid content by several folds (Kajiwara et al. 1997). This was not the case when *idi* was expressed in *P. rhodozyma*, which utilizes mevalonate for IPP and DMAPP synthesis (Visser et al. 2005). This different effect of *idi* over-expression in *E. coli* and *P. rhodozyma* reflects differences in the utilized pathways. In the mevalonate pathway, IPP is the direct precursor of DMAPP and a functional Idi is absolutely required. In the MEP pathway, IPP and DMAPP are synthesized by the same enzyme from the same precursor.

After the transformation of *P. rhodozyma* with *crtYB*, a variation in phenotype can be found (Verdoes et al. 2003). This depends on the number of integrated copies. When appropriate transformants are selected, special care must be taken to reach pathway stability. With respect to genome integration, the transformants showed to be stably maintaining the plasmid for at least 15 generations at non-selective growth (Wery et al. 1997). In order to analyse the stability of pathway modification, a β-carotene-accumulating strain was transformed with a ketolase gene and the colour change from yellow to red was taken as selection criterion. Red colonies obtained directly from the transformation plate were cultivated in the absence of antibiotics. Yellow, orange and red colonies appeared in the second generation with zero, low or high amounts of ketocarotenoids, respectively (C. Jin, unpublished results). To obtain stable, highly canthaxanthin-accumulating red-pigmented strains, it is important to grow the transformant for several rounds with high selection pressure. The transgene distribution immediately after transformation must be due to the life cycle of *P. rhodozyma*. Although it is a basidiomycetous yeast, it seems that the cell is not dikaryotic. It is still open whether *Phaffia* grows in a haploid (Wery et al. 1997) or diploid (Hermosilla et al. 2003) state. The latter is supported by a range of heterozygous and homozygous knockout mutants which were obtained by directed mutagenesis of carotenogenic genes (Niklitschek et al. 2008).

The first engineering of the complete astaxanthin biosynthesis pathway in a fungus was with non-carotenogenic *C. utilis* (Miura et al. 1998b). This was achieved stepwise by first establishing lycopene synthesis, optimization of carotenoid production and finally directing the pathway to astaxanthin. Lycopene accumulated to 1.1 $mg\,g^{-1}$ dry weight after the genes *crtE*, *crtB* and *crtI* codon optimized for *C. utilis* were introduced. Enhancement and redirection of the isoprenoid pathway into carotenogenesis by overexpression of the catalytic domain of HMG–CoA reductase and disruption of one copy of the squalene synthase gene in combination increased the lycopene content to 7.8 $mg\,g^{-1}$ dry weight (Shimada et al. 1998). As proof of concept, lycopene in *C. utilis* transformants could be further converted to β-carotene and astaxanthin after transformation with the lycopene cyclase gene (*crtY*) from *Erwinia uredovora* and the hydroxylase gene (*crtZ*) and the ketolase gene (*crtW*) from *Paracoccus* sp. N81106 (NBRC101723; formerly called *Agrobacterium aurantiacum*) (Miura et al. 1998a).

Verwaal et al. (2007) have chosen *S. cerevisiae* as a host for heterologous carotenoid synthesis with *P. rhodozyma* carotenogenic genes. A maximum yield of total carotenoids was achieved by the genomic integration of *crtYB*, *crtI* and *crtE* and the additional overexpression of *crtI* as well as the catalytic domain of HMG–CoA reductase. The resulting strain produced 5.9 mg β-carotene per gram of dry weight and represented a suitable strain for optimizing downstream biosynthesis pathways towards astaxanthin. According to this finding, Ukibe et al. (2009) constructed a *S. cerevisiae* strain episomaly expressing *crtYB* and *crtI* from *P. rhodozyma* as well as the GGPP synthase from *S. cerevisiae* (*BTS1*) yielding 390 μg β-carotene per gram of dry weight. β-Carotene was then converted to astaxanthin after the introduction of other plasmids carrying the genes for astaxanthin synthase (*asy*) and astaxanthin synthase reductase (*asr*).

Oleaginous yeasts are also suitable host strains for the production of lipophilic compounds due to their high lipid storage capacity. Recently, the carotenoid-producing *Yarrowia lipolytica* has been generated by metabolic engineering (Sharpe et al. 2008; Trueheart et al. 2009).

Conclusions

The ketocarotenoid astaxanthin is the second most important carotenoid with growing global market value. Up-to-date biotechnological production of natural astaxanthin has not been cost competitive with the chemical synthesis of this compound, thus only accounting for about 3% market share. Among a few organisms capable of synthesizing astaxanthin de novo, the heterobasidiomycetous yeast *P. rhodozyma* is a very promising candidate for both targeted strain development and improved bioprocesses as it is well accessible by metabolic pathway and bioprocess engineering. It is therefore conceivable that biotechnological astaxanthin production will eventually result in cost-competitive processes, especially for the human market.

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