

MiniReview

Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*

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Received 29 January 2003; received in revised form 13 June 2003; accepted 14 June 2003

First published online 10 July 2003

Abstract

This review describes the different approaches that have been used to manipulate and improve carotenoid production in *Xanthophyllomyces dendrorhous*. The red yeast *X. dendrorhous* (formerly known as *Phaffia rhodozyma*) is one of the microbiological production systems for natural astaxanthin. Astaxanthin is applied in food and feed industry and can be used as a nutraceutical because of its strong antioxidant properties. However, the production levels of astaxanthin in wild-type isolates are rather low. To increase the astaxanthin content in *X. dendrorhous*, cultivation protocols have been optimized and astaxanthin-hyperproducing mutants have been obtained by screening of classically mutagenized *X. dendrorhous* strains. The knowledge about the regulation of carotenogenesis in *X. dendrorhous* is still limited in comparison to that in other carotenogenic fungi. The *X. dendrorhous* carotenogenic genes have been cloned and a *X. dendrorhous* transformation system has been developed. These tools allowed the directed genetic modification of the astaxanthin pathway in *X. dendrorhous*. The *crtYB* gene, encoding the bifunctional enzyme phytoene synthase/lycopene cyclase, was inactivated by insertion of a vector by single and double cross-over events, indicating that it is possible to generate specific carotenoid-biosynthetic mutants. Additionally, overexpression of *crtYB* resulted in the accumulation of β -carotene and echinone, which indicates that the oxygenation reactions are rate-limiting in these recombinant strains. Furthermore, overexpression of the phytoene desaturase-encoding gene (*crtI*) showed an increase in monocyclic carotenoids such as torulene and HDCO (3-hydroxy-3',4'-didehydro- β , ψ -carotene-4-one) and a decrease in bicyclic carotenoids such as echinone, β -carotene and astaxanthin.

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Keywords: Carotenoids; *Phaffia rhodozyma*; Production; Mutagenesis; Recombinant-DNA technology

1. *Xanthophyllomyces dendrorhous* and its carotenoid-biosynthetic pathways

X. dendrorhous is the teleomorph state of *Phaffia rhodozyma* [1]. It is a red/pink-pigmented yeast, which was isolated from tree-exudates in mountainous regions of Japan and Alaska in the late 1960s by Herman J. Phaff and colleagues [2]. This heterobasidiomycete is capable of both fermenting sugars and producing carotenoids [3]. The latter serve as antioxidants to protect *X. dendrorhous* from oxidative damage caused by active oxygen species [4,5].

Astaxanthin, an optically active compound, is the prin-

cipal carotenoid produced by *X. dendrorhous*. It represents about 85% of the total carotenoid content [6]. While most organisms known to produce astaxanthin synthesize the (3*S*,3'*S*)-isomer, *X. dendrorhous* produces the opposite isomer having the (3*R*,3'*R*)-configuration [7]. Astaxanthin is formed via the mevalonate pathway, which starts at acetyl-CoA and proceeds via mevalonate to isopentenylpyrophosphate (IPP), the general precursor of all isoprenoids. Subsequently eight molecules of IPP are condensed to form the colorless carotenoid phytoene. Via four dehydrogenation and two cyclization reactions phytoene is converted into β -carotene. Finally β -carotene is oxidized to yield astaxanthin (Fig. 1) [6]. The existence of a monocyclic carotenoid-biosynthetic pathway in *X. dendrorhous* was proposed by An et al. [8]. The monocyclic pathway diverges from the dicyclic pathway at neurosporene and proceeds through β -zeacarotene, γ -carotene, torulene, 3-hydroxy-3',4'-didehydro- β , ψ -carotene-4-one

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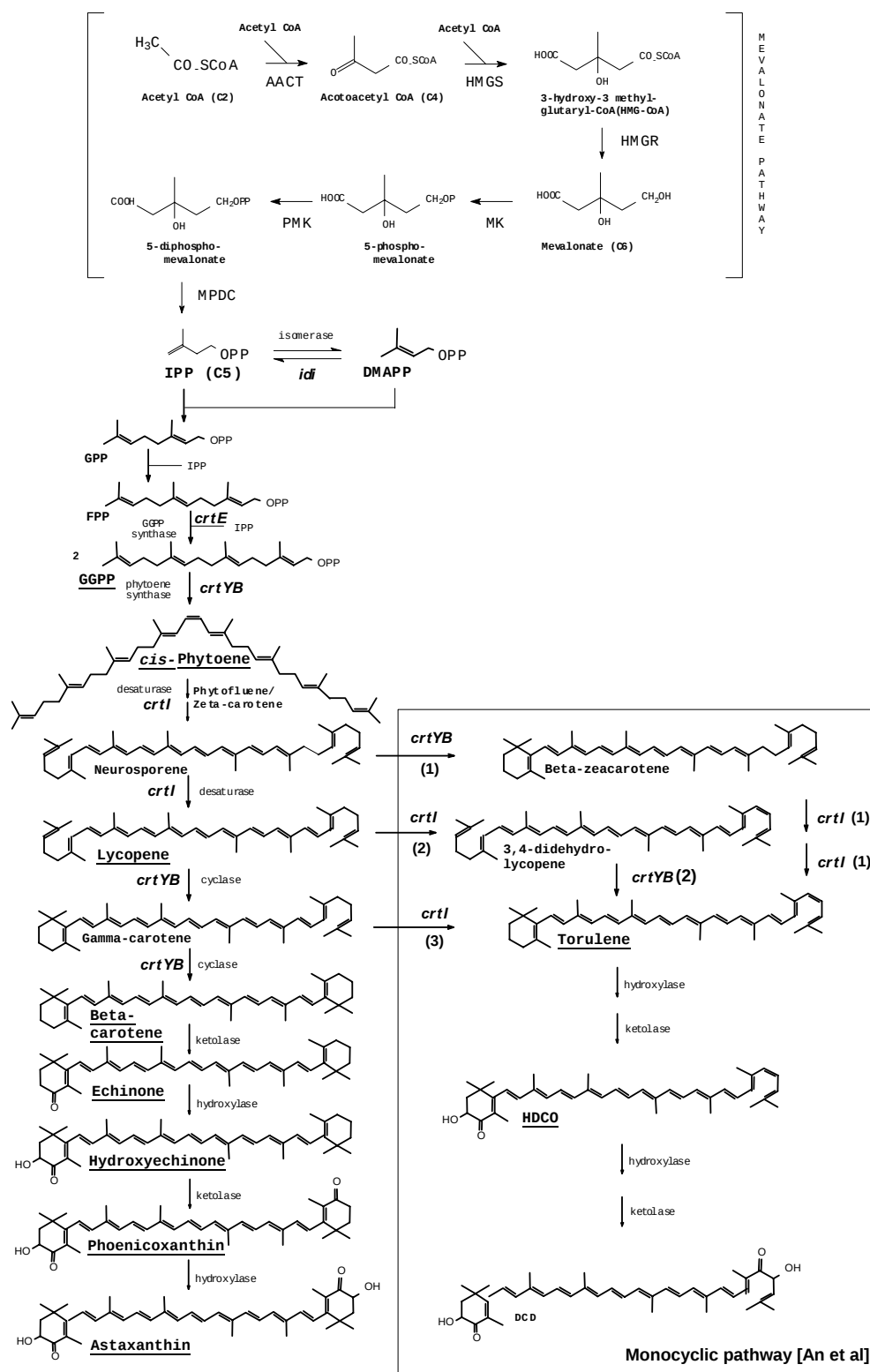


Fig. 1. Dicyclic and monocyclic carotenoid-biosynthetic pathways in *X. dendrorhous* as proposed by Andrewes et al. [6] and An et al. [8] (boxed area), respectively. AACT, acetoacetyl-CoA thiolase. HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase. HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase. MK, mevalonate kinase. PMK, phosphomevalonate kinase. MPDC, mevalonate-pyrophosphate decarboxylase. IPP, isopentenyl-pyrophosphate. GPP, geranyl-pyrophosphate. FPP, farnesyl-pyrophosphate. GGPP, geranylgeranyl-pyrophosphate. HDGO, 3-hydroxy-3',4'-didehydro- β,ψ -carotene-4-one. DCD, 3,3'-dihydroxy- β,ψ -carotene-4,4'-dione. Carotenoids that are mentioned in the text are underlined. The numbers in the boxed area, i.e. (1), (2) and (3), indicate the three potential routes to torulene. Carotenogenic genes: see Table 2.

(HDCO) to the end product 3,3'-dihydroxy- β,ψ -carotene-4,4'-dione.

Astaxanthin is a commercially valuable carotenoid with industrial applications. Traditionally, astaxanthin is used as a feed supplement in aquaculture industries. Since the animals involved are unable to synthesize astaxanthin de novo, this compound has to be supplemented to the feed to ensure, e.g., proper flesh pigmentation and thereby allowing successful marketing and appreciation by the consumer. A well-known example is the distinctive red-orange color of the flesh of pen-raised salmon.

Furthermore, astaxanthin is a powerful antioxidant demonstrating several beneficial effects on human health (see, e.g., [9,10]).

2. Regulation of fungal carotenogenesis

A great deal of knowledge on the molecular biology and regulation of fungal carotenoid biosynthesis has been obtained by investigation of fungi such as *Neurospora crassa* (produces neurosporaxanthin), *Phycomyces blakesleeanus* (β -carotene), *Mucor circinelloides* (β -carotene) and *Fusarium fujikuroi* (neurosporaxanthin). Previously, the late structural genes that encode carotenogenic enzymes (e.g. phytoene synthase/lycopene cyclase, phytoene desaturase) have been isolated and characterized from *N. crassa* [11–13] and *P. blakesleeanus* [14], while more recently carotenogenic genes have been cloned from *M. circinelloides* [15,16], *P. blakesleeanus* [17] and *F. fujikuroi* [18]. Transcription of these genes is induced by (blue) light by only partially understood regulatory mechanisms and results in increased carotenoid biosynthesis. Several regulatory genes that act in photoregulated carotenogenesis in *N. crassa* and *P. blakesleeanus* have been described and reviewed as well [19,20]. Furthermore, lack of *crgA* function in *M. circinelloides* causes the accumulation of carotenoids both in the light and the dark, indicating that *crgA* encodes a negative regulator of light-inducible carotenogenesis in this fungus [21,22].

The accumulation of higher amounts of intermediate carotenes in *carB*- or *carRA*-disrupted mutants when compared to the carotene contents in wild-type strains also suggests a mechanism of feedback regulation in carotene biosynthesis by *P. blakesleeanus* and *F. fujikuroi* [18,23,24]. Additionally, when Mucoracean fungi such as *P. blakesleeanus* enter the sexual cycle, carotenoid biosynthesis is strongly enhanced. This process is mediated by compounds referred to as trisporic acids, which are derivatives of β -carotene that are exchanged by mycelia of different mating types [20].

In the case of *X. dendrorhous* little is known about regulation of carotenogenesis. Astaxanthin biosynthesis does not seem to be photo-inducible in wild-type strains of *X. dendrorhous* [25]. However, carotenogenesis has been shown to be photo-inducible in a number of *X. dendro-*

rhous mutant strains, primarily by blue light [26]. Furthermore, high light intensities inhibit cell growth and decrease carotenoid formation [25]. Further research is required to elucidate the role of light in (regulation of) carotenogenesis in *X. dendrorhous*.

The biological role of astaxanthin in *X. dendrorhous* is to protect the cell from oxidative damage caused by, e.g., reactive oxygen species or peroxy radicals. It acts as a scavenger/antioxidant molecule [5]. Changes in the levels of these damaging compounds also change carotenoid formation. Singlet oxygen induces carotenoid accumulation, possibly by gene activation [27]. Peroxy radicals, on the other hand, decrease the astaxanthin content by oxidative degradation. As a result, β -carotene levels are increased. These and other data suggest feedback regulation of carotenogenesis by the end product astaxanthin [27].

In many organisms, mevalonate synthesis, which is an early step in terpenoid biosynthesis, is a key point of regulation [28] of the corresponding pathway. This is probably also true for fungi, including *X. dendrorhous*. In fact, addition of mevalonate to a culture of *X. dendrorhous* stimulated both astaxanthin and total carotenoid biosynthesis four times [29]. This indicates that the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by HMG-CoA reductase is a potential bottleneck on the road to modified strains with higher astaxanthin content. Recent examples of enhanced carotenoid accumulation and increased levels of HMG-CoA reductase (as well as HMG-CoA synthase) messenger RNA have been published for *N. crassa* and *P. blakesleeanus* [30,31].

3. Strategies for the improvement of carotenoid yield in *X. dendrorhous*

The astaxanthin content of wild strains of *X. dendrorhous* is approximately 200–400 $\mu\text{g g}^{-1}$ of dry yeast [32,33]. The yeast grows well under industrial conditions. The level of astaxanthin in *X. dendrorhous* has to be increased by a factor 10–50 to become competitive with industrial chemically synthesized astaxanthin [33]. Natural astaxanthin from the alga *Haematococcus pluvialis* and likely also from *X. dendrorhous* is produced to supply specialty markets. However, the demand for natural astaxanthin, and consequently the astaxanthin price and competition position versus synthetic astaxanthin might increase in the near future [34]. Therefore, many research efforts have been focused on the improvement of astaxanthin production. One approach is the optimization of culture media and fermentation conditions. This includes the addition of a specific precursor [29,35], the optimization of parameters such as the levels of glucose, oxygen, and phosphate as well as the carbon/nitrogen ratios [35,36], and the selection and use of low-cost culture media such as molasses, peat or eucalyptus hydrolysates and several juices (see [37] and references therein). Importantly, fer-

mentative conditions, e.g. limited oxygen or high glucose concentrations, decrease the astaxanthin production rate drastically, while the astaxanthin production rate increases with increasing oxygen uptake under aerobic conditions [36].

In general, the improvement of the astaxanthin levels by fermentation is not satisfactory for commercial exploitation. Another approach is the isolation of astaxanthin-hyperproducing strains and/or carotenoid-biosynthetic mutants. This can be achieved by using random mutagenesis or by the manipulation of the expression of one or more astaxanthin-biosynthetic genes or regulatory genes in *X. dendrorhous*.

4. Classical genetic approaches to improve astaxanthin yield

A relatively easy way to obtain strains with improved astaxanthin production is by random mutagenesis of wild strains and selection for higher carotenoid contents. Ultra-violet light and mutagenic compounds such as ethyl methanesulfonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and others have been used to create carotenoid-hyperproducing strains.

However, genetic instability is a major drawback of using mutagenesis to create carotenoid-hyperproducing mutants. For example, EMS-induced mutants were shown to revert at high frequency [38]. Despite this problem, several research groups have successfully isolated stable *Xanthophyllomyces* mutant strains with improved astaxanthin biosynthesis (Table 1). However, most of these stable mutants show a (strong) reduction in growth rate and/or biomass yield. It is difficult to tell which astaxanthin-hyperproducing mutant is the best in terms of absolute astaxanthin yield, since data on mutant growth rates are not always included in the literature, cultivation conditions vary between different laboratories and cost calculations are missing.

Additionally, carotenoid-hyperproducing *Xanthophyllomyces* hybrids have been constructed by protoplast fusion of parental strains that produce approximately 1600 μg (g dry weight, dw)⁻¹ carotenoid [44]. The hybrids were stable

and produced >2000 μg (g dw)⁻¹ carotenoid. However, no information on astaxanthin content or growth was given in this report.

X. dendrorhous strains with high astaxanthin production capacity as well as corresponding optimized culture conditions for high astaxanthin production are used in industry. However, details on these strains and fermentation processes are not made public because of the industrial competition on the carotenoid market. Nevertheless, stable *Xanthophyllomyces* mutant strains that produce >3000 to 4000 μg (g dw)⁻¹ of yeast have been reported to produce astaxanthin economically on a commercial production scale in working volumes of at least 1500 l. Specific fermentation conditions ensure the formation of high-cell-density cultures. Enhanced and stable astaxanthin accumulation are accomplished in the maturation phase by slowly feeding the yeasts with a rapidly metabolized energy source such as glucose, which is then replaced by a slowly metabolized energy source, e.g. glycerol. Furthermore, during cultivation these yeast cells are exposed to a low-intensity light source [45,46].

Selection of astaxanthin-hyperproducing mutants from parental strains on agar plates is possible but difficult, especially when a large number of colonies need to be analyzed. Therefore, more sensitive procedures to select carotenoid-hyperproducing mutants have been developed. A simple, yet powerful positive screening method involves the use of carotenogenesis inhibitors (e.g. β -ionone and diphenylamine) in the growth medium [40,42]. Carotenoid-hyperproducing mutants show resistance to these compounds. This property makes them easy to distinguish from the parental strain where carotenoid biosynthesis is inhibited. On the other hand, An et al. [38] have followed a negative screening approach. Here, carotenoid-hyperproducing strains were identified on the basis of their higher susceptibility than the parent strain to antimycin A, a respiratory inhibitor. Furthermore, as a model system for the non-selective enrichment of mutants that are increased in secondary metabolite production, An et al. [47] have successfully employed quantitative flow cytometry and cell sorting to isolate astaxanthin-hyperproducing mutants.

Another major drawback of the mutagenesis approach

Table 1
Improved astaxanthin production by mutants of *X. dendrorhous*

Mutant strain	Astaxanthin (μg (g dw) ⁻¹)	Increase (fold up) ^a	Decreased growth rate and/or biomass yield	Reference
ant-1	900	2	yes	[38]
ant-1-4	900–1200	2–4	yes	[38]
unknown	2000–2500	5–6	unknown	[38]
J4-3	1392	7	yes	[39]
N9	1834	9	yes	[39]
NTG-3-7	725	2.5	unknown	[40]
D49.7	912	2	no	[41]
NCHU-FS301	1516	2.5	yes	[42]
PR-2-171	650	1.5	yes	[43]

^aRelative to the parental strain and rounded off to 0.5.

is the introduction of secondary mutations, which may have an effect on the physiology, viability, or metabolic capacity of a cell. Furthermore, using this approach researchers have not been able to isolate all specific mutants in the astaxanthin pathway of *X. dendrorhous*, e.g. mutants that accumulate lycopene or one of the oxygenated derivatives of β -carotene such as echinone, 3-hydroechinenone or phoenicoxanthin. A more specific approach for the engineering of a pathway would be the application of recombinant-DNA technology. However, this implies the development of a transformation procedure for *X. dendrorhous* and the isolation of the astaxanthin-biosynthetic genes.

5. The use of recombinant-DNA technology to improve astaxanthin yield

A transformation system for *X. dendrorhous* has been developed and optimized [48]. The promoters of the actin- and the glyceraldehyde dehydrogenase (*gpd*)-encoding genes of *X. dendrorhous* were used to express the kanamycin resistance gene of transposon Tn5, conferring G418 (geneticin) resistance in *X. dendrorhous*. For stable integration in the genome and to improve the frequency of integration, a part of the ribosomal DNA of *X. dendrorhous* was introduced in the transformation vectors. Under optimized conditions the transformation efficiency was 1000 transformants μg^{-1} plasmid DNA [48].

From the possible strategies for the isolation of the astaxanthin-biosynthetic genes of *X. dendrorhous* a heterologous complementation approach was selected [49]. *Escherichia coli*, which lacks the potential to produce carotenoids, was transformed with a plasmid that carried a set of carotenoid-biosynthetic genes (*crt*) of the bacterium *Erwinia uredovora* [50]. When the complete cluster of six *crt* genes is introduced in *E. coli* the orange-colored carotenoid zeaxanthin- β -diglucoside is synthesized. Misawa et al. [51] have constructed a full collection of plasmids in which one or more genes of the *E. uredovora crt* gene cluster are inactivated. Such strains were used as hosts to screen a cDNA library of *X. dendrorhous*. Complementation will result in a color of the colony and thus positive clones can easily be selected by visual selection (Fig. 2A). It was expected that, based on the analysis of carotenoid content in *X. dendrorhous* [6] and *E. uredovora*, similar enzymatic activities should be present in both organisms. Using this approach the *X. dendrorhous* genes encoding geranylgeranyl-pyrophosphate (GGPP) synthase (*crtE*), phytoene synthase (*crtYB*), phytoene desaturase (*crtI*) and lycopene cyclase (*crtYB*) were isolated. Surprisingly, we identified that one single gene, designated *crtYB*, is able to carry out two non-sequential conversion steps in the biosynthetic pathway of astaxanthin. The gene product CrtYB condensates two molecules of GGPP into phytoene and cyclizes the linear carotenoid lycopene into β -caro-

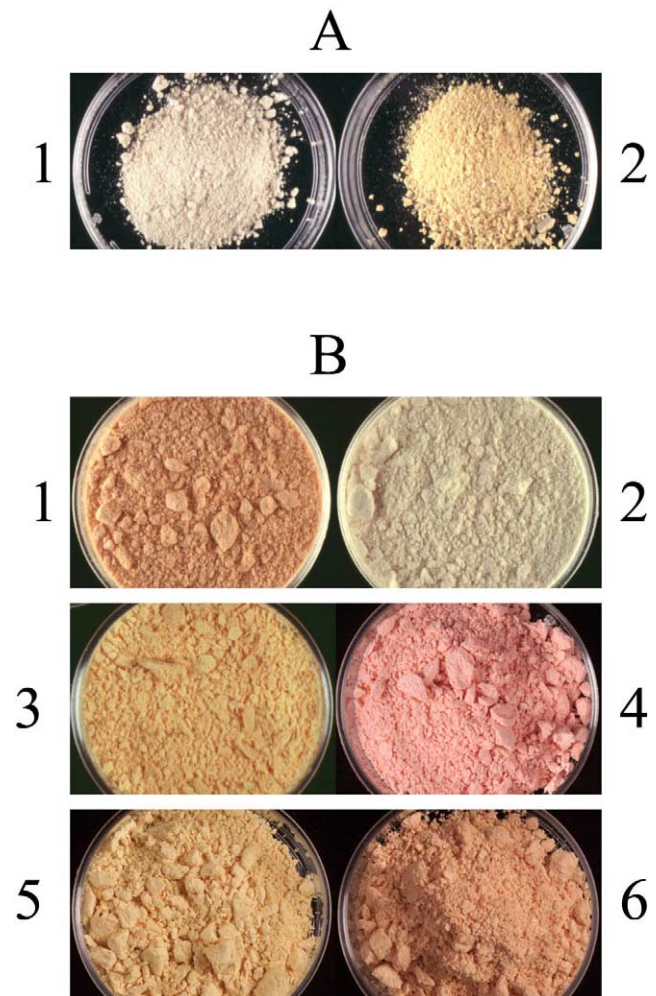


Fig. 2. A: Cloning of *X. dendrorhous* carotenogenic genes by a heterologous complementation approach. 1, *E. coli*(pACCRT Δ crtE) harbors the plasmid-borne zeaxanthin- β -diglucoside pathway of *E. uredovora*, which is interrupted in the *crtE* gene and results in a white-colored strain. This strain was used as a host to screen a *X. dendrorhous* cDNA library. 2, *E. coli*(pACCRT Δ crtE/pPRcrtE) was selected from the majority of white-colored colonies based on its yellow-orange color and contained the *crtE* cDNA of *X. dendrorhous*. B: Carotenoids produced by wild-type, mutant and genetically engineered *X. dendrorhous* strains. 1, wild-type strain CBS 6938. 2, strain CBS 6938, which *crtYB* gene is interrupted. 3, strain CBS 6938, which *crtYB* gene is overexpressed. 4, strain CBS 6938, which *crtI* gene is overexpressed. 5, strain PR-1-104 (a CBS 6938 mutant [43]) that accumulates β -carotene. 6, strain PR-1-104, which *crtI* gene is overexpressed.

tene. This novel type of carotenoid-biosynthetic gene encoding a bifunctional enzyme was later on also found in other carotenoid-producing fungi (Table 2).

Also a lycopene-accumulating *E. coli* strain, XL-blue MRF[pACCRT-EIB], was used to screen the cDNA library of *X. dendrorhous*. An unexpected color change from pale red into dark red was observed. The responsible polypeptide showed clear homology to IPP – dimethylallyl diphosphate (DMAPP) isomerase of non-carotenoid-producing yeasts. With the introduction of the *idi* gene of

X. dendrorhous in a carotenoid-producing *E. coli* strain carotenoid production could be improved by a factor 1.3–3 [52]. Apparently, IPP isomerase activity is a rate-limiting step in carotenogenic *E. coli* cells. In *X. dendrorhous*, IPP is formed via the mevalonate pathway. IPP isomerase converts IPP to DMAPP, which is used in subsequent prenyl transferase reactions. Therefore, as has been shown for *Saccharomyces cerevisiae* [58], the *X. dendrorhous idi* gene is likely to be essential. In *E. coli*, the situation is different; IPP is produced by the non-mevalonate (or pyruvate/glyceraldehyde-3-phosphate) pathway [59]. Additionally, the *E. coli idi* gene is not essential [60]. DMAPP may therefore be produced by another (novel type of) IPP isomerase or from an unknown precursor, which is also used to synthesize IPP. Therefore, carotenogenesis in *E. coli* is physiologically different from that in *X. dendrorhous*, implying that also the role and regulation of IPP isomerase in *X. dendrorhous* regarding isoprenoid fluxes will probably be different between these two microorganisms.

Recently the *ast* gene has been isolated by Hoshino and others [57]. They have used a complementation approach in a carotenoid-biosynthetic mutant of *X. dendrorhous*. Complementation was achieved by the introduction of a genomic library in a β -carotene-accumulating mutant. In one of the transformants the astaxanthin production was restored. The complementing sequence was isolated and a homology search with the deduced amino acid showed that the product of the *ast* gene represents a cytochrome-P450 type enzyme. It is clear that the enzyme is involved in the conversion of β -carotene. However, it is

unclear whether the enzyme is able to add both the keto and the hydroxyl groups to the two rings of the β -carotene molecule, as an in vitro enzymatic reaction has not been described yet. The characteristics of the cloned carotenoid-biosynthetic genes of *X. dendrorhous* and the encoded proteins are summarized in Table 2.

In *X. dendrorhous* isopentenyl diphosphate (IPP), the universal precursor of carotenoid (terpenoid) biosynthesis is produced via the mevalonate pathway (Fig. 1). Recently, the genes of the mevalonate pathway and the gene encoding farnesyl diphosphate synthase have been cloned by Hoshino et al. [61]. They cloned partial gene fragments containing a part of the encoding genes of HMG-CoA synthase (*hmc*), HMG-CoA reductase (*hmg*), mevalonate kinase (*mvk*), mevalonate diphosphate decarboxylase (*mpd*), and farnesyl diphosphate synthase (*fps*) by using a degenerated polymerase chain reaction (PCR) method. By probing a genomic library of *X. dendrorhous*, with these partial sequences, the genes, including expression signals, were identified.

6. Metabolic engineering of *X. dendrorhous* by recombinant-DNA techniques

The *crtYB* locus was selected as model to develop a gene-specific inactivation approach in *X. dendrorhous*. Two plasmids were constructed to inactivate the endogenous *crtYB* gene by integration via site-homologous recombination. The 5' end of the cDNA clone encoding CRTYB was cloned in the integrative transformation vec-

Table 2
Characteristics of the astaxanthin-biosynthetic genes of *Xanthophyllomyces dendrorhous*

Enzyme	Conversion: substrate $\Rightarrow$ product	Gene	Introns	No. of amino acids (mol. mass, kDa)	Homology ^a	Accession number	Reference
IPP isomerase	IPP $\rightleftharpoons$ DMAPP	<i>idi</i>	4	251 (28.7)	<i>Aspergillus fumigatus</i> (56; 129/227)	Y15811	[52,53]
GGPP synthase	FPP $\Rightarrow$ GGPP	<i>crtE</i>	8 ^b	376 (42.1)	<i>Mucor circinelloides</i> (55; 94/169)	A63889	[54]
Phytoene synthase	GGPP $\Rightarrow$ phytoene	<i>crtYB</i> ^c	4	673 (74.7) ^d	<i>Mucor circinelloides</i> (34; 134/394) ^e	AJ133646	[55]
Phytoene desaturase	Phytoene $\Rightarrow$ lycopene	<i>crtI</i>	11	602 (67.6)	<i>Fusarium fujikuroi</i> (47; 269/566)	Y15007	[56]
Lycopene cyclase	Lycopene $\Rightarrow$ β -carotene	<i>crtYB</i> ^c	4	673 (74.7) ^d	<i>Mucor circinelloides</i> (34; 134/394) ^e	AJ133646	[55]
Astaxanthin synthetase (P450)	β -carotene $\Rightarrow$ xanthophyll ^f (astaxanthin?)	<i>ast</i>	17	557 (62.6)	<i>Oryza sativa</i> ; rice (29; 84/285)	AX034666	[57]

^aHomology search has been carried out using BLAST2 at EBI. The highest identity scores are indicated (%) as well as the number of identical amino acids per stretch of amino acids from the homologous sequence. For example, 129/227 indicates 129 identical amino acids in a stretch of 227 amino acids.

^bVerdoes, unpublished results.

^cThe gene encodes a bifunctional protein displaying both phytoene synthase and lycopene cyclase activity.

^dThe molecular mass of the complete protein is indicated. The N-terminal domain (295 aa) encodes mainly the lycopene cyclase and the C-terminal domain the phytoene synthase activity.

^eWhen the two separate regions were used the highest identity for the phytoene synthase domain was found with *Gibberella fujikuroi* (46%, 65/141) and *Phycomyces blakesleeana* (32%, 85/264) when the lycopene cyclase-encoding domain was used.

^fThe gene complements a β -carotene-accumulating strain of *X. dendrorhous*. The specific conversion of β -carotene has not been determined yet.

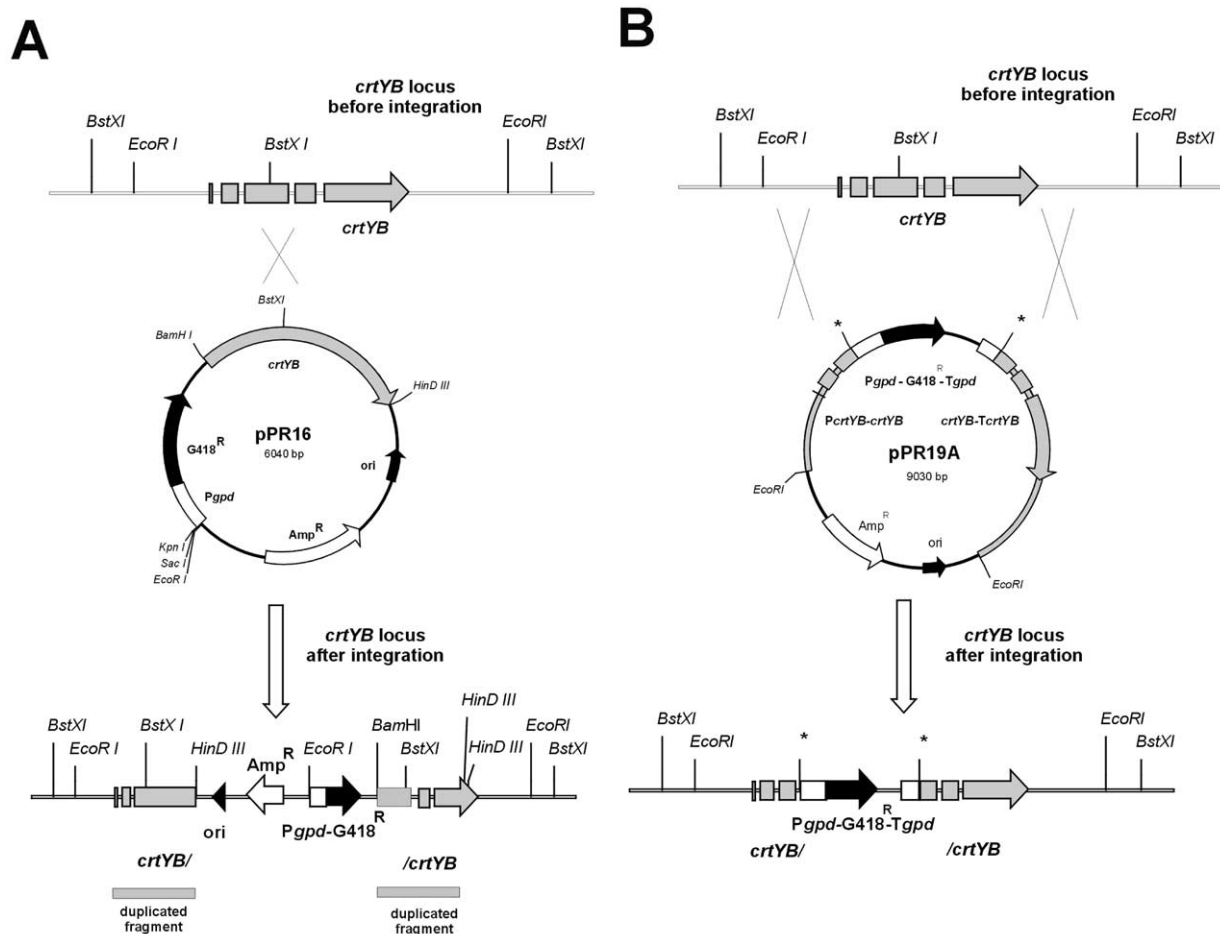


Fig. 3. Schematic view of the gene-specific inactivation approach in *X. dendrorhous*. The *crtYB* locus was selected as a model. A: Gene-specific integration of vector pPR16 by a single cross-over event. B: Gene-specific integration of vector pPR19A by a double cross-over event. The vectors pPR16 and pPR19A were linearized by endonucleases *BstXI* and *EcoRI*, respectively, prior to electroporation. *, the digested *EcoRI* and *BstXI* sites were blunted by the Klenow fragment of *E. coli* DNA polymerase I and bacteriophage T4 DNA polymerase, respectively, prior to ligation.

tor pPR1TN, yielding pPR16 (Fig. 3A). The second vector, called pPR19F, was constructed by the insertion of the G418 marker cassette in the *crtYB* gene (Fig. 3B). The integration should occur by a single recombination event with pPR16 and by a double cross-over event with pPR19. In the latter case, the inactive gene copy replaces the endogenous *crtYB*. The linearized vectors pPR16 and pPR19 were introduced by electroporation in *X. dendrorhous* strains CBS 6938. Several tens of G418 resistant colonies with a white-colored phenotype (Fig. 2B) were isolated. High-performance liquid chromatography (HPLC) analysis of carotenoid extracts of these recombinant strains was used to show that no carotenoids were produced. Southern analysis showed that the hybridization patterns matched the expected patterns of transforming plasmid integrated at the *crtYB* locus. These results demonstrated that it is possible to design specific carotenoid-biosynthetic mutants of *X. dendrorhous* by recombinant-DNA techniques.

In *Thermus thermophilus* the conversion of GGPP into phytoene by the enzyme phytoene synthase is the rate-

limiting step in carotenoid synthesis. A transformant, carrying the corresponding gene on a multicopy plasmid, produced three times more carotenoid than the host strain [62]. To analyze the same situation for astaxanthin biosynthesis and carotenoid composition in *X. dendrorhous* we started a study in which the endogenous phytoene synthase/lycopene cyclase-encoding gene was overexpressed in the wild-type *X. dendrorhous* strain CBS6938 as well as in a carotenoid-biosynthetic mutant of *X. dendrorhous*. The latter strain, named PR-1-104, was isolated after classical mutagenesis and accumulates β -carotene [43]. To overexpress the *crtYB* gene multiple copies of the gene were introduced by integrative transformation. In addition, the expression of the *crtYB* gene was controlled by the promoter region of the *gpd* gene. The vector, called pPR13, and a negative control vector (pPR2TN, [55]) were introduced in the two host strains by electroporation. For each plasmid several hundred G418-resistant colonies were isolated. Southern blot analysis of chromosomal DNA indicated the integration of multiple copies of the transforming plasmids in these transformants. The color of the

colony was changed from pink into orange in transformants of CBS 6938 carrying additional copies of the *crtYB* gene (Fig. 2B). The transformants of the β -carotene-accumulating strain displayed, compared to the control PR-1-104[pPR2TN], a brighter yellow color. HPLC analysis indicated a small increase in the total carotenoid production in derivatives of CBS 6938 carrying multiple copies of the *crtYB* gene (Table 3). However, compared to the control strain, the specific and relative amount of astaxanthin is reduced whereas the specific and relative amounts of echinone and, to a lesser extent, β -carotene are increased. In derivatives of strains PR-1-104 the only carotenoid that is produced is β -carotene. In all transformants with multiple copies of the *crtYB* gene the β -carotene production is improved (less than 50%).

In a second study multiple copies of the phytoene desaturase-encoding gene of *X. dendrorhous* were introduced. The gene product, CRTI, is a dehydrogenase that introduces four additional double bonds into phytoene, yielding the red-colored carotenoid lycopene (Fig. 1). An expression cassette consisting of the promoter and terminator regions as well as a cDNA copy of the phytoene desaturase-encoding gene (*crtI*) was synthesized by recombinant PCR. This cassette was cloned in the integrative transformation vector pPR2TN yielding pPR40. Introduction of this vector in *X. dendrorhous* strain CBS 6938 resulted in a collection of transformants in which the phenotype varied from pink to dark red (Fig. 2B). HPLC analysis of carotenoid extracts showed a significant and unexpected increase in the total amount of monocyclic

carotenoids such as torulene and HDCO and a decrease in the specific amounts of bicyclic carotenoids, e.g. echinone, β -carotene and astaxanthin (Table 3). Analysis of carotenoid extracts from transformants of the β -carotene-accumulating strain (PR-1-104) carrying multiple copies of pPR40 showed an increase in the relative amount of the monocyclic carotenoid torulene. There is a negative correlation between the amount of torulene and both the total amount of carotenoids and the specific amount of β -carotene (Table 3).

Thus, the flux in the astaxanthin-biosynthetic pathway can be manipulated by changing the amounts and activities of carotenogenic enzymes. Overexpression of the *crtYB* gene, encoding the bifunctional carotenogenic enzyme, in CBS 6938 resulted in the accumulation of intermediates β -carotene and echinone and a decrease of carotenoids that are formed as minor products from lycopene such as torulene and HDCO (Table 3). These results indicate that when the *crtYB* gene is overexpressed the oxygenation reactions (e.g. of β -carotene and echinone) are the rate-limiting steps in the pathway to astaxanthin. In the non-transformed strain, the dominating reaction is the cyclization of lycopene, which directs the metabolic flux towards astaxanthin. However, when the gene encoding phytoene desaturase is overexpressed, the five-step desaturation to 3,4-didehydrolycopene is intensified, resulting in an accumulation of torulene and HDCO as subsequent products (Table 3).

Recently An et al. [8] have proposed the presence of a second, monocyclic carotenoid pathway in *X. dendrorhous*.

Table 3

Carotenoid composition of *X. dendrorhous* strains CBS 6938 and PR-1-104 with multiple copies of the phytoene synthase/lycopene cyclase-encoding gene (*crtYB*; pPR13) or phytoene desaturase-encoding gene (*crtI*; pPR40) of *X. dendrorhous*

	CBS 6938						
	[pPR2TN] #1	[pPR13F] #1	[pPR13F] #9	[pPR13F] #18	[pPR2TN] #1	[pPR40F] #3	[pPR40R] #4
Total amount carotenoids in $\mu\text{g (g dw)}^{-1a}$	256 (100)	301 (118)	273 (107)	465 (182)	265 (100)	187 (71)	185 (70)
Specific amounts in $\mu\text{g (g dw)}^{-1b}$:							
Astaxanthin	81 (33)	47 (16)	71 (26)	95 (21)	132 (50)	47 (25)	45 (24)
Phoenicoxanthin	40 (19)	43 (14)	44 (16)	60 (13)	– (0)	– (0)	– (0)
HO-echinone	20 (8)	4(1)	– (0)	– (0)	18 (7)	14 (8)	16 (9)
Echinone	31 (12)	113 (38)	78 (28)	180 (39)	41 (16)	11 (6)	11 (6)
β -carotene	28 (11)	68 (23)	50 (18)	113 (24)	30 (11)	6 (3)	6 (3)
HDCO	39 (15)	18 (6)	31 (11)	17 (4)	42 (16)	101 (54)	101 (55)
Torulene	2 (5)	7 (2)	– (0)	– (0)	3 (1)	7 (4)	9 (5)
	PR-1-104 ^c						
	[pPR2TN] #1	[pPR13F] #1	[pPR13F] #2	[pPR2TN] #1	[pPR40F] #4	[pPR40F] #11	
Total amount carotenoids in $\mu\text{g (g dw)}^{-1a}$	308 (100)	41(134)	372 (121)	410 (100)	406 (99)	207 (52)	
Specific amounts in $\mu\text{g (g dw)}^{-1b}$:							
β -carotene	308	412	372	370 (90)	344 (84)	136 (65)	
Torulene	–	–	–	40 (10)	63 (16)	71 (35)	

^aAll strains were cultivated in duplicate and the indicated numbers are the average of two cultures. In parentheses, the relative amounts are indicated (the strain with the empty cloning vector pPR2TN is set 100 and was included in each cultivation experiment).

^bIn parentheses the relative distribution in % is indicated; – indicates: not detected.

^cPR-1-104 is a classical mutant of CBS 6938 [43] and it produces β -carotene only.

This was based on the analysis of carotenoid-hyperproducing mutants and the use of specific inhibitors of the carotenoid synthesis. The fact that torulene is the end product in a β -carotene-accumulating strain carrying multiple copies of the phytoene desaturase-encoding gene suggests that the enzymes that convert β -carotene into astaxanthin are the same as the ones that convert torulene into HDCO (Fig. 1). Apparently these enzymes have a broad substrate range and can accept both monocyclic and bicyclic carotenoids.

7. Discussion and outlook

As outlined above, several approaches have been used to improve astaxanthin biosynthesis by *X. dendrorhous*. Although the results are encouraging, more research is needed for further improvement.

Regulation of isoprenoid biosynthesis in *S. cerevisiae* has been shown to be very complex involving multiple levels of feedback inhibition that affect transcription, translation and protein stability [63,64]. This is probably also valid for *X. dendrorhous*. One should realize that each genetic modification in a pathway can cause the deregulation of that pathway and others. Thus, in addition to, e.g., the overexpression of genes encoding rate-limiting enzymes or the disruption of pathway branch points, the natural regulatory networks that control metabolite fluxes have to be adjusted to the new recombinant isoprenoid pathway as well. This approach, which is called metabolic control engineering, was successfully applied to lycopene production in *E. coli* [65].

It is therefore desirable to determine the effect of a genetic modification on complete cellular processes such as transcription, translation and protein stability, and metabolite concentrations and fluxes. This holistic picture will show the effect of the genetic modification and it will indicate what should or can be done to further optimize the carotenoid production system. Contrary to that of *S. cerevisiae*, the genome sequence of *X. dendrorhous* is not known, which is essential to analyze the complete transcriptome and proteome. Nevertheless, genomics techniques will be very helpful in the analyses of the effect of genetic modifications on carotenoid biosynthesis in *X. dendrorhous*. To start with, partial transcript profiles can be generated by using the cDNA-AFLP technique [66]. Furthermore, cell protein compositions can be analyzed by two-dimensional protein gel electrophoresis and protein spots of interest can be identified. Therefore, it is possible to create a large set of data that is very useful in the design of efficient carotenoid-producing *X. dendrorhous* strains.

It is anticipated that ultimately by joint efforts and using combinations of both classical and modern methodologies the carotenoid content in *X. dendrorhous* can be altered significantly and can be directed to produce a specific carotenoid in higher amounts.

Acknowledgements

This paper is dedicated with great appreciation to the late Prof. Herman Jan Phaff (1913–2001) as the founder of *Phaffia rhodozyma* research and as devoted contributor to yeast research in general.

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