Appl Microbiol Biotechnol
DOI 10.1007/s00253-016-7769-0

Engineering of the carotenoid pathway in *Xanthophyllomyces dendrorhous* leading to the synthesis of zeaxanthin

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Received: 1 April 2016 / Revised: 19 June 2016 / Accepted: 1 August 2016

Abstract Zeaxanthin is an essential nutrient for prevention of macular degeneration. However, it is limited in our diet. For the production of zeaxanthin, we have engineered zeaxanthin synthesis into a carotenoid mutant of *Xanthophyllomyces dendrorhous* which is blocked in astaxanthin synthesis and accumulates β-carotene instead. Two strategies were followed to reach high-yield zeaxanthin synthesis. Total carotenoid synthesis was increased by over-expression of genes *HMGR*, *crtE*, and *crtYB* encoding for limiting enzymes in the pathway leading to and into carotenoid biosynthesis. Then bacterial genes *crtZ* were used to extend the pathway from β-carotene to zeaxanthin in this mutant. The increase of total carotenoids and the formation of zeaxanthin is dependent on the number of gene copies of *crtYB* and *crtZ* integrated into the *X. dendrorhous* upon transformation. The highest zeaxanthin content around 500 μg/g dw was reached by shaking flask cultures after codon optimization of *crtZ* for *Xantho phyllomyces*. Stabilization of carotenoid and zeaxanthin formation in the final transformant in the absence of selection agents was achieved after passing through a sexual cycle and germination of basidiospores. The values for the transformant before and after stabilization were very similar resembling about 70 % of total carotenoids and corresponding to a conversion rate of 80 % for hydroxylation of β-carotene to zeaxanthin. The stabilized transformant allowed experimental small-scale fermentation yielding *X. dendrorhous* cells with a zeaxanthin content similar to the shaking flask cultures. Our result demonstrates the potential of *X. dendrorhous* for its development as a zeaxanthin producer and its suitability for large-scale fermentation.

Keywords Carotenoid biosynthesis · Pathway engineering · *Xanthophyllomyces dendrorhous* · Zeaxanthin

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Introduction

Lutein, zeaxanthin, and carotenoids with an unsubstituted β-ionone ring like β-carotene are essential carotenoids of a functional vision apparatus of humans. All three carotenoids cannot be synthesized by them and have to be taken up with their diet (Krinsky et al. 2003). The cleavage product of β-carotene is a precursor of the visual chromophore retinal (von Lintig et al. 2005) whereas lutein and zeaxanthin in combination protect the retina from excess blue light and ultraviolet radiation. The amount of both carotenoids is inversely associated with macular degeneration of elderly people which leads to blindness (Krinsky et al. 2003; SanGiovanni and Neuringer 2012). Lutein is present in substantial quantities in green vegetables whereas the dietary supply of zeaxanthin is limited to the consumption of corn and orange peppers (Perry et al. 2009).

There is a developing market for zeaxanthin as food supplement (Sandmann 2014). Many carotenoids are provided by chemical synthesis. In contrast, chemical synthesis on an industrial scale has not been established for zeaxanthin. Therefore, microbial production systems for zeaxanthin are of general interest. Fungi are unable to synthesize zeaxanthin (Sandmann and Misawa 2002). Nevertheless, it is possible to engineer zeaxanthin synthesis. First attempts have been made with yeast (Chang et al. 2015). It has been shown that it is advantageous to use carotenogenic hosts for carotenoid production due to their storage capacity for lipophilic carotenoids. One of the yeast of choice is *Xanthophyllomyces dendrorhous*

Published online: 16 August 2016

(Schmidt et al. 2011). This basidiomycete yeast synthesizes astaxanthin (Andrewes and Starr 1976) and was genetically engineered for high astaxanthin production (Gassel et al. 2013). In addition to wild-type strains, mutants are available which are blocked at the stage of β-carotene (Girard et al. 1994) (see Fig. 1 for pathway) which can be converted to zeaxanthin in a single enzymatic step.

In the present investigation, we utilized this carotenogenic potential and the carotenoid storage capacity of *X. dendrorhous* to redirect carotenogenesis toward zeaxanthin. Starting from a β-carotene mutant, engineering of a hydroxylation step extended the pathway to the desired product and enhancement of precursor supply to the carotenoid pathway resulted in an increase of zeaxanthin yield.

Material and methods

Strains, cultivation, and fermentation conditions

X. dendrorhous strain PR1-104 derived from CBS 6938 (Girard et al. 1994) and transformants were grown over 7 days in YM medium (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1.0 % glucose) with white light illumination in shaking cultures (50 ml in 500-ml baffled Erlenmeyer flasks, 180 rpm) at 20 °C or in a 2-l stirred fermenter (Biostat A plus, Braun, Germany). Fermenter cultures started with an initial filling of 1.0 l medium. The process was run at 21 °C with an air flow rate of 1 ml/min. The pH was controlled with 2 M NaOH and 2 M HCl at 5.5. Dissolved oxygen was kept above 40 % air saturation (with the stirring speed varying from 300 to 600 rpm) and determined continuously with an oxygen electrode. Foam formation was suppressed by the addition of Antifoam 204 (Sigma-Aldrich, USA). After consumption of the initial glucose, a 25 % glucose solution was fed to the fermenter for 34 h with a rate of 6.5 ml/h. Glucose concentration was determined with test strips (Medi-Test Glucose, Macherey-Nagel, Germany) and kept below 2 g/l. Cell concentration (freeze-dried cell mass per l) was determined from samples taken at different time points as indicated. Total process time for both shaking and fermenter cultures was 168 h. For selection of transformants on agar plates, geneticin (G-418 sulfate, 100 μg/ml), hygromycin (60 μg/ml), or nourseothricin (30 μg/ml) was used. *Escherichia coli* strains DH5a and JM110 used for genetic manipulations were grown in LB medium containing ampicillin (100 μg/ml).

Stabilization of transformants by sporulation

Transformant PR1-104-(YB)2-HMGR-E-$(Zo)_1$ was cultivated overnight in 5 ml YPD medium (1.0 % yeast extract, 2.0 % peptone, 2.0 % glucose). Then, the cells were washed with sterile water and 1 ml of the solution was streaked on agar plates containing 0.5 % ribitol as the only carbon source which is known to induce sexual life cycle and sporulation of *X. dendrorhous* (Kucsera et al., 1998). Cells were incubated at 10 °C in the dark. After 5 weeks, slender holobasidia and basidiospores were observed by microscopy. After another 3 weeks, yellow colonies appeared which were transferred to 5 ml YPD medium and further cultivated as described above.

Carotenoid extraction, quantification, and HPLC analysis

Cells of *X. dendrorhous* were harvested and freeze-dried. Subsequently, the pulverized cells (20 mg) were mixed with approx. 500 μl glass beads (0.25–0.5 mm), 675 μl methanol, and 75 μl of a 60 % potassium hydroxide solution for saponification. After breaking in a cell mill (Retsch MM 400) for 8 min at a frequency of 30 per second, the extract was transferred into a separator funnel and the residual glass beads were washed three times with methanol. After addition of 10 ml of 50 % diethyl ether in petrol (bp 40–60 °C), 1 ml of water, and 1 ml of ethanol, the upper phase was collected, dried in a stream of nitrogen, and re-suspended in acetone. Carotenoids were quantified from three independently grown cultures and concentration values given together with their standard deviations. Analytical HPLC was performed in an isocratic way on a reversed phase 15 × 0.4 cm Nucleosil 100 C18, 3 μ column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as mobile phase with a flow rate of 1 ml/min at 20 °C. Spectra were recorded online with a photodiode array detector 994 (Waters, Milford, USA). Carotenoid identification was carried out with authentic standards.

Determination of *crtYB* and *crtZ* copy numbers

Genomic DNA was isolated from *X. dendrorhous* after breaking the cells with glass beads in the cell mill for 5 min at a frequency of 30 per second using classical phenol-chloroform extraction and isopropanol precipitation as described by Lecellier and Silar (1994). The purity was checked on 1 % agarose gels and the amounts quantified after ethidium bromide staining by densitometry. qRT-PCR was carried out in the Rotor Gene PCR Cycler 3000 (Corbett Life Science) with the Kapa Sybr Fast Universal Mix (Peqlab, Erlangen, Germany). For the *crtYB*, *crtZ*, $crtZ_o$, and *act* genes, the primers YB_fw: 5′-AGG GCT GAT CCC TCG ATA CC-3, YB_rv: 5′-GTC TCG ATA GGC GTC TTC CG-3′, Z_fw: 5′-TGG ATT GGC GCA GGT ATG AC-3′, Z_rv: 5′-ACC GTT TGA GGT AGC CCT TG-3′, Z.o_fw: 5′-GTC CAT CGA CGA TTC CCT AC-3′, Z.o_rv: 5′-CCC AGA GGA ATC CGA AAG AG-3′, act_fw: 5′-TGG CTC CTT GGC TCA TAT CC-3′, and act_rv: 5′-AGC ATC ATC TCC GGC GAA TC-3′ were used. The PCR cycles were 1 cycle of denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for

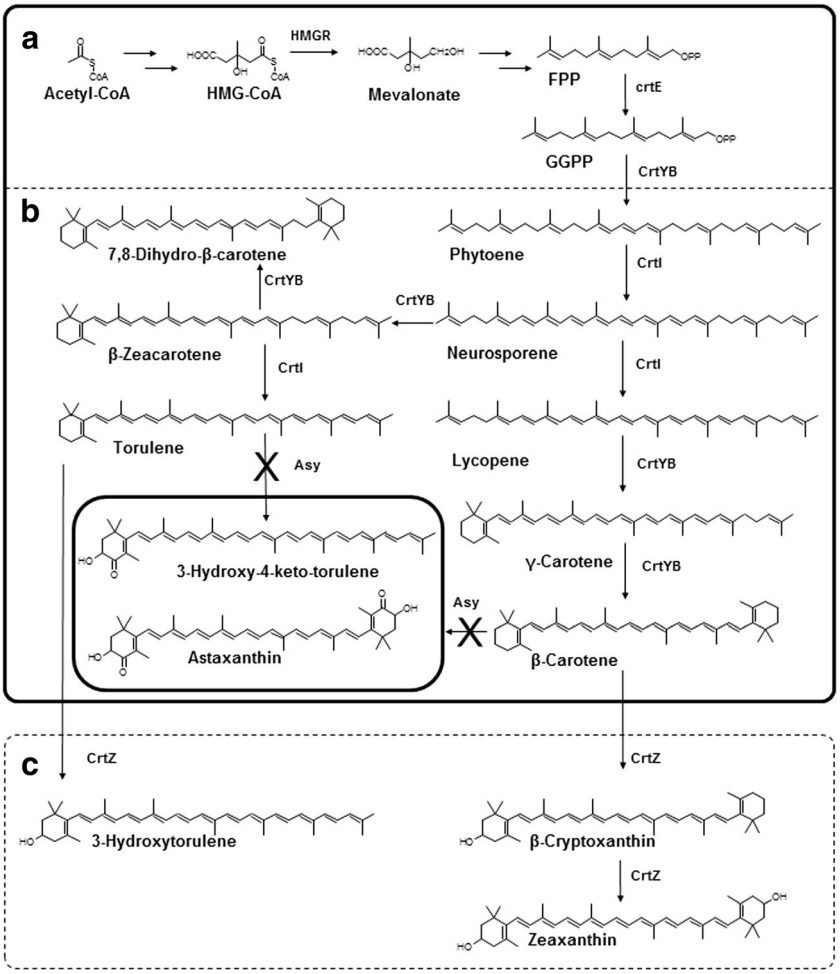


Fig. 1 The early steps (**a**) and specific reactions of carotenoid biosynthesis leading to astaxanthin or β-carotene in wild-type *Xanthophyllomyces dendrorhous* or in a mutant with blocked astaxanthin synthase (**b**). The reactions catalyzed by the β-carotene hydroxylase transgene *crtZ* are marked by a *dotted box* (**c**). The products of the over-expressed genes and inactive astaxanthin synthase (Asy) in the mutant are indicated

3 s, and extension at 60 °C for 20 s and a final melt curve with a range from 50 to 59 °C in 0.5 °C steps. For relative quantification according to Pfaffl (2001), ΔCT values and efficiency of amplification of the target gene were used. The copy numbers of the *act* gene were taken as reference normalized to two for a diploid yeast.

Plasmid construction and transformation

The plasmid pPR2TNo-crtZ was obtained by modification of pPRcDNA1crtZ (Ojima et al. 2006). From this plasmid, the G418 resistance cassette was cut out (*Pst*I/*Pst*I) and replaced by a cassette with the *nat* gene from *Streptomyces noursei* encoding nourseothricin acyl transferase (Krügel et al. 1988). After linearizing with *Hin*dIII, a second *crtZ* gene cassette was inserted yielding pPR2TNo-crtZ-crtZ. For the construction of plasmid pPR2TNo-crtZ$_o$, the vector set described by Gassel et al. (2014) was used. First, a codon-optimized version of the *crtZ* gene from *Brevundimonas* sp. strain SD212 (Choi et al. 2006) named *crtZ$_o$* was synthesized (GenBank Accession No. KX063854), cut with *Eco*RI and *Sal*I, and cloned into pUC8ΔEcoRI-HNNH for fusion with the promoter and terminator of the glyceraldehyde phosphate dehydrogenase gene. Then, the whole cassette was cut out by restriction with *Not*I and ligated with pPR2TNo yielding pPR2TNo-crtZ$_o$. The construction of the other plasmids used in this study was described elsewhere (see Table 1 for details and references). Transformation by electroporation was performed as described in Visser et al. (2005) with 1 μg plasmid DNA. After phenol-chloroform purification, plasmid DNA was linearized by digestion with *Cla*I. All integration plasmids are based on pPR2TN (Visser et al. 2005) possessing different selection markers and a large part of the ribosomal DNA sequences from *X. dendrorhous* for multi copy integration into the chromosomal ribosomal DNA of *X. dendrorhous*.

Results

For the transformation of a β-carotene mutant into a zeaxanthin producer, only the gene for a single enzyme is sufficient as indicated in Fig. 1c. Several structurally different β-carotene hydroxylases from bacteria, algae, and plants exist (Cui et al. 2013). For transformation of *X. dendrorhous*, we

Table 1 Plasmids used for genomic integration of carotenoids genes in *Xanthophyllomyces dendrorhous* PR1-104

Plasmid	Resistance	Carotenoid gene(s)	Reference
pPRcDNA1crtZ	Geneticin	*crtZ* (from *Pantoea ananatis*)	Ojima et al. 2006
pPR13F	Geneticin	*crtYB*	Verdoes et al. 2003
pPR2TN-YB-YB	Geneticin	*crtYB*, *crtYB*	Ledetzky et al. 2014
pPR2TNH-HMGR-crtE	Hygromycin	*HMGR*, *crtE*	Gassel et al. 2014
pPR2TNo-crtZ	Nourseothricin	*crtZ* (from *Pantoea ananatis*)	This work
pPR2TNo-crtZ-crtZ	Nourseothricin	*crtZ*, *crtZ* (from *Pantoea ananatis*)	This work
pPR2TNo-crtZ$_o$	Nourseothricin	*crtZo* (from *Brevundimonas* SD212; optimized for *X. dendrorhous*)	This work

first used a previously constructed plasmid with the *crtZ* gene from the bacterium *Pantoea ananatis* (formerly *Erwinia uredovora*) (Ojima et al. 2006).

In contrast to the wild type which accumulated mainly astaxanthin together with small amounts of keto-hydroxy-torulene and β-carotene (Fig. 2a), the β-carotene mutant PR1-104 synthesized two isomers of β-carotene as major products and torulene (Fig. 2b) as a by-product (Fig. 1b). Upon transformation with the *crtZ* gene, several hydroxylated carotenoids were synthesized (Fig. 2c). This included

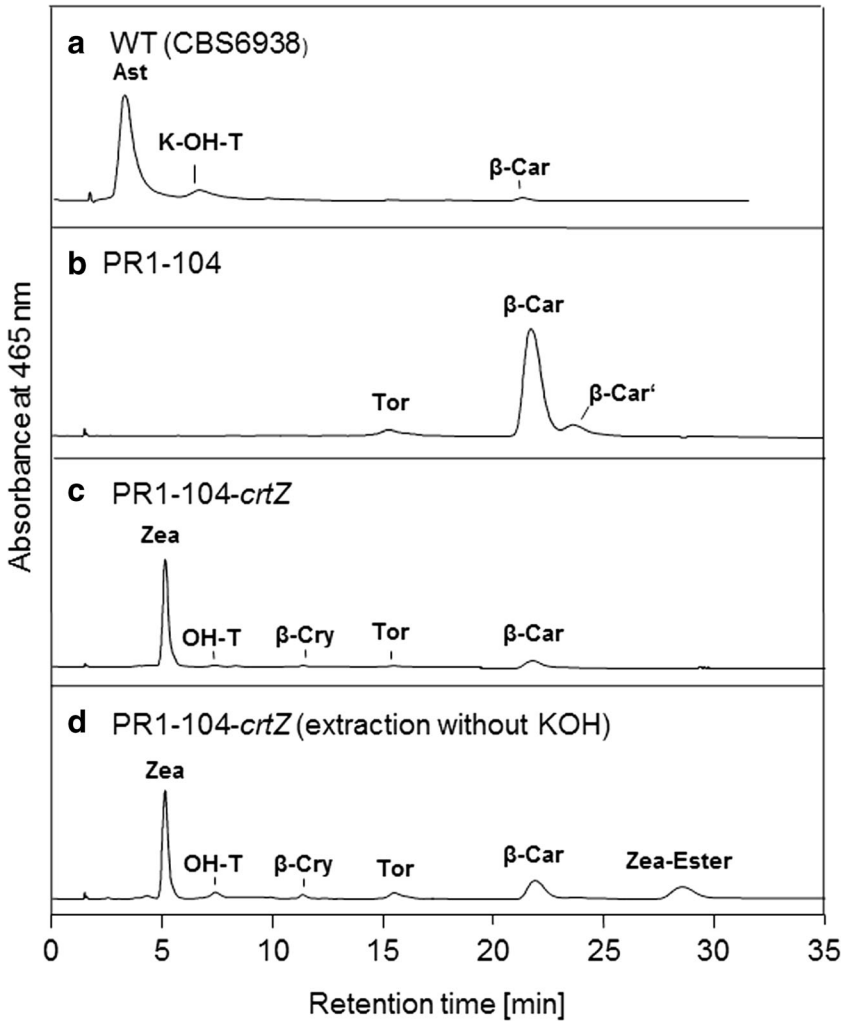


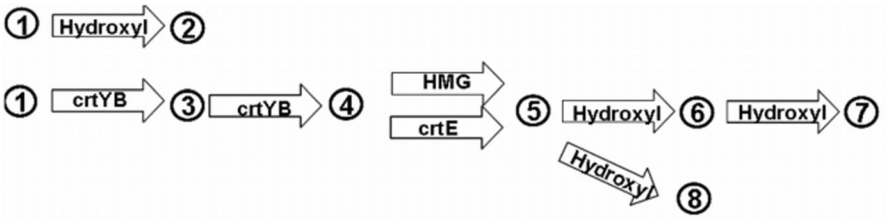
Fig. 2 HPLC separation of carotenoid extracts from *Xanthophyllomyces dendrorhous* wild type (WT) (**a**) and β-carotene accumulating mutant PR1-104 (**b**) and from the latter transformed with the β-carotene hydroxylase gene *crtZ* without (**c**) or after saponification (**d**). *Zea* zeaxanthin, *OH-T* hydroxy-torulene, *β-Cry* β-cryptoxanthin, *Tor* torulene, *β-Car* β-carotene, *β-Car'* β-carotene cis isomer, *Zea-Ester* zeaxanthin fatty acid este, *As* t astaxanthin, *K-OH-T* keto-hydroxy-torulene

zeaxanthin with the highest peak, the mono-hydroxylated intermediate β-cryptoxanthin, and the hydroxylated torulene. This extended pathway is shown in Fig. 1c. In non-saponified carotenoid extracts from this transformant, a zeaxanthin fatty acid ester is detectable (Fig. 2d).

Precursors for carotenoid synthesis are provided via the mevalonate pathway in *X. dendrorhous* (Fig. 1a). It has previously been shown that their synthesis is limited by 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMGR) and geranylgeranyl pyrophosphate synthase encoded by the *crtE* gene (Gassel et al. 2014). The gateway enzyme regulating the flux into carotenogenesis is phytoene synthase catalyzed by the product of the *crtYB* gene (Verdoes et al. 2003). Therefore, we followed a dual engineering strategy for the generation of a zeaxanthin producer with high yield. At first, the carotenoid synthesis activity of the β-carotene mutant was enhanced, then conversion of β-carotene into zeaxanthin was optimized (for strategy, see Table 2A). This included transformation with plasmids pPR13F (Verdoes et al. 2003) and pPR2TN-YB-YB with a either a single or two copies of *crtYB*. The transformants PR1-104-$(YB)_1$ and PR1-104-$(YB)_2$ carried three or four additional copies of *crtYB* in their genomes which resulted in an increasing β-carotene content (Table 2). The carotenoid composition compared to the wild type changed since torulene formation was suppressed and small amounts of 7,8-dihydro-β-carotene were synthesized (Fig. 3a). The latter transformant was further improved with respect to β-carotene accumulation by transformation with plasmid pPR2TNH-HMGR-CrtE for simultaneous integration of *HMGR* and *crtE*. The resulting transformant PR1-104-$(YB)_2$-HMGR-E synthesized 3.4 times more carotenoids than the non-transformed mutant and its β-carotene content was 2.6-fold increased.

As a next step, PR1-104-YB_2-HMGR-E was again transformed with different *crtZ* containing plasmids containing either one or two copies of this gene. Upon transformation with the single-copy *crtZ* plasmid, only one copy of this gene was integrated into the genome resulting in the formation of only trace amounts of zeaxanthin for PR1-104-$(YB)_2$-HMGR-E-$(Z)_1$ (Fig. 3c, Table 2B). Using the plasmid with two *crtZ* copies, four copies of *crtZ* were genome integrated. Now, zeaxanthin appeared as the major carotenoid (Table 2B)

Table 2 Sequential transformation (A) and carotenoid composition of resulting transformants grown in shaking flasks (B)



# PR strains	Carotenoids (µg/g dw)							Integrated copies	
	TCar	Zeax	HO-Tor	β-Cry	Tor	β-Car	D-Car	crtYB	crtZ
1. 104	235±7	0	0	0	9±1	226±8	0	0	0
2. 104-Z	248±30	207±22	4±1	4±0	4+1	29±8	0	0	–
3. 104-$(YB)_1$	467±9	0	0	0	0	312±25	154±33	3	0
4. 104-$(YB)_2$	549±21	0	0	0	0	406±21	143±3	4	0
5. 104-$(YB)_2$-HMG-E	800±22	0	0	0	0	576±8	223±16	4	0
6. 104-$(YB)_2$-HMG-E-$(Z)_1$	799±32	19±10	0	10±3	0	530±32	240±53	4	1
7. 104-$(YB)_2$-HMG-E-$(Z)_2$	792±52	200±19	0	14±5	0	341±47	236±80	4	4
8. 104-$(YB)_2$-HMG-E-$(Zo)_1$	760±31	517±17	0	28±11	0	131±28	84±9	4	4

The numbering in parts A and B correspond to each other.

TCar, total carotenoids; Zeax zeaxanthin; HO-Tor, 3-HO-torulene; β-Cry, β-cryptoxanthin, Tor, torulene; β-Car, β-carotene;

D-Car, 7,8-dihydro-β-carotene; crtYB, phytoene synthase / lycopene cyclase gene, crtZ, β-carotene hydroxylase gene; Zo, β-carotene hydroxylase codon optimized; HMG, hydroxyl-methylglutaryl-CoA reductase gene; crtE, geranylgeranyl pyrophosphate synthese gene; - not determined.

The numbering in parts A and B corresponds to each other. Strain #9 stabilized for zeaxanthin formation by selection from basidiospores of #8. All cultures in shaking flasks contain corresponding selection agents except for #8* and #9*

TCar total carotenoids, *Zeax* zeaxanthin, *HO-Tor* 3-HO-torulene, *β-Cry* β-cryptoxanthin, *Tor* torulene, *β-Car* β-carotene, *D-Car* 7,8-dihydro-β-carotene, *crtYB* phytoene synthase/lycopene cyclase gene, *crtZ* β-carotene hydroxylase gene, *Zo* codon-optimized β-carotene hydroxylase, *HMGR* hydroxyl-methylglutaryl-CoA reductase gene, *crtE* geranylgeranyl pyrophosphate synthase gene, – not determined

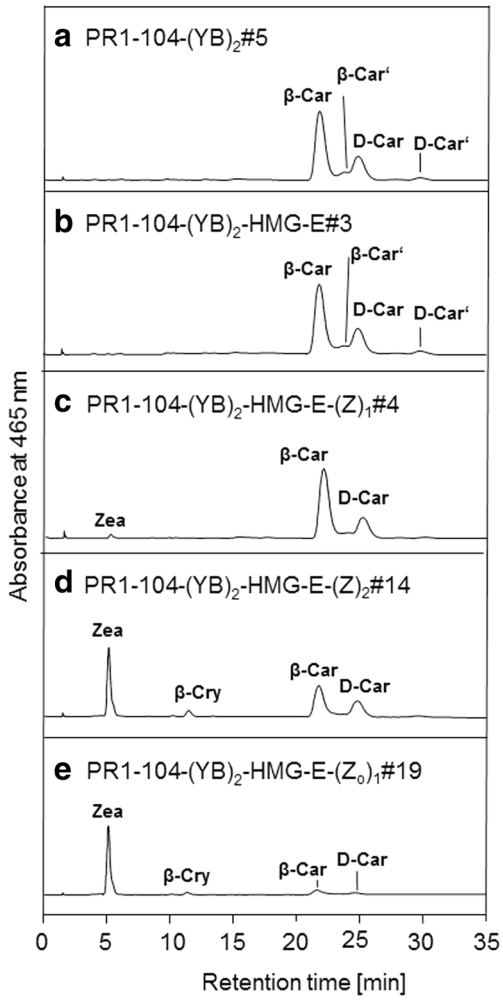


Fig. 3 HPLC profile of carotenoids in *Xanthophyllomyces dendrorhous* mutant PR1-104 with stepwise over-expression of the *crtYB* gene (**a**), *crtE* gene (**b**), single-transformed (**c**) or double-transformed (**d**) genuine *crtZ* gene, or single-transformed with a codon-optimized *crtZ* gene (**e**). Abbreviations as in Fig. 2; *D-Car* 7,8-dihydro-β-carotene

together with small amounts of the intermediate β-cryptoxanthin (Fig. 3d). In this transformant, the zeaxanthin share with 25 % of total carotenoid was less satisfactory. In order to increase this conversion rate, a plasmid with *crtZ* from *Brevundimonas* SD212 codon optimized for *X. dendrorhous* was constructed for the transformation of *X. dendrorhous* since it showed superior zeaxanthin formation in a heterologous host (Choi et al. 2006). Its use resulted in a new transformant PR1-104-$(YB)_2$-HMGR-E-$(Zo)_1$ with 517 μg/g dw zeaxanthin when grown as shaking flask cultures. This represents 68 % of total carotenoids and corresponds to a conversion rate for β-carotene of 80 % (Table 2B). All cultures of this directly obtained final transformant had to be supplemented with the selection agents for each of the integrated plasmids (see Table 1). Otherwise, total carotenoid content was almost half and conversion of β-carotene to zeaxanthin decreased to 27 %. We attempted to stabilize total carotenoid and zeaxanthin formation by passing through a sexual reproductive cycle with sexual conjugation between cells of the same strain and basidiospore formation. Finally, cultures from germinating basidiospores were collected and high-pigmented colonies selected. When the resulting strain PR1-104-$(YB)_2$-HMGR-E-$(Zo)_1$S with the highest zeaxanthin content was grown in the absence of any selection marker, contents of total carotenoids and of zeaxanthin were within 80 to 90 % compared to the non-stabilized culture in a medium with selection marker (Table 2B). In addition, all resistances were maintained. However, the copy numbers of the *crtYB* and the *crtZ* genes decreased from ten to eight and from four to two, respectively (Table 2B). This relates to the moderate decrease of total carotenoids and the conversion rate for β-carotene.

Experimental small-scale fermenter studies were carried out mainly to determine the degree of stability of zeaxanthin formation with the stabilized transformant under production conditions in the absence of selection pressure. A representative experiment is shown in (Fig. 4). Since *X. dendrorhous* is a mesophilic fungus, fermenter temperature was kept at 21 °C. Under our conditions, a cell density of about 24 g/l was reached compared to about 5 mg/l in shaking flasks (Fig. 4). After starting with a glucose concentration of 10 g/l, glucose feeding (25 % solution at a rate of 6.5 ml/h) between 18 and 52 h of growth kept the concentration around 2 g/l before it depleted afterwards. At the end of glucose feeding, cells changed from the linear to the stationary growth phase. Concurrently, oxygen saturation increased from 40 to 70 %. Although cell growth was stalled, zeaxanthin formation doubled during the stationary phase to 10.8 mg/l. This corresponds to 460 μg/g dw. Also, conversion of β-carotene to zeaxanthin was increased (Fig. 4) due to the higher oxygen concentration in the fermenter. This exploratory fermentation study provided basic conditions to start from in future large-scale fermentations.

Discussion

Similar to wild-type *X. dendrorhous*, a β-carotene accumulating mutant could be used to exploit the carotenoid biosynthesis potential for zeaxanthin production. Although the last step of the pathway in this mutant PR1-104, the formation of astaxanthin is missing (Fig. 1b), the rest of the pathway must be regulated similar to wild type since over-expression of the genes *HMGR*, *crtE*, and *crtYB* had the same effect resulting in enhanced carotenoid biosynthesis (Gassel et al. 2014).

The use of a bacterial β-carotene hydroxylase gene was successful for the extension of the carotenoid pathway to zeaxanthin. The efficiency of β-carotene conversion was a limited step which is depended on the number of genome integrated *crtZ* genes. Also, the optimization of the codons for *X. dendrorhous* improved zeaxanthin formation (Table 2B). This higher activity compared to the activity of the

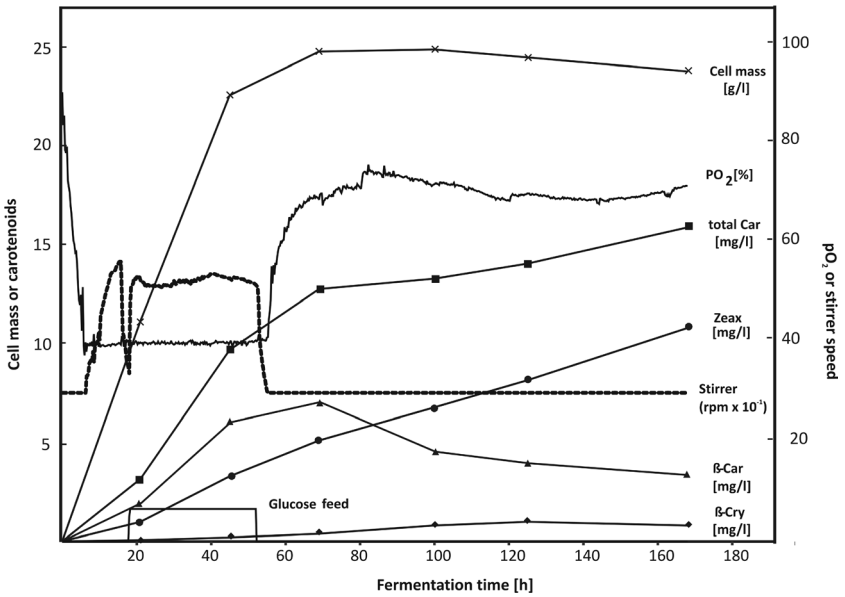


Fig. 4 Growth and carotenoid production of *Xanthophyllomyces dendrorhous* transformant PR1-104-$(YB)_2$-HMGR-E-$(Zo)_1$S in a bioreactor. Glucose feed was performed with a 25 % solution at a rate of 6.5 ml/h

transformant with non-optimized *crtZ* (and the same number of integrated copies) indicates that enhanced translation of *crtZ* led to a less limited protein synthesis which corresponds to higher enzymatic activity in this transformant. Due to very low accumulation of the mono-hydroxylated intermediate β-cryptoxanthin, we can assume that the second hydroxylation step converting this carotenoid to zeaxanthin (Fig. 1c) is faster than the initial one.

The carotenoid accompanying astaxanthin is keto-hydroxy-torulene which in the mutant lacking oxygenase activity corresponds to unsubstituted torulene (Figs. 1b and 2a, b). Upon enhancement of the pathway by over-expression of *crtYB*, the torulene by-product disappeared (Fig. 3a). This is due to the special feature of *crtYB* which encodes a phytoene synthase together with a lycopene cyclase (Verdoes et al. 1999). Upon over-expression, the higher lycopene cyclase activity in our mutant diverts the flow within the carotenoid pathway toward β-carotene away from the route to torulene (Fig. 1b). Another effect of the higher lycopene cyclase activity is cyclization of neurosporene via β-zeacarotene to 7,8-dihydro-β-carotene. In non-saponified carotenoid extracts of the β-carotene mutant transformed with *crtZ*, a small portion of zeaxanthin fatty acid ester was detectable. This indicates that a non-specific esterase in *X. dendrorhous* is capable of metabolizing the newly formed zeaxanthin to some extent.

For the *crtYB* and the *crtZ* genes, we used transformation plasmid containing one or two gene copies for genome insertion. Since our integration plasmids possess a site for multiple integration at the rDNA (Wery et al. 1997), several additional copies of a gene can be integrated into the genome of *X. dendrorhous*. The number of newly integrated copies that was reached was four for both genes *crtYB* and *crtZ* (Table 2B). In case of *crtYB*, this copy number resembles the same maximum number obtained with wild-type *X. dendrorhous* (Ledetzky et al. 2014). For both genes, we found a tendency that higher numbers of integrated copies increase metabolic activity. However, the relationship is not linear. Especially after recombination, lower copy numbers still resulted in substantial total carotenoid and zeaxanthin formation (Table 2B). Since we selected a line with the highest zeaxanthin formation from the basidiospores, we assume that the position of integration may be as important for transcription as the copy number.

Finally, our engineering strategy to increase metabolite flow into carotenoid biosynthesis which involved pathway extension by a hydroxylase and the application of a stabilization procedure through a sexual cycle resulted in a fermentable *X. dendrorhous* transformant. Total carotenoid and zeaxanthin concentrations and conversion of β-carotene to zeaxanthin were comparable in the flask and in the fermenter cultures (Table 2, Fig. 4). In the fermenter, growth behavior and cell productivity of our transformant corresponded to other studies with wild-type non-transformed *X. dendrorhous* (Yamane et al. 1997).

There is a debate but no direct proof whether homothallic *X. dendrorhous* is a haploid (Wery et al. 1997) or diploid (Kucsera et al. 1998) growing yeast. Although genome data indicated haploid vegetative growth for the *X. dendrorhous* strain from which our zeaxanthin mutant was derived (Sharma et al. 2015), instability in the absence of selection markers and successful stabilization of our transformant can best be explained for diploid vegetative *X. dendrorhous* cells. Thus, instability may be caused by mitotic recombination (LaFave and Sekelsky 2009) within the heterozygous cells eliminating the cassette with the integrated carotenogenic gene and the resistance gene. In a sexual cycle, homozygous lines for the integrated genes (carotenogenic genes as well as resistance genes) with a stable phenotype are generated through meiosis via a tetraploid zygote and segregation from

germinating diploid basidiospores (Kucsera et al. 1998). The decrease of the copy numbers for both the *crtYB* and the *crtZ* genes (Table 2B) indicates that a recombination process has occurred.

The amount of zeaxanthin accumulation is superior to foods like orange pepper with about 170 μg/g dw, yellow maize with 5 μg/g dw (Perry et al. 2009), and transgenic potato with 40 mg/g dw (Römer et al. 2002). The only known organisms with a higher zeaxanthin content than our transformant are bacteria like the cyanobacterium *Synechococcus* PCC7942 which contains 1.7 mg/g dw (Schäfer et al. 2006) or *Muricauda* species with 1.5 mg/g dw (Prabhu et al. 2013). The highest zeaxanthin producer to date is an engineered *E. coli* strain with 11 mg/g dw (Li et al. 2015). Similar concentration is within reach for *X. dendrorhous* by optimized media and fermenter conditions. This has been shown with *X. dendrorhous* transformants engineered for enhanced astaxanthin synthesis which yielded up to 22-fold higher carotenoid content compared to shaking cultures (Gassel et al. 2013). Furthermore, high astaxanthin-producing mutants with a total carotenoid about 20-fold higher than the wild type and the PR1-104 mutant are available and can be further engineered for higher carotenoid production (Gassel et al. 2014). Similar mutants could be used to modify the carotenoid pathway to zeaxanthin formation. With this type of mutants, there are good prospects to generate a stable zeaxanthin producer with much higher yield by following the engineering strategies developed in this publication.

Acknowledgments This work was supported by the ERA IB project PROCAR by the German Federal Ministry of Education and Research (BMBF) (FKZ 031A569). Additional funding was through the LOEWE Project "Integrative Pilzforschung."

Compliance with ethical standards

Funding This study was funded by BMBF (FKZ 031A569).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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