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Genetic engineering of the complete carotenoid pathway towards enhanced astaxanthin formation in *Xanthophyllomyces dendrorhous* starting from a high-yield mutant

Sören Gassel · Jürgen Breitenbach · Gerhard Sandmann

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Abstract The yeast *Xanthophyllomyces dendrorhous* is one of the rare organisms which can synthesize the commercially interesting carotenoid astaxanthin. However, astaxanthin yield in wild-type and also in classical mutants is still too low for an attractive bioprocess. Therefore, we combined classical mutagenesis with genetic engineering of the complete pathway covering improved precursor supply for carotenogenesis, enhanced metabolite flow into the pathway, and efficient conversion of intermediates into the desired end product astaxanthin. We also constructed new transformation plasmids for the stepwise expression of the genes of 3-hydroxymethyl-3-glutaryl coenzyme A reductase, geranylgeranyl pyrophosphate synthase, phytoene synthase/lycopene cyclase, and astaxanthin synthase. Starting from two mutants with a 15-fold higher astaxanthin, we obtained transformants with an additional 6-fold increase in the final step of pathway engineering. Thus, a maximum astaxanthin content of almost 9 mg per g dry weight was reached in shaking cultures. Under optimized fermenter conditions, astaxanthin production with these engineered transformants should be comparable to *Haematococcus pluvialis*, the leading commercial producer of natural astaxanthin.

Keywords Astaxanthin · Carotenoid pathway · Pathway engineering · *Xanthophyllomyces dendrorhous*

S. Gassel · J. Breitenbach · G. Sandmann (✉)
Institute of Molecular Bioscience, J.W. Goethe Universität, Max von Laue Str. 9, 60438 Frankfurt, Germany
e-mail: sandmann@bio.uni-frankfurt.de

Introduction

The carotenoid astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) is a commercially important feed ingredient in salmon and trout farming. Its health-promoting activities to humans are due to its high antioxidative potential—a reason why astaxanthin is gaining importance in the health food sector. To date, the market is dominated by chemically synthesized astaxanthin. However, biological systems are currently developed and improved. There are only very few organisms known which are able to synthesize astaxanthin or its fatty acid and glycosylated derivatives. They include strains from the bacterium *Paracoccus* (Misawa et al. 1995), the alga *Haematococcus pluvialis* (Margalith 1999), only one fungus *Xanthophyllomyces dendrorhous* (=sexual state of *Phaffia rhodozyma*) (Andrews et al. 1976), and among plants only those of the genus *Adonis* (Seybold and Goodwin 1959). Among them, *H. pluvialis* is the primary source for natural astaxanthin which is approved as food supplement (Lorenz and Cysewski 2000). Commercially grown cells of this alga contain astaxanthin in about 1.5 to 3 % of their dry matter whereas astaxanthin in wild-type strains of *X. dendrorhous* is below 0.1 % (Johnson and Schroeder 1995). However, chemical mutants have been generated with substantially increased astaxanthin content (see Rodriguez-Saiz et al. 2010 and Schmidt et al. 2011 for review). For further improvement of astaxanthin formation, it is an advantage of *X. dendrorhous* that its carotenoid biosynthesis pathway can be genetically manipulated. All necessary pathway genes have been cloned. They include those encoding a phytoene synthase/lycopene cyclase (Verdoes et al. 1999a), a phytoene desaturase (Verdoes et al. 1999b), and an astaxanthin synthase (Ojima et al. 2006). After transformation and plasmids

had been developed (Wery et al. 1998), first attempts have been made to evaluate and to overcome limiting steps in the pathway. It could be demonstrated that overexpression of the gateway enzyme phytoene synthase, CrtYB, stimulated carotenogenesis (Verdoes et al. 2003) as did the overexpression of the enzyme geranylgeranyl pyrophosphate synthase CrtE which is especially limited under high oxygen conditions (Breitenbach et al. 2011).

As a strategy for the increase of astaxanthin formation in *X. dendrorhous*, a combination of classical mutagenesis and genetic pathway engineering was suggested (Schmidt et al. 2011). The feasibility of this approach has been recently demonstrated with promising results by further increasing carotenoid synthesis in two high-yield astaxanthin-producing mutants by engineering the metabolite flow into the carotenoid pathway (Gassel et al. 2013). Therefore, in the present investigation, we started from these *X. dendrorhous* mutants and overexpressed systematically the limiting enzymes step by step thus increasing the metabolite provision for the pathway and improving the intermediate conversion to the end product astaxanthin. The resulting astaxanthin yields were the highest reached to date in laboratory cultures and may be further increase under optimized fermentation conditions (Gassel et al. 2013).

Materials and methods

Strains and cultivation

X. dendrorhous (=sexual state *P. rhodozyma*) strain ATCC96594 (=CBS6938) was grown in shaking cultures (50 ml in 500 ml baffled Erlenmeyer flasks, 180 rpm, over 8 days) at 20 °C. A modified GSM medium (de Boer et al. 1993) with a 10-fold increased $CuSO_4$ and a 4.8-fold increased biotin content was used. This medium and the reddish pigment astaxanthin mutants AXG-13 and AXJ-20 with 15-fold improved astaxanthin accumulation relative to the wild type obtained by mutagenesis with nitrosoguanidine have been described recently (Gassel et al. 2013). Transformation of *X. dendrorhous* was as described (Visser et al. 2005). Selection of transformants was with geneticin (G418 sulfate, 100 μ/ml), hygromycin (60 μg/ml), or nourseothricin (30 μg/ml) on agar plates.

Escherichia coli strains DH5α and JM110 used for genetic manipulations and plasmid amplification were grown in LB medium with the antibiotic ampicillin (100 μg/ml).

Genetic manipulations

Plasmid pUC8ΔEcoRI-HNNH was constructed starting with the elimination of the *Eco*RI site from pUC8 (Hanna et al. 1984) by linearization with *Eco*RI, blunting and religation yielding pUCΔEcoRI. The promoter–terminator cassette was constructed by fusion PCR with pPR2TN as template and primers used before (Ojima et al. 2006) with some modifications: PGPD1 shortened for ATATT, PGPD2, and TGPD1 without modification and TGPD2 in which the AGCTAAGCTT end was replaced by GCGGCCGC creating a *Not*I restriction site. This promoter–terminator fusion product was then ligated into the T-overhang vector pMON38201 (Borovkov and Rivkin 1997), cut out with *Hin*dIII, and ligated into the *Hin*dIII site of pUCΔEcoRI. Plasmids pPR2TNH with hygromycin as resistance marker and pPR2TNN with nourseothricin resistance were obtained by modification of pPR2TN (Visser et al. 2005). In this plasmid, the G418 (geneticin) resistance cassette containing the *nptII* gene from *Micromonospora rhodorangea* was cut out (*Pst*I/*Pst*I) and replaced by the *hph* gene from *E. coli* encoding hygromycin phosphotransferase (Gritz and Davies 1983) or the *nat* gene from *Streptomyces noursei* encoding nourseothricin acyl transferase (Krügel et al. 1988), respectively. After transformation of *X. dendrorhous*, gene integration was controlled by PCR.

Carotenoid extraction and analysis

Cells of *X. dendrorhous* were harvested and freeze-dried. Carotenoids were subsequently extracted from these cells with dimethyl sulfoxide by heating for 15 min at 60 °C. After centrifugation, the extract was transferred into a separator funnel, diethyl ether was added, and the mixture was kept on ice for 3 min before water was added for phase separation. The upper phase was collected to which step by step acetone, 10 % (*v*/*v*) diethyl ether in petroleum ether (bp 60–80 °C), and water were added. Again, the upper phase was collected, washed with water, dried in a stream of N_2, and resuspended in acetone. Details of the extraction procedure are given elsewhere (Visser et al. 2005). High-performance liquid chromatography (HPLC) separation of carotenoids was performed in an isocratic way on a 15-cm Nucleosil 100 C18, 5 μ column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase at 20 °C. Spectra were recorded online with a photodiode array detector 440 (Kontron, Straubenhard, Germany). Carotenoid identification and quantification was carried out with authentic standards. They were generated by genetic engineering of *E. coli* leading to the reconstruction of pathway to the desired carotenoids astaxanthin, HO-canthaxanthin, keto-hydroxy-torulene, HO-echinenone, echinenone, torulene, and β-carotene (Sandmann 2002). Carotenoids were quantified from five independently grown cultures and are given together with their standard deviations.

Results

Transformation vectors

Integration into the genome is generally preferred for engineering of organisms to increase stability, reduce the effect of plasmid burden, and to avoid the need for selective agents in final industrial processes. However, there is a limitation of integration vectors available for *X. dendrorhous*. Previously, the integration plasmid pPR2TN with G418 as selection marker has been used (Visser et al. 2005). To expand the availability of selectable markers and allow integration of several inserts into one strain, we constructed a new set of plasmids by exchanging the G418 resistance for hygromycin and nourseothricin resistance obtaining pPR2TNH and pPR2TNN, respectively (Fig. 1). The three resistance genes are under the control of the glyceraldehyde phosphate dehydrogenase (GAPDH) promoter and terminator, and the rest of the plasmid are identical. Multicopy integration is achieved through the 18S-ribosomal DNA sequence from *X. dendrorhous*. The multiple cloning regions of these plasmids comprise the *Eco*RI/*Xho*I/*Not*I restriction site. For insertion of any gene or cDNA of interest, we also constructed a pUC8-based modification plasmid pUC8ΔEcoRI-HNNH (Fig. 1). By insertion into its *Eco*RI/*Xho*I sites, the GAPDH promoter and terminator are fused to the reading frame. The flanking *Bam*HI, *Not*I, and *Hin*dIII sites allow the excision of the whole cassette and the insertion of up to two gene cassettes into any of the three integration plasmids. Thus, tandem genes in three plasmids allow the insertion of six genes for pathway manipulation of *X. dendrorhous*. All three plasmids were used for genetic pathway engineering by insertion of genes of the early step of prenyl pyrophosphate synthesis and late steps of the carotenoid biosynthesis pathway.

In order to overcome the limitation of carotenoid biosynthesis in *X. dendrorhous* which initially is phytoene synthesis catalyzed by CrtYB and then astaxanthin synthesis from β-carotene catalyzed by Asy after *crtYB* overexpression (Verdoes et al. 2003), we constructed pPR2TN-YB-ASY with geneticin as selection marker for the simultaneous integration of the *crtYB* and the *asy* gene as a first step. Starting from pPR2TN (Fig. 1), the entire promoter–terminator cassettes with the *asy* cDNA from pPR2TN2BPAT (Ojima et al. 2006) and the *crtYB* gene cassette from pPR13F (Verdoes et al. 2003) were inserted. Both gene products, *crtYB* and *asy*, should enhance the metabolite flow into the carotenoid pathway by higher phytoene synthase activity, a more active cyclization and a better conversion of β-carotene and all ketolated intermediates into astaxanthin (Fig. 2).

Metabolite flow into carotenogenesis can be improved by overexpression of the key enzymes hydroxymethyl glutaryl coenzyme A reductase of the mevalonate pathway in *X. dendrorhous* (Shimada et al. 1998) and by overexpression of geranylgeranyl pyrophosphate synthase (CrtE) for substrate provision to phytoene synthase (Breitenbach et al. 2011). The *crtE* gene and the 3-hydroxymethyl-3-glutaryl coenzyme A (HMG-CoA) reductase gene (*HMG*) from *X. dendrorhous* (Verwaal et al. 2007) were first cloned into pUC8ΔEcoRI-HNNH (Fig. 1) for fusion with the promoter and terminator of glyceraldehyde phosphate dehydrogenase and the whole cassettes cut out. Insertion of both cassettes into pPR2TNH with hygromycin as selection marker (Fig. 1) yielded pPR2TNH-HMG-CrtE. The hydroxymethyl glutaryl coenzyme A reductase gene was used as a truncated form lacking the membrane-binding region. For additional increase of Asy activity, plasmid pPR2TNN-asy with nourseothricin as selection marker was obtained by insertion of the *asy* cDNA cassette with the *asy* cDNA into pPR2TNN. This resulting plasmid was used in the third transformation step.

Transformants of mutants AGX-13 and AXJ-20

Two high-astaxanthin mutants, AXG-13 and AXJ-20, were selected for carotenoid pathway engineering since it has been shown that phytoene synthase and astaxanthin synthase were still limiting in both of them (Gassel et al. 2013). Therefore, plasmid pPR2TN-YB-asy with G418 selection had been constructed and was used for the transformation of both mutants as a first step. Several transformants were selected and analyzed for carotenoid composition. The best lines with respect to astaxanthin contents are shown in Figs. 3 and 4. The best crtYB-asy transformants from AXG-13 and AXJ-20 were AXG#5 with 3.1 mg/g dw and AXJ#37 with 3.7 mg/g dw astaxanthin, respectively. Enhanced provision of precursors for carotenogenesis via the mevalonate pathway was attempted by overexpressing the HMG-CoA reductase gene (*HMG*) in combination with the geranylgeranyl pyrophosphate synthase gene *crtE* as outlined in Fig. 2. Therefore, in the next stage of pathway engineering, we used plasmid pPR2TNH-HMG-CrtE with hygromycin selection to enhance precursor supply for the carotenoid pathway. For this purpose, AXG#5 and AXJ#37 were used. These transformations resulted in a further increase of the astaxanthin yield reaching 5.2 mg/g dw in AXG#521 and in AXG#522, 4.7 mg/g dw in AXJ#3726 and 5.8 mg/g dw in AXJ#3728. Detailed analysis of the carotenoid composition of the double transformants AXG#522 and AXJ#3728 showed that only 52 and 61 % of the astaxanthin precursor carotenoids were finally metabolized into astaxanthin (Table 1). In addition, HO-canthaxanthin and HO-echinenone which are substrates of Asy accumulate in the double transformants to a higher percentage compared to the single transformants. This

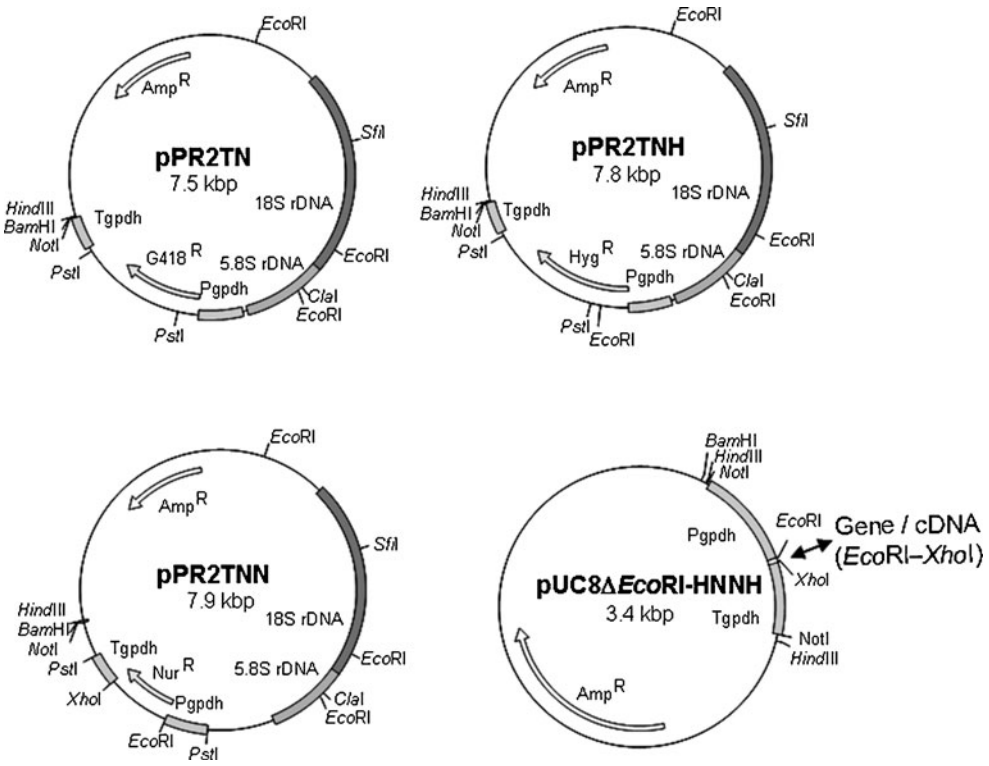

Fig. 1 Modification and transformation vectors for the genetic engineering of *X. dendrorhous*. P_{GAPDH} promoter of glyceraldehyde phosphate dehydrogenase, T_{GAPDH} terminator of glyceraldehyde phosphate dehydrogenase, $G418^R$ G418 resistance gene, Hyg^R hygromycin resistance gene, Nur^R nourseothricin resistance gene, Amp^R ampicillin resistance gene, *rDNA* ribosomal DNA

result indicated that although Asy was overexpressed in the first transformation round, there was still a limitation in the activity of this enzyme. In order to strengthen the metabolization of β-carotene, 3-HO-canthaxanthin, echinenone, and 3-HO-echinenone into astaxanthin, plasmid pPR2NN-asy containing the *asy* cDNA with nourseothricin selection was used for additional integration and expression in AXG#522 and AXJ#3728. After this third transformation round, the selected transformants showed a higher astaxanthin content. They were AXG#5221 with 8.0, AXG#5223 with 6.5 mg/g dw, AXJ#37284 with 8.7 mg/g dw and AXJ#37287 with 7.8 mg/g dw astaxanthin (Figs 3 and 4). In all of them, the higher additional expression of Asy decreased the intermediate levels resulting in a higher percentage of astaxanthin per total carotenoids and a higher absolute amount in the cells. Data for AXG#5221 and AXJ#37287 are shown in Table 1. Integration of additional copies of *asy* in the third transformation round was demonstrated by Southern hybridization with a probe against the nourseothricin resistance gene as integration marker.

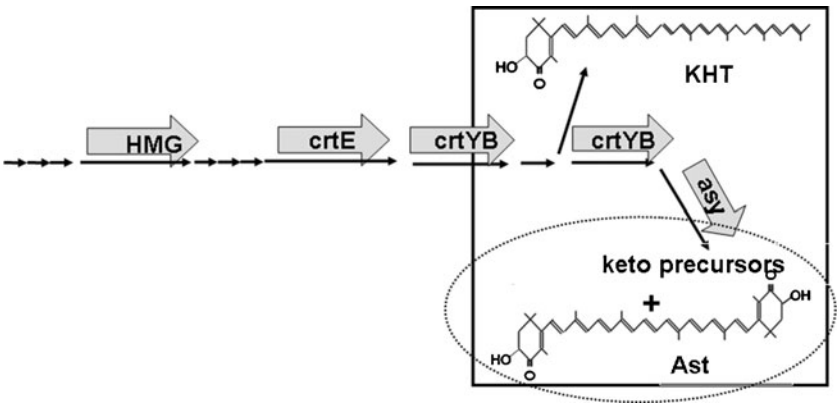

Fig. 2 Strategies for genetic modification of carotenoid biosynthesis for enhanced astaxanthin formation in *X. dendrorhous* by expression of limiting genes in the pathway. The specific carotenoid pathway is *boxed* the reactions catalyzed by astaxanthin synthase encoded by the *asy* gene is *circled*. From the early mevalonate pathway and formation and interconversion of prenyl pyrophosphates, the overexpressed genes were those for 3-hydroxymethyl-3-glutaryl coenzyme A reductase (*HMG*) and geranylgeranyl pyrophosphate synthase (*crtE*). Gene *crtYB* encodes a bifunctional phytoene synthase/lycopene cyclase. *KHT* keto-hydroxytorulene, *Ast* astaxanthin

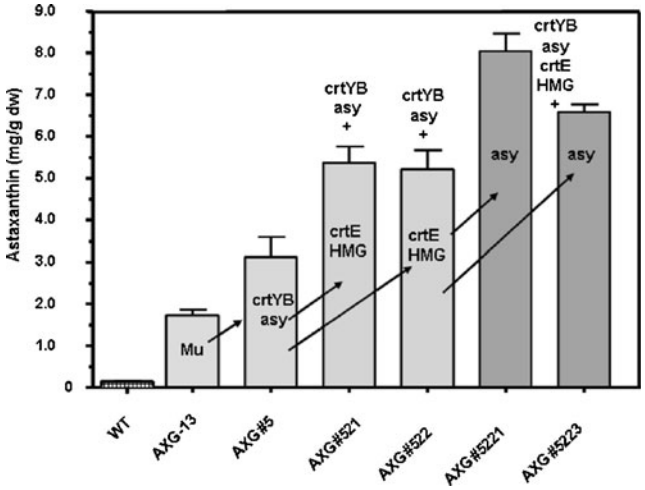

Fig. 3 Astaxanthin formation after several modification steps of mutant AXG-13 resulting from chemical mutagenesis and selection for high astaxanthin content. Concentrations are means of determinations±standard deviation from five individual cultures. *Arrows* indicate which of the strains and *lines* was used for stepwise transformation starting with AXG-13; gene combinations on *top* represent the transgenic background of the recipient strain. *Mu* mutant, *crtYB* phytoene synthase/lycopene cyclase gene, *asy* astaxanthin synthase gene, *crtE* geranylgeranyl pyrophosphate synthase gene, *HMG* 3-hydroxymethyl-3-glutaryl coenzyme A reductase gene

Discussion

Over decades, attempts have been made to generate high-yield astaxanthin mutants by chemical mutagenesis (Rodriguez-Saiz et al. 2010; Schmidt et al. 2011). Although significant progress has been made, the levels reached were not sufficient

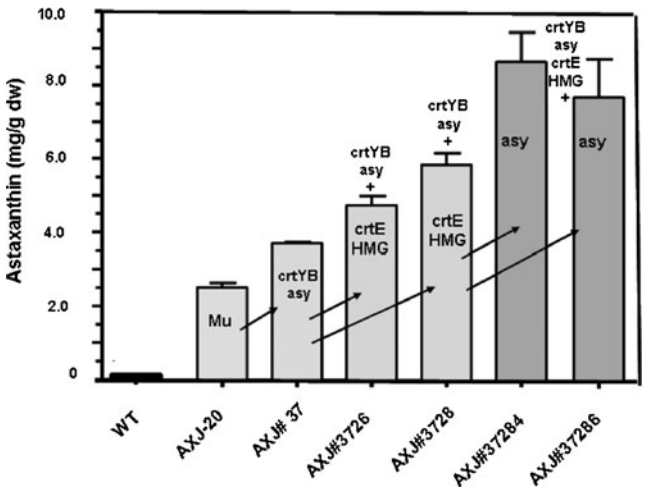

Fig. 4 Astaxanthin formation after several modification steps of mutant AXJ-20 resulting from chemical mutagenesis and selection for high astaxanthin content. Concentrations are means of determinations±SD from five individual cultures. *Arrows* indicate which of the strains and *lines* was used for stepwise transformation starting with AXJ-20; gene combination on *top* represents the transgenic background of the recipient strain. *Mu* mutant, *crtYB* phytoene synthase/lycopene cyclase gene, *asy* astaxanthin synthase gene, *crtE* geranylgeranyl pyrophosphate synthase gene, *HMG* 3-hydroxymethyl-3-glutaryl coenzyme A reductase gene

Table 1 Carotenoid distribution in transformants of *X. dendrorhous* mutants AXG-13 and AXJ-20

	AXG-13		AXJ-20	
	#522	#5221	#3728	#37284
Astaxanthin content (mg/g dw)	5.2±0.8	9.0±0.7	5.8±0.6	8.6±1.4
% Distribution				
Astaxanthin	52	64	61	69
HO-canthaxanthin[a]	14	0	9	0
Keto-hydroxy-torulene	5	7	6	6
HO-echinenone[a]	14	13	12	10
Echinenone[a]	5	6	4	4
Torulene	0	1	0	7
β-Carotene[a]	9	7	6	4

Astaxanthin content as means of determinations±SD from five individual cultures

[a] Substrate or intermediates which can be converted by astaxanthin synthase into astaxanthin

for commercial astaxanthin production. Two of those mutants, AXG-13 and AXJ-20, have been recently analyzed. In addition to their increased astaxanthin content, it was shown that phytoene synthase and astaxanthin synthase were still limiting in both of them (Gassel et al. 2013). Therefore, they were used for genetic pathway engineering aiming for an additional increase of the astaxanthin production. As outlined in Fig. 2, these metabolic limitations of the carotenoid pathway in *X. dendrorhous* were overcome by integration of additional copies of the phytoene synthase/lycopene cyclase gene *crtYB* and the astaxanthin synthase cDNA *asy* via a single transformation vector. This left the two other tandem integration vectors with other selection markers (Fig. 1) open for further genetic modifications. One of them was used to provide precursor metabolites of the mevalonate pathway since an enhanced carotenoid pathway needs an efficient metabolite supply by overexpressing geranylgeranyl pyrophosphate synthase CrtE (Breitenbach et al. 2011) in combination with the HMG-CoA reductase gene (*HMG*). The latter is the key enzyme of the mevalonate pathway (Shimada et al. 1998) providing precursors to the terpenoid pathways including carotenoid and sterol biosynthesis in *X. dendrorhous*. This additional transformation step increased total carotenoid synthesis in both *X. dendrorhous* mutants. However, the enzyme Asy although already overexpressed has become a bottleneck of the carotenoid pathway in the resulting double transformant. The endogenous astaxanthin synthase Asy and the product of the copies from the first transformation round were not sufficient for efficient metabolization of β-carotene and intermediates of the Asy catalyzed reactions (Table 1). The final transformation with another *asy* containing plasmid pPR2TNN-asy assured a constant metabolic flow from the mevalonate pathway via

prenyl pyrophosphates into carotenogenesis and to the desired end product astaxanthin.

In conclusion, the combination of classical mutagenesis with genetic pathway engineering was highly effective to obtain an extremely high astaxanthin accumulation in *X. dendrorhous*. This production level is regarded to be attractive for commercial exploitation (Johnson and Schroeder 1995) especially when the amount of astaxanthin of engineered transformants can be increased additionally up to 3-fold by optimized fermenter cultivation compared to shaking cultures as previously shown (Gassel et al. 2013). Starting from two mutants with a 15-fold higher astaxanthin content than the wild-type, this production could be topped by another 6-fold increase due to pathway engineering. It was possible to genetically modify the entire pathway of both *X. dendrorhous* mutants, the provision of metabolites for carotenoid synthesis and the carotenoid towards the end product astaxanthin. This indicates that the mutations in AXG-13 and AXJ-20 most likely affected upregulation of astaxanthin synthesis by a process which is not understood yet. Nevertheless, we demonstrated here that the combined strategy of random mutagenesis hitting an unknown regulatory system followed by systematic genetic pathway improvement was highly successful for the development of astaxanthin production strains. The potential for commercial use as a cell factory for astaxanthin of this type of genetically engineered *X. dendrorhous* strains has now to be proven in optimized fermenter cultures by development of a routine and cost-competitive bioprocess. This should also include a detailed investigation on the metabolic fluxes within the carotenoid pathway.

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