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Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmids

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Abstract Carotenoids are of great commercial interest and attempts are made to produce different carotenoids in transgenic bacteria and yeasts. Development of appropriate systems and optimization of carotenoid yield involves transformation with several new genes on suitable plasmids. Therefore, the non-carotenogenic bacterium *Escherichia coli* JM101 was transformed in our study with several genes that mediated the biosynthetic production of the carotenoid zeaxanthin in this host. Selection of plasmids for the introduction of five essential genes for zeaxanthin formation showed that a pACYC-derived plasmid was the best. Multiplasmid transformation generally decreased production of zeaxanthin. By cotransformation with different plasmids, limitations in the biosynthetic pathway were found at the level of geranylgeranyl-pyrophosphate synthase and β-carotene hydroxylase. In our study a maximum zeaxanthin content of 289 μg/g dry weight was obtained. This involved the construction of a plasmid that mediated high-level expression of β-carotene hydroxylase. The level of expression was demonstrated on protein gels and solubilization by the mild detergent Brij 78 revealed that a significant portion of the expressed enzyme is located in the *E. coli* membranes where it can exert its catalytic function. Based on the results obtained, new strategies for vector construction and strain selection were proposed which could increase the present concentrations drastically. Optimal growth conditions of the transfomed *E. coli* strains for carotenoid formation were found at a temperature of 28 °C and a cultivation period of 2 days.

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Introduction

Carotenoids are naturally occurring pigments, the majority being C_{40} terpenoids. They occur as hydrocarbons and oxygenated derivatives thereof, which can be found in bacteria, fungi, algae and higher plants (Goodwin and Britton 1988). In these organisms, carotenoids are powerful photoprotecting agents. In photosynthesis they are essential to protect the photosynthetic apparatus from damage by excess light and, furthermore, participate in harvesting the light energy (Cogdell 1988). Also in non-photosynthetic organisms and in humans, carotenoids are important components of antioxidant systems (Polazza and Krinski 1992). Because of these properties, carotenoids have been proposed to act as anticancer agents (Krinski 1994).

From a commercial point of view, there is an increasing demand of special carotenoids as food colorants, as precursors of vitamin A and as animal feed. Some carotenoids like astaxanthin and β-carotene are produced by chemical synthesis (Bernhard 1989). However, both carotenoids are also produced by fermentation of yeast and growth of algae in bioreactors (Nonomura 1989). This approach, however, is restricted by the number of useful species and also the carotenoid yield cannot compete with chemical synthesis. In addition to astaxanthin and β-carotene, other carotenoids could be produced biotechnologically by exploiting the carotenoid genes cloned in recent years from different species (Sandmann 1994). First attempts have already been made to synthesize carotenoids in non-carotenogenic bacteria and yeast by using recombinant gene techniques (Sandmann et al. 1990; Misawa et al. 1991; Ausich 1994). So far the whole gene cluster from an *Erwinia* species has been used to mediate the synthesis

of carotenoids in *Escherichia coli*. The content of different carotenoids in the transformant was rather low (less than 100 μg/g dry weight). Furthermore, several intermediates accumulated at the expense of the end-product of the pathway (Sandmann et al. 1990). A similar problem was encountered in different yeast transformants (Ausich 1994).

For high-yield carotenoid production, optimization should focus at different levels. First, sufficient precursors should be available. Then a balanced level of carotenogenic enzymes should be expressed for efficient precursor conversion without loss of metabolites in pools of intermediates and finally the strain of choice should possess a high storage capacity for carotenoids. In the present publication we extended the previous studies by starting to optimize the enzymic levels in *E. coli* for production of zeaxanthin (=β,β-carotene-3,3′-diol). The introduction of five different genes from *Erwinia uredovora* (Misawa et al. 1990) is necessary and sufficient to mediate the synthesis of this carotenoid. The formation of zeaxanthin was chosen as an example because it is of industrial importance especially as it is the direct precursor of astaxanthin, which is the most valued carotenoid (Johnson and Schroeder 1995). In order to minimize the accumulation of intermediates and increase the yield of the desired end-product, zeaxanthin, different plasmids were constructed and used to optimize the formation of this carotenoid and to evaluate the potential of this recombinant system. Investigations on the enzymic limitation within the carotenoid-biosynthetic pathway and demonstration of the levels of expressed β-carotene hydroxylase were also included.

Materials and methods

Growth, strains and plasmids

E. coli strains JM101 and BL21DE3 were grown in Luria-Bertani medium (Sambrook et al. 1989) supplemented with ampicillin (100 μg/ml) for transformants with pUC-derived plasmids, chloramphenicol (34 μg/ml) for pACYC184-derived plasmids or kanamycin (25 μg/ml) for co-transformants with mGP1-2, which carries the M13 origin of replication (Tabor and Richardson 1987). The source of plasmids is indicated in Table 1. Plasmid pCRT-Z, containing the *Erwinia uredovora crtZ* gene, was constructed by inserting the *Sph*I(5599)-*Eco*RI(6505) fragment of pCAR25 (Misawa et al. 1990) into the *Eco*RI/*Sph*I site of pUC18. pT7-7crtZ was constructed by polymerase chain reaction (PCR) amplification of the whole reading frame of *crtZ* from *E. uredovora* with pCRT-Z as template, using the following start and end primers: A CGT CAT ATG CTG TGG ATT TG and A TTA ACG CTG CTG CTG GCC. Both primers were designed to replace the second codon of *crtZ* for Leu, TTG, by CTG and to create a new *Pst*I cloning site behind the codon region of *crtZ*. The DNA fragment obtained was cloned into the *Nde*I/*Pst*I sites of vector pT7-7 (Tabor and Richardson 1985). For construction of pUCCRT-B, the entire reading frame of *crtB* from *E. uredovora* was amplified by PCR, using pCAR25 (Misawa et al. 1990) as template and the primers CAT GCG GTC GAA ACG ATG GCA and CTG TCA AAG CTT CAG GCG ACG, creating a *Sal*I restriction site in front of the start codon and a *Hind*III restriction site behind the coding region. The DNA fragment was cloned in the corresponding sites of vector pUC8 (Vieira and Messing 1982).

Carotenoid analysis

Carotenoids were extracted from freeze-dried *E. coli* cells with methanol containing 6% KOH and heating for 20 min at 60 °C. They were then separated and quantified by HPLC using a Spherisorb ODS-1 column and acetonitrile/methanol/2-propanol (85:10:5) as eluent (Ernst and Sandmann 1988). The zeaxanthin and β-carotene used as standards to identify and to quantify the amounts produced were purchased from Roth, Karlsruhe, Germany. β-Cryptoxanthin was a gift from BASF, Ludwigshafen, Germany.

Proteins

Sodium dodecyl sulphate(SDS)/polyacrylamide gel electrophoresis was carried out on 17.5% gels as described (Laemmli 1970). Cells resuspended in 50 mM TRIS/HCl buffer, pH 8, containing 10 mM EDTA and 1 mM dithiothreitol, were broken in a French pressure cell at 80 MPa. β-Carotene hydroxylase was solubilized after addition of 0.05 mM Brij 78, incubation for 30 min at 4 °C and centrifugation at 20 000 *g*. The relative amounts of solubilized β-carotene hydroxylase were estimated after separation of the solution on SDS/polyacrylamide gels and staining with Coomassie brilliant blue by scanning and integration with a densitometric software. The relative value obtained was used to calculate the absolute concentration of β-carotene hydroxylase from the total amount of protein applied to the lane.

Table 1 Plasmids used for the production of zeaxanthin in *Escherichia coli* JM101. Origins of replication were M13 for mGP1-2, p15A for pACCAR16ΔcrtX as well as pACCRT25ΔcrtX with copy numbers around 15, and pMB1 for all the other plasmids with copy numbers around 500. *GGPPS* geranylgeranyl-pyrophosphate synthase; *PSY* phytoene synthase; *PD* phytoene desaturase; *LCY* lycopene cyclase; *BCH* β-carotene hydroxylase; *oex*. overexpressed

Plasmid	Expressed enzyme	Source
mGP1-2	T7 RNA polymerase	Tabor and Richardson 1987
pUCCRT-E	GGPPS (oex.)	Wiedemann et al. 1993
pUCCRT-B	PSY (oex.)	This work
pUCCRT-I	PD (oex.)	Fraser et al. 1992
pUCCRT-Y	LCY (oex.)	Schnurr et al. 1996
pACCAR16ΔcrtX	GGPPS, PSY, PD, LCY	Misawa et al. 1995
pCAR25ΔcrtX	GGPPS, PSY, PD, LCY, BCH	Misawa et al. 1990[a]
pACCAR25ΔcrtX	GGPPS, PSY, PD, LCY, BCH	Misawa et al. 1995
pCRT-Z	BCH	This work
pT7-7crtZ	BCH (oex.)	This work

[a] Plasmid pCAR25ΔcrtX corresponds to pCAR25delB (Misawa et al. 1990)

Results

Plasmid selection for zeaxanthin production

As transformation of the non-carotenogenic *E. coli* with the whole carotenogenic gene cluster resulted in rather low formation of carotenoids (Sandmann et al. 1990), different plasmids for increased synthesis of zeaxanthin were constructed. Strain JM101 was transformed with different plasmid combinations and the carotenoid content analyzed (Table 2). Either a pUC-derived vector, pCAR25ΔcrtX, or a pACYC184-derived vector, pACCAR25ΔcrtX, both of which carry all five genes (*crtE*, *crtB*, *crtI*, *crtY* and *crtZ*) essential for the synthesis of zeaxanthin, were used. Zeaxanthin formation was highest with 276 μg/g dry weight when the pACYC184-derived plasmid was introduced. The pUC-derived plasmid mediated the formation of only about one-third of this amount of zeaxanthin. Conversion of precursor carotenoids into zeaxanthin was very effective in both cases. Only small amounts of the monohydroxy derivative, β-cryptoxanthin, were detectable and accumulation of β-carotene was below the detection limits. In addition to single plasmid transformants, *E. coli* was also transformed with a combination of two plasmids. One, in each case, was pACCAR16ΔcrtX, which initiates the formation of β-carotene only, and the other was either pCRT-Z, with the single β-carotene hydroxylase gene, or pCAR25ΔcrtX with all the genes for the synthesis of zeaxanthin. In the latter case, double transformation with plasmids containing large gene clusters, pACCRT16ΔcrtX and pCAR25ΔcrtX, had a negative effect on the overall formation of carotenoids. When pCAR25ΔcrtX was replaced by pCRT-Z, formation of total carotenoids was comparably high with 442 μg/g dry weight. However, only 40% of this amount was converted to zeaxanthin. The rest was more or less equally distributed between β-cryptoxanthin and β-carotene.

As the highest zeaxanthin value was obtained with JM101/pACCAR25ΔcrtX, this transformant was grown at two temperatures, 28 °C and 37 °C, for different cultivation periods (Fig. 1). The zeaxanthin content of the transformant increased steadily and reached a maximum after 29 h, in the case of the 37 °C culture, or after 45 h when *E. coli* was grown at a temperature of 28 °C. Longer incubation times did not improve the production of zeaxanthin. Comparing the growth temperature of 37 °C with 28 °C, we found after 20 h a 1.7- to 2.7-fold higher production with the latter growth

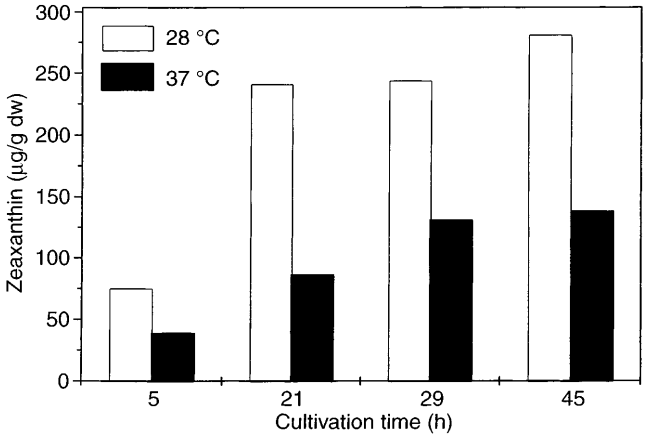

Fig. 1 Time course of zeaxanthin formation (μg/g dry weight) by *Escherichia coli* JM101/pACCAR25ΔcrtX growing at 28 °C or 37 °C

temperature, depending on the increasing age of the culture. Therefore, the optimal condition for zeaxanthin formation in this *E. coli* transformant is cultivation for 2 days at 28 °C.

Evaluation of enzymatic limitations within the carotenoid pathway

In the course of this investigation, attempts were made to find out whether there is a limitation of the catalytic activity of any of the four enzymes involved in the synthesis of β-carotene, the substrate for the hydroxylase. To answer this question, *E. coli* carrying pACCAR25ΔcrtX was cotransformed with plasmids that mediate overexpression of any of these enzymes (Table 3). Furthermore, a control cotransformant with

Table 3 Zeaxanthin formation in *E. coli* JM101 containing pACCAR25ΔcrtX and co-transformed with an additional plasmid which overexpresses any enzyme from the carotenoid pathway prior to the hydroxylase reaction. Values are means of five different determinations. *GGPP* geranygeranyl pyrophosphate

Zeaxanthin (μg/g dry weight)	Additional plasmid	Overexpressed enzyme
138 ± 30	pUC18	None
169 ± 28	pUCCRT-E	GGPP synthase
142 ± 21	pUCCRT-B	Phytoene synthase
134 ± 28	pUCCRT-I	Phytoene desaturase
148 ± 24	pUCCRT-Y	Lycopene cyclase

Table 2 Formation of zeaxanthin and intermediates in *E. coli* JM101 transformants cultivated for 2 days at 28 °C. Results are means of five different determinations. *ND* not detectable

Plasmids	Content (μg/g dry weight)		
	Zeaxanthin	β-cryptoxanthin	β-carotene
pCAR25ΔcrtX	100 ± 16	2 ± 1	n.d.
pACCAR25ΔcrtX	276 ± 52	5 ± 1	n.d.
pACCAR16ΔcrtX + pCRT-Z	177 ± 39	137 ± 22	128 ± 19
pACCRT16ΔcrtX + pCAR25ΔcrtX	105 ± 20	7 ± 1	10 ± 1

pUC18 instead was included. Comparing the formation of this double transformant with *E. coli* carrying pACCRT25ΔcrtX alone (Table 2, line 2), it is evident that the additional pUC vector decreased the potential of strain JM101 to synthesize zeaxanthin by about half. An increase of zeaxanthin yield above the control was only observed when the plasmid for high-level expression of geranylgeranyl-pyrophosphate synthase was introduced on a second vector.

Expression of β-carotene hydroxylase and isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent zeaxanthin formation

In order to overcome the limited conversion of β-carotene and β-cryptoxanthin to zeaxanthin under the conditions allowing the highest formation of total carotenoids (Table 2, line 3), an overexpressing construct of the coding region of *crtZ* was produced by an in-frame fusion in plasmid pT7-7, resulting in the new plasmid pT7-7crtZ. This pT7-7 expression system was chosen to obtain high levels of β-carotene hydroxylase enzyme, for the following reasons. It is possible to clone the reading frame to begin with its own ATG start codon. This avoids N-terminal extensions of the expressed enzyme, which may have a negative effect on its activity. Furthermore, the regulation of protein expression is very tight and inducible by IPTG because of the combination of two necessary components. In addition to pT7-7crtZ, containing the open reading frame behind the T7 RNA polymerase promoter, the T7 RNA polymerase gene behind the IPTG-inducible *lacZ* promoter must be present either in the genome of the modified *E. coli* strain BL21DE3 or on mGP1-2 when *E. coli* JM101 is used for synthesis of zeaxanthin.

For evaluation of the expression of the hydroxylase, pT7-7crtZ was introduced into *E. coli* BL21DE3 and the degree of expression of β-carotene hydroxylase mediated by this plasmid and by pCRT-Z and pACCAR25ΔcrtX was compared by separation of total proteins on a SDS/polyacrylamide gel. In order to obtain the portion of β-carotene hydroxylase that is not sequestered in inclusion bodies and is therefore potentially active in the catalytic reaction, the amount of this enzyme that is solubilized by Brij 78 was estimated after separating solubilisates from broken cells of transformants carrying either pT7-7crtZ or pACCAR25ΔcrtX. The first transformant yielded a total β-carotene hydroxylase content of 8.1 mg/g dry weight from which 2.6 mg/g dry weight could be solubilized. In the transformant with pACAR25ΔcrtX a total of 56 μg/g dry weight was expressed, from which 29 μg/g dry weight was solubilized. The amount of hydroxylase expressed from pCRT-Z was below visible detection.

IPTG- and growth-phase-dependent formation of zeaxanthin in the presence of pT7-7crtZ was evaluated. Carotenoid formation was determined in a transformant carrying pACCRT16ΔcrtX (mediating the synthesis of the substrate β-carotene), pT7-7crtZ for overexpression of the hydroxylase and a third vector mGP1-2 for IPTG-inducible T7 RNA polymerase (Tabor and Richardson 1987). Table 4 shows the dependence of zeaxanthin formation on IPTG induction of β-carotene hydroxylase. IPTG was added at different growth phases to the culture. When given simultaneously with inoculation, the lowest zeaxanthin content of 133 μg/g dry weight was found. The highest value of 289 μg/g dry weight was obtained when expression of the hydroxylase was induced by addition of IPTG at the end of the log phase. A control transformant with all the necessary genes for zeaxanthin synthesis on one plasmid, namely pACCRT25ΔcrtX, together with pT7-7 without inserted *crtZ* and mGP1-2 had a zeaxanthin content of 224 μg/g dry weight, which resembles more than 80% of what is found in a transformant with pACCRT25ΔcrtX alone. Comparison of this control instead to the transformants with pT7-7crtZ (Table 4, line 4) which has a very similar plasmid background, increases the zeaxanthin content to 129%.

Table 4 Isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent formation of zeaxanthin in *E. coli* JM101 transformed simultaneously with the plasmids pT7-7crtZ, pACCAR16ΔcrtX and mGP1-2. Values are means of three different determinations. IPTG was added at 0.1 mM, the contents of β-cryptoxanthin and β-carotene were below the detection limit

Phase of IPTG addition	Zeaxanthin (μg/g dry weight)
Simultaneous with inoculation	133 ± 29
Beginning of log phase	189 ± 36
Within log phase	187 ± 32
End of log phase	289 ± 49
Stationary phase	252[a] ± 40

[a] Control zeaxanthin value for transformant with the three plasmids pACCAR25ΔcrtX, pT7-7 and mGP1-2 after addition of IPTG in the late logarithmic phase was 224 μg/g dry weight

Discussion

In the present work the non-carotenogenic bacterium *E. coli* was used as a model system to study and optimize the production of zeaxanthin. This involved the introduction of different combinations of plasmids with appropriate genes into *E. coli* in order to shift all carotenoid intermediates totally into the product zeaxanthin. For this purpose *E. coli* was transformed with a combination of different plasmids carrying all the necessary genes for the synthesis of this carotenoid. Selection of the appropriate vector for introduction of the carotenogenic genes leading to zeaxanthin synthesis in *E. coli* clearly showed that, with a pACYC-derived vector, synthesis of zeaxanthin is 2.8-fold higher than with a pUC-derived plasmid (Table 2). Simultaneous transformation with pUC- and pACYC-related vectors also resulted in lower zeaxanthin values than with a single pACYC-derived plasmid alone. It is rather difficult to find a reason for this observation. In contrast to

pACYC, pUC is a high-copy-number plasmid mediating the synthesis of large amounts of β-lactamase (Lamotte et al. 1994). This may exhaust the *E. coli* metabolism in a way that negatively affects the synthesis of carotenoids. Optimization of growth conditions for zeaxanthin production was carried out with JM101 containing a pACYC-derived carotenogenic plasmid (Fig. 1). We found that production was best at slow growth at 28 °C and in the stationary phase after 2 days. Further selection of high-productivity *E. coli* strains and optimization of the growth medium will be future steps to increase zeaxanthin production.

Furthermore, we also wanted to find possible limitations in the biosynthetic pathway of zeaxanthin formation. For this purpose we overexpressed the enzymes involved from the synthesis of geranylgeranyl pyrophosphate to β-carotene (Table 3) and to zeaxanthin (Table 4) one after the other. High-level expression of the proteins and the corresponding enzyme activity have already been documented (Fraser et al. 1992; Wiedemann et al. 1993; Schnurr et al. 1996). A substantial increase (1.2-fold) in zeaxanthin synthesis was obtained only when the expression of geranylgeranyl-pyrophosphate synthase was enhanced. This is the foreign enzyme that channels the farnesyl pyrophosphate made by *E. coli* into carotenoid biosynthesis. A limitation of other enzymes in the pathway to β-carotene, as reported for phytoene synthase in the carotenogenic *Thermus thermophilus* (Hoshino et al. 1994), was not observed. For high-level expression of β-carotene hydroxylase an overexpressing construct was made in pT7-7 to avoid the production problems with pUC vectors. A transformant with the resulting vector pT7-7crtZ expressed about 100-fold more β-carotene hydroxylase not sequestered in inclusion bodies than a transformant with pACCAR25ΔcrtX. In transformants carrying pCRT-Z the level of β-carotene hydroxylase was below the detection limit.

Transformation of our carotenoid production strain *E. coli* JM101 with three different plasmids was necessary to evaluate the effect of high-level expression of β-carotene hydroxylase mediated by pT7-7crtZ (Table 4). This combination of plasmids had a generally negative effect on zeaxanthin production, as indicated by a control experiment with all the genes for zeaxanthin synthesis on one pACYC184-derived plasmid, together with or without the additional plasmids pT7-7 and mGP1-2 (compare legend of Table 4 and Table 1, line 2). Nevertheless, the highest value was obtained with pT7-7crtZ upon induction by IPTG. Formation of zeaxanthin was related to the expression levels of β-carotene hydroxylase. When pACCAR25ΔcrtX, with a lower expression of total and soluble enzyme than pT7-7crtZ, was used, less zeaxanthin was found. In the case of pCRTZ the *crtZ* gene was cloned under its own promoter lacking a putative second promoter sequence (Misawa et al. 1990). Therefore, the expression level of the hydroxylase was below visual detection on SDS gels. When this plasmid was used, zeaxanthin yield was low and the precursors β-cryptoxanthin and β-carotene accumulated (Table 2).

All our results clearly indicate that, for high production of zeaxanthin, a push of metabolites into the newly introduced carotenoid biosynthesis pathway and a pull by the final enzyme into the end-product zeaxanthin is important. The highest zeaxanthin value obtained in this study was 289 μg/g dry weight. This is more than obtained previously with *E. coli* (76.2 μg/g dry wt. Sandmann et al. 1990) and also for β-carotene synthesized in *Zymomonas mobilis* (220 μg/g dry wt. Misawa et al. 1991). Only β-carotene production in *Agrobacterium tumefaciens* was higher with 350 μg/g dry weight. Our results indicate that the limit of zeaxanthin production in *E. coli* JM101 has not yet been reached. The potential of our system can be enhanced by avoiding multiplasmid transformants, e.g. with a new single pACYC184-derived plasmid containing all five essential genes for the synthesis of zeaxanthin in *E. coli* including those for geranylgeranyl-pyrophosphate synthase and β-carotene hydroxylase under a strong promoter. Starting from a zeaxanthin concentration of 276 μg/g dry weight for JM101/pACCAR25ΔcrtX, a 1.2-fold increase with higher geranylgeranyl-pyrophosphate synthase expression (Table 3) and a 1.3-fold stimulation with more β-carotene hydroxylase (Table 4), the expected formation can be roughly estimated to be then in the range of 500 μg/g dry weight. Under conditions of overproduced limiting enzymes, manipulation by amino acid exchanges in order to increase enzyme activity (Ausich 1994) should be of minor importance. As already pointed out, another aspect of increasing the zeaxanthin production beyond this level is the selection of more appropriate *E. coli* strains. Apart from enhanced metabolite channelling into carotenoids, which is another strategy to increase carotenoid yield (unpublished results), one has to consider the storage of the highly lipophilic zeaxanthin in *E. coli* (Johnson and An 1991). The only lipophilic structures available in the *E. coli* transformants for sequestering the synthesized zeaxanthin is the membrane system in the cell. Therefore, the amount of membranes could develop to another limiting factor for carotenoid production. Finally, a systematic screening for strains with increased amounts of membranes will certainly be advantageous.

In conclusion, our study implies subsequent experimental steps to increase zeaxanthin formation further in *E. coli*. These include the assembly of new plasmids containing all the carotenogenic genes, including those encoding geranylgeranyl-pyrophosphate synthase and β-carotene hydroxylase, as a high-expression construct, as well as selection for strains with a higher terpenoid metabolism and also a higher amount of membranes for storage of carotenoids. In addition, we have already started work to increase the input of precursors into the carotenoid pathway.

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