Heterologous Synthesis of Xanthophyll in Model Microorganisms

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Xanthophyll is an oxidated version of carotenoid. It presents significant value to the pharmaceutical, food, and cosmetic industries due to its specific antioxidant activity and variety of colors. Chemical processing and conventional extraction from natural organisms are still the main sources of xanthophyll.

xanthophyll biosynthetic pathway model microorganisms

1. Introduction

Carotenoids are a class of secondary metabolites with a tetraterpene structure that are widely found in plants, algae, yeast, archaea, and some bacteria species [1][2][3][4][5]. Carotenoids are classified into two categories depending on whether they contain oxygen (**Figure 1**): carotene and xanthophyll [6][7]. The structure and function of xanthophyll present abundant diversity attributed to the oxygen group, which exists in various forms, such as hydroxyl-, keto-, expo-xanthophyll, and expo-xanthophyll derivatives [8]. So far, more than 600 xanthophylls (including isomers) have been found or identified among approximately 1000 carotenoids (https://coconut.naturalproducts.net/ (accessed on 3 March 2023).

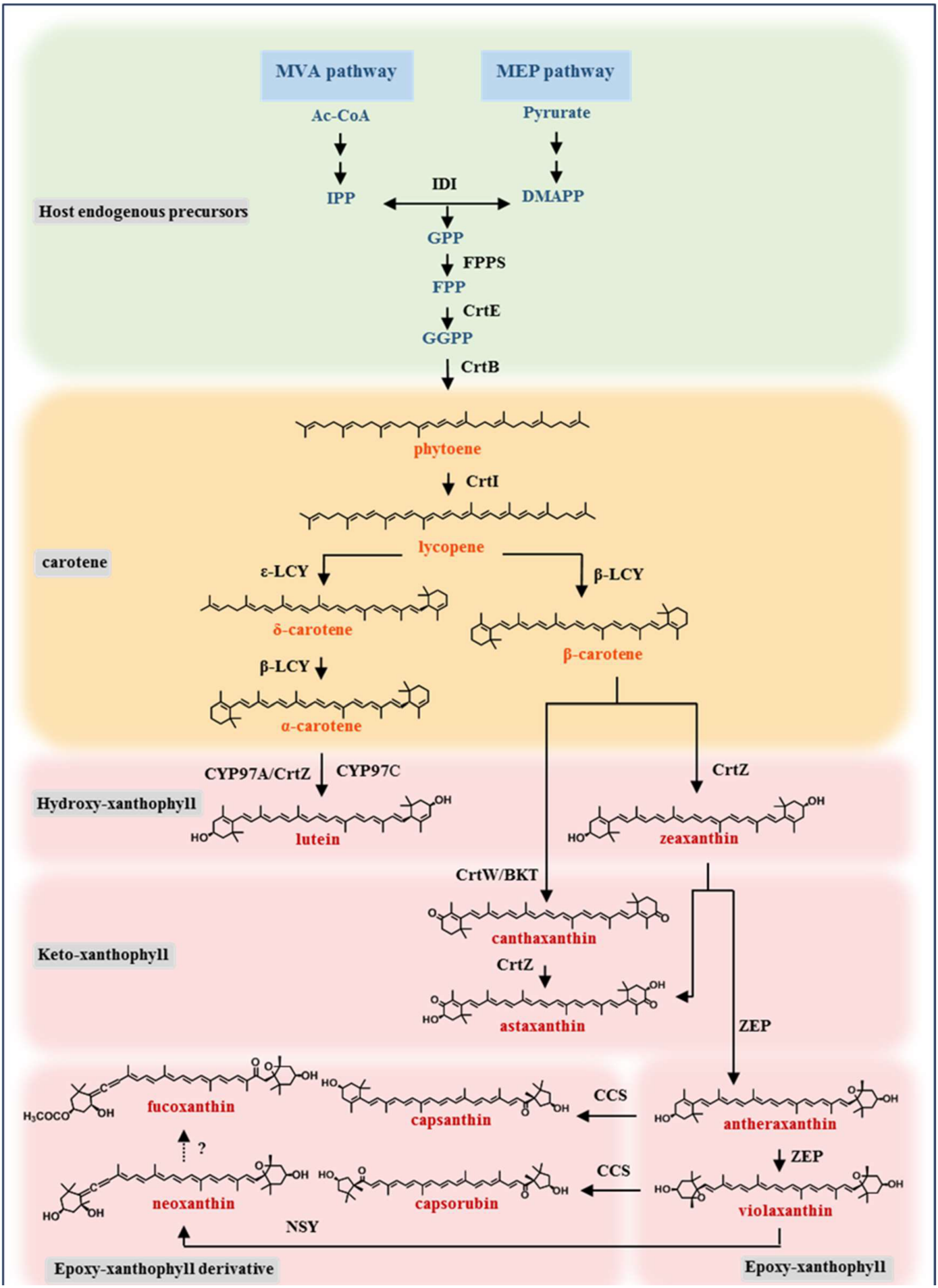
MVA pathway
MEP pathway
Ac-CoA
Pyrurate
IPP
IDI
DMAPP
Host endogenous precursors
GPP
FPPS
FPP
CrtE
GGPP
CrtB
phytoene
CrtI
lycopene
carotene
ε-LCY
β-LCY
δ-carotene
β-carotene
β-LCY
α-carotene
CYP97A/CrtZ
CYP97C
CrtZ
Hydroxy-xanthophyll
lutein
zeaxanthin
CrtW/BKT
canthaxanthin
Keto-xanthophyll
CrtZ
astaxanthin
ZEP
fucoxanthin
capsanthin
CCS
antheraxanthin
?
ZEP
neoxanthin
capsorubin
CCS
violaxanthin
NSY
Epoxy-xanthophyll derivative
Epoxy-xanthophyll

Figure 1. Xanthophyll biosynthesis pathway. Abbreviations: MVA, mevalonate; MEP, 2-C-methyl-D -erythritol-4-phosphate; IPP, isoprene diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IDI, IPP isomerase; FPPS, farnesyl diphosphate synthase; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; ε-LCY, lycopene ε-cyclase; β-LCY, lycopene β-cyclase; CYP97A, α-carotene β-ring hydroxylase; CYP97C, α-carotene ε-ring hydroxylase; CrtZ, β-carotene hydrolase; CrtW, β-carotene ketolase (from bacteria); BKT, β-carotene ketolase (from algae); ZEP, zeaxanthin epoxidase; CCS, capsanthin/capsorubin synthase; NSY, neoxanthin synthase. The dotted line indicates that the enzyme catalyzing the reaction has not been identified.

Xanthophyll has been used commercially in a wide variety of industries, such as food, feed, pharmaceuticals, and cosmetics [8][9][10]. Currently, chemical processing and conventional extraction from natural organisms are the mainly source of xanthophyll. Despite the low cost and high yield of chemical synthesis methods, the formation of unnatural chiral isomer reduces the bioavailability of the product. In addition, petrochemical-refined xanthophyll is not allowed to be used in food and pharmaceutical products. The yield of xanthophyll extracted from plants or algae is too low to meet industrial needs [11][12]. In recent decades, metabolic engineering based on model microbes for the production of nature bioactivity compounds has experienced extensive development [13][14][15], which brings a promising potential for the industrial synthesis of natural xanthophyll.

The elucidation of the natural synthetic pathways and key enzymes of several important xanthophylls has laid the foundation for xanthophyll synthesis by the metabolic engineering of model microorganisms [16][17][18][19][20]. The natural synthetic pathway of xanthophyll is a cascade reaction that begins with the oxidative modification of the α- or β-carotenoid terminal group (**Figure 1**). The hydroxylation of α- and β-carotene terminal groups enables the production of lutein and zeaxanthin, respectively [9][21][22][23]. Lutein and zeaxanthin, two yellow hydroxyl-xanthophylls, are used to treat aging-related macular degeneration because they are the only and necessary carotenoids for the macular function of the human eye [2][21]. The β-carotene and zeaxanthin can be ketonylated to produce canthaxanthin and astaxanthin, two orange or red keto-xanthophylls [24][25]. They are used as feed additives in aquaculture and poultry farming to give fish or egg yolks a red color [1][11][26]. Astaxanthin is also widely used in nutraceuticals, pharmaceuticals, and cosmetics due to its excellent antioxidant activity [11][27]. Epoxy-xanthophyll, antheraxanthin, and violaxanthin are derived from another branch of the metabolic pathway of zeaxanthin [28]. The terminal epoxide groups of antheraxanthin and violaxanthin can be further modified to produce capsanthin, capsorubin, neoxanthin, etc. [29][30][31][32][33][34][35][36]. These different modifications allow xanthophyll to show a broader color variation and stronger antioxidant activity compared to carotene [24][37][38][39][40].

2. General Strategy for the Xanthophyll Production by Metabolic Engineering of Model Microorganisms

Briefly, the microbial metabolic engineering of xanthophyll involves the docking of the exogenous pathway of xanthophyll synthesis with the endogenous core metabolism of the microbial host, allowing the flows of carbon and energy to the synthesis of xanthophyll.

In model microorganisms, glucose or other carbon sources undergo glycolysis to produce acetyl coenzyme A (acetyl-CoA) and pyrurate. The acetyl-CoA and pyrurate then pass through the ergosterol synthesis pathway of the host cell, MVA (in plants, yeast, and algae) or MEP (in plants and bacteria) [16][17], to produce geranylgeranyl diphosphate (GGPP), an intermediate metabolite of the ergosterol pathway. GGPP is the connect point between core microbial metabolism and exogenous carotinoid synthesis pathways [41]. The subsequent synthesis of carotene and xanthophyll requires various heterologous carotenogenic enzymes and cofactors to convert GGPP further [18][19][20].

Experimental manipulation is used to introduce the genes of carotegenesis enzymes and cofactors into the microorganism and utilize the transcriptional, translational, and metabolic regulatory functions of the microbial host to control the expression of these genes. The key genetic elements involved in this process include highly active key enzyme genes, cofactor genes matching the key enzymes, various promoters and terminators, etc. The key enzyme genes and cofactor genes will be described in detail in Section 3, and the structures of expression cassettes consisting of the most effective promoters, genes, and terminators are listed in **Table 1**.

3. Progress in Metabolic Engineering Synthesis of Various Xanthophylls

3.1. Hydroxy-Xanthophyll

Hydroxy-xanthophyll mainly refers to zeaxanthin, lutein, β-cryptoxanthin, zeinoxanthin, etc. The β-cryptoxanthin and zeinoxanthin are the intermediate metabolites of zeaxanthin and lutein [9][21][23], respectively, and the metabolic engineering of model microorganisms taking these intermediate metabolites as the end products has not yet been reported, so this section focuses on the engineering biosynthesis of zeaxanthin and lutein.

Zeaxanthin is the first xanthophyll in the β-carotene branch of the xanthophyll synthetic pathway and is the precursor of various high-value xanthophyll, such as astaxanthin, violaxanthin etc. (**Figure 1**). Unfortunately, although the production of zeaxanthin in various microbial engineering hosts has shown an increasing trend in recent years, it is still too low, with a maximum yield of only 18.7 mg/g [42], and it is the speed-limiting step of the engineering synthesis of downstream xanthophyll.

The reported model microorganisms for zeaxanthin synthesis include *E. coli*, *S. cerevisiae*, and *Y. lipolytica*. Zeaxanthin engineering synthesis is generally achieved by first introducing the exogenous β-carotene pathway into the host to construct a carotene chassis strain; the heterologous CrtZ is then expressed in this chassis strain to hydroxylate the β-ring at each end of β-carotene to produce zeaxanthin. The CrtZ gene has been cloned from a variety of algae and bacteria and applied to the metabolic engineering synthesis of zeaxanthin. In *E. coli*, compared with those from *Pantoea agglomerans* and *Haematococcus pluvialis*, the CrtZ of *Pantoea ananatis* has the highest β-carotene hydroxylation activity [42][43][44][45]. In *Y. lipolytica*, the catalytic activity of CrtZ from *Brevundimonas Vesicularis*, *H. lacustris* and *P. ananatis* was compared [46], and *P. ananatis* CrtZ had the highest activity, as in *E. coli*. In *S. cerevisiae*, the synthesis of zeaxanthin was mainly used as a reporting approach to study the feasibility

of experimental methods such as multi gene co-transformation [47][48]; research aimed at improving the yield of zeaxanthin is rare. The discovery, optimization, and heterologous functional expression of highly active CrtZ are critical and urgent problems for zeaxanthin synthesis.

In addition to the above CrtZs, CrtZ proteins sequences of other species obtained by gene prediction or protein sequence alignment can be found on the NCBI GenBank database published in recent years, such as *Massilia varians* (GenBank: BDT58294.1), *Xanthomonadaceae bacterium* (GenBank: RZA32178.1), *Oxalobacteraceae bacterium* (GenBank: USX26554.1), etc. Their identities of protein sequences aligning against the CrtZ of *P. ananatis* are generally in the range of 60–80%, which is not lower than the identities between the above reported CrtZs. The potential of these putative CrtZ applications for the metabolic engineering of zeaxanthin also deserves further exploration.

Another important representative of hydroxyl-xanthophyll is lutein. The metabolic engineering of lutein is more complex and difficult than zeaxanthin due to the asymmetric cyclization of lycopene. α- carotene is the direct precursor of lutein, and the β-ring and ε-ring on the two ends of α- carotene are catalyzed by lycopene β-cyclase and ε-cyclase, respectively (**Figure 1**). The hydroxylation of the β-ring and ε-ring are catalyzed by two cytochrome P450 enzymes, CYP97A and CYP97C, respectively. These hydroxylation reactions require ferredoxin-NADP+ reductase (FNR), with ferredoxin redox partner, NADPH, and flavin adenine dinucleotide (FAD) as cofactors [49][50][51].

Studies have shown that the heterologously expressed activity of β-cyclase is higher than that of ε-cyclase, leading to a preference for lycopene for the formation of β-carotene with two β-rings [52][53][54]. Therefore, in addition to the hydroxylation, the asymmetric cyclization of lycopene is also a hindrance to lutein production in model microorganisms. The reported engineering microorganisms producing lutein are only *E. coli* and *S. cerevisiae*, and the yield is very low. In *E. coli*, through the screening of ε-cyclase from three sources (*Lactuca sativa*, *Tagetes erecta*, and *Marchantia polymorpha*) and CYP97C from nine sources (*Chlamydomonas reinhardtii*, *H. pluvialis*, *Brassica napus*, *Chenopodium quinoa*, *Oryza sativa*, *L. sativa*, *Nicotiana tabacum*, *Helianthus annuus*, and *M. polymorpha*), only the enzymes from *M. polymorpha* have lutein biosynthetic activity [52][55]. In *S. cerevisiae*, the synthesis of lutein can be achieved by the co-expression of β-cyclase from *Xanthophyllomyces dendrorhous*, ε-cyclase from *Tagetes erecta*, and CYP97A and CYP97C from *Arabidopsis thaliana* [53][54].

3.2. Keto-Xanthophyll

Among all keto-xanthophylls (including astaxanthin, canthaxanthin, echinenone, etc.), astaxanthin has received the most attention due to its strongest antioxidant activity; this promotes the most in-depth research and the highest fermentation yield, and has shown great industrialization potential. Under controlled bioreactor fermentation, the maximum yield can reach tens of mg/g DCW [56], which could be comparable to a yield of approximately 40 mg/g DCW of the industrial algae natural-producer *H. pluvialis* [57].

The most commonly used model microorganisms for the production of astaxanthin include *E. coli*, *S. cerevisiae*, and *Y. lipolytica* [24][58]. *Kluyveromyces maximus* [59][60], *P. pastoris* [61][62], *Corynebacterium glutamicum* [63][64] etc., as hosts have also been reported.

The first step in astaxanthin synthesis by metabolic engineering is the heterologous functional expression of CrtW and CrtZ. Since both enzymes involve two substrates, there is an issue of substrate preference. Therefore, the yield of astaxanthin is closely related to the enzymatic activity, the substrate preference, and the combination of the two enzymes. Accordingly, the first step of almost all metabolic engineering research on the heterologous production of astaxanthin is the screening and adaptability analysis of these two enzymes. In *E. coli*, the combination of CrtW from *Brevundimonas* sp. SD212 and CrtZ from *Pantoea* sp. (including *P. agglomerans* and *P. ananatis*), or the CrtZ from *Paracoccus* sp. PC1 as a supplement to CrtZ from *Pantoea* sp., produces the highest astaxanthin yield [65][66][67][68][69][70][71]. The CrtZs from *Pantoea* sp. and *Paracoccus* sp. PC1 have different substrate preferences; a high conversion efficiency from β-carotene and canthaxanthin to astaxanthin can be achieved when the two enzymes have a combinatorial expression at a specific copy number ratio [65]. In *S. cerevisiae*, the higher astaxanthin production comes from the combination of the mutant BKT (H165R/V264D/F298Y/M1T/N188D/L271R) and CrtZ (L288R) of *H. pluvialis*, or the combination of the CrtW of *B. vesicularis* and CrtZ of *Agrobacterium aurantiacum* [72][73][74][75][76][77][78][79]. In *Y. lipolytica*, Wang [25] and Ma [80] et al. reported that the combination of the CrtZ from *Paracoccus* sp. and the CrtW from *H. pluvialis* yielded a higher astaxanthin production. Furthermore, the result of Wang et al. also showed that astaxanthin cannot be detected with the co-expression of CrtW and CrtZ from *H. pluvialis* in *Y. lipolytica*, On the contrary, Zhu [63] and Tramontin [81] reported that a higher astaxanthin production was produced by these CrtW and CrtZ from *H. pluvialis*. This contradiction needs to be further verified. In general, no matter the type of host, the bacteria CrtW and CrtZ usually have higher activity, followed by algae enzymes, and *X. dendrorhous* and plants (such as *Adonis aestivalis*) have very low or almost no activity [78][82].

Almost all reports indicate that CrtZ is a rate-limiting enzyme when the astaxanthin synthetic pathway is introduced in a model microbial host, which means the conversion from β-carotene to zeaxanthin, or from canthaxanthin and echinenone to astaxanthin, which is a key step for limiting the production of astaxanthin. Thus, in addition to the CrtZs listed in **Table 1**, the functional identification of other putative CrtZs mentioned in 2.1 may also be of significance for the synthesis of astaxanthin.

Canthaxanthin is another keto-xanthophyll that is widely used commercially. At present, only *E. coli* [83] and *S. cerevisiae* [84] have been reported as engineering microorganisms that take canthaxanthin as the target product. Theoretically, the synthesis of canthaxanthin does not require CrtZ, and its production should be higher than astaxanthin. Indeed, comparing the experimental results of Ye et al. [76][84], the yield of canthaxanthin (approximately 10–15 mg/g) in *S. cerevisiae* was higher than that of axtathophyll (5.7 mg/g) when almost the same strategies and shake flask culture conditions were used. This further demonstrates the rate-limiting effect of CrtZ.

3.3. Epoxy-Xanthophyll

Epoxy-xanthophyll mainly includes violaxanthin and antheraxanthin. Antheraxhanthin was mentioned mainly as the intermediate metabolite of violaxanthin, so the researchers only focused on the engfigureineering biosynthesis of violaxanthin in this section. The biosynthesis of violaxanthin by metabolic engineering is achieved by prolonging the pathway of zeaxanthin with the introduction of ZEP into a zeaxanthin-producing microbial host. Although the cloning and heterologous expression of ZEP have been studied for nearly 30 years [85][86][87][88], as far as the metabolic engineering of violaxanthin is concerned, it has only been described in detail in recent years.

ZEP belongs to FAD-dependent monooxygenase and catalyzes the epoxidation of zeaxanthin in the presence of NADPH, FAD, ferredoxin (FD), and ferredoxin-NADPH oxidoreductase (FNR) [89][90]. Therefore, the synthesis of violaxanthin in an engineering host requires the reducing power and the redox pair ether from the endogenous metabolism of the host or the functional heterologous expression of relative genes. In general, the reducing power of host cells cannot meet the needs of an efficient synthesis of violaxanthin, and not all redox pairs can transfer the electron to ZEP due to the specificity of ZEP for the redox partner. Therefore, the first challenge of violaxanthin metabolic engineering is the heterologous expression of highly active ZEP and its matching redox partner. The reported microorganism hosts for violaxanthin synthesis include *E. coli* and *S. cerevisiae*. In *E. coli*, the activity of ZEPs from seven higher-plants (*Capsicum annuum*, *A. thaliana*, etc.), one liverwort (*M. polymorpha*), and one algae (*Phaeodactylum tricornutum*) was compared [91]. The *C. annuum* ZEP had the highest activity, whereas that of *Prunus ameniaca*, *Zea mays*, *M. polymorpha*, and *P. tricornutum* showed extremely low activity in *E. coli*. Although the NADPH and electron transport system of *E. coli* can make ZEP active, the yield of violaxanthin is low. Exogenous redox partners, spinach ferredoxin and ferredoxin oxidoreductase, can improve the ZEP activity, whereas *Nostoc* sp. severely decreases its activity. The *Bacillus subtilis* glucose dehydrogenase as the NADPH-regenerating enzyme can increase the synthesis of violaxanthin in *E. coli*. Furthermore, the *E. coli* strains and ribosome-binding site (RBS) sequences also impacted the yield of violaxanthin. The best *E. coli* strain, JM101, had a yield of 231 μg/g of violaxanthin; however, the ratio of violaxanthin to total carotenoids was only 1.4–21%, which was still low. In *S. cerevisiae*, compared with the ZEPs of *A. thaliana* and *Solanum lycopersicum*, the *Haematococcus lacustris* ZEP showed the highest activity [92]. The redox partner from *A. thaliana* can improve the violaxanthin yield, whereas the yeast mitochondrial ferredoxin-like protein and its reductase has no effect on the yield. In the best violaxanthin-producing *S. cerevisiae*, the final yield of violaxanthin reached 7.3mg/g, corresponding to 58.4% of total carotenoids.

From the above, in *E. coli* and in *S. cerevisiae*, a considerable amount of upstream carotene remained, indicating that the catalytic efficiency of the heterologously expressed ZEP was low. The characterization and screening of ZEP and the matching redox partner from different species in various microbial hosts need to be further explored.

3.4. Epoxy-Xanthophyll Derivative

Epoxy-xanthophyll derivatives include capsanthin, capsorubin, neoxanthin, fucoxanthin, diadinoxanthin, etc. Capsanthin, capsorubin, and neoxanthin are usually considered as the last metabolites of the carotenoid synthesis pathway in higher plants. The epoxy-xanthophyll derivatives have more complex terminal groups, and they may

have multiple terminal groups, such as hydroxyl, ketone, epoxy, allenic, cyclopentane, cyclohexane, etc. (**Figure 1**).

Except for neoxanthin synthase (NSY) and the capsanthin/capsorubin synthase (CCS), the synthases for other epoxy-xanthophyll derivatives have not yet been identified or characterized. The NSY catalyze the opening of cyclohexenyl 5–6 epoxides at one end of violaxanthin to form an allenic group through a transient carbocation [31]. Although the NSY from *Lycopersicum esculentum* [31], *S. tuberosum* [93], *Arabidopsis* [32], and *Chinese Kale* [33] has been cloned, and the activity of *L. esculentum* NSY [94] conversing from violaxanthin to neoxanthin has been verified in *E. coli* (the substrate violaxanthin was added to the system and the yield of neoxhanthin was not mentioned), to date, neoxanthin production by microbial metabolic engineering has not been reported in detail.

Table 1. Non-native xanthophyll-producing microorganisms with the highest yield or titer [a].

Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
lutein	*E. coli*	ε-LCY	*M. polymorpha*	P_{tac}-*IDI*-*CrtE*-*CrtB*-*CrtI*-*MpLCYb*-*MpLCYe*-*CrtZ*-T_{rrnB} and P_{T7}-*MpCYP97C*-T_{T7}- P_{T7}-*MpLCYe*-T_{T7}	Electroporation	Selection of ε-LCY and CYP97C from different species, decreasing the activity of β-LCY and increasing the copy number of ε-LCY gene		2.6	[52]
		β-LCY	*M. polymorpha*						
		CYP97C	*M. polymorpha*						
		CrtZ	*P. ananatis*						
	S. cerevisiae	ε-LCY	*A. thaliana*	P_{TER1}-*tHMG1*-T_{CYC1}, P_{PGK1}-*CrtE03M*-T_{ADH1}, P_{PGK1}-*CrtYB11M*-T_{ADH1}, P_{TEF}-*CrtI*-T_{CYC1},	Chemical transformation	Selection of ε-LCY from different species, regulation of ratios of CYP97A3 and RFNR1/FD3, and hierarchical	4.53	19.92	[54]
		CrtYB	*X. dendrorhous*						

Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
				P_{ACT1}-*Gal4M9*-T_{ADH1}, P_{GAL1}-*CrtYB*-T_{CYC1}, T_{CYC1}-*CYP97A3*-P_{GAL1}-P_{GAL10}-*LUT1*-T_{ADH1}-T_{PGK1}-*FD3*-P_{GAL2}-P_{GAL7}-*RFNR1*-T_{TPS1}, P_{TEF1}-$PM^{SeV\text{-}C}$-*At-LCYE*-T_{CYC1}, P_{GAL1}-*CYP97A3*-T_{CYC1}	dynamic regulation based on the temperature-responsive promoter			
		CYP97A3	*A. thaliana*						
		Lut1	*A. thaliana*						
		RFNR1	*A. thaliana*						
		FD3	*A.s thaliana*						
zeaxanthin	*E. coli*	CrtZ	*P. ananatis*	P_{T5}-*CrtEIBipi*-TTR, P37-*CrtY- 2CrtZ*-T_{rrnB}, $pZSP_{IA44}$-$MevT_{TIGR}$-$MevB_{TIGR}$ *IS*-2	Electroporation	Introduction and dynamic control of the MVA pathway of *S. cerevisiae* to increase the precursors supply and prevent the accumulation of	18.7	58.05	[42]

Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
						toxic metabolites			
	S. cerevisiae	CrtZ	*P. ananatis*	P_{PDC1}-*CrtE*-T_{PDC1}, P_{TPI1}-*CrtB*-T_{TPI1}, P_{GPM1}-*CrtI*-T_{GPM1}, P_{GPD}-*CrtY*-T_{GPD}, P_{FBA1}-*CrtZ*-T_{FBA1}	Chemical transformation	Zeaxanthin as a reporter gene for identification of promoter strength		0.74	[47]
	Y. Lipolytica	CrtZ	*P. ananatis*	P_{TEF1N}-*CrtE*-T_{xpr2}, P_{TEF1N}-*CrtB*-T_{xpr2}, P_{TEF1N}-*CrtI*-T_{xpr2}, P_{TEF1N}-*CarRP*-T_{xpr2}, P_{TEF1N}-*CrtZ*-T_{xpr2}	Frozen-EZ Yeast Transformation II Kit	High-copy-number integration of CrtZ gene into ribosomal DNA region		21.98 in YPD medium (3.2 in YNB medium)	[46]
astaxanthin	*E. coli*	CrtZ	*P. ananatis*	P_{TM2}-*CrtEBIA*, P_{T7}-*RLZ*-*CrtZ*-*RLW*-*CrtW*		Screening and regulation of promoters and RBSs	15.1	62	[71]
		CrtW	*Brevundimonas* sp. SD212						

Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
	S. cerevisiae	CrtZ	*A. aurantiacum*	A high β-carotene producing strain with P_{FBA1}-*CrtZ*-T_{ADH1}, P_{TDH3}-*CrtW*-T_{TDH2}	Homologous recombination	Selection and optimization of combinations of CrtW and CrtZ from different species	6.05		[72]
		CrtW	*Alcaligenes* sp.						
	Y. Lipolytica	CrtZ	*H. pluvialis*	P_{TEF}-*carRP*-T_{XPR2}, P_{TEF}-*thmgR*-T_{XPR2}, P_{TEF}-*GGS1*-T_{XPR2}, P_{TEF}-*carB*-T_{XPR2}, P_{TEF}-*CrtW*-$_{linker\text{-}RIDD}$-T_{XPR2}-P_{TER}-*CrtZ*-$_{linker\text{-}RIAD}$-T_{XPR2}	Chemical transformation	Selection of CrtW and CrtZ from different species and fine-tuning their transcription	17.5		[56]
		BKT	*H. pluvialis*						
	P. pastoris	CrtZ		P_{AOX1}-*CrtI*-T_{CYC1}, P_{AOX1}-*CrtE*-*CrtZ*-T_{CYC1}, P_{AOX1}-*CrtYB*-*CrtW*-T_{CYC1}	CRISPR/Cas9	Astaxanthin as a reporter gene for marker-less integration of multigene pathways into *Pichia pastoris* via CRISPR/Cas9		Approximately 2.5	[62]
		CrtW							

Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
a	b *K. marxianus*	CrtZ	*H. pluvialis*	P_{KlLac4}-*CrtZ*-T_{KlLac4}, $P_{ScGapDH}$-*CrtE*-T_{ScGap}, P_{ScPGK}-*CrtZ*-T_{ScPGK}, $P_{KlGapDH}$-*kanMX*-T_{ScGap}, P_{ICL}-*CrtI*-T_{35S}, P_{KlPGK}-*BKT*-T_{ScPGK}, P_{KlADH1}-*CrtYB*-T_{ScGap}, P_{ScADH1}-*tHMG*-T_{ScADH1}	Homologous recombination	Increasing the copy number of Hpchyb and BKT genes and modifying the Hpchyb by site-directed mutagenesis	3.125 in YPL medium, 5.701 in YPG medium		[60]
		BKT	*C. reinhardtii*		[95]	[30]			
[98]	*C. glutamicum*	CrtZ	*F. pelagi* +	P_{tuf}-*CrtZ*-linker-*CrtW*	electroporation with xenogeneic plasmid DNA	Fusion expression of CrtZ and CrtW, increasing the expression of upstream enzymes, mediated medium composition	3.1		[99][54]
		CrtW	*B. aurantiaca*						
canthaxanthin	*E. coli*	BKT	*Anabaena variabilis*	P_{Trc}-*CrtW*	Electroporation	Overexpression of host genes	Approximately 10.65	24.84	[83]

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capsorubin was only a minor product [37]. Therefore, it can be speculated that CCS may have a preference for violaxanthin over antheraxanthin. Any metabolic engineering of capsanthin or capsorubin in yeast has not yet been reported.

From the above, it is known that the metabolic engineering of epoxy-xanthophyll derivatives is still at the beginning stage. The cloning and characterization of key enzymes, substrate preference, and cofactor specificity need to be further investigated. From the catalytic properties of CCS, it can be speculated that the key enzymes, such as the NSY, fucoxanthin, or diadinoxanthin synthetic enzymes, may also involve multiple substrates or multiple products, which implies that the metabolic engineering of epoxy-xanthophyll derivatives is more difficult to control.

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Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
						increases the carbon flux into the canthaxanthin biosynthetic pathway			
	S. cerevisiae	OBKTM29 (mutant BKT)	*H. pluvialis*	P_{GAL1}-*mBKT*-T_{CYC1}-P_{GAL10}-*CrtE03*-T_{ADH2}, T_{CYC1}-$PM^{SeV\text{-}C}$-*mBKT*-P_{GAL1}-P_{GAL10}-*CrtYB*-T_{ADH2}, T_{CYC1}-*PDR3*-P_{GAL1}-P_{GAL10}-*CrtYB*-T_{ADH2}	Homologous recombination and CRISPR/cas9	Subcellular re-localization of OBKTM29 and its copy number adjustment both in the cytoplasm and on the periplasmic membrane, pleiotropic drug resistance (PDR) regulator overexpression	approximately 20–30	168	[84]
violaxanthin	*E. coli*	CrtZ	*P. ananatis*	P_{lac}-*CrtE*-*CrtY*-*CrtI*-*CrtB*-*CrtZ*, P_{lac}-*ZEP*, P_{T7}-*gdh*		Selection of ZEP from different species and optimization of *E. coli* strain, expression vector, and	0.231		[91]
		ZEP	*C. annuum*						

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Xanthophyll	Engineering Microbial Hosts	Key Enzymes	Natural Origin Species	Key Expression Cassettes [b]	Methods or Principles of Host Transformation	Key Strategies	Yield (mg/g DCW)	Titer (mg/L)	Ref.
		glucose dehydrogenase (gdh)	*B. subtilis*			ribosome-binding site (RBS) sequence			
		CrtZ	*P. ananatis*	P_{TDH3}-*CrtYB*-T_{CYC1}, P_{TDH3}-*CrtI*-T_{CYC1}, P_{TDH3}-*CrtE*-T_{CYC1}, P_{TEF1}-*CrtZ*-linker-*trZEP*-T_{ADH1}, P_{TEF1}-*trRFNR1*-T_{ADH1}, P_{PGK1}-*trFD3*-T_{CYC1}	Modified homologous recombination	Selection of CrtZ, ZEP and redox partner from different species and their truncated variants, increasing gene copy number of upstream carotenogenic genes	7.3		[92]
	S. cerevisiae	ZEP	*H. lacustris*						
		RFNR1	*A. thaliana*						
		FD3	*A. thaliana*						
capsanthin	*E. coli*	CrtZ	*P. ananatis*	P_{lac}-*HpIDI*-*CrtE*-*CrtY*-*CrtI*-*CrtB*-*CrtZ*, $P_{tac/T7}$-CCS_{M40}-*CaZEP*		A particularly high expression of CCS		0.5	[93]
		ZEP	*C. annuum*						
		CCS	*C. annuum*						

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