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REVIEW

Pathway engineering strategies for production of beneficial carotenoids in microbial hosts

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Abstract Carotenoids, such as lycopene, β-carotene, zeaxanthin, canthaxanthin and astaxanthin have many benefits for human health. In addition to the functional role of carotenoids as vitamin A precursors, adequate consumption of carotenoids prevents the development of a variety of serious diseases. Biosynthesis of carotenoids is a complex process and it starts with the common isoprene precursors. Condensation of these precursors and subsequent modifications, by introducing hydroxyl- and keto-groups, leads to the generation of diversified carotenoid structures. To improve carotenoid production, metabolic engineering has been explored in bacteria, yeast, and algae. The success of the pathway engineering effort depends on the host metabolism, specific enzymes used, the enzyme expression levels, and the strategies employed. Despite the difficulty of pathway engineering for carotenoid production, great progress has been made over the past decade. We review metabolic engineering approaches used in a variety of microbial hosts for carotenoid biosynthesis. These advances will greatly expedite our efforts to bring the health benefits of carotenoids and other nutritional compounds to our diet.

Keywords Astaxanthin · β-Carotene · Carotenoid · Lycopene · Metabolic engineering · Zeaxanthin

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Introduction

Carotenoids are tetraterpenoids containing 40 carbons. Carotenoids such as lycopene, α-carotene, and β-carotene do not contain an O atom and are often referred to as carotenes. Carotenoids such as canthaxanthin, zeaxanthin, astaxanthin and lutein, contain oxygen and are referred to as xanthophylls (Fig. 1). The health benefits of carotenoids are thought to derive from their activity as antioxidants (Krinsky and Johnson 2005). Carotenoids are suggested to be protective against cancer, coronary artery disease, and ocular diseases. In particular, lycopene intake is inversely related to prostate cancer risk (Giovannucci et al. 1995), intake of foods rich in carotenoids, especially β-carotene, is associated with reduced risk of cardiovascular disease (Mayne 1996); increased plasma levels of α-carotene, β-carotene, and lycopene tend to be inversely related with ischemic stroke (Hak et al. 2004); and increased plasma levels of lutein and zeaxanthin are associated with a decreased risk of age-related macular degeneration (Eye Disease Case-Control Study Group 1993). Because of their antioxidant properties and colors, carotenoids enjoy a wide

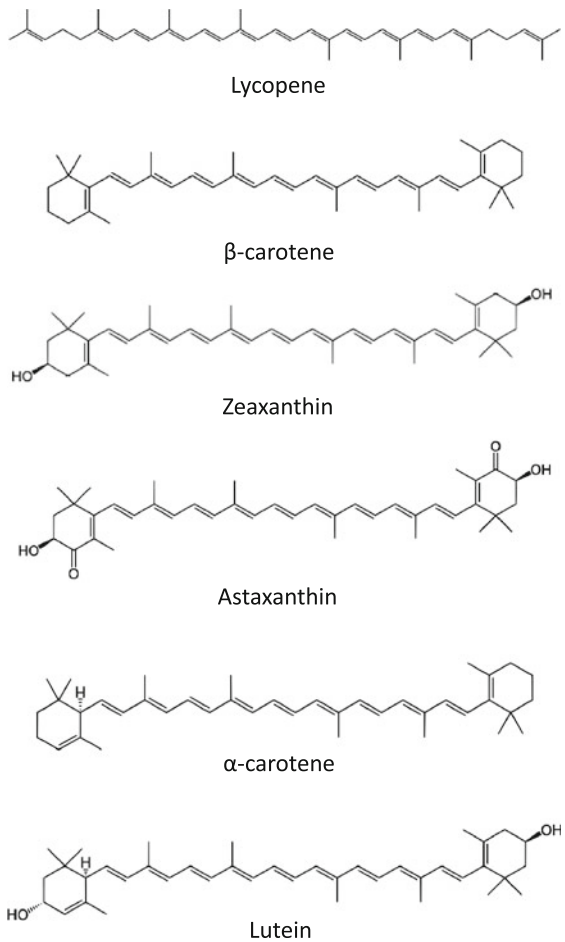

Fig. 1 Chemical structures of common beneficial carotenoids

variety of applications. They are used in nutritional supplements, fortified and natural food, and cosmetics. They are also used as coloring agents in eggs, shrimp, and fish. The total value of carotenoids in 2010 was $1 billion (BCC Research 2011). This figure can reach $1.4 billion within this decade. Currently, both lycopene and lutein are mainly extracted from natural sources such as tomato and marigold (Fernández-Sevilla et al. 2010; Vílchez et al. 2011). In addition, microalgae can be an effective alternative for natural source of caroteneoids. On the other hand, major carotenoids such as β-carotene, canthaxanthin, and astaxanthin are mainly derived from petroleum through chemical synthesis. Metabolic engineering of clinically important molecules represents a significant opportunity to improve public health through biotechnology (Ye and Bhatia 2012).

Carotenoid biosynthetic pathway

Carotenoids are derived from the five-carbon isoprene units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). There are two distinct pathways leading to the biosynthesis of these common precursors: the mevalonate pathway (MVA) and the non-mevalonate pathway (Fig. 2). The non-mevalonate pathway is also known as the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Lange et al. 2000; Hunter 2007). Plant plastids and most bacteria use the MEP pathway, while all eukaryotes use the mevalonate pathway in the cytoplasm (Lichtenthaler 2007; Okada 2011). The MVA pathway also exists in some bacteria (Lange et al. 2000). The MEP pathway starts with the condensation of pyruvate and glyceraldehyde 3-phosphate to form the 1-deoxyxylulose-5-phosphate (DXP) as the first intermediate. The 1-deoxyxylulose 5-phosphate reductoisomerase (DXR) catalyzes subsequent conversion of DXP to MEP. Further reactions are required before the formation of IPP and DMAPP. For the MVA pathway, the first step is the formation of acetoacetyl-CoA from acetyl-CoA. In the second reaction, acetoacetyl-CoA is converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Reduction of HMG-CoA to mevalonate is catalyzed by HMG reductase (HMGR). Up-regulation of the *HMGR* gene results in a significant increase in the carbon flux from the isoprenoid pathway. HMGR is a rate-limiting step of the MVA pathway.

The isoprene units IPP and DMAPP from the isoprenoid pathways can be condensed into the C_{10} molecule geranyl pyrophosphate (GPP) which is then converted to the C_{15} compound farnesyl pyrophosphate (FPP) with addition of an isoprene unit (Fig. 3). Another round of condensation forms the C_{20} geranylgeranyl pyrophosphate (GGPP). This step is catalyzed by CrtE, the GGPP synthase. The early part of the carotenoid biosynthesis starts with the formation of the first C_{40} carotenoid phytoene from two GGPP molecules. This step is catalyzed by phytoene synthase (PSY, CrtB). Desaturation and isomerization reactions lead to the formation of lycopene. The terminal isoprene groups of lycopene are cyclized to form β-carotene by the enzyme lycopene β-cyclase (LYC-b), while a joint reaction of both lycopene ε-cyclase (LYC-e) and LYC-b results in biosynthesis of α-carotene. Hydroxylation of each ring on α-carotene at the C-3 position produces lutein (Kim and DellaPenna 2006).

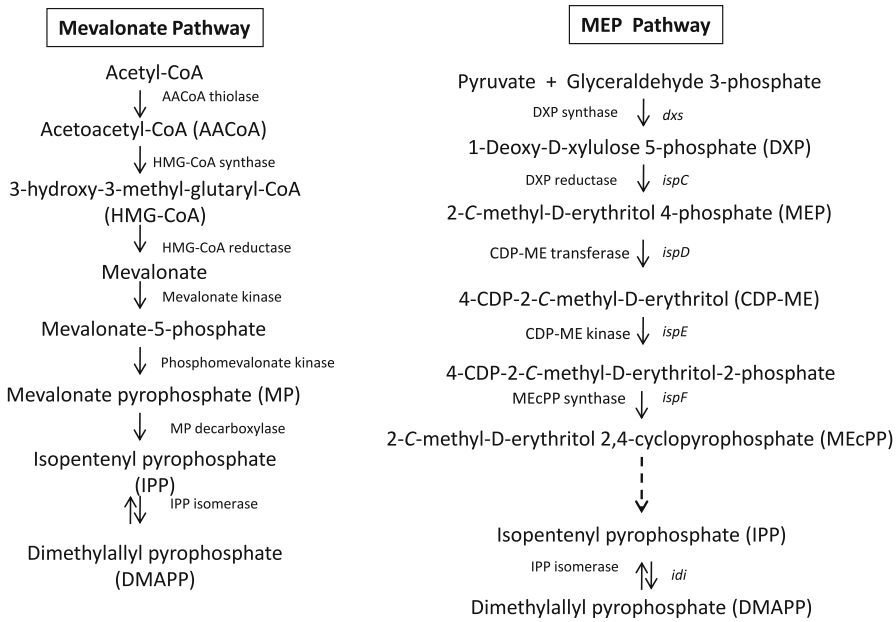

Fig. 2 Isoprenoid biosynthetic pathways

Starting from β-carotene, a variety of carotenoids can be synthesized due to the introduction of hydroxyl and keto groups on the β-ionone ring structure. In some organisms such as carotenogenic bacteria and algae, the β-carotene hydroxylase (BCH or CrtZ) adds the hydroxyl group at the 3,3′- positions and the β-carotene ketolase (BKT, CRTW, or CRTO) adds the keto groups at the 4,4′-positions (Misawa 2011). These reactions form beneficial carotenoids including zeaxanthin and astaxanthin. In carotenogenic red yeast *Xanthophyllomyces dendrorhous*, the formation of astaxanthin from β-carotene is catalyzed by a single enzyme, the astaxanthin synthase (Asy or CrtS), a cytochrome P450 monooxygenase (Alvarez et al. 2006; Schmidt et al. 2011). In the astaxanthin-producing *Adonis* flowers, a new pathway for astaxanthin biosynthesis has been discovered (Cunningham and Gantt 2011). The first step of the pathway is the activation of the number 4 carbon of the β-ionone ring by a carotenoid β-ring 4-dehydrogenase (CBFD), followed by the dehydrogenation of the carbon to form the keto group. This step is catalyzed by the 4-hydroxy-β-ring 4-dehydrogenase (HBFD). The third step of the reaction is the formation of hydroxyl group at the number 3 carbon. This step is catalyzed by the same CBFD enzyme. Identification of these two novel enzymes provides a tool for pathway engineering of astaxanthin production.

Strategies to optimize isoprenoid and carotenoid pathways for lycopene and β-carotene biosynthesis

Optimization of carbon flux to lycopene and β-carotene in *E. coli*

Since the isoprenoid pathway provides the common precursors for the biosynthesis of carotenoid and related compounds, increasing the carbon flux through this pathway is an important method for achieving desirable rate, titer, and yield for lycopene and β-carotenoids. In *E. coli*, several approaches have been used. The first approach involves optimization of the endogenous MEP pathway by balancing the expression of *dxs*, *idi*, and *ispDF* genes with downstream pathways (Ajikumar et al. 2010). The second approach uses the MVA pathway from yeast *S. cerevisiae* to get around the host regulatory mechanism (Martin et al. 2003). The subsequent improvement of this approach involves replacing the rate-limiting step, HMGR, with the corresponding gene from bacteria in conjunction with cofactor optimization (Pitera et al. 2007; Ma et al. 2011). The third approach explores the MVA pathways from bacteria (Yoon et al. 2009). This strategy combines different properties of the MVA pathways from bacterial sources. Specifically, the MVA pathway is

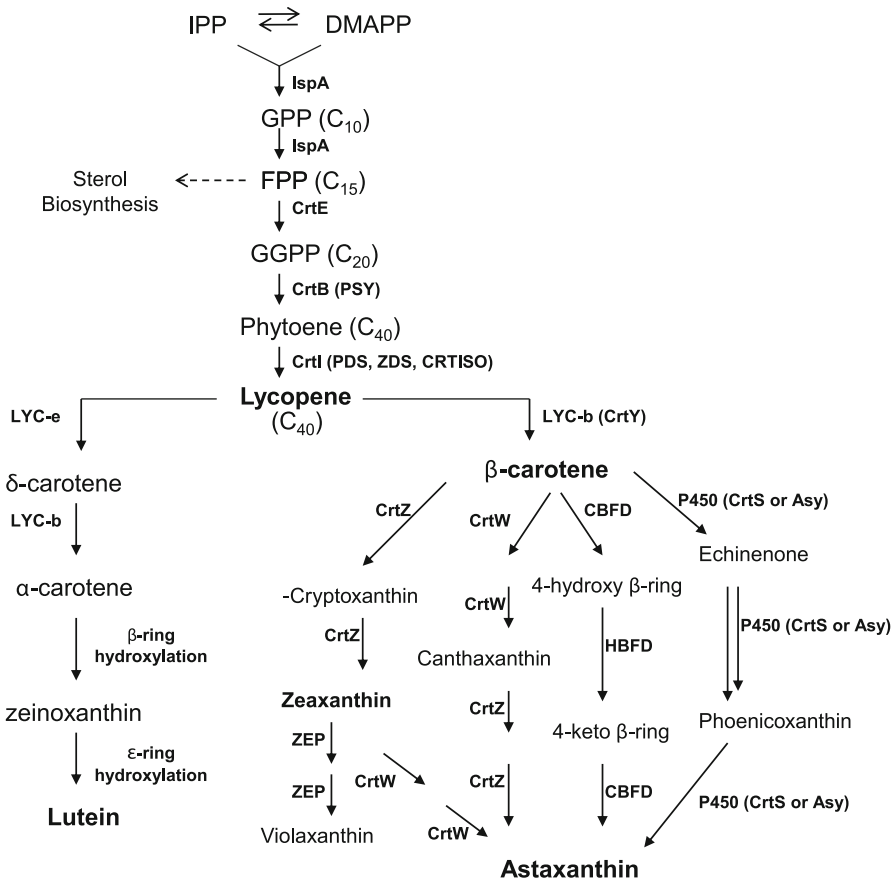

Fig. 3 General pathways for biosynthesis of beneficial carotenoids. *IPP* isopentenyl diphosphate; *DMAPP* dimethylallyl diphosphate; *GPP* geranyl pyrophosphate; *FPP* farnesyl pyrophosphate; *GGPP* geranylgeranyl pyrophosphate; *IspA* FPP synthase; *CrtE* GGPP synthase; CrtB (*PSY*) phytoene synthase; CrtI carotene desaturase; *LYC-b* lycopene β-cyclase (CrtY); *LYC-e* lycopene ε-cyclase; *CBFD* carotenoid β-ring 4-dehydrogenase; *HBFD* carotenoid 4-hydroxy-β-ring 4-dehydrogenase; *CrtZ* carotenoid β-ring 3(3′)-hydroxylase; *CrtW* carotenoid β-ring 4(4′)-ketolase (4(4′)-oxygenase; *P450* cytochrome P450 monooxygenase (CrtS or Asy)

divided into two modules. The top portion produces the mevalonate from acetyl-CoA. The bottom portion synthesizes the IPP and DMAPP from mevalonate. Initially, the bottom portion of the MVA pathway from S*treptococcus pneumonia*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Saccharomyces cerevisiae* were compared in the *E. coli* strain containing β-carotene biosynthetic genes. In this comparison, the mevalonate from the top portion of the MVA pathway was supplemented exogenously. The experiment showed that the bottom MVA pathway of *S. pneumoniae* produced the greatest amount of β-carotene. The top portions of MVA pathway from various bacteria were subsequently compared and the top MVA pathway of *E. faecalis* was the most efficient for mevalonate production. Based on these results, the whole MVA pathway was constructed by combining the bottom and top portions of MVA pathway of *S. pneumoniae* and *E. faecalis*, respectively. The final recombinant *E. coli* strain produced β-carotene at a titer of 465 mg/L, which was higher than the titer of 390 mg/L obtained with the strain using the MEP pathway.

Carbon sources greatly influence the production of carotenoids, such as lycopene, in metabolically-engineered *E. coli* strain bearing the MVA pathway (Kim et al. 2011). Due to catabolite repression, lycopene production was lower with glucose or L-arabinose as a carbon source than with glycerol. In contrast, when glucose and L-arabinose were supplemented in a fed batch culture experiment with glycerol as the main carbon source, significant increases in cell mass and titer were observed. The cell mass, lycopene concentration, specific lycopene content, and lycopene productivity reached 42 g/L, 1,350 mg/L, 32 mg/g cells, and 40 mg/L per hour, respectively. This is the highest concentration and productivity of lycopene reported in the literature. Although the detailed mechanism was not elucidated, supplementation of glucose and L-arabinose synergistically stimulated the engineered mevalonate pathway and thus improved the carbon flux in the transgenic strain. The concentration and

productivity of lycopene in this strain were 1.5- and 5.1-fold greater, respectively, than those in the carotenogenic fungi, *Blakeslea trispora* (Tereshina et al. 2010). *B. trispora* has been commercially used for lycopene production (Vitatene, Leon, Spain). Based on this comparison, the constructed *E. coli* strain can be a commercially viable option.

Engineering non-carotenogenic yeasts for lycopene and β-carotene production

Although the budding yeast *S. cerevisiae* does not naturally make carotenoids, there are several advantages to using it as a host for carotenoid production. This food yeast is widely used in the brewing and food industries. It is generally recognized as safe (GRAS). Being a model organism, its genetic system, physiology, and regulatory networks have been characterized. The fermentation technology for large-scale production is also established. Unlike bacteria, there is no issue of bacterial phage contamination during large-scale fermentation.

In an early effort to produce β-carotene in *S. cerevisiae*, bacterial genes *crtE*, *crtB*, *crtI*, and *crtY* were over-expressed in episomal expression vectors. This approach succeeded in engineering the yeast to produce β-carotene, but the production level was low (103 μg/g dry wt) (Yamano et al. 1994). Using a different strategy, expression of carotenogenic genes from red yeast *X. dendrorhous* was studied (Verwaal et al. 2007). These genes included *crtE*, *crtI* and *crtYB*, which encode a bifunctional phytoene synthase and lycopene cyclase. Instead of using plasmids, carotenoid genes were successively integrated into the yeast chromosome to improve strain stability. To further improve carbon flux, isoprenoid genes encoding tHMG1 and GGPP synthase were also introduced, resulting in a higher carbon flux as shown by an increase in carotenoid production. The β-carotene concentration reached 5.9 mg/g dry wt in the final strain. Optimal expression of genes in both isoprenoid and carotenoid pathways is necessary to increase the β-carotene content in *S. cerevisiae*.

Sterol biosynthesis in yeast requires the same common isoprenoid precursor FPP (Fig. 3). Another strategy to improve the carbon flux for carotenoid biosynthesis in yeast is a combination of an increase in the expression of the MVA pathway with the inhibition of sterol biosynthesis. This approach was demonstrated with the over-expression of the HMG-CoA reductase coupled with the addition of ergosterol inhibitors (Yan et al. 2011). The experiment showed that over-expression of the HMG-CoA reductase gene or the addition of inhibitor ketoconazole at 100 mg/L resulted in a 135 or 15.6 % increase in β-carotene content, respectively. The combination of these two approaches achieved a >200 % increase in β-carotene concentration, reaching up to 6.3 mg/g dry cell wt. Although ergosterol inhibitors are not practical, genetic approaches can be used to reduce the flux to sterol biosynthesis.

The production of carotenoids has also been explored in non-carotenogenic yeast *Pichia pastoris* (Araya-Garay et al. 2011; Bhatayaa et al. 2009). *P. pastoris* can grow to high cell densities with various organic materials, including methanol. When genes involved in lycopene production were expressed on plasmids, the constructed strain was capable of producing 4.6 mg lycopene/g dry cell wt, with a concentration of 73.9 mg lycopene/L. When the genes involved in β-carotene biosynthesis were introduced through chromosomal integration, lycopene and β-carotene at 1,14 and 340 μg/g dry cell wt, respectively, were obtained. Both experiments demonstrate the possibility of engineering *P. pastoris* for carotenoid biosynthesis, providing an alternative yeast production host.

The oleaginous yeast, *Yarrowia lipolytica,* is another alternative production host for carotenoids. *Yarrowia lipolytica* is a well-established industrial strain for a variety of useful compounds. An EU-sponsored LipoYeasts project is developing the oleaginous yeast, *Y. lipolytica,* into a microbial production platform that will direct its lipid metabolism towards production of industrially-valuable compounds such as wax esters, isoprenoid-derived compounds (carotenoids, polyenic carotenoid ester), polyhydroxyalkanoates, and free hydroxylated fatty acids (Sabirova et al. 2011). In this project, different lipid feedstocks (petroleum, alkane, vegetable oil, fatty acid) are being evaluated. In addition to the many potential feedstocks, genetic feasibility, and ability for industrial scale-up, *Y. lipoliptica* contains a large lipid body under certain growth conditions. This property provides a repository for carotenoids. Industrial companies have invested effort in developing *Yarrowia* as a production host for various carotenoids (Bailey et al. 2011).

Metabolic engineering for zeaxanthin biosynthesis

Engineering the zeaxanthin pathway in bacteria

Biosynthesis of zeaxanthin is the result of hydroxylation of the β-ionone rings on β-carotene (Figs. 1, 2). In bacteria, five genes, *crtE*, *crtI*, *crtB*, *crtY*, and *crtZ*, are required for zeaxanthin biosynthesis. To engineer zeaxanthin pathway with a high carbon flux, three MEP pathway genes (*idi*, *ispA*, and *dxs*) were cloned under the control of the L-rhamnose-inducible promoter in a shuttle vector (Beuttler et al. 2011). High-level expression of these three genes can increase the biosynthesis of products derived from the MEP pathway (Ajikumar et al. 2010; Wang et al. 2009). In addition, all five genes for zeaxanthin production were also cloned in the same operon in the shuttle vector. The recombinant *E. coli* strain harboring this plasmid produced zeaxanthin up to 2.4 mg/g dcw in Terrific Broth (TB) medium as compared to 1.0 mg/g dcw production in Luria-Bertani (LB) broth. When the same plasmid was transferred into wild type *P. putida* via conjugation, the frequency of transformation was low and the transconjugants were not stable. It was determined that production of lycopene was toxic to the *P. putida*. To overcome this problem, lycopene-tolerant mutants were obtained through transposon mutagenesis. In one mutant strain harboring the zeaxanthin plasmid, the highest concentration of zeaxanthin reached 7 mg/g dcw with a volumetric yield of 51.3 mg/L. This result was again achieved in TB medium, indicating that this medium was more appropriate for zeaxanthin production for both *E. coli* and *P. putida*. The specific components responsible for increasing the zeaxanthin production remain to be elucidated; TB contains increased amounts of yeast extract and peptone. Glycerol is often added, which can increase zeaxanthin production (Kim et al. 2011). To alleviate the potential inhibitory effects of carotenoid production in *P. putida*, a set of hydrophobic substances were added to extract the carotenoids in situ. These compounds included choline, lecithin, Amberlite XAD-7, and oleic acid. The best results were obtained with lecithin and oleic acid. The addition of lecithin enhanced the volumetric yield by a factor of 4.7, reaching up to 239 mg/L. This experiment demonstrated *P. putida* as a microbial host for zeaxanthin production.

Engineering biosynthetic pathways for astaxanthin production

Pathway optimization for astaxanthin production in *E. coli*

Astaxanthin is primarily used as dietary supplement and feed additive in aquaculture. Chemically-synthesized astaxanthin dominates the market for trout and salmon farming. The production cost by the chemical process is $1000/kg (Li et al. 2011). The production cost from the green alga *Haematococcus pluvialis* is $3000/kg. Astaxanthin from natural sources is not economically competitive and only has niche applications (Schmidt et al. 2011). Though improvement in growth or fermentation processes for natural strains could reduce costs, metabolic engineering of microbes or plants for astaxanthin biosynthesis provides another option. In this regard, *E. coli* serves as a model system for technology development.

Biosynthesis of astaxanthin requires the hydroxylation and ketolation of the β-ionone rings of β-carotene (Figs. 1, 3). It has been shown that many bacterial hydroxylase CrtZ and ketolase CrtW are bifunctional (Misawa 2011). They can accept β-carotene as well as hydroxylated or ketolated products as substrate, resulting in the formation of different carotenoid intermediates. A key strategy to engineer pathways for astaxanthin production from β-carotene is to ensure proper activities of both hydroxylase CrtZ and ketolase CrtW to reduce intermediate accumulation. This includes the choice of appropriate enzymes and their balanced expressions. In a plasmid-free system in *E. coli*, the β-carotene ketolase gene *crtW148* from *Nostoc punctiforme* and the β-carotene hydroxylase gene *crtZ* from *Pantoea ananatis* were expressed (Lemuth et al. 2011). Using the λ-Red recombineering, all the *crt* genes were stably integrated in the chromosome. Expression of *crtEBIY* genes from *P. ananatis* and the *crtW148* gene were controlled by the IPTG inducible *tac*-promoter. To achieve a variable expression, *crtZ* was expressed under control of the rhamnose-promoter. To obtain a high ratio of activity of ketolase to hydroxylase, minimal medium with glucose as the carbon source was used. After induction by IPTG for 24 h, there were significant amounts of ketolated intermediates adonirubin (13 %) and canthaxanthin (12 %). However, there was little hydroxylated intermediate zeaxanthin

in the medium. After 48 h, the strain produced astaxanthin as the predominant carotenoid (95 %) at a concentration of 1.4 mg/g dcw. The results indicated that the β-carotene was mainly converted to ketolated intermediates by CrtW before hydroxylation by the CrtZ in this system, due to the higher ketolase activity.

In a similar study with a single-vector system, the *crtZ* gene was placed under the control of the *trc* promoter while the *crtW* gene was regulated by pBAD (Scaife et al. 2009). The *trc* promoter is a hybrid of the *lac* and *trp* promoters and is stronger than the pBAD promoter in the absence of inducer. As a result, the hydroxylase activity was relatively high in this configuration. The *E. coli* strain with this vector had a low level of the astaxanthin and the intermediate concentrations were high. In the alternative configuration, pTrcCrtW-pBADCrtZ, the ketolase activity was higher. This strain had an increased selectivity for astaxanthin production (about 90 %) with low concentration of intermediates. Appropriate activities of β-carotene ketolase and hydroxylase are important for astaxanthin biosynthesis.

The balance of activities for both ketolase and hydroxylase can also be achieved through protein engineering. Mutagenesis of the ketolase can lead to change of enzyme properties or expression levels (Ye et al. 2006; Tao et al. 2006). One of the strategies for screening CrtW mutants was based on color change of *E. coli* colonies. In this system, the background strain contained two plasmids. The first plasmid bore the *crtZEYIB* gene cluster for zeaxanthin production. The second plasmid was a lower copy number plasmid containing the *crtW* gene. The activity of the CrtW enzyme was relatively low due to low expression level, and the *E. coli* strain produced astaxanthin with the predominant products as zeaxanthin and adonixanthin. With the addition of L-arabinose, the *E. coli* colonies appeared yellow for at least 24 h. Colonies with improved CrtW activity showed an increase in astaxanthin production, resulting in a change of color from yellow to orange. Successful identification of CrtW variants demonstrates the usefulness of this approach to improve the ketolase. Further improvement of the ketolase or other enzymes for carotenoid biosynthesis, using protein engineering, is a strategy for strain construction in different production hosts.

Engineering methane-utilizing bacteria for astaxanthin production

Astaxanthin production in a methane-utilizing bacterium was investigated to explore alternative feedstocks for biotechnology development (Ye et al. 2007). Obligate methanotrophic bacteria, such as *Methylomonas* sp. can use either methane or methanol as the sole carbon source. To obtain a stable strain, the initial step in constructing the carotenoid pathway was to introduce the canthaxanthin gene cluster, *crtWidiEYIB*, in the chromosome. Subsequent conversion to astaxanthin was optimized by integrating different copies of *crt* genes, particularly *crtW* and *crtZ.* In the strain containing only a single copy of both *crtW* and *crtZ* genes, 64 % of the total carotenoid was astaxanthin, while the partial ketolated intermediate adonixanthin was 32 %. In the strain containing one copy of *crtW* and two copies of *crtZ*, the amount of adonixanthin increased to 60 %. This result indicated that the ketolase activity was limiting in both strains. In contrast, in the strain containing two copies of the *crtW* genes and a single copy of *crtZ,* the amount of partially hydroxylated intermediate adonirubin was increased, suggesting that the hydroxylation step was limiting. Production of astaxanthin as the major product was achieved by integrating two complete sets of gene clusters for astaxanthin biosynthesis, which included two copies of *crtW* and *crtZ.* Under batch fermentation conditions with methane as the carbon source, this final strain produced astaxanthin at a titer up to 4 mg/g dcw, and 90 % of the carotenoid was astaxanthin. In addition, the bioavailable form of astaxanthin, all *E*-isomer, was 89 % of the total astaxanthin. The generated strain had the desirable properties for aquaculture applications, though a higher titer is required for a cost-effective process.

Astaxanthin production in the engineered strain was affected by O_2 availability. At low O_2 levels, astaxanthin biosynthesis was lower. To overcome this problem, bacterial hemoglobin was expressed in *Methylomonas* to improve astaxanthin production (Tao et al. 2007). Three truncated hemoglobin genes, thbN1, thbN2, and thbO, were isolated from the genome of *Methylomonas sp.* strain 16a. Co-expression of these hemoglobin genes along with the *crtWZ* genes in strain 16a resulted in a higher astaxanthin production than did expression of the *crtWZ* genes

alone. The mechanism of astaxanthin enhancement is likely the improvement of the oxygen-requiring hydroxylase and ketolase activities by hemoglobin.

The expression levels of carotenoid genes are impacted by locations after integration in the chromosome. Continuing improvement of *Methylomonas* for astaxanthin production required the identification of appropriate loci. To take advantage of the color selection, three promoterless carotenoid transposons were constructed and used to identify locations that could support astaxanthin production (Sharpe et al. 2007). Ten- to 20-fold increases in carotenoid content were found in five chromosomal locations, the *fliCS*, *hsdM*, *ccp-3*, *cysH*, and *nirS* regions. Successful application of these carotenoid transposons demonstrates that metabolic engineering of microorganisms can be greatly advanced by identifying chromosomal locations of the host that permit optimal expression of target genes.

Engineering yeasts for astaxanthin production

The carotenogenic red yeast *X. dendrorhous* naturally produces astaxanthin. To develop an economic process that can compete with chemical synthesis, several strategies to increase astaxanthin titers in this organism have been explored. These include genetic engineering of the astaxanthin pathway, random mutagenesis, and optimization of fermentation (Schmidt et al. 2011). One example of genetic engineering for yield improvement is the expression of cDNA encoding the GGPP synthase (CrtE; Fig. 3). Expression of this gene diverts the carbon flux from sterol biosynthesis to carotenoid production (Breitenbach et al. 2011). The increased level of GGPP synthase led to a higher titer. Under optimal physiological conditions for carotenoid biosynthesis, dim light and extra air supply of the shaking culture, the titer of 451 μg/g dry cell wt was obtained within 4 days of growth. This demonstrates that metabolic engineering can be performed in natural astaxanthin-producing yeast.

To engineer non-carotenogenic yeast *S. cerevisiae* for astaxanthin production, phytoene desaturase (CrtI) and bifunctional phytoene synthase/lycopene cyclase (CrtYB) genes of the red yeast *X. dendrorhous* were used (Ukibe et al. 2009). The constructed strain resulted in the accumulation of a small amount of β-carotene (about 18 μg/g dry cell wt). Overexpression of geranylgeranyl pyrophosphate (GGPP) synthase from *S. cerevisiae* (the *BTS1* gene product) led to a 22-fold increase in β-carotene content (390 μg/g dry cell wt), due to accelerated conversion of farnesyl pyrophosphate to GGPP. For astaxanthin production, the *X. dendrorhous* astaxanthin synthase gene (*CrtS*), a cytochrome P450 enzyme, was introduced. The strain, however, did not produce astaxanthin. Only when *CrtS* was co-expressed with *X. dendrorhous* cytochrome P450 reductase gene *CrtR*, the recombinant strain produced astaxanthin (3 μg/g dry cell wt). As an alternative approach, bacterial genes *crtW* and *crtZ* were expressed in *S. cerevisiae* for astaxanthin production. A higher level of astaxanthin (29 μg/g dry cell wt) was obtained, suggesting that the bacterial CrtW and CrtZ enzymes were functional in yeast. The yield of astaxanthin production in engineered *S. cerevisiae* was still substantially lower than that of *X. dendrorhous*. Further optimization of the overall pathway is necessary. The β-carotene content in the transgenic *S. cerevisiae* strain was more than 10-fold greater than the astaxanthin content. Improvement in the β-carotene hydroxylase and ketolase steps should increase the specific yield for astaxanthin.

Conclusion

Growing evidence for human health benefits of carotenoids has driven the metabolic engineering effort to produce these molecules in a sustainable and cost-effective manner. Microbial systems, especially industrial strains, offer advantages of easy genetic manipulation, a better understanding of cell metabolism, fast growth, and existing fermentation technologies for large-scale production. Great progress has been made in increasing the carbon flux for lycopene production in *E. coli* by expressing the MVA pathway in combination with optimization of carbon sources (Kim et al. 2011). In fact, the titer and productivity in the recombinant strain are very close to, if not better than, the current commercial process. This demonstrates the potential of metabolic engineering for carotenoid production in microbes.

The overall biosynthetic pathway for carotenoid production is well-established (Fig. 2). Carbon flux through the isoprenoid pathway leading to the biosynthesis of IPP and DMAPP can be increased by overcoming rate limiting steps, depending on the

organism and the pathway used. The carotenoid biosynthetic pathway leads to the formation of colorful carotenoids that can be visualized. As a result, carotenoid biosynthesis provides a model system to develop and validate metabolic engineering technologies. These new technologies will expedite the effort to deliver the health benefits of carotenoids.

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