Metabolic engineering for the microbial production of isoprenoids: Carotenoids and isoprenoid-based biofuels

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Isoprenoids are the most abundant and highly diverse group of natural products. Many isoprenoids have been used for pharmaceuticals, nutraceuticals, flavors, cosmetics, food additives and biofuels. Carotenoids and isoprenoid-based biofuels are two classes of important isoprenoids. These isoprenoids have been produced microbially through metabolic engineering and synthetic biology efforts. Herein, we briefly review the engineered biosynthetic pathways in well-characterized microbial systems for the production of carotenoids and several isoprenoid-based biofuels.  

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1. Introduction  

Isoprenoids, also called terpenoids or terpenes, are the most abundant and highly diverse (structurally and functionally) group of natural products synthesized in almost all living organisms. Many isoprenoids have been used for pharmaceuticals, nutraceuticals, flavors, cosmetics, food additives and biofuels. Isoprenoids are usually classified into groups according the number of carbons: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenes (carotenoids, C40). 

All isoprenoids derive from isopentenyl diphosphate (IPP) and
its isomer dimethylallyl diphosphate (DMAPP) (Fig. 1). They can be produced by two metabolic pathways, the mevalonate pathway (MVA or MEV) [1] and the 1-deoxy-o-xylulose-5-phosphate (DXP) pathway (also called the 2-C-methyl-D-erythritol 4-phosphate pathway, MEP pathway) [2]. The MVA pathway is mainly present in archaea, fungi, plant cytoplasm and other eukaryotes. The DXP pathway is mostly found in bacteria and plant plastids. The MVA pathway initiates with the condensation of two acetyl-CoAs by thiolase to produce acetoacetyl-CoA. Subsequently, another acetyl-CoA is condensed with acetoacetyl-CoA to synthesize 3-hydroxy-3-
methyl-glutaryl-CoA (HMG-CoA) by HMG-CoA synthase. Then, mevalonic acid is formed from HMG-CoA using NADPH as a cofactor by HMG-CoA reductase. Two kinases, mevalonate kinase (MK) and phosphomevalonate kinase (PMK), sequentially catalyze the phosphorylation of mevalonate to produce mevalonate-5-diphosphate (Mev-PP). The final step of the MVA pathway to form IPP is the ATP-driven decarboxylation catalyzed by mevalonate diphosphate decarboxylase (PMD). The isomerization of IPP by isopentenyl diphosphate isomerase (Idi) leads to DMAPP formation and the initiation of isoprenoid biosynthesis (Fig. 1). The overall stoichiometry of the MVA pathway for synthesizing IPP from glucose is given by equation (1).

\[
1.5 \text{Glucose} + 2 \text{NADPH} + 6 \text{NAD} = \text{IPP} + \text{CO}_2 + 2 \text{ADP} + 2 \text{NADP} + 6 \text{NADH}
\]

The DXP pathway consists of seven enzymatic steps that convert glyceraldehyde 3-phosphate (G3P) and pyruvate to IPP and DMAPP in a ratio of 3:1 [3]. The DXP pathway starts with the condensation of glyceraldehyde 3-phosphate (G3P) and pyruvate to IPP and DMAPP (Fig. 1). This step is crucial and is known as the rate-limiting step of the DXP pathway. DXP is then converted to 2-C-methyl-D-erythritol-4-phosphate (MEP), 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-MEP), 2-C-methyl-D-erythritol-4-cyclodiphosphate (MEcPP), 4-hydroxy-3-methyl-butenyl 1-diphosphate (HM-BP), and IPP and DMAPP via the series of enzymatic reactions. The overall stoichiometry of the DXP pathway for synthesizing IPP from glucose is given by equation (2).

\[
\text{Glucose} + 2 \text{ATP} + 3 \text{NADPH} + \text{NAD} = \text{IPP} + \text{CO}_2 + 2 \text{ADP} + 3 \text{NADP} + \text{NADH}
\]

From equations (1) and (2), it can be found that the theoretical maximum IPP yield on glucose via the DXP pathway [5/(6 = 0.83 C-mol/C-mol)] is higher than that via the MVA pathway [5/(9 = 0.56 C-mol/C-mol)]. However, the DXP pathway needs one mol more NADPH and 2 mol more ATP than the MVA pathway, indicating that the DXP pathway requires more energy and reducing equivalents.

As most isoprenoids were originally discovered in plants, their demand from plant extraction because of the slow growth rate and the DXP pathway requires more energy and reducing equivalents. The overall stoichiometry of the DXP pathway for synthesizing IPP from glucose is given by equation (1).

Carotenoids are an important group of natural and liposoluble pigments with multiple physiological and nutritional functions. They were found in plants, fungi, algae and bacteria, displaying yellow, orange or red color. Carotenoids are widely used as food colorants, food and cosmetics additives, health supplements, animal feeds and nutraceuticals. At least 700 carotenoids have been characterized [4]. Carotenoids can be classified into C30, C40 and C50 carotenoids [5]. More than 95% of known carotenoids are C40 carotenoids [6]. The global carotenoids market in 2015 was $1.23 billion, with an expected increase to $1.81 billion by 2022 [7].

## 2. Carotenoid products

Carotenoids are an important group of natural and liposoluble pigments with multiple physiological and nutritional functions. They were found in plants, fungi, algae and bacteria, displaying yellow, orange or red color. Carotenoids are widely used as food colorants, food and cosmetics additives, health supplements, animal feeds and nutraceuticals. At least 700 carotenoids have been characterized [4]. Carotenoids can be classified into C30, C40 and C50 carotenoids [5]. More than 95% of known carotenoids are C40 carotenoids [6]. The global carotenoids market in 2015 was $1.23 billion, with an expected increase to $1.81 billion by 2022 [7].

### 2.1. Lycopene

Lycopene is one of the most widely used carotenoids in the healthcare product market owing to its excellent performance as an antioxidant and its great potential in the reduction of prostate cancer risk in humans. With the development of metabolic engineering, the heterologous expression of the lycopene biosynthetic pathway in *Escherichia coli* and *Saccharomyces cerevisiae* has become a promising strategy for lycopene production.

The following strategies have been used to improve lycopene production in *E. coli* 1) overexpression of the rate-limiting enzyme’s genes; 2) removal of the competing pathways; 3) introduction of a heterologous MVA pathway; 4) cofactor engineering; 5) genome modifications, including promoter replacement and chromosomal evolution [8]. A shot-gun approach was used to screen native genes that should be overexpressed in lycopene production. Overexpression of the *dxs, appY, ctrl* and *rpoS* genes improved lycopene production [9]. Flux scanning based on enforced objective flux (FSEOF) was successfully employed for the identification of gene amplification targets for improving lycopene production. Co-overexpression of the *dxs, idi* and *mdh* genes enhanced lycopene production [10]. The deletions of *gdtA* and *gpmAB* improved lycopene production [10]. Flux balance analysis also revealed that the deletions of *aceF, fdhF* and *gdtA* enhanced lycopene production by 40% [11]. Zhou et al. reported that the ZFW knockout increased lycopene production by 130X [12]. Introduction of a heterologous MVA pathway into *E. coli* increased the IPP supply, leading to an increase in lycopene production [13–16]. Introduction of a heterologous MVA pathway increased lycopene content up to 198 mg/g dry cell weight (DCW) from 68 mg/g DCW [15]. Our group also reported that the chromosomal heterologous expression of the optimized *S. cerevisiae* MVA pathway can further improve lycopene production [16]. The promoter engineered *E. coli* Lycop 20 produced lycopene at 529.45 mg/L and 20.25 mg/g DCW in a fed-batch culture [16]. Combined modulating expression of *sucAB, sdtABCD* and *talB* with the regulatory part for increasing ATP and NADPH availability, and *dxs, idi* and *crbT* with the RBS libraries resulted in a significant increase in lycopene production to 3.52 g/L with a content of 50.6 mg/g DCW [17]. Zhu et al. applied the targeted engineering strategy to construct an engineered *E. coli* harboring the MVA and DXP pathway that produced lycopene at 1.23 g/L (34.3 mg/g DCW) in a 100-L fed-batch fermentation [18]. Kim et al. constructed an engineered *E. coli* co-expressing the DXP and MVA pathway that produced lycopene at 1.35 g/L (32 mg/g DCW) in a 2-L fed-batch fermentation [19]. Plasmid-based overexpression of genes has been the principal strategy for metabolic engineering. However, plasmid-based expression systems are not suitable because of genetic instability and the requirement for constant selective pressure to ensure plasmid maintenance. Thus, a chemically induced chromosomal evolution (CIChE), which is a plasmid-free and high gene copy expression system for engineering *E. coli*, was first developed to overcome these drawbacks by Tyo [20]. We applied the CIChE strategy to construct a lycopene hyper-producer *E. coli* that does not carry a plasmid or an antibiotic marker. The CIChE strategy is another host strain of metabolic engineering for
Table 1
Production of isoprenoids by engineered microorganisms.

| Isoprenoids produced | Host                  | Approach                                                                 | Culture conditions | Yield/Titer                | References |
|----------------------|-----------------------|--------------------------------------------------------------------------|-------------------|---------------------------|------------|
| Lycopene             | *E. coli*             | Systematic (model-based) methods; Combinatorial (transposition-based) methods; Gene knockout. | Shake-flask fermentation | 18 mg/g DCW              | [11]       |
| Lycopene             | *E. coli*             | Central metabolic genes knockouht; Amplification of MEP pathway genes     | Shake-flask fermentation | 7.55 mg/g DCW             | [12]       |
| Lycopene             | *E. coli*             | Overexpression of native *dxs*; Other optimization methods (promoters, vectors, strains) | Shake-flask fermentation | 16.8 mg/L             | [13]       |
| Lycopene             | *E. coli*             | Introduction of a heterologous MVA pathway; Overexpression *Bacillus licheniformis id* | Shake-flask fermentation | 198 mg/g DCW             | [15]       |
| Lycopene             | *E. coli*             | Optimization of MVA pathway; Promoter engineering                        | Fed-batch fermentation | 20.25 mg/g DCW           | [16]       |
| Lycopene             | *E. coli*             | Increase ATP and NADPH; Engineering TCA modules; Overexpression of *dxs/id*/*ctE* | Fed-batch fermentation | 50.62 mg/g DCW           | [17]       |
| Lycopene             | *E. coli*             | Application of the targeted engineering strategy                          | Fed-batch fermentation | 34.3 mg/g DCW           | [18]       |
| Lycopene             | *E. coli*             | Co-expression of the DXP and MVA pathway                                 | Fed-batch fermentation | 32 mg/g DCW             | [19]       |
| Lycopene             | *E. coli*             | Application of CiChE                                                    | Shake-flask fermentation | 33.43 mg/g DCW           | [21]       |
| Lycopene             | *E. coli*             | Optimization of the lycopene biosynthetic genes; Overexpression the MEP pathway (*dxs-id*-*id*/*dpID*) | Shake-flask fermentation | 448 mg/g DCW             | [22]       |
| Lycopene             | *S. cerevisiae*       | Combination of directed evolution and metabolic engineering strategy    | Fed-batch fermentation | 24.41 mg/g DCW           | [23]       |
| Lycopene             | *S. cerevisiae*       | Combination of host engineering and pathway engineering                  | Fed-batch fermentation | 55.56 mg/g DCW           | [25]       |
| Lycopene             | *Y. lipolytica*       | Deletion of POX1 and GUT2                                                | Shake-flask fermentation | 16 mg/g DCW             | [26]       |
| Lycopene             | *S. avermitilis*      | Activation of the silent lycopene synthetnic gene cluster                | Shake-flask fermentation | 82 mg/g DCW             | [27]       |
| β-carotene           | *E. coli*             | Plasmid-expressing the lower MVA pathway and id* from *S. cerevisiae*; Plasmid-expressing the upper MVA pathway from *Enterococcus faecalis*, *Bacillus subtilis* *ds* and *fni*, and *GPPS2* from *Abies grandis*; Plasmid-expressing the β-carotene synthetic pathway. | Fed-batch fermentation | 60 mg/g DCW             | [28]       |
| β-carotene           | *E. coli*             | Combined engineering of the MEP, the β-carotene synthetic, the TCA and the pentose phosphate (PP) modules by artificial modulation parts | Fed-batch fermentation | 3.2 g/L             | [29]       |
| β-carotene           | *E. coli*             | Optimizing the biosynthetic pathway                                      | Fed-batch fermentation | 2.0 g/L             | [30]       |
| β-carotene           | *S. cerevisiae*       | Decentralized assembly strategy                                          | Fed-batch fermentation | 7.41 mg/g DCW           | [31]       |
| β-carotene           | *S. cerevisiae*       | Using the inducer/inhibitor-free sequential control strategy to sequentially control the expression of the carotenoid pathway, the MVA pathway and the competitive squalene pathway by glucose in the culture broth, | Fed-batch fermentation | 20.79 mg/g DCW, 1156 mg/L | [32]       |
| Zeaxanthin            | *E. coli*             | Optimization of the zeaxanthin biosynthetic pathway                      | Shake-flask fermentation | 11.95 mg/g DCW           | [38]       |
| Zeaxanthin            | *E. coli*             | Introduction of a dynamically controlled TIGR-mediated MVA pathway       | Fed-batch fermentation | 23.16 mg/g DCW           | [39]       |
| Astaxanthin           | *E. coli*             | Chromosomal expressing the optimized synthetic pathway                   | Shake-flask fermentation | 7.50 mg/g DCW           | [41]       |
| Astaxanthin           | *E. coli*             | RBS-modulated expression of the astaxanthin biosynthetic genes          | Shake-flask fermentation | 5.8 mg/g DCW           | [42]       |
| Astaxanthin           | *E. coli*             | Plasmid-overexpression of *Punioea anamatic* *crzEB*, *P. agglomerans* *crzX, Brevundimonas* sp. SD212 *crzW* and *E. coli* *idi* | Fed-batch fermentation | 8.64 mg/g DCW           | [43]       |
| Astaxanthin           | *S. cerevisiae*       | Introduction of codon-optimized Haematococcuspluvialis *crz2* and idr2   | Shake-flask fermentation | 4.7 mg/g DCW           | [44]       |
| Astaxanthin           | *S. cerevisiae*       | Combinatorial metabolic engineering and protein engineering              | Shake-flask fermentation | 8.10 mg/g DCW           | [45]       |
| Astaxanthin           | *C. glutamicum*       | Balanced expression of *crzW* and *crZ*                                | Shake-flask fermentation | 0.4 mg/L/Lh          | [46]       |
| Isoprene              | *E. coli*             | Introduction of MVA pathway, codon and RBS optimization, deleted nine relevant genes to express *ispD* | Shake-flask fermentation | 1832 mg/L               | [48]       |
| Isoprene              | *E. coli*             | Chromosomal expressing the MVA lower pathway; Plasmid-expression of the MVA upper pathway; Plasmid-expression of *mkk* from *Methanosaricina* *mazeri* and isoprene synthase gene from *Populus alba* | 14 L fed-batch fermentation | 60 g/L             | [49]       |
| Isoprene              | *E. coli*             | Overexpression of MEP and MVA pathway; Plasmid-expression *mkk* from *Methanosaricina* *mazeri* and isoprene synthase gene from *Populus alba* | Fed-batch fermentation | 24 g/L            | [50]       |
| Isoprene              | *S. cerevisiae*       | Fed-batch fermentation                                                  | 2527 mg/L            |              | [51]       |
carotenoid production. Xie et al. applied a combined directed evolution and metabolic engineering strategy to construct an engineered *S. cerevisiae* that produced 1.61 g/L (24.41 mg/g DCW) of lycopene in a fed-batch fermentation [23]. Some distantly located genetic loci may have potential interactions with the target pathway. The deletions of these distant genes (YPL062W, YJL064W, ROX1 and DOS2) improved carotenoid production in *S. cerevisiae* [24,25]. Chen et al. constructed an engineered *S. cerevisiae* by combining host engineering (distant genetic loci and cell mating types) with pathway engineering (enzyme screening and gene fine tuning) for lycopene production, which produced 1.65 g/L (55.56 mg/g DCW) of lycopene in a 5-L bioreactor fed-batch fermentation [25]. *Varrovia lipolytica* is another yeast that has been successfully used for lycopene production. The deletion of *POX1* and *GUT2*, which led to an increase in the size of lipid bodies, significantly enhanced lycopene production (16 mg/g DCW) in *Y. lipolytica* [26].

*Streptomyces avermitilis* has also been successfully used for lycopene production. After activating the silent lycopene synthetic gene cluster in *S. avermitilis*, 82 mg/g DCW of lycopene was produced in a shake flask fermentation [27].

2.2. β-Carotene

β-Carotene is a carotenoid compound that has been widely used in the industrial production of not only pharmaceuticals but also nutraceuticals, animal feed additives, functional cosmetics, and food colorants. β-Carotene functions as provitamin A, and it is responsible for the synthesis of retinoids. β-Carotene is the cyclization product of lycopene by lycopene β-cyclase (CrtYB) (Fig. 1). The heterologous expression of the β-carotene biosynthetic genes in non-carotenogenic microbiology, e.g., *E. coli* and *S. cerevisiae*, has become a main alternative means of β-carotene production. *E. coli* co-overexpressing the optimized MEP pathway (*Bacillus subtilis* dxs and fni, and GPPS2 from *Abies grandis*) and the MVA pathway produced 3.2 g/L of β-carotene in a fed-batch fermentation [28]. ATP and NADPH are two important cofactors for terpenoid compounds. Combined engineering of the MEP, the β-carotene synthetic, the TCA and the pentose phosphate (PP) modules by artificial modulation parts resulted in a significant increase in the β-carotene yield. The final strain, *E. coli* CAR005, produced 2.1 g/L β-carotene with a yield of 60 mg/g DCW [29]. After integrating the β-carotene biosynthetic pathway into the *E. coli* genome and optimizing the
MEP, central metabolite pathway and β-carotene biosynthetic pathway, the engineered *E. coli* produced 2.0 g/L of β-carotene in fed-batch fermentation [30]. Yu’s group developed a decentralized assembly strategy to construct a controllable multigene pathway, and then they applied this strategy to construct a controllable β-carotene biosynthetic pathway in *S. cerevisiae*. The resulting strain produced 7.41 mg/g DCW of β-carotene [31]. They then established an inducer/inhibitor-free sequential control strategy in *S. cerevisiae* by combining a modified GAL regulation system and a HXT1 promoter-controlled squalene synthetic pathway [32]. They applied this strategy to sequentially control the expression of the carotenoid pathway, the MVA pathway and the competitive squalene pathway [32]. Production, which reached 1.6 mg/g DCW [36]. It has been reported that CrtZ is the rate-limiting step in zeaxanthin biosynthesis and a dsx group to carbons 3 and 3'. The hydroxylation of each ring of β-carotene by β-carotene hydroxylase (CrtZ) produces zeaxanthin (Fig. 1). Co-overexpression of the *dsx* and *idi* genes in engineered *E. coli* harboring the zeaxanthin biosynthetic pathway had an additive effect on zeaxanthin production, which reached 1.6 mg/g DCW [36]. It has been reported that CrtZ is the rate-limiting step in zeaxanthin biosynthesis and a higher expression level of *crtZ* should be required for zeaxanthin production [37,38]. We compared *Pantoea ananatis*, *Pantoea agglomerans* and *Haematomonas plus culvis* and *crtZ* and reported that *P. ananatis* *crtZ* is superior to those from *P. agglomerans* or *H. plus culvis* for zeaxanthin production [38]. *E. coli* BETIA-1 containing pZSBA-2 (pZSBA-2 P37-crtZPAN) produced 11.95 mg/g DCW of zeaxanthin. To balance the expression of the multigene, the tunable intergenic region (TIGR)-mediated MVA pathway was introduced into the zeaxanthin-producing strain, *E. coli* ZEAX, leading to an increase in zeaxanthin production [39]. However, IPP and FPP are toxic when they accumulate in *E. coli*. To avoid the accumulation of IPP or FPP, a dynamically controlled TIGR-mediated MVA pathway was introduced into the zeaxanthin-producing strain, *E. coli* ZEAX, markedly enhancing its zeaxanthin production, which achieved 722.46 mg/L (23.16 mg/g DCW) in a 5.0-L fed-batch fermentation [39].

2.3. Zeaxanthin

Zeaxanthin (3,3′-dihydroxy-β-carotene) is a yellow oxygenated carotenoid composed of 40 carbon atoms that is used as a food additive and as a feed additive for fish (color enhancement for the flesh) and poultry (yolk and skin pigmentation) [33]. Zeaxanthin plays a critical role in preventing age-related macular degeneration and cancer and may protect against age-related cataract formation [34,35]. The hydroxylation of each ring of β-carotene by β-carotene hydroxylase (CrtZ) produces zeaxanthin (Fig. 1). Co-overexpression of the *dsx* and *idi* genes in engineered *E. coli* harboring the zeaxanthin biosynthetic pathway had an additive effect on zeaxanthin production, which reached 1.6 mg/g DCW [36]. It has been reported that CrtZ is the rate-limiting step in zeaxanthin biosynthesis and a higher expression level of *crtZ* should be required for zeaxanthin production [37,38]. We compared *Pantoea ananatis*, *Pantoea agglomerans* and *Haematomonas plus culvis* and *crtZ* and reported that *P. ananatis* *crtZ* is superior to those from *P. agglomerans* or *H. plus culvis* for zeaxanthin production [38]. *E. coli* BETIA-1 containing pZSBA-2 (pZSBA-2 P37-crtZPAN) produced 11.95 mg/g DCW of zeaxanthin [38]. To balance the expression of the multigene, the tunable intergenic region (TIGR)-mediated MVA pathway was introduced into the zeaxanthin-producing strain, *E. coli* ZEAX, leading to an increase in zeaxanthin production [39]. However, IPP and FPP are toxic when they accumulate in *E. coli*. To avoid the accumulation of IPP or FPP, a dynamically controlled TIGR-mediated MVA pathway was introduced into the zeaxanthin-producing strain, *E. coli* ZEAX, markedly enhancing its zeaxanthin production, which achieved 722.46 mg/L (23.16 mg/g DCW) in a 5.0-L fed-batch fermentation [39].

2.4. Astaxanthin

Astaxanthin is a highly valued keto-carotenoid with strong antioxidant activity and singlet oxygen quenching ability. The pathway from β-carotene to astaxanthin is a crucial step in the synthesis of astaxanthin. This pathway requires two bifunctional enzymes: β-carotene hydroxylase CrtZ to add hydroxyl functional groups to carbons 3 and 3' of β-carotene and β-carotene ketolase CrtW to add keto functional groups to carbons 4 and 4' of β-carotene (Fig. 1). The two enzymes are bifunctional proteins with respect to their substrate specificity. CrtZ can convert not only β-carotene to zeaxanthin but also canthaxanthin to astaxanthin. CrtW is capable of converting not only β-carotene but also zeaxanthin. Consequently, the heterologous expression of *crtZ* and *crtW* in a β-carotene-producing strain results in the accumulation of eight intermediates (eichenone, canthaxanthin, adonirubin, β-cryptoxanthin, zeaxanthin, adonixin, 3-hydroxyechinenone and 3'-hydroxyechinenone), which affects the percentage of astaxanthin that is produced relative to the total carotenoid content. The CrtW and CrtZ enzymes from different sources show different activities and substrate specificities. Thus, optimal astaxanthin biosynthesis requires careful control of the carbon flux along a cooperative function of these two proteins. It has been suggested that astaxanthin biosynthesis proceeds from β-carotene through hydroxylatation first and then onto ketolation [40]. To increase the astaxanthin percentage relative to the total carotenoid content, we compared the conversion efficiency to astaxanthin in four CrtWs, which had higher efficiency for astaxanthin production reported in literature, with recombinant *E. coli* cells that synthesizes zeaxanthin due to the presence of the *P. ananatis* *crtenBHYZ* and found that the *Brevundimonas* sp. SD212 *crtW* and *P. ananatis* *crtZ* genes are the best combination for astaxanthin production [41]. After tune-fining the crt genes, an astaxanthin producer *E. coli* ASTA-1 that does not carry a plasmid or antibiotic marker was constructed. The engineered strain *E. coli* ASTA-1 produced 7.50 mg/g DCW of astaxanthin with an astaxanthin ratio of 96.6% relative to the total carotenoid content in a shake flask fermentation [41]. The ratio of astaxanthin to the total carotenoids (96.6%) is the highest value reported to date. Balanced expression of the astaxanthin biosynthetic genes with a compact set of ribosome binding sites led to an astaxanthin accumulation of 5.8 mg/g DCW in *E. coli* [42]. Ma et al. identified and characterized the astaxanthin-producing ability of *Sphingomonas* sp. ATCC 55669 by complete genome sequencing, and then compared the astaxanthin biosynthetic efficiency of the crt genes from different microorganisms in *E. coli*. The resulting *E. coli* plasmid-expressing *P. ananatis* *crtenBHYZ*, *P. agglomerans* *crtYZ*, *B. trichocarpa* and *P. agglomerans* *crtZ* produced 8.64 mg/g DCW [43].

An astaxanthin producing *S. cerevisiae* was constructed by integrating two copies of the codon-optimized *H. plus culvis* *crtZ* and *bkt* in β-carotene producing *S. cerevisiae*. The engineered *S. cerevisiae* produced 4.7 mg/g DCW of astaxanthin in a shake-flask culture [44]. The group recently applied combinatorial metabolic engineering and protein engineering to markedly enhance astaxanthin production *S. cerevisiae*, which reached 8.10 mg/g DCW in shake-flask cultures [45].

Recently, *Corynebacterium glutamicum* has been engineered for astaxanthin production, and it reached 1.6 mg/g DCW [46].

In addition, the titer of astaxanthin is much lower than that of other carotenoids (lycopene, β-carotene and zeaxanthin). Because very few carotenoids were detected in our engineered strain *E. coli* ASTA-1, we guess that the lower astaxanthin yield may be because the recombinant enzyme (β-carotene hydroxylase and ketolase) or product of their enzymatic reaction affects the formation of the carotenoid precursors upstream of phytoene. Therefore, further efforts focused on astaxanthin production should be carried out.

3. Isoprenoid-based biofuels

Methyl branching and cyclic structures are commonly observed in isoprenoids. The methyl branching structure lowers the freezing point significantly. The cyclic structures increase the energy density and are generally considered valuable features for jet fuels. In recent years, some isoprenoids have been tested and produced as potential diesel and gasoline fuel alternatives because of their lower hygroscopy, higher energy content and good fluidity at low temperatures. 

3.1. Hemiterpenoid-based biofuels

Isoprene (C₅H₈) is the simplest isoprenoid. It is used to produce millions of tons of rubber annually and has been suggested as a liquid fuel [47]. Co-overexpression of *Populus trichocarpa* codon-optimized isoprene synthase gene *ISPS* and the MVA pathway
genes in the 9-gene knockout E. coli AceCo improved isoprene production, reaching 1832 mg/L in a shake-flask culture [48]. Plasmid-expression of the upper pathway of MVA in concert with the P. alba isoprene synthase gene ISPS plus the mevalonate kinase and phosphogluconolactonase gene in E. coli integrated the lower pathway of MVA from S. cerevisiae and resulted in production of 60 g/L isoprene with a mass yield of isoprene from glucose in a 14-L fed-batch fermentation [49]. Fed-culture of the engineered E. coli overexpressing the synergistic dual pathway of MVA and MEP resulted in the production of 24.0 g/L isoprene with a yield of 0.267 g/g [50]. The isoprene synthase gene has also been introduced in S. cerevisiae for isoprene production. In recent years, organelle engineering of yeast has attracted increased attention in the biosynthesis of chemicals. Dual metabolic engineering of the cytoplasmic and mitochondrial acetyl-CoA increased isoprene production in S. cerevisiae, reaching 2527 mg/L in a fed-batch fermentation [51]. A two-level expression system was developed for the GAL4-controlled ISPS by overexpression of GAL4 [52]. Combining the two-level expression system and directed evolution of ISPS in S. cerevisiae led to the production of 3.7 g/L in a fed-batch fermentation [52].

Ester of isoprenoid alcohols (C5, C10 and C15) have the potential to be used as replacements for petroleum-based diesels. B. subtilis nudF and E. coli nudB have been introduced into E. coli for isoprenol/isoprenol production [53,54]. Overexpression of some isopentenol tolerance-enhancing genes, such as metR and mrdB, improved the production of isopentenol in E. coli [55]. A novel IPP-bypass MVA pathway was reported for isopentenol production in E. coli. The IPP-bypass MVA pathway contains the decarboxylation of mevalonate phosphate by PMD and the hydroxylation of isopentenyl monophosphate (IP) by E. coli phosphatase AphA [56]. George et al. constructed an E. coli with a high yield in 3-methyl-3-buten-1-ol production [57]. A titer of 2.23 g/L isoprenol was obtained by using an oleyl alcohol overlay in the engineered E. coli. This is the highest yield achieved from an engineered strain.

3.2. Monoterpene–based biofuels

Monoterpeneoids are C10 compounds built from two isoprenoid units (one IPP and one DMAP). Monoterpeneoids can be divided into three major subgroups based on their structural features: 1) acyclic monoterpeneoids, such as myrcene and ocimene; 2) monocyclic monoterpeneoids, such as limonene, menthol, and carvone; 3) bicyclic monoterpeneoids, such as pinene, sabinene, and camphor.

Co-overexpression of the MVA pathway, A. grandis GPPS2 and the Quercus ilex L. myrcene synthase gene in E. coli resulted in the production of 58.19 mg/L myrcene [58]. E. coli harboring the MVA pathway, A. grandis GPPS2 and the Salvia pomifera sabinene synthase gene sabs1 produced 2.65 g/L sabinene in a fed-batch fermentation [59].

A novel biosynthetic pathway of α-pinene was assembled in E. coli BL21(DE3) with the heterologous MVA pathway, codon-optimized GPPS from A. grandis and codon-optimized α-pinene synthase Pt30 from Pinus taeda [60]. The final producing strain YM28 produced 5.44 mg/L in a shake-flask fermentation and 0.97 g/L α-pinene in a fed-batch fermentation. Sarria et al. combinatorially expressed three pinene synthase (PS) and three GPPS from conifers in engineered E. coli harboring the MVA pathway. They achieved approximately 28 mg/L pinene using the best combination (PS and GPPS from A. grandis). Furthermore, they designed GPPS-PS protein fusions to reduce GPP product inhibition and toxicity by substrate channeling, producing 32.4 mg/L pinene in a shake-flask fermentation [61]. PS is the rate-limiting enzyme for pinene biosynthesis. To significantly improve the activity of PS, α-pinene synthase Pt1 from P. taeda was evolved to obtain a PS mutant PSΔ380A. They expressed the PS mutant and GPPS from A. grandis in the engineered E. coli harboring the MVA pathway and achieved 150 mg/L pinene in a shake-flask fermentation [62]. An engineered E. coli expressing the MVA pathway, codon-optimized GGPS from A. grandis and codon-optimized limonene synthase from Mentha spicata on one plasmid produced 400 mg/L limonene in a shake-flask fermentation [63].

Monoterpeneoids have been reported to be highly toxic, resulting in low microbial production of monoterpene. Overexpression of the efflux pump or tolerance-enhancing genes has become a common strategy for improving monoterpene production [64,65]. Overexpression of the efflux pump gene (YP-692684) from Alcanivorax borkumensis significantly improved tolerance and enhanced limonene production [64]. This tolerance engineering strategy has also successfully been applied for improving the production of isopentenol, olefin and other biofuels [55,64–66].

3.3. Sesquiterpene–based biofuels

Sesquiterpenoids are one of the largest groups of isoprenoid natural products and have a wide range of activities from antimicrobial agents (such as phytotoxins capsidiol) to alarm pheromone (such as farnesol). Structurally, sesquiterpenoids can be acyclic, monocyclic, bi- or even tricyclic with different TPS that catalyze FPP into a large variety of sesquiterpenoids. Sesquiterpenoids are 15 carbons, close to the average length of diesel (C16), but with a branched, rather than a straight-chain structure. Among sesquiterpenoids, farnesol, farnesene and bisabolene have been proposed as diesel fuels and produced from IPP.

Combination expression of the heterologous MVA pathway and the fused proteins of IspA/AFS led to an approximate 317-fold increase over the initial production of farnesene in E. coli. The final engineered E. coli produced approximately 380 mg/L farnesene in a shake-flask fermentation [67]. In vitro studies on the purified protein components of MVA and the downstream FPP pathway have revealed that Idi played a key role in α-farnesene synthesis in vitro [68]. Based on the in vitro studies, farnesene production was optimized through overexpression of Idi with IspA and AFS in E. coli expressing the synthetic MVA pathway. After 96 h of induction, farnesene production reached a concentration of approximately 1.1 g/L in a shake-flask fermentation [68]. Meadows et al. constructed an artificial cytosolic acetyl coenzyme biosynthetic pathway with a reduced ATP requirement, which contained Dickeya zae aldehyde dehydrogenase (acetylating) (ADA), Leuco-nostoc mesenteroides xylulose-5-phosphate specific phosphoketolase (PK) and Clostridium kluveri phosphotransacetylase (PTA) [69]. Combining the artificial acetyl coenzyme biosynthetic pathway with the NADH-consuming HMG-CoA reductase from Silicibacter pomeroyi in S. cerevisiae enhanced farnesene production, which reached 130 g/L in 200, 000-L bioreactor fed-batch fermentation [69].

Bisabolene, a monocyclic sequiterpene, has been identified as a precursor to a potential D2 diesel fuel. To obtain higher titers of bisabolene, bisabolene synthase (BIS) genes from Arabidopsis thaliana, Pseudotsuga menziesii, A. grandis and Picea abies have been screened in E. coli (harboring the entire MVA pathway in a single plasmid) and S. cerevisiae [70]. Overexpression of the codon-optimized AgBIS in an engineered E. coli expressing the optimized MVA pathway resulted in production of 912 mg/L of bisabolene. The same level of bisabolene was also obtained in the engineered S. cerevisiae with an overproduction of FPP [70]. Kirby et al. reported a novel route from ribulose 5-phosphate (Ru5P) to DXP (nDXP) and uncovered two nDXP genes: ribB(G108S) and yqiO. Expression of a Dxr-RibB(G108S) fusion improved bisabolene titers more than 4-fold [71]. Using a carotenoid-based phenotypic screen of the yeast
deletion collection, the genes that affected isoprenoid synthesis in yeast were identified. Combinations of these deletions and other MVA pathway modifications improved the titers of bisabolene more than 20-fold to 800 mg/L in a flask and 5.2 g/L in a fermentation process [72].

Farnesol is an important C15 isoprenyl alcohol. Co-overexpression of the heterologous MVA pathway and ispA in E. coli led to the production of 135 mg/L farnesol [73]. Overexpression of IspA and the membrane phosphatase PgpB, along with a heterologous MVA pathway in E. coli, increased farnesol production to 526.1 mg/L in a shake flask fermentation [74]. A farnesol production of 145 mg/L was attained by S. cerevisiae ATCC 200589 with overexpression of HMG-CoA reductase (Hmg1) in a fermentation culture for 7 days [75].

4. Conclusions

Carotenoids and isoprenoid-based biofuels are two classes of important isoprenoids. With advances in metabolic engineering and synthetic biology, engineered microorganisms have become a primary alternative for their production. Most studies on carotenoid production are focused on the regulation of carbon flux. Our results from the comparative proteomes demonstrated that zeaxanthin overproduction may be associated with not only precursor availability but also cofactor availability, oxidative stress response, and membrane storage capacity [39]. Morphology engineering for increasing membrane storage capacity may be another strategy for improving carotenoid production.

Some isoprenoids have been proposed as biofuels. However, the levels of isoprenoid-based biofuels are lower than the order of magnitude of those of carotenoids. Moreover, the titers of monoterpenes are lower than those of hemiterpenes and sesquiterpenes. Toxicity and enzyme activity may be major factors. Tolerance engineering and the evolution of enzymes may be effective strategies for improving yields.

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