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

Fu-Xing Niu [a, b, 1], Qian Lu [a, b, 1], Yi-Fan Bu [a, b], Jian-Zhong Liu [a, b, *]

[a] Biotechnology Research Center and Biomedical Center, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China
[b] South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China

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ABSTRACT

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.

Contents

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

* Corresponding author. Biotechnology Research Centre, School of Life Science, Sun Yat-Sen University, Guangzhou 510275, China.
E-mail address: lssljz@mail.sysu.edu.cn (J.-Z. Liu).
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[1] Contributed equally to this work.

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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-D-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-

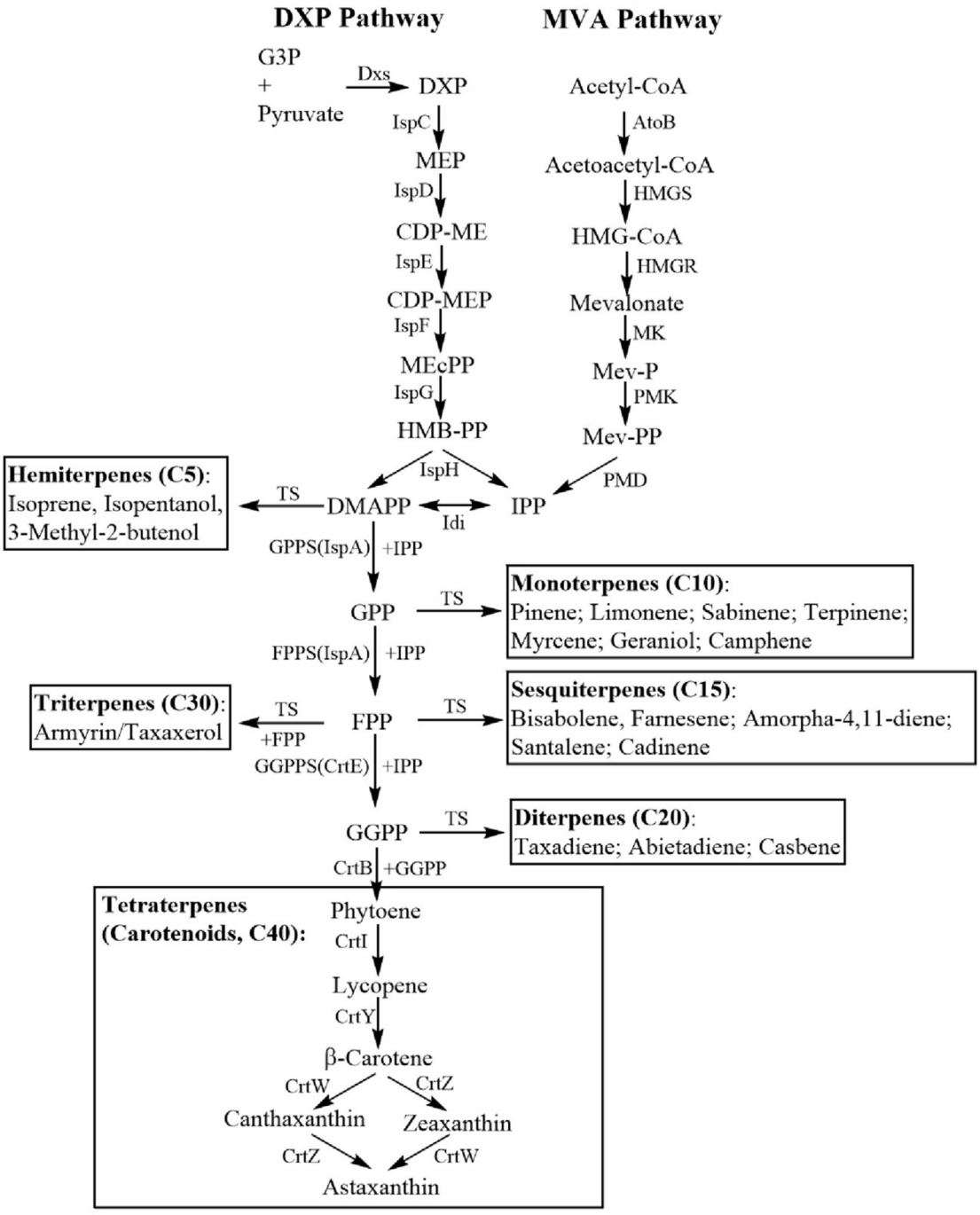

Fig. 1. Isoprenoid biosynthetic pathway. G3P: Glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose-5-phosphate; MEP: 2-C-methyl-D-erythritol-4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; MEcPP: 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HMB-PP: 4-hydroxy-3-mehtyl-butenyl 1-diphosphate; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA; Mev-P: Mevalonate 5-phosphate; Mev-PP: Mevalonate diphosphate; IPP: Isopentenyl diphosphate; DMAPP: Dimethylallyl diphosphate; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranylgeranyl diphosphate; Dxs: 1-deoxy-D-xylulose-5-phosphate synthase; IspC: 1-D-deoxy-D-xylulose 5-phosphate reductoisomerase; IspD: 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase; IspE: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; IspF: 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; IspG: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; IspH: 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase; Idi: Isopentenyl diphosphate isomerase; AtoB: Acetoacetyl-CoA synthase; HMGS: Hydroxymethylglutaryl-CoA synthase; HMGR: Hydroxymethylglutaryl-CoA reductase; MK: Mevalonate kinase; PMK: Phosphomevalonate kinase; PMD: Mevalonate diphosphate decarboxylase; GPPS: GPP synthase; FPPS: FPP synthase; GGPPS: GGPP synthase; TS: terpene synthase.

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, mecalonate 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).

$$1.5\ \text{Glucose} + 2\ \text{NADPH} + 6\ \text{NAD} = \text{IPP} + \text{CO}_2 + 2\ \text{ADP} + 2\ \text{NADP} + 6\ \text{NADH} \quad (1)$$

The DXP pathway consists of seven enzymatic steps that convert glyceraldehyde 3-phosphate (G3P) and pyruvate to IPP and DMAPP in a ratio of 5:1 [3]. The DXP pathway starts with the condensation of G3P and pyruvate to produce DXP by DXP synthase (Dxs). 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-2,4-cyclodiphosphate (MEcPP), 4-hydroxy-3-mehtyl-butenyl 1-diphosphate (HMB-PP), 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).

$$\text{Glucose} + 2\ \text{ATP} + 3\ \text{NADPH} + \text{NAD} = \text{IPP} + \text{CO}_2 + 2\ \text{ADP} + 3\ \text{NADP} + \text{NADH} \quad (2)$$

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 extraction from plant materials remains a major production route. However, it becomes increasingly difficult to meet their growing demand from plant extraction because of the slow growth rate and low isoprenoid content of plants. Microbes are an excellent alternative for overcoming this limitation, as they grow fast, require little land/water resources, and naturally produce the building blocks of all isoprenoids: IPP and DMAPP (Fig. 1). In recent years, quite a few isoprenoid compounds were overproduced in engineered microorganisms, including carotenoids, isopentanol, pinene, farnesene, limonene and bisabolene, etc. (Table 1).

Herein, we briefly reviewed recent advances in the metabolic engineering of microorganisms for isoprenoid production, focusing on carotenoids and isoprenoid-based biofuels.

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*, *crl* 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 *gdhA* and *gpmAB* improved lycopene production [10]. Flux balance analysis also revealed that the deletions of *aceF*, *fdhF* and *gdhA* enhanced lycopene production by 40% [11]. Zhou et al. reported that the *zwf* knockout increased lycopene production by 130% [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*, *sdhABCD* and *talB* with the regulatory part for increasing ATP and NADPH availability, and *dxs*, *idi* and *crtB* 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 *E. coli* produced lycopene at 33.43 mg/g DCW [21]. De Mey's group developed a new combinatorial multigene pathway assembly approach based on Single Strand Assembly methods and Golden Gate Assembly, and they applied this approach to optimize the lycopene biosynthetic genes in *E. coli* overexpressing the MEP pathway to obtain a lycopene hyper-producer *E. coli* that produced 448 mg/g DCW of lycopene in a shake flask fermentation [22]. This yield is the highest value reported so far.

S. cerevisiae 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 knockout; 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; Overexpressing *Bacillus licheniformis idi*	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\idi\crtE*	Fed-batch fermentation	50.602 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; Overexpressing the MEP pathway (*dxs-idi-ispDF*)	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 synthetic gene cluster	Shake-flask fermentation	82 mg/g DCW	[27]
β-cartoene	*E. coli*	Plasmid-expressing the lower MVA pathway and *idi* from *S. cerevisiae*, Plasmid-expressing the upper MVA pathway from *Enterococcus faecalis*, *Bacillus subtilis dxs* and *fni*, and *GPPS2* from *Abies grandis*; Plasmid-expressing the β-cartoene synthetic pathway.	Fed-batch fermentation	60 mg/g DCW	[28]
β-cartoene	*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-cartoene	*E. coli*	Optimizing the biosynthetic pathway	Fed-batch fermentation	2.0 g/L	[30]
β-cartoene	*S. cerevisiae*	Decentralized assemble strategy	Shake-flask fermentation	7.41 mg/g DCW	[31]
β-cartoene	*S. cerevisiae*	Using the inducer/inhibiter-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 *Pantoea ananatis crtEIB*, *Pantoea agglomerans crtYZ*, *Brevundimonas* sp. *SD212 crtW* and *E. coli idi*		8.64 mg/g DCW	[43]
Astaxanthin	*S. cerevisiae*	Introduction of codon-optimized Haematococcuspluvialis *crtZ* and *bkt*	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 *crtW* and *crtZ*	Shake-flask fermentation	0.4 mg/L/h	[46]
Isoprene	*E. coli*	Introduction of MVA pathway, codon and RBS optimization, deleted nine relevant genes to express *ispS*	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 *mvk* from *Methanosarcina mazei* 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-expressing *mvk* from *Methanosarcina mazei* and isoprene synthase gene from *Populus alba*	Fed-batch fermentation	24 g/L	[50]
Isoprene	*S. cerevisiae*		Fed-batch fermentation	2527 mg/L	[51]

Table 1 (*continued*)

Isoprenoids produced	Host	Approach	Culture conditions	Yield/Titer	References
		Dual metabolic engineering of cytoplasmic and mitochondrial acetyl-CoA utilization			
Isoprene	*S. cerevisiae*	Combining the two-level expression system and directed evolution of ISPS	Fed-batch fermentation	3.7 g/L	[52]
Isopentenol	*E. coli*	Introduction of MVA pathway; Expressing *BsNudF* gene	Shake-flask fermentation	1.3 g/L	[54]
Isopentenol	*E. coli*	Constructing the MVA IPP-bypass pathway	Shake-flask fermentation	705 mg/L	[56]
Isopentenol	*E. coli*	RBS engineering of *nudB;* Expressing the Idi-NudB fusion protein	Shake-flask fermentation	2.23 g/L	[57]
Myrcene	*E. coli*	Co-overexpression of MVA pathway, *AgGPPS* and *ms* from *Quercus ilex* L.	Shake-flask fermentation	58.19 mg/L	[58]
Myrcene	*E. coli*	Introducing the MVA lower pathway; Expressing the MVA upper pathway in combination with *AgGPPS* and *SabS1*	Fed-batch fermentation	2.65 g/L	[59]
Pinene	*E. coli*	Introduction of MVA pathway; Expressing AgGPPS-Pt30 fusion protein	Fed-batch fermentation	0.97 g/L	[60]
Pinene	*E. coli*	Introduction of MVA pathway; Expressing AgPS-AgGPPS fusion protein	Shake-flask fermentation	32.4 mg/L	[61]
Pinene	*E. coli*	Introduction of MVA pathway; Expressing PSmut-AgGPPS fusion protein	Shake-flask fermentation	150 mg/L	[62]
Limonene	*E. coli*	Introduction of MVA pathway, Expressing the AgGPPS–LS fusion protein	Shake-flask fermentation	435 mg/L	[63]
Farnesene	*E. coli*	Introduction of MVA pathway; Expressing the codon-optimized FS-IspA fusion protein	Shake-flask fermentation	380 mg/L	[67]
Farnesene	*E. coli*	Application of *In vitro* reconstitution and targeted proteomics; Overexpression of *Idi* with *IspA* and *AFS* in *E. coli* expressing synthetic MVA pathway	Shake-flask fermentation	1.1 g/L	[68]
Farnesene	*S. cerevisiae*	Introduction of the artificial acetyl coenzyme biosynthetic pathway (contained *Dickeya zeae* aldehyde dehydrogenase (acylating), *Leuconostoc mesenteroides* xylulose-5-phosphate specific phosphoketolase and *Clostridium kluyveri* phosphotransacetylase) with the NADH-consuming HMG-CoA reductase from *Silicibacter pomeroyi*	200,000 L bioreactor fed-batch fermentation	130 g/L	[69]
Bisabolene	*E. coli*	Co-expressing the codon-optimized *AgBIS* and the optimized MVA pathway	Shake-flask fermentation	912 mg/L	[70]
Bisabolene	*S. cerevisiae*	Co-expressing the codon-optimized *AgBIS* and the optimized MVA pathway	Shake-flask fermentation	994 mg/L	[70]
Bisabolene	*S. cerevisiae*	Screening the yeast knockout libraries; Co-expressing the MVA pathway and *BIS* gene	Fed-batch fermentation	5.2 g/L	[72]
Farnesol	*E. coli*	Co-expressing *ispA* and the MVA pathway	Shake-flask fermentation	135.5 mg/L	[73]
Farnesol	*E. coli*	Overexpressing *ispA*, *pgpB* and the MVA pathway	Shake-flask fermentation	526.1 mg/L	[74]
Farnesol	*S. cerevisiae*	Overexpressing the truncated HMG-CoA reductase	5L fed-batch fermentation	145 mg/L	[75]

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]. *Yarrowia 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 (CrtY) (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 metabolic 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/inhibiter-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 by glucose in the culture broth, resulting in marked increase in β-carotene production, which reached 20.79 mg/g DCW [32].

2.3. Zeaxanthin

Zeaxanthin (3,3′-dihydroxyl-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 *Haematococcus pluvialis crtZ* and reported that *P. ananatis crtZ* is superior to those from *P. agglomerans* or *H. pluvialis* for zeaxanthin production [38]. *E. coli* BETA-1 containing pZSBA-2(P37-crtZ$_{PAN}$) 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 (echinenone, canthaxanthin, adonirubin, β-cryptoxanthin, zeaxanthin, adonixanthin, 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 hydroxylation 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 crtEBIYZ* 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 crtEIB*, *P. agglomerans crtYZ, B. sp. SD212 crtW* and *E. coli idi* produced 8.64 mg/g DCW [43].

An astaxanthin producing *S. cerevisiae* was constructed by integrating two copies of the codon-optimized *H. pluvialis 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 (C_5H_8) 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 increasing 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 P_{GAL1}-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 isoprentol/isoprenol production [53,54]. Overexpression of some isopentenol tolerance-enhancing genes, such as *metR* and *mdlB*, 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 stain.

3.2. Monoterpenoid–based biofuels

Monoterpenoids are C10 compounds built from two isoprenoid units (one IPP and one DMAP). Monoterpenoids can be divided into three major subgroups based on their structural features: 1) acyclic monoterpenes, such as myrcene and ocimene; 2) monocyclic monoterpenes, such as limonene, menthol, and carvone; 3) bicyclic monoterpenes, 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 GGPS from *A. grandis* and codon-optimized α-pinene synthase Pt30 from *Pinus taeda* [60]. The final producing strain YJM28 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. combinatorically 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 GGPS 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 PS^{D380A}. 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. grandi* and codon-optimized limonene synthase from *Mentha spicata* on one plasmid produced 400 mg/L limonene in a shake-flask fermentation [63].

Monoterpenes have been reported to be highly toxic, resulting in low microbial production of monoterpene. Overexpression of 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. Sesquiterpenoid–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 phytoalexins capsidiol) to alarm pheromones (such as farnesene). Structurally, sesquiterpenoids can be acyclic, monocyclic, bi- or even tricyclic with different TPS that catalyze FPP into a large variety of sesquiterpenes. 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 bisabolane have been proposed as diesel fuels and produced from IPP.

Combinational 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 zeae* aldehyde dehydrogenase (acylating) (ADA), *Leuconostoc mesenteroides* xylulose-5-phosphate specific phosphoketolase (PK) and *Clostridium kluyveri* 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 *yajO*. Expression of a DxrRibB(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 demonstrate 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 member 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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