Chapter 5

Biosynthesis of Carotenoids and Apocarotenoids by Microorganisms and Their Industrial Potential

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Additional information is available at the end of the chapter

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Abstract

Carotenoids are a large group of natural pigments, ranging from red, to orange, to yellow colors. Synthesized by plants and some microorganisms (e.g., microalgae, fungi and bacteria), carotenoids have important physiological functions (e.g., light harvesting). Apocarotenoids are carotenoid-derived compounds and play important roles in various biological activities (e.g., plant hormones). Many carotenoids and apocarotenoids have high economic value in feed, food, supplements, cosmetics and pharmaceutical industries. Despite high commercial values, they are severely undersupplied because of low abundance in natural hosts (usually a few milligrams per kilogram of raw materials). Furthermore, plants or microbes usually produce mixtures of these molecules with very similar physical and chemical properties (such as α- and β-carotenes). All these features render the extraction from natural hosts rather difficult and also very costly both from process economics and sustainable land-use viewpoints. Chemical synthesis is also expensive due to structural complexity (e.g., astaxanthin has many unsaturated bonds and two chiral regions). Biotechnology via the rapidly advancing metabolic engineering and synthetic biology approaches has led to alternative ways to attain several carotenoids and apocarotenoids at relatively high titers and yields using fast-growing microorganisms. This chapter briefly reviews the biosynthesis of carotenoids and apocarotenoids by microorganisms and their industrial potential.

Keywords: metabolic engineering, fermentation, carotenoids, astaxanthin, lycopene, carotene, retinol and ionone

1. Introduction

Carotenoids are natural red, orange or yellow pigments widely distributed in nature. The vivid color of carotenoids contributes to the beauty of many flowers, fruits and animals. For

example, the loveliness of yellow marigolds comes mainly from lutein, a yellow carotene; the redness of watermelons and tomatoes is because they are rich in lycopene, a red carotene; and the scarlet plumage of flamingos stems from another red carotenoid, astaxanthin. The beautiful colors of plants are also responsible for attracting insects and animals for their pollination and seed dispersal [1]. The carotenoid color originates in the structure of multiple conjugated double bonds. This unique structure enables two essential features of carotenoids: the light-harvesting capability and a powerful anti-oxidant effect by the quenching of free radicals, singlet oxygen and reactive oxygen species. In photosynthetic organisms, carotenoids are indispensable for photosynthesis and photo-protection [2]. In non-photosynthetic organisms including animals, the anti-oxidant activity not only protects cells from oxidative damages (e.g., oxidative DNA damage [3]) but can provide additional benefits for humans such as anti-inflammatory and anti-cancer effect [4]. In addition, carotenoids play an important health role as pro-vitamin A compounds. About 30–50 carotenoids are believed to have vitamin A activity including two well-known compounds: β-carotene and α-carotene [2].

Vitamin A includes retinol, retinal and retinoic acid, which are all apocarotenoids. Apocarotenoids are a group of oxidative products of carotenoids. While carotenoids contribute to the visual beauty of flowers and fruits, apocarotenoids are famous for the pleasant aromas and give rise to fragrance and palatable flavors of many flowers and fruits (such as rose, violet, tomato and raspberry) [5–7]. These apocarotenoid aromas, in a similar manner to the colored carotenoids, attract pollinators and promote plant-insect interactions [8]. In addition, some apocarotenoids act as hormones. For example, the plant growth hormone, abscisic acid, has multiple functions in plant development processes including bud dormancy and response to environmental stress and plant pathogens [5]. Strigolactones are another important subclass of apocarotenoids, functioning as shoot-branching inhibitors and promoting the formation of symbiotic association between plants and fungi [9, 10].

Due to the color, aroma, remarkable nutrition and health benefits, carotenoids and apocarotenoids have been widely used in food, feed, nutritional, pharmaceutical and personal care industries. The market demands for carotenoids and apocarotenoids are rising rapidly as increasing clinical research studies report various health and pharmaceutical benefits [11–13]. The global carotenoid market is projected to reach 1.53 billion USD by 2021 [14]. The regular uptake of food with a high content of carotenoids (e.g., β-carotene) or retinoids is vital to alleviate vitamin A deficiency. Vitamin A deficiency can lead to severe aftermath including blindness, decreased immune function and even death [15]. Lutein and zeaxanthin are critical for eye health by preventing age-related macular degeneration [16]. Astaxanthin has even more benefits such as potent anti-oxidant activities, promoting immune response, reducing eye fatigue, enhancing muscle performance and so on [11]. Because of low exceptional fragrance property, α-ionone and β-ionone are widely used in cosmetics such as perfumes [17]. Crocin is another valuable apocarotenoid and is responsible for the red pigmentation of saffron, a high-value spice with retail prices ranging between 2000 and 7000 euros/kg [18].

Despite carotenoids and apocarotenoids being widely distributed in nature, their cellular contents are extremely low. For example, 100 tons of raspberries, or 20 hectares of agricultural area, could only yield 1 g of α-ionone [19]. Similarly, it requires the manual harvest of stigmas from as many as 110,000–170,000 flowers to obtain 1 kg of saffron [20], justifying the high cost of

these molecules. Chemically synthetized carotenoids, despite being less expensive, have been reported to have hazardous effects to human health and are increasingly unpopular with consumers [19]. Currently, microbial-derived commercial carotenoids are those derived from native producer strains which have not been genetically engineered but in some cases have undergone classical mutagenesis followed by selection to screen for improved production characteristics. These include the β-carotene production strains of the microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2]. Recent advances in microbial biotechnology have made the microbial production of carotenoids and apocarotenoids potentially more efficient and cost-effective, using metabolic engineering strategies in industrial workhorse strains such as *Escherichia coli* and *Saccharomyces cerevisiae*, for which the fermentation strategies are well established.

To date, 1117 natural carotenoids and apocarotenoids have been reported, which consist of C30, C40, C45 and C50 carotenoids [22]. Among them, C40 carotenoids and their derived apocarotenoids are the most abundant with 1093 different structures. In this chapter, I will cover only a few of the commercially interesting C40 carotenoids and apocarotenoids that will illustrate the challenges and potentials of this biosynthetic alternative supply chain.

2. Biosynthesis of carotenoids and apocarotenoids in nature

To understand how carotenoids and apocarotenoids can be produced in microbes, it is essential to elucidate the biosynthetic enzymes which constitute these metabolic pathways.

Carotenoids are a subclass of terpenoids (or isoprenoids); thus, as other terpenoids, they share the common C5 building blocks, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). In nature, there exist two independent biosynthetic pathways to produce IPP/DMAPP: the mevalonate (MVA) pathway [23] and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate (DXP) or the non-MVA pathway [24].

The MEP pathway starts from the condensation of pyruvate and glyceraldehyde-3-phosphate, which are catalyzed by DXP synthase (*dxs*), to produce DXP, which is subsequently reduced into MEP by DXP reductase (*dxr*). MEP is converted into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDPME) by CDPME synthase (*ispD*). CDPME is subsequently transformed into 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) through two intermediates, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDPMEP) and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEC) by CDPME kinase (*ispE*), MEC synthase (*ispF*) and HMBPP synthase (*ispG*), respectively. Finally, HMBPP reductase catalyzes HMBPP into a 5-6:1 ratio of IPP and DMAPP, while IPP isomerase (*idi*) inter-converts IPP and DMAPP to adjust the ratio according to the cellular requirements (**Figure 1**).

In the MVA pathway, two molecules of acetyl-CoA are condensed into one molecule of acetoacetyl-CoA by acetyl-CoA acetyltransferase (*atoB*). Acetoacetyl-CoA is converted into mevalonate via an intermediate (S)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase and HMG-CoA reductase, respectively. IPP is produced from mevalonate by another three enzymes, mevalonate kinase (*mk*), phosphomevalonate kinase (*pmk*) and

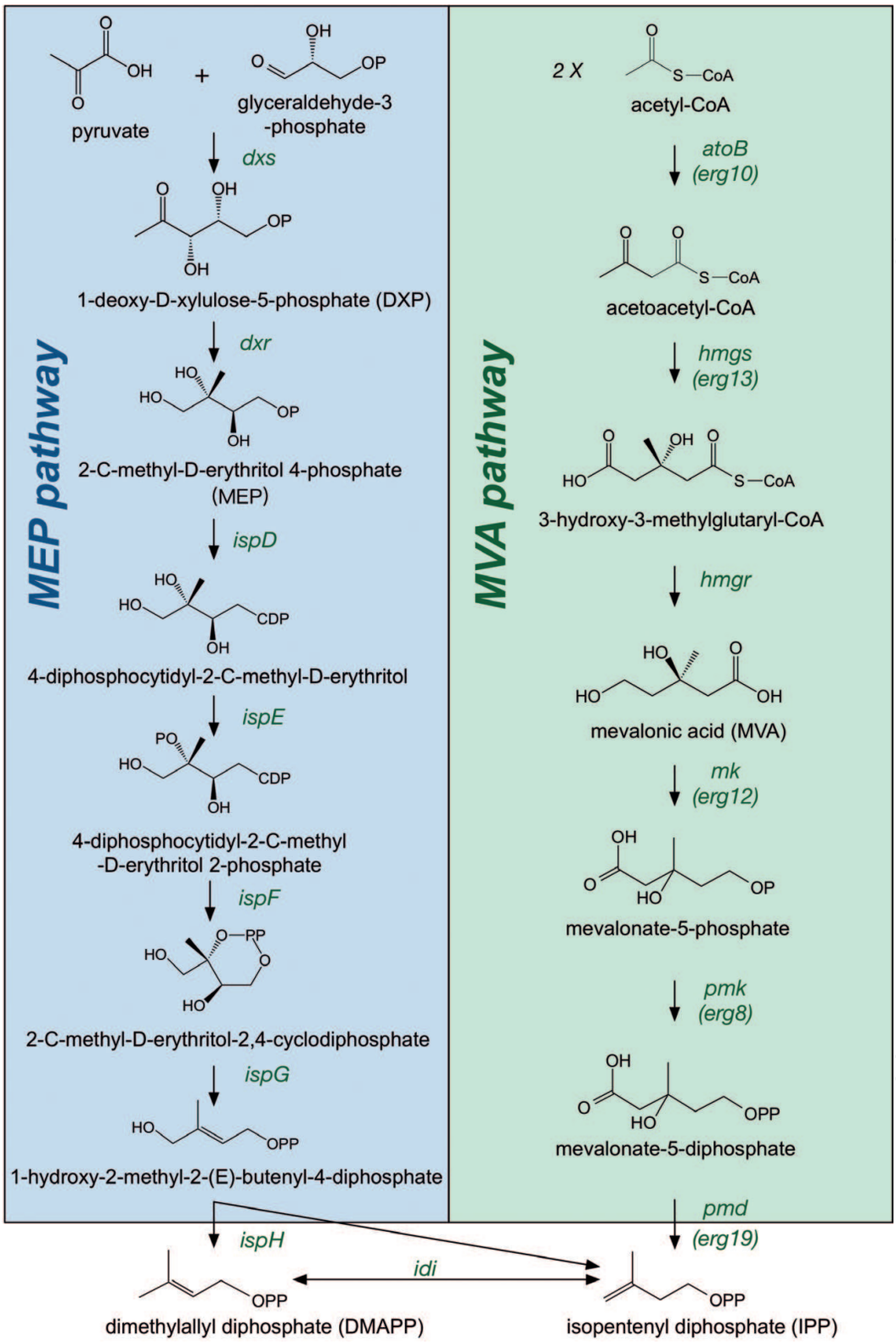

Figure 1. Biosynthetic pathway of terpenoid precursors. Carotenoids are a subclass of terpenoids. In nature, two major biosynthetic pathways of terpenoids exist, one is the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, and the other is the mevalonate (MVA) pathway. Abbreviations: *dxs* (DXP synthase), *dxr* (DXP reductase), *ispD* (4-diphosphocytidyl-2-C-methyl-D-erythritol, or CDPME synthase), *ispE* (CDPME kinase), *ispF* (2-C-methyl-D-erythritol-2,4-diphosphate synthase), *ispG* (1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase), *ispH* (1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase), *atoB* (acetoacetyl-CoA thiolase), *hmgs* (hydroxymethylglutaryl-CoA, or HMG-CoA synthase), *hmgr* (HMG-CoA reductase), *mk* (mevalonate kinase), *pmk* (phosphomevalonate kinase), *pmd* (phosphomevalonate decarboxylase), and *idi* (IPP isomerase).

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phosphomevalonate decarboxylase (*pmd*). Thus, while the MEP pathway produces mixtures of DMAPP and IPP, the MVA pathway produces only IPP and requires *idi* to generate DMAPP (**Figure 1**).

Most bacteria including cyanobacteria use exclusively the MEP pathway, whereas most eukaryotes and archaea possess only the MVA pathway. Interestingly, plants have both pathways: the MVA pathway located in the plant cytoplasm and the MEP pathway located in plastids. This is consistent with the hypothesis that chloroplasts originate from cyanobacteria endosymbionts [25]. Both pathways have been engineered to produce terpenoids including carotenoids. The MEP pathway has a higher theoretical yield than the MVA pathway [26] due to its adoption of a variety of cofactors (ATP, NADPH, CTP and flavodoxin, etc.) whereas the MVA pathway mainly uses ATP. However, in practice, it is easier to manipulate the MVA pathway and its theoretical yield has been achieved for certain products [27–30]. In contrast, the practical yield of the MEP pathway is often limited by the low activity of ispG and ispH enzymes and their special requirement of iron-sulfur cofactors. To the best of my knowledge, the highest reported yields of terpenoids synthesized by the MEP pathway in literature are less than 20% of its theoretical yield [30].

The two pathway metabolites IPP and DMAPP are condensed to give geranyl diphosphate (GPP, C10) or farnesyl diphosphate (FPP, C15), catalyzed by GPP synthase (*gpps*) or FPP synthase (*fpps*), respectively. Geranylgeranyl diphosphate (GGPP) synthase catalyzes the addition

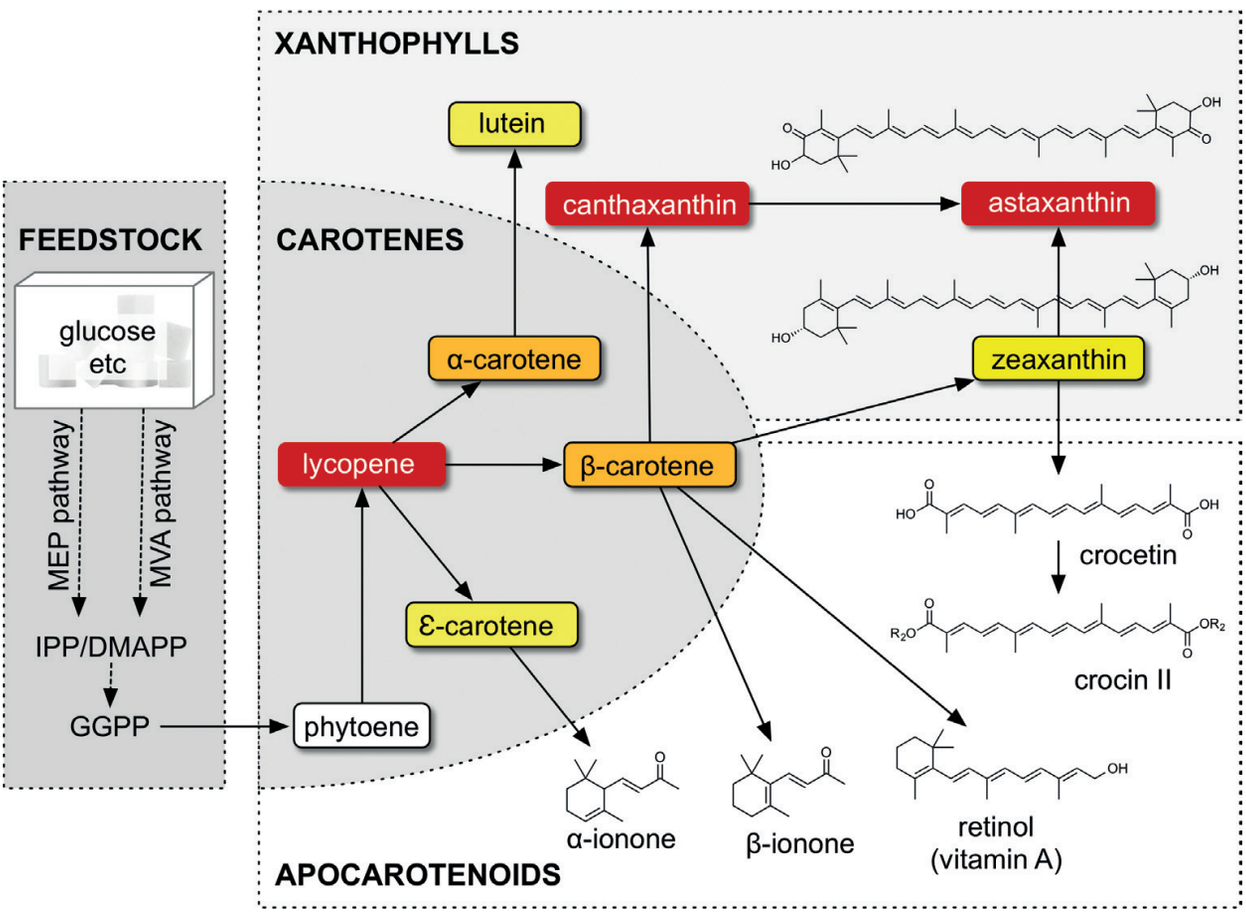

Figure 2. Biochemical pathway of carotenoids and apocarotenoids.

of another IPP into FPP to yield GGPP (C20). Finally, phytoene synthase (*crtB*) catalyzes the first committed step of carotenoid biosynthesis, the formation of one molecule of phytoene (C40) from two molecules of GGPP (**Figure 2**). Phytoene is a colorless acyclic carotene with only three conjugated double bonds. All the C40 carotenoids are derived from phytoene, which accounts for over 90% of total carotenoids to date [22]. Based on molecular structures, carotenoids are classified into two groups: carotenes and xanthophylls. Carotenes are hydrocarbon carotenoids with only carbon and hydrogen atoms (e.g., lycopene and β-carotene), whereas xanthophylls are oxygenated carotenoids by hydroxylation, ketolation and epoxidation (e.g., astaxanthin, lutein, **Figure 2**) [31]. In plants, algae, fungi and bacteria, apocarotenoids are derived from the oxidation of carotenoids or other apocarotenoids with carotenoid cleavage enzymes (such as carotenoid cleavage dioxygenases or CCDs and apocarotenoid cleavage oxygenases or ACOs) [32]. Some apocarotenoid examples are shown in **Figure 2**.

3. Production of carotenoids in engineered microbes

3.1. Biosynthesis of carotenes

As a colorless carotene, phytoene is the common precursor to all the C40 carotenoids (**Figure 2**). It exhibits excellent anti-UV activity [33] and is clinically proved to have activities of skin whitening and wrinkle reduction [34]. Hence, there are increasing cosmetic products developed based on phytoene. Phytoene is an intermediate carotenoid in plants and exists only as a minor product; hence, it is expensive to extract phytoene from plant materials. Consequently, it is promising to engineer microbes to produce higher concentrations of phytoene and more importantly, to produce it at high purity without other carotenoids. By deleting the *crtI* gene, encoding phytoene desaturase (see below), from an engineered lycopene-producing strain of *Escherichia coli* previously developed in our laboratory [19], it was relatively simple to generate strains of producing more than 50 mg/L of high-purity phytoene in simple low-cell density shake flasks [35]. Although this carotene with a high-potential market in cosmetics could be relatively simple to transfer to the industry, this is only just the beginning to attract interest, as witnessed by a French company Deinove (www.deinove.com) [36]. Despite certainly being more efficient than the use of the tomato strain developed to this end, there could still be considerable progress made by optimizing the engineered strains such as that used in our study and coupling this to high cell density fermentation processes to achieve a more cost-effective process.

Lycopene, a red color pigment most commonly associated with tomatoes, belongs to one of the top six commercial carotenoids. It is produced from the dehydrogenation of phytoene catalyzed by different types of phytoene desaturases (*crtI*, *PDS* or *ZDS*, **Figure 2**). Lycopene has been used as animal feed, food coloring and nutritional products. Some clinical studies have suggested that lycopene functions in reducing the risk of prostate cancers [37, 38]. In recent years, multiple research groups reported relatively high concentrations of lycopene produced in *E. coli* and yeasts. In *E. coli*, Kim et al. have used a mixture of carbon sources containing glucose, glycerol and arabinose to produce lycopene at 1.35 g/L [39]. Our laboratory initially optimized the MEP pathway which enabled the *E. coli* strain to produce at 20 mg/g

dry cell weight (DCW) of lycopene [40] and more recently reconstituted the MVA pathway in *E. coli* to produce lycopene in a glucose-defined medium, reaching 1.5 g/L in a simple non-optimized fed-batch process [19]. Xie et al. evolved the bifunctional enzyme crtYB to acquire only the phytoene synthase function. By applying this mutated enzyme and optimizing the copy number of crt genes, the engineered *Saccharomyces cerevisiae* strain produced 1.61 g/L of lycopene [41]. In addition, *Yarrowia lipolytica*, the oleaginous yeast, has also been engineered to produce lycopene but at slightly lower yields [42, 43].

Moving further along the carotenoid biosynthetic pathway, lycopene is usually cyclized into β-carotene or α-carotene by a lycopene cyclase (**Figure 2**). Both α- and β-carotenes are yellow pigments; β-carotene is more commonly marketed, being one of the most important commercial carotenoids. As mentioned earlier, β-carotene is a direct precursor of vitamin A (**Figure 2**). It has been widely used as a colorant, nutritional supplement, animal feed and in pharmaceutical and personal care industries. Chemically synthesized β-carotene is less popular among consumers than that extracted from natural sources or so-called 'bio-based' sources. At the same time, naturally derived β-carotene has gradually taken over the market. Currently, β-carotene is produced mainly in the microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2]. As summarized in **Table 1**, many groups have engineered fast-growing microorganisms and achieved high titers of β-carotene. Yang et al. have applied a hybrid MVA pathway in *E. coli* to overproduce β-carotene at 3.2 g/L [44]. Zhao et al. have engineered the central metabolic pathway to increase cofactor supply in an *E. coli* strain, which enabled the strain to produce at 2.1 g/L of β-carotene [45]. *Y. lipolytica* has shown potential as a better host for producing β-carotene; 4 g/L of β-carotene was achieved in an *Y. lipolytica* strain by integrating multiple copies of key enzymes (hmgr in **Figure 1** and the bi-functional enzymes phytoene synthase/lycopene cyclase carRP) [46]. Recently, based on an engineered lipid overproducing strain of *Y. lipolytica*, Larroude et al. have rewired it to produce at 6.5 g/L and 90 mg/g DCW of β-carotene [47]. These results are relatively better than those previously achieved [48–50]. It would not be surprising if some of these examples would lead to the successful commercialization notably of novel β-carotene sources in the near future.

3.2. Biosynthesis of xanthophylls

The modification of carotenes by enzymes such as hydroxylases and ketolases leads to the synthesis of xanthophylls (**Figure 2**). Due to the polarity introduced by oxygen, xanthophylls have different physical properties and physiological activities. For example, unlike carotenes, most xanthophylls do not possess provitamin A activity but do have higher anti-oxidant activities. The reason is that, in addition to the polyene structure, the functional groups of xanthophylls such as keto groups in the β-ionone rings can also quench singlet oxygen resides [31].

Among various xanthophylls, astaxanthin is the most important commercial product. Astaxanthin is a red pigment with numerous health benefits. As a potent anti-oxidant, astaxanthin protects the tissue against UV-light damage [51–53] and exhibits anti-cancer activity [54, 55] and anti-inflammatory properties [56]. In double-blind, randomized controlled trials, astaxanthin lowered oxidative stress in obese subjects and improved cognition and promoted proliferation of nerve stem cells [57]. Astaxanthin also improves integrated immune response [58], reveals anti-aging effects by protecting red blood cells in both aging and young people

No.	Hosts	Carotenes	Titer (mg/L)	Content (mg/g DCW)	Culture conditions	References
1	*Escherichia coli*	Phytoene	50	35	1–2 days, in flasks	[35]
2	*Escherichia coli*	Lycopene	224	34.5	1–2 days, in flasks	[48]
3	*Escherichia coli*	Lycopene	/	20	1–2 days, in flasks	[40]
4	*Escherichia coli*	Lycopene	1500	35	2 days, in bioreactors	[19]
5	*Escherichia coli*	Lycopene	1350	32	2 days, in bioreactors	[39]
6	*Saccharomyces cerevisiae*	Lycopene	1610	24.4	5–6 days, in bioreactors	[41]
7	*Saccharomyces cerevisiae*	Lycopene	1650	54.6	5–6 days, in bioreactors	[49]
8	*Yarrowia lipolytica*	Lycopene	/	16	7–8 days, in flasks	[42]
9	*Yarrowia lipolytica*	Lycopene	213	21.1	10 days, in bioreactors	[43]
10	*Blakeslea trispora*	β-Carotene	5600	/	7 days, in bioreactors	[50]
11	*Escherichia coli*	β-Carotene	2100	60	3–4 days, in bioreactors	[45]
12	*Escherichia coli*	β-Carotene	3200	/	2–3 days, in bioreactors	[44]
13	*Yarrowia lipolytica*	β-Carotene	6500	90	5–6 days, in bioreactors	[47]
14	*Yarrowia lipolytica*	β-Carotene	4000	50	10–11 days, in bioreactors	[46]

Table 1. Microbial production of carotenes in literature.

[59, 60] and relieves eye fatigue especially beneficial for persons spending too much time on the computer and smartphones [61]. In addition, astaxanthin supplement can prevent atherosclerotic cardiovascular disease [62, 63] and diabetes [64, 65]. More importantly, besides all these benefits, astaxanthin is clinically proven to be safe for human and animals. Therefore, astaxanthin has been widely used in fish feeding, food, nutritional, medicinal and cosmetic industries. The current global annual market of astaxanthin is around 250 tons worth $447 million [66], and it is growing rapidly. Synthetic astaxanthin, like β-carotene, is less popular with consumers and yields a mixture of three isomers, RR, RS and SS, at the ratio of 1:2:1 and appears to be less available for assimilation than the natural forms. Astaxanthin produced by the microalga *Haematococcus pluvialis* has a higher cost and lower purity than synthetic astaxanthin so additional work is required before good natural astaxanthin can be marketed effectively. Furthermore, astaxanthin in microalgae, shrimp and fish exists as an ester form rather than in the free form, which limits its nutraceutical applications.

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Due to the wide application of astaxanthin, many researchers have been working hard to engineer microbes to produce high titer and yield of astaxanthin. It is not trivial to optimize the biotransformation of β-carotene to astaxanthin as the biosynthetic pathway is rather complex with many intermediates and a complex network of enzymatic reactions [67]. By screening different β-carotene hydroxylases and ketolases, there has been success to improve astaxanthin production from sub-milligram to milligram per gram DCW [67, 68]. Further optimization of the metabolic pathway leading to astaxanthin synthesis has led to improved yields which are now promising for commercialization. For example, Zhou et al. developed a *S. cerevisiae* strain that overproduced astaxanthin at 47.2 mg/L and 8.1 mg/g DCW, where they used a direct evolution approach to generate a triple mutant of beta-carotene ketolase with higher activity [69]. Lin et al. integrated a multicopy of the key biosynthetic genes of astaxanthin (*hpchyb* and

No.	Hosts	Carotenoids	Titer (mg/L)	Content (mg/g DCW)	Culture conditions	References
1	*Saccharomyces cerevisiae*	Astaxanthin	/	4.7	3–4 days in flasks	[72]
2	*Saccharomyces cerevisiae*	Astaxanthin	/	0.029	5 days in flasks	[73]
3	*Escherichia coli*	Astaxanthin	/	2.64	2 days in flasks	[74]
4	*Escherichia coli*	Astaxanthin	/	0.31	2 days in flasks	[68]
5	*Escherichia coli*	Astaxanthin	2.1	1.41	2 days in flasks	[75]
7	*Escherichia coli*	Astaxanthin	2.9	1.99	2 days in flasks	[67]
8	*Corynebacterium glutamicum*	Astaxanthin	/	1.6	2 days in flasks	[76]
9	*Xanthophyllomyces dendrorhous, previously as Phaffia rhodozyma*	Astaxanthin	1.6	0.29	3 days in flasks	[77]
10	*Xanthophyllomyces dendrorhous*	Astaxanthin	/	9.0	8 days in flasks	[78]
11	*Xanthophyllomyces dendrorhous*	Astaxanthin	561	5.0	4–5 days in bioreactors	[79]
12	*Saccharomyces cerevisiae*	Astaxanthin	47.2	8.1	3–4 days in flasks	[69]
13	*Kluyveromyces marxianus*	Astaxanthin	/	9.90	3 days in bioreactors	[70]
14	*Yarrowia lipolytica*	Astaxanthin	54.6	3.5	3–4 days in plates	[66]
15	*Escherichia coli*	Astaxanthin	320	15.0	2 days in bioreactors	[71]
16	*Xanthophyllomyces dendrorhous*	Zeaxanthin	10.8	0.5	7.5 days in flasks	[80]
17	*Escherichia coli*	Zeaxanthin	/	11.9	2 days in bioreactors	[81]
18	*Escherichia coli*	Zeaxanthin	722	23.2	2.5 days in bioreactors	[82]

Table 2. Microbial production of astaxanthin and zeaxanthin in literature.

bkt) into the *Yarrowia lipolytica*. As a result, they were able to achieve about 9.97 mg/g DCW of astaxanthin [70]. By developing and using an efficient multidimensional heuristic process and colorimetric medium screening approach, our laboratory has achieved one of the best results of astaxanthin using *E. coli*, 320 mg/L and 15 mg/g DCW [71]. As summarized in **Table 2**, the engineered *S. cerevisiae* [69], *Y. lipolytica* [66], *Kluyveromyces marxianus* [70] and *E. coli* [71] have produced promisingly high titers and yields of astaxanthin. The recently achieved titers and yields [66, 70, 71] are from 10-fold to 100-fold higher than those previously reported in *S. cerevisiae* [72, 73], *E. coli* [74, 75], *Corynebacterium glutamicum* [76] and *Xanthophyllomyces dendrorhous*, previously as *Phaffia rhodozyma* [77–79].

Zeaxanthin is another important xanthophyll with high commercial values. Lutein, an isomer of zeaxanthin, is typically found in plants (such as corn), whereas zeaxanthin is present in cyanobacteria and some non-photosynthetic bacteria [2]. Although both lutein and zeaxanthin are used as colorants and potentially in pharmaceutical and nutraceutical industries, the demand for alternative sources of zeaxanthin is more urgent than lutein. Till now, *X. dendrorhous* has been engineered to produce 10 mg/L of zeaxanthin [80]. The first attempt to produce zeaxanthin in *E. coli* achieved 11.9 mg/ g DCW in bioreactors [81]. A few years later, the same group applied a dynamic control system to *E. coli* in which 720 mg/L of zeaxanthin was produced [82] (**Table 2**).

4. Production of apocarotenoids in engineered microbes

As shown in **Figure 2**, carotenoids can be further converted into apocarotenoids by CCDs or other oxygenases. Here, three apocarotenoids of high commercial values are highlighted here. Retinol, or vitamin A, is one of the most important apocarotenoids to humans. Retinol exhibits an essential function in vision, bone development and also promotes skin health as an anti-oxidant [83]. The other two are aromatic molecules, α-ionone, which naturally exists in raspberry, and β-ionone, which is found in many flowers, for example, rose, osmanthus and violet [84]. The chemical synthesis of these three molecules is not very difficult and contributes significantly to the current market share. However, consumers prefer natural derivatives and are willing to pay higher prices for natural ingredients [19]. As mentioned in the introduction, the extremely low concentrations in natural plant materials make their extraction an extremely expensive process. Consequently, the fermentation of engineered microbes is a promising alternative route.

4.1. Biosynthesis of retinol or vitamin A

As an important nutritional compound and an active cosmetic ingredient, retinol market size is estimated at 1.6 billion dollars [85]. Jang et al. pioneered retinol production in metabolically engineered *E. coli* [85]. Unlike carotenoids that are stored intracellularly in the lipid structures of microbes, apocarotenoids are smaller and thus can pass the cell membrane into the culture media. Consequently, a two-phase culture system was applied to capture extracellular retinol and improve its production by minimizing its degradation [85]. The same group later identified a gene (*ybbo*) that has retinal reductase activity that converts retinal into retinol.

Consequently, overexpression of the YBBO enzyme improved the final yield (76 mg/L) and purity (88%) of retinol in the final products [86]. Based on our lycopene chassis strain, we developed a 'plug-n-play' system that could easily adapt our *E. coli* strain into different apocarotenoids, such as α-, β-ionones and retinol [19] with promising results obtained (**Figure 3**).

4.2. Biosynthesis of α- and β-ionone

Both α-ionone and β-ionone have exceptional aroma activities as their odor threshold is at the sub-ppb range [7, 87]. Hence, they have been widely used as fragrance molecules in cosmetics and perfumes. As consumers prefer natural ingredients, the market demand for natural ionone is increasing dramatically. In addition, there is a chiral center for α-ionone. Natural α-ionone from plants (such as raspberry) is (R)-(+)-(E)-alpha-ionone. In contrast, synthetic α-ionone has two isomers (R and S). The R-enantiomer has a unique and strong floral flavor and aroma, described as a violet-like, fruit-like or raspberry-like flavor, while the S-enantiomer is woody or β-ionone like. Lashbrooke et al. did a proof-of-principle production of α-ionone at about 300 ng/L [88]. By coupling the modular metabolic engineering approach and enzyme engineering methods (N-terminal truncation and protein fusion), we developed an *E. coli* strain to produce 'natural identical' α-ionone at almost 500 mg/L, about 1400 times higher than that previously reported [19] (**Table 3**). Similarly, Phytowelt (www.phytowelt.com), a German company, has also developed an *E. coli*-based process to produce α-ionone, demonstrating that it has attracted more commercial interest.

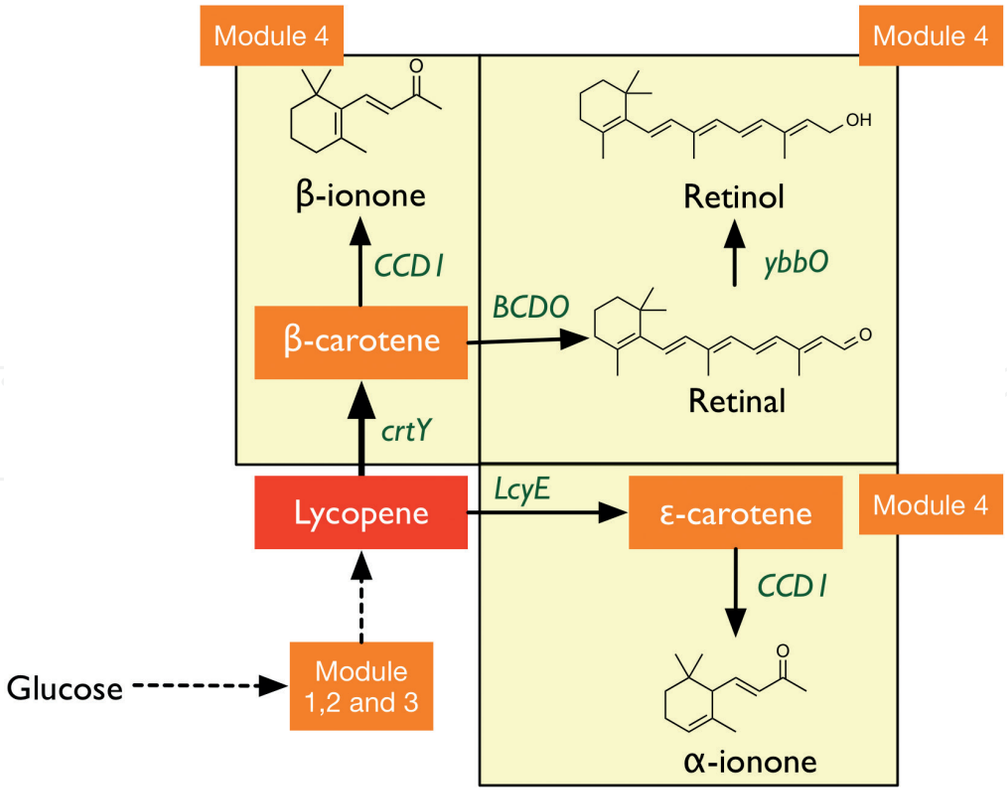

Figure 3. A 'plug-n-play' platform for biosynthesis of apocarotenoids. Adapted from author' s paper [19]. crtY—lycopene beta-cyclase; CCD1—carotenoid cleavage dioxygenase; BCDO (or blh)—β-carotene dioxygenase; ybbO—NADP+-dependent aldehyde reductase.

No.	Hosts	Apocarotenoids	Titer (mg/L)	Specific titer (mg/g DCW)	Culture conditions	References
1	*Escherichia coli*	Retinol	54	6.3	2–3 days in flasks	[85]
2	*Escherichia coli*	Retinol	76	9.8	2–3 days in flasks	[86]
3	*Escherichia coli*	Retinol	28	10.0	2 days in flasks	[19]
4	*Saccharomyces cerevisiae*	β-Ionone	0.22	/	2–3 days in flasks	[87]
5	*Saccharomyces cerevisiae*	β-Ionone	6	1.0	2–3 days in bioreactors	[90]
6	*Escherichia coli*	β-Ionone	500	16.0	2 days in bioreactors	[19]
7	*Escherichia coli*	α-Ionone	340 ng/L	/	2 days in flasks	[88]
8	*Escherichia coli*	α-Ionone	480	7.0	2 days in bioreactors	[19]

Table 3. Microbial production of retinol, α- and β-ionones in literature.

Although several groups have attempted to produce β-ionone using yeast or *E. coli*, their yields are relatively low. Simkin et al. firstly engineered *E. coli* cells to synthesize β-ionone but with only detectable trace amounts being reported [89]. Beekwilder et al. engineered *Saccharomyces cerevisiae* for the production of β-ionone; however, the titer achieved was only 0.22 mg/L [87]. López et al. inserted extra copies of geranylgeranyl diphosphate synthase gene and CCD1 gene from the plant *Petunia hybrid*, which enabled their *S. cerevisiae* strain to produce about 6 mg/L of β-ionone when grown in a bioreactor [90]. To date, the best-reported β-ionone strain was from our laboratory, where the engineered *E. coli* strain produced 500 mg/L of β-ionone [19] (**Table 3**).

5. Challenges and potential for the commercialization of microbial production of carotenoids and apocarotenoids

In general, the chief challenge for commercializing microbial production of chemicals is relatively high cost. The cost depends mainly on titer, rate (or productivity) and yield (or 'TRY') [91]. Hence, researchers are inventing and exploring different approaches to engineer microbes to obtain TRY figures of merit. Until then, it would not be cost effective or competitive to other sources (such as chemical synthesis). The good news is that carotenoids and apocarotenoids are high-value specialty chemicals; thus, their requirements for commercialization are less stringent as compared to fuels and commodity chemicals. For example, the current processes of β-carotene production in microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2] are already profitable. Many recent cases of microbial production of carotenoids have reached

TRY figures [46, 47, 71] higher than existing processes. It is not surprising that some of them will be translated into more cost-effective industrial processes. More importantly, scientists and engineers are working together to continue improving microbial strains and fermentation processes. Breakthrough by innovation and collective knowledge will markedly reduce product cost and make it more competitive. In addition, the recent trend of consumers' preference to 'natural' or 'bio-based' ingredients will make microbial-derived carotenoids and apocarotenoids more appealing.

6. Conclusion

Amid diverse natural products, carotenoids and apocarotenoids are particularly interesting. This is not only due to their bright color and pleasant fragrances but also their light-harvesting capability, the electron/energy transferring ability, the potent anti-oxidant properties, the hormone function, vitamin A activity and numerous other health benefits to both human and other life forms on the Earth. Increasingly, clinical studies have supported the concept that the regular uptake of carotenoids can prevent many serious diseases. The list of benefits and applications keeps growing and with the market for commercial exploitation it can be confidently expected to increase. In light of this and the extremely low levels found in plant materials, it is urgent to find solutions enabling these valuable molecules to be supplied in a sustainable and cost-effective manner. In the past decade, the metabolic engineering of microorganisms has progressed remarkably for the production of carotenoids and apocarotenoids. Some of these processes are being commercialized already but the scope to further extend this family of molecules is high, adding an increasingly solicited pipeline of natural products to compete with chemical synthesis.

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