Microbial Production of Zeaxanthin

Chi-Ching Lee and Yasemin Şefika Küçükata

Contents

Introduction 2
Biosynthetic Pathway and Genetic Manipulation Pathway for Zeaxanthin Production 3
Zeaxanthin Production by Zeaxanthin-Accumulating Native Microorganisms 5
Bacteria 8
Microalgae 13
Zeaxanthin Production by Metabolic Engineered Non-native Microorganisms 20
Bacteria 24
Fungi 30
The Improvement of Zeaxanthin Production in the Future Strategies 32
Conclusions and Perspectives 33
References 34

Abstract

A high amount of zeaxanthin, lutein, and meso-zeaxanthin are stored in the human macula, and they are associated with the risk reduction of human eye diseases such as age-related macular degeneration, glaucoma, and cataracts. The xanthophyll zeaxanthin and lutein usually coexist naturally and can be consumed from various fruits and vegetables. Zeaxanthin can be found in yellow-orange color fruits and vegetables like yellow corn, papaya, peaches, carrots, and mandarin oranges. Spirulina is also a microscopic and filamentous cyanobacterium containing a high concentration of zeaxanthin. Currently, zeaxanthin has been mainly produced by extraction and isolation from plants, but it is high-cost and energy-consuming. Therefore, the biosynthesis of zeaxanthin by microorganisms has been studied and developed recently. Many studies demonstrate that bacteria and microalgae are the most common naturally occurring zeaxanthin-accumulating microorganisms. *Escherichia coli* or yeast are broadly reported as

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engineered microorganisms for zeaxanthin production by regulating the biosynthetic pathway and the overexpression of the constructed gene. This chapter will discuss biosynthetic pathways of zeaxanthin, zeaxanthin production by natural zeaxanthin-accumulating method, and metabolic engineered microorganisms. At the end of the chapter, perspectives concerning the innovative strategies for further developing zeaxanthin production and its trends to improve the metabolically engineering microorganisms will be discussed.

Keywords

Zeaxanthin · Carotenoid · Lutein · Astaxanthin · Metabolic engineering · *Flavobacterium* · *Chlorella* · *Saccharomyces cerevisiae* · Mucor circinelloides · *Xanthophyllomyces dendrorhous*

Introduction

Carotenoids are the most common naturally occurring terpenoid pigments widely distributed in nature and serve important biological functions, and the demand for pigment produced from natural sources has developed rapidly in recent years (Eguchi et al. 2005). Carotenoids have the most extensive interest of all pigments in the market. They give yellow and red coloring to fruits, vegetables, and some plants and can be synthesized by microorganisms (Masetto et al. 2001). Natural carotenoids come about two major classes: (1) carotenes, such as b-carotene and a-carotene, and (2) xanthophylls, the carotenoid derivatives that have been oxygenated (Sajilata et al. 2008). Carotenoids like lutein and zeaxanthin are classified as generally recognized as safe (GRAS) for human consumption, allowing them to be used as food additives (Bernstein et al. 2016). Zeaxanthin, a stereoisomer of lutein, often coexists with lutein. Lutein and zeaxanthin are distinguished from other carotenoids by the presence of hydroxyl groups on both ends of the molecule. The hydroxyl groups allow them to orient within cell membranes and lipoproteins in ways other carotenoids cannot (Okur 2019). Zeaxanthin has several protective effects in anti-inflammatory, antioxidant, and anti-apoptotic mechanisms, as well as some neurological disorders (Bouyahya et al. 2021). Lutein and zeaxanthin are used for chicken skin pigments, pet food, animal and fish feed, and pharmaceutical purposes (Ilter et al. 2017). They are also used to improve the color of the yolk and flesh in poultry and aquaculture feed (Joshi and Singhal 2016). The health benefits of natural xanthophylls like zeaxanthin are in the interest of many experts such as physicians and dietitians. It is known that lutein and zeaxanthin are the only dietary carotenoids that make it to the human retina (Bernstein et al. 2016). Studies have shown that zeaxanthin has a critical role in delaying cataracts and age-related macular degeneration (Nwachukwu et al. 2016). The chemical structure of zeaxanthin (β,β-carotene-3,3′-diol) contains 11 conjugated double bonds with the chemical formula of $C_{40}H_{56}O_2$ and a molecular weight of 568.8 da. It is an oxygen-containing carotenoid derivative discovered in maize as oxygenated carotenoid zeaxanthin that

can occur naturally in marigold flowers (Zhang et al. 2018). Traditionally derived from plants such as marigold flowers, zeaxanthin co-occurs with lutein (Sarnaik et al. 2018). Saffron, another source of zeaxanthin, has volatile aromatic compounds of more than 150, and most of them are zeaxanthin, lycopene, and a-b-carotenes (Surgun Acar et al. 2017). Orange pepper and several yellow maize cultivars are food sources high in zeaxanthin (Breitenbach et al. 2019). Because of the difficulty in distinguishing zeaxanthin from lutein due to their similar molecular structures, an isolated production system for zeaxanthin is required (Sarnaik et al. 2018). The application of zeaxanthin has been developed in a variety of food, cosmetics, and nutraceutical business. Zeaxanthin is extensively used as pigmentation in the poultry and aquaculture industry. Due to the numerous zeaxanthin health benefits, nutraceutical products containing this primary functional bioactive compound have huge market potential. In this chapter, the biological synthesis pathways of zeaxanthin will be described. For microbial production of zeaxanthin, unmodified natural microorganisms as native hosts and the metabolic engineered non-native microbial host will be extensively discussed in various microbial species.

Biosynthetic Pathway and Genetic Manipulation Pathway for Zeaxanthin Production

Microorganisms are considered to offer tremendous promise in gaining high-amount and cost-effective zeaxanthin because of their higher stability, accessibility, and processability than plants. The genera *Paracoccus* and *Flavobacterium* have been presented to produce high content of zeaxanthin. *Vitellibacter* sp. and *Formosa* sp. also were developed to be exploited for zeaxanthin production (Sowmya and Sachindra 2016). Non-photosynthetic *Flavobacterium* sp. can produce enormous levels of zeaxanthin, which is more than 95% of the all carotenoid content in these microorganisms (Bhosale et al. 2004). The bacteria in the family *Flavobacteriaceae are the native zeaxanthin producer, and the major microorganisms include Aquibacter zeaxanthinifaciens* CC-AMZ-304^T (Hameed et al. 2014), *Gramella planctonica* CC-AMWZ-3^T (Shahina et al. 2014), *Hyunsoonleella jejuensis* (Yoon et al. 2010), and members of the *Flavobacteriaceae* genus *Muricauda* (Prabhu et al. 2013), *Kordia aquimaris* CC-AMZ-301^T (Hameed et al. 2013), and *Zeaxanthinibacter enoshimensis* TD-ZE3^T (Asker et al. 2007b). *Sphingomonas jaspsi* TDMA-16^T (Asker et al. 2007c) in the family *Sphingomonadaceae* and a novel genus and species *Nubsella zeaxanthinifaciens* TDMA-5^T (Asker et al. 2008) also were found to be promising microorganisms for zeaxanthin production (Zhang et al. 2018). A variety of microalgae, such as *Synechococcus* spp. (Schäfer et al. 2006; Sarnaik et al. 2018), *Chlorella saccharophila* (Singh et al. 2013, 2015), and *Nannochloropsis* spp. (Liau et al. 2011; Mitra and Mishra 2019), are also able to produce zeaxanthin.

Zeaxanthin is predominantly produced by microbes from terpenoids. The mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway are two main metabolic pathways that produce terpenoids (Fig. 1). The

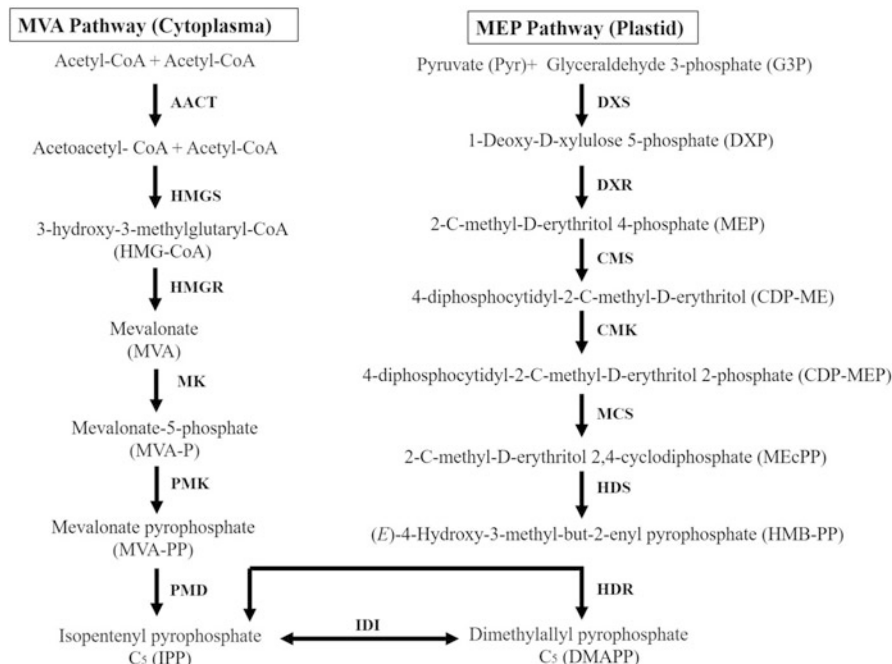

Fig. 1 Isoprenoid biosynthetic pathways. The mevalonate (MVA) pathway and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway are two primary isoprenoid biosynthetic pathways. AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate-5-kinase; PMK, phosphomevalonate kinase; PMD, mevalonate pyrophosphate decarboxylase. IDI, isopentenyl diphosphate isomerase; DXS, 1-deoxyxylulose-5-phosphate synthase; DXR, DXP reductoisomerase; CMS, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MCS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, HMB-PP synthase; HDR, HMB-PP reductase (Xu et al. 2016)

MVA pathway takes place in the cytoplasm and requires acetyl-CoA from the glycolytic pathway. Acetoacetyl-CoA thiolase (AACT) uses two molecules of acetyl-CoA to produce acetoacetyl-CoA, which is then condensed with another acetyl-CoA to synthesize 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) activated by HMG-CoA synthase (HMGS). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to MVA. Mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate pyrophosphate decarboxylase (MPD) catalyze sequential processes that convert MVA to isopentenyl pyrophosphate (IPP). Finally, isopentenyl diphosphate isomerase (IDI) converts IPP to dimethylallyl diphosphate (DMAPP). Archaea, fungi, higher plant cytoplasm, and other eukaryotes all have MVA pathways. Nevertheless, the MEP pathway also allows most bacteria, green algae, and plant plastids to synthesize terpenoids (Phillips et al. 2008). In the MEP pathway (Fig. 1), pyruvate and D-glyceraldehyde 3-phosphate are combined to produce 1-deoxy-D-xylulose 5-phosphate (DXP), which is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). The

synthesis of MEP is catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) after a sequence of chemical rearrangements and decreases DXP. MEP is converted into IPP or DMAPP by the sequential catalysis of 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS; Isp D), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK; Isp E), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS; Isp F), (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (HDS; Isp G), and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate reductase (HDR; Isp H) (Buhaescu and Izzedine 2007).

Geranyl diphosphate synthase catalyzes the condensation of IPP with DMAPP to produce ten-carbon geranyl diphosphate (GPP) (Fig. 2). Farnesyl diphosphate synthase (FPPS) condenses GPP with one molecule of IPP to produce fifteen-carbon farnesyl diphosphate (FPP), and then one molecule of FPP condenses with one molecule of IPP to synthesize twenty-carbon geranylgeranyl pyrophosphate (GGPP) by the action of GGPP synthase (CrtE). Phytoene synthase (CrtB) catalyzes the condensation of two molecules of GGPPs into a phytoene. The enzyme phytoene dehydrogenase (CrtI) converts phytoene to lycopene, which is then synthesized into carotene by two cyclization processes catalyzed by lycopene cyclase (CrtY). Lastly, the production of zeaxanthin from β-carotene is catalyzed by β-carotene hydroxylase (CrtZ). The zeaxanthin biosynthesis pathway in most cyanobacteria, such as *Synechococcus*, *Acaryochloris*, and *Prochlorococcus*, is comparable to that of *Erwinia* sp. and *Agrobacterium aurantiacum*. The early processes that lead to phytoene synthesis from IPP are comparable to those observed in *Erwinia* sp. and *Agrobacterium aurantiacum*. The enzymes phytoene desaturase (CrtP), ζ--carotene desaturase (CrtQ), and *cis*-carotene isomerase (CrtH) are used to catalyze the conversion of phytoene to the lycopene, following which *trans*-lycopene is cyclized to β-carotene by lycopene β-cyclase (CrtL-b). Ultimately, the hydroxylation of β-carotene to produce zeaxanthin is catalyzed by β-carotene hydroxylase (CrtR) (Schäfer et al. 2006). A considerable variety of zeaxanthin-producing bacteria and their genes have been discovered and described, developing the way for zeaxanthin production by conventional fermentation optimization and metabolic engineering (Weaver et al. 2015). MVA and MEP pathways, two of the biosynthesis pathways for producing zeaxanthin, have shown that isoprenoid flux is essential for improving carotenoid productivity, thus leading to genetic modifications in the zeaxanthin production pathways (Eguchi et al. 2005). The recent genetic studies for bacterial carotenoid biosynthetic pathways can lead to novel in vivo options for producing zeaxanthin, canthaxanthin, and astaxanthin. In Fig. 3, the chemical structures of carotenoids associated with the zeaxanthin biosynthetic pathway are described.

Zeaxanthin Production by Zeaxanthin-Accumulating Native Microorganisms

Lately, the microbial production of zeaxanthin has engaged the attention of scientists. However, while β-carotene and astaxanthin are dominant carotenoids produced by microbes, zeaxanthin is synthesized by only a few microorganisms (Sajilata et al. 2008). *Paracoccus* sp. provides a substitute and cost-effective source of zeaxanthin

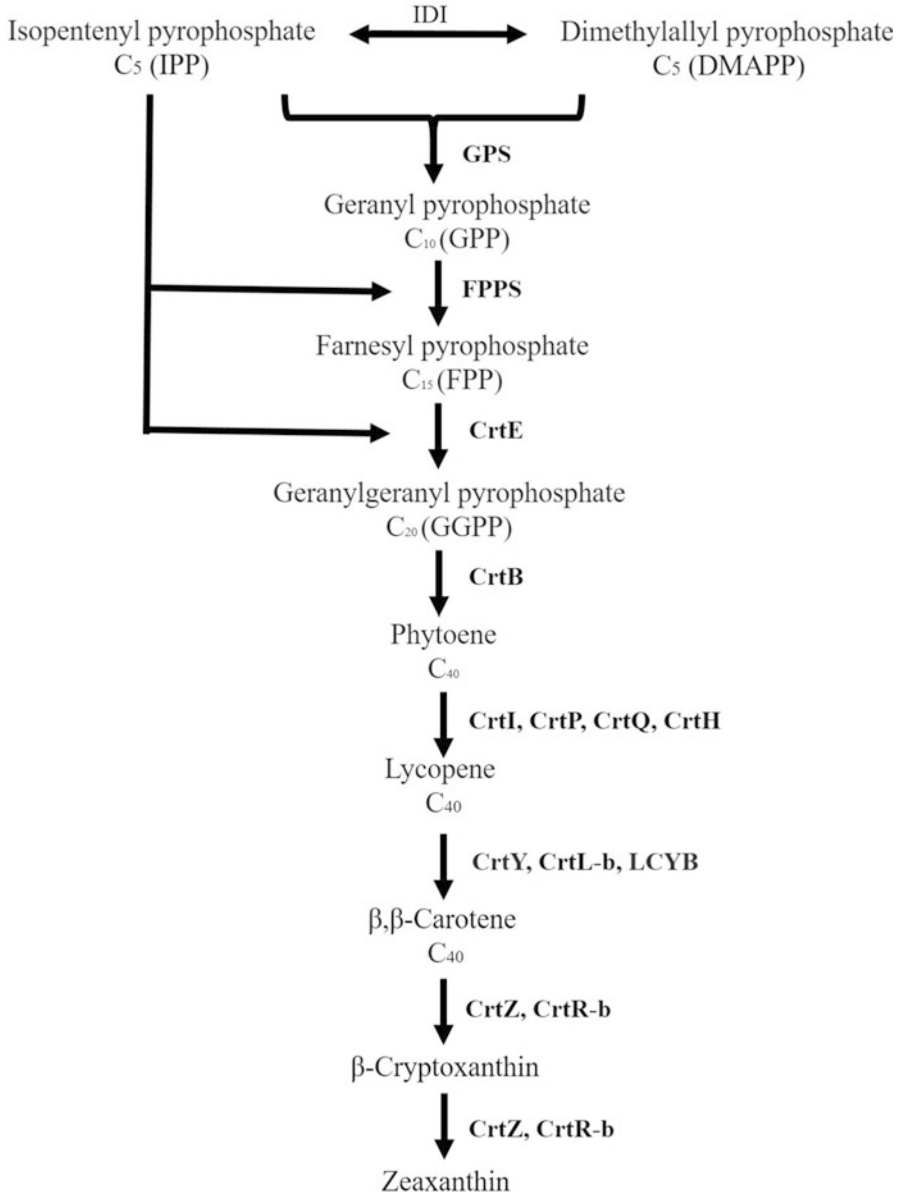

Fig. 2 Zeaxanthin biosynthetic pathways in bacteria and microalgae. IDI, isopentenyl diphosphate isomerase; GPS, geranyl pyrophosphate synthase; FPPS, farnesyl pyrophosphate synthase; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, bacterial phytoene desaturase; CrtP, algal phytoene desaturase; CrtQ, algal ζ--carotene desaturase; CrtH, algal *cis*-carotene isomerase; CrtY, bacterial lycopene β-cyclase; LCYB, plant lycopene β-cyclase; CrtL-b, algal lycopene β-cyclase; CrtZ, bacterial β- carotene hydroxylase; CrtR-b, algal β-carotene hydroxylase (Zhang et al. 2018)

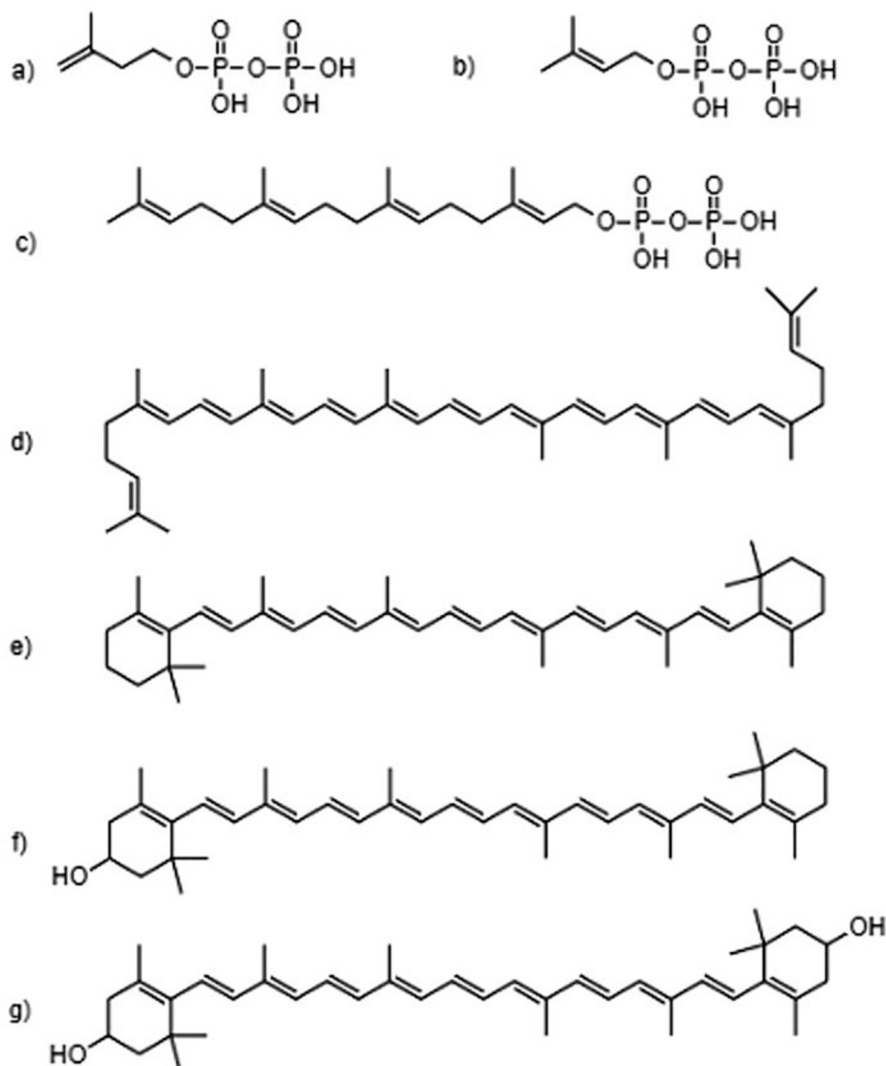

Fig. 3 Chemical structures of carotenoids: (**a**) isopentenyl pyrophosphate (IPP), (**b**) dimethylallyl pyrophosphate (DMAPP), (**c**) geranylgeranyl pyrophosphate (GGPP), (**d**) lycopene, (**e**) β-carotene, (**f**) β-cryptoxanthin, (**g**) zeaxanthin

due to its rapid growth rate and capacity to produce larger concentrations of carotenoids. The bacterial strain *Paracoccus zeaxanthinifaciens* ATCC 21588 is particularly intriguing because zeaxanthin is the only carotenoid produced by this strain (Joshi and Singhal 2016). Apart from yeast and bacteria, it is possible to produce zeaxanthin from various microalgae (Ilter et al. 2017).

Nitrogen and carbon sources are two of the essential variables in zeaxanthin synthesis. Optimizing nutrient medium with the addition of glucose or sucrose, amino acids, and some metal ions for *Flavobacterium* sp. can provide up to 500 mg/L of zeaxanthin. The oxygen supplied to the growth medium is another crucial component in forming microbial pigments (Sajilata et al. 2008). Improving and optimizing pigment production requires enhancing carbon and nitrogen sources, salts, and vitamins. Besides, the availability of oxygen, adequate amount of nutrients, agitation, and ventilation are essential for producing zeaxanthin with high efficiency (Vila et al. 2020). Natural carotenoid search grew rapidly throughout the late 1990s, as public awareness of health issues grew, as did a preference for organically produced pigments over chemically synthesized ones. Microalgae, yeasts, filamentous fungi, and bacteria were all considered potential options. Thus, the industrial production of carotenoids became a target for a variety of lucrative industries such as foods and nutraceuticals (Ram et al. 2020a).

Bacteria

Flavobacterium spp.

Flavobacterium sp. is known as the best microbial producer of zeaxanthin, and it constitutes a vast part of microbial carotenoids. Several studies have found that, in addition to environmental influences, different medium ingredients influence zeaxanthin production (Masetto et al. 2001). *Flavobacterium* sp. is reported to produce zeaxanthin as its sole carotenoid among the sources of microbial xanthophylls, and zeaxanthin production by *Flavobacterium* can be 95–99% (Sajilata et al. 2008). The influence of selected tricarboxylic acid cycle intermediates on the zeaxanthin synthesis from *Flavobacterium multivorum* was examined for improving the production of zeaxanthin. By applying a two-level factorial design, the influences of malic acid, isocitric acid, and α-ketoglutarate were evaluated. The concentration of these three tricarboxylic acid cycle intermediates was adjusted in shake flask culture by investigative synthesis profiles of various carotenoid compounds to improve the medium at several growth stages. The best cultivation conditions were 6.02 mM malic acid, 6.20 mM isocitric acid, and 0.02 mM α-ketoglutarate to obtain a sixfold increase in zeaxanthin content during the late logarithmic growth phase resulting in the greatest astaxanthin productivity of 10.65 μg/mL after 44-h cultivation. The specific rate of zeaxanthin production can achieve 198·9 μg/g per hour, which is a much higher production rate than 17.8 μg/g per hour in a basal culture medium. Scale-up trials in large bioreactors utilizing the improved medium might lead to additional improvements in *Flavobacterium multivorum* for zeaxanthin production (Bhosale et al. 2004). Forty-seven *Flavobacterium* strains were isolated from the supraglacial layers of four glaciers in mainland China. 0 to 20 cm depth of surface ice in the glacier tongues was corrected and sampled. The influence of light illumination in these 47 zeaxanthin-producing glacial *Flavobacterium* strains was investigated because carotenoids are often produced by glacial bacteria. Most strains grew faster under light exposure, while only two strains died under 50 μmol photon m^{-2} s^{-1}. The significant effect of light stimulation was observed only when strains were

cultured in either anoxic or oligotrophic environments at 7 and 14 °C. Zeaxanthin was highly produced by these 45 strains under the light simulation because the genome of zeaxanthin biosynthesis was discovered in these 45 strains, 37 of which also had the proteorhodopsin gene *prd*. The zeaxanthin production of glacial *Flavobacterium* strains was significantly improved by light stimulation, which may offer higher photoprotection and maintenance of membrane integrity when these microorganisms live in freezing conditions (Liu et al. 2021).

Mesoflavibacter spp.

A rod-shaped, aerobic Gram-negative orange non-flagella bacterium *Mesoflavibacter aestuarii* strain KYW614^T was discovered, identified, and isolated from a seawater sample from Gwangyang Bay, Republic of Korea. After the polyphasic taxonomic analysis, the result displayed that the isolate is a new species found in the genus *Mesoflavibacte*r. Accordingly, the name *Mesoflavibacter aestuarii* strain KYW614^T is given. The zeaxanthin production in strain KYW614^T was 12 mg/g dry cell weight, which was 8.8 times higher in comparison with 1.37 mg/g dry cell weight in *Mesoflavibacter zeaxanthinifaciens* KCTC 32286^T (Lee et al. 2014). Several novel zeaxanthin-accumulating bacteria were isolated, identified, and analyzed from the water samples in Japan. A Gram-negative halophilic and mesophilic aerobic bacterium TD-ZX30^T floating and rod-shaped was isolated from seawater near Kamakura City on Japan's Pacific coast. The growth temperature range of TD-ZX30^T was from 16 °C to 44 °C. A new genus and species *Mesoflavibacter zeaxanthinifaciens* TD-ZX30^T strain was named and proposed. The TD-ZX30^T belongs to a separate lineage in the *Flavobacteriaceae* family, with 93.9% sequence similarity to the closest microorganism, *Olleya marilimosa*. However, a variety of chemotaxonomic and phenotypic traits separated TD-ZX30^T from other species in the family *Flavobacteriaceae*. The result exhibited that zeaxanthin as a major carotenoid can be produced by TD-ZX30^T strain with a yield of 910 μg/g dry cell weight, and the other two zeaxanthin isomers can produce 11 and 230 μg/g dry cell weight of zeaxanthin (Asker et al. 2007a).

Muricauda spp.

Several novel zeaxanthin-accumulating bacteria were also isolated, identified, and examined from the coastline of India and Taiwan. A zeaxanthin-producing thermophilic bacterium *Muricauda lutaonensis* CC-HSB-11^T strain was discovered and isolated in a coastline hot spring of Green Island, Taiwan. The optimized medium was used for cultivating this strain to obtain 3.12 mg/L zeaxanthin in the bioreactor. The supercritical carbon dioxide antisolvent precipitating technique was used to micronize zeaxanthin, and its yield was 53.4%. The strategy takes advantage of *Muricauda lutaonensis* CC-HSB-11^T's inherent capacity to produce zeaxanthin, and the research revealed the viability of using an antisolvent precipitating technique to generate microparticles using a bacterial strain (Hameed et al. 2011). Two novel bacteria YUAB-SO-11 and YUAB-SO-45 were discovered, isolated, and identified on the sandy beaches of Malabar Coast, located on the southwestern coastline of India. Based on the 16S rDNA profile, two zeaxanthin-producing strains YUAB-SO-11 and YUAB-SO-45 were tightly linked to a member of the genus *Muricauda.* This

strain had a highly significant degree of sequence similarity with *Muricauda aquimarina* JCM 11811^T and *Muricauda olearia* JCM 15563^T exhibiting approximately 98.9% and 99.2% similarity, respectively. For the highest astaxanthin production, two strains YUAB-SO-11 and YUAB-SO-45 were cultivated under the optimal condition in marine broth supplemented with 2% sodium chloride at 30 °C, pH 7.0, 125 rpm for 5 days. The highest values of astaxanthin production were 1.2 mg/g cell dry weight in strain YUAB-SO-11 and 1.02 mg/g cell dry weight in strain YUAB-SO-45. The study showed that both of these strains might represent a novel highly zeaxanthin-producing species. Besides, zeaxanthin synthesis can be enhanced by the addition of 0.1 M glutamic acid, which is an intermediate in the citric acid cycle. The zeaxanthin production of these two strains YUAB-SO-11 and YUAB-SO-45 can achieve 1.47 mg/g and 1.18 mg/g, increasing by 18.4% and 14%, respectively (Prabhu et al. 2013). Later, *Muricauda aquimarina* JCM 11811^T, *Muricauda flavescens* JCM 11812^T, *Muricauda lutimaris* KCTC 22173^T, and *Muricauda lutaonensis* KCTC 22339^T, as well as two environmental isolates mentioned above, YUAB-SO-11 and YUAB-SO-45 obtained from Ullal and Cochin beaches located in the southwestern coastline in India, were employed. Zeaxanthin was one of the most abundant carotenoids in *Muricauda* strains. The optimal *Muricauda flavescens* JCM 11812^T strain yielded the highest astaxanthin at 4.4 mg/L (2.9 mg/g dry cell weight) in marine broth at 32 °C for 3 days (Prabhu et al. 2014).

Sphingomonas spp.

Three colorful bacteria CHOB06-6, KODA19-6, and MAKB08-4 were discovered, isolated, and identified from various sponges in the Gulf of Thailand based on the 16S rDNA analytical profile. Two sponge-associated zeaxanthin-producing strains CHOB06-6 and MAKB08-4 were tightly linked to *Sphingomonas phyllosphaerae* FA2^T, which was found and identified from the plant rhizosphere of a leguminous tree, *Acacia caven*, in Argentina. *Sphingomonas* (*Blastomonas*) *natatoria* KODA19-6 appeared to yield the highest zeaxanthin at 0.73 mg/L with a yield of 4.9 mg/g in the optimal cultivated condition a pH 7.6 and 30 °C in a modified Zobell medium for 4 days. The zeaxanthin productivity of *Sphingomonas natatoria* KODA19-6 was 6.27 μg/L per hour and the most favorable at pH 6.0 for its growth with 0.6 g/L (Thawornwiriyanun et al. 2012). A freshwater sample obtained at Misasa, located in Tottori, Japan, was examined, and a Gram-negative aerobic orange-yellowish pleomorphic bacterium strain TDMA-16^T was isolated. A new genus and species *Sphingomonas jaspsi* sp. TDMA-16^T strain was named and proposed based on polyphasic taxonomic analysis. This TDMA-16^T strain can efficiently produce a variety of carotenoids, including zeaxanthin and other carotenoids, with a yield of 1.7 mg/g dry cell weight (Asker et al. 2007b).

Other Native Bacteria

The primary carotenoids generated by an *Algibacter* strain were mostly determined by the culture conditions. 98% of the total carotenoids produced by an *Algibacter* strain AQP096 was zeaxanthin under conventional cultivation in marine broth at

26 °C for 2 days. 3.47 mg/g dry cell weight of zeaxanthin was produced. Besides, an *Algibacter* mutant strain AQP096-16 under UV irradiation mutagenesis was observed to generate an 11.41 mg/g dry cell weight of zeaxanthin (Mohamed et al. 2013). Another aerobic yellow Gram-negative rod-shaped bacterium strain TDMA-5^T was also discovered from a freshwater sample at Misasa located in Tottori, Japan. A new genus and species *Nubsella zeaxanthinifaciens* TDMA-5^T was also named. A variety of chemotaxonomic and phenotypic traits separated TD-$ZX30^T$ from other species in the family *Sphingobacteriaceae*. This strain can produce zeaxanthin as a main carotenoid pigment compound (Asker et al. 2008). A seawater sample from the Pacific coastline of Japan near Enoshima Island, Japan, was investigated, and a Gram-negative aerobic yellow rod-shaped bacterium TD-$ZE3^T$ strain was isolated. A new genus and species *Zeaxanthinibacter enoshimensis* TD-$ZE3^T$, which can release oxidase and catalase, was also named. This is a unique zeaxanthin-producing marine microorganism from the *Flavobacteriaceae* family (Asker et al. 2007c). Another seawater sample was obtained on the seashore of Jeju Island, South Korea, and a new yellow Gram-negative rod-shaped non-flagellated aerobic bacterium strain $CNU004^T$ was identified from this seawater sample. A new genus and species oxidase- and catalase-producing *Hyunsoonleella jejuensis* $CNU004^T$ was also named. This strain can produce zeaxanthin as a main carotenoid pigment compound (Yoon et al. 2010). A zeaxanthin-producing *Arthrobacter gandavensis* MTCC 25325 was isolated and identified from a freshwater sample in Bor Talav, Bhavnagar, India, under screening potential bacterial strains for carotenoid accumulation. The chemometric techniques were used to optimize the influential parameters on zeaxanthin production, including carbon source, pH, temperature, harvesting time, agitation speed, and inoculum size. The highest zeaxanthin yield was found to be 1.51 mg/g dry cell weight under the optimized culture conditions containing pH 6.0, 10% (v/v) inoculum size, and1.5% (w/v) glucose on the third day of cultivation, agitation at 120 rpm under 40 °C (Ram et al. 2020b).

In addition, several novel zeaxanthin-accumulating bacteria were also isolated, identified, and examined from the water samples in Taiwan. A rod-shaped Gram-negative aerobic bacterial strain *Siansivirga zeaxanthinifaciens* CC-SAMT-1^T was discovered and isolated from a coastline seawater sample in the China Sea, Taiwan. This strain had a significant degree of sequence similarity with species of the genera *Mariniflexile* and *Gaetbulibacter* showing approximately 95.7% and 96.0% similarity, respectively. Strain CC-SAMT-1^T produced all-*trans* zeaxanthin as a major xanthophyll carotenoid with a yield of 6.5 mg/g dry biomass. A new genus and species zeaxanthin-producing *Siansivirga zeaxanthinifaciens* CC-SAMT-1^T was also named (Hameed et al. 2012). A coastal surface seawater sample near Taichung harbor, Taiwan, was examined. A rod-shaped, non-spore-forming, Gram-negative, non-flagellated aerobic bacterium strain CC-AMZ-301^T was found, and it can produce predominant carotenoid all-trans-zeaxanthin. The marine CC-AMZ-304^T strain generated 67% all-*trans*-zeaxanthin as the main xanthophyll pigment, substantial levels of 14% *cis*-zeaxanthin, and 15% several unidentified carotenoids. This strain CC-AMZ-304^T is reported to be a distinct species in the genus *Kordia*. Therefore, a name for novel species zeaxanthin-producing Kordia aquimaris

CC-AMZ-301^{T} (JCM 18556^{T}or BCRC 80464^{T}) was proposed (Hameed et al. 2013). Later, from the same area, a seaside surface seawater sample near Taichung harbor, Taiwan, was collected and investigated. A yellow strain CC-AMZ-304^{T} was found, and it can produce predominant carotenoid all-trans-zeaxanthin. The CC-AMZ-304^{T} strain generated 65% all-*trans*-zeaxanthin as the main xanthophyll pigment, substantial levels of 17% *cis*-isomeric zeaxanthin, and 15% several unidentified carotenoids. This strain CC-AMZ-304^{T} is reported to be a distinct species of a new genus in the family *Flavobacteriaceae*. Therefore, a name for the novel genus and species zeaxanthin-producing Aquibacter zeaxanthinifaciens CC-AMZ-304^{T} was also proposed (Hameed et al. 2014). Under various culture conditions and nutrient content, the marine carotenoid-producing bacterium *Formosa* sp. KMW from the family *Flavobacteriaceae* was determined. The yield of total carotenoids synthesized climbed during cultivation. After 6 days of fermentation, the zeaxanthin concentration enhanced to 51.4% of total carotenoids. The highest total carotenoid yield was 0.97 mg/L, while the cells were cultivated at pH 7.0 under light and shaking conditions. The intermediates of the tricarboxylic acid cycle displayed no effect on carotenoid synthesis. In addition, carotenoid production was much higher with a yield of 0.69 mg/L in the absence of sugars and also had 0.66 mg/L carotenoids followed in the presence of glucose. Besides, the highest carotenoid content was presented when *Formosa* sp. KMW was grown in 0.82 mg/L glutamic acid and 0.81 mg/L cysteine. When the vortexing method was used during carotenoid extraction, a higher carotenoid output was observed (Sowmya and Sachindra 2015).

The marine zeaxanthin-producing bacterium previously known as *Flavobacterium* sp. strain R-1512 (LMG 21293 T or ATCC 21588) underwent a thorough taxonomic re-assessment. A novel species of the genus *Paracoccus* was constituted by this strain and two other formerly discovered marine isolates, which are *Paracoccus* sp. strain MBIC 3966 and *Flavobacterium* strain R-1506. A new genus and species *Paracoccus zeaxanthinifaciens* sp. nov. is proposed, with ATCC 21588 T strain which was named and proposed based on polyphasic taxonomic analysis (Berry et al. 2003). The influence of different medium ingredients in zeaxanthin production *Paracoccus zeaxanthinifaciens* ATCC 21588 was determined by utilizing a combination of statistical and mathematical methodologies. The most important influential input factors were determined primarily by the Taguchi design, and they were pyridoxine hydrochloride, yeast extract, methyl palmitate, and glucose. These characteristics were investigated utilizing a central composite design to construct response surface methodology and artificial neural network models. The hybrid of artificial neural networks and the genetic algorithm was shown to be preferable in comparison with the hybrid of response surface methodology and genetic algorithm. It is because the percent prediction error was 1.55% for the hybrid of artificial neural network and genetic algorithm in comparison to 5.09% for the hybrid of response surface methodology and genetic algorithm. The result revealed that the optimal composition of the culture medium was 0.18 mg/L pyridoxine hydrochloride, 24.3 g/L glucose, 8 g/L methyl palmitate, and 30 g/L yeast extract. These conditions were predicted by the hybrid of artificial neural network and genetic algorithm, and it can yield 11.63 mg/L in an average total zeaxanthin

production. Under the optimal conditions mentioned above, there was 36% of total zeaxanthin production released extracellularly (Joshi and Singhal 2016). A surface seawater sample was obtained in coastline Kending, Taiwan, and an aerobic rod-shaped Gram-negative strain CC-AMWZ-3^T with non-spore-forming was found, isolated, and identified. Based on the 16S rDNA profile, the zeaxanthin-producing strains CC-AMWZ-3^T were tightly linked to a member of the genus *Gramella.* In the pairwise comparison of 16S rRNA gene sequence similarities, this strain shared a highly significant degree of sequence similarity with *Gramella echinicola* KMM 6050^T and other *Gramella* species, showing approximately 93.3% and 92.4 to 96.0% similarity, respectively. This strain also built divergent phyletic lineage during phylogenetic analysis. After the polyphasic taxonomic analysis, the result demonstrated that the isolate is a novel species found in the genus *Gramella*. Accordingly, the name *Gramella planctonica* strain CC-AMWZ-3^T is suggested. The CC-AMWZ-3^T strain produced all-trans-zeaxanthin as the main xanthophyll pigment (more than 72%), low levels of cis-isomeric zeaxanthin (13%), and several unidentified carotenoids (14%) (Shahina et al. 2014). In Table 1, zeaxanthin production in unmodified naturally-zeaxanthin-accumulating native bacteria is described.

Microalgae

A variety of carotenoids can be found and extracted from numerous natural sources like plants and algae. Besides, carotenoid compounds can be manufactured by chemical synthesis and also be produced by many microorganisms. Zeaxanthin production was observed and developed in a variety of microalgae, such as green algae *Neospongiococcum excentricum* (Liao et al. 1995), *Dunaliella salina* mutant *zea1* (Jin et al. 2003), *Dunaliella parva* (Young and Britton 1990), *Dunaliella bardawil* (Ben-Amotz et al. 1982), cyanobacterial *Microcystis aeruginosa* (Chen et al. 2005), blue-green algae *Cyanophora paradoxa*, and *Glaucocystis nostochinearum.* Yellow-green algae *Pleurochloris commutate* (Goodwin 1992), *as well as* red algae *Corallina* ojicinalis, *Corallina elongata*, and *Jania* sp. (Palermo et al. 1991). The red algae Rhodophytes and blue-green algae *Cyanobacteria* are the two major phyla of algae for zeaxanthin production (Bourdon et al. 2021).

Chlorella spp.

A green microalgae *Chlorella saccharophila* was isolated from the seawater on the seashore of New Zealand and cultivated heterotrophically in a culture medium with glucose or glycerol as carbon sources. Biomass production was observed to be higher with 2.14 g/L in cultures treated with glucose than 0.378 g/L biomass in cultures supplemented with glycerol. Conversely, when microalgae were grown in the media supplemented with glycerol, total carotenoid production was 0.406 mg/g, which was higher as compared to 0.21 mg/g of carotenoids with glucose. The optimized production medium included 50% v/v artificial seawater, 1 g yeast extract, 0.1 g peptone, and 1 g glycerol or glucose. After microalgae were

Table 1 Zeaxanthin production in unmodified zeaxanthin-accumulating native bacteria

Microorganisms	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Algibacter sp. mutant AQP096-16	3.47 mg/g; 11.41 mg/g	The growth conditions were at 25 °C, pH 7.5, for 48 h with the continuous aeration shaking at 200 rpm in 25 ml shake Erlenmeyer flask	Mohamed et al. 2013
Aquibacter zeaxanthinifaciens CC-AMZ-304^T	All-*trans*-zeaxanthin (65%) *cis*-zeaxanthin (17%) in total carotenoids	The strain was cultured in marine broth at 30 °C, pH 7.0, in the presence of 3% NaCl for 48 h	Hameed et al. 2014
Arthrobacter gandavensis MTCC 25325	1.51 mg/g	The highest zeaxanthin yield was found under the optimized culture conditions containing pH 6.0, 10% (v/v) inoculum size, and1.5% (w/v) glucose on the third day of cultivation, agitation at 120 rpm under 40 °C	Ram et al. 2020b
Flavobacterium multivorum ATCC 55238	10.65 mg/L	The culture was in shake flasks with the optimized medium at 30 °C on a rotary shaker at 250 rpm for 44 h	Bhosale et al. 2004
Forty-five glacial *Flavobacterium* strains	The overrepresentation of carotenoid compounds	The glacial strains were cultivated in 5 ml of PYG broth containing 0.4% glucose at 14 °C under light fluxes of 10 and 50 μmol photons m^{-2} s^{-1} in an incubator	Liu et al. 2021
Formosa sp. KMW	51.4% of 0.97 mg/L total carotenoids	The growth conditions were at 37 °C, pH 7.0, for 7 days with continuous aeration shaking at 200 rpm in marine broth under white light illumination, 0.82 mg/L glutamic acid, and 0.81 mg/L cysteine without sugars	Sowmya and Sachindra 2015
Gramella planctonica CC-AMWZ-3^T	More than 72% of the total carotenoid is all-*trans*-zeaxanthin	The strain was cultured in the marine medium at 30 °C, pH 7.0, in the presence of 2% NaCl for 48 h	Shahina et al. 2014

(continued)

Table 1 (continued)

Microorganisms	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Hyunsoonleella jejuensis CNU004^T	Major carotenoid compounds	The strain was cultivated in the marine agar at 30 °C, pH 7.0 in the aerobic condition for 7 days	Yoon et al. 2010
Kordia aquimaris CC-AMZ-301^T	All-*trans*-zeaxanthin (67%) *cis*-zeaxanthin (14%) in total carotenoids	The strain was cultivated in the marine medium at 30 °C, pH 7.0, in the presence of 2% NaCl for 2 days	Hameed et al. 2013
Mesoflavibacter zeaxanthinifaciens TD-ZX30^T	0.91 mg/g	The culture was in the marine agar plate at 37 °C for 2 days	Asker et al. 2007a
Mesoflavibacter aestuarii KYW614^T	12 mg/g	The strain was cultivated in marine agar at 25 °C, pH 7.0 in the aerobic condition for 3 days	Lee et al. 2014
Muricauda lutaonensis CC-HSB-11^T	3.12 mg/L	Culture media was in commercial marine broth at 40 °C, pH 7.4, stirrer speed at 150 rpm, 1 per unit volume of growth medium per minute, dissolved oxygen >15% in a 2-L bioreactor for 72 h	Hameed et al. 2011
Muricauda aquimarina YUAB-SO-11 and YUAB-SO-45	1.47 mg/g in YUAB-SO-11 and 1.18 mg/g in YUAB-SO-45	Strains YUAB-SO-11 and YUAB-SO-45 were subjected to grow under pH 7.0, 30 °C, salinity (NaCl 2% *w*/*v*), and 125 rpm in marine broth for 5 days	Prabhu et al. 2013
Muricauda flavescens JCM 11812^T	2.9 mg/g; 4.4 mg/L	Strains were cultured in marine broth at 32 °C,150 rpm for 72 h	Prabhu et al. 2014
Nubsella zeaxanthinifaciens TDMA-5^T	Major carotenoid compounds	The strain was cultivated in Luria–Bertani (LB) solid medium at 30 °C, pH 7.0, in the aerobic condition for 3 days	Asker et al. 2008
Paracoccus zeaxanthinifaciens ATCC 21588	11.63 mg/L	The strain was grown at 35 °C, pH 7.5, for 72 h with a continuous rotary shaker at 180 rpm in a 250 mL Erlenmeyer flask containing the 50 mL culture medium. The	Joshi and Singhal 2016

(continued)

Table 1 (continued)

Microorganisms	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
		optimal culture medium contained 0.18 mg/L pyridoxine hydrochloride, 24.3 g/L glucose, 8 g/L methyl palmitate, and 30 g/L yeast extract	
Siansivirga zeaxanthinifaciens CC-SAMT-1^T	6.5 mg/g	The strain was cultivated in marine broth at 30 °C, pH 7.0, in the aerobic condition for 2 days.	Hameed et al. 2012
Sphingomonas jaspsi TDMA-16^T	1.7 mg/g in total carotenoids	The strain was cultivated in nutrient agar at 37 °C, pH 7.0, in the anaerobic condition for 2 days	Asker et al. 2007b
Sphingomonas natatoria KODA19-6	4.9 mg/g; 0.73 mg/L	0.62 mg/L with a yield of 4.1 mg/g in the optimal cultivated condition at pH 7.6, 30 °C and 150 rpm in a modified Zobell medium for 4 days	Thawornwiriyanun et al. 2012
Zeaxanthinibacter enoshimensis TD-ZE3^T	Major carotenoid compounds	The strain was cultivated in nutrient agar at 30 °C, pH 8.0, in the aerobic condition for 2 days	Asker et al. 2007c

incubated at 20 °C, pH 6.5 with shaking at 150 rpm for 192 h, the maximal carotenoid yield can reach 11.32 mg/g zeaxanthin, 5.07 mg/g β-carotene, and 16.39 mg/g total carotenoid. The results also demonstrated that ultrasonication was determined to be critical for the optimal extraction technique after several mechanical and chemical approaches to carotenoid extraction were compared (Singh et al. 2013). Later, ultrasonication was used for zeaxanthin production of *Chlorella saccharophila* GTC under a variety of conditions. The influence of zeaxanthin production in this strain was investigated by four factors, including power, pulse, the ratio of solvent and cell dry weight, time, and their interaction, followed by response surface methodology for optimization. There was a significant combination which is p-value less than 0.05 with a considerable influence on zeaxanthin production for power and time, as well as power and the ratio of solvent and cell dry weight. Optimal conditions of extraction for zeaxanthin production were determined as pulse length of 19.7 s, power of 27.82% out of total power of 500 watts, time of 13.48 min, as well as a ratio of solvent and cell dry weight of 67.38 μL/mg. The maximum zeaxanthin yield was 11.2 mg/g after optimal conditions of extraction. This approach contributed to a lowering period while maintaining carotenoid production (Singh et al. 2015).

The variability and amount of carotenoids in the microalga *Chlorella pyrenoidosa* were determined by high-performance liquid chromatography. The microalga cells were ground, extracted, and saponified for all the analysis. The gradient elution was designed by utilizing a mobile phase of two eluents with methanol–acetonitrile water (84:14:2, v/v/v) and methylene chloride (100%). By using a C30 column with a flow rate of 1 mL/min under absorbance detection at 450 nm, 32 carotenoids were recovered in 49 min. The content of all-*trans*-lutein was 125.03 mg/g, which was a significantly high amount and followed by *cis* isomers of lutein (27.97 mg/g), all-*trans*-α-carotene (2.47 mg/g), and zeaxanthin (2.17 mg/g) (Inbaraj et al. 2006). A CZ-*bkt1*mutant of *Chlorella zofingiensis* was selected to produce high levels of zeaxanthin. CZ-*bkt1* mutant was developed by treating algal cells with 0.5–10 mg/mL *N*-methyl-*N*′-nitro-*N* nitrosoguanidine as a chemical mutagen in the dark for 1 h, followed by a color-based colony-screening method. The colorimeter can measure color of the colonies in different microorganisms and can be measured and examined by colorimeters with various optical analytical methods (Lee et al. 2020). CZ-*bkt1* was shown to be a deficient carotenoid ketolase, resulting in zeaxanthin accumulation instead of the ketocarotenoid astaxanthin downstream of the carotenoid pathway. CZ-*bkt1* mutant was induced to generate zeaxanthin by four stress conditions, such as 2% sodium chloride, high light irradiation with the intensity of 460 μmol photons m^{-2} s^{-1}, glucose stress, and nitrogen deprivation with medium replacing potassium nitrate with potassium chloride. Under a combined treatment of high-light irradiation and nitrogen deficiency, the CZ-*bkt1* mutant can produce 7.00 mg/g of zeaxanthin and a yield of 12.39 mg/L. This mutant can also have a yield of 36.79 mg/L zeaxanthin with a combination of three treatments, including high-light irradiation, nitrogen deficiency, and glucose addition (Huang et al. 2018).

Nannochloropsis spp.

The extraction and recovery of zeaxanthin from microalgae *Nannochloropsis oceanic was investigated* with six extraction solvent systems, including chloroform/methanol (2:1), chloroform/methanol (1:2), dichloromethane/methanol (2:1), hexane/isopropanol (3:2), hexane/methanol (3:2), and ethyl acetate/hexane (2:3). The culture was grown in a 14-L photobioreactor containing a modified Conway medium under a light intensity of 60 μmol photons m^{-2} s^{-1} and an 18 h/6 h (light/dark) cycle photoperiod regime for 15 days. The major carotenoid of *Nannochloropsis oceanica* was confirmed as bioactive carotenoid zeaxanthin. The zeaxanthin concentration among these six solvent systems ranged from 14.7 mg/g dry cell biomass in ethyl acetate/hexane (2:3) to 30.2 mg/g dry cell biomass in chloroform/methanol (1:2). Maximum zeaxanthin recovery was also found in chloroform/methanol (1:2) (Mitra and Mishra 2019). Zeaxanthin was recovered from *Nannochloropsis oculata* by using conventional solvent extraction and column elution chromatography coupled with modified supercritical fluid extraction. Besides, the extraction yield of zeaxanthin was measured. *Nannochloropsis oculata* was grown in seawater in five-ton polypropylene tanks, and 500 g of dry substance algae were collected. The zeaxanthin concentration in the elution fraction was 400 mg/g, which was more than 20 times higher than 13.17 mg/g of zeaxanthin in the supercritical fluids extraction and 20.1 mg/g of zeaxanthin in Soxhlet dichloromethane extract (Liau et al. 2011).

Neospongiococcum excentricum

Zeaxanthin-producing microalgae identified from a group that includes a microorganism that contains all of the key features of *Neospongiococcum excentricum* HZ 1236/274, ATCC No. 74108; HZ 1236/437, ATCC No. 74109; HZ 1236/538, ATCC No. 74107. The most favorable fermentation conditions were microalgae cultivation at 36 °C, pH 6.0, less than 0.04 atm of carbon dioxide partial pressure, and more than about 10% of dissolved oxygen content. Under this optimized condition, *Neospongiococcum excentricum* ATCC No. 74107 (HZ1236/538) and ATCC No. 74109 (HZ1236/437) can be capable of producing zeaxanthin at the level of 3 mg/g dry cell weight, which is approximately 40% of the total carotenoids produced by these two strains (Orndorff et al. 1994). In addition, genome enhancement heavily depends on more traditional procedures such as random mutagenesis and screening because of the absence of engineering simulation and modeling. A microalgae *Neospongiococcum excentricum* ATCC 40335 and its mutants were used for zeaxanthin production. This wild-type photosynthetic microorganism was mutated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine under mutagenesis. The selection and screening strategy took the use a relationship between chlorophyll concentration and carotenoid level of this microorganism. Carotenoids and chlorophyll are known to be related since both are involved in photosynthesis. The culture condition was preferably 15 to 20 g/L glucose and pH 5.7 to 6.2 at 35 °C. Glucose is usually the desirable carbon source in the medium during fermentation. The culture medium also comprises nitrogen, carbon, iron, phosphate, magnesium, sulfate, and other trace metals. Under the optimized culture conditions, the xanthophyll content was at least 0.65% of dry cell weight, the lutein content was 0.445% of dry cell weight, and the zeaxanthin content was 0.023% of dry cell weight (Liao et al. 1995).

Other Microalgae

Three novel stable mutants (*zea1*, *zea2*, and *zea3*) of the unicellular halotolerant green alga *Dunaliella salina* were selected, isolated, and characterized. These three mutants can produce high content of zeaxanthin but lacking neoxanthin, violaxanthin, and antheraxanthin because mutants are impaired in the zeaxanthin epoxidation. Biochemical examinations revealed that zeaxanthin substitutes for neoxanthin, violaxanthin, and antheraxanthin consistently and quantitatively in the *zea1* strain. There are a variety of bioactive compounds found in blue-green algae like phycocyanins (Ashaolu et al. 2021). The zeaxanthin production of *zea1* mutant was 5.90 mg/g dry cell weight which was significantly higher than 0.23 mg/g dry cell weight in the wild-type strain under low-light (100 μmol photons m^{-2} s^{-1}) physiological culture conditions in 1-L hypersaline medium with 25 mM $NaHCO_3$ at 28 °C (Jin et al. 2003). The bioactive xanthophyll zeaxanthin was isolated and purified from the cyanobacterium *Microcystis aeruginosa by* high-speed counter-current chromatography. After saponifying the microalgal culture, raw zeaxanthin was extracted by organic solvents. A two-phase solvent system composed of *n*-hexane-ethyl acetate-ethanol-water with a volume ratio of 8:2:7:3, v/v/v/v was selected and injected into high-speed counter-current chromatography. From 150 mg of the crude zeaxanthin in a 10 mL sample solution dissolved in this solvent

combination with a one-step separation, the zeaxanthin purification can achieve at 96.2%, and zeaxanthin recovery was 91.4% (Chen et al. 2005). After screening 13 species of microalgae, *Monoraphidium braunii* exhibited the highest yield of zeaxanthin and lutein. The optimized culture conditions of this microalgae were cultivated in a modified Arnon medium with 4 mM K_2HPO_4 and 20 mM $NaNO_3$ at 28 °C in batch culture with 1-L Roux flasks under mercury halide lamps illuminated laterally at 460 μmol photons m^{-2} s^{-1} for 13 to 14 days. Maximal specific growth rate and zeaxanthin accumulation were attained at values of zeaxanthin of 0.09 per hour and 8.4 mg/L. Meanwhile, lutein production also reached the highest yield at 2.4 mg/L (Cordero et al. 2011).

The stimulation of light intensity for zeaxanthin production in the cyanobacterium *Synechococcus* PCC7942 was confirmed. Light-dependent phytoene formation was found by inhibiting the process at the stage of phytoene synthesis. The first reaction to high-light in *Synechococcus* is associated with an elevation of the β-carotene hydroxylase transcript, which is required for rapid conversion of β-carotene to zeaxanthin. Later, the biosynthetic ability in the carotenoid pathway is enhanced through coordinating transcriptional up-regulation of the first three enzymes, phytoene synthase, phytoene, and β-carotene desaturase, to facilitate the carotenogenic pathway and through to zeaxanthin as the end product carotenoid. Cultures were pre-grown under a light intensity of 15 μmol m^{-2} s^{-1} and then cultivated for 5 h under the light of 50 or 400 μmol m^{-2} s^{-1}. After 5-hour light treatment under 400 μmol m^{-2} s^{-1} light intensity, the zeaxanthin production achieved a maximum of 1.7 μg/g cell dry weight, which shows 58% of total carotenoids in *Synechococcus* PCC7942 (Schäfer et al. 2006). The influence of light and temperature was investigated on a red microalga *Porphyridium purpureum* under nitrogen-replete modes during batch cultivation for carotenoid production and other bioactive substances. This red microalga was batch-cultivated in 150 mL nitrogen-replete F/2-RSE medium containing a high initial 1 g/L $NaNO_3$ in 250 mL glass Erlenmeyer flasks for 10 days. Nitrate deficiency reduces growth performance as well as other bioactive compound levels, which may be associated with membrane disintegration. Nonetheless, it increases carbohydrate formation. With combined increasing light from low light (30 μmol m^{-2} s^{-1}) to high light (200 μmol m^{-2} s^{-1}) and temperature from 10 °C to 30 °C, zeaxanthin content and productivity ranged from 411 to 60 μg/L and 269 to 63 μg/g dry cell weight. The highest productivity of zeaxanthin was observed within the optimum growth temperature range of 20–25 °C and under low light intensity (30 μmol m^{-2} s^{-1}) although the microalgae growth rate was decreased at this light intensity (Guihéneuf and Stengel 2015). The influence of temperature and light was also determined on the growth rate, biomass generation, and polysaccharides synthesis of a thermotolerant cyanobacterium *Cyanobacterium aponinum*. At temperatures between 28 °C and 45 °C, eight different light intensities ranging from 15 to 650 μmol photons m^{-2} s^{-1} were examined. When light intensity increased, the specific growth rate and biomass productivity of *Cyanobacterium aponinum* increased significantly. The maximum biomass yield (approximately 92 mg/L per day) was achieved at 40 °C, whereas the highest polysaccharides synthesis rate (approximately 10 mg/L

per day) was observed at 35 °C. The high levels of illumination can improve the zeaxanthin yield at maximum final content of 3.17 mg/g dry cell weight under high light intensity with 650 μmol photons m^{-2} s^{-1} at 35 °C for 3-day cultivation (Gris et al. 2017).

In Table 2, zeaxanthin production in zeaxanthin-accumulating native microalgae was described.

Zeaxanthin Production by Metabolic Engineered Non-native Microorganisms

Metabolic engineering, which involves using recombinant DNA methods to modify metabolic pathways in living cells to synthesize desirable bioactive compounds with higher yields and productivity, facilitates the generation of huge amounts of valuable carotenoids in vivo. *Escherichia coli* uses the MEP pathway for its growth functions. It is described that the creation of genetically engineered *Escherichia coli* by disrupting a gene encoding a specific enzyme involved in the MEP pathway and introducing the genes encoding the key enzymes in the mevalonate pathway can result in high production of carotenoids like lycopene, β-carotene, as well as zeaxanthin (Eguchi et al. 2005). Many foodborne outbreaks involving leafy greens like spinach and lettuce have been linked to Shiga toxin-producing *Escherichia coli* (Lee et al. 2016). Some other microorganisms can produce zeaxanthin, such as *Erwinia herbicola*, *Synechocystis* sp., *Xanthophyllomyces dendrorhous*, and *Pseudomonas putida*, and microalgae *Haematococcus pluvialis*, *Chlorella zofingiensis*, and *Chlorella protothecoides* are also considered alternative carotenoid producers (Sajilata et al. 2008; Erdal and Ökmen 2013; Singh et al. 2015). Lack of sufficient knowledge on the production of pigments by genetically engineered bacteria is one of the main challenges linked with macro-scale production and optimization of bacterial pigments. Using genetic and metabolic engineering for specific pigment production by bacteria needs full understanding of genome biology (Srivastava et al. 2022). As more bacteria are examined, the structure of zeaxanthin biosynthetic pathways would become more characterized, allowing metabolic engineering of microbes to create high-valued and yielding carotenoids (Dufossé 2016). High production costs, microbial contamination, and a lack of information on metabolic pathways make further breakthroughs in pigment synthesis difficult. To stabilize the extracellular matrix, three-dimensional gel complexes utilizing oleogelators have been established (Demirci et al. 2020). It is expected that developing technologies in genetic engineering will make it possible to reach more profitable and more efficient methods in bacterial pigment production.

Advancements in recombinant technologies provided xanthophyll production by naturally present microbial sources. Microbial carotenoids have become competitive with chemically manufactured products due to the advantages of genetic engineering (Sarnaik et al. 2018). The metabolic capability of the carotenoid pathway and its effectiveness in converting intermediates, including the direct precursor into the desired end product zeaxanthin, determine the yield of a zeaxanthin-producing

Table 2 Zeaxanthin production in zeaxanthin-accumulating native microalgae

Microalgae	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Chlorella saccharophila	11.32 mg/g	Microalgae was cultured in 100 mL vessels at 20 °C, pH 6.5, with shaking at 150 rpm for 192 h. The optimized production medium was 50% v/v artificial seawater, 10 g/L g yeast extract, 1 g/L peptone and 10 g/L glycerol or glucose	Singh et al. 2013
Chlorella saccharophila	11.2 mg/g	Optimal conditions of zeaxanthin extraction were pulse length of 19.7 s, power 27.82% out of total power of 500 W, time of 13.48 min, as well as a ratio of solvent and dry cell weight of 67.38 μL/mg	Singh et al. 2015
Chlorella pyrenoidosa	2.17 mg/g	Microalgae-derived tablets are available commercially from Vedan Enterprise Corp., Taiwan. The gradient elution was designed by utilizing two eluents of methanol-acetonitrile–water (84:14:2, v/v/v) and methylene chloride (100%)	Inbaraj et al. 2006
Chlorella zofingiensis CZ-bkt1 mutant	7.00 mg/g; 36.79 mg/L	Strains were first grown in 10 mL Kuhl broth medium at 25 °C, shaking at 150 rpm, light intensity with 30 μmol photons m^{-2} s^{-1} for 4 days and then inoculated at 10% (v/v) into 50 or 100 mL fresh Kuhl medium for the next 4 days	Huang et al. 2018
Cyanobacterium aponinum	3.17 mg/g	The microalgae were grown in BG-11 medium buffered at pH 8 with 1 M HEPES in 250-mL Erlenmeyer flasks at 28 to 45 °C, under light intensities ranging from 15 to 650 μmol photons m^{-2} s^{-1} for 3 days	Gris et al. 2017
Dunaliella salina three mutants	5.90 mg/g	Cells were cultivated photoautotrophically in 1-L flat bottles in the hypersaline medium containing 25 mM $NaHCO_3$ as the inorganic carbon source at 28 °C under continuous low- and high-light	Jin et al. 2003

(continued)

Table 2 (continued)

Microalgae	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
		intensities of 100 and 2000 μmol photons m^{-2} s^{-1}	
Monoraphidium braunii	8.4 mg/L	The microalgae were grown in the modified Arnon medium with 4 mM K_2HPO_4 and 20 mM $NaNO_3$ at 28 °C in batch culture with 1-L Roux flask under mercury halide lamps illuminated laterally 460 μmol photons m^{-2} s^{-1} for 13 to 14 days	Cordero et al. 2011
Microcystis aeruginosa	15 mg/mL, 96.2% purity	Microalgae was cultured at 28 °C, pH 7.0 with shaking at 150 rpm for 12 days in the media supplemented with 40 g/L glucose and 10 g/L $NaNO_3$. The optimal combined organic solvents were *n*-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v/v/v) in high-speed counter-current chromatography	Chen et al. 2005
Nannochloropsis oceanica strain CCNM 1081	30.2 mg/g	The culture was grown in a 14-L photobioreactor containing the modified Conway medium under a light intensity of 60 μmol photons m^{-2} s^{-1} and an 18 h: 6 h (light: dark) cycle photoperiod regime for 15 days	Mitra and Mishra 2019
Nannochloropsis oculata	2.66 mg/g	Microalgae were grown in seawater in 5-ton polypropylene tanks, and 500 g of dry substance algae were collected	Liau et al. 2011
Neospongiococcum excentricum ATCC 74107, and ATCC 74109	3.0 mg/g	The strains were cultivated at 36 ° C, pH 6.0, less than 0.04 atm of carbon dioxide partial pressure, and more than about 10% of dissolved oxygen content	Orndorff et al. 1994
Nospongiococcum excentricum ATCC 40335	0.023% of total dry cell weight	The culture conditions were preferably 15 to 20 g/L glucose and pH 5.7 to 6.2 at 35 °C. The culture medium also comprises nitrogen, carbon, iron, phosphate, magnesium, sulfate, and other trace metals	Liao et al. 1995

(continued)

Table 2 (continued)

Microalgae	Highest zeaxanthin yield (mg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Porphyridium purpureum	0.269 mg/g dry cell weight; 0.411 mg/L	This red microalga was batch-cultivated in 150 mL nitrogen-replete F/2-RSE medium containing a high initial 1 g/L $NaNO_3$ in 250 mL glass Erlenmeyer flasks, 10–30 °C, 30, 100, and 200 μmol photons m^{-2} s^{-1} for 10 days	Guihéneuf and Stengel 2015
Synechococcus PCC7942	0.0017 mg/g	The strain was cultivated in the BG11 medium containing 30 μg/mL methionine at 37 °C. Cultures were pre-grown under light intensity 15 μmol m^{-2} s^{-1} for 2 to 3 days and then cultivated for 1 or 5 h under the light of 50 or 400 μmol m^{-2} s^{-1}	Schäfer et al. 2006
Synechococcus elongatus PCC 7942	9.02 mg/g (autotrophy); 1.8 mg/L/day (mixotrophy)	The cultures were cultivated in BG-11 medium in the 250 mL Erlenmeyer flasks with continuous aeration shaking at 120 rpm under natural diurnal light (maximum 1200 μmol m^{-2} s^{-1}) at 28 °C	Sarnaik et al. 2018

organism (Breitenbach et al. 2019). The use of wild-type strains for carotenoid production is limited for commercial needs because of producing relatively low concentrations of the necessary carotenoid compounds, insufficient supplies, and related high costs (Ram et al., 2020a). Although zeaxanthin production is increased with media and genetic modifications, it is still difficult to meet the industrial need for bacterial zeaxanthin production. Further research must be done to boost zeaxanthin efficiency in bacteria (Shen et al. 2016). Genetic engineering is more precise, targeted, efficient, and safe than other traditional approaches (Srivastava et al. 2022). Major carotenoids like β-carotene, canthaxanthin, zeaxanthin, and astaxanthin are primarily generated from petroleum by chemical synthesis. It is possible to increase B-carotene levels by mediating one to two transgenes (Ha et al. 2019). Metabolic engineering of clinically significant molecules represents a tremendous possibility for biotechnology to benefit public health (Ye and Bhatia 2012). There are two critical issues for zeaxanthin production via metabolic engineering. One of them is integrating plant-derived zeaxanthin biosynthesis genes into microorganisms and characterizing new zeaxanthin biosynthesis genes. Second, a comprehensive investigation of the zeaxanthin synthase routes is required (Zhang et al. 2018). β-carotene is the pioneer in producing zeaxanthin as well as astaxanthin. Depending on the end product, the pathways for zeaxanthin formation from β-carotene can be modified by

inactivating astaxanthin synthase genes. On the other hand, the quantity of β-carotene hydroxylase genes integrated into microorganisms is important (Breitenbach et al. 2019). It is necessary to maintain strain improvement and find better producers for maximum industrial production to increase yield. In Table 3, the list of metabolic engineering strategies for zeaxanthin production is presented in bacteria, yeasts, and molds.

Bacteria

Escherichia coli

To improve the availability of precursor prenyl pyrophosphates for carotenoid synthesis, genetic engineering of early non-mevalonate terpenoid pathway was conducted in *Escherichia coli*. *Escherichia coli* JM101 transformed with the different plasmids was cultivated in Luria–Bertani medium in the presence of 0.1 mM Isopropyl ß-D-1-thiogalactopyranoside for 2 days with the appropriate antibiotics such as chloramphenicol, ampicillin, tetracycline, or kanamycin. Carotenogenesis was increased by 3.5-fold after transformation with the overexpression of gene *idi* (encoding isopentenyl pyrophosphate synthase) from *Xanthophyllomyces dendrorhous*, gene *dxs* (encoding 1-deoxy-D-xylulose 5-phosphate synthase), and gene *dxr* (encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase). The synthesis of ß-carotene and zeaxanthin was increased by 1.6 mg/g dry cell weight when *idi* was co-transformed with either *dxs* or *dxr* from *Escherichia coli* while the recombinant *Escherichia coli* contained a plasmid combination pACCAR25△crtX, pUC BM20dxs C, and pRK-idi (Albrecht et al. 1999). *Escherichia coli-produced* Shiga toxins in various water environments have different abilities for attachment and biofilm formation (Lee et al. 2015). Effective antibacterial nanoparticles against foodborne pathogens have been widely proven using surface-functionalized antimicrobials (Bahrami et al. 2022). The effect of overexpressing *Escherichia coli* TOP10 F′ D-1-deoxyxylulose 5-phosphate synthase (DXS) in conjunction with carotenoid enzymes expressed by *Erwinia uredovora* was investigated to evaluate the flux interactions between the DXS-mediated isoprenoid pathway and a carotenoid biosynthetic source in *Escherichia coli*. In the existence of the *Erwinia* carotenoid gene cluster, overexpression of *Escherichia coli* DXS produced remarkably rich-colored colonies. Lycopene reached a concentration of 1.33 mg/g dry cell weight, whereas zeaxanthin reached a concentration of 0.59 mg/g dry cell weight. During 11-day cultivation, zeaxanthin-producing colonies proliferated approximately twice as rapidly as lycopene-producing colonies (Matthews and Wurtzel 2000). Tunable intergenic regions (TIGRs) were utilized to regulate the expression of all genes in the MEV pathway from *Saccharomyces cerevisiae*. Then an isopentenyl diphosphate/farnesyl diphosphate-responsive promoter P*gadE* was used to continuously modulate the TIGR-mediated MEV pathway to inhibit the generation of hazardous metabolites by overexpression of the *Escherichia coli* genes *idi* and *ispA* (encoding geranyl diphosphate/farnesyl diphosphate synthase) to increase the production of farnesyl diphosphate. The zeaxanthin concentration and yield in shaker flask cultures

Table 3 Zeaxanthin production in metabolic engineered non-native microorganisms

Microorganisms	Metabolic engineering strategy	Highest zeaxanthin yield (mg or μg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Escherichia coli JM101	Co-transformation and co-overexpression of *idi* from *X. dendrorhous* and either *dxs* or *dxr* from *Escherichia coli* in the recombinant *Escherichia coli* containing a plasmid combination pACCAR25∆crtX + pUC BM20dxs C + pRK-idi	1.57 mg/g	The strain transformed with the different plasmids was cultivated in Luria–Bertani medium in the presence of 0.1 mM Isopropyl ß-D-1-thiogalactopyranoside for 2 days with the appropriate antibiotics such as chloramphenicol, ampicillin, tetracycline, or kanamycin	Albrecht et al. 1999
Escherichia coli TOP10 F′	Co-overexpression of *E. coli dxs* with *Erwinia uredovora* gene clusters encoding carotenoid biosynthetic enzymes	0.59 mg/g	Cultures were grown in 50 ml Luria–Bertani broth with antibiotics in 125-ml Erlenmeyer flasks at 37 °C with shaking at 240 rpm for 30 h, then held for another additional 30 h at room temperature. For cultivation on Luria–Bertani solid medium, cells were grown at 37 °C for 8 h and then at room temperature for 2 to 11 days	Matthews and Wurtzel 2000
Escherichia coli ZEAX	Tunable intergenic regions were utilized for overexpression of genes *HMGS* and *tHMGR* from *Saccharomyces cerevisiae* and genes *idi* and *ispA* from *Escherichia coli* in zeaxanthin-producing *Escherichia coli* ZEAX	23.16 mg/g or 722.46 mg/L	Fed-batch fermentation was carried out in a 5-L fermenter containing 3 L of SBMSN medium and 500 g/L glucose at 30 °C, pH 7.0, an airflow of 3 L/min and agitation rate of 400 rpm for 64 h	Shen et al. 2016

(continued)

Table 3 (continued)

Microorganisms	Metabolic engineering strategy	Highest zeaxanthin yield (mg or μg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Mucor circinelloides mutant (MS12)	*Mucor circinelloides* expression signals were used to modulate the gene *crtW* and *crtZ* of astaxanthin biosynthesis found in heterologous *Agrobacterium aurantiacum*	0.01 mg/g	*Mucor* strains were cultivated in the minimal medium containing 0.05% yeast nitrogen base without amino acids, 0.15% $(NH_4)_2SO_4$, 5% glucose, 0.15% glutamic acid, and supplemented with 200 μg/mL leucine or 400 μg/mL uracil for 4 days under continuous light	Papp et al. 2006
Mucor circinelloides mutant (MS12)	Heterologously overexpressing the *Paracoccus* sp. N81106 genes *crtZ* and *crtW* (encoding β- carotene ketolase) from *Xanthophyllomyces dendrorhous*	0.03 mg/g	*Mucor* strains were cultivated in the minimal medium containing 0.05% yeast nitrogen base without amino acids, 0.15% $(NH_4)_2SO_4$, 1% glucose, 0.15% sodium glutamate, 2% agar, and supplemented with 500 μg/mL leucine and uracil for 4 days at 25 °C under continuous light	Csernetics et al. 2014
Mucor circinelloides	The expression of cDNA from the *Xanthophyllomyces dendrorhous* Y2476 *crtS* gene in *Mucor circinelloide* was used to construct zeaxanthin overproducing transformants	No data shown	The strain was cultured in 25 mL fermentation medium within a 500-mL flask for 120 to 168 h at 17–20 °C and 250 rpm under illumination	Rodríguez-Sáiz et al. 2012

Pseudomonas putida D7L3	An L-rhamnose-inducible promoter was used to construct a novel plasmid containing three genes *idi*, *ispA*, *dxs* from *Escherichia coli* JM109 and five carotenogenic genes *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* from *Pantoea ananatis* in *Pseudomonas putida* D7L3 mutant	7 mg/g or 51.3 mg/L; 239 mg/L (The addition of lecithin)	Strains were grown in 50–200 ml Luria–Bertani medium or terrific broth or MEK medium containing 30 μg/ml kanamycin in baffled flasks shaken at 160 rpm at 25 °C for 48–72 h	Beuttler et al. 2011
Saccharomyces cerevisiae YZE-19	Overexpression of five genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Pantoea ananatis was carried out in yeast strains* with estradiol induction	75 μg/g, or 1.5 μg/ml, or 15 ng/OD 600 cells	*Yeast strains were grown in* either synthetic dropout medium or YPA medium containing 1% of yeast extract, 2% of peptone and 0.01% of adenine hemisulphate supplemented with 2% of glucose at 30 °C with shaking at 250 rpm for a 72-h incubation	Liang et al. 2013
Synechococcus elongatus PCC 7942	β-carotene oxygenase gene *CrtR* was cloned from *Synechococcus elongatus* PCC 7002 via homologous recombination. An operon construct was developed in a heterologous gene *GalP* (Hexose-H+ symporter) from *Escherichia coli* MG1655	9.02 mg/g (autotrophy) 1.8 mg/L/day (mixotrophy)	The cultures were cultivated in BG-11 medium in the 250 mL Erlenmeyer flasks with continuous aeration shaking at 120 rpm under natural diurnal light (maximum 1200 μmol m^{-2} s^{-1}) at 28 °C	Sarnaik et al. 2018
Synechocystis sp. strain PCC 6803	The *psbAII* locus was utilized as an integrating substrate by regulating the *psbAII* promoter to overexpress genes associated with carotenoid biosynthesis in this strain. The gene *ipi*, *crtR*, *crtP*, and *crtB* were inserted into *Synechocystis* replacing the gene *psbAII* coding sequences	0.98 μg/mL per OD730 unit	The strain was cultivated at 30 °C in the modified BG-11 medium buffered with 10 mM TES-NaOH (pH 8.0), 4.5 mM ammonia, and 50 μg/mL kanamycin at a light flux density of 50 μmol of photons · m^{-2} · s^{-1} for 4 days	Lagarde et al. 2000

(continued)

Table 3 (continued)

Microorganisms	Metabolic engineering strategy	Highest zeaxanthin yield (mg or μg/g dry cell weight, or mg/L sample)	Cultivation conditions	Reference
Xanthophyllomyces dendrorhous mutant AXG13	Integrating and overexpressing β-carotene hydroxylase genes by knock-out / knock-in pathway engineering	5.23 mg/g	Transformants were cultivated, at 20 °C under white light illumination in 500-mL baffled Erlenmeyer flasks at shaking 180 rpm with 50 mL YMD medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose	Breitenbach et al. 2019
Xanthophyllomyces dendrorhous mutant PR1–104	Overexpression of *HMGR*, *crtE*, and *crtYB* and then introduction of codon-optimized gene *crtZ* from *Brevundimonas* sp. SD212 into *Xanthophyllomyces dendrorhous* mutant PR1–104 with deactivated astaxanthin synthase	517 μg/g	Transformants were cultivated in shaking culture at 180 rpm under white light illumination at 20 °C in 500-ml baffled Erlenmeyer flasks with 50 mL yeast mold medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose for 7 days. 2-L stirred fermenter culture was run at 21 °C, pH 5.5 with an air flow rate of 1 ml/min, in the presence of a 25% glucose solution with 6.5 ml per hour, above 40% dissolved oxygen, stirring speed from 300 to 600 rpm for 34 h	Pollmann et al. 2017

after introducing the continuously regulated TIGR-mediated MEV pathway into *Escherichia coli* were 2.1 times and 2.0 times higher in comparison with those of the strain carrying the stably controlled non-TIGR-mediated MEV pathway. In a 5-L fed-batch fermentation, the content and concentration of zeaxanthin in *Escherichia coli* ZEAX (pZSP$_{gadE}$-MevT$_{TIGR}$-MevB$_{TIGR}$IS-2) achieved 722.46 mg/L and 23.16 mg/g dry cell weight, respectively, in 56 h and the zeaxanthin productivity was 12.9 mg/L per hour (Shen et al. 2016).

Synechocystis sp.

Overexpression of the β-carotene hydroxylase gene was attempted to increase zeaxanthin production from *Synechocystis* sp., resulting in 2.5 times the efficiency. Plasmid administration of the β-carotene hydroxylase genes *crtR* and *crtZ* to some wild strains resulted in a significant increase in zeaxanthin production (Singh et al. 2015). The *psbA* genes encode the D1 protein of photosystem II, which is required for oxygenic photosynthesis. The locus of photosystem II protein D1 (psbAII) was utilized as an integrating substrate by regulating the *psbAII* promoter to overexpress genes associated with carotenoid biosynthesis in *Synechocystis* sp. strain PCC 6803. The gene coding for the yeast isopentenyl diphosphate isomerase (ipi), β-carotene hydroxylase (crtR), phytoene desaturase (crtP), and phytoene desaturase (crtB) was inserted into *Synechocystis* replacing the gene *psbAII* coding sequences. The myxoxanthophyll and zeaxanthin concentration of the mutant strain increased by 50% by overexpression of *crtP* and *crtB* while the β-carotene and echinenone amounts remained unaltered. In the *crtR*2 strain, zeaxanthin content can achieve up to 0.98 μg/mL/OD730 unit. In comparison to the wild-type strain, *crtR* overexpression resulted in a 2.5-time boost in zeaxanthin formation in the equivalent overexpressing mutant. The level of β-carotene and echinenone is decreased by a factor of two in this mutant strain, and zeaxanthin represented the predominant pigment, which was more than 50% of the total carotenoid content (Lagarde et al. 2000).

Other Bacterial Non-native Host

Zeaxanthin production was examined in the *Pseudomonas putida* KT2440 strain. A mutant *Pseudomonas putida* D7L3 was screened from this strain KT2440. An L-rhamnose-inducible promoter was used to develop a novel plasmid containing three genes *idi*, *ispA*, and *dxs* of *Escherichia coli* JM109 and five carotenogenic genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* of *Pantoea ananatis* in this lycopene-tolerant mutant *Pseudomonas putida* D7L3. The results presented that a 7 mg/g cell dry weight of zeaxanthin was obtained after optimizing growth conditions and product processing, equating to a product content of 51 mg/L zeaxanthin. The addition of lecithin to *Pseudomonas putida* during cell culture boosted volumetric yield to 239 mg/L zeaxanthin by a ratio of 4.7 compared to 51 mg/L of zeaxanthin production under lecithin-free cultivation (Beuttler et al. 2011).

The cyanobacterium *Synechococcus elongatus* PCC 7942 can synthesize zeaxanthin as one of the primary cellular carotenoids but lacks pathway genes of lutein biosynthesis. By cloning the β-carotene oxygenase gene *CrtR* from *Synechococcus*

elongatus PCC 7002 via homologous recombination, strain PCC 7942 (*Synechococcus* 79R48) was modified genetically to increase carotene flow towards zeaxanthin production. This approach substantially increased the zeaxanthin production at a milligram per gram dry cell weight in the transformants. Furthermore, an operon construct was developed in which a heterologous gene *GalP* (Hexose-H+ symporter) from *Escherichia coli* MG1655 was cloned downstream of *CrtR* in *Synechococcus* 79RG48 to increase zeaxanthin titer at a milligram per liter, allowing intrinsically compel photoautotrophic WT PCC 7942 cells to absorb extracellular glucose in transformants to enhance biomass productivity. When the culture grew in the autotrophic mode and mixotrophy cultivation, zeaxanthin production was 9.02 mg/g dry cell weight and 8.09 mg/g dry cell weight, respectively, which was two times the enhancement over the wild-type strain. The productivities of the transformants were 1.18 mg/L per day in an autotrophic mode and 1.8 mg/L per day in the mixotrophic mode compared to 0.58 mg/L per day in the zeaxanthin production of wild-type parental cells. The results demonstrated that the recombinant transformants caused an increase in zeaxanthin synthesis without lutein production under the cyanobacterial model (Sarnaik et al. 2018).

Fungi

Mucor circinelloides

Studies have shown that after integrating the *ctrS* gene (encoding astaxanthin synthetase) into the natural zeaxanthin-producing mold *Mucor circinelloides* produced zeaxanthin as the dominant carotenoid. The expression of cDNA from the red yeast *Xanthophyllomyces dendrorhous* crtS gene in the mold *Mucor circinelloide* was used to construct zeaxanthin overproducing transformants (Rodríguez-Sáiz et al. 2012). In addition, overexpression of some gene clusters (*crtE*, *crtY*, *crtI*, *crtB*, and *crtZ*) stimulated the carbon flow, resulting in the formation of isoprenoid precursors from glucose, thus increasing the production of zeaxanthin. It has been observed that zeaxanthin accumulation can reach the highest amount in *E. coli* by rearranging these *crt* genes (Zhang et al. 2018). *Mucor circinelloides* expression signals were used to modulate the gene *crtW* (encoding β-carotene ketolase) and *crtZ* (encoding β-carotene hydroxylase) of astaxanthin biosynthesis found in *Agrobacterium aurantiacum*. Polyethylene-glycol-induced genetic transformation in a specific *Mucor circinelloides* strain was accomplished using expression vectors encoding the bacterial genes. The predominant carotenoid accumulated by most *Mucor* species is β-carotene. Transformants with different levels of carotene production were identified and studied. A *leuA* $^{-}$ *pyrG* $^{-}$ mutant MS12 derived from the wild-type *Mucor circinelloides* strain CBS277.49 was used in the transformation, and *Escherichia coli* DH5α was used for cloning and plasmid amplification. *Mucor* strains were cultivated in a minimal medium containing 0.05% yeast nitrogen base without amino acids, 0.15% $(NH_4)_2SO_4$, 5% glucose, 0.15% glutamic acid, and supplemented with 200 μg/mL leucine or 400 μg/mL uracil for 4 days under continuous light. Among four mutant strains (MS12, MS12-Z, MS12-W, MS12-

ZW), MS12-Z transformants harboring pPT50 yielded substantially more zeaxanthin (10 μg/g dry cell weight) and β-cryptoxanthin (41 μg/g dry cell weight) than other MS12 mutants, indicating that the bacterial gene was expressed (Papp et al. 2006). An increase of carotenoid was accumulated by expressing the *crtZ* gene from *Paracoccus* sp. N81106 in canthaxanthin-producing mutants of *Mucor circinelloides*. The *crtZ* gene was placed on self-replicating plasmids and then inserted into the *Mucor* genome using the restriction enzyme-mediated integration approach. In the transformation studies, the *Mucor circinelloides* strains MS12 + pCA8lf/1 and MS12 + pPT51'R3/2, which have the $leuA^{+}$, $pyrG^{-}$, $crtW^{+}$ genotype and are derived from the $leuA^{-}$, $pyrG^{-}$ mutant MS12, were utilized. These strains are uracil auxotrophs and have the *crtW* gene from *Paracoccus* sp. N81106 (previously *Agrobacterium aurantiacum*). This *crtW* gene was inserted into the genome by using homologous double recombination (MS12 + pCA8lf/1) or restriction enzyme-mediated integration (MS12 + pPT51'R3/2) techniques. *Escherichia coli* TOP10F^{-} was used for cloning and plasmid amplification. *Mucor* strains were cultivated in a minimal medium containing 0.05% yeast nitrogen base without amino acids, 0.15% $(NH_4)_2SO_4$, 1% glucose, 0.15% sodium glutamate, 2% agar, and supplemented with 500 μg/mL leucine and uracil for 4 days at 25 °C under continuous light. In the MS12 + pPT51'R3/2 + pPT50 strain, the highest zeaxanthin production was 30 μg/g dry cell weight in the presence of glucose (Csernetics et al. 2014).

Saccharomyces cerevisiae

A multi-gene pathway in brewer's yeast *Saccharomyces cerevisiae* was designed by assembling and regulating with estradiol induction under enormously high sensitivity (10 nM). For the enhancement of zeaxanthin biosynthesis, overexpression of five genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Pantoea ananatis was carried out in the yeast Saccharomyces cerevisiae*. *Yeast strains were grown in* either synthetic dropout medium or YPA medium containing 1% of yeast extract, 2% of peptone, and 0.01% of adenine hemisulfate supplemented with 2% of glucose at 30 °C with shaking at 250 rpm for a 72-h incubation. The results demonstrated that in two different zeaxanthin-producing yeast strains, YZE-19 and YZE-PA, YZE-19 produced nearly twice as much zeaxanthin as YZE-PA, and 50 times more than the intrinsic pathway after induction for 72 h. Under monitoring at wavelength 450 nm on high-performance liquid chromatography, the zeaxanthin concentration was 1.5 μg/ml, which equates to 15 ng/OD 600 cells or 75 μg/g dry cell weight (Liang et al. 2013).

Xanthophyllomyces dendrorhous

Xanthophyllomyces dendrorhous is one of the first yeast species whose carotenoid biosynthesis pathways have been attempted to modify. Because fungi lack the hydroxylation ability to convert ß-carotene to zeaxanthin, random mutagenesis does not operate in yeasts as it does in algae. As a result, zeaxanthin synthesis in *Xanthophyllomyces dendrorhous* can only be achieved through genetic engineering. Changes in the carotenoid pathway can transform *Xanthophyllomyces dendrorhous*

into a high yield zeaxanthin-producing strain through genetic adjustments (Breitenbach et al. 2019). A carotenoid mutant of *Xanthophyllomyces dendrorhous* was constructed to accumulate β-carotene instead of astaxanthin for zeaxanthin biosynthesis. For enhancing total carotenoid production, overexpression of the genes *HMGR* (encoding HMG-CoA reductase), *crtE* (encoding geranylgeranyl pyrophosphate synthase), and *crtYB* (encoding lycopene cyclase), which encode for restricting these enzymes in the pathway inducing carotenoid biosynthesis. Bacterial genes *crtZ* were utilized to increase the cascade from β-carotene to zeaxanthin in this mutant. The number of *crtYB* and *crtZ* gene copies incorporated into the *Xanthophyllomyces dendrorhous* during transformation determines the amount of total carotenoid production and the zeaxanthin synthesis. Transformants were cultivated in shaking culture at 180 rpm under white light illumination at 20 °C in 500-ml baffled Erlenmeyer flasks with 5o mL yeast mold medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose for 7 days. 2-L stirred fermenter culture was run at 21 °C, pH 5.5 with an airflow rate of 1 ml/min, fed a 25% glucose solution with 6.5 ml per hour, above 40% dissolved oxygen, stirring speed from 300 to 600 rpm for 34 h. The results displayed that the highest zeaxanthin production was observed to be 0.517 mg/g dry cell weight in a new transformant PR1-104-(YB)2-HMGR-E-(Zo)1 with codon-optimized β-carotene hydroxylase in shaking flask cultures. This equates to a conversion rate of 80% for β-carotene, which provides 68% of total carotenoids (Pollmann et al. 2017).

The Improvement of Zeaxanthin Production in the Future Strategies

Commercial production of zeaxanthin from plants and microorganisms is sensitive to climatic and geographical variations. On the other side, synthetic zeaxanthin includes unwanted by-products and contributes to the presence of toxic wastes (Sarnaik et al. 2018). Traditional extraction has limitations, such as low extraction yields and a high concentration of contaminants. On the other hand, chemically manufactured zeaxanthin is not well-liked by consumers who prefer natural products (Joshi and Singhal 2016). Providing those needs, genetic engineering can meet consumer demand for natively produced compounds and industrial production of high-efficiency zeaxanthin. Commercial demand for zeaxanthin is growing due to its health benefits, and fermentative zeaxanthin production using microbial zeaxanthin producers is being investigated (Lee et al. 2018). Unlike other carotenoids like b-carotene, astaxanthin, and lycopene, which are produced by a wide range of microorganisms, zeaxanthin is produced by only a few bacteria, cyanobacteria, and microalgae (Vila et al. 2020). Because plant-derived carotenoids are expensive, scientists have become increasingly interested in bacterial carotenoid production in recent years because of its economic viability and budget (Ram et al. 2020a). In this chapter, zeaxanthin production was divided into two sections, the first of which described zeaxanthin-accumulating native bacteria and microalgae (Tables 1 and 2) and the second of which described metabolically engineered microbes (Table 3). The

optimized fermentation conditions for zeaxanthin production were also addressed. The highest zeaxanthin yield was achieved by utilizing metabolically engineered *Escherichia coli* demonstrating the possibility and importance of metabolic engineering for zeaxanthin synthesis. For zeaxanthin production via metabolic engineering, genes from higher plants in the zeaxanthin biosynthetic pathway are suggested to be overexpressed in microorganisms, and these novel genes for zeaxanthin production can be characterized. Nonetheless, no genes from higher plants were constructed in the development of zeaxanthin-accumulating microorganisms. Hence, bacteria like *Mesoflavibacter aestuarii* and *Algibacter* sp. are able to produce a significant amount of zeaxanthin, which offers promising gene resources for zeaxanthin production via metabolic engineering. Knocking out several genes coding for antioxidative ability and oxidative stress enzymes in the engineered microorganism host like *Escherichia coli* is a promising strategy to increase zeaxanthin production. These engineered host strains with low antioxidative ability would be induced to overexpress heterologous zeaxanthin biosynthetic pathway to enhance antioxidative ability within the metabolically engineered microorganism host because the growth rate of these engineered strains could be significantly reduced in comparison with the wild-type strains. Moreover, the native MEP pathway was shown to be inferior to the heterologous MVA pathway in the zeaxanthin synthesis of *Escherichia coli*. It is assumed that the pyruvate transit into the MEP pathway may occupy just a small portion of the overall pyruvate flux (Shen et al. 2016). Eliminating the cause of the restricted MEP and MVA pathway would tremendously enhance zeaxanthin synthesis in *Escherichia coli*. Therefore, MEP and MVA pathways should be studied further for zeaxanthin synthesis.

Conclusions and Perspectives

The potential of microbes to be used as a source of zeaxanthin was well-presented. Zeaxanthin can be produced by natural zeaxanthin-accumulating microorganisms and metabolic engineered microorganisms. In nature, zeaxanthin can be found in a variety of plants, including corn, persimmons, paprika, and saffron. Commercial application of zeaxanthin has been developed for decades due to its numerous human health benefits such as improvement of cognitive function, prevention of age-related macular degeneration, chemoprevention of cancer, hepatoprotection, and diabetes treatment. Among natural zeaxanthin-accumulating microorganisms, many species of bacteria were isolated and identified as zeaxanthin producers. The highest bacterial zeaxanthin production was found to be 12 mg/g dry cell weight in *Mesoflavibacter aestuarii* KYW614T (Lee et al. 2014), followed by 11.41 mg/g dry cell weight in *Algibacter* mutant strain AQP096-16 (Mohamed et al. 2013). In addition, another main source of zeaxanthin is microalgae, and their highest zeaxanthin yield was observed to be 30.2 mg/g dry cell weight in a single-celled microalga *Nannochloropsis oceanica* (Mitra and Mishra 2019). Besides, for zeaxanthin production via metabolic engineering in the non-native host, the highest zeaxanthin yield and productivity were 23.16 mg/g dry cell weight and 722.46 mg/L in

Escherichia coli ZEAX under several gene overexpression in tunable intergenic regions (Shen et al. 2016). A number of scientific studies have reported that zeaxanthin production can be significantly enhanced by the optimization of fermentation conditions in unmodified naturally-zeaxanthin-accumulating microorganisms. Furthermore, the ability of zeaxanthin productivity has also been developed and improved genetically in various microbial hosts under a combination strategy of optimized fermentation conditions and metabolic engineering modification in the genome or the biosynthetic pathway. The microbial production of zeaxanthin has been well-discussed based on various current research reports and scientific studies. Zeaxanthin synthesis from metabolically modified non-native microbes is emerging to compete with conventional production based on efficiency, cost, and productivity. Furthermore, microbial-derived zeaxanthin will soon replace synthetic and natural-produced zeaxanthin since these biosynthetic approaches are ecologically favorable and long-lasting in sustainability and ecosystems.

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