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Research review paper

Metabolic engineering for the microbial production of marine bioactive compounds

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ABSTRACT

Many marine bioactive compounds have medicinal and nutritional values. These bioactive compounds have been prepared using solvent-based extraction from marine bio-resources or chemical synthesis, which are costly, inefficient with low yields, and environmentally unfriendly. Recent advances in metabolic engineering allowed to some extent more efficient production of these compounds, showing promises to meet the increasing demand of marine natural bioactive compounds. In this paper, we review the strategies and statuses of metabolic engineering applied to microbial production of marine natural bioactive compounds including terpenoids and their derivatives, omega-3 polyunsaturated fatty acids, and marine natural drugs, and provide perspectives.

Contents

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1. Introduction

Marine bioactive compounds are playing increasingly important roles in the development of functional foods and drugs based on their antimicrobial, antitumor, antioxidant and other properties (Yamanaka et al., 2014; Lechner et al., 2011; Ambati et al., 2014). Marine bioactive compounds were discovered in many different marine organisms such as sharks (Xu et al., 2016b) and other fishes (Mühlroth et al., 2013), shrimps and crabs (Giuffrida et al., 2016), algae (Lemoine and Schoefs, 2010), sponges (Abdelmohsen et al., 2014), bacteria (Ukibe et al., 2009), cyanobacteria (Ongley et al., 2013), and others.

For some marine bioactive compounds traditionally isolated from the natural producers, attempts were made to produce them by metabolic engineered microorganisms. For example, attempts were made to produce squalene, which was traditionally obtained from the liver of deep-sea shark, by metabolically engineered microorganisms (Xu et al., 2016b). Rapid advances in systems biology and bioinformatics have allowed much more rapid discovery of novel biosynthetic pathways of marine bioactive compounds, facilitating the attempts to produce these bioactive compounds by metabolic engineering and synthetic biology (Lane and Moore, 2010).

Bio-based production of various chemicals, fuels and materials from renewable non-food biomass has become increasingly successful. A number of review papers on the strategies of metabolic engineering for the production of building block chemicals and polymers (Ahn et al., 2016; Lee et al., 2011a; Chung et al., 2015) and biofuels (Cho et al., 2015; Cheon et al., 2016; Choi et al., 2014) are available. Also, metabolic engineering of microorganisms for the production of secondary metabolites has been reviewed (Gustavsson and Lee, 2016; Kim et al., 2012b; Lee et al., 2011b; Hwang et al., 2014; Weber et al., 2015). Although there have been a few review papers on production of marine bioactive compounds by metabolic engineering, they are rather limited to several key products: astaxanthin (Ye et al., 2015), eicosapentaenoic acid (EPA; Hong et al., 2011), and omega-3 polyunsaturated fatty acids (omega-3 PUFAs; Gong et al., 2014). To the best of our knowledge, there is no comprehensive review covering production of diverse marine bioactive compounds by metabolic engineering and synthetic biology. In particular, there has been no review on metabolic engineering studies on the production of marine natural drugs. Although most of the discovered marine natural drugs still cannot be produced by metabolic engineering, there have been increasing cases reported on metabolic engineering of native organisms or heterologous hosts for the production of marine natural drugs such as enterocin (Bonet et al., 2014), lyngbyatoxin (Videau et al., 2016) and patellamides (Long et al., 2005). In this paper, we review the works on metabolic engineering and synthetic biology towards the production of marine bioactive compounds, which are for convenience classified as terpenoids and their derivatives, omega-3 PUFAs, and marine natural drugs (Fig. 1). We have to admit that most of the examples described below are product-specific case studies, yet without adopting systems-level metabolic engineering strategies or more recently available synthetic biology tools. Nonetheless, it is hoped that readers will be able to quickly update themselves through this review on what the state-of-the-art works are available for future studies.

2. Terpenoids and their derivatives

Terpenoids are natural compounds built up from isoprene subunits (Ajikumar et al., 2008). Squalene ($C_{30}H_{50}$) naturally accumulating in the liver of deep-sea shark is a marine terpenoid (Donald et al., 1997).

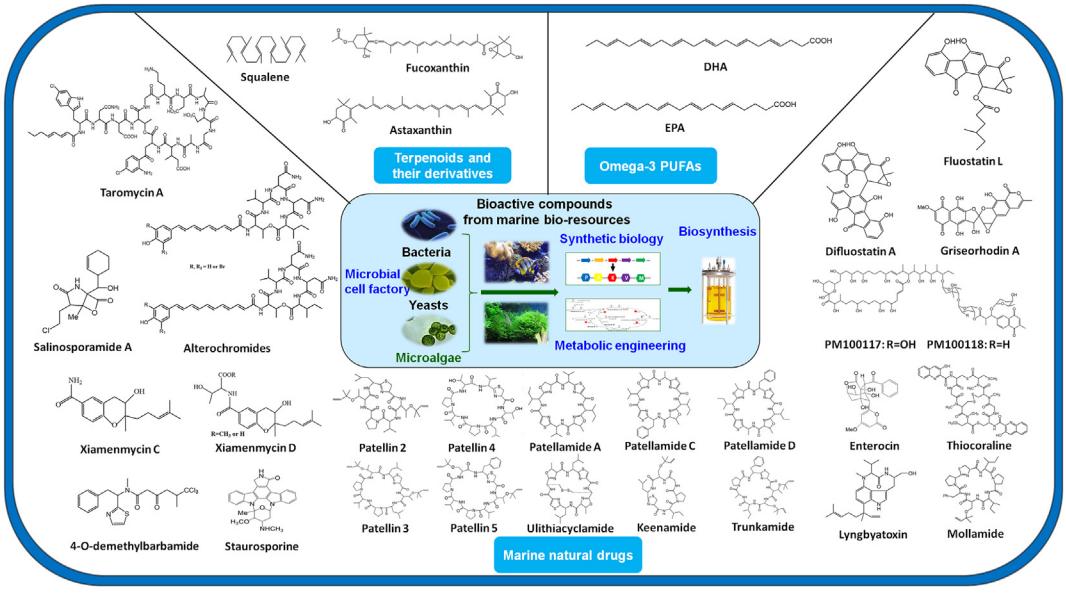

Fig. 1. Marine bioactive compounds produced by bacteria, yeasts and microalgae developed by metabolic engineering and synthetic biology. The bioactive compounds can be grouped into terpenoids and their derivatives, omega-3 PUFAs and marine natural drugs.

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Carotenoids, derivatives from terpenoids, are natural pigments of yellow-to-red colors found ubiquitously in many organisms. Astaxanthin ($C_{40}H_{52}O_4$) and fucoxanthin ($C_{42}H_{58}O_6$) are carotenoid derivatives mainly found in marine organisms (Giuffrida et al., 2016; Mikami and Hosokawa, 2013).

2.1. Squalene

Squalene, a linear triterpene natural product formed by the mevalonate (MVA) pathway or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, is traditionally isolated from the liver oils of deep-sea sharks (Xu et al., 2016b). Therefore, we classify squalene as a marine bioactive compound, despite of its wide distribution in microorganisms, plants and animals. Squalene has found many applications in medicine, food, and cosmetic industries, due to its antioxidant, antistatic and anti-carcinogenic properties (Ghimire et al., 2016).

2.1.1. Squalene biosynthetic pathway

Squalene is synthesized from the isopentenyl block units by the MVA or MEP pathway, the latter being also called the 1-deoxyxylulose-5-phosphate (DXP) pathway (Lange et al., 2000). As shown in Fig. 2A, the MVA pathway starts with the formation of acetoacetyl-CoA from two acetyl-CoA molecules catalyzed by acetoacetyl-CoA synthase or β-ketothiolase. Next, acetoacetyl-CoA and another acetyl-CoA are condensed to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. HMG-CoA is then reduced to MVA in the presence of NADPH by HMG-CoA reductase. Subsequently, two successive phosphorylation reactions catalyzed by MVA kinase and phospho-MVA kinase convert MVA to MVA 5-diphosphate, which is decarboxylated by MVA 5-diphosphate decarboxylase to form isopentenyl diphosphate (IPP). IPP is then converted to dimethylallyl diphosphate (DMAPP) by isopentanyl diphosphate isomerase (IDI). Farnesyl diphosphate synthase condenses IPP and DMAPP to form geranyl diphosphate, and also condenses another IPP with geranyl diphosphate to form farnesyl diphosphate (FPP). Finally, squalene synthase catalyzes the conversion of 2 molecules of FPP to squalene using NADPH as cofactor (Spanova and Daum, 2011).

The MEP pathway starts with the formation of DXP from glyceraldehyde-3-phosphate and pyruvate catalyzed by DXP synthase. DXP is then rearranged and reduced to MEP by DXP reductoismerase (DXR) or its

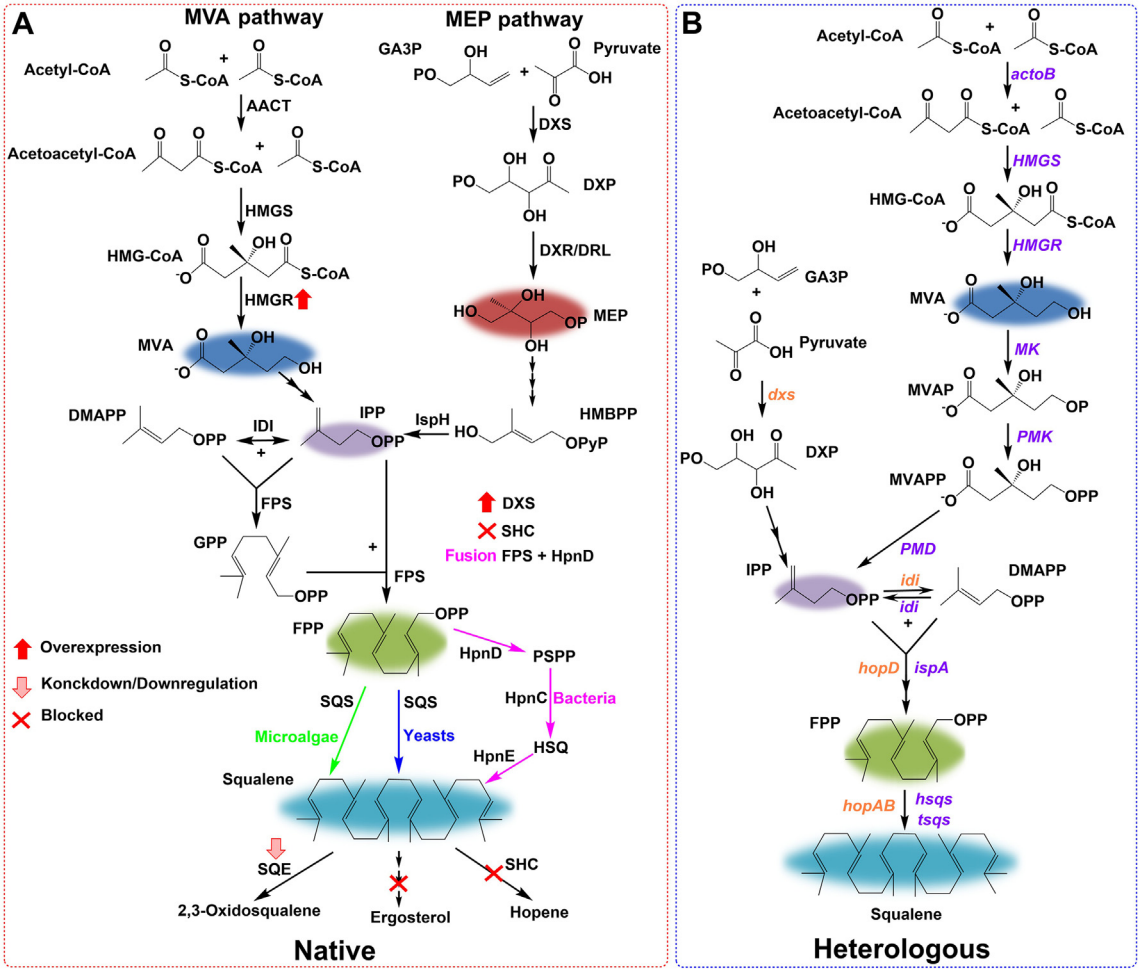

Fig. 2. Squalene biosynthetic pathway and the squalene production by metabolic engineering and synthetic biology. (A) Squalene biosynthetic pathway in microalgae (green), yeasts (blue) and bacteria (purple) and squalene production by metabolic engineering in native organisms. The engineering strategies and corresponding genes in different organisms are as follows: knockdown of *sqe* (Kajikawa et al., 2015) and deletion of *shc* (Englund et al., 2014) in microalgae; overexpression of *dxs*, deletion of *shc*, and fusion of *fps* and *hpnD* (Xu et al., 2016a) in bacteria; overexpression of *HMGR* (Donald et al., 1997; Tokuhiro et al., 2009; Mantzouridou and Tsimidou, 2010), reduction of *SQE* (Garaiova et al., 2014), blocking of squalene to ergosterol in yeasts (Kamimura et al., 1994). (B) Squalene production in heterologous hosts. Genes in MVA or MEP squalene biosynthetic pathway were overexpressed in *E. coli* (Ghimire et al., 2009; Katabami et al., 2015). MVA, mevalonate; MEP, 2-C-methyl-D-erythritol 4-phosphate; AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, HMG-CoA reductase; IPP, isopentenyl pyrophosphate; IDI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GA3P, 3-phosphoglycerate; DXP, 1-deoxyxylulose-5-phosphate; DXS, 1-deoxyxylulose-5-phosphate synthase; DXR, DXP reductoismerase; DRL, DXR-like enzyme; HMBPP, 4-hydroxy-3-methyl-but-2-enyl pyrophosphate; SQS, squalene synthase; PSPP, presqualene diphosphate; HSQ, (*R*)-12-hydroxysqualene; SQE, squalene epoxidase; SHC, squalene hopene cyclase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 1
Squalene production in native organisms and heterologous hosts.

	Strain	Strategy	Fermentation mode/volume	Titer (mg l^{-1})	Yield (μg g^{-1} DCW)	Reference
Native	*Saccharomyces cerevisiae*	Disrupt a gene required in the conversion of squalene to ergosterol by homologous recombination	Shake flask/100 ml	18	5000	Kamimura et al., 1994
	S. cerevisiae	Overexpression of the catalytic domain of the HMG-R	Shake flask/10 ml	10-fold of wild-type	N.A.	Donald et al., 1997
	S. cerevisiae	Overexpression of the hydroxymethylglutaryl-coenzyme A reductase (*HMG1*) gene, which encodes the major rate-limiting enzyme of the mevalonate pathway	Bioreactor/10 l	191.9	N.A.	Tokuhiro et al., 2009
	S. cerevisiae	Integration of the *HMG2* gene with a K6R stabilizing mutation in Hmg2p under the galactose promoter into the chromosomal HO locus	Shake flask/100 ml	N.A.	18,500	Mantzouridou and Tsimidou, 2010
	S. cerevisiae	Specific point mutation of *ERG1* gene with reduced activity of squalene epoxidase	Shake flask/N.A.	N.A.	>1000 μg/10^9 cells	Garaiova et al., 2014
	Synechocystis sp.	Deletion of the *slr2089* gene encoding squalene hopene cyclase, an enzyme converting squalene into hopene	Shake flask/25 ml	0.67 mg OD_{750}^{-1} l^{-1}, >70-times of wild-type	N.A.	Englund et al., 2014
	Chlamydomonas reinhardtii	Knockdown of squalene epoxidase (CrSQE), which expression level reduced by 59–76%	N.A.	N.A.	900–1100	Kajikawa et al., 2015
	Rhodopseudomonas palustris	Delete *shc* gene, fuse *crt*E gene with *hpn*D gene, and co-express *dxs* gene	Shake flask/50 ml	N.A.	15,800 112-fold of the wild-type	Xu et al., 2016a
Heterologous	*Escherichia coli*	Overexpression of *dxs* (encoding 1-deoxy-D-xylulose 5-phosphate synthase) and *idi* (encoding isopentenyl diphosphate isomerase) of *E. coli*, and *hopA* and *hopB* (encoding squalene/phytoene synthases) together with *hopD* (encoding farnesyl diphosphate synthase) from *Streptomyces peucetius*	Shake flask/N.A.	11.8	N.A.	Ghimire et al., 2009
	E. coli	Expression of *hpnC*, *hpnD*, and *hpnE* genes from *Zymomonas mobilis*	N.A.	N.A.	N.A.	Pan et al., 2015
	E. coli	Expression of *hpnC*, *hpnD*, and *hpnE* genes from *R. palustris*	N.A.	N.A.	N.A.	Pan et al., 2015
	E. coli	Co-expression of *Thermosynechococcus elongatus* squalene synthase together with chimeric mevalonate pathway containing a truncated mutant of HMG-CoA reductase (*tHMGR*), HMG-CoA synthase (*HMGS*), mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), and mevalonate diphosphate decarboxylase (*PMD*), from *S. cerevisiae* and acetyl-CoA acetyltransferase (*atoB*), isoprenyl diphosphate isomerase (*idi*), and farnesyl diphosphate synthase (*ispA*) from *E. coli*	Shake flask/N.A.	150	55,000	Katabami et al., 2015
	E. coli	Co-expression of human squalene synthase together with chimeric mevalonate pathway containing a truncated mutant of HMG-CoA reductase (*tHMGR*), HMG-CoA synthase (*HMGS*), mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), and mevalonate diphosphate decarboxylase (*PMD*), from *S. cerevisiae* and acetyl-CoA acetyltransferase (*atoB*), isoprenyl diphosphate isomerase (*idi*), and farnesyl diphosphate synthase (*ispA*) from *E. coli*	Shake flask/N.A.	230	54,000	Katabami et al., 2015

isozyme DXR-like enzyme (DRL; Sangari et al., 2010). MEP is converted to IPP in the following several steps. The remaining steps from IPP to squalene are similar to those in the MVA pathway (Xu et al., 2016b).

2.1.2. Squalene production by metabolic engineering of native organisms

There have been several attempts to produce squalene by metabolic engineering of native microorganisms as summarized in Table 1. Most works have been done with *Saccharomyces cerevisiae*. Squalene is an intermediate in the ergosterol biosynthesis pathway of *S. cerevisiae*. By homologous recombination, a gene required for the conversion of squalene to ergosterol was disrupted in *S. cerevisiae*, which increased the squalene titer up to 5000 μg g^{-1} DCW (Kamimura et al., 1994). The HMG-CoA reductase (HMGR) encoded by *HMG1* is a rate-limiting enzyme of the MVA pathway. The engineered *S. cerevisiae* overexpressing the catalytic domain of the HMGR could produce squalene at the titer 10-fold higher than that obtained with the wild-type strain (Donald et al., 1997). Overexpression of *HMG1* in *S. cerevisiae* increased the squalene concentration up to 191.9 mg l^{-1} (Tokuhiro et al., 2009). By integration of an extra *HMG2* gene with a K6R stabilizing mutation in Hmg2p under the galactose promoter into the chromosomal HO locus of *S. cerevisiae*, 18,500 μg g^{-1} DCW of squalene was produced (Mantzouridou and Tsimidou, 2010). Introducing a specific point mutation in the gene *ERG1* caused reduced activity of squalene epoxidase expressed by *S. cerevisiae*, which allowed accumulation of over 1000 μg squalene per 10^9 cells (Garaiova et al., 2014). As mentioned above, there have been many works on the metabolic engineering of *S. cerevisiae* for squalene production. However, these studies rather focused on single step rather than the whole biosynthetic pathway. It will be thus important to optimize the whole metabolic and regulatory networks through further studies to develop *S. cerevisiae* strains capable of enhanced production of squalene.

Other than *S. cerevisiae*, photosynthetic microorganisms have also attracted attention for the production of squalene by metabolic engineering. In the cyanobacterium *Synechocystis* PCC 6803, the *slr2089* gene encoding squalene hopene cyclase, catalyzing the conversion of squalene into hopene, was deleted to produce 0.67 mg OD_{750}^{-1} l^{-1} squalene, which is over 70-times of that obtained with the wild-type strain (Englund et al., 2014). Metabolic engineering of another photosynthetic bacterium *Rhodopseudomonas palustris* for the production of squalene has also been reported. Deletion of the *shc* gene (encoding squalene hopene cyclase), fusion of the *crtE* (encoding geranylgeranyl pyrophosphate synthase) and *hpnD* (encoding an enzyme catalyzing formation of presqualene diphosphate (PSPP) from two molecules of FPP) genes, and overexpression of the *dxs* gene (encoding DXP synthase) allowed production of 15,800 μg g^{-1} DCW of squalene, which was 112-fold higher than that produced by the wild-type strain (Xu et al., 2016a).

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A microalgal strain *Chlamydomonas reinhardtii* was also metabolically engineered to produce squalene. Knockdown of squalene epoxidase (CrSQE) caused its expression level reduction by 59%–76%, which increased the squalene production in *C. reinhardtii* to 900–1100 μg g^{-1} DCW (Kajikawa et al., 2015). This is the only report on squalene production by engineered microalgae, and this field will further advance when better genetic manipulation systems are developed for microalgae.

The highest squalene concentration was obtained by overexpressing *HMG1*, which indicated that hydroxymethylglutaryl-coenzyme A reductase was one of the key rate-limiting enzymes in the squalene biosynthetic pathway in *S. cerevisiae* (Tokuhiro et al., 2009). Increase of squalene concentration by 112-fold in the engineered photosynthetic bacterium *R. palustris* exhibited the good potential of engineering more than one steps in the metabolic pathway (Xu et al., 2016a).

2.1.3. Squalene production by metabolic engineering of heterologous hosts

There have been several attempts on the heterologous production of squalene by metabolic engineering of microorganisms as well (Table 1). *Escherichia coli* was metabolically engineered by overexpression of the native *dxs* (encoding DXP synthase) and *idi* (encoding IDI), and *Streptomyces peucetius hopD* (encoding FPP synthase), *hopA* and *hopB* (encoding squalene/phytoene synthases). This resulted in production of 11.8 mg l^{-1} of squalene in engineered *E. coli* (Ghimire et al., 2009). Expression of *hpnC* (encoding an enzyme converting PSPP to hydroxysqualene), *hpnD*, and *hpnE* (encoding an enzyme reducing hydroxysqualene to squalene) genes from either *Zymomonas mobilis* or *R. palustris* enabled the engineered *E. coli* accumulate squalene using farnesyl diphosphate as substrate (Pan et al., 2015). Co-expression of *Thermosynechococcus elongates* squalene synthase together with chimeric MVA pathway containing HMG-CoA synthase (encoded by *HMGS*), a truncated mutant of HMG-CoA reductase (encoded by *tHMGR*), MVA kinase (encoded by *MK*), phosphomevalonate kinase (encoded by *PMK*), and MVA diphosphate decarboxylase (encoded by *PMD*) from *S. cerevisiae* and IDI (encoded by *idi*), acetyl-CoA acetyltransferase (encoded by *atoB*), and FPP synthase (encoded by *ispA*) from *E. coli* resulted in production of 150 mg l^{-1} (55,000 μg g^{-1} DCW) squalene. Similarly, co-expression of human squalene synthase together with the same chimeric MVA pathway in *E. coli* resulted in production of 230 mg l^{-1} (54,000 μg g^{-1} DCW) squalene (Katabami et al., 2015; Fig. 2B).

Until now, the heterologous production of squalene was mainly investigated in *E. coli*. In the work of Katabami et al., 230 mg l^{-1} squalene was produced by the engineered *E. coli* by overexpressing genes from human, *S. cerevisiae* and also *E. coli* itself. Codon optimization of all the heterologous genes was carried out in this study, which turned out to be a feasible way to obtain high concentration of squalene (Katabami et al., 2015). This work suggests that the engineered heterologous host can produce more target products than the engineered native producers (highest 191.9 mg l^{-1} in *S. cerevisiae*; Tokuhiro et al., 2009).

2.2. Astaxanthin

Astaxanthin (3, 3′-dihydroxy-β, β-carotene-4, 4′-dione) is a red, cyclic C40 carotenoid. As one of the strongest natural anti-oxidants, astaxanthin can be used for protecting skin, promoting hair growth, anti-aging, alleviating sports fatigue, and preventing cancer, cardiovascular disease, diabetes, and others (Liu and Osawa, 2007; Giuffrida et al., 2016; Ambati et al., 2014; Higuera-Ciapara et al., 2006; Fassett and Coombes, 2012). Traditionally, astaxanthin has been extracted from shrimp wastes (Mao et al., 2017), *Haematococcus pluvialis* (Dong et al., 2016) and *Xanthophyllomyces dendrorhous* (Martín et al., 2008).

2.2.1. Astaxanthin biosynthetic pathway

Astaxanthin is accumulated in many microorganisms such as marine bacteria *Agrobacterium aurantiacum* (Misawa et al., 1995), *Erwinia uredovora* (Misawa et al., 1990) and *Paracoccus haeundaensis* (Lee and Kim, 2006), the alga *H. pluvialis* (Lemoine and Schoefs, 2010) and the yeast *X. dendrorhous* (Martín et al., 2008). Astaxanthin biosynthetic pathway comprises two parts: the β-carotene synthesis and transformation of β-carotene to astaxanthin (Misawa and Shimada, 1998). The pathway for β-carotene synthesis is highly conserved (Fig. 3A). First, a building block IPP together with its isomer DMAPP are synthesized by the MVA or MEP pathway as described before. Second, FPP synthase and CrtE catalyze the formation of geranylgeranyl pyrophosphate (GGPP) from three molecules of IPP and one molecule of DMAPP. Third, two GGPP molecules are transformed to phytoene by phytoene synthase. Fourth, phytoene is converted into lycopene by phytoene desaturase. Fourth, lycopene cyclase catalyzes the formation of β-carotene from lycopene (Ye et al., 2015). Astaxanthin formation from β-carotene varies in different organisms. In marine bacteria *E. uredovora* (Misawa et al., 1990), *A. aurantiacum* (Misawa et al., 1995) and *P. haeundaensis* (Lee and Kim, 2006), β-carotene 3, 3′-hydroxylase encoded by *crtZ* and β-carotene 4, 4′-ketolase encoded by *crtW* are needed for astaxanthin formation from β-carotene. In the photoautotrophic green alga *H. pluvialis*, β-carotene 3, 3′-hydroxylase and β-carotene 4, 4′-ketolase encoded by the *bkt* and *crtR-b* genes, respectively, are used (Lemoine and Schoefs, 2010). In the heterotrophic yeast *X. dendrorhous*, astaxanthin synthase encoded by *CrtS* and cytochrome P450 reductase encoded by *CrtR* participate in the conversion of β-carotene to astaxanthin (Martín et al., 2008; Alcaíno et al., 2008).

2.2.2. Astaxanthin production by metabolic engineering of native organisms

Attempts on astaxanthin production by metabolic engineering of naturally astaxanthin-producing microorganisms are summarized in Table 2. Many works have been done with the yeast *X. dendrorhous*. The efficient transformation system for *X. dendrorhous* was developed, and further metabobic engineering of *X. dendrorhous* for astaxanthin production was explored in the past two decades (Wery et al., 1998). Overexpression of the astaxanthin biosynthetic pathway genes *CrtYB* (encoding phytoene synthase), *CrtI* (encoding phytoene desaturase), *CrtE* and *CrtS* have all been tried, and the corresponding astaxanthin yield of 95, 48, 451, and 179 μg g^{-1} DCW were obtained, respectively (Verdoes et al., 2003; Breitenbach et al., 2011; Contreras et al., 2013). In the work of Hara et al. (2014), an alcohol dehydrogenase promoter, P*adh4*, was screened from *X. dendrorhous*, and used for *CrtE* overexpression. This resulted in production of 430 μg g^{-1} DCW (2.45 mg l^{-1}) of astaxanthin. Upon stepwise transformation, the copy numbers of *CrtYB* encoding the limiting enzyme phytoene synthase could be increased. The resulting metabolically engineered *X. dendrorhous* strain produced 533 μg g^{-1} DCW of astaxanthin (Ledetzky et al., 2014). Double deletion of *CYP61* encoding C-22 sterol desaturases related to ergosterol synthesis blocked the pathway competing for astaxanthin accumulation. This allowed increase of astaxanthin yield to 280 μg g^{-1} DCW (Yamamoto et al., 2016). So far, however, the best astaxanthin production was achieved by metabolic engineering coupled with mutagenesis. Overexpression of *CrtS* in the astaxanthin-overproducing mutant *X. dendrorhous* MK19 strain promoted both cell growth and astaxanthin yield. Fermentation of the engineered mutant strain in 5 l bioreactor produced 27.8 mg l^{-1} (1200 μg g^{-1} DCW) of astaxanthin (Chi et al., 2015). By the overexpression of *CrtYB* and the astaxanthin synthase *Asy* in an astaxanthin-overproducing mutant, the maximum astaxanthin concentration could reach up to 9700 μg g^{-1} DCW (Gassel et al., 2013). Also, stepwise expression of *HMG* encoding HMGR, *CrtE* encoding geranylgeranyl pyrophosphate synthase, *CrtYB* encoding phytoene synthase/lycopene cyclase, and *Asy* encoding astaxanthin synthase in an astaxanthin-overproducing mutant resulted in production of 9000 μg g^{-1} DCW of astaxanthin (Gassel et al., 2014).

Besides *X. dendrorhous*, microalgae have also attracted attention for the production of astaxanthin by metabolic engineering. The wild-type *H. pluvialis* could accumulate astaxanthin to a high titer (Guerin et al., 2003), and thus it is an excellent native host strain for further metabolic engineering studies. In the microalga *H. pluvialis*, the gene

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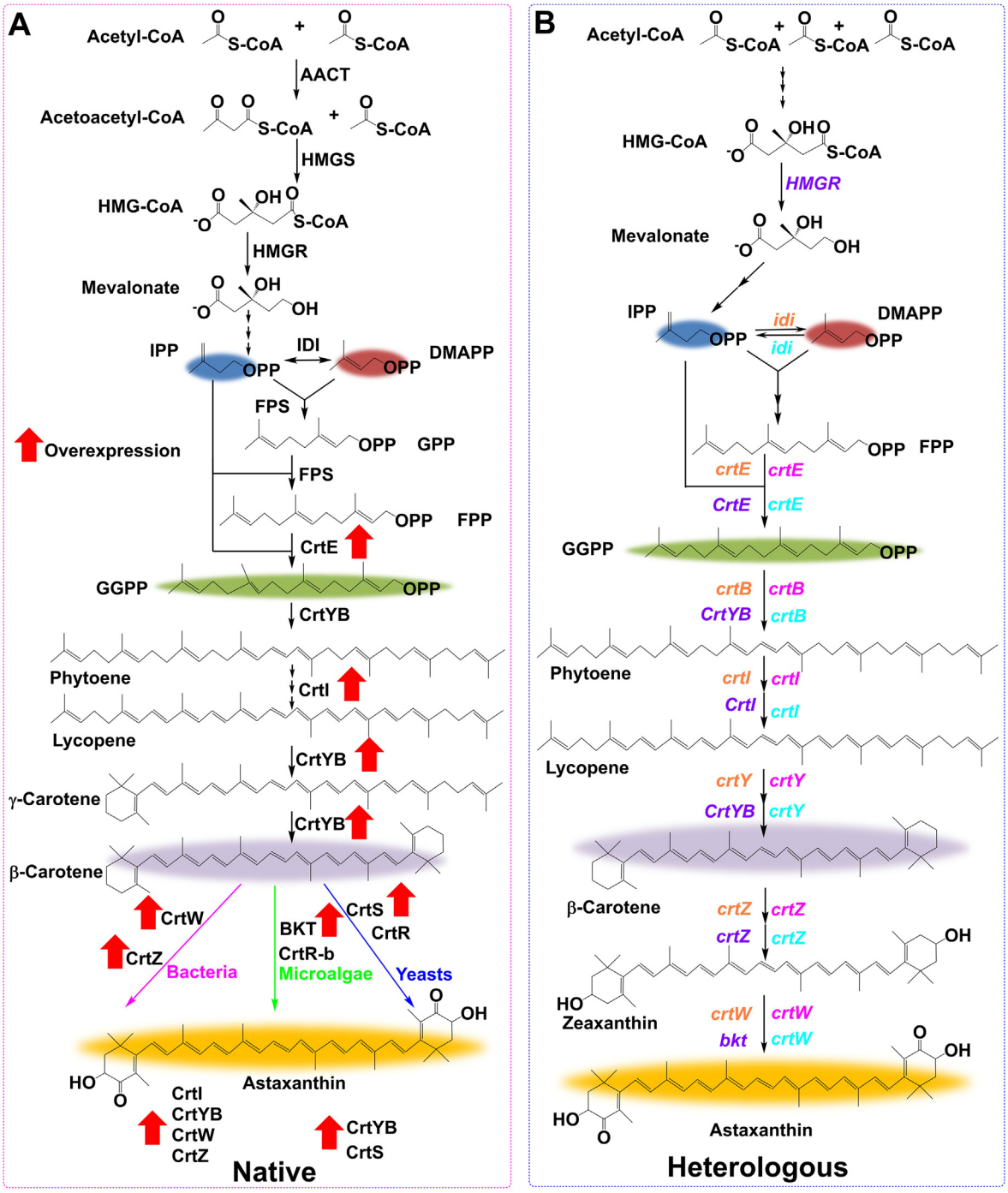

Fig. 3. Astaxanthin biosynthetic pathway and the astaxanthin production by metabolic engineering and synthetic biology. (A) Astaxanthin biosynthetic pathway in microalgae (green), yeasts (blue) and bacteria (purple) and astaxanthin production by metabolic engineering in native organisms. Overexpression of *crtI* (Steinbrenner and Sandmann, 2006) and *bkt* (Kathiresan et al., 2015) in microalage, *crtI*, *crtYB*, *crtW* and *crtZ* in bacteria (Ide et al., 2012), and *CrtE* (Breitenbach et al., 2011; Hara et al., 2014), *CrtI* (Verdoes et al., 2003), *CrtYB* (Gassel et al., 2013; Ledetzky et al., 2014) and *CrtS* (Contreras et al., 2013; Gassel et al., 2013; Chi et al., 2015) in yeasts was carried out. (B) Astaxanthin production in heterologous hosts. Astaxanthin pathway genes of different sources were overexpressed in different hosts such as *E. coli* (Ma et al., 2016), *Saccharomyces cerevisiae* (Zhou et al., 2015), *Candida utilis* (Miura et al., 1998) and *Methylomonas* sp. (Ye et al., 2007). AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, HMG-CoA reductase, IPP, isopentenyl pyrophosphate; IDI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; FPS, farnesyl pyrophosphate synthase; CrtE, GGPP synthase; GGPP, geranylgeranyl pyrophosphate; CrtYB, phytoene synthase and lycopene cyclase; CrtI, phytoene desaturase; CrtW, β-carotene 4,4′-ketolase; CrtZ, β-carotene 3,3′-hydroxylase; BKT, β-carotene 3,3′-hydroxylase; CrtR-b, β-carotene 4,4′-ketolase; CrtS, astaxanthin synthase; CrtR, cytochrome P450 reductase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

encoding phytoene desaturase was modified to change the leucine codon to an arginine codon at position 504 by site-directed mutagenesis (Steinbrenner and Sandmann, 2006). The resistance of the modified enzyme to the bleaching herbicide norflurazon was increased by 43-fold. After overexpression of the modified phytoene desaturase gene in *H. pluvialis*, 11,400 μg g^{-1} DCW astaxanthin was produced. By overexpression of the *bkt* gene encoding β-carotene ketolase in *H. pluvialis*, the astaxanthin titer could be increased to 4990 μg g^{-1} DCW (Kathiresan et al., 2015). Metabolic engineering of another microalga *Aurantiochytrium* sp. SK4 for the production of astaxanthin has also been reported. The hemoglobin gene from *Vitreoscilla stercoraria* was overexpressed in *Aurantiochytrium* sp. SK4, which significantly

Table 2
Astaxanthin production in native organisms and heterologous hosts.

	Strain	Strategy	Fermentation mode/volume	Titer (mg l^{-1})	Yield (μg g^{-1} DCW)	Reference
Native	*Xanthophyllomyces dendrorhous*	*CrtYB* overexpression	Shake flask/30 ml	N.A.	95	Verdoes et al., 2003
	X. dendrorhous	*CrtI* overexpression	Shake flask/30 ml	N.A.	48	Verdoes et al., 2003
	X. dendrorhous	*CrtE* overexpression	Shake flask/150 ml	N.A.	451	Breitenbach et al., 2011
	X. dendrorhous	*CrtS* overexpression	Shake flask/N.A.	N.A.	179	Contreras et al., 2013
	X. dendrorhous	Chemical mutagenesis + overexpression of *CrtYB* and *asy*	Bioreactor/1.3 l	N.A.	9700	Gassel et al., 2013
	X. dendrorhous	Chemical mutagenesis + stepwise expression of *HMG*, *CrtE*, *CrtYB*, *Asy*	Shake flask/50 ml	N.A.	9000	Gassel et al., 2014
	X. dendrorhous	*CrtE* overexpression under the newly screened strong promoter P*adh4*	Shake flask/80 ml	2.45	430	Hara et al., 2014
	X. dendrorhous	Stepwise transformation of multiple copies of *CrtYB*	Shake flask/50 ml	N.A.	533	Ledetzky et al., 2014
	X. dendrorhous	*CrtS* Overexpression in the astaxanthin-overproducing mutant	Bioreactor/5 l	27.8	1200	Chi et al., 2015
	X. dendrorhous	Double deletion of *CYP61* encoding C-22 sterol desaturases	Shake flask/80 ml	1.65	280	Yamamoto et al., 2016
	Haematococcus pluvialis	Overexpression of the modified phytoene desaturase gene	Flask shaken manually once a day/N.A.	N.A.	11,400	Steinbrenner and Sandmann, 2006
	H. pluvialis	*bkt* overexpression	Flask shaken manually once a day/N.A.	N.A.	4990	Kathiresan et al., 2015
	Paracoccus sp.	Random mutagenesis + overexpression of *crtW*, *crtZ*, *crtY*, *crtI*, and *crtB*	Bioreactor/1.2 l	480	8000	Ide et al., 2012
	Aurantiochytrium sp.	Overexpression of the hemoglobin of *Vitreoscilla stercoraria*	Shake flask/80 ml	N.A.	131	Suen et al., 2014
	Sphingomonas sp.	Overexpression of *idi* from *E. coli*	Shake flask/N.A.	N.A.	6.91	Ma et al., 2016
Heterologous	*E. coli*	Overexpression of *bkt* from *H. pluvialis* and *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* from *Erwinia uredovora*	N.A./N.A.	N.A.	120	Kajiwara et al., 1995
	E. coli	Overexpression of *crtW* from *Agrobacterium aurantiacum* and *crtE*, *crtX*, *crtB*, *crtI*, *crtY* and *crtZ* from *E. uredovora*	N.A./12 l	N.A.	N.A.	Yokoyama et al., 1998
	E. coli	Overexpression of *idi* from *E. coli*, *gps* from *Archaeoglobus fulgidus*, and cluster *crtBIYZW* from *A. aurantiacum*	Shake flask/N.A.	N.A.	1419	Wang et al., 1999
	E. coli	Overexpression of *crtW148* from *Nostoc punctiforme* and *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* from *E. uredovora*	N.A./N.A.	N.A.	N.A.	Steiger and Sandmann, 2004
	E. coli	Overexpression of *crtW* from *Nostoc* sp. and *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* from *Pantoea agglomerans*	Shake flask/N.A.	3.89	1931	Scaife et al., 2009
	E. coli	Integration of *crtW148* from *N. punctiforme*, *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* from *E. uredovora* to the *E. coli* chromosome	Shake flask/N.A.	2.07	1410	Lemuth et al., 2011
	E. coli	Comparison of overexpression of 12 genes encoding β-Carotene hydroxylase	Shake flask/50 ml	N.A.	313	Scaife et al., 2012
	E. coli	Overexpression of *idi* from *E. coli*, *gps* from *A. fulgidus*, and cluster *crtBIYZW* from *A. aurantiacum*, Spanning proteins expression space using ribosome-binding site combinatorics	Shake flask/N.A.	N.A.	5800	Zelcbuch et al., 2013
	E. coli	Overexpression of *crtEIB* from *Pantoea ananatis*, *crtYZ* from *P. agglomerans*; *crtW* from *Brevundimonas* sp.; *idi* from *E. coli*	Shake flask/200 ml	N.A.	6600	Ma et al., 2016
	E. coli	Overexpression of *crtEIB* from *P. ananatis* with $crtE_{sp1}$ from *Sphingomonas* sp., overexpression of *crtYZ* from *P. agglomerans*, *crtW* from *Brevundimonas* sp., *idi* from *E. coli*	Shake flask/200 ml	N.A.	4440	Ma et al., 2016
	E. coli	Overexpression of *crtEIB* from *P. ananatis*, *crtYZ* from *P. agglomerans* with $crtZ_{sp1}$ from *Sphingomonas* sp., *crtW* from *Brevundimonas* sp., *idi* from *E. coli*	Shake flask/200 ml	N.A.	1220	Ma et al., 2016
	E. coli	Overexpression of *crtEIB* from *P. ananatis*, *crtYZ* from *P. agglomerans* with $crtZ_{sp2}$ from *Sphingomonas* sp., *crtW* from *Brevundimonas* sp., *idi* from *E. coli*	Shake flask/200 ml	N.A.	2790	Ma et al., 2016
	Candida utilis	Overexpression of *crtE*, *crtB*, *crtI*, *crtY* from *E. uredovora* and *crtZ*, *crtW* from *A. aurantiacum*	N.A./N.A.	N.A.	400	Miura et al., 1998
	S. cerevisiae	Overexpression of *CrtI*, *CrtYB*, *BTS1*, *CrtS*, *CrtR* from *X. dendrorhous*	N.A./N.A.	N.A.	3	Ukibe et al., 2009
	S. cerevisiae	Overexpression of *CrtI*, *CrtYB*, *BTS1* from *X. dendrorhous*, *crtW* from *Paracoccus* sp. and *crtZ* from *P. ananatis*	N.A./N.A.	N.A.	29	Ukibe et al., 2009
	S. cerevisiae	Overexpression of *crtZ*, *bkt* from *H. pluvialis* in a β-carotene-producing *S. cerevisiae* constructed before by integration of *CrtE*, *CrtYB*, *CrtI* from *X. dendrorhous*, and *tHMG1* from *S. cerevisiae*	Shake flask/50 ml	N.A.	4700	Zhou et al., 2015; Xie et al., 2014
	Synechococcus sp.	Overexpression of *crtO* from *H. pluvialis*	N.A./N.A.	0.14	N.A.	Harker and Hirschberg, 1997
	Synechocystis sp.	Insertion of a β-carotene di-ketolase gene *crtW148* from *N. punctiforme* and an additional copy of the β-carotene hydroxylase gene *crtR* from *Synechocystis*, and disruption of the β-carotene mono-ketolase gene *crtO*	N.A./N.A.	1.11 mg l^{-1} d^{-1}	N.A.	Albers, 2016
	Rhodovulum	Overexpression of *crtI*, *crtY* from *Erythrobacter longus*, *crtW*, *crtZ* from	N.A./N.A.	N.A.	2	Mukoyama et

(continued on next page)

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Table 2 (*continued*)

Strain	Strategy	Fermentation mode/volume	Titer (mg l^{-1})	Yield (μg g^{-1} DCW)	Reference
sulfidophilum	*Paracoccus* sp.				al., 2006
Methylomonas sp.	Overexpression of *crtE*, *idi*, *crtYIB* cluster from *Brevundimonas vesicularis*, *crtW*, *crtZ* from *Paracoccus* sp.	Bioreactor/1.6 l	N.A.	2400	Ye et al., 2007
Methylomonas sp.	Overexpression of *crtE, idi, crtYIB* cluster from *B. vesicularis*, *crtW*, *crtZ* from *Paracoccus* sp., integration of *thbN1* (from *Methylomonas*)-*crtW* (from *Sphingomonas* sp.) -*crtZ* (from *Novosphingobium aromaticivorans*) cassette to *Methylomonas* sp. chromosome	Bottle/10–20 ml	N.A.	N.A.	Tao et al., 2007
Corynebacterium glutamicum	Overexpression of *crtY*, *crtZ* from *P. ananatis*. *crtW*, *crtZ* from *Fulvimarina pelagi*	Shake flask/50 ml	N.A.	1600	Henke et al., 2016
C. reinhardtii	Overexpression of the zeaxanthin epoxidase gene *zep* from *Chlorella zofingiensis*	Shake flask/1 l	N.A.	500	Couso et al., 2012

increased the biomass concentration, and also astaxanthin production by 9-fold to 131 μg g^{-1} DCW (Suen et al., 2014).

Some bacteria such as *Paracoccus* sp. and *Sphingomonas* sp. have also been metabolically engineered to produce astaxanthin. Random mutagenesis and overexpression of the *crtW*, *crtZ*, *crtY* (encoding lycopene cyclase), *crtI* and *crtB* (encoding phytoene synthase) genes were conducted to improve astaxanthin production in *Paracoccus* sp. Fermentation of this engineered strain produced astaxanthin to 480 mg l^{-1} (8000 μg g^{-1} DCW; Ide et al., 2012). Metabolic engineering of another bacterium *Sphingomonas* sp. ATCC 55669 by the overexpression of the *idi* gene from *E. coli* allowed production of 6.91 μg g^{-1} DCW of astaxanthin (Ma et al., 2016).

There have been numerous studies on the metabolic engineering of *X. dendrorhous* to promote astaxanthin production. Among these works, the best result was obtained through combination of conventional mutagenesis and metabolic engineering (9700 μg g^{-1} DCW, Gassel et al., 2013). Through mutagenesis, astaxanthin concentration (1609 μg g^{-1} DCW) obtained with the mutant was about 15-fold higher than that (108 μg g^{-1} DCW) produced by wild-type strain. Although the astaxanthin concentration was only increased by 5-fold after metabolic engineering, the final concentration of astaxanthin (9700 μg g^{-1} DCW) by combining metabolic engineering and mutagenesis was 89.8-fold higher than that obtained with the initial wild-type strain (Gassel et al., 2013). Similarly, another high performance strain for astaxanthin production was obtained by random mutagenesis together with metabolic engineering (8000 μg g^{-1} DCW; Ide et al., 2012). The astaxanthin concentration was increased by 16.7-fold to 16 mg l^{-1} by mutagenesis, and increased by 2.5-fold to 58 mg l^{-1} by metabolic engineering. Further increase of astaxanthin concentration to 480 mg l^{-1} (7.6-fold increase) could be achieved through fermentation optimization (Ide et al., 2012). These results suggest that integrating traditional mutagenesis, metabolic engineering and also fermentation optimization is obviously important to increase the product titer and productivity.

2.2.3. Astaxanthin production by metabolic engineering of heterologous hosts

Astaxanthin production has also been attempted in many heterologous hosts such as *E. coli* (Zelcbuch et al., 2013), *S. cerevisiae* (Zhou et al., 2015), photosynthetic microorganisms (Albers, 2016), and others (Henke et al., 2016). The first heterologous production of astaxanthin was carried out in *E. coli* expressing *bkt* from *H. pluvialis* and *crtE*, *crtY*, *crtI*, *crtB* and *crtZ* from *E. uredovora*. This engineered *E. coli* produced 120 μg g^{-1} DCW of astaxanthin (Kajiwara et al., 1995). From then on, many works (Table 2) were conducted to heterologously express the astaxanthin biosynthetic pathway genes from different organisms such as *A. aurantiacum*, *Archaeoglobus fulgidus*, *Nostoc punctiforme*, *Pantoea agglomerans* in *E. coli* (Yokoyama et al., 1998; Wang et al., 1999; Steiger and Sandmann, 2004; Scaife et al., 2009). Integration of *crtW148* from *N. punctiforme*, *crtE*, *crtI*, *crtB*, *crtY* and *crtZ* from *E. uredovora* into the *E. coli* chromosome resulted in production of 1410 μg g^{-1} DCW (2.07 mg l^{-1}) of astaxanthin (Lemuth et al., 2011). Twelve β-carotene hydroxylase genes were compared for astaxanthin production, and among which the best one gave a titer of 313 μg g^{-1} DCW (Scaife et al., 2012). Also, *E. coli* was metabolically engineered by overexpression of the native *idi*, *gps* (encoding GGPP synthase) from *A. fulgidus* and *crtBIYZW* cluster from *A. aurantiacum*, and astaxanthin was produced at the concentration of 1419 μg g^{-1} DCW (Wang et al., 1999). When combinatorial examination of the ribosome-binding site was performed to expand the protein expression space of the enzymes encoded by *idi*, *crtE*, *crtB*, *crtI*, *lcyB* (encoding lycopene cyclase), *crtW* and *crtZ*, the engineered *E. coli* produced 5800 μg g^{-1} DCW of astaxanthin (Zelcbuch et al., 2013). In more recent work by Ma et al. (2016), the genes for astaxanthin biosynthesis in the bacterium *Sphingomonas* sp. were characterized. Several different combinations of astaxanthin pathway genes from different sources were compared for astaxanthin production (Table 2). The highest titer of 6600 μg g^{-1} DCW was obtained using the metabolically engineered *E. coli* expressing the native *idi*, *Pantoea ananatis crtEIB*, *P. agglomerans crtYZ*, and *Brevundimonas* sp. *crtW* (Ma et al., 2016; Fig. 3B).

Other than *E. coli*, yeasts such as *S. cerevisiae* (Ukibe et al., 2009) and *Candida utilis* (Miura et al., 1998) have also attracted attention for the heterologous production of astaxanthin by metabolic engineering. In the yeast *S. cerevisiae*, two approaches were taken for heterologous production of astaxanthin (Ukibe et al., 2009). In the case of overexpressing the whole astaxanthin pathway containing *CrtYB*, *CrtI*, *BTS1* (encoding GGPP synthase), *CrtS*, *CrtR* from *X. dendrorhous* in *S. cerevisiae*, 3 μg g^{-1} DCW of astaxanthin was produced. On the other hand, through overexpressing *CrtI*, *CrtYB*, *BTS1* from *X. dendrorhous*, *crtW* from the marine bacterium *Paracoccus* sp. and *crtZ* from *P. ananatis*, the concentration of astaxanthin was increased to 29 μg g^{-1} DCW (Ukibe et al., 2009). In another study, overexpression of *crtZ* and *bkt* from *H. pluvialis* in a β-carotene-producing *S. cerevisiae* strain (Xie et al., 2014) constructed previously by integration of *X. dendrorhous CrtE*, *CrtYB* and *CrtI*, and the native *tHMG1* resulted in the production of 4700 μg g^{-1} DCW of astaxanthin (Zhou et al., 2015). In the food-grade yeast *C. utilis* was also engineered for astaxanthin production. When the *E. uredovora crtI*, *crtB*, *crtE* and *crtY* genes together with the *crtZ* and *crtW* genes from the marine bacterium *A. aurantiacum* were overexpressed, 400 μg g^{-1} DCW of astaxanthin was produced (Miura et al., 1998).

Metabolic engineering of photosynthetic microorganisms for the heterologous production of astaxanthin has also been reported. In the cyanobacterium *Synechococcus* PCC7942, the β-carotene oxygenase gene *crtO* from green alga *H. spluvialis* was overexpressed to produce 0.14 mg l^{-1} of astaxanthin (Harker and Hirschberg, 1997). In another cyanobacterium *Synechocystis* sp., the astaxanthin productivity of 1.11 mg l^{-1} d^{-1} was obtained through the insertion of a β-carotene di-ketolase gene (*crtW148*) from *N. punctiforme* and an additional copy of the β-carotene hydroxylase gene (*crtR*) from *Synechocystis*, and disruption of the β-carotene mono-ketolase gene (*crtO*) (Albers, 2016). In photosynthetic bacterium *Rhodovulum sulfidophilum*, astaxanthin biosynthetic pathway was constructed through the introduction of the *crtI* (phytoene dehydrogenase) and *crtY* (lycopene

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cyclase) genes from *Erythrobacter longus* and the *crtW* (β-carotene oxygenase) and *crtZ* (β-carotene hydroxylase) genes from *Paracoccus* sp. This metabolically engineered *R. sulfidophilum* strain produced astaxanthin up to 2 μg g^{-1} DCW (Mukoyama et al., 2006).

Overexpression of the *crtE*, *idi* and *crtYIB* genes from *Brevundimonas vesicularis*, and the *crtW* and *crtZ* genes from *Paracoccus* sp. enabled the obligate methanotrophic bacterium *Methylomonas* sp. strain 16a produce antaxanthin as high as 2400 μg g^{-1} DCW in a 1.6 l bioreactor fermentation (Ye et al., 2007). Also, by integration of the *thbN1* (encoding hemoglobin) from *Methylomonas* sp., *crtW* from *Sphingomonas* sp. and *crtZ* from *N. aromaticivorans* into the chromosome of antaxanthin-producing *Methylomonas* sp. strain, this genome-engineered strain accumulated astaxanthin to 80% of the total carotenoids (Tao et al., 2007). More recently, an industrial workhorse strain *Corynebacterium glutamicum* was also engineered to produce astaxanthin. The engineered *C. glutamicum* strain overexpressing the *P. ananatis crtY* and *crtZ* genes together with the *Fulvimarina pelagi crtW* and *crtZ* genes produced astaxanthin up to 1600 μg g^{-1} DCW (Henke et al., 2016).

Moreover, metabolic engineering of green microalga for the heterologous production of astaxanthin has been reported. The zeaxanthin epoxidase gene *zep* was characterized in the green microalga *Chlorella zofingiensis*. By overexpression of *zep* in zeaxanthin-producing green microalga *C. reinhardtii* without zeaxanthin epoxidase activity, 500 μg g^{-1} DCW of astaxanthin could be produced (Couso et al., 2012).

Heterologous production of astaxanthin has been carried out in many kinds of microorganisms. A principle strategy employed has been overexpressing the pathway genes such as *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, *crtW*, *idi* and *gps* from many different microorgainsms. The highest concentration of astaxanthin was obtained in an engineered *E. coli* strain overexpressing *crtEIB* from *Pantoea ananatis*, *crtYZ* from *P. agglomerans*, *crtW* from *Brevundimonas* sp., and native *idi* (Ma et al., 2016). An astaxanthin-synthesizing platform was reconstructed in *E. coli* to identify the genes *crtE* and *crtZ* from a newly found astaxanthin-producing strain *Sphingomonas* sp. ATCC 55669. Six different chimeric astaxanthin synthetic pathways with different candidate genes were constructed and compared. The engineered strain with the best *crtE* and *crtZ* could produce 6600 μg g^{-1} DCW of astaxanthin (Ma et al., 2016). This work demonstrated the importance of identifying new genes and pathways for increasing target product titer by metabolic engineering. Further enhancement of astaxanthin production will be possible by employing more synthetic biology tools, such as building high-efficiency synthetic pathways guided by *in vitro* reconstitution (Tan et al., 2016).

2.3. Other carotenoids

Fucoxanthin is a carotenoid having anti-cancer, antioxidant, anti-obesity and anti-inflammation activities, and is dominantly produced in brown algae (Mikami and Hosokawa, 2013). In the marine microalga *Phaeodactylum tricornutum*, the integration of additional copies of its own *dxs* (encoding DXP synthase) and *psy* (encoding phytoene synthase) genes increased the fucoxanthin concentration to 24,200 μg g^{-1} DCW and 18,400 μg g^{-1} DCW, respectively (Eilers et al., 2016).

Zeaxanthin is a carotenoid wide-spread in cyanobacteria, microalgae, fruits, vegetables, corn and other organisms. As an antioxidant, zeaxanthin can repair photo-oxidative damage. Furthermore, zeaxanthin is a key contributor to high visual acuity as well as central vision, and can protect human eyes from high energy blue light (Nwachukwu et al., 2016). An engineered marine *Synechocystis* sp. PCC 6803 strain was constructed by overexpression of *crtR* and disruption of *crtO*, which produced 0.95 μg ml^{-1} OD_{730}^{-1} of zeaxanthin and 0.19 μg ml^{-1} OD_{730}^{-1} of β-carotene. When the *crtP* (encoding phytoene desaturase) and *crtB* genes were overexpressed while the *crtO* gene was disrupted, the concentrations of zeaxanthin and β-carotene obtained were 0.62 μg ml^{-1} OD_{730}^{-1} and 0.68 μg ml^{-1} OD_{730}^{-1}, respectively (Lagarde et al., 2000). Heterologous expression of β-carotene hydroxylase gene from a chlorophycean microalga *C. reinhardtii* in a halotolerant marine microalga *Dunaliella salina*, zeaxanthin could be produced up to 200 μg g^{-1} DCW. In addition, production of another bioactive compound violaxanthin was also improved to reach 3130 μg g^{-1} DCW (Simon et al., 2016) (Table 3).

3. Omega-3 polyunsaturated fatty acids (PUFAs)

Omega-3 PUFAs have attracted a great attention because of their use in fish farming in addition to the positive effects on human health. Until now, most omega-3 PUFAs have been mainly extracted from seafood (Mühlroth et al., 2013). Docosahexaenoic acid (DHA, 22:6-$\Delta^{4,7,10,13,16,19}$) and EPA (20:5-$\Delta^{5,8,11,14,17}$) are two important omega-3 PUFAs biosynthesized by marine organisms. DHA and EPA have been suggested for their use in neural development and preventing neurodegeneration (Demaison and Moreau, 2002). Omega-3 PUFAs have been proven to have positive effects on preventing and treating many diseases including inflammatory bowel diseases, major depression and several types of cancer (Diamond et al., 2008; McNamara, 2006). Many human health benefits of DHA and EPA have been reviewed elsewhere (Swanson et al., 2012).

3.1. Omega-3 PUFAs biosynthetic pathway

Omega-3 PUFAs can be biosynthesized by the aerobic desaturation and elongation pathway mainly found in eukaryotic organisms or by the anaerobic polyketide pathway (Metz et al., 2001) in some marine bacteria such as *Photobacterium profundum* (Allen and Bartlett, 2002).

Table 3
Production of marine carotenoids (except astaxanthin) by metabolic engineering and synthetic biology.

Compound	Strain	Strategy	Fermentation mode/volume	Titer (μg l^{-1})	Yield (μg g^{-1} DCW)	Reference
Fucoxanthin	*Phaeodactylum tricornutum*	Integration of additional copies of *dxs*	Shake flask/N.A.	N.A.	24,200	Eilers et al., 2016
Fucoxanthin	*P. tricornutum*	Integration of additional copies of *psy*	Shake flask/N.A.	N.A.	18,400	Eilers et al., 2016
Zeaxanthin	*Synechocystis* sp. PCC 6803	Overexpression of *crtR* + disruption of *crtO*	N.A./N.A.	N.A.	0.95 μg ml^{-1} D_{730}^{-1}	Lagarde et al., 2000
Zeaxanthin	*Synechocystis* sp. PCC 6803	Overexpression of *crtP* and *crtB* + disruption of *crtO*	N.A./N.A.	N.A.	0.62 μg ml^{-1} D_{730}^{-1}	Lagarde et al., 2000
β-Carotene	*Synechocystis* sp. PCC 6803	Overexpression of *crtR* + disruption of *crtO*	N.A./N.A.	N.A.	0.19 μg ml^{-1} D_{730}^{-1}	Lagarde et al., 2000
β-Carotene	*Synechocystis* sp. PCC 6803	Overexpression of *crtP* and *crtB* + disruption of *crtO*	N.A./N.A.	N.A.	0.68 μg ml^{-1} D_{730}^{-1}	Lagarde et al., 2000
Zeaxanthin	*Dunaliella salina*	Heterologous expression of β-carotene hydroxylase gene from the chlorophycean microalga *C. reinhardtii*	N.A./N.A.	N.A.	200	Simon et al., 2016
Violaxanthin	*D. salina*	Heterologous expression of β-carotene hydroxylase gene from the chlorophycean microalga *C. reinhardtii*	N.A./N.A.	N.A.	3130	Simon et al., 2016

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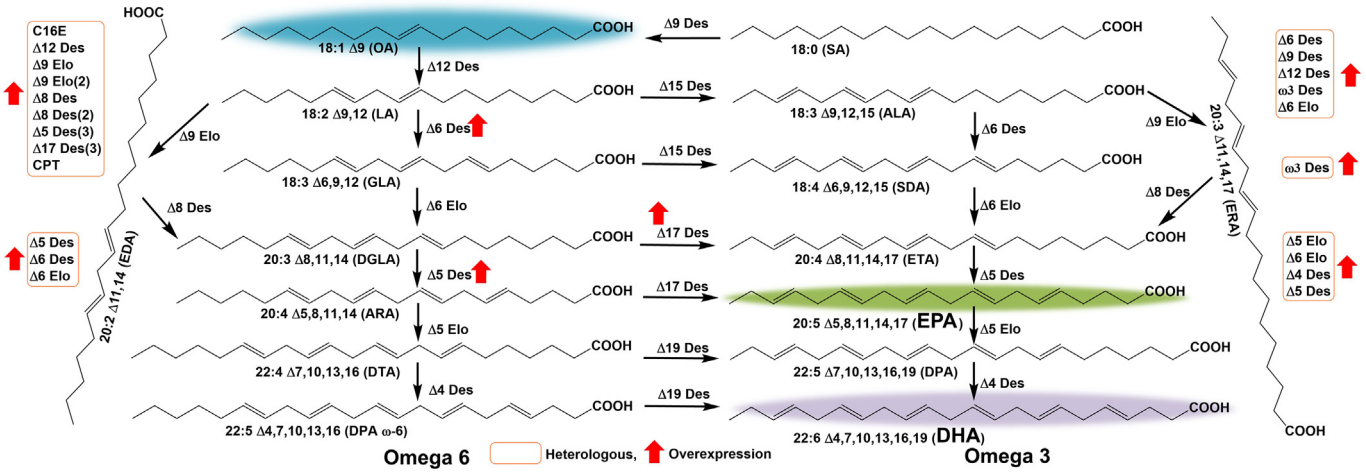

Fig. 4. Omega-3 PUFAs biosynthetic pathway and the omega-3 PUFAs production in native organisms / heterologous hosts by metabolic engineering and synthetic biology. Overexpression of Δ5 Elo, Δ6 Des (Hamilton et al., 2014) in native organisms and Δ5 Elo, Δ6 Elo, Δ4 Des, Δ5 Des (Meyer et al., 2004) in heterologous hosts were conducted for DHA production. Overexpression of ω3 Des (Ando et al., 2009), Δ5 Des (Kobayashi et al., 2011; Peng et al., 2014), Δ17 Des (Okuda et al., 2015), Δ6 Des (Shi et al., 2016) in native organisms and Δ5 Des, Δ6 Des, Δ6 Elo, ω3 Des, Δ9 Des, Δ12 Des, C16E, Δ9 Elo, Δ9 Elo(2), Δ8 Des, Δ8 Des(2), Δ5D(3), Δ17D(3), CPT, etc. (Beaudoin et al., 2000; Domergue et al., 2002; Pereira et al., 2004; Domergue et al., 2005; Tavares et al., 2011; Xue et al., 2013; Li et al., 2009) in heterologous hosts were conducted for EPA production. OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, di-homo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Two distinct and converging pathways exist in aerobic desaturation and elongation pathway: namely, the conventional △6-pathway and the alternative △8-pathway. The conventional △6-pathway begins with the synthesis of γ-linolenic acid (GLA, 18:3$\Delta^{6,9,12}$) and stearidonic acid (SDA, 18:4$\Delta^{6,9,12,15}$) through △6-desaturation of LA (ω-6) and ALA (ω-3), which is catalyzed by △6-desaturase. Next, elongation of GLA and SDA in the presence of △6-elongase yields di-homo-γ-linolenic acid (DGLA, 20:3$\Delta^{8,11,14}$) and eicosatetraenoic acid (ETA, 20:4$\Delta^{8,11,14,17}$), respectively. ARA (ω-6) and EPA (ω-3) are formed from DGLA and ETA, respectively, by △5-desaturase. In the alternative △8-pathway, biosynthesis of omega-3 PUFAs starts with the △9-elongation of the same substrates as in the △6-pathway (LA and ALA) by △9-elongase, to form eicosadienoic acid (EDA, 20:2$\Delta^{11,14}$) and eicosatrienoic acid (ERA, 20:3$\Delta^{11,14,17}$), respectively. DGLA and ETA are subsequently generated through desaturation of these two △9-elongated fatty acids catalyzed by △8-desaturase. ARA and EPA are formed by △5-desaturation of DGLA and ETA, respectively, in the same way as in the conventional △6-pathway. In DHA-synthesizing microbes, △5/C20-elongase catalyzes △5-elongation of EPA to form docosapentaenoic acid (DPA, 22:5$\Delta^{7,10,13,16,19}$), followed by △4-desaturation of DPA for DHA (*n*-3) generation (Gong et al., 2014; Fig. 4).

In the anaerobic polyketide pathway (Fig. 5), PUFA biosynthesis starts with the condensation of malonyl-CoA and acetyl-acyl carrier protein (ACP) to form β-ketobutyryl-ACP, which is catalyzed by 3-ketoacyl synthase. Then, β-ketobutyryl-ACP is reduced to β-hydroxybutyryl-ACP by the NADPH-dependent 3-ketoacyl-ACP reductase. The bifunctional enzyme 3-hydroxyacyl-ACP dehydratase/isomerase catalyzes the dehydration of β-hydroxybutyryl-ACP to form trans-2-

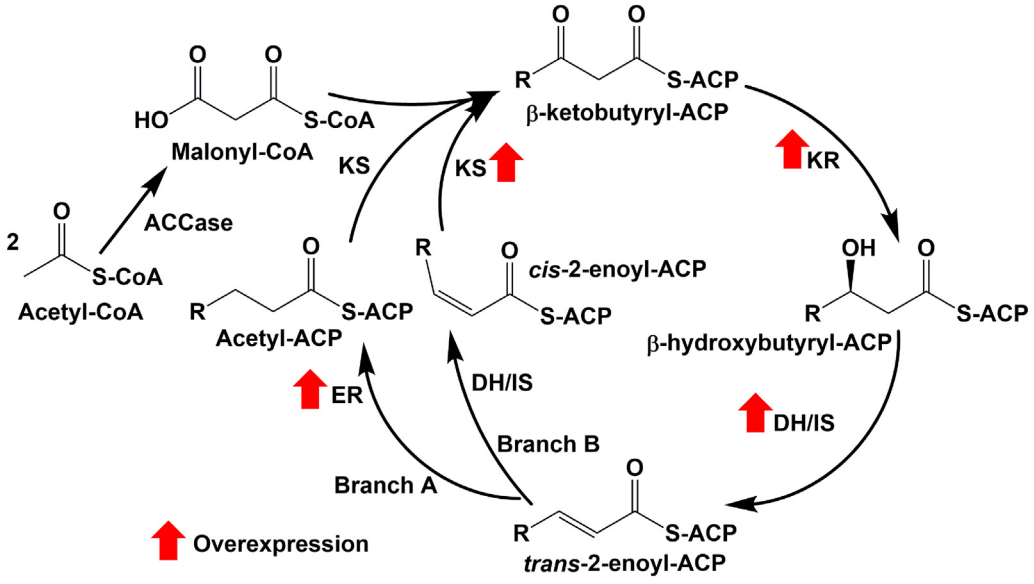

Fig. 5. Proposed polyketide pathway for omega-3 PUFAs biosynthesis with putative intermediates. In every cycle, 2 carbon atoms are added to the PUFAs chain. Branch A and Branch B are flowed through in different cycles with the result that one double bond was located at every 3 carbon atoms. For metabolic engineering production of DHA and EPA, heterologous expression of KS, KR, DH/IS, ER encoded by the gene cluster containing *pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE* in different hosts such as *E. coli* (Peng et al., 2016; Orikasa et al., 2006a), *Lactococcus lactis* subsp. *cremoris* (Amiri-Jami et al., 2014), and *Pseudomonas putida* (Gemperlein et al., 2016) were carried out. ACP, acyl carrier protein; ACCase, acetyl-CoA carboxylase; KS, β-ketoacyl-ACP synthase; KR, β-ketoacyl-ACP reductase; DH/IS, β-hydroxyacyl-ACP dehydratase/isomerase; ER, enoyl reductase.

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enoyl-ACP. In the end of the cycle, the double bond in the trans-2-enoyl-ACP intermediate is reduced by enoyl reductase, which also acts in some of the cycles. In each cycle, 2 carbon atoms are added to the fatty acyl chain, while each double bond is located at every 3 carbon atoms along the acyl chain. EPA and DHA are generated from their precursors after several cycles of the above biocatalytic process (Cao et al., 2012). In the bacteria such as *Shewanella baltica*, *Moritella marina* and *Colwellia psychrerythraea*, the enzymes of the polyketide pathway are encoded by the *pfa* operon containing the *pfaA* (encoding a multifunctional protein including domains of 3-ketoacyl synthase, malonyl-CoA: acyl carrier protein (ACP) acyltransferase, and six repeats of acyl carrier protein, 3-ketoacyl-ACP reductase), *pfaB* (encoding acyltransferase), *pfaC* (encoding a protein with domains of 3-ketoacyl synthase, chain length factor, and two 3-hydroxydecanoyl-ACP dehydratases), *pfaD* (encoding enoyl reductase) and *pfaE* (encoding phosphopantetheinyl transferase) genes (Amiri-Jami et al., 2015; Orikasa et al., 2006b; Peng et al., 2016).

3.2. DHA

3.2.1. DHA production by metabolic engineering of a native organism

P. tricornutum, a unicellular marine diatom, was metabolically engineered to produce DHA. In the wild-type strain, EPA could be produced up to 35% of the total fatty acids, but DHA level was rather low. Co-expression of △5-elongase OtElo5 and △6-desaturase OtD6 from the marine pitoalga *Ostreococcus tauri* in *P. tricornutum* UTEX646 increased the DHA content to 11% of total fatty acids, while the EPA content decreased to 18% of total fatty acids (Hamilton et al., 2014).

3.2.2. DHA production by metabolic engineering of heterologous hosts

There have been several attempts to produce DHA by metabolic engineering of heterologous microorganisms as summarized in Table 4. *E. coli* was metabolically engineered by the co-expression of the *pfaE* gene and the DHA biosynthetic cluster (pHDA3) containing the *pfaA*, *pfaB*, *pfaC* and *pfaD* genes from a marine bacterium *M. marina*. This engineered *E. coli* strain produced DHA to 5.2% of total fatty acids (Orikasa et al., 2006b). Furthermore, an engineered *E. coli* strain overexpressing a 35 kb cluster containing the *S. baltica* EPA/DHA biosynthetic genes was able to produce DHA and EPA up to 0.4% and 14% of total fatty acids, respectively, at 15 °C (Amiri-Jami and Griffiths, 2010). In another study, an engineered *E. coli* Nissle strain expressing the EPA/DHA gene cluster (*pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*) from *S. baltica* could produce EPA to the level of 31,360 μg g^{-1} DCW and DHA to <0.2% of the total extracted fatty acids (Amiri-Jami et al., 2015). When the *pfaABCD* genes and also the *C. psychrerythraea pfaE* gene were co-expressed in *E. coli*, DHA could be produced to a concentration of 2.4 mg l^{-1} (3400 μg g^{-1} DCW) in a medium containing 2 mg l^{-1} of cerulenin (Peng et al., 2016).

Besides *E. coli*, *Lactococcus lactis* subsp. *cremoris* has also been metabolically engineered for the heterologous production of DHA. The unnecessary genes located at the upstream and downstream of the EPA/DHA gene cluster from *S. baltica* were removed, and the remaining 20-kb gene cluster including the *pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE* genes was expressed in L. *lactis* subsp. *Cremoris*. This engineered L. *lactis* strain produced 1350 μg g^{-1} DCW of DHA and 120 μg g^{-1} DCW of EPA (Amiri-Jami et al., 2014). *Pseudomonas putida* was also metabolically engineered to produce DHA by expressing the artificial *pfa* gene cluster from *Aetherobacter fasciculatus*. The engineered *P. putida* strain produced 1400 μg g^{-1} DCW (3.0 mg l^{-1}) of DHA (Gemperlein et al., 2016).

Until now, most of the DHA heterologous production works have been conducted using bacteria as hosts through the overexpression of anaerobic polyketide pathway genes, such as *pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE* from different sources. An exceptional case was metabolic engineering of *S. cerevisiae* by overexpressing genes from the aerobic desaturation and elongation pathway carried out by Meyer et al. (2004). The △6-elongase from *Thalassiosira pseudonana*, △5-elongases from *O. tauri*, △5-desaturase from *P. tricornutum* and △4-desaturase from *Euglena gracilis* were co-expressed in *S. cerevisiae*, and DHA was produced up to 0.5% of total fatty acids using stearidonic acid as a substrate (Meyer et al., 2004). It is thus plausible that the metabolically engineered oleaginous yeast *Yarrowia lipolytica* will be able to produce DHA to a higher titer.

3.3. EPA

3.3.1. EPA production by metabolic engineering of native organisms

Research works on EPA production by metabolic engineering of native microorganisms are summarized in Table 5. Thraustochytrids are marine protists accumulating PUFAs with EPA as a minor constituent. Expression of fatty acids desaturase △5 from *Thraustochytrium aureum* in an EPA-producing bacterium *Aurantiochytrium limacinum* resulted in production of EPA up to 2.85% of total fatty acids (Kobayashi et al., 2011). *P. tricornutum* overexpressing its native △5 desaturase gene could produce EPA up to 38,900 μg g^{-1} DCW, which is 58% higher than that produced by the wild-type strain (Peng et al., 2014).

Table 4
DHA production in native organisms and heterologous hosts.

	Strain	Strategy	Fermentation mode/volume	Titer (mg l^{-1})	Yield (μg g^{-1} DCW)	Reference
Native	*P. tricornutum*	Co-expression of △5 elongase (OtElo5) and △6 desaturase (OtD6) from *Ostreococcus tauri*	N.A.	N.A.	18% EPA and 11% DHA of total fatty acids	Hamilton et al., 2014
Heterologous	*E. coli*	Co-expression of *pfaE* gene and DHA biosynthetic gene cluster (pHDA3) containing *pfaA, pfaB, pfaC, pfaD* from marine bacterium *Moritella marina*	Shake flask/50 ml	N.A.	5.2% of total fatty acids	Orikasa et al., 2006b
	E. coli	Overexpression of a 35 kb cluster containing EPA/DHA biosynthetic gene cluster from *Shewanella baltica*	Shake flask/200 ml	N.A.	0.4% DHA and 14% EPA of total fatty acids	Amiri-Jami and Griffiths, 2010
	E. coli	Expression of EPA/DHA biosynthetic gene cluster (*pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) from marine bacterium *S. baltica*	Shake flask/200 ml	N.A.	EPA of 31,360 and DHA <0.2% of total fatty acids	Amiri-Jami et al., 2015
	E. coli	Co-expression of *pfaABCD* and *pfaE* from *Colwellia psychrerythraea* with 2 mg/l cerulenin treatment	Shake flask/N.A.	2.4	3400	Peng et al., 2016
	S. cerevisiae	Co-expression of the △6 elongase from *Thalassiosira pseudonana*, △5 elongases from *O. tauri*, △5 desaturase from *P. tricornutum* and △4 desaturase from *Euglena gracilis*, in the presence of stearidonic acid as substrate	N.A.	N.A.	0.5% of total fatty acids	Meyer et al., 2004
	Lactococcus lactis subsp. *cremoris*	The unnecessary genes located upstream and downstream of the EPA/DHA biosynthetic gene cluster from *S. baltica* were removed, and the resulting 20-kb gene cluster (including *pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) was then expressed	Shake flask/200 ml	N.A.	1350 (DHA) 120 (EPA)	Amiri-Jami et al., 2014
	Pseudomonas putida	Overexpression of artificial *pfa* gene cluster from *Aetherobacter fasciculatus*	Shake flask/50 ml	3.0	1400	Gemperlein et al., 2016

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Table 5
EPA production in native organisms and heterologous hosts.

	Strain	Strategy	Fermentation mode/volume	Titer (mg l^{-1})	Yield (μg g^{-1} DCW)	Reference
Native	*Mortierella alpina*	Overexpression of the ω3 desaturase gene	N.A.	N.A.	42.1% of the total fatty acids	Ando et al., 2009
	Aurantiochytrium limacinum	Expression of fatty acids desaturase △5 from *Thraustochytrium aureum* supplied with 0.1 mM ETA	Shake flask/5 ml	N.A.	2.85% of total fatty acids	Kobayashi et al., 2011
	P. tricornutum	Overexpression of the △5 desaturase gene	N.A.	N.A.	38,900	Peng et al., 2014
	M. alpina	Expression of △17 desaturase gene (*sdd17m*) from *Saprolegnia diclina* and cultured under ordinary temperature	Bioreactor/5 l	1800	N.A.	Okuda et al., 2015
	M. alpina	Expression of △6 desaturase gene from *Micromonas pusilla* and peony seed meal was used as source of α-linolenic acid	Bioreactor/5 l	588.5	N.A.	Shi et al., 2016
Heterologous	*E. coli*	Overexpression of five ORFs (*pfaA-E*) essential for EPA biosynthesis and the phosphopantetheinyl transferase (PPTase) from *Shewanella* sp.	N.A.	N.A.	22% of the total fatty acids	Orikasa et al., 2004
	E. coli	Heterologous expression of an EPA biosynthetic gene cluster from *Shewanella oneidensis*	N.A.	N.A.	0.689% of the total fatty acids	Lee et al., 2006
	E. coli	Co-expression of *pfaA*, *pfaB*, *pfaC*, *pfaD* from *Shewanella pneumatophori*, and *pfaE* from *M. marina*	Shake flask/N.A.	N.A.	11.6% of total fatty acids	Orikasa et al., 2006a
	E. coli	Co-expression of EPA biosynthesis genes from *Shewanella* sp. and a high-performance catalase gene (*vktA*) from *Vibrio rumoiensis*	Shake flask/N.A.	7.3	N.A.	Orikasa et al., 2007
	E. coli	Overexpression of EPA biosynthetic gene cluster from *S. oneidensis* and regulated by a strong *lacZ* promoter	N.A.	N.A.	7.5% of total fatty acids	Lee et al., 2008
	S. cerevisiae	Co-expression of △5 desaturase from *M. alpina*, △6 desaturase from *Borago officinalis* and △6 specific elongase from *Caenorhabditis elegans*, in the presence of α-linolenic acid as substrate	N.A.	N.A.	0.2% of total fatty acids	Beaudoin et al., 2000
	S. cerevisiae	Co-expression of △6 and △5 desaturases from *P. tricornutum* and △6-specific elongase from *Physcomitrella patens*, in the presence of α-linolenic acid as substrate	N.A.	N.A.	0.23% of total fatty acids	Domergue et al., 2002
	S. cerevisiae	Expression of a novel ω3 fatty acids desaturase from *S. diclina*	N.A.	N.A.	6.25% of total fatty acids	Pereira et al., 2004
	S. cerevisiae	Co-expression of △6 desaturase from the *O. tauri*, △6 elongase from *P. patens* and △5 desaturase from *P. tricornutum*	N.A.	N.A.	4.5% of total fatty acids	Domergue et al., 2005
	S. cerevisiae	Overexpression of △6 Des, △9 Des, △12 Des, ω3 Des, and △6 Elo from *Paramecium tetraurelia*	N.A.	N.A.	0.49% of the total fatty acids	Tavares et al., 2011
	Yarrowia lipolytica	Strain Y4305 was constructed by introduction of 30 copies of 9 different genes [encoding C16E, △12 Des, △9 Elo, △9 Elo(2), △8 Des, △8 Des(2), △5 Des(3), △17 Des(3), CPT] from different organisms	N.A.	N.A.	150,000, 56.6% of total fatty acids	Xue et al., 2013
	Y. lipolytica	A complex constructing process to obtain Strain Z5567 containing 41 copies of 19 different genes from different organisms	N.A.	N.A.	250,000, 50% of total fatty acids	Hong et al., 2011
	Pichia pastoris	Co-expression of △6 desaturase, △6 fatty acids elongase, and △5 desaturase from *P. tricornutum*	N.A.	N.A.	0.1% of the total fatty acids	Li et al., 2009
	Synechococcus sp.	Expression of EPA biosynthetic gene cluster from *Shewanella putrefaciens*	N.A./N.A.	N.A.	640	Takeyama et al., 1997
	Synechococcus sp.	Expression of EPA biosynthetic gene cluster from *Shewanella* sp.	N.A./N.A.	3.86	N.A.	Yu et al., 2000

Mortierella alpine, an oleaginous fungus, was also metabolically engineered to produce EPA. *Agrobacterium tumefaciens*-mediated transformation of *M. alpina* 1S-4 was established. Using this novel transformation method, overexpression of an omega-3 desaturase gene in *M. alpina* increased the EPA titer from <7% to 42.1% of total fatty acids (Ando et al., 2009). When the △17 desaturase gene (*sdd17m*) from *Saprolegnia diclina* was expressed in *M. alpina*, EPA could be produced to a concentration of 1800 mg l^{-1} (Okuda et al., 2015). Expresion the *Micromonas pusilla* △6 desaturase gene in *M. alpina* strain resulted in production of 588.5 mg l^{-1} of EPA in a medium containing 50 g l^{-1} peony seed meal (Shi et al., 2016).

3.3.2. EPA production by metabolic engineering of heterologous hosts

There have been several attempts to produce EPA by metabolic engineering of heterologous microorganisms as well (Table 5). The polyketide pathway for EPA biosynthesis has been heterologously overexpressed in *E. coli* (Orikasa et al., 2004; Orikasa et al., 2007; Lee et al., 2008). An engineered *E. coli* strain overexpressing five open reading frames (ORFs) *pfaA-E* essential for EPA biosynthesis and the phosphopantetheinyl transferase (PPTase) from *Shewanella* sp. was able to produce EPA up to 22% of total fatty acids (Orikasa et al., 2004). Heterologous expression of *Shewanella oneidensis* EPA biosynthetic gene cluster in *E. coli* resulted in production of EPA to 0.689% of the total fatty acids (Lee et al., 2006). When the *Shewanella pneumatophori pfaA*, *pfaB*, *pfaC* and *pfaD* genes together with the *M. marina pfaE* gene were expressed in *E. coli*, EPA could be produced to 11.6% of total fatty acids (Orikasa et al., 2006a). Co-expression of the *Shewanella* sp. EPA biosynthetic genes and the *Vibrio rumoiensis* catalase gene (*vktA*) in *E. coli* resulted in production of 7.3 mg l^{-1} of EPA (Orikasa et al., 2007). When the *S. oneidensis* EPA biosynthetic gene cluster was overexpressed under a strong *lacZ* promoter in *E. coli*, EPA could be produced up to 7.5% of total fatty acids (Lee et al., 2008).

Besides *E. coli*, yeasts including *S. cerevisiae* (Tavares et al., 2011), *Y. lipolytica* (Hong et al., 2011), and *Pichia pastoris* (Li et al., 2009), have also been metabolically engineered for the heterologous production of EPA. Co-expression of △5 desaturase from *M. alpina*, △6 desaturase from *Borago officinalis* and △6-specific elongase from *Caenorhabditis elegans* in *S. cerevisiae* enabled production of EPA up to 0.2% of total fatty acids in the presence of α-linolenic acid as a substrate (Beaudoin et al., 2000). When the △6 and △5 desaturase genes from *P. tricornutum* and the △6-specific elongase gene from *Physcomitrella patens* were co-expressed in *S. cerevisiae*, EPA could be produced up to 0.23% of total fatty acids in the presence of α-linolenic acid (Domergue et al., 2002). An engineered *S. cerevisiae* strain expressing a novel *S. diclina* omega-3

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fatty acids desaturase gene was able to produce EPA up to 6.25% of total fatty acids (Pereira et al., 2004). In another study, an engineered *S. cerevisiae* strain co-expressing the Δ6 desaturase gene from *O. tauri*, the Δ6 elongase gene from *P. patens* and the Δ5 desaturase gene from *P. tricornutum* could produce EPA up to 4.5% of total fatty acids (Domergue et al., 2005). Overexpression of the Δ6-Des, Δ9-Des, Δ12-Des, ω3-Des and Δ6-Elo genes from *Paramecium tetraurelia* in *S. cerevisiae* resulted in production of EPA to 0.49% of total fatty acids (Tavares et al., 2011). Production of EPA by *P. pastoris* was demonstrated by the co-expression of the Δ6 desaturase gene, Δ6 fatty acids elongase gene, and Δ5 desaturase gene from *P. tricornutum*. The engineered *P. pastoris* produced EPA to the level of 0.1% of total fatty acids (Li et al., 2009).

As an oleaginous yeast, *Y. lipolytica* is an excellent host for EPA production. In particular, the engineeried *Y. lipolytica* Y4305 and Z5567 strains have been used for commercial production of EPA (Xue et al., 2013; Hong et al., 2011). An engineered *Y. lipolytica* Y4305 for commercial production of EPA was constructed by 8-step metabolic engineering. Strain Y4305 was engineered to contain 30 copies of 9 different genes [encoding C16E, Δ12D, Δ9E, Δ9E(2), Δ8D, Δ8D(2), Δ5D(3), Δ17D(3), CPT] from different organisms, which produced EPA to an impressively high level (56.6% of total fatty acids, representing 15% of DCW). Furthermore, the total saturated fatty acids, C16:0 and C18:0, were only 4.1% of the total fatty acids (Xue et al., 2013). Another commercial strain *Y. lipolytica* Z5567 for EPA production contained 41 copies of 19 different genes and produced EPA at 50% of the total fatty acids, equivalent to about 25% of DCW (Hong et al., 2011).

Moreover, metabolic engineering of cyanobacterium for the heterologous production of EPA has also been attempted. Expression of the *Shewanella putrefaciens* EPA biosynthetic gene cluster in marine cyanobacterium *Synechococcus* sp. resulted in the production of 640 μg g^{-1} DCW of EPA (Takeyama et al., 1997). In another work similary performed, heterologous expression of the *Shewanella* sp. EPA biosynthetic gene cluster in *Synechococcus* sp. resulted in production of 3.86 mg l^{-1} EPA (Yu et al., 2000).

By using *E. coli* as a host, the heterologous production pathway of EPA was constructed by overexpressing the anaerobic polyketide pathway involving *pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE* genes from different sources. When yeasts are used as host strains, many genes in aerobic desaturation and elongation pathway from different sources were overexpressed. The excellent commercial strains *Y. lipolytica* Y4305 (Xue et al., 2013) and *Y. lipolytica* Z5567 (Hong et al., 2011) demonstrate that successful metabolic engineering requires a suitable host (such as oleaginous yeast) and the design of system-wide engineering strategies involving many genes.

4. Marine natural drugs

There are numerous marine bioactive compounds exhibiting antimicrobial, antitumor and cytotoxic activities. These compounds are of various molecular types including peptides, polyketides, alkaloids, and benzopyrans (Ross et al., 2015; Piel et al., 2000; Li et al., 2013). These marine bioactive compounds can be used as natural drugs, and have recently been attracting much attention. In this section, we review production of marine natural drugs by metabolic engineering of different marine microorganisms including cyanobacteria, actinobacteria and others (Table 6; Kim et al., 2012a; Yamanaka et al., 2014; Tianero et al., 2012).

4.1. Marine natural drugs from cyanobacteria

Cyanobacteria are important producers of many marine natural drugs. Metabolic engineering of cyanobacteria for the enhanced production of marine natural drugs such as 4-O-demethylbarbamide (Kim et al., 2012a), lyngbyatoxin (Zhang et al., 2016), lyngbyatoxin (Long et al., 2005) and trunkamide (Tianero et al., 2012) has been performed.

Barbamide is a molluscicidal agent, the biosynthetic pathway of which was characterized in a marine cyanobacterium *Moorea producens*. A new barbamide congener 4-O-demethylbarbamide (<1 μg l^{-1}) was detected in *Streptomyces venezuelae* heterologously expressing the barbamide biosynthetic gene cluster from *M. producens* (Kim et al., 2012a).

Lyngbyatoxin is an indole alkaloid and a cyanotoxin isolated from the cyanobacterium *M. producens* (Ongley et al., 2013). An engineered *E. coli* expressing the whole lyngbyatoxin biosynthetic pathway from *M. producens* could produce 25.6 mg l^{-1} of lyngbyatoxin (Ongley et al., 2013). Overexpression of the *M. producens ltxA-C* genes encoding the lyngbyatoxin biosynthetic pathway using P*glnA* as promoter in the freshwater cyanobacterium *Anabaena* sp. PCC 7120 resulted in production of 3.2 mg l^{-1} (2307.2 μg g^{-1} DCW) of lyngbyatoxin (Videau et al., 2016). The marine *Streptomyces* lyngbyatoxin biosynthetic gene cluster *tleABC* was heterologously expressed in the hosts *S. lividans* and *S. avermitilis*, which resulted in production of 2.0 mg l^{-1} and 0.2 mg l^{-1} of lyngbyatoxin, respectively (Zhang et al., 2016).

Patellamides are didemnid ascidian natural products with potent bioactivities. *Prochloron* sp. is a photosynthetic endosymbiont of the ascidian *Lissoclinum patella*, The *Prochloron* sp. patellamide biosynthetic gene cluster was cloned and successfully expressed in *E. coli*. This engineered *E. coli* strain could produce 77 μg l^{-1} of patellamide D in shake-flask culture; also, ascidiacylamide (94 μg l^{-1}) was produced (Long et al., 2005). In another study, an engineered *E. coli* strain expressing the *Prochloron didemni* patellamide biosynthetic gene cluster could produce 20 μg l^{-1} of patellamide A and a trace amount of patellamide C (Schmidt et al., 2005).

Patellin and trunkamide are both cyanobactins naturally biosynthesized by cyanobacteria. Trunkamide is a prenylated antitumor preclinical candidate (Donia et al., 2008). When the *Prochloron* sp. *tru* cluster for patellin biosynthesis was heterologously expressed in *E. coli*, patellin 2 and patellin 3 were produced to a total concentration of 100 μg l^{-1}. On the other hand, trunkamide production was detected in a 10 l fermentation of an engineered *E. coli* heterologously expressing the *Prochloron* sp. *tru* cluster with *truE2* replaced by *truE1* (Donia et al., 2008). In their followup study, the *Prochloron* sp. *tru* operon was heterologously expressed under the constitutive *lac* promoter in *E. coli* and the *truE* precursor peptide gene was co-expressed in another vector. This engineered *E. coli* strain produced all three cyanobactins: 13.7 μg l^{-1} of patellin 2, 10.3 μg l^{-1} of patellin 3 and 9.9 μg l^{-1} of trunkamide (Donia et al., 2011). In another study, multiple non-proteinogenic amino acids and tandem mutations were incorporated into the cyanobactin ribosomal peptide pathway, which was then heterologously expressed in *E. coli*. The engineered *E. coli* strain produced many kinds of peptides such as mollamide (700 μg l^{-1}), keenamide (400 μg l^{-1}), patellin 2 (500 μg l^{-1}), patellin 4 (170 μg l^{-1}), patellin 5 (500 μg l^{-1}), trunkamide (350 μg l^{-1}), ulithiacyclamide (24 μg l^{-1}) and patellamide (78 μg l^{-1}) (Tianero et al., 2012).

4.2. Marine natural drugs from actinobacteria

Marine actinobacteria, especially marine *Streptomyces*, are another important resource of marine natural drugs (Lechner et al., 2011; Piel et al., 2000). There have already been many reports on metabolic engineering of marine actinobacteria for the production of marine natural drugs, such as, enterocin (Bonet et al., 2014), griseorhodin A (Li and Piel, 2002), indolocarbazoles (Li et al., 2013), PM100117 and PM100118 (Salcedo et al., 2016b), salinosporamide A (Lechner et al., 2011), taromycin A (Yamanaka et al., 2014), thiocoraline (Lombó et al., 2006) and Xiamenmycin C/D (You et al., 2013).

Enterocin is a bacteriostatic polyketide found in both terrestrial and marine bacteria (Aymerich et al., 1996; Piel et al., 2000). The *Streptomyces maritimus enc* cluster encoding enterocin was heterologously expressed in the *Streptomyces lividans* K4-114 strain. This engineered strain could produce enterocin as the major polyketide metabolite

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Table 6
Production of marine natural drugs by metabolic engineering and synthetic biology.

Compound	Strain	Strategy	Fermentation mode/volume	Titer ($\mu g\ l^{-1}$)	Yield ($\mu g\ g^{-1}$ DCW)	Reference
4-O-demethylbarbamide	*Streptomyces venezuelae*	Heterologous expression of the barbamide biosynthetic gene cluster from *Moorea producens*	N.A./N.A.	<1	N.A.	Kim et al., 2012a
Alterochromide	*E. coli*	Heterologous expression of the ~34 kb alterochromide lipopeptides pathway gene cluster from *Pseudoalteromonas piscicida*	Shake flask/50 ml	N.A.	N.A.	Ross et al., 2015
Ascidiacylamide	*E. coli*	Heterologous expression of the patellamide biosynthetic gene cluster from *Prochloron* sp.	Shake flask/40 ml	94	N.A.	Long et al., 2005
Enterocin	*Streptomyces lividans* K4–114	Heterologous expression of the *enc* cluster for enterocin biosynthesis from the marine bacterium *Streptomyces maritimus*	N.A./N.A.	N.A.	N.A.	Piel et al., 2000
Enterocin	*Streptomyces*	Overexpression of type II polyketide synthase pathway from *Salinispora pacifica* in *Streptomyces*	N.A./N.A.	N.A.	N.A.	Bonet et al., 2014
Fluostatin	*Streptomyces coelicolor*	Heterologous expression of the *fls* gene cluster encoding atypical angucycline fluostatins biosynthetic pathway from the marine-derived *Micromonospora rosaria* SCSIO N160	N.A./N.A.	N.A.	N.A.	Yang et al., 2015
Griseorhodin A	*S. lividans*	Heterologous expression of the *grh* gene cluster for griseorhodin biosynthesis from the marine derived *Streptomyces* sp. J95	N.A./N.A.	N.A.	N.A.	Li and Piel, 2002
Indolocarbazole	*S. coelicolor*	Heterologous expression of the indolocarbazole biosynthetic gene cluster from the marine-derived *Streptomyces sanyensis*	N.A./N.A.	N.A.	N.A.	Li et al., 2013
Lyngbyatoxin	*E. coli*	Overexpression of the whole lyngbyatoxin biosynthetic pathway from *M. producens*	Shake flask/50 ml	25.6	N.A.	Ongley et al., 2013
Lyngbyatoxin	*Anabaena* sp.	Overexpression of *ltxA-C* from *M. producens* under the promoter P*glnA*	Shake flask/100 ml	3.2	2307.2	Videau et al., 2016
Lyngbyatoxin	*S. lividans*	Overexpression of *tleABC* from *Streptomyces blastmyceticus*	N.A./N.A.	2.0	N.A.	Zhang et al., 2016
Lyngbyatoxin	*Streptomyces avermitilis*	Overexpression of *tleABC* from *S. blastmyceticus*	N.A./N.A.	0.2	N.A.	Zhang et al., 2016
Mollamide Keenamide Patellin 2 Patellin 4 Patellin 5 Trunkamide Ulithiacyclamide Patellamide	*E. coli*	The cyanobactin ribosomal peptide natural product pathway was manipulated to incorporate multiple tandem mutations and non-proteinogenic amino acids, then heterologous expressed in *E. coli*	Bioreactor/10 l	700 400 500 170 500 350 24 78	N.A.	Tianero et al., 2012
Patellamides A	*E. coli*	Heterologous expression of the patellamide biosynthetic gene cluster from *Prochloron didemni*	Shake flask/1 l	20	N.A.	Schmidt et al., 2005
Patellamides C	*E. coli*	Heterologous expression of the patellamide biosynthetic gene cluster from *P. didemni*	Shake flask/1 l	N.A.	N.A.	Schmidt et al., 2005
Patellamide D	*E. coli*	Heterologous expression of the patellamide biosynthetic gene cluster from *Prochloron* sp.	Shake flask/40 ml	77	N.A.	Long et al., 2005
Patellin 2/ Patellin 3	*E. coli*	Heterologous expression of the *tru* cluster for patellin synthesis from *Prochloron* sp.	Bioreactor/10 l	100	N.A.	Donia et al., 2008
Patellin 2 Patellin 3 Trunkamide	*E. coli*	The *tru* operon from *Prochloron* sp. under the constitutive *lac* promoter was co-expressed with *truE* precursor peptide gene from *Prochloron* sp.	N.A./N.A.	13.7 10.3 9.9	N.A.	Donia et al., 2011
PM100117/PM100118	*Streptomyces caniferus*	Deletion of *gonCP* encoding a putative cytochrome P450	N.A./N.A.	N.A.	N.A.	Salcedo et al., 2016b
PM100117/PM100118	*S. caniferus*	Deletion of putative glycosyltranferase genes	N.A./N.A.	N.A.	N.A.	Salcedo et al., 2016a
Salinosporamide A	*Salinispora tropica*	Overexpression of *salR2* encoding salinosporamide A-specific pathway regulator	N.A./N.A.	65,000	N.A.	Lechner et al., 2011
Taromycin A	*S. coelicolor*	Heterologous expression of a silent lipopeptide biosynthetic gene cluster from *Saccharomonospora* sp.	Shake flask/1 l	1000	N.A.	Yamanaka et al., 2014
Thiocoraline	*Streptomyces* sp.	Overexpression of a region of about 53 kb, containing 26 ORFs for thiocoraline synthesis in *Micromonospora* sp.	N.A./N.A.	N.A.	N.A.	Lombó et al., 2006
Trunkamide	*E. coli*	Heterologous expression of the *tru* cluster from *Prochloron* sp. with the *truE2* replaced by *truE1*	Bioreactor/10 l	N.A.	N.A.	Donia et al., 2008
Xiamenmycin C/D	*Streptomyces xiamenensis*	Ribosome engineering: screening for spontaneous rifampicin resistance + integration of *rpsL* gene to confer streptomycin resistance	Bioreactor/33 l	N.A.	N.A.	You et al., 2013

(Piel et al., 2000). In another work, heterologous expression of type II polyketide synthase pathway from marine actinomycete *Salinispora pacifica* in *Streptomyces* also enabled enterocin production (Bonet et al., 2014).

Griseorhodin A, a telomerase inhibitor, is a bacterial natural product belonging to aromatic polyketides. The *grh* gene cluster for griseorhodin biosynthesis was characterized in the marine *Streptomyces* sp. J95 strain. Integration of the *grh* cluster in the chromosome of *S. lividans* ZX1 enabled production of a trace amount of griseorhodin A (Li and Piel, 2002).

Indolocarbazoles has attracted attention due to their biological activities and potential therapeutic applications. The *Streptomyces sanyensis* indolocarbazole biosynthetic gene cluster was heterologously expressed in *S. coelicolor*, which resulted in the production of a minute amount of staurosporine, one kind of indolocarbazole, detectable by HPLC-MS (Li et al., 2013).

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PM100117 and PM100118 are glycosylated polyketides with antitumor properties, and are produced by a marine actinobacterium *Streptomyces caniferus*. Deletion of the *gonCP* gene encoding a putative cytochrome P450 resulted in the production of two derivatives of PM100117/PM100118, both of which exhibited improved antitumor activities (Salcedo et al., 2016b). Similarly, some novel derivatives of PM100117/PM100118 were produced by *S. caniferus* by deleting a putative glycosyltranferase gene (Salcedo et al., 2016a).

As a potent 20S proteasome inhibitor, the chlorinated natural product salinosporamide A is currently utilized as an anticancer agent. The *salR2* gene encoding salinosporamide A-specific pathway regulator was characterized and overexpressed in the marine bacterium *Salinispora tropica*, which increased production of salinosporamide A to a titer of 65,000 $\mu g\ l^{-1}$ (Lechner et al., 2011).

Taromycin A is a dichlorinated lipopeptide antibiotic. A silent lipopeptide biosynthetic gene cluster (67 kb) from a marine actinobacterium *Saccharomonospora* sp. CNQ-490 was directly cloned and refactored in *S. coelicolor*. This resulted in successful production of 1 $mg\ l^{-1}$ of taromycin (Yamanaka et al., 2014).

Thiocoraline, a twofold-symmetric bicyclic nonribosomal octathiodepsipeptide with antitumor activity, was produced by an actinobacterium *Micromonospora* sp. isolated from marine invertebrate (Baz et al., 1997). A 53 kb thiocoraline biosynthesis gene cluster was isolated and characterized in *Micromonospora* sp. ML1. *Streptomyces* sp. expressing this gene cluster containing 26 ORFs has been shown to produce thiocoraline (Lombó et al., 2006).

Benzopyran derivatives have various bioactivities. For example, xiamenmycin, a benzopyran compound, was found to suppress inflammation (Liu et al., 2013) and to inhibit fibrosis (You et al., 2013). Ribosome engineering was carried out in *Streptomyces xiamenensis* isolated from deep-sea sediments to discover new anti-fibrotic compounds. A mutant M1-5R22 with spontaneous rifampicin resistance was screened, and then the *rpsL* gene responsible for streptomycin resistance was integrated into the chromosome of mutant M1-5R22 to obtain mutant M6. This engineered M6 strain could produce two novel benzopyran compounds xiamenmycin C and xiamenmycin D. Xiamenmycin C exhibited better anti-fibrotic activity than xiamenmycin (You et al., 2013).

4.3. Marine natural drugs from other marine bacteria

Besides actinobacteria, there are also some other marine bacteria producing natural drugs, such as the alterochromide-producing *Pseudoalteromonas piscicida* (Ross et al., 2015) and the fluostatin-producing *Micromonospora rosaria* (Yang et al., 2015).

Alterochromides are natural lipopeptides with antibacterial and cytotoxic properties. The *P. piscicida* alterochromide lipopeptides biosynthetic gene cluster (~34 kb) was heterologously expressed in *E. coli*, which allowed detection of trace amount of alterochromide production by LCMS UV (Ross et al., 2015).

Fluostatins, natural compounds belonging to an atypical angucyclines family, exhibit varying degrees of dipeptidyl peptidase inhibition, antitumor, and antibacterial activities. The *fls* gene cluster encoding atypical angucycline fluostatins was characterized in *M. rosaria* SCSIO N160. Heterologous expression of the *fls* gene cluster in *Streptomyces coelicolor* YF11 resulted in the discovery of two novel compounds, fluostatin L and difluostatin A (Yang et al., 2015).

In the case of marine natural drugs, there have only been limited metabolic engineering studies. One successful example of metabolic engineering for marine natural drugs production was conducted in a native organism (Lechner et al., 2011). Characterization and expression of the salinosporamide A-specific pathway regulatory gene *salR2* in the marine bacterium *S. tropica* enabled the engineered strain to produce salinosporamide A to a level that is 2-fold higher than that produced by the wild-type strain. Microbial marine natural drugs have also been produced by metabolically engineered heterologous microorganisms. Heterologous expression of the lyngbyatoxin biosynthetic pathway from *M. producens* in *E. coli* and *Anabaena* sp. was carried out (Ongley et al., 2013; Videau et al., 2016). Similarly, the lyngbyatoxin biosynthetic pathway from *S. eticus* was heterologously expressed in *S. lividans* and *S. avermitilis*, respectively (Zhang et al., 2016). The highest lyngbyatoxin concentrations obtained with these engineered *E. coli* and *Streptomyces* were 25.6 $\mu g\ l^{-1}$ and 2.0 $\mu g\ l^{-1}$, respectively. These results demonstrate the potential of *E. coli* and *Streptomyces* as hosts for the heterologous production of marine natural drugs. Further progress in our understanding on the metabolic pathways and regulatory networks involved in marine natural drugs biosynthesis will allow better design of strategies for metabolic engineering and synthetic biology towards enhanced production of these natural drugs of marine source.

5. Concluding remarks and perspectives

Metabolic engineering and synthetic biology have demonstrated great potential for enhanced production of novel bioactive compouds. This is best exemplified by the successful reconstruction of the engineered *Y. lipolytica* strain Y4305 (containing 30 copies of 9 different genes from different organisms) for commercial production of EPA (Xue et al., 2013). For many marine natural products, however, production titers are still quite low due to their complex structures and complicated biosynthetic pathways. Based on many great works described above, the following general strategies can be suggested for production of marine bioactive compounds by metabolic engineering and synthetic biology. First, the metabolic pathways and enzymes (genes) involved in biosynthesis of many of these compounds need to be better understood as they have not yet been thoroughly studied. Also, alternative pathways and enzymes potentially more efficient in biosynthesizing the marine bioactive compounds of interest need to be identified. Different isozymes of important pathway enzymes can be compared for their effects on product biosynthesis. As in other metabolic engineering studies, directed evolution of key enzymes for improved charactersitcs and identification of rate-controlling step enzymes are needed. Better host strains can be chosen based on the availability of precursors or intermediate metabolites of the final product. Indeed, these strategies have been employed for developing metabolically engineered strains for the production of astaxanthin, squalene, DHA and EPA discussed above. Refactoring of the biosynthetic gene clusters through synthetic biology will be increasingly needed. Such metabolic engineering and synthetic biology studies are scarce for the production of many marine natural drugs, mainly because their biosynthetic pathways and enzymes involved in have not been well characterized. Synthetic biology will provide a promising solution for the enhanced production of valuable marine bioactive compounds such as ganglioside, caerulomycin, diketopiperazine, curacin, apratoxin, and others. This is because most of the marine natural compounds are produced through a multi-step, complex biosynthetic pathways. Modular construction, pathway refactoring and flux optimization need to be performed in a best selected host strain, which will be followed by systems metabolic engineering (Lee et al., 2012; Lee and Kim, 2015) for optimization of product formation while considering fermentation as well as downstream processes. Vigorous efforts for establishing the genetic manipulation systems in marine bacterium provide new avenues for application of robust metabolic engineering tools as CRISPR/Cas system (Choi and Lee, 2016). Once more diverse marine bioactive compounds are produced, they can be exteremly valuable for examining their potential synergistic actions as demonstrated for the multi-component multi-target approaches of traditional oriental medicine (Kim et al., 2015). All such effort will undoubtedly enable production of more diverse marine bioactive compounds of complicated structure for their use in medicinal, nutritional, cosmetic and many other applications in the near future.

Abbreviations

EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid

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Omega-3 PUFAs	omega-3 polyunsaturated fatty acids
MVA	mevalonate pathway
MEP	2-C-methyl-D-erythritol 4-phosphate pathway
AACT	acetoacetyl-CoA thiolase
DXP	1-deoxyxylulose-5-phosphate pathway
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
IPP	isopentenyl diphosphate
DMAPP	dimethylallyl diphosphate
IDI	isopentanyl diphosphate isomerase
FPP	farnesyl diphosphate
DXP	1-deoxyxylulose-5-phosphate
DXS	1-deoxyxylulose-5-phosphate synthase
DXR	DXP reductoismeras
HMGS	HMG-CoA synthase
HMGR	HMG-CoA reductase
GGPP	geranylgeranyl pyrophosphate
GA3P	3-phosphoglycerate
DRL	DXR-like enzyme
HMBPP	4-hydroxy-3-methyl-but-2-enyl pyrophosphate
SQS	squalene synthase
PSPP	presqualene diphosphate
HSQ	(*R*)-12-hydroxysqualene
SQE	squalene epoxidase
SHC	squalene hopene cyclase
OA	oleic acid
LA	linoleic acid
GLA	γ-linolenic acid
AA	arachidonic acid
ALA	α-linolenic acid
SDA	stearidonic acid
DGLA	di-homo-γ-linolenic acid
ETA	eicosatetraenoic acid
EDA	eicosadienoic acid
ERA	eicosatrienoic acid
DPA	docosapentaenoic acid
ACP	acyl carrier protein
ACCase	acetyl-CoA carboxylase
KS	β-ketoacyl-ACP synthase
KR	β-ketoacyl-ACP reductase
DH/IS	β-hydroxyacyl-ACP dehydratase/isomerase
ER	enoyl reductase

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