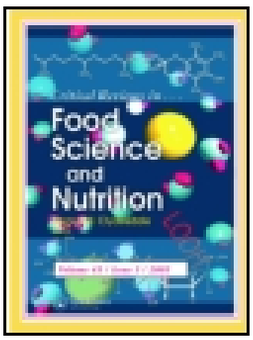

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High-value bioproducts from microalgae: strategies and progress

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Abstract

Microalgae have been considered as alternative sustainable resources for high-value bioproducts such as lipids (especially triacylglycerides [TAGs]), polyunsaturated fatty acids (PUFAs), and carotenoids, due to their

relatively high photosynthetic efficiency, no arable land requirement, and ease of scale-up. It is of great significance to exploit microalgae for the production of high-value bioproducts. How to improve the content or productivity of specific bioproducts has become one of the most urgent challenges. In this review, we will describe high-value bioproducts from microalgae and their biosynthetic pathways (mainly for lipids, PUFAs, and carotenoids). Recent progress and strategies for the enhanced production of bioproducts from microalgae are also described in detail, and these strategies take advantages of optimized cultivation conditions with abiotic stress, chemical stress (addition of metabolic precursors, phytohormones, chemical inhibitors, and chemicals inducing oxidative stress response), and molecular approaches such as metabolic engineering, transcriptional engineering, and gene disruption strategies (mainly RNAi, antisense RNA, miRNA-based knockdown, and CRISPR/Cas9).

Keywords: microalgae; high-value bioproducts; omics; abiotic stress; metabolic engineering; gene knockout; carotenoid; lipid; LC-PUFA.

1. Introduction

Bioproducts or bio-based products are materials, chemicals and energies derived from renewable biological resources. Microalgae have been considered as an alternative sustainable resource for high-value bioproducts such as lipids (especially triacylglycerides (TAGs)), polyunsaturated fatty acids (PUFAs), sterols, carbohydrates, proteins, polysaccharides, and terpenoids including carotenoids. The diverse bioproducts from microalgae can be widely used for pharmaceuticals, nutraceuticals, food colorants, animal feed, and biofuels. Oleaginous microalgae, such as *Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., and *Dunaliella tertiolecta* can accumulate large amounts of neutral lipids, which can be up to 20 ~ 80% of the dry cell weight (DCW) (Liang and Jiang 2013). Microalgal lipids (mainly neutral lipids, i.e. TAGs) have been considered as the promising sources for biodiesel production. In the presence of a catalyst, TAGs can act with alcohol by transesterification to generate fatty acid methyl ester (FAME, biodiesel) and the by-product glycerol (Liang and Jiang 2013). Some microalgal species, such as *Crypthecodinium cohnii* (Sijtsma *et al.* 2010), *Nannochloropsis oceanica* (Udayan and Arumugam 2017), and *Phaeodactylum tricornutum* (Balamurugan *et*

al. 2017), can produce high-value long-chain PUFAs (LC-PUFAs), such as ω-3 PUFAs docosahexaenoic acid (DHA, $22:6^{\Delta4,7,10,13,16,19}$), eicosapentaenoic acid (EPA, $20:5^{\Delta5,8,11,14,17}$), and α-linolenic acid (ALA, $18:3^{\Delta9,12,15}$) as well as ω-6 PUFA arachidonic acid (ARA, $20:4^{\Delta5,8,11,14}$). These essential fatty acids are beneficial to human health. DHA and EPA can be effective in preventing or treating many diseases including cardiovascular disease, brain disorder, cancer, and diabetes (Cao *et al.* 2012, Gong *et al.* 2014). The microalgal strains *Dunaliella salina* and *Haematococcus pluvialis* have been commercially used for the production of β-carotene (Raja *et al.* 2007, Ye *et al.* 2008) and astaxanthin (Lorenz and Cysewski 2000), respectively. In addition, the cyanobacterium *Arthrospira* (*Spirulina*) and the green algae *Chlorella* can be exploited as nutraceuticals and health food additives (Leu and Boussiba 2014). Phycocyanin extracted from cyanobacteria can be used in the fields of foods and cosmetics, and medicine (Eriksen 2008).

Microalgae have relatively high photosynthetic efficiency and do not require arable land and can be cultivated in marine environments including fresh water, salt water, or even waste water. They are sustainable alternatives for the production of various biomaterials, biochemicals, and biofuels from microbial cell factories such as bacteria or fungi, due to their ability to use CO_2 and light without any organic carbon as substrates. Therefore, it is of great significance to exploit microalgae for the production of high-value bioproducts. How to improve the content or productivity of specific bioproducts has become one of the most urgent challenges.

Microalgae display great adaptability to different abiotic stress factors (such as temperature, light, salinity, and nutrient deprivation) and produce high-value metabolites. Microalgae can be exploited for the production of desired metabolites under certain abiotic stress conditions (Paliwal *et al.* 2017). In addition, the productivity of microalga-based processes can also be improved by chemical additives to enhance cell growth and accumulation of specific bioproducts (Yu *et al.* 2015). With the progress of genomics, transcriptomics, and proteomics providing increasingly growing data, researches on functional characterization of key genes or regulators, identification of metabolic pathways, and elucidation of cell physiology of microalgae have made a major breakthrough. Molecular approaches such as metabolic engineering, transcriptional engineering (Bajhaiya *et al.* 2016b), and gene knockout strategies are emerging as the potential strategies to enhance the accumulation of high-value bioproducts. In this review, we will describe high-value bioproducts from

microalgae and their biosynthetic pathways (mainly for lipids, PUFAs, and carotenoids). Recent progress and strategies for the enhanced production of bioproducts from microalgae are also described in detail, and these strategies take advantages of optimized cultivation conditions with abiotic stress, chemical stress (addition of metabolic precursors, phytohormones, chemical inhibitors, and chemicals inducing oxidative stress response), and molecular approaches such as metabolic engineering, transcriptional engineering, and gene disruption strategies (mainly RNA-based knockdown and CRISPR/Cas9) (Figure 1).

2. Biosynthetic pathways of some high-value bioproducts from microalgae

Microalgae can trap light energy as energy source and assimilate CO_2 as carbon source. CO_2 enters in chloroplasts via the Calvin cycle to produce glyceraldehyde 3-phosphate (GAP). GAP can be available for the synthesis of pyruvate via glycolytic pathway. Pyruvate can be used as the precursor for the synthesis of glucose, starch, fatty acids, and terpenoids. With the progress of genomics, transcriptomics, and proteomics on microalgal research providing increasingly growing data, researches on functional characterization of key genes or regulators, and identification of metabolic pathways in microalgae have made a major breakthrough. The biosynthetic pathways of fatty acid and TAG in microalgae have been well described (Liang and Jiang 2013) (Figure 2). Acetyl-CoA carboxylase (ACC) catalyzes the rate-limiting step for fatty acid biosynthesis. Acyl-ACP thioesterase (FAT) can terminate the fatty acid elongation cycle via hydrolyzing an acyl group on a fatty acid. The *de novo* pathway of TAG biosynthesis takes place in the endoplasmic reticulum of eukaryotes or in the cytoplasm of prokaryotes, also called Kennedy pathway, consisting of four sequential steps. Three acyltransferases, i.e. glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT), catalyze the *sn*-specific esterification of glycerol-3-phosphate (G3P). While LC-PUFAs can be synthesized in endoplasmic reticulum via alternating desaturation and elongation steps acting on saturated fatty acids (SFAs) (Khozingoldberg *et al.* 2011).

Microalgae can generate C5 isoprenoid precursors, i.e. isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), via the mevalonic acid (MVA) pathway in the cytosol or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids, which have been described in detail in a previous review (Moise *et al.* 2013) (Figure 2). Isoprenoids can be classified into six groups according to the number of carbons: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20),

triterpenes (C30), and tetraterpenes (carotenoids, C40) (Niu *et al.* 2017). Farnesyl pyrophosphate (FPP, C15) is a key precursor in the biosynthesis of sesquiterpenes, triterpenes, and sterols (Piironen *et al.* 2000, Fabris *et al.* 2014). In addition, geranylgeranyl pyrophosphate (GGPP, C20) is an essential precursor for a variety of products including gibberellins, chlorophylls, diterpenes, tetraterpenes, and carotenoids (Rodríguez-Concepción 2010, Liang *et al.* 2015). The biosynthetic pathway of carotenoids and sterols in microalgae have been well described (Fabris *et al.* 2014, Lu *et al.* 2014, Liang *et al.* 2017b) (Figure 2). Phytoene synthase (PSY), two desaturases (phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS)), two isomerases (15-cis-ζ-carotene isomerase (ZISO) and carotenoid isomerase (CRTISO)), two cyclases (lycopene β-cyclase (LcyB) and lycopene ε-cyclase (LcyE)), and two types of hydroxylases (β-carotene hydroxylase (BCH) and P450-type carotenoid hydroxylases (CYP97s)), and β-carotene ketolase (BKT) are involved in the carotenoid biosynthesis (Liang *et al.* 2017b). Then lycopene, β-carotene, lutein, zeaxanthin, and astaxanthin can be synthesized. Among them, astaxanthin can be found in a few microalgae. FPP, the substrate of squalene synthase (SQS), initiates the biosynthesis of sterols, which are vital structural and regulatory components. Then squalene is epoxidized to squalene 2,3-epoxide by squalene epoxidase (SQE). The cyclization of squalene 2,3-epoxide to cycloartenol in green algae and plant is catalyzed by distinct oxidosqualene cyclases (OSCs), cycloartenol synthase (CAS) and lanosterol synthase (LAS), respectively (Fabris *et al.* 2014, Lu *et al.* 2014) . The synthesis of sterols varies depending on the phylogeny.

3. Algal omics in response to different abiotic stresses

Effects of different abiotic factors (such as light irradiation, temperature, nutrient, and salinity) or different cultivation conditions on the production of metabolites from microalgae have been widely evaluated and reviewed (Chen *et al.* 2017, Paliwal *et al.* 2017). The accumulation of high-value bioproducts from microalgae can be triggered by a number of abiotic stresses such as high light, temperature stress, salt stress, and nutrient deprivation. Two-stage cultivation for the production of high-value products in microalgae is a widely used and efficient strategy, in which in the first stage cells are grown in nutrient-sufficient conditions to maximize the biomass productivity, while in the second stage, stress conditions induce the accumulation of the desired bioproducts (Markou and Nerantzis 2013). However, the underlying molecular mechanisms of these stress responses remain to be explored. Recently, algal omics (genomics, transcriptomics, proteomics,

metabolomics, and lipidomics) have played an important role in the identification of key genes, transcription factors, and regulators involved in the biosynthetic pathways of bioproducts, analysis of the dynamics of gene, protein, and metabolite abundance, and unraveling regulatory networks in microalgae (Guarnieri and Pienkos 2015). Here, we present the advances in the accumulation of bioproducts under different cultivation conditions and algal omics technologies on revealing stress response mechanisms (Table 1).

3.1. Carbon source and light irradiation

Generally, microalgae cultivation can be performed in three modes: photoautotrophy, heterotrophy, and mixotrophy (Giordano and Wang 2018). Microalgae can use sunlight as energy and inorganic carbon (CO_2 and/or HCO_3^-) as carbon source for photoautotrophy. Heterotrophic culture of microalgae can use organic carbon substrates (such as glucose, acetate, and glycerol) as energy and carbon source without light, resulting in high biomass density. Mixotrophic cultivation of microalgae is a combination of photoautotrophy and heterotrophy to use both inorganic and organic carbon substrates, which can enhance the biomass productivity (Li 2014). It was reported that the shift from starch-rich heterotrophy (with glucose, this stage for high biomass density) to lipid-rich photoautotrophy (supply light, without glucose) resulted in the accumulation of lipid (~ 35% DCW) and protein (~ 40% DCW) in *Chlorella pyrenoidosa* FACHB-9 (Fan *et al.* 2015). Transcriptomic analysis revealed that this shift up-regulated genes involved in carbon fixation, photosynthesis, fatty acid biosynthesis, and starch catabolism, thus carbon skeletons from photosynthesis and starch degradation may be directly converted into lipid and protein biosynthesis.

The supply of high-dose CO_2 in microalgal cultivation can enhance photosynthetic efficiency, subsequently with increases of biomass and bioproducts like lipids and carotenoids. With 10% CO_2 (v/v) aeration in the cultivation of *Chlorella sorokiniana* LS-2, although the fatty acid biosynthetic genes were down-regulated, genes involved in carbon fixation, carbohydrate metabolism and the TCA cycle were up-regulated. Therefore, elevated CO_2 enhanced biosynthesis of acetyl-CoA, and then drove more carbon flow into fatty acid biosynthesis, leading to an increase of overall TAG generation (Sun *et al.* 2016). Under high

light (250 μmol photons/m^2/s) with 15% CO_2, astaxanthin yield of *H. pluvialis* mutant red cells was 1.7 times higher than that of a wild strain aerated with air (Cheng *et al.* 2017). High concentration of CO_2 enhanced pyruvate metabolic pathway for more precursors to astaxanthin, and up-regulated genes involved in astaxanthin biosynthesis and fatty acid biosynthesis, thus promoting astaxanthin esterification and deposition (Cheng *et al.* 2017). Extra CO_2 supply is considered as a promising strategy for scale-up production of bioproducts.

High light intensity acts as a stimulant in the production of neutral lipid (such as TAG) and/or carotenoid in microalgae. The lipid content of *Chlamydomonas* sp. JSC4 under high light intensity (optimal, 300 μmol photons/m^2/s) was significantly higher than that under low light intensity (30 μmol photons/m^2/s), resulting in the lipid productivity of 312 mg/L/d (Ho *et al.* 2015). By using ^{13}C-labeling metabolomic technology, high light intensity increased the levels of TAG-synthesis-related metabolites and the turnover rate of most metabolites for lipid synthesis. On the other hand, higher light intensity (500 μmol photons/m^2/s) led to less PUFAs and more SFAs and monounsaturated fatty acids (MUFAs) (Ho *et al.* 2015). After 2 d of exposure to high irradiance (400 μmol photons/m^2/s), the total carotenoid content of *H. pluvialis* cells increased by 3.15-fold, compared with the control (20 μmol photons/m^2/s), and genes involved in IPP synthesis and astaxanthin biosynthesis were up-regulated (Gwak *et al.* 2014). Moreover, the TAG content was enhanced by 5.42-fold after 4 d under high irradiance, which was due to the up-regulation of genes involved in fatty acid biosynthesis and TAG biosynthesis. (Gwak *et al.* 2014). Additionally, an increase in reactive oxygen species (ROS) scavenging activity and reduction of excess ROS generation enabled *H. pluvialis* cells to survive under high irradiance.

3.2. Nutrient deficiency

Nitrogen starvation is the most applied nutrient stress for biofuel production in microalgae although the mechanisms of stimulating lipid accumulation under nitrogen starvation remain to be elucidated. While nitrogen starvation led to a decrease in chlorophyll a and b, and total carotenoid levels, the levels of total fatty acids (TFAs) increased to 3.8-fold after 144 h of nitrogen starvation in *C. reinhardtii*, with an increased percentage of the SFA palmitate (C16:0) and the MUFA oleate (C18:1Δ9), as compared with nitrogen-replete condition (Wase *et al.* 2014). By using GC-MS-based metabolomics and iTRAQ-labeled proteomics analysis,

the proteins involved in the glycolysis, TCA cycle, starch, lipid metabolism, nitrogen assimilation, amino acid metabolism, and oxidative phosphorylation were enhanced, in contrast, the enzymes of Calvin cycle, light harvesting complex, glyoxylate cycle, one carbon metabolism, pentose phosphate pathway, and ribosomes were reduced under nitrogen starvation (Wase *et al.* 2014). When algal cells were transferred from nitrogen-replete to nitrogen-deprived conditions, it was possible that the metabolic pathway shifts play a role in regulating the pace of nitrogen reassimilation, supply of carbon skeletons, and/or conversion of excess carbon into fatty acid biosynthesis. In addition, TAG synthesis of *Nannochloropsis oceanica* strain IMET1 increased by approximately 400-fold within 96 h of nitrogen depletion (Li *et al.* 2014). By mRNA-Seq and ESI/MS-based lipidomics, many genes involved in carbohydrate and protein degradation as well as genes encoding plastid and mitochondrial membrane transporters were up-regulated, suggesting that these pathways convert photosynthetic carbon precursors from carbohydrate and protein metabolism into fatty acid synthesis and finally TAG synthesis under nitrogen deprivation (Li *et al.* 2014).

In contrast, phosphorus starvation seemed to have little effect on lipid accumulation in microalgae (Chen *et al.* 2011, Liang *et al.* 2017a). While in most diatoms, silicon is essential for cell wall synthesis and growth, and silicon starvation can lead to growth arrest and the formation of lipid droplets. After short-term of silicon starvation, the cellular FAME content in *T. pseudonana* nearly increased by 3-fold, with a decrease in cellular membrane lipid biosynthesis. Transcriptomic analysis revealed that transcription levels of large numbers of genes are coordinated with cell cycle progression and photophysiological shifts during silicon starvation (Smith *et al.* 2016). It was reported that the maximal rate of H_2 production in *C. reinhardtii* could be observed at 48 h of sulfur deprivation. Proteomic analysis revealed that during the process of sulfur-depleted H_2 photoproduction, proteins involved in pentose phosphate pathway and fermentative metabolism were increased, while proteins involved in Calvin cycle and TCA cycle were decreased (Chen *et al.* 2010).

3.3. Temperature stress

In most of the microalgae, low temperature usually leads to the accumulation of polar lipid (i.e., phospholipid), while high temperature results in higher production of nonpolar lipids (neutral lipid, such as TAG) (Paliwal *et al.* 2017). Lipidomic analysis of *C. reinhardtii* under heat stress (60 min at 42 °C) revealed that a strong decrease in specific polyunsaturated membrane lipids occurred with an increase in

polyunsaturated TAGs and diacylglycerols (DAGs) (Légeret *et al.* 2016). It seemed that TAG biosynthesis in nitrogen-starved cells is mostly derived from *de novo* synthesized fatty acids, while in heat-stressed cells, TAG is synthesized by conversion of polyunsaturated membrane lipids and recycling of their polyunsaturated acyl chains. By using transcriptomic analysis, it was evident that heat stress inhibited ribosome formation and protein synthesis, up-regulated *DGTT1* and *MLDP*, and down-regulated several lipid hydrolyzing genes (Légeret *et al.* 2016). A recent report showed that DHA content of *Aurantiochytrium* sp. SD116 can be significantly increased under cold stress (Ma *et al.* 2017). Proteomic analysis using iTRAQ technology revealed that cold stress inhibits glycolysis and the TCA cycle, leading to less energy and NAPDH supply for anabolism, and upregulated PUFA synthase, resulting in an increase in the content of PUFAs (such as docosapentaenicaicid (DPA) and DHA), which played a vital role in maintaining cell membrane fluidity (Ma *et al.* 2017).

3.4. Oxidative stress and salt stress

Some chemicals capable of inducing oxidative stress responses by generating ROS have been used to boost microalgal growth and accumulation of high-value bioproducts (Yu *et al.* 2015). ROS, including hydroxyl radicals (•OH), superoxide anion (O_2•$^-$), and singlet oxygen ($^1O_2$), have been used as signal molecules to induce the production and accumulation of many bioproducts. By treatment of acetate and Fe^{2+}, oxidative stress was induced in *H. pluvialis* cells. Cellular astaxanthin could be accumulated after the oxidative stress and reached its peak level after 6 or more d of stress treatment (Wang *et al.* 2004). Proteomic analysis indicated that some proteins involved in photosynthesis and nitrogen assimilation were decreased under oxidative stress. It appeared that the early oxidative stress response involves multiple antioxidative enzymatic defense processes, while the accumulation of the antioxidant astaxanthin is the predominant response of the cells to the long-term oxidative stress (Wang *et al.* 2004).

It was reported that increasing salinity from 1.0 M to 2.0 M for *Amphora subtropica* or decreasing salinity from 3.0 M to 2.0 M for *Dunaliella* sp. can increase lipid and carotenoid contents (BenMoussa-Dahmen *et al.* 2016). The contents of SFAs and MUFAs in *Dunaliella* sp. were increased with the increasing NaCl concentration from 0.4 to 4.0 M (BenMoussa-Dahmen *et al.* 2016). A fresh water microalga, *Scenedesmus* sp. IITRIND2, were cultivated under 100% seawater salinity. Salt stress enhanced its

lipid content by 52%, and led to an increase by ~3.2 fold in lipid productivity (Arora *et al.* 2017). High salinity induced oxidative stress and led to the reduction of photosynthetic pigments and the enhancement of ROS, osmolytes, and activity of antioxidant enzymes.

4. Chemical stress to enhance the accumulation of high-value bioproducts in microalgae

In addition to various efforts in cultivation optimization under optimized stress conditions and other suitable condition, another effective way is to apply chemical stress to enhance the accumulation of high-value bioproducts. According to the enhancing mechanisms of chemicals, they can be classified into four types: chemicals directly used as metabolic precursors, phytohormones and analogs regulating microalgal metabolisms, chemical inhibitors regulating biosynthetic pathway, and chemicals inducing oxidative stress response. Here we mainly focus on the recent advances in using chemical stimulants or inhibitors for the accumulation of the desired bioproducts in microalgae (Table 2), and the molecular mechanisms of the stimulatory or inhibition roles of these chemicals.

4.1. Chemicals used as metabolic precursors

Microalgae can not only use inorganic carbon as a carbon source, but also use organic carbon substrates such as glucose, acetate, and glycerol as carbon sources (Chen and Jiang 2017). Using sodium acetate as a carbon source led to the maximum lipid content (42.5% DCW) in *Chlorella vulgaris* under nitrogen starvation condition (Najafabadi *et al.* 2015). Using glycerol (the major byproduct from biodiesel production) as the main carbon source resulted in a high oil content (50% DCW) in *Chlorella protothecoides* by a semi-continuous mode, and the biomass also contained relevant amounts of other interesting bioproducts such as vitamin A, riboflavin, and lutein (Cerón-García *et al.* 2013). Using volatile fatty acids (VFAs, derived from food wastes) as carbon sources in heterotrophic cultivation led to the highest lipid content of 48.7% DCW in *C. protothecoides* (Fei *et al.* 2015). The lipid contents produced by using the organic carbon substances mentioned above as carbon sources were similar to those by using glucose. But due to their lower price, those organic carbon substrates are considered as a promising suitable carbon source for algal lipid production.

For DHA accumulation, the DHA content of TFAs in *Schizochytrium* sp. HX-308 was increased from 35 to 60% by the addition of 4 g/L malic acid at the rapid lipid accumulation stage (Ren *et al.* 2009). Furthermore, the addition of 40 mL ethanol/L at the late lipid accumulation stage led to an apparent increase of 35% in total lipid content (Ren *et al.* 2009). In another study, the use of either acetate or ethanol as a carbon source resulted in a remarkable increase in DHA productivity by the marine alga *Crypthecodinium cohnii* (Sijtsma *et al.* 2010). Malic acid, acetate or ethanol can reinforce the supply of acetyl-CoA or/and NADPH for lipogenesis.

For carotenoid accumulation, the addition of 100 mM pyruvate in *Chlorella zofingiensis* enhanced the yield of astaxanthin with a 28.2% increase. Supplementation with citrate and malic acid also had the similar stimulatory effects on astaxanthin accumulation (Chen *et al.* 2009). Pyruvate might serve as a precursor for isopentenyl pyrophosphate (IPP), the carotenoid precursor in *C. zofingiensis*; while the stimulatory effects of citrate and malic acid on astaxanthin biosynthesis may be due to their conversions to pyruvate.

4.2. Phytohormones and their analogs regulate microalgal metabolisms

Phytohormones, also known as plant growth regulators (PGRs), such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), gibberellin, and auxin can contribute to plant growth and development. Some phytohormones can promote cell division, accelerate ripening and senescence, stimulate cell elongation in the hypocotyls, and induce plant disease resistance, drought resistance, salt resistance, and temperature resistance (Lin *et al.* 2017). The treatment with PGRs in microalgae for high-value metabolites seems to be a useful tool. However, the mechanisms of action of these PGRs in microalgal cells remain to be fully explored.

ABA treatments on *C. reinhardtii* caused a 10~13% increase of FAME yield (Park *et al.* 2013). Treatments with 1~20 μM ABA in *Chlorella saccharophila* were able to increase TAG accumulation compared with the control (Contreras-Pool *et al.* 2016). The fatty acid composition of ABA-treated *Scenedesmus quadricauda* showed a 11.17% increase in SFA content when compared with the nitrogen-starved cells (Sulochana and Arumugam 2016). Supplementation with 40 μM SA resulted in a 1.3-fold increase in the TFA content of *P. tricornutum* after 4 d of exposure (Xu *et al.* 2017). Exogenous application of JA in *C. vulgaris* at early stationary growth phase promoted microalgal growth with a 51%

increase of cell density relative to the control, and JA also transiently enhanced the total oil production by 54% (Jusoh *et al.* 2015). Under fulvic acid (a PGR) treatment, the lipid and protein contents of *Monoraphidium* sp. FXY-10 increased rapidly from 44.6% to 54.3% and from 31.4% to 39.7%, respectively. Fulvic acid treatment significantly up-regulated *ME*, *accD*, and *GPAT*, and down-regulated *PEPC* (Che *et al.* 2017). It seemed that fulvic acid significantly increased microalgal lipid accumulation by changing the intracellular ROS accumulation, gene expression, and enzyme activities of ACC, ME and phosphoenolpyruvate carboxylase (PEPC). Auxin treatment coupled with salt stress resulted in a considerable improvement both in growth and lipid production in *D. tertiolecta*. At the first stage auxin addition enhanced biomass accumulation of *D. tertiolecta* by 40%, and salt stress at the second stage led to lipid increase from 24% to 70% with optimal concentrations combination of auxin and NaCl (Elarroussi *et al.* 2015). The addition of 40~60 μM auxin could improve lipid productivity up to 3-fold in *Scenedesmus abundans*, and 40~60 μM gibberellin led to 7-fold increase in lipid productivity of *Chlorella ellipsoidea* (González-Garcinuño *et al.* 2016). Treatment with 40 ppm indole-3 acetic acid (IAA) in *Nannochloropsis oceanica* CASA CC201 led to high lipid accumulation of 60.9% DCW, and increased the EPA percentage to 10.76%, which was much higher than the control (1.87%) (Udayan and Arumugam 2017).

Low concentrations of indole-3-butyric acid (IBA) had a stimulatory effect on chlorophyll *a* and carotenoids accumulation, in contrast, higher concentrations of IBA induced the accumulation of phycocyanin, allophycocyanin, and phycoerythrin in the treated *Nostoc linckia* (Mansouri and Talebizadeh 2017). As for astaxanthin production, algal cells treated separately with methyl jasmonate and gibberellins A3 resulted in more astaxanthin accumulation than the controls (Lu *et al.* 2010). Moreover, it was reported that SA, JA, gibberellin A3, and 2,4-epibrassinolide could increase astaxanthin productivity in *H. pluvialis* (Gao *et al.* 2012a, Gao *et al.* 2012b, Gao *et al.* 2013a, Gao *et al.* 2013b). In addition, the astaxanthin content increased by 86.89% under the treatment of 5 mg/L fulvic acid in *H. pluvialis* LUGU (Zhao *et al.* 2015).

4.3. Chemical inhibitors regulate biosynthetic pathway

Compared with nitrogen deprivation, the treatment of sodium azide resulted in a 60~80% increase in TAG yield of *C. desiccata* with minor growth retardation and moderate inhibition of photosynthesis and respiration

(Zalogin and Pick 2014a). It was found that sodium azide can induce TAG accumulation mainly due to the photoinactivation of nitrate reductase, which is the major enzyme controlling nitrogen assimilation in plants and algae (Zalogin and Pick 2014b). Sesamol, a strong antioxidant phenolic compound, was added in the marine microalga *C. cohnii*, and caused the increase of DHA content (% of TFAs) and DHA productivity by 11.25% and 20%, respectively (Liu *et al.* 2015). Besides its potent antioxidation activity, sesamol can inhibit the activity of malic enzyme, which is one of the most important NADPH sources in oleaginous microalgae, and thus decreases the synthesis of *de novo* fatty acids. On the other hand, the precursors may be redirected to the biosynthesis of DHA via another pathway with less NADPH, resulting in the possible increase in DHA content. Treatment with 100 ppm triethylamine elevated the lipid content of *D. tertiolecta* by 80%, but the biomass production and chlorophyll content were notably reduced (Xue and Jiang 2017).

In a recent study, addition of 50~150 ppm triethylamine could trigger lycopene production in *Dunaliella bardawil* (Liang *et al.* 2016). Triethylamine, as a kind of lycopene cyclase inhibitor, can inhibit the expression levels of *LcyB* and *LcyE*, and up-regulate the upstream carotenogenic genes. Nicotine and imidazole can be also used as lycopene cyclase inhibitors. Low concentrations of nicotine significantly decreased β-carotene, but triggered the accumulation of lycopene in *Chlorella regularis* Y-21 and *Dunaliella salina* CCAP 19/18, possibly due to the inhibition of lycopene cyclases (Ishikawa and Abe 2004, Fazeli *et al.* 2009). The addition of 2-methylimidazole in *D. salina* resulted in an increase in lutein content by 1.7-fold and an associated decrease in β-carotene content, indicating that 2-methylimidazole prefers to inhibit LcyB activity, thus shifting the pathway from β-carotene to the α-carotene direction (Yildirim *et al.* 2017). Addition of diphenylamine enhanced β-carotene content to 1.46-fold in *H. pluvialis* exposed to high light in nutrient sufficient condition (Vidhyavathi *et al.* 2009), as diphenylamine can inhibit β-carotene ketolase (BKT), thus leads to the accumulation of β-carotene. Addition of the squalene epoxidase (SQE) inhibitor terbinafine decreased the sterol content yet significantly enhanced the carotenoid levels and fatty acid production in *N. oceanica* (Lu *et al.* 2014). It was reported that addition of terbinafine caused the accumulation of squalene in *C. reinhardtii* (Kajikawa *et al.* 2015a). Sometimes, the addition of chemical inhibitors is used to confirm some biosynthetic pathway. By using terbinafine and Ro 48-8071, specific inhibitors of SQE and OSC enzymes, it

was confirmed that squalene 2,3-epoxide is a sterol pathway intermediate (Figure 2) in *P. tricornutum* (Fabris *et al.* 2014).

4.4. Chemicals induce oxidative stress response

The addition of 0.1 mM hydrogen peroxide (H_2O_2) to the cell culture leading to the generation of •OH which in turn enhanced the yield of astaxanthin from 9.9 mg/L to 12.58 mg/L in *C. zofingiensis* (Ip and Chen 2005). It was reported that 0.1 mM H_2O_2 increased the total astaxanthin accumulation in *Chlorococcum* sp. from 5.8 to 6.5 mg/L DCW. In addition, 0.5 mM Fe^{2+} added with 0.1 mM H_2O_2 further promoted astaxanthin formation to 7.1 mg/L DCW (Ma and Chen 2001). By inducing an oxidative stress, Fe^{2+} could stimulate β-carotene synthesis in *D. salina*, especially when acting in the presence of acetate (Mojaat *et al.* 2008). The astaxanthin productivity in *H. pluvialis* could be enhanced to 2.03-fold with 3% (v/v) ethanol addition. It seemed that ethanol may cause the generation of ROS. In addition, ethanol up-regulated *IPI-1* and *IPI-2* genes encoding isopentenyl pyrophosphate isomerase (IPI) at transcriptional level, promoting isoprenoid synthesis and precursors supply to carotenoid formation (Wen *et al.* 2015).

5. Molecular approaches to enhance the accumulation of bioproducts in microalgae

5.1. Metabolic engineering in microalgae

Metabolic engineering of microalgae to enhance the accumulation of high-value bioproducts has become increasingly feasible with the advance in the understanding of microalgal genes regulation and the manipulation of microalgal metabolic pathways by gene overexpression and/or knockdown (Liang and Jiang 2013, Goncalves *et al.* 2016b). Here we summarize the recent advances in metabolic engineering strategies to enhance the production of lipids (Table 3) and other bioproducts (Table 4) in microalgae by gene overexpression. As for gene knockdown/disruption, we will discuss in detail in Section 5.3.

5.1.1. Metabolic engineering for lipid production in microalgae

Overexpression of the endogenous glycerol kinase gene (*GK*) through glycerol recycling in the oleaginous marine diatom, *Fistulifera solaris* JPCC DA0580 could achieve a 12% increase in lipid productivity (Muto *et al.* 2015). Overexpression of *GPAT* gene from *Lobosphaera incisa* could reach up to 50% TAG content in *C.*

reinhardtii (Iskandarov *et al.* 2016). Overexpression of *GPAT* in the model diatom *P. tricornutum* increased 1.81-fold TAG content, moreover, ultrastructural observation showed increased lipid droplets in the cytosol and TAG-rich plastoglobuli in the plastids (Balamurugan *et al.* 2017). In a separate report, it showed that overexpression of endogenous *DGAT2* which plays an important role in TAG biosynthesis resulted in a 35% increase in neutral lipid content in *P. tricornutum* (Niu *et al.* 2013). TAG biosynthesis was accelerated, and the neutral lipid content was significantly enhanced by 69% by overexpressing *DGAT2* in oleaginous microalga *N. oceanica* (Li *et al.* 2016). Overexpression of a type-I *DGAT* (*NoDGAT1A*) from *Nannochloropsis oceanica* IMET1 led to ~39% increase in TAG content upon nitrogen depletion, and also enhanced TAG accumulation by ~2.4-fold under nitrogen-replete conditions without compromising cell growth (Wei *et al.* 2017). Furthermore, in the endogenous glycerol-3-phosphate dehydrogenase (*GPDH*)-overexpressing *P. tricornutum* cells, there was a 60% increase in neutral lipid content (Yao *et al.* 2014).

Overexpression of the *AccD* and *ME* genes in *D. salina* could lead to a 12% increase in total lipid content (Talebi *et al.* 2014). Malic enzyme (ME) catalyzes the decarboxylation of malate to form pyruvate accompanied with NADPH, which is vital for fatty acid biosynthesis (Liang and Jiang 2015, Liang *et al.* 2017a). DGAT and ME have been considered as the most promising targets for genetic engineering for lipid production. Overexpression of *PtME* markedly increased the total lipid content by 2.5-fold, and reached up to 57.8% of DCW in *P. tricornutum* (Xue *et al.* 2015). While overexpression of *PtME* in the green microalga *Chlorella pyrenoidosa* led to a significant increase in the neutral lipid content by 3.2-fold, and reached up to 40.9 % DCW (Xue *et al.* 2016). In the *G6PD* (glucose-6-phosphate dehydrogenase) overexpressing microalga *P. tricornutum*, the G6PD enzyme activity increased with the enhancement of NADPH production. Consequently, the lipid content increased by 2.7-fold and reached up to 55.7% DCW (Xue *et al.* 2017). It seems that G6PD can be used as a target for lipid accumulation via metabolic engineering by enhancing NADPH supply.

Overall, the availabilities of more acetyl-CoA precursor (such as overexpression of *ACC* gene) and more NAPDH (such as overexpression of *ME* gene) increase the throughput of fatty acid synthesis. For another,

up-regulation of the downstream pathway (such as overexpression of *GPAT* or *DGAT* gene) creates a driving force by consuming acyl-CoA intermediates and increasing the rate of storage of TAG in lipid bodies.

5.1.2. Metabolic engineering for the accumulation of other bioproducts in microalgae

For carotenoid accumulation, overexpression of *PSY* gene from *D. salina* (*DsPSY*) in *C. reinhardtii* resulted in a 1.25-fold increase of *DsPSY* transcript level, and a 2.6-fold increase of carotenoids content compared with the untransformed cells (Couso *et al.* 2011). The overexpression of *PSY* from *C. zofingiensis* (*CzPSY*) in *C. reinhardtii*, by nuclear transformation, led to an increase in the *CzPSY* transcript level, and in violaxanthin and lutein contents which were 2.0- and 2.2-fold higher than in untransformed cells (Cordero *et al.* 2011). The introduction of *PSY* increased the fucoxanthin content by 1.45-fold relative to the levels in wild-type diatom *P. tricornutum* (Kadono *et al.* 2015). Transformation of *H. pluvialis* with a modified PDS (PDS-L504R, with a leucine-arginine change at position 504) enhanced 32.6 % astaxanthin levels compared to wild-type cells (Steinbrenner and Sandmann 2006). The introduction of PDS-L516F, which exhibited a 33 % higher desaturation activity than the unaltered enzyme, resulted in 32.1 % more total carotenoids and 54.1 % more astaxanthin in *C. zofingiensis* (Liu *et al.* 2014). Interestingly, introduction of a *BKT* gene from *H. pluvialis* in *D. salina*, which cannot naturally produce astaxanthin, contributed to the presence of astaxanthin and canthaxanthin with maximum contents of 3.5 and 1.9 μg/g DCW, respectively (Anila *et al.* 2016). It was shown in a previous report that overexpression of the endogenous *BKT* gene in *H. pluvialis* led to 2~3-fold higher total carotenoids and astaxanthin contents, and 8~10-fold higher contents of intermediates like echinenone and canthaxanthin than in the control cells (Kathiresan *et al.* 2015). Overexpression of β-carotene hydroxylase (*BCH*) gene from *C. reinhardtii* in *D. salina* increased the violaxanthin content by 3-fold, and the zeaxanthin content by 2-fold in the transformed cells (Simon *et al.* 2016). In higher plants, the cauliflower *Or* (orange) gene encodes a plastid-associated protein containing a DnaJ cysteine-rich domain that can mediate high levels of β-carotene accumulation (Lu *et al.* 2006). It was suggested that the Or protein can trigger chromoplast differentiation for carotenoid accumulation. It was reported that overexpression of the cauliflower *Or* gene resulted in carotenoid accumulation and chromoplast formation in transgenic potato tubers (Lopez *et al.* 2008, Li *et al.* 2012a). In microalgae, homologous *Or* genes have been identified, but their

roles remain to be explored. Manipulation of *Or* genes may also be a novel genetic tool to induce carotenoid accumulation in microalgae.

For the accumulation of PUFAs, it was reported that overexpression of the Δ5-elongase gene (*OtElo5*) from *Ostreococcus tauri* resulted in an 8-fold increase in DHA content in *P. tricornutum*, and co-expression of an acyl-CoA-dependent Δ6-desaturase (OtD6Pt) with OtElo5 displayed a further increase in DHA levels (Hamilton *et al.* 2014). Overexpression of *GPAT* in *P. tricornutum* led to a significant increase in PUFAs, particularly EPA and DHA (Balamurugan *et al.* 2017). Overexpression of a type 2 DGAT in *P. tricornutum* led to a 76 % increase in EPA content (Niu *et al.* 2013). It was also shown that overexpression of *BnDGAT2* from *Brassica napu* in *C. reinhardtii* led to a decrease in the levels of SFAs, but enhanced the level of PUFAs, especially ALA, with an increase up to 12 % (Ahmad *et al.* 2015). For glycerol production, overexpression of sedoheptulose-1,7-bisphosphatase gene (*SBPase*, encoding a rate-limiting enzyme of the Calvin cycle) from *C. reinhardtii* increased glycerol production by about 37 % in a glycerol-producing halotolerant *D. bardawil* under high salinity conditions (Fang *et al.* 2012). The glycerol concentration in the *GPDH*-overexpressing *P. tricornutum* cells increased by 6.8-fold compared with the wild type (Yao *et al.* 2014).

5.2. Transcriptional Engineering in microalgae

Transcription factors (TFs) control gene expression by interacting with RNA polymerase and binding to *cis*-acting elements in target gene promoters. Regulatory proteins such as TFs and transcriptional regulators (TRs) as well as *cis*-acting elements play important roles in regulating gene transcription, especially in responses to abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2005, Saibo *et al.* 2008). In higher plants, transcription factors, such as basic leucine-zipper (bZIP), MYB-type, dehydration-responsive-element binding (DREB) proteins, NAC, AP2/ERF, and WRKY transcription factors, regulate abiotic stress-induced multiple gene expression (Saibo *et al.* 2008, Yadav *et al.* 2013). Recently, omics methods are used to identify TFs and TRs in microalgae (Guarnieri *et al.* 2013, Gargouri *et al.* 2015). A MYB-type transcription factor LCR1 (low-CO_2 stress response) was found to regulate CO_2-responsive genes and phosphate starvation signaling in *C. reinhardtii* (Rubio *et al.* 2001, Yoshioka *et al.* 2004). The MYB-related transcription factor ROC40 played a role in nitrogen starvation-induced lipid accumulation (Goncalves *et al.* 2016a). NRR1 (nitrogen response regulator 1) from *C. reinhardtii* can play a regulatory role in nitrogen assimilation and TAG accumulation in

nitrogen-deprivation conditions (Boyle *et al.* 2012). It was reported that PSR1 (phosphorus stress response 1) from *C. reinhardtii* can regulate carbon storage metabolism by controlling specific genes involved in lipid and starch metabolisms, and therefore seems to function as a global regulator of microalgal carbon storage (Ngan *et al.* 2015, Bajhaiya *et al.* 2016a). As shown in Table 5, overexpression of PSR1 increases TAG accumulation without growth inhibition (Ngan *et al.* 2015), and PSR1 overexpression can also increase starch biosynthesis (Bajhaiya *et al.* 2016a). A dual-specificity tyrosine-phosphorylation-regulated kinase, TAR1 (TAG accumulation regulator 1) positively regulates TAG accumulation in N and S deficiency but negatively regulates photosynthesis in N deficiency (Kajikawa *et al.* 2015b). All these TFs and TRs are the potential targets for transcriptional engineering to improve oil yield in microalgae.

Overexpression of a DOF-type transcription factor gene in *C. reinhardtii* resulted in about 2-fold of total lipids (Ibáñez-Salazar *et al.* 2014). Overexpression of *DOF4* from soybean (*Glycine max*) significantly increased the lipid content of *Chlorella ellipsoidea* by 46.4~52.9% without growth inhibition (Zhang *et al.* 2014). This increase in lipid content could be attributed to the large number of genes with up-regulated expression, in particular, the *ACC* gene (Zhang *et al.* 2014). Overexpression of *NsbHLH2* led to the increased biomass and FAME productivity in *Nannochloropsis salina*, and the transformant NsbHLH2 3-6 showed increased biomass productivity by 36% under the normal condition, and FAME productivity by 33% under nitrogen limitation condition (Kang *et al.* 2015). Transcriptional engineering seems to be a promising technique for enhanced microalgal lipid production, which may modify multiple genes of a metabolic pathway simultaneously (Bajhaiya *et al.* 2016b). As for knockout of TF or TR, it was found that knockout of a transcriptional regulator $Zn(II)_2Cys_6$ (ZnCys) in *Nannochloropsis gaditana* improved the allocation of total carbon to lipids from 20% DCW (wild type) to 40~55% DCW (mutant) in nutrient-replete conditions (Ajjawi *et al.* 2017).

In higher plants, some TFs and TRs involved in carotenoid metabolism have been characterized. It has been reported that in *Arabidopsis thaliana*, AtRAP2.2 (a member of AP2 transcription factor family) could bind to *PSY* and *PDS* upstream regulatory sequences to modify carotenoid biosynthesis (Welsch *et al.* 2007), and phytochrome-interacting factors (PIFs, subfamily of basic-helix–loop–helix (bHLH) transcription factors) down-regulated the expression of *PSY* gene and carotenoid accumulation (Toledo-Ortiz *et al.* 2010). In

microalgae, TFs or TRs involved in carotenoid metabolism are yet to be experimentally confirmed although some *cis*-acting elements of carotenogenic genes in *D. bardawil* have been identified. Salt-regulated element, GT1GMSCAM4, in the *DbPSY*, *DbLycB*, *DbGGPS*, *DbCRTISO* and *DbBCH* genes, may be interacted by certain TFs that could be used as targets for transcriptional engineering (Lao *et al.* 2014, Liang and Jiang 2017, Liang *et al.* 2017c). It seemed that WRKY transcription factors in *D. bardawil* may act as activators to regulate the expression of carotenogenic genes under salt stress (Liang and Jiang 2017).

5.3. Gene disruption strategies in microalgae

5.3.1. CRISPR technology for genome editing in microalgae

Recently, CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated nuclease 9) system, which is an immune system discovered in bacteria and archaea, has become a rapid, powerful and precise tool for genome editing in many organisms, including microalgae (Ng *et al.* 2017, Banerjee *et al.* 2018). CRISPR/Cas9 system requires Cas9 protein to cut the DNA, and also requires CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) or single synthetic guide RNA (sgRNA) to target to specific sequence by inducing double strand breaks (DSBs). Two mechanisms, homologous recombination (HR) or nonhomologous end joining (NHEJ), can be used for repairing DSBs (Ng *et al.* 2017). So far, It was showed that the green alga *C. reinhardtii* (Jiang *et al.* 2014, Baek *et al.* 2016, Shin *et al.* 2016), oleaginous alga *Nannochloropsis* sp. (Ajjawi *et al.* 2017), the diatom *P. tricornutum* (Nymark *et al.* 2016) and *Thalassiosira pseudonana* (Hopes *et al.* 2016) can be manipulated for genome editing by CRISPR/Cas9.

Initial attempts to apply CRISPR-Cas9 technology to *C. reinhardtii*, failed to yield cells containing edited genes. Then only a single rapamycin-resistant transformant with a modified *FKB12* (rapamycin sensitivity gene) target site was yielded in 16 independent transformation experiments involving $>10^9$ *Chlamydomonas* cells (Jiang *et al.* 2014). With extremely low targeting efficiency, it seemed to be difficult to use CRISPR/Cas9 system for genome editing in microalgae. To solve this problem, delivery of Cas9 ribonucleoproteins (RNPs) comprising the Cas9 protein and sgRNAs was used to avoid cytotoxicity and off-targeting associated with vector-driven expression of Cas9 (Baek *et al.* 2016, Shin *et al.* 2016). Targeted mutagenic efficiency by CRISPR/Cas9 was improved up to $3.3\sim8.9\times10^{-8}$ at the *MAA7*, *CpSRP43* and *ChlM* loci in *C. reinhardtii* (Shin *et al.* 2016). The direct delivery of Cas9 RNPs may have quite a lot of advantages

including improved mutagenic efficiency, minimized off-targeting effect, less toxicity of Cas9, and no laborious cloning work compared to the transgenic techniques (Woo *et al.* 2015, Shin *et al.* 2016). A highly efficient (with a mutation frequency of 31%) CRISPR/Cas9 based system has been developed for creating stable targeted gene knockouts in the marine diatom *P. tricornutum* (Nymark *et al.* 2016). The chloroplast signal recognition particle 54 (CpSRP54) was targeted for CRISPR/Cas9-based disruption in *P. tricornutum*, which led to a greater decrease in Fv/Fm (photosynthetic efficiency) in the CpSRP54 mutants than in wild-type strains exposure to high intensity blue light for 1 h (Nymark *et al.* 2016). A high percentage of bi-allelic mutations (≤ 61.5%) targeted to the urease gene were observed in another diatom, *Thalassiosira pseudonana*, with the CRISPR/Cas9 construct, resulting to a significant reduction in growth rate and cell size of these bi-allelic mutants in urea compared to growth in nitrate (Hopes *et al.* 2016).

As for CRISPR technology for genome editing for enhancing the accumulation of bioproducts in microalgae, only a few studies have been mentioned (Table 6). By delivering preassembled Cas9 protein-gRNA RNPs, the sequential *ZEP* (zeaxanthin epoxidase) and *CpFTSY* (chloroplast thylakoid membrane-bound protein) two-gene knockout (Δ*ZEP*/Δ*CpFTSY*) could produce increased zeaxanthin and showing improved photosynthetic productivity in *C. reinhardtii* (Baek *et al.* 2016). As for knockout of TF or TR, CRISPR/Cas9 was used for knocking out 20 putative negative regulators of TFs from *Nannochloropsis gaditana* during nitrogen deprivation. It was found that knockout of a transcriptional regulator ZnCys in *N. gaditana* improved the allocation of total carbon to lipids by 20~35% DCW in nutrient-replete conditions (Ajjawi *et al.* 2017). Another CRISPR-based technology, CRISPR-interference (CRISPRi) has also been applied in microalgae to manipulate the down-regulation of genes in certain pathways for desired bioproducts. The CRISPRi system uses deactivated Cas9 (dCas9) which lacks nuclease activity. dCas9 still retains the ability to complex with the sgRNA and target to the homologous locus for gene interference instead of gene editing (Khatodia *et al.* 2016). Knockdown of phosphoenolpyruvate carboxylase gene (*CrPEPC1*) in *C. reinhardtii* by CRISPRi enhanced lipid content and lipid productivity by 74.4% and 94.2%, respectively (Kao and Ng 2017).

5.3.2. Other gene knockdown strategies for improvement the accumulation of bioproducts in microalgae

Other strategies of targeted gene knockdown for lipid improvements have been proposed. RNA interference (RNAi) is a post-transcriptional process in which RNAs inhibit gene expression or translation by sequence-directed degradation of specific RNAs. Knockdown of *CrPEPC1* by RNAi decreased PEPC activity by 39~50%, but increased TAG level by 20% (Deng *et al.* 2014). On the contrary, overexpression of *CrPEPC1* in *C. reinhardtii* increased PEPC activity by 157~184 %, and decreased TAG level by 37 % (Deng *et al.* 2014). PEPC catalyzes the conversion of phosphoenolpyruvate (PEP) to oxaloacetate, which can enter into the TCA cycle to provide the substrate and energy for protein synthesis. PEP also can be converted into pyruvate, which can be transformed to acetyl-CoA. ACC uses acetyl-CoA as substrate catalyzing the committing step for fatty acid biosynthesis. That is to say, the carbon flux from glycolysis can be used for the synthesis of protein or fatty acid depending on the relative activity of PEPC and ACC (Liang and Jiang 2013). Recently, the expression of two *CrPEPC* genes (*CrPEPC1* and *CrPEPC2*) was down-regulated by artifcial microRNA (amiRNA)-mediated knockdown technology, resulting in a dramatic increase in the TFA content by 28.7~48.6% (Wang *et al.* 2017a). amiRNA-mediated knockdown method needs to design amiRNAs through substitution of sequences in miRNA backbone, and then specifically down-regulates a gene of interest by means of targeted RNA degradation and translational inhibition (Zhao *et al.* 2009).

Quite a number of genes can be used as target genes of knockout/knockdown for lipid enhancement. Repression of *MLDP* (encoding major lipid droplet protein) using RNAi resulted in a significant increase in lipid droplet (LD) size without affecting TAG content in *C. reinhardtii* (Moellering and Benning 2010). While in *P. tricornutum*, knockdown of *PtLDP1* (encoding LD-associated protein) by RNAi decreased lipid and TAG contents and reduced the LD size (Wang *et al.* 2017b). In contrast, overexpression of *PtLDP1* gene enhanced lipid content and increased the LD size (Wang *et al.* 2017b). Knockdown of a lipase-encoding gene (*CrLIP1*) using amiRNA technology caused a slight increase in the total TAG level (Li *et al.* 2012b). It was speculated that CrLIP1 promotes TAG turnover in *C. reinhardtii* by degrading the DAG generated from TAG hydrolysis. Targeted knockdown of a multifunctional lipase/phospholipase/acyltransferase by antisense and RNAi approaches led to less membrane degradation and an increase in lipid content by 3.2~4.1-fold without

affecting growth in the diatom *T. pseudonana* (Trentacoste *et al.* 2013). It was suggested that this lipase enzyme plays a role in membrane lipid turnover and lipid homeostasis. Another multifunctional enzyme, CrPDAT (phospholipid: diacylglycerol acyltransferase), with acyltransferase and lipase activities, was reported to mediate TAG synthesis from chloroplast membrane turnover and degradation (Yoon *et al.* 2012) AmiRNA silencing of CrPDAT affected the membrane lipid composition and depressed TAG synthesis in *C. reinhardtii* (Yoon *et al.* 2012).

Knockdown of citrate synthase gene (*CrCIS*) by RNAi enhanced the TAG level by 169.5% in *C. reinhardtii*, while overexpression of *CrCIS* gene decreased the TAG level by 45% (Deng *et al.* 2013). It was suggested that the gene silencing of *CrCIS* caused photosynthetic carbon flux to enter fatty acid synthesis instead of the TCA cycle, thereby controlling the lipid synthesis. Due to antisense knockdown of pyruvate dehydrogenase kinase (PDK), neutral lipid content of transgenic *P. tricornutum* cells increased up to 82% (Ma *et al.* 2014). PDK phosphorylates and deactivates PDC (pyruvate dehydrogenase complex), which catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA.

The nitrate reductase gene (*NR*)-RNAi knockdown transformant of *P. tricornutum* showed an increase in lipid content by 40% (Levitan *et al.* 2015). It was indicated that knockdown of *NR* can result in a redirection from photosynthetic carbon to lipids. By targeted knockout of *NR* gene using TALEN (transcription activator-like effector nuclease) technology, the TAG content of *P. tricornutum* increased immediately following the addition of NO_3^-, which was in agreement with the up-regulated gene expression of key TAG biosynthetic genes, while genes involved in chlorophyll biosynthesis, carbon fixation and metabolism were down-regulated (McCarthy *et al.* 2017). The TALEN fuses a TALE DNA-binding domain to the FokI nuclease domain and can be engineered to cut specific DNA sequences (Lenka *et al.* 2016).

Inactivation of ADP-glucose pyrophosphorylase (AGPase, Figure 2) by X-ray mutagenesis and UV mutagenesis in a *Chlamydomonas* starchless mutant led to a 10-fold increase in TAG content, suggesting that the photosynthetic carbon from starch is driven towards TAG synthesis (Li *et al.* 2010). Targeted genome modification of UDP-glucose pyrophosphorylase gene (*UGPase*) by TALEN enhanced a 45-fold increase in TAG accumulation in *P. tricornutum* (Daboussi *et al.* 2014), indicating that inactivation of *UGPase* gene

involved in the carbohydrate storage pathway lead to an increase in TAG accumulation. Another report showed that antisense knockdown of *UGPase* in *P. tricornutum* led to a significant decrease in chrysolaminaran content and an increase in lipid synthesis (Zhu *et al.* 2016). While antisense knockdown of chrysolaminarin synthase gene in *T. pseudonana* cells reduced the accumulate chrysolaminarin and led to a 3-fold increase in TAG level, with minimal detriment to growth (Hildebrand *et al.* 2017).

It was reported that gene silencing of stearoyl-ACP desaturase (SAD) by amiRNA enhances the stearic acid (C18:0) content in *C. reinhardtii* (De *et al.* 2017). SAD catalyzes the conversion of stearic acid into oleic acid. The reduction of activity of this enzyme could potentially lead to the accumulation of stearic acid. Although genetic modification in *C. vulgaris* by introducing the disrupted omega-3 desaturase (ω-3 FAD) gene through homologous recombination led to higher production of palmitic acid (C16:0) with a reduction in oleic acid (C18:1), no remarkable changes were observed in ALA (C18:3n3) composition (Lau *et al.* 2017). The enzyme ω-3 FAD catalyzes the conversion of C18:2 to C18:3n3. Phytoene, a colorless carotenoid could be accumulated in *Dunaliella salina* V-101 by down-regulating *PDS* gene expression using RNAi and antisense technology (Srinivasan *et al.* 2017). It was reported that the carotenoid levels were reduced in *P. tricornutum* by amiRNA targeted against the endogenous *PSY* gene, a key component of carotenoid biosynthetic pathway (Kaur and Spillane 2015).

6. Limitations and challenges in production of high-value bioproducts from microalgae

Most of the above microalgae-based technologies for production of bioproducts, such as stress manipulation strategies, omics-based technology, and molecular approaches, are feasible at the laboratory scale. However, comprehensive integration of the microalgae-based processes remains challenging especially on a large scale and economic feasibility. Microalgae-based processes need to face the challenges of lowering the costs of biomass harvesting and efficient extraction through engineering and culture management improvements, reducing emission of waste, and screening strains for high productivity (Khan *et al.* 2018). Harvesting of microalgal biomass from cultivation systems accounts for 20~30% of the total cost of production (Taparia *et al.* 2016). On a large scale, microalgae can be cultured in open ponds. Open culture systems are comparatively

inexpensive, but contamination becomes a serious issue. Microalgae grown in sea water exhibit high performance with reduction of cultivation cost in open ponds as well as photo-bioreactors (Rashid *et al.* 2014). Selective cultivation of microalgae under extreme conditions such as high salinity is a method to decrease the contamination issue in open ponds (Hannon *et al.* 2010, Paliwal *et al.* 2017). Further improvements in microalgae cultivation are possible by selecting appropriate species, which possess high growth rate, high productivity of specific bioproducts, and are resistant to the environment.

Molecular approaches have shown great potentials to enhance the accumulation of bioproducts from microalgae, however, the major risks of genetically modified (GM) microalgae related to the human health and environmental impacts are considered as limitations. Release of GM microalgae into the environment could have potential negative ecological effects such as displacing native microalgae, destroying food chains, change in microalgal diversity in the ecosystem, hazardous algal bloom formation, and production of toxins and allergens (Singh *et al.* 2016, Beacham *et al.* 2017). Some microalgae are consumed as human food supplement or animal feed. Therefore, release of GM microalgae can cause harm to human health (Singh *et al.* 2016).

7. Conclusions

Microalgae have promising potential to produce a variety of high-value bioproducts. Suitable stress conditions such as nitrogen starvation, high light, and high salinity could stimulate synthesis and accumulation of high-value bioproducts from microalgae. Two-stage cultivation to favor biomass production at the first stage and to induce the accumulation of high-value bioproducts at the second stage is an efficient strategy. Recent progress in omics-based technologies (genomics, transcriptomics, proteomics, metabolomics, and lipidomics) have boosted the research on microalgal gene regulation and molecular mechanisms under stress conditions, which are helpful to further improve the accumulation of high-value bioproducts via molecular approaches. Furthermore, metabolic engineering, transcriptional engineering, CRISPR system, and RNA-based knockdown strategies show great potentials for enhanced production of high value bioproducts from microalgae. We believe that coupled with omics-based bioinformatics, stress-based strategies (abiotic stress and chemical stress), and molecular approaches (Figure 1), optimized cultivation processes will be manipulated to enhance the production of high-value bioproducts efficiently in microalgae. By overcoming

the associated challenges and limitations, microalgae-based technologies for production of bioproducts from laboratory scale to commercial level will be developed and fine-tuned in the near future.

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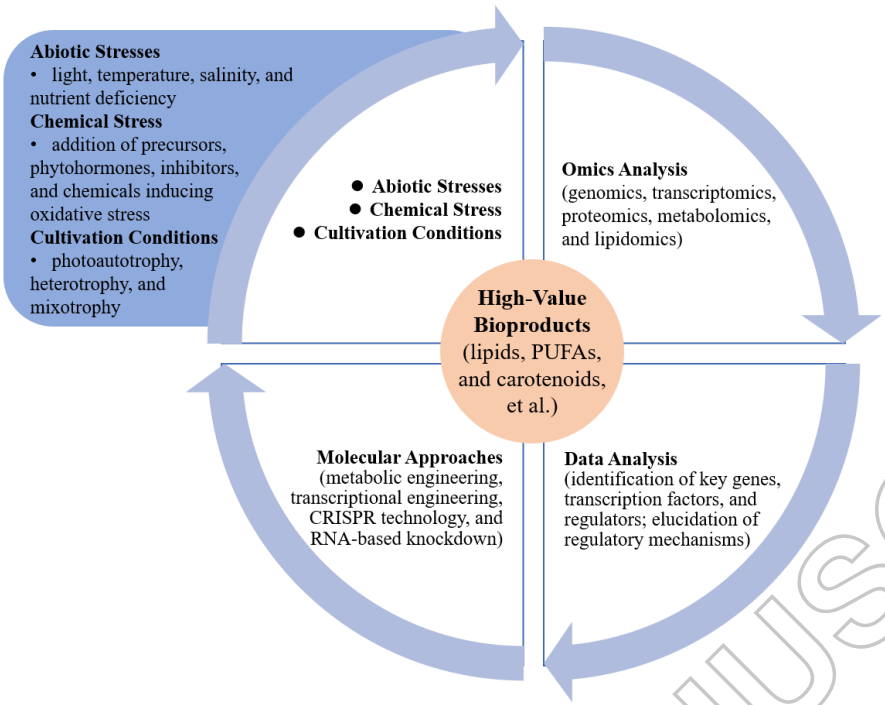

Figure 1 High-value bioproducts from microalgae based on stress manipulation strategies, omics-based technology, and molecular approaches.

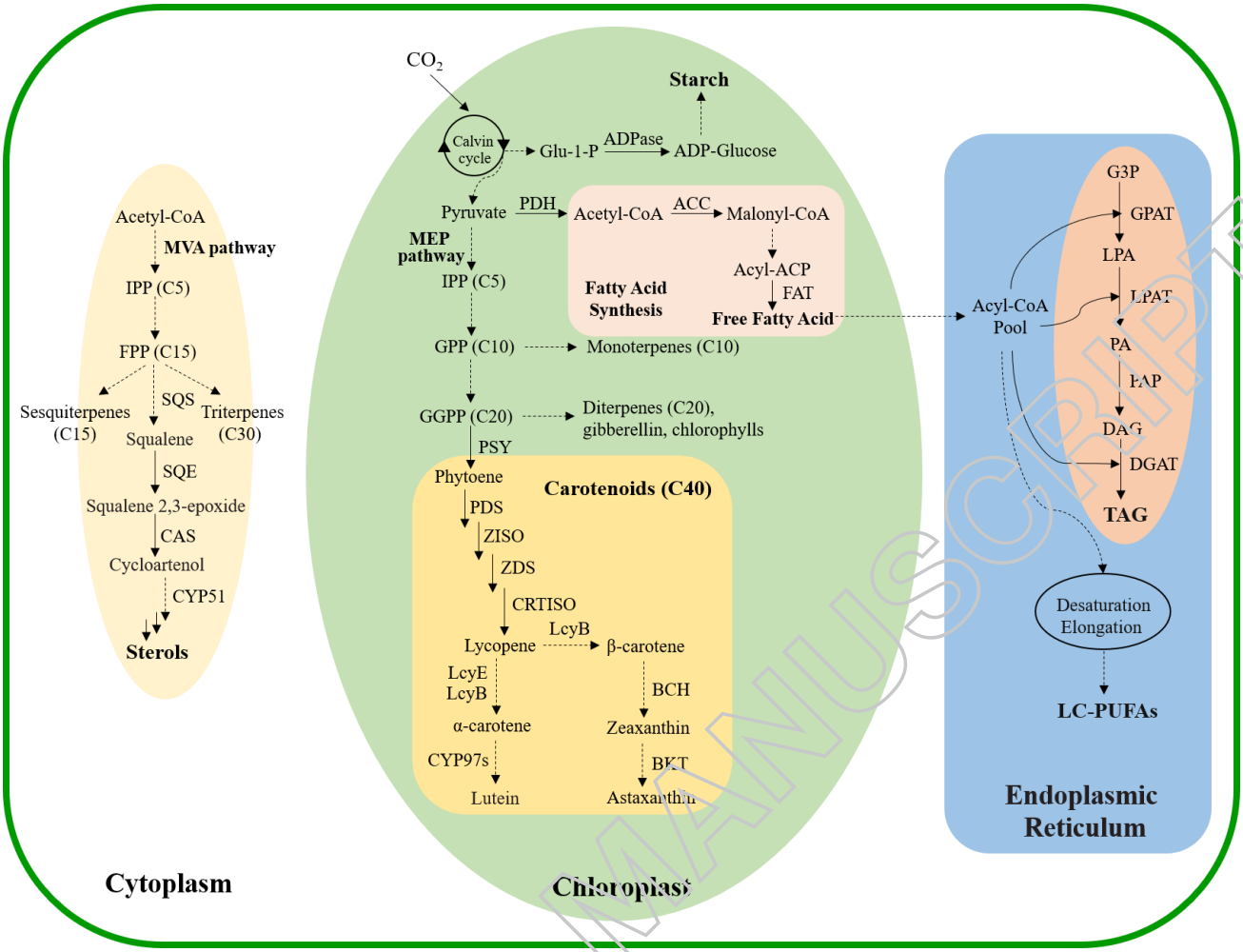

Figure 2 Biosynthetic pathways of some bioproducts from microalgae. ACC, acetyl-CoA carboxylase; AGPase, ADP-glucose pyrophosphate, BCH, β-carotene hydroxylase; BKT, β-carotene ketolase; CAS, cycloartenol synthase; CRTISO, carotenoid isomerase; CYP51, cytochrome P450 sterol-14-α demethylase; CYP97s, cytochrome P450-type carotenoid hydroxylases; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FAT, Acyl-ACP thioesterase; FPP, farnesyl pyrophosphate; G3P, glycerol-3-phosphate; Glu-1-P, glucose 1-phosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPAT, glycerol-3-phosphate acyltransferase; IPP, isopentenyl diphosphate; LcyB, lycopene β-cyclase; LcyE, lycopene ε-cyclase; LPA, lysophosphatidic acid; LPAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PDH, pyruvate dehydrogenase; PDS, phytoene desaturase; PSY, phytoene synthase; SQE, squalene epoxidase; SQS, squalene synthase; TAG, triacylglyceride; ZDS, ζ-carotene desaturase; ZISO, 15-cis-ζ-carotene isomerase.

Table 1 Effects of different abiotic factors or different cultivation conditions on the production of bioproducts in microalgae.

Omics techniques	Species	Cultivation conditions	Effects (compared with the control)	Refs.
		Carbon source		
Genomics and transcriptomics	*C. pyrenoidosa* FACHB-9	Heterotrophy shift to photoautotrophy; heterotrophy as the control	Lipid (~35% DCW), protein (~40% DCW) Up-regulate genes involved in carbon fixation, photosynthesis, fatty acid biosynthesis, and starch catabolism	(Fan *et al.* 2015)
Transcriptomics	*C. sorokiniana* LS-2	Aerated with 10% CO_2; with air as the control	+ TAG Up-regulate genes involved in carbon fixation, carbohydrate metabolism and the TCA cycle	(Sun *et al.* 2016)
Transcriptomics	*H. pluvialis* mutant	High light, 15% CO_2, high light with air as the control	2.7× astaxanthin yield Enhance pyruvate metabolic pathway and fatty acid biosynthesis; up-regulate carotenogenic genes	(Cheng *et al.* 2017)
		High irradiance /high light		
Metabolomics	*Chlamydomonas* sp. JSC4	High light (300 μmol/m^2/s); low light (30 μmol/m^2/s) as the control	+ lipid content, lipid productivity (312 mg/L/d) Increase the levels of TAG-synthesis-related metabolites and the turnover rate of most metabolites for lipid synthesis	(Ho *et al.* 2015)
Transcriptomics	*H. pluvialis*	High irradiance (400 μmol/m^2/s);	3.15× total carotenoid content,	(Gwak *et al.*

and lipidomics		without high irradiance (20 μmol/m^2/s) as the control	5.42× TAG content Up-regulate genes involved in IPP synthesis, astaxanthin biosynthesis, *de novo* fatty acid biosynthesis, and TAG biosynthesis, and ROS scavenging system	2014)
		Nutrient deficiency		
Proteomics and metabolomics	*C. reinhardtii*	Nitrogen starvation; nitrogen repletion as the control	3.8× TFA levels Enhance the proteins involved in glycolysis, TCA cycle, starch, lipid metabolism, and nitrogen assimilation; reduce enzymes of glyoxylate cycle, Calvin cycle, photosynthetic and light harvesting complex	(Wase *et al.* 2014)
Transcriptomics and lipidomics	*N. oceanica* strain IMET1	Nitrogen depletion; nitrogen repletion as the control	~400× TAG synthesis Up-regulate genes involved in carbohydrate and protein degradation as well as genes encoding plastid and mitochondrial membrane transporters	(Li *et al.* 2014)
Transcriptomics	*T. pseudonana*	Silicon starvation; without treatment as the control	~3× FAME content Transcription levels of genes were coordinated with cell cycle progression and photophysiological shifts	(Smith *et al.* 2016)
Proteomics	*C. reinhardtii*	Sulfur deprivation; without treatment as the	+ H_2 production Up-regulate proteins involved in pentose phosphate pathway and fermentative metabolism;	(Chen *et al.* 2010)

		control	down-regulate proteins involved in Calvin cycle and TCA cycle	
		Heat/cold stress		
Transcriptomics and lipidomics	*C. reinhardtii*	Heat stress (42°C); without heat treatment as the control	3× TAGs, + DAGs, - membrane lipids Inhibit ribosome formation and protein synthesis; up-regulate *DGTT1* and *MLDP*; down-regulate several lipid hydrolyzing genes	(Légeret *et al.* 2016)
Transcriptomics and proteomics	*Aurantiochytrium* sp. SD116	15°C cold stress; 25°C as the control	+ DHA content Inhibit glycolysis and the TCA cycle; up-regulate PUFA synthase	(Ma *et al.* 2017)
		oxidative stress		
Proteomics	*H. pluvialis*	With acetate and Fe^{2+}; without acetate and Fe^{2+} as the control	+ astaxanthin Down-regulate some proteins involved in photosynthesis and nitrogen assimilation; up-regulate antioxidative enzymes	(Wang *et al.* 2004)
		High salinity		
——	*Scenedesmus* sp. IITRIND2	100% seawater salinity, without sea salts as the control	+52% lipid content, ~3.2× lipid productivity Reduce photosynthetic pigments, elevate ROS, osmolytes, and activity of antioxidant enzymes	(Arora *et al.* 2017)

×, multiply increased; +, increased; -, decreased.

Table 2 Effect of the addition of chemicals on the accumulation of high-value bioproducts.

Chemicals	Species	Effects	Refs.
Chemicals used as metabolic precursors			
Sodium acetate	*C. vulgaris*	Lipid content (42.5% DCW)	(Najafabadi *et al.* 2015)
Glycerol	*C. protothecoides*	Lipid content (50% DCW)	(Cerón-García *et al.* 2013)
Volatile fatty acids	*C. protothecoides*	Lipid content (48.7% DCW)	(Fei *et al.* 2015)
Malic acid	*Schizochytrium* sp. HX-308	60% DHA/TFA	(Ren *et al.* 2009)
Ethanol	*Schizochytrium* sp. HX-308	+35% lipid content	(Ren *et al.* 2009)
Acetate	*C. cohnii*	+DHA productivity	(Sijtsma *et al.* 2010)
Ethanol	*C. cohnii*	+DHA productivity	(Sijtsma *et al.* 2010)
Pyruvate	*C. zofingiensis*	+28.2% astaxanthin yield	(Chen *et al.* 2009)
Malic acid	*C. zofingiensis*	+ astaxanthin content	(Chen *et al.* 2009)
Citrate	*C. zofingiensis*	+ astaxanthin content	(Chen *et al.* 2009)
Phytohormones and analogs regulating microalgal metabolisms			
ABA	*C. reinhardtii*	+10~13% FAME yield	(Park *et al.* 2013)
ABA	*C. saccharophila*	+ TAG accumulation	(Contreras-Pool *et al.* 2016)
ABA	*S. quadricauda*	+11.17% SFA content	(Sulochana and Arumugam 2016)
SA	*P. tricornutum*	1.3× TFA content	(Xu *et al.* 2017)
JA	*C. vulgaris*	+54% total oil production	(Jusoh *et al.* 2015)
Fulvic acid	*Monoraphidium* sp. FXY-10	54.3% lipid content, 39.7% protein content	(Che *et al.* 2017)

Auxin + salt stress	*D. tertiolecta*	+40% biomass, +36% lipid	(Elarroussi *et al.* 2015)
Auxin	*S. abundans*	3× lipid productivity	(González-Garcinuño *et al.* 2016)
Gibberellin	*C. ellipsoidea*	7× lipid productivity	(González-Garcinuño *et al.* 2016)
IAA	*N. oceanica* CASA CC201	Lipid content (60.9% DCW), EPA (10.76%)	(Udayan and Arumugam 2017)
IBA	*N. linckia*	+ carotenoids, + phycocyanin	(Mansouri and Talebizadeh 2017)
Methyl jasmonate	*H. pluvialis*	+ astaxanthin	(Lu *et al.* 2010)
Gibberellin A3			
SA	*H. pluvialis*	+ astaxanthin productivity	(Gao *et al.* 2012a, Gao *et al.* 2012b, Gao *et al.* 2013a, Gao *et al.* 2013b)a
JA			
Gibberellin A3			
2,4-epibrassinolide			
Fulvic acid	*H. pluvialis* LUGU	+86.89% astaxanthin content	(Zhao *et al.* 2015)
Metabolic inhibitors regulating biosynthetic pathway			
Sodium azide	*C. desiccata*	+60~80% TAG yield	(Zalogin and Pick 2014a)
Sesamol	*C. cohnii*	+11.25% DHA content, +20% DHA productivity	(Liu *et al.* 2015)
Triethylamine	*D. tertiolecta*	+80% lipid content	(Xue and Jiang 2017)
Triethylamine	*D. bardawil*	+ lycopene	(Liang *et al.* 2016)
Nicotine	*C. regularis* Y-21	+ lycopene	(Ishikawa and Abe 2004)
Nicotine	*D. salina* CCAP 19/18	+ lycopene	(Fazeli *et al.* 2009)

2-methylimidazole	*D. salina*	1.7× lutein content	(Yildirim *et al.* 2017)
Diphenylamine	*H. pluvialis*	1.46× β-carotene content	(Vidhyavathi *et al.* 2009)
Terbinafine	*N. oceanica*	- sterol content, + carotenoid levels, + fatty acid production	(Lu *et al.* 2014)
Chemicals inducing oxidative stress response			
H_2O_2	*C. zofingiensis*	+ astaxanthin yield	(Ip and Chen 2005)
H_2O_2, Fe^{2+}	*Chlorococcum* sp.	+ astaxanthin	(Ma and Chen 2001)
Fe^{2+}, acetate	*D. salina*	+ β-carotene	(Mojaat *et al.* 2008)
Ethanol	*H. pluvialis*	2.03× astaxanthin productivity	(Wen *et al.* 2015)

×, multiply increased; +, increased; -, decreased.

Table 3 Researches about lipid production by gene overexpression in microalgae.

Genes	Source-species	Receiver-Algal species	Notes	Refs.
GK	*F. solaris*	*F. solaris*	+12% lipid productivity.	(Muto *et al.* 2015)
GPAT	*L. incisa*	*C. reinhardtii*	Up to 50 % TAG content	(Iskandarov *et al.* 2016)
GPAT	*P. tricornutum*	*P. tricornutum*	1.81× TAG content	(Balamurugan *et al.* 2017)
DGAT2	*P. tricornutum*	*P. tricornutum*	+35% neutral lipid content	(Niu *et al.* 2013)
DGAT2	*N. oceanica*	*N. oceanica*	+69% neutral lipid content	(Li *et al.* 2016)
DGAT1A	*N. oceanica* IMET1	*N. oceanica* IMET1	+39% TAG content	(Wei *et al.* 2017)
GPDH	*P. tricornutum*	*P. tricornutum*	+60% neutral lipid content	(Yao *et al.* 2014)
AccD, ME	*B. napus, C. reinhardtii*	*D. salina*	+12% lipid content	(Talebi *et al.* 2014)
ME	*P. tricornutum*	*P. tricornutum*	2.5× total lipid content, up to 57.8% DCW	(Xue *et al.* 2015)
ME	*P. tricornutum*	*C. pyrenoidosa*	3.2× neutral lipid content, up to 40.9% DCW	(Xue *et al.* 2016)
G6PD	*P. tricornutum*	*P. tricornutum*	2.7× lipid content, up to 55.7% DCW	(Xue *et al.* 2017)

×, multiply increased; +, increased.

Table 4 Researches about the accumulation of other bioproducts by overexpressing genes in microalgae.

Genes	Source-species	Receiver-Algal species	Notes	Refs.
PSY	*D. salina*	*C. reinhardtii*	2.6× total carotenoids	(Couso *et al.* 2011)
PSY	*C. zofingiensis*	*C. reinhardtii*	2.2× total carotenoids	(Cordero *et al.* 2011)
PSY	*P. tricornutum*	*P. tricornutum*	1.45× fucoxanthin	(Kadono *et al.* 2015)
PDS	*H. pluvialis*	*H. pluvialis*	+32.6% astaxanthin	(Steinbrenner and Sandmann 2006)
PDS	*C. zofingiensis*	*C. zofingiensis*	+32.1% total carotenoids, +54.1% astaxanthin	(Liu *et al.* 2014)
BKT	*H. pluvialis*	*D. salina.*	3.5 μg/g DCW astaxanthin, 1.9 μg/g DCW canthaxanthin	(Anila *et al.* 2016)
BKT	*H. pluvialis*	*H. pluvialis*	2~3× total carotenoids and astaxanthin, 8~10× echinenone and canthaxanthin	(Kathiresan *et al.* 2015)
BCH	*C. reinhardtii*	*D. salina*	3× violaxanthin, 2× zeaxanthin	(Simon *et al.* 2016)
OtElo5	*O. tauri*	*P. tricornutum*	8× DHA content	(Hamilton *et al.* 2014)
OtD6Pt and *OtElo5*	*O. tauri*	*P. tricornutum*	+ + DHA content	(Hamilton *et al.* 2014)
GPAT	*P. tricornutum*	*P. tricornutum*	+ EPA, + DHA	(Balamurugan *et al.* 2017)
DGAT2	*P. tricornutum*	*P. tricornutum*	+76% EPA content	(Niu *et al.* 2013)
DGAT2	*B. napus*	*C. reinhardtii*	+12% ALA	(Ahmad *et al.* 2015)
SBPase	*C. reinhardtii*	*D. bardawil*	+37% glycerol production	(Fang *et al.* 2012)
GPDH	*P. tricornutum*	*P. tricornutum*	6.8× glycerol concentration	(Yao *et al.* 2014)

×, multiply increased; +, increased.

Table 5 Transcriptional engineering of TFs or TRs for the accumulation of high-value products in microalgae.

Transcription factor	Source-species	Receiver-species	Notes	Refs.
PSR1	*C. reinhardtii*	*C. reinhardtii*	+ TAG	(Ngan *et al.* 2015)
PSR1	*C. reinhardtii*	*C. reinhardtii*	+ starch	(Bajhaiya *et al.* 2016a)
DOF	*C. reinhardtii*	*C. reinhardtii*	~2× total lipid content	(Ibáñez-Salazar *et al.* 2014)
DOF4	*G. max*	*C. ellipsoidea*	+46.4~52.9% lipid content	(Zhang *et al.* 2014)
bHLH2	*N. salina* CCMP1776	*N. salina* CCMP1776	+33% FAME productivity	(Kang *et al.* 2015)
ΔZnCys	*N. gaditana*	*N. gaditana*	+20~35% lipid content in DCW	(Ajjawi *et al.* 2017)

×, multiply increased; +, increased; Δ, knockout.

Table 6 Researches about the accumulation of bioproducts by gene disruption in microalgae.

Genes	Algal species	Gene disruption Methods	Notes	Refs.
Δ*ZEP*/Δ*CpFTSY*	*C. reinhardtii*	CRISPR/Cas9	+ zeaxanthin, + photosynthetic productivity	(Baek *et al.* 2016)
Δ*ZnCys*	*N. gaditana*	CRISPR/Cas9	+ 20~35% lipid content in DCW	(Ajjawi *et al.* 2017)
Δ*CrPEPC1*	*C. reinhardtii*	CRISPRi	+74.4% lipid content, + 94.2% lipid productivity	(Kao and Ng 2017)
Δ*PEPC1*	*C. reinhardtii*	RNAi	+ 20% TAG level	(Deng *et al.* 2014)
Δ*CrPEPC1*, Δ*CrPEPC2*	*C. reinhardtii*	amiRNA	+ 28.7~48.6% TFA content	(Wang *et al.* 2017a)
Δ*MLDP*	*C. reinhardtii*	RNAi	+ LD size, no change in TAG content	(Moellering and Benning 2010)
Δ*PtLDP1*	*P. tricornutum*	RNAi	- lipid contents, - LD size	(Wang *et al.* 2017b)
Δ*CrLIP1*	*C. reinhardtii*	amiRNA	+ total TAG level	(Li *et al.* 2012b)
Δ*lipase*	*T. pseudonana*	antisense, RNAi	3.2~4.1× lipid content	(Trentacoste *et al.* 2013)
Δ*CrPDAT*	*C. reinhardtii*	amiRNA	- TAG synthesis	(Yoon *et al.* 2012)
Δ*CrCIS*	*C. reinhardtii*	RNAi	+ 169.5% TAG level	(Deng *et al.* 2013)
Δ*PDK*	*P. tricornutum*	antisense	Up to 82% lipid content	(Ma *et al.* 2014)
Δ*NR*	*P. tricornutum*	RNAi	+ 40% lipid content	(Levitan *et al.* 2015)

Δ*NR*	*P. tricornutum*	TALEN	+ TAG content	(McCarthy *et al.* 2017)
Δ*AGPase*	*C. reinhardtii*	X-ray mutagenesis, UV mutagenesis	10× TAG content	(Li *et al.* 2010)
Δ*UGPase*	*P. tricornutum*	TALEN	45× triacylglycerol accumulation	(Daboussi *et al.* 2014)
Δ*UGPase*	*P. tricornutum*	Antisense	- chrysolaminaran content, + lipid synthesis	(Zhu *et al.* 2016)
Δchrysolaminarin synthase gene	*T. pseudonana*	Antisense	- chrysolaminaran content, 3× TAG level	(Hildebrand *et al.* 2017)
Δ*SAD*	*C. reinhardtii*	amiRNA	+ stearic acid (C18:0)	(De *et al.* 2017)
Δ*ω-3 FAD*	*C. vulgaris*	homologous recombination	+ palmitic acid (C16:0), - oleic acid (C18:1)	(Lau *et al.* 2017)
Δ*PDS*	*D. salina* V-101	RNAi, antisense	+ phytoene	(Srinivasan *et al.* 2017)
Δ*PSY*	*P. tricornutum*	amiRNA	- carotenoid level	(Kaur and Spillane 2015)

×, multiply increased; +, increased; -, decreased; Δ**,** knockout.