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REVIEW ARTICLE

Micro Algal pigments: An introduction to their biosynthesis, applications and genetic engineering

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ABSTRACT: Algae are an enormous biological group, forming 50% of photosynthetic organisms. In addition to having chlorophyll for the absorption of light photons, algae are rich in red, orange, and yellow carotenoids, which mainly protect cells against harmful radiation and free radicals. Moreover, these organisms have phycobiliproteins (red and blue pigments), which are involved in capturing and passing light energy to chlorophylls during photosynthesis and have a wide range of antioxidant properties. Algae also play a key role in substituting artificial colorants with natural colorants due to the adverse side-effects of chemical colorants, especially since natural colors are commonly used by individuals and various industries. Recently, algal pigments have been widely used in medical, nutraceutical, cosmeceutical, and pharmaceutical industries owing to their antioxidant, antidiabetic, anti-obesity, anti-inflammatory, antiaging, antimalarial, and neuroprotective properties. The growing demand for algal bioproducts highlights the importance of evaluating the trends influential factors in their production. The current review study provided an introduction to algal pigment classification, distribution, function, application, and biological production. In addition, we have discussed crucial biochemical pathways, enzymes, and gene/biotechnological modifications, such as transformation and expression regulation, which noticeably affect the metabolism of their sink and source.

KEYWORDS: Algae, Pigment Biotechnology, Photosynthetic Pigments, Chlorophyll, Carotenoids, Phycobiliprotein.

INTRODUCTION

Algae are a large and diverse group of eukaryotes [1] and extremely diverse polyphyletic organisms, which contain multiple species ranging from unicellular microalgae to multicellular macroalgae. Macroalgae are eukaryotic and multicellular organisms with varying sizes from millimeters to meters. Some of these organisms have plant-like structures similar to roots, shoots, leaves, and even flagellums [10, 18]. Seaweeds with different color varieties represent the macroalgae that are mostly found in coastal areas.

Brown algae (*Phaeophyceae*) with approximately 4,000 species, red algae (*Rhodophyta*) with approximately 5,000 species, and green algae (*Chlorophyceae*) with approximately 7,000 species are the most important species of macroalgae. Macroalgae are extensively utilized in Asian food products as they contain valuable bioactive compounds, such as laminarin, alginic acid, alginate, agar, fucoidan, mannitol, and fucoxanthin [64, 78, 82]. China and Indonesia are the main producers of macroalgae (21-23 million tons) [79, 82]. On the other

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hand, microalgae are unicellular microorganisms with about 20,000 species and a size of 1-400 micrometers. These organisms are classified into different species, the most important of which are green algae (*Chlorophyceae*), blue-green algae (*Cyanophyceae* [cyanobacteria]), golden algae (*Chrysophyceae*), yellow-green algae (*Xanthophyceae*), and diatom (*Bacillariophyceae*).

Microalgae are extensively used in pharmaceutical products and food ingredients owing to their anticancer, antimicrobial, and anti-inflammatory properties [29]. Despite the comparatively costly commercialization of microalgae products due to their expensive cultivation and harvesting process setup, the production of microalgae increased steadily from 500 tons per year in 1984 to 20,000 tons per year in 2016 [85-88]. This increment is attributed to several advantages of microalgae, such as having no competition with crops for freshwater and farmable land as they could be cultivated in salty water and on non-fertile land. Furthermore, microalgae have a high growth rate and significant metabolite contents based on the dry weight index [14]. Microalgae could also improve air quality via reducing carbon dioxide and greenhouse gas emissions. Another advantage is that microalgae biomass residue could be used as a nitrogen source fertilizer for crops or a protein-rich animal feed. In addition, they could be cultured based on most wastewaters and contain a broader range and greater concentration of photosynthetic pigments, which result in the successful ecological adoption of microalgae to divers aquatic environments with different light quality and intensity (unlike higher plants) [2, 3], thereby serving as a remarkable source of natural pigments [4-6]. In the current review study, we have discussed various aspects of microalgae pigments.

Chlorophylls, carotenoids, and phycobiliproteins (PBPs) are the ley photosynthetic pigments found in algae [3]. In general, the most important pigments in macroalgae are chlorophyll a and b, carotenoids, and xanthophylls, while microalgae primarily contain chlorophyll a, phycocyanin, and phycoerythrin. However, these compounds are often integrated into the chloroplast lamellae structure, while they might be homogeneously distributed as chromatoplasm, especially in blue-green algae. These pigments may be built with long-chain tetrapyrrole rings (e.g., chlorophyll and PBPs) and isoprene-based rings (e.g., carotenoids) depending on the chemical structure of their subunits. The conjugated ring systems of chlorophyll and PBPs involve pigments in the absorption of the visible range of light and transduction of energy levels via resonance during photosynthesis [2, 4, 7].

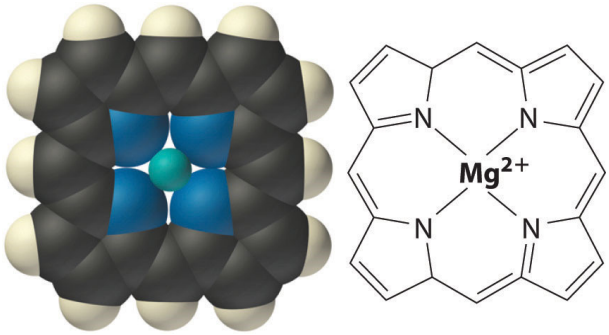

Figure 1. The structure of chlorophyll's core.

Among various algal metabolites and pigments, phycocyanin (blue pigment from *Spirulina*), β-carotene (yellow pigment from *Dunaliella*), and astaxanthin (yellow-to-red pigment from *Haematococcus*) are natural, nontoxic, and non-carcinogenic colorants that are gaining popularity, acceptability, and importance over synthetic pigments [7-11].

Chlorophylls (Chl)

Chlorophyll, with the general formula of $C_{(35-55)}H_{(28-72)}MgN_4O_{(5-7)}$, is the most common pigment in photosynthetic organisms (from cyanobacteria and aquatic species to algae and higher plants), which is localized in all light harvesting complexes and the reaction centers of photosystems I and II [2,12-14].

Algae contain five classes of chlorophylls, which are categorized as a, b, c, d, e, and f and have hydrophobic properties. As is shown in Fig. 1, these classes are composed of four -CH bridged pyrrole rings with a central metal ion (generally a magnesium), enclosed by four nitrogen atoms and connected to the tetrapyrrole ring [15, 16]. This structure contains several associated double bonds, which are responsible for absorbing visible wavelengths, especially at 670-680 nanometers (red) and 435-455 nanometers (blue) [16]. Chlorophylls also have a long hydrophobic tail, which anchors the hydrophobic segments within the thylakoid membrane (Raven et al., 2005). Chlorophyll molecules have different tails, which determine their key properties and absorption peak of solar light [17].

Chlorophyll b (Chl b) has a yellowish color in its natural state and absorbs blue light, especially at the wavelengths of 453 and 625 nanometers. However, its chemical formula is $C_{55}H_{70}MgN_4O_6$ and has a formyl group in its

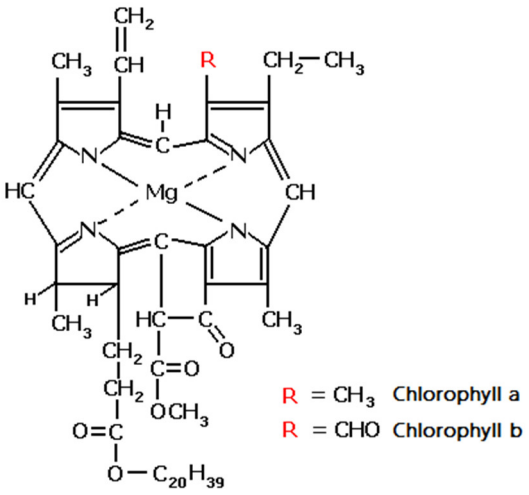

Figure 2. Chlorophyll a and b.

second ring instead of a methyl group unlike Chl a (Fig. 2) [16, 18]. Chl b is found in green algae and higher plants and assists Chl a in the photosynthesis process [12, 19].

Chlorophyll c (Chl c) is a brownish-golden pigment, and its absorption peak is at 445 and 625 nanometers. Chl c cooperates with chlorophyll a in photosynthesis and is abundantly found in diatoms and marine algae [12]. This chlorophyll is divided into three subclasses, which are c1 ($C_{35}H_{30}MgN_4O_5$), c2 ($C_{35}H_{28}MgN_4O_5$), and c3 ($C_{36}H_{28}MgN_4O_7$"). C1 is considered to be the most common form of Chl c [12].

Chlorophyll d (Chl d) absorbs the extreme red end of the spectrum of sunlight, especially at the wavelengths of 450 and 690 nanometers *in-vitro* [12, 20, 21]. Chl d was first identified in red algae (*Rhodophyta*). The chemical formula is $C_{54}H_{70}MgN_4O_6$, and Chl d has a formyl group instead of a divinyl group in the ring A of porphyrin in contrast to Chl a [22].

Chlorophyll e (Chl e) is the rarest chlorophyll and is found in *Vaucheria hamata* and *Tribonema bombycinum*. The functioning mechanism of this compound remains unclear [12].

Chlorophyll f (Chl f) is the most recently discovered chlorophyll in cyanobacteria, which can absorb the lowest light from the extreme end of the infrared spectrum for photosynthesis. The absorption and fluorescence peaks of Chl f are 706 and 722 nanometers, respectively [12, 23]. With the chemical formula of $C_{55}H_{70}MgN_4O_6$, Chl f has a CHO at the C_2 position of its porphyrin ring in contrast to Chl b [24].

Biosynthesis and Pathway of Chlorophylls

Chlorophylls are the most abundant pigments in nature, and their biosynthesis constitutes a major metabolic activity in photosynthetic organisms. The C5 pathway in plastids is responsible for synthesizing tetrapyrrole pigments. Moreover, this pathway produces crucial precursors and aminolevulinic acid (ALA), which is an intermediary C5 compound [25].

In the following section, we have described the process of chlorophyll synthesis via the C5 pathway and the genes and proteins that are essentially involved and could be genetically manipulated to enhance/regulate its biosynthesis (Fig. 3) [12, 26, 27]:

1. Glutamate activation by t-RNA^{Glu}
2. Formation of glutamyl-1 semialdehyde through the reduction of glutamyl t-RNA^{Glu} (crucial enzyme: glutamyl t-RNA^{Glu} reductase)
3. Conversion of glutamate-1 semialdehyde into ALA
4. Producing monopyrrole porphobilinogen from two ALAs (via ALA dehydratase enzyme)
5. Formation of hydroxymethylbilane (tetrapyrrole) through the deamination of four porphobilinogen molecules (crucial enzyme: hydroxymethylbilane synthase)
6. Formation of uroporphyrinogen III (first cyclic compound of the chlorophyll biosynthesis pathway) from hydroxymethylbilane (catalyzed by uroporphyrinogen III synthase)
7. Catalysis of coproporphyrinogen III through the decarboxylation of the acetate chains of uroporphyrinogen III
8. Formation of protoporphyrinogen IX through the oxidative decarboxylation of coproporphyrinogen III
9. Catalysis of magnesium ion insertion via the magnesium chelatase enzyme and ATP hydrolysis
10. Conversion of magnesium-protoporphyrin IX to magnesium-protoporphyrin IX monoethyl ester by S-adenosyl-L-methionine through the esterification of the 6th position of the propionic acid side chain and using S-adenosyl-L-methionine as a cofactor
11. Constitution of magnesium-2, 4-divinylpheoporphyrin a5 (MgDVP) via β-oxidation and cyclization of the 6th position of ring C by magnesium protoporphyrin IX oxidative cyclase (crucial)
12. Conversion of protochlorophyllide into chlorophyllide a and dihydroporphyrin or chlorin (macrocycle) through a photoreduction process catalyzed by NADPH protochlorophyllide oxidoreductase (a chromophore in the macrocycle causes the green color of Chl a.)

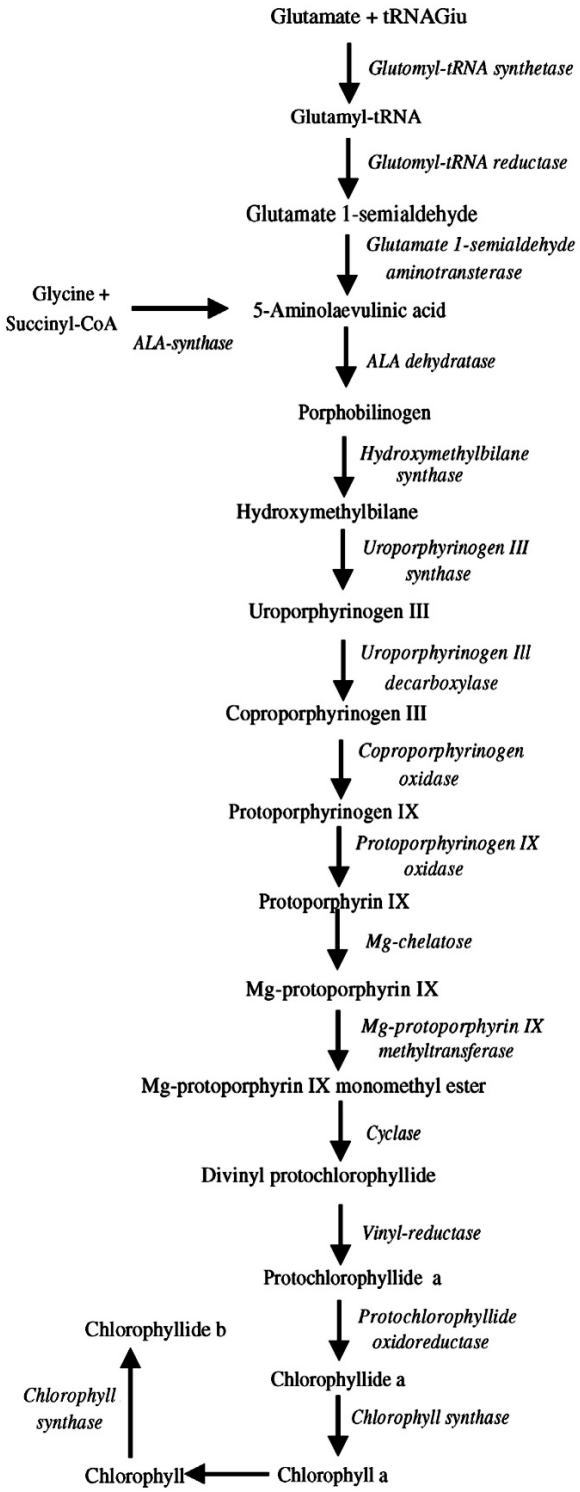

Figure 3. Chlorophyll biosynthesis pathway [106].

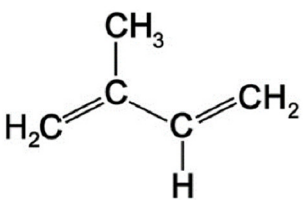

Figure 4. Isoprene unit.

Carotenoids

Carotenoids are isoprenoids that are synthesized by all photosynthetic organisms and are produced by eight isoprene units (C_5H_8) (Fig. 4). They have a single long hydrocarbon chain and contain 40 carbon atoms [4]. Carotenoids are water-insoluble pigments and consorted with chloroplast lipids [28]. They are often classified into carotenes and xanthophylls based on the chemical elements found in their structure [29]. Carotenes are merely composed of hydrocarbon, while xanthophylls contain various functional groups, such as hydroxyl, epoxide, ketone and methoxy. In other words, xanthophylls are produced by hydroxylation and the subsequent oxidation of carotenes [30]. These oxygen-containing functional groups affect the biochemical functions of carotenoids, making xanthophylls more polar and hydrophile than carotenes [31, 32].

Biosynthesis and Pathways of Carotenoids

The biosynthesis of carotenoids occurs in the cytoplasm and the chloroplast via a conventional metabolic pathway although it is reported that its details differ in various species [33]. Isopentenyl pyrophosphate (IPP) is the precursor for carotenoid synthesis, which has two pathways for biosynthesis; the mevalonate dependent pathway, which is in the cytosol, and the 1-deoxy-D-xylulose-5-phosphate dependent pathway (DOXP or MEP pathway), which is often in the chloroplast [34]. IPP synthesis in *Haematococcus pluvialis* and *Chlamydomonas reinhardtii* (unicellular green microalgae) is assumed to occur exclusively through the non-mevalonate DOXP pathway in plastids [35, 36].

In the following section, we have described the further proposed steps for carotenoid biosynthesis, as well as the critical genes and proteins as proper candidates for genetical manipulation studies (Fig. 5).

1. Production of geranyl-geranyl diphosphate (GGPP) through the condensation of three IPP molecules and one molecule of dimethylallyl diphosphate by GGPP synthase. GGPP is also the precursor for several other groups of metabolites, including chlorophylls, ubiquinones, and tocopherols.
2. Formation of a 40-carbon phytoene (precursor of all carotenoids) through the condensation of two GGPPs; This step is catalyzed by phytoene synthase (PSY), which is a critical enzyme in carotenoid biosynthesis. Green algae such as *Ostreococcus* and *Micromonas* have two classes of PSYs, while species such as *C.*

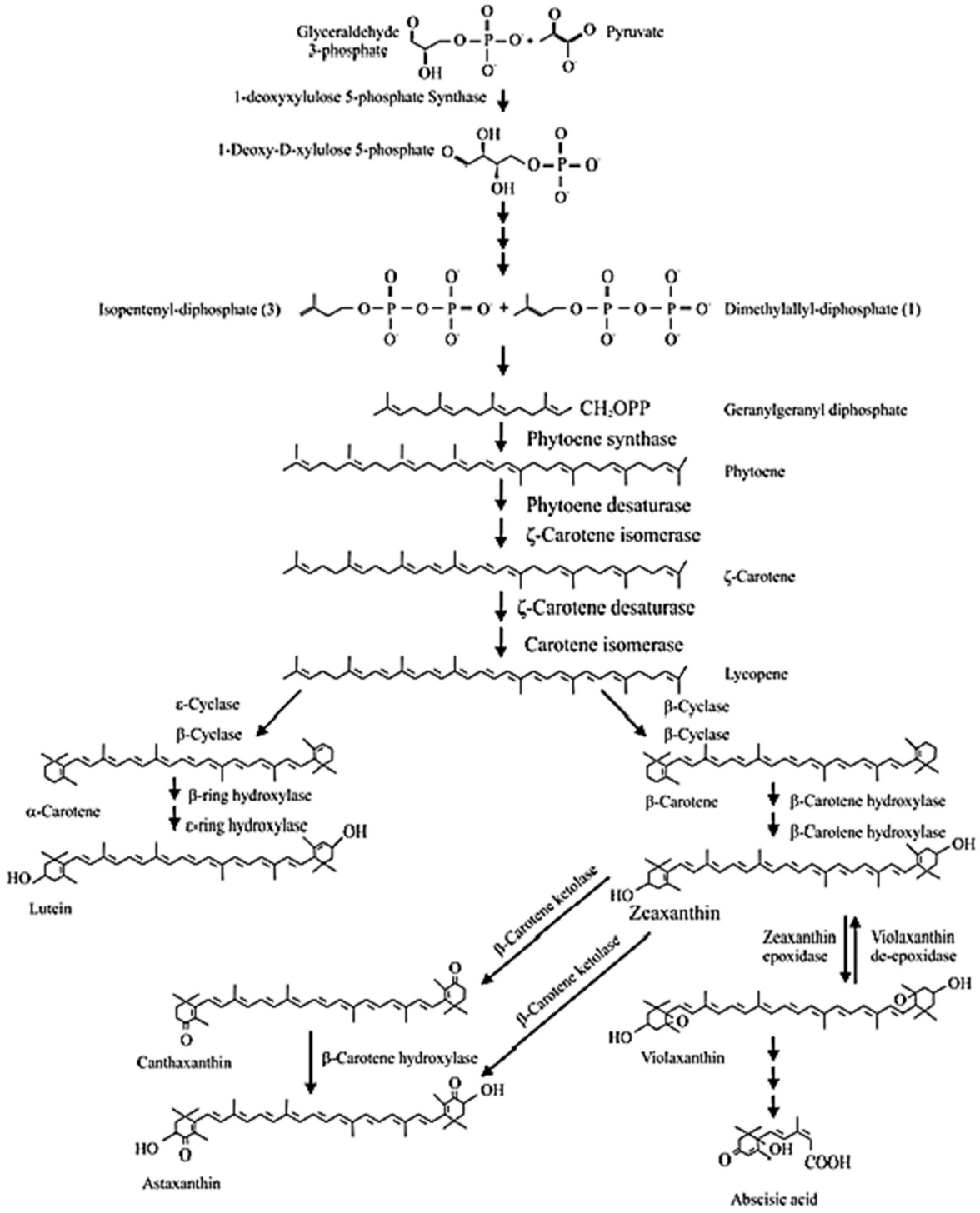

Figure 5. Carotenoid and its derivatives biosynthesis pathways [107].

reinhardtii and *C. vulgaris* have only one class of PSYs (Tran et al., 2009). Phytoene undergoes sequential reactions to form lycopene; in bacteria, only one phytoene desaturase catalyzes this conversion (encoded by *CrtI* gene). In plants, a minimum of four enzymatic reactions is required to complete this step with various enzymes, including phytoene desaturase (PDS), zeta-carotene desaturase (ZDS), zeta carotene isomerase (ZISO), and carotenoid isomerase (CRTISO).

3. Conversion of phytoene into lycopene;

3-1. Production of poly-*cis*-compounds from phytoene by PDS

3-2. Isomerization of poly-*cis*-compounds to zeta carotene (ζ-carotene) by ZISO

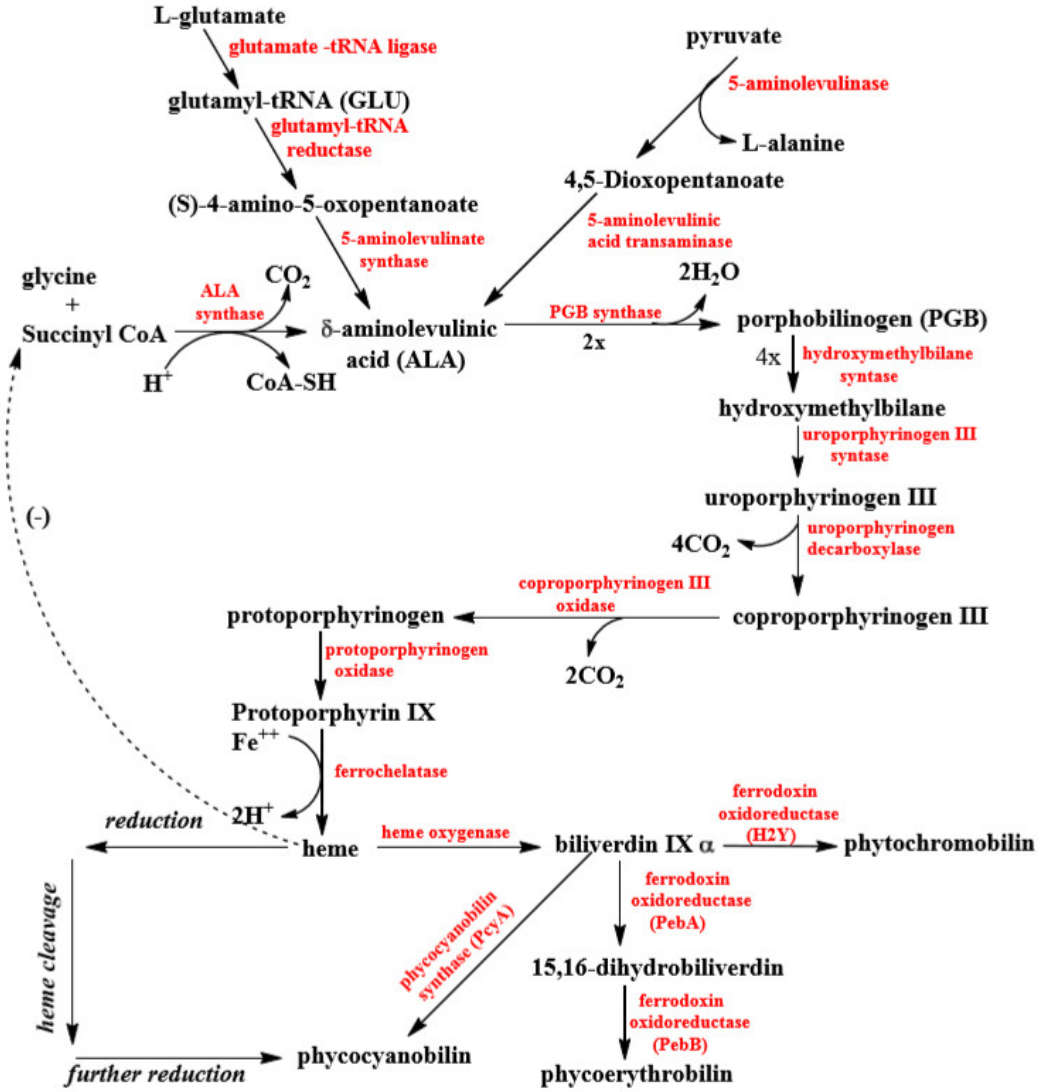

Figure 6. Phycobilins biosynthetic pathways in algae and plant [46]

3-3. Dehydrogenation of ζ-carotene to phytofluene by ZDS

3-4. Isomerization of phytofluene to lycopene by CRTISO

Higher plants and green algae have additional carotenoids such as α-carotene and β-carotene, as well as their derivatives, including lutein (α-carotene derivative), zeaxanthin, vilaxanthin, canthaxanthin, and astaxanthin (β-carotene derivatives).

When lycopene β-cyclase (β-CYC) introduces a β-ring at one end and ε-cyclase (ε-CYC) adds a ε-ring to the other end of lycopene, α-carotene is produced. The hydroxylation of the β and ε rings of α-carotene by the β-ring hydroxylase and ε-ring hydroxylase, respectively lead to the production of lutein. Zeinoxanthin, which is a β-ring hydroxylated α-carotene, is the transitional substrate of this process.

Lutein is a key photosynthetic pigment in the xanthophyll family, and it is reported that PDS is the key enzyme involved in its biosynthesis. *Chlorella protothecoides* and *Scenedesmus almeriensis* have also been proposed as the potential microalgae producers of lutein [37, 38]. Factors such as pH, EC, light, temperature, and availability of nitrogen and oxidizing substances are considered to be most influential in the lutein content of algae [28]. When β-CYC introduces a β-ring at both ends of lycopene through a consecutive two-step reaction, β-carotene is produced. If both beta rings of β-carotene are hydroxylated, zeaxanthin is yielded. Zeaxanthin could be epoxidized once to form antheraxanthin and twice to form violaxanthin [39, 40].

Phycobiliproteins (PBPs)

PBPs are the main light-harvesting chromoproteins, which capture and pass the light energy on to chlorophylls during photosynthesis and are mainly found in *Rhodophyta*, *Cyanobacteria*, and certain type of marine algae [41, 42]. PBPs are water-soluble and highly fluorescent proteins belonging to open-chain tetrapyrroles with linear prosthetic groups (bilins), which are covalently bound via cysteine amino acid residues [43]. These protein pigments are categorized based on properties such as structure, absorption spectra (consensus maximum absorbance, Lambda max [λ_{max}]), and color. Based on absorbance wavelength, cyanobacteria and red algae PBPs are classified into four categories, as follows:

1. Phycoerythrin (PE) is purple, and its λ_{max} is within the range of 490-570 nanometers;
2. Phycoerythrocyanin (PEC) is orange, and its λ_{max} is within the range of 560-600 nanometers;
3. Phycocyanin (PC) is blue, and its λ_{max} is within the range of 610-625 nanometers;
4. Allophycocyanin (APC) has a bluish green color, and its λ_{max} is within the range of 650-660 nanometers.

Cryptomonads are classified into two PBP categories, which are as follows [44]:

1. PE; λ_{max}= 540-570 nm
2. PC; λ_{max}= 610-650 nm

PBPs are also classified into two large groups based on their color; red PBP is known as phycoerythrin, and phycocyanin is blue PBP. Moreover, phycocyanins are divided into three subcategories [45], which are allophycocyanin (APC), C-phycocyanin (C-PC), and R-phycocyanin (R-PC). PEs are also subdivided into B-PE and R-PE [46, 47].

Biosynthesis Pathways of Phycobilins

Phycobilins are biosynthesized from heme, and their biosynthesis is initiated by a significant increase (2-3-fold) in the heme pigment content [48]. Following that, heme is converted into biliverdin IXα through the action of heme oxygenase. Three main phycobilins are directly produced from biliverdin, including phycoerythrobilin, phycocyanobilin, and phytochromobilin. An outline of this production process has been described in the following section (Fig. 6).

At the first step, biliverdin is reduced to 15,16-dihydrobiliverdin IXα by biliverdin 15,16-reductase, which is reduced to phycoerythrobilin by bilin 2,3-reductase. The next step is the isomerization of phycoerythrobilin to phycocyanobilin through the action of phycobilin isomerase. In addition, ferredoxin oxidoreductase catalyzes the production of phytochromobilin in the final phase [49, 50].

In addition to the phycobilins originating from heme (phycocyanobilin, phycoerythrobilin, and photochromobilin), there is another group of phycobilins, including phycocyanobilin (PVB) and phycourobilin (PUB), which contain a vinyl group at their C3 and are produced by phycoerythrobilin lyase-isomerase [49].

Influential Factors in Algal Pigment Production

In addition to genes and proteins as significant influential factors in algal pigment, the most important environmental determinants in this regard are light intensity, wavelength, photoperiods, temperature, pH, nitrogen supplements, heavy metals, and salinity stress [10, 51, 52].

Light

Light intensity and quality are the most significant environmental factors affecting pigment production. For instance, the far-red, red, green, and blue spectra of light in microalgae act as photomorphogenic signals, thereby affecting their relative pigment composition. According to the literature, individual light regime affects the productivity of algae [2, 53]. The structure and composition of phycobilisome could also change by light intensity, alterations in the total irradiance, and absence of spectral shifts [10]. In cyanobacteria, low light stimulates PBP production [54]. In *Dunaliella salina* and *Spirulina platensis*, chlorophyll production and content per cell increase at low light intensity, while the irradiance production of chlorophyll decreases with increased light [10].

Light intensity also has a significant effect on the enhanced production of carotenoids and accumulation of β-carotene in *Dunaliella salina* [55]. Therefore, it seems that the higher irradiation of blue-green microalgae could significantly improve the production of β-carotene. In *Nostoc muscorum*, high light intensity (13-28 klux) has been shown to stimulate carotenoid production, while low light intensity (1 klux) leads to the accumulation of PBPs [56]. Astaxanthin accumulation has demonstrated the same pattern in *Haematococcus pluvialis* [57]. *Anacystis nidulans*, *Synechococcus* sp., *Calothrix* 7601, *Nostoc*

UAM 206, and *N. muscorum* have also shown increased phycobiliprotein production with high red light intensity [58]. In nitrogen-fixing cyanobacteria, red light only enhanced the C-phycocyanin content significantly [59].

Temperature

The temperature of microorganism culture media is another significant environmental factor influencing several metabolic mechanisms, including enzymatic activity, membrane fluidity, and the electron transport chain [60]. With regard to algae, optimal growth and a tolerable temperature range vary in different strains [52]. In blue-green microalgae, the temperature of 25-30°C causes the accumulation of carotenoids (e.g., β-carotene), while the temperature of 28°C facilitates astaxanthin and lutein biosynthesis [56, 61]. The temperature of 25-36°C is considered to be optimal for phycobiliprotein production in various algal strains [62].

pH

The pH effects on organisms indirectly. In fact, pH influences and alters the solubility and the bioavailability of nutrients. In related to the microalgae pigments, the pH 8-9 lead to maximum production of chlorophyll and PBPs. For instance, it has been reported that the pH of 8 led to the optimum production of PBPs in *Anabaena* sp. and *Synechocystis* sp., as well as the production of carotenoids in *Scenedesmus almeriensis* [9]. Furthermore, the pH of 9 is considered optimal for the production of phycocyanin, phycoerythrin, and allophycocyanin in *Nostoc* sp., as well as the production of chlorophyll in *Spirulina platensis.* The pH value of 6.5-7.5 is also regarded as the optimum range for carotenoid production; such examples are astaxanthin in *Chlorella zofingiensis*, carotenoid in *Chlorella protothecoides*, lutein in *Muriellopsis* sp., and *Chlorococcum citriforme, Neospongiococcus gelatinosum*, and β-carotene in *Dunaliella salina* [9].

Salinity

Salt concentration and osmotic pressure have a crucial impact on the chlorophyll and carotenoid content of microalgae. These parameters become more significant when cultivated in a saline environment [55, 63]. As a general principle, maximum carotenoid production is obtained at the enhanced concentration of salts, whereas the low concentration of salts increases chlorophyll and PBP production. Previous findings have also indicated that increased salinity could decrease the chlorophyll content, while enhancing the β-carotene production in blue-green microalgae. For instance, *Dunaliella salina* produced 54.12 mg/g of β-carotene at three ppt (parts per trillion) of salinity, and *Oscillatoria* sp. produced 66.70 mg/g of phycoerythrin at 15 ppt. According to Hemlata and Fatma [52], PBP production in *Anabaena* showed an increase of up to 135.73 mg/g at 10 ppt of salt.

Nutrient Supplement

Several factors may influence microalgal growth rate, biomass, and pigment production. Culture medium and its composition play a pivotal role in this regard. Nitrogen and iron are the most influential nutrients in algal growth media. Nitrogen is an essential macronutrient to microalgal growth and is involved in the synthesis of carbohydrates, lipids, and protein. Moreover, nitrogen has a positive and direct impact on chlorophyll production since the Chl molecule structure contains four nitrogen atoms [64].

According to the literature, nitrogen repletion is a proper strategy for increasing protein, neoxanthin, β-carotene, and diadinoxanthin in the algal biomass, while its starvation directly enhances algal lipid production [65] and indirectly decreases the β-carotene content in algae as the formation of free radicals increases with the reduction of nitrogen, and the free radicals cause the breakdown of beta carotene. Nitrate, nitrite, urea, and ammonium are the nitrogen forms that could be used by microalgae. However, using nitrate ($NO3^-$) is more common compared to ammonium salts ($NH4^+$) as nitrate is more stable with the shift of pH, and ammonia ($NH4^+$) concentrations of more than 25 μM are toxic for microalgae [66, 67].

Some genus of microalgae (unlike macroalgae) are able to fix nitrogen. Such examples are *Anabaena*, *Nostoc*, *Aulosira*, *Tolypothrix*, and *Scytonema*, which are commonly used in rice cultivation in Asian countries, especially China and India. It is estimated that the mentioned genus could fix 20 kilograms of nitrogen per Ha/year, which is one-third of the nitrogen requirements of traditional rice cultivars [64]. Notably, the iron content of the culture medium positively affects β-carotene and astaxanthin production [68-70].

Microalgal Pigment Applications

As mentioned earlier, microalgal cells contain numerous pigments, such as chlorophylls, carotenoids, and PBPs.

These pigments have various applications in bio-technological studies, as well as in food, cosmetic, diagnostic, and pharmaceutical industries. Owing to their nontoxic, non-carcinogenic, antioxidative, and immune-boosting properties, their industrial applications are on the rise. Some of their applications and advantages have been described in the following section.

Chlorophylls have long been used as a traditional therapeutic medicine for wound healing, controlling calcium oxalate crystal, and preventing bacteria advancement [71]. It is acknowledged that these compounds are effective in the treatment of cancer and cardiovascular diseases, while could also decrease 'bad breath' and colostomy odor [72, 73]. The intravenous injection of chlorophyll is a common approach in the treatment of pancreatitis patients in order to reduce inflammation and pain. In the early stages of lung and skin cancer, the intravenous injection of chlorophylls, along with talaporfin and laser therapy, is also prescribed to prevent disease progression [74].

Since the chemical structure of chlorophylls is similar to hemoglobin, it may facilitate the rate of carbon dioxide and oxygen interchange [75]. This may explain the positive effects of these compounds on rapid wound healing and the formation of new tissues [72, 76]. Furthermore, this property renders chlorophyll a key compound in the treatment of postoperative ulcers in rectal surgeries, increasing the rate of healing by 25% [73]. Chlorophyll derivatives (especially pheophorbide b and pheophytin b) are considered to be potent protective antioxidants, which could scavenge the mutagens in the gastrointestinal tract [77].

Carotenoids have widespread applications as feed, food/cosmetic additives, and colorants [40]. The positive health properties of carotenoids are mainly associated with their antioxidant activities, which reduce the risk of AIDS, diabetes, cataract, macular degeneration, and neurodegeneration [11, 78-80].

Astaxanthin is a significant free radical scavenger, has substantial antioxidant activity, and inhibits lipid peroxidation. The Food and Drug Administration (FDA) has approved the use of astaxanthin as a food colorant in fish and animal feed [81]. *H. pluvialis* is an astaxanthin is a rich algal reservoir, and its extract could act as a cell growth inhibitor by stimulating apoptosis in a dose- and time-dependent manner [58].

Fucoxanthin is a xanthophyll, which is found in diatoms and golden-brown unicellular microalgae and has anticancer, antioxidant, antiangiogenic, antidiabetic, anti-obesity, anti-inflammatory, and antimalarial properties [81]. It has also exhibited anticancer effects on patients with colon cancer, leukemia, liver cancer, prostate cancer, breast cancer, gastric cancer, and urinary bladder cancer. The anticancer properties of fucoxanthin are attributed to the reduction of cell viability by arresting the cell cycle during the G_0/G_1 phase [82] or apoptosis induction through chemoprevention effects, such as the impact on EJ-1 human bladder cancer. On the other hand, previous findings have confirmed the anti-obesity activity of fucoxanthin, especially in combination with pomegranate seed oil (xanthigen). Since obesity is an important public health concern and leads to cardiovascular diseases, hypertension, hyperlipidemia, and type II diabetes, the development of fucoxanthin production has gained importance in an unprecedented manner [83, 84].

According to the literature, fucoxanthin could also regulate the production of inflammatory mediators such as nitric oxide and prostaglandin E2 through inhibiting the activation of nuclear factor-jB (NFjB) and mitogen-activated protein kinase phosphorylation in lipopolysaccharide-stimulated inflammatory responses [85]. Inhibiting the production of these inflammatory mediators is a potential target in the treatment of inflammatory diseases [86].

Canthaxanthin is a lipid-soluble natural xanthophyll with remarkable antioxidant activity compared to β-carotene [87]. Canthaxanthin is used as a food additive for farmed shrimp and salmon fish to improve the skin color. Zeaxanthin and lutein are also categorized as xanthophylls and have great positive potential in maintaining eye health [88].

Phycocyanin is a water-soluble pigment [89] with potential anticancer activity. It stimulates apoptosis through G_2/M cell cycle arrest, and its anticancer activity against human pancreatic adenocarcinoma has been confirmed *in-vitro* and *in-vivo* [72].

Gene Manipulation and Transformation

Biochemical pathways could be modified through the regulation of their associated transcriptional and translational genes. Genes could be 'knocked out' or 'knocked in' through genetic engineering to achieve the desired results. This approach is more rapid and more accurate compared to random mutagenesis as it precisely targets the appropriate gene, thereby increasing productivity and reducing costs, which make the approach a proper option for daily use. On the other hand, genetic

Table 1. Enhancement of pigment synthesis using genetic engineering of microalgal strains

Micro / macro algal strain	Engineered Gene(s)	Result	Reference
Chlamydomonas reinhardtii	Overexpression of Phytoene synthase	Increased the production of violaxanthin, lutein, and neoxanthin	[92]
Chlamydomonas reinhardtii	Overexpression of PSY	Increased the production of violaxanthin and lutein	[108]
Chromochloris zofingiensis	Expression and analysis of cartenogenic genes	Increased the production of carotenoid	[108]
Chlamydomonas reinhardtii	Silencing the chlorophyllide *a* oxygenase (*CAO*) gene	Increased the production of photosynthesis and chlorophyll *b*	[109]
Chlamydomonas reinhardtii	Overexpression of Phytoene desaturase (CrPDS-L505F)	Increased the resistance to herbicide norflurazon and the production of carotenoids and zeaxanthin	[110]
Chlorella zofingiensis	Overexpression of PDS	Increased the production of carotenoid	[111]
Phaeodactylum tricornutum	Transformation of *dxs* and *psy* genes	Increased the content of fucoxanthin	[112]
Dunaliella salina	Transformation of *bkt* gene	Increased the production of the astaxanthin and canthaxanthin	[113]
Scenedesmus sp. CPC2	transformation of *psy* gene	Increased the production of β-carotene	[114]
Chlamydomonas reinhardtii	Silencing the Zeaxanthin epoxidase *(ZEP)* gene	Increased the zeaxanthin content	[115]
Haematococcus pluvialis	overexpression of *PDS* gene	Increased the accumulation of astaxanthin	[95]

engineering is used to manipulate genes to obtained useful products more easily and accurately. The genetic manipulation of microalgae follows the basic step in general genetic engineering, which involves the selection of a proper host and gene of interest, the optimized transformation technique, post-transformation selection, and screening. With regard to microalgae, transformation techniques and selection are the most important steps, and more particular optimization is essential for each genus [90, 91].

The genetic study of microalgae is considered paramount from two perspectives. First, microalgae may be proper candidate models for metabolic and genetic engineering studies as they are unicellular, have a simple genetic organization, and reproduce rapidly. Second, the pigment production of microalgae could be significantly modulated and improved through genetic engineering (Table 1) [91].

Although the regulatory mechanisms that control algal pigment biosynthesis remain unclear, the genes involved in pigment biosynthesis have been identified in recent years [90]. Some algal genomes have been entirely sequenced (Table 2), and some are under investigation. A project known as 10KP (10,000 Plants) Genome Sequencing is underway with the aim of sequencing the genome of 10,000 species (including 1,000 green algae) by 2023, which is speculated to accelerate algal research and engineering products [90]. Based on the genome sequencing data provided so far, the following conclusions could be drawn [4]:

I. Most of the genes involved in the algal pigment pathway originate from cyanobacteria.

II. PSY is a key regulatory enzyme in algal pigment biosynthesis. The *psy* gene encodes the PSY enzyme in algae, cyanobacteria, and plants, and genomics studies have demonstrated that they share a common ancestor.

III. Two of the most important bottleneck pathways that could be proposed for further genetic studies are as follows:

1. Genetic engineering to address feedback inhibition

2. Genetic engineering to address the limitations of the pigment storage space

Phycobilin is the only pigment that does not involve the issue of space as it is accumulated on the cytoplasmic side of the thylakoid membrane [4]. *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Chlorella vulgaris* are known to encode a unique *psy* gene, while the *D. salina* genome contains two classes of a PSY-encoding gene [40]. In addition, it is reported that *psy* is up-regulated under stress [92, 93]. For instance, *psy* expression has been shown to increase and lead to a four-fold increment in astaxanthin production in *Chlorella zofingiensis* under nitrogen starvation [82].

Another limitation of algal metabolic engineering for carotenoid production is the interference of biosynthetic enzymes and formation of a metabolic sink for managing the increased flux of the desired metabolite. According to successive reports, the optimal solution in this regard is

Table 2. Algae with completely sequenced genome [90]

Micro / Macro algal strain	Genome size (Mb)	Acc. No.
Nannochloropsis salina CCMP1776	24.4	AFGQ01002567
Phaeodactylum tricornutum CCAP1055/1	27.5	NC_011669
+Nannochloropsis gaditana CCMP526	33.98	NW_005803952
Chlorella vulgaris strain NJ-7 NJ-7_scaffold	39.08	VATV01000001
Chlorella sorokiniana ASM313072v1	58.53	PKFC00000000
Chlamydomonas reinhardtii CC-503	111.1	NW_001843471.1
Tetradesmus obliquus	158	FNXT00000000
Dunaliella salina CCAP 19/18	343.7	NSFN00000000

overexpressing enzymes, which catalyze the final reactions to a greater extent than the other enzymes in the biosynthetic pathway [91]. Notably, the nuclear overexpression of endogenous mutated PDS has resulted in 32.1% increase in the total carotenoids in *Chlorella zofingiensis* and 26% increase in astaxanthin in *Haematococcus pluvialis* [94]. However, transforming *Chlamydomonas reinhardtii* with *psy* from *D. salina* and its overexpression have led to a 2.6-fold increase in lutein [92]. Meanwhile, the expression of the nuclear *pds* gene in the chloroplast of *H. pluvialis* has increased astaxanthin accumulation by up to 90% [95].

Managing the flux of pigment precursors toward other metabolic branches could contribute to the overproduction of carotenoids [79]. For instance, the down-regulation of lycopene epsilon cyclase (LCYE) has led to the overproduction of β-carotene since the conversion of lycopene into α-carotene reduced. Similarly, it is reported that the down-regulation of LCYE and cyp97c in *Chlorella zofingiensis* suppresses the downstream competing pathway, thereby ensuring the supply of β-carotene as an astaxanthin precursor [90].

The formation or expansion of a metabolic sink may increase the flux of the desired metabolite through defeating feedback inhibition. In *D. salina* and *H. pluvialis,* lipid accumulation could increase the biosynthesis of β-carotene and astaxanthin since lipid is their metabolic sink [96]. In the past decade, extensive research has been focused on increased lipid production through the genetic engineering of its pathway. Some of these studies have been summarized in Table 3.

The mere formation of a sink may not guarantee the accumulation of biopigments due to the lack of apt transporters leading the biopigments to the sink. In *H. pluvialis*, β-carotene is transported to the cytoplasm where it is converted into astaxanthin [97]. When the additional astaxanthin sink increases in the cytoplasm, it is occupied by β-carotene and could not be converted into astaxanthin efficiently [90, 91]. These unexpected results could be due to the lack of a systematic view regarding the genome of algae, and next-generation sequencing studies may be advantageous in resolving similar cases. The potential of genetic engineering with regard to microalgae seems to be unlimited and will greatly facilitate the commercialization of various products from engineered microorganisms, thereby resolving various economic and sustainability issues [90, 91, 98].

Conclusion and Research Implications

Algae have considerable diversity and renewable properties, such as bioremediation [51, 99] and production of lipids and carbon fixation, while they could also synthesize a wide range of bioactive substances that have human health benefits in food, cosmetics, pharmaceutical industries. Antioxidant, anticancer, antiangiogenic, anti-obesity, anti-inflammatory, wound healing, and drug delivery compounds could help cope with depleting resources and population growth [90]. On the other hand, microalgae and cyanobacteria are assumed to the 'green gold' of the future owing to their bio-sustainable energy and bio-pharmaceutical production capabilities. Therefore, their metabolites (e.g., lipids, protein, and pigments) have recently attracted the attention of researchers worldwide [100,101].

One of the most applicable aspects of algae as an emerging sustainable platform for the production of value-added metabolites and proteins is that they are autotrophic and contain chloroplasts, which acts as a bio-factory with a minimal genetic system and could be manipulated precisely. In fact, this is the most distinctive feature of algae compared to the regular heterotrophic organisms used in industrial biotechnology (e.g., bacteria and yeast) [102]. Nevertheless, some obstacles must be overcome before microalgae could become an economically viable platform. Suggestions in this regard are engineering the metabolic pathways *in-vitro* and *in-vivo* to enhance pigment production and extraction price and conducting extensive research regarding the stability, compatibility, and possible toxicological effects of algae.

Table 3. Microalgae genetic engineering Studies conducted to enhance biosynthesis of lipids (as pigment sink)

Micro / Macro algal strain	Genetic modification	Result	Reference
Phaeodactylum tricornutum	Overexpression of Malic enzyme (PtME)	Increased total and neutral lipid content	[116]
Nannochloropsis salina	Overexpression of transcription factor basic helix-loop-helix	Increased in biomass and fatty acid under defined condition	[117]
Nannochloropsis oceanica	Overexpression of microsomal-like Δ12-desaturase	Increased lipid content under Nitrogen starvation	[118]
Chlamydomonas reinhardtii	Overexpression of *PSR1*(Transcription factor)	Increased lipid accumulation without any stress	[119]
Nannochloropsis oceanica	Overexpression of diacylglycerol acyltransferase (DGAT2)	Increased neutral lipid content and enhanced TAG biosynthesis	[120]
Scenedesmus obliquus	transformed and overexpressed of DGAT2 gene DGTT1	Increased the biomass concentration and enhanced lipid content	[121]
Chlamydomonas reinhardtii	Overexpression of Pi starvation response1 (PSR1)	Increased starch content but reduced neutral lipid content	[122]
Mortierella alpina	Overexpression of Delta-6 desaturase (FADS6)	Enhanced the production of Eicosapentaenoic acid (EPA)	[123]
Nannochloropsis oceanica	Overexpression of malonyl CoA-acyl carrier protein trans acylase (MCAT)	Increased the neutral lipid content	[124]
Nannochloropsis oceanica	overexpression of NoDGAT1A & knockdown of *NoDGAT1A*	Increased the TAG accumulation & declined TAG synthesis	[125]
Nannochloropsis salina	Heterologous expression of AP2	Increased the neutral and total lipid content	[126]
Nannochloropsis oceanica	Overexpression of DGAT2	Increased the accumulation of TAG	[127]
Chlamydomonas reinhardtii	Transformation of acyl-ACP thioesterases (TE)	Increased the lipid content	[128]
Phaeodactylum tricornutum	Overexpression of bZIP	Introducing the bZIP as a TCA regulator under stress condition	[129]
Schizochytrium sp.	Overexpression of malonyl-CoA: ACP transacylase (MAT)	Increased the polyunsaturated fatty acids (PUFAs) and lipids	[130]
Mychonastes afer	Expression of 3-ketoacyl-coA synthase gene *in Saccharomyces cerevisiae* BY4741	Increased the lipid content especially nervonic acid in defined condition	[131]
Phaeodactylum tricornutum	Overexpression of glycerol-3-phosphate acyltransferase (GPAT1) and lysophosphatidic acid acyltransferase (LPAT1)	Increased the photosynthetic activity and lipid content	[132]
Nannochloropsis salina	Overexpression of basic leucine zipper (bZIP TF)	Improved both growth and lipid accumulation	[133]
Chlamydomonas reinhardtii	Overexpression of a DNA-binding-with-one-finger TF	Increased the fatty acid production in defined medium	[134]
Nannochloropsis oceanic	Overexpression of multiple fatty acid desaturases (FAD)	Enhanced the production of EPA and ω *3* long-chain polyunsaturated fatty acids (LCPUFAs)	[135]
Nannochloropsis oceanic	Generating random mutant strain using Transposome complex Tn5 anti-biotic resistance contained cassette	Increased accumulation of intracellular lipids	[136]
Schizochytrium sp.	Overexpression of malic enzyme and a codon-optimized gene encoding for an elongase enzyme *ELO3*	Enhanced the production of Docosahexaenoic acid (DHA) and odd-chain fatty acids (OCFAs)	[137]
Synechococcus sp.	RNAi of Synpcc7942_0537 (*fabB/F*) and Synpcc7942_1455 (*fabH*)	Elevated the level of short chain fatty acids (SCFAs)	[31]
Chlamydomonas reinhardtii	Cloning and Overexpression *of crDOF*	Increased the content of intracellular lipid	[138]
Nannochloropsis oceanic	Overexpression of bZIP1	Boosted the production and secretion of lipids	[139]
Chlamydomonas reinhardtii	Knocking out of Phospholipase A2 (PLA2) gene	Increased the production of lipid	[140]

The recent advancement in genome sequencing projects, DNA synthesis, omics approaches, and systematic biology have largely contributed to biotechnology and the bioengineering of microalgae for industrial applications, thereby virtually predicting the possible outcomes and providing insight into the experimental genome [98].

With the completion of the human genome project, researchers could easily develop applied medical and biological strategies through observing genomic factors and their interaction [103]. The improvement of gene expression levels could be attained by applying synthetic promoters, genes, adapted codon usage, and multiple gene expression in the chloroplast or nuclear genome, which are based on enriched genomic sequences. Furthermore, genome sequencing and transcriptional profiling studies could help achieve the target aspects more accurately and efficiently. The possibility of a genetic engineering approach to microalgae seems to be ultimate, and the necessary tools could be utilized for the commercialization of pigments from altered microorganisms and solving economic and sustainability issues [98].

The availability of synthetic toolboxes and comprehensive genomic and multi-omics databases as the most recent advances in microalgae and cyanobacteria have led to a deeper understanding of the genes that are possible to be manipulated. In general, these genes are associated with the production of chlorophyll, beta-carotene, astaxanthin, lutein, phycocyanin, terpenes, polyunsaturated fatty acids, biopolymer precursors, commercial enzymes, alcohols, and therapeutic/ nutraceutical compounds [101]. Moreover, the construction of a stoichiometric model of metabolism through in silico genomics and proteomics has minimized labor-intensive lab works. Nevertheless, the availability of mutant libraries is limited to *Chlamydomonas reinhardtii* as a microalgae model and *Synechococcus* spp. as a cyanobacteria model. Therefore, the construction of the insertional mutant libraries of synthetic promoters, ribosome binding sites, transcription terminators, plasmids, and the transcriptional regulators that are essential to the development of applied genetic technologies in microalgae and cyanobacteria are the new step toward determining the most proper pathway of increasing the production of pigments and other bioproducts [101].

Based on the final report of the National Alliance for Advanced Biofuels and Bioproducts (NAABB), the main purpose of developing new strains must be safer and more sustainable algal products [104]. Furthermore, the combination of natural and transgenic microalgae strains could decrease the production costs by 85% [105].

Algal organisms should be extensively studied given the wide-ranging applications of algal pigments, the value of these organisms in enhancing the oxygen-to-carbon ratio in the environment, and their ability to maintain stability through the development of micro- and macro-photosynthetic branches of the ecosystem. Therefore, introducing and reviewing the previous studies in this regard could help with the more effective development of their applications. For instance, studying the polymorphism of PSY, PDS, ZDS, ZISO, CRTISO, β-CYC, and ε-CYC proteins or their encoding genes may improve the knowledge of their biological adaptation/evolution and/or lead to the ameliorating manipulation of carotenoid production. Due to the all-encompassing scopes of this platform, practical studies are highly recommended, and engineering research could also be more focused on these organisms.

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رنگدانه های ریزجلبکی: بیوسنتز، کاربرد و مهندسی ژنتیک

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چکیده

جلبک‌ها گروه بزرگی از موجودات هستند که در تشکیل ۵۰ درصد اکسیژن جو نقش دارند. علاوه بر حضور کلروفیل بعنوان جاذب فوتون‌های نور، جلبک‌ها غنی از کاروتنوئیدهای قرمز، نارنجی و زرد رنگی هستند که می‌توانند آنها را در مقابل پرتوهای خطرناک و رادیکال‌های آزاد حفاظت کند. همچنین این موجود دارای فیکوبیلی پروتئین (رنگدانه های قرمز و آبی) هستند که در دریافت نور و انتقال آن به کلروفیل طی فرآیند فتوسنتز نقش داشته و ویژگی‌های آنتی اکسیدان فراوانی نیز دارند. با توجه به اثرات جانبی رنگدانه‌های سنتزی، رنگدانه‌های طبیعی جلبک‌ها نقش مهمی در جایگزینی با این ترکیبات دارند. اخیراً رنگدانه‌های جلبکی بعلت دارا بودن ویژگی‌های آنتی‌اکسیدانی، ضد دیابتی، ضد چاقی، ضد التهابی، ضد پیری، ضد مالاریا و حفاظت عصبی کاربرد گسترده‌ای در پزشکی و صنایع غذایی، آرایشی و دارویی داشته‌اند. تقاضای رو به رشد محصولات جلبکی اهمیت تولید این رنگدانه‌ها را پررنگ نموده است. مقاله پیش رو به مطالعه طبقه‌بندی رنگدانه‌های جلبکی، توزیع، عملکرد، کاربرد و تولید زیستی آنها پرداخته است. همچنین مسیرهای بیوشیمیایی، آنزیم‌ها، اصلاحات ژنی یا بیوتکنولوژیکی مانند ترنسفورماسیون و تنظیم بیان که متابولیسم مخزن و منبع آنها را درگیر می‌کند نیز بررسی شده است.

کلمات کلیدی: جلبک، بیوتکنولوژی رنگدانه، رنگدانه های فتوسنتزی، کلروفیل، کلرتنوئید، فیکوبیلی پروتئین