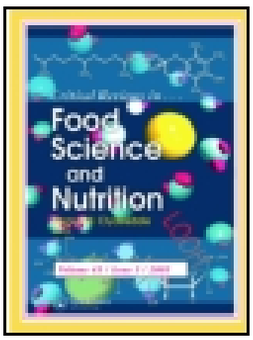

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REVIEW

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Enhancing production of microalgal biopigments through metabolic and genetic engineering

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ABSTRACT

The versatile use of biopigments in food, feed, cosmetic, pharmaceutical and analytical industries emphasized to find different and renewable sources of biopigments. Microalgae, including cyanobacteria, are becoming a potential candidate for pigment production as these have fast-growing ability, high pigment content, highly variable and also have "Generally recognized as safe" status. These algal groups are known to produce different metabolites that include hormones, vitamins, biopolythene and biochemicals. We discuss here the potential use of microalgal biopigments in our daily life as well as in food and cosmetic industries. Pigment like carotenoids has many health benefits such as antioxidant, anti-inflammatory properties and also provide photo-protection against UV radiation. This review details the effect of various abiotic and biotic factors such as temperature, light, nutrition on maximizing the pigment content in the microalgal cell. This review also highlights the potential of microalgae, whether in present native or engineered strain including the many metabolic strategies which are used or can be used to produce a higher amount of these valuable biopigments. Additionally, future challenges in the context of pigment production have also been discussed.

KEYWORDS

Microalgae; metabolic engineering; genetic engineering; carotenoids; phycobiliproteins

Introduction

Increase in the population worldwide has raised the need for food, energy, shelter, and natural resources. Microalgae is one of the most unutilized resources which recently drag attention as renewable sources of various natural metabolites including protein, lipids, fuel, biomass and biochemically active compounds (pigment and vitamins) and the biomass which can produce different types of organic solvents e.g., ethanol (Rizwan et al. 2018; Chew et al. 2017). Microalgae have one of the broadest and widely distributed group of the microorganisms, which have about 2×10^6species (Katiyar et al. 2017). "Microalgae" term includes cyanobacteria and eukaryotic photosynthetic microorganism, in which only about 1% of the species are known to date. These algae gained attention due to their fast-growing ability or a high growth rate. These microalgae have also been utilized as human food, animal feedstock and for the production of biodiesel in the field of renewable energy sources and different aspects are reported recently including use of gene editing and systems biology (Jagadevan et al. 2018; Kumar et al. 2017; Banerjee et al. 2018; Anand et al. 2017). Microalgae require solar energy and few essential elements (C, N, P, S, K, etc.) for their division as well as biomass accumulation (Mata, Martins, and Caetano 2010). These algae require essential nutrients in a specific composition, and lack of any essential nutrient will decline the growth rate and also biomass accumulation. Microalgae are considered superior over a different type of plants and organic sources for the production of a variety of biopigments because of the characteristics *viz* (1) Microalgae can be cultivated on the non-arable land, so it avoids competition with agricultural lands (Pathak et al. 2018); (2) These can grow and flourish under different conditions and round the year and increase their harvest capabilities (Varshney et al. 2015). Finally, their short life cycle, simple structural and cellular organization makes them easy to handle and manipulate according to the requirements.

Use of biopigments has many health as well as industrial benefits with large economic potential as shown in Fig. 1. As awareness for synthetic color and dye is increasing, people globally, are switching towards natural resources of colors and dyes. Microalgae are an effective, promising, renewable and excellent source of biopigments (Yusuf, Shabbir, and Mohammad 2017). The wide range of pigments that can be produced is β carotene, lutein, zeaxanthin, lycopene, chlorophyl, and phycobiliproteins. Some of the pigments (carotenoids) are essential for the growth of the microalgae as these pigments protect from ROS (reactive oxygen species) and high light intensity. Mutant species of

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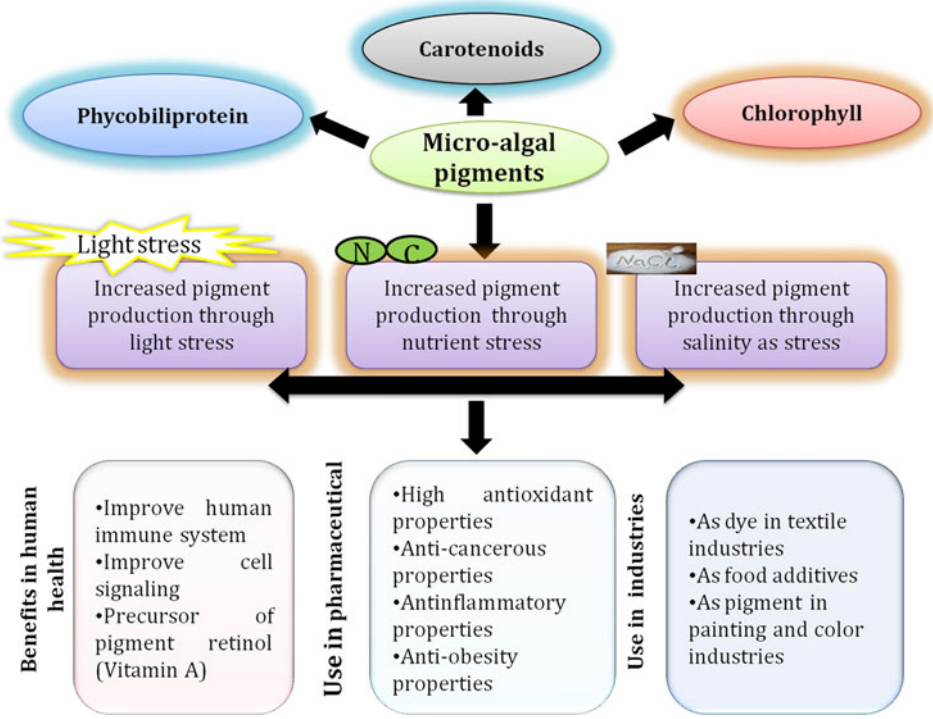

Figure 1. Overview of different applications of biopigments.

Chlamydomonas reinhardtii leads to autophagy due to disruption of enzymes like phytoene desaturase and phytoene synthase which were involved in carotenoids synthesis when exposed to high light intensity. Many microalgal species are identified for the higher pigment production such as *β*-carotene by *Dunaliella salina,* astaxanthin by *Hematococcus pluvialis and Chlorella spp*. (Liu et al. 2018), fucoxanthin by *Muriellopsis spp.* (Shukla and Kumar 2018), Zeaxanthin by *Dunaliell/a salina,* (Kim et al. 2017), phycoerythrin by *Porphyridium spp*. (Thoisen, Hansen, and Nielsen 2017), Phycoerythrin by *Anabaena variabilis* (Chakdar and Pabbi 2012), etc. Pigments like *β*-carotene, astaxanthin, lutein etc. are involved in the treatment and prevention of many diseases. Phycobiliproteins (phycoerythrin and phycocyanin) are used as biochemical tags and marker in immunological testing as well as research purpose (Dasgupta 2015).

All these pigments have potential application as coloring agents in the dye and textile industry as described in Table 1. Furthermore, pigments such as carotenoids especially *β* carotene, astaxanthin, etc. and phycobiliproteins have potential health benefits. Astaxanthin is the most potent antioxidant present in nature. Many clinical trials have been done to cure or prevent some health disorders like joint-tendon health, eye and brain health, cardiovascular health, etc (Zhang et al. 2014). Health benefits of fucoxanthin include anti-inflammatory, anti-obesity, and anti-cancerous, etc. It reduces adipose tissue in the mouse as proved in the diet with pomegranate seed and brown seed contain fucoxanthin that significantly decrease women weight in a clinical trial (McClure et al. 2018). Pigment production in microalgae is more evenly affected by environmental factors which include light intensity, wavelength and nutrient availability in the medium. Light sources significantly effect on chlorophyl, phycobiliproteins and primary carotenoids and for example, low light intensity, increase the concentration (Cuellar-Bermudez et al. 2015).

Metabolic and genetic engineering are advanced tools which utilize the cellular genetic knowledge and molecular biological technique to enhance productivity and identify the rate-limiting step of the metabolic pathway. These techniques target the rate-limiting step of any metabolic pathway or enzymes to enhance the yield of the desired product. This review includes various uses of biopigments produced by microalgae in the food and pharmaceutical industries and their other biotechnological application. We also discuss biosynthesis of different pigments, especially carotenoids. This review also focuses on the metabolic and genetic regulation of carotenoids production as well as different strategies to enhance their production.

As biopigments market is increasing day by day, modern approaches have been applied to enhance their production. Metabolic and genetic engineering is one of the reliable approaches to enhance pigment production by manipulating the rate-limiting steps of a metabolic pathway. Microalgae which also include cyanobacteria which are prokaryotic can be more easily engineered through different engineering techniques. Cyanobacteria are known to produce various kinds of metabolite and bioactive compounds. Mainly metabolic engineering used in cyanobacteria focuses on increase product formation, direct secretion, and optimization of the process. The aspect of metabolic engineering have intensely studied in case of cyanobacteria for biohydrogen product, biofuel, butanol (Lan and Liao 2011), isoprene (Bentley, Zurbriggen, and Melis 2014), sucrose (Reinsvold et al. 2011), fatty alcohols (Yao et al. 2014). Many genes involved in pigment production especially astaxanthin were heterologously

Table 1. Pigments and their application.

Pigment	Microalgae strains	Application	Reference
β-carotene	*Dunaliella* salina, *Scenedesmus almeriensis, Dunaliella bardawil*	The precursor of Vitamin A, anti-oxidant property, prevent macular degeneration, asthma, pharmaceutical, cosmetics	Xu et al. 2018
Astaxanthin	*Haematococcus* pluvialis, *Chlorella* spp., *Chlorococcus* spp.	Protect from UV rays, Anti-oxidants, Food colorant, Enhance immune Functions, anti-aging property, pharmaceutical, nutraceutical, cosmeceutical	Ambati et al. 2014
Lutein	*Chlorella fusca, Chlorococcum citroforme, Scenedesmus obliquus*, *Muriellopsis* spp.	Feed additive, food colorant, help to regulate cardiovascular diseases, cancers, cognitive function, and age-related macular degeneration (AMD) in human	Chan, Li, and Hu 2013; Sarada et al. 2017; Blanco et al. 2007
Zeaxanthin	*Scenedesmus almeriensis Nannochloropsis oculata*	Food additive, pharmaceutical, food colorant	Granado-Lorencio et al. 2009
Canthaxanthin	*Chlorella* spp. *Scenedesmus* spp.	As an additive to poultry feed, prevent some blood-related disorder diseases	Gouveia et al. 2008
Fucoxanthin	*Phaeodactylum* tricornutumx 4	Anti-oxidant, anti-viral, anti-obesity, antidiabetes, anticancer, anti-osteoporotic anti-inflammatory, nutraceutical	McClure et al. 2018; Sathasivam and Ki, 2018
Lycopene	*Chlorella marina*	anti-oxidant, anti-cancerous, antiatherogenic	Bhalamurugan, Valerie, and Mark 2018
Violaxanthin	*Chlorella ellipsodea*	Anti-inflammatory activity	Sathasivam and Ki, 2018
Chlorophylls	Present in all ubiquitously in all photosynthetic organism	Used in the food industry as a natural ingredient of pigment, Antioxidant activity	Singh et al. 2015
Phycocyanin	*Spirulina* spp. *Arthrospira platensis*	Used as fluorescent reagents hepatoprotective activity, antioxidant, anti-inflammatory Neuroprotective	de Morais et al., 2018; Sonani et al. 2016
Phycoerythrin	*Porphyridium spp., Agardhiella subulata, Polysiphonia morrowii*	Used as fluorescent reagents Phycoerythrin-labeled streptavidin used to identify biotin labeled DNA and protein probes.	Sekar and Chandramohan, 2008; del Pilar Sánchez-Saavedraet al. 2018

transferred in *E. coli*. Park et al. (2018) developed a metabolically engineered *E.coli* to produce a high concentration of astaxanthin. It produced 6.26 mg/g DCW which further increase through IPTG induction up to 7.12 mg/gDCW. *Kluyveromyces marxianus* strain Sm23 was also developed through metabolic engineering by integration astaxanthin biosynthesis genes *bkt* and *Hpchyb* from *Haematococcus pluvialis*. It produced about 9972 µg/g DCW in a 5 L fermentor (Lin et al. 2017). Cyanobacteria has about 126 genome sequence available which allow us to manipulate them to develop green cloning cell (Shih et al. 2013). Pigment synthesis has been regulated in multiple steps and many genes. Metabolic and genetic engineering together used to develop microalgae to enhance production of various secondary metabolites. This review discusses on use of metabolic and genetic engineering to produce various biopigments especially carotenoids through microalgae.

Potential role of biopigments from microalgae

Microalgae have a widespread application in the food chain in the marine ecosystem. It includes prokaryotes (e.g., cyanobacteria) and eukaryotes (e.g., green algae) with size ranging from 0.2 to 2.0 µm and has a simple structure which gives them the advantage to modify the cell structure or metabolism according to the environments (Kiran, Kumar, and Deshmukh 2014). These capabilities allow microalgae to proliferate rapidly and avoid harsh environmental factors such as cold, heat, salinity and ultraviolet radiation (Wang et al. 2015). Commercially biopigments were extracted from the higher plants, but as the demand for biopigments is increasing day by day due to their varied potential application as explained earlier, there is a search for new and economically viable pigment resources. Microalgae provide alternative sources of natural pigments and their abilities to grow fast, and non-seasonal growth has allowed the humanity in overcoming the difference between demand and production ratio (Kose and Oncel 2017).

Carotenoids are most commercialized biopigments because of its many health and industrial applications. These lipophilic compounds are known to have an antioxidant property which helps to protect the cell against oxidative damages. These biopigments are also involved in many biological pathways and cell signaling mechanisms (Christaki et al. 2013; Matos et al. 2017). Most of the organisms including humans are unable to produce or synthesize carotenoids molecules, so there is a need for these pigments from the external sources such as plant and microalgae (Khan, Shin, and Kim 2018). About 600 types of naturally occurring carotenoids molecules have been identified among various plant and other photosynthetic organisms (Paliwal et al. 2016).

Among them, β-carotene, lutein, and astaxanthin are the most important ones because of their potential use in food industries. The demand for natural β carotene is increasing in comparison to synthetic one, as many properties shown by the natural pigment are not present in synthetic pigments. Jayappriyan et al. (2013) reported that the natural β-carotene increases the rate of apoptosis in prostrate cancerous cells as compared to the synthetic version. Thus, nowadays people are giving more attention towards the natural sources of carotenoids other than plant, so microalgae are one promising source for pigment production (Mata, Martins, and Caetano 2010; Mehta et al. 2018).

Table 2. Distribution and biosynthesis of major microalgal pigments.

Pigments							
Group	Chemical backbone	Type	Biosynthetic precursor	Immediate precursor	Enzymes catalyzing conversion from immediate precursor	Distribution	Reference
Carotenes	Isoprene	α-carotene	IPP or DMAPP	δ-carotene	β-cyclase	Cryptophyta and some Chlorophycean members	Takaichi 2011, Chakdar and Pabbi, 2017
		β-carotene		Lycopene	β-cyclase	Cyanophyta, Rhodophyta, Chlorophyta, Glaucophyta, Haptophyta, Chlorarachinophyta, Euglenophyta,	
Xanthophylls	Isoprene (oxygenated derivative of carotenes)	Astaxanthin	IPP or DMAPP	Violxanthin	β-carotene oxygenase	Some Chlorophytes	Boussiba, 2000; Takaichi 2011
		Lutein		α-carotene	β-hydroxylase ε-hydroxylase	Macrophytic rhopdophytes and some Chlorophytes	
		Violaxanthin		Zeaxanthin	Zeaxanthin epoxidase Violaxanthin de-epoxidase	Chlorophyta and some phaeophycean members	
		Zeaxanthin		β-carotene	Carotene β-hydroxylase	Cyanophyta, Rhodophyta, Glaucophyta and some Phaeophycean members	
Phycobilins	Tetrapyrrole	Phycoerythrobilin Phycocyanobilin	ALA	15, 16-dihydrobiliverdin IXα Phycoerythrobilin	– –	Rhodophyta, Cyanophyta Cyanophyta, Rhodophyta, Glaucophyta and some Cryptophytes	Chakdar and Pabbi, 2017, 2016;
Scytonemin	Indole and phenol	–	Tryptophan and tyrosine	Scytonemin monomer derived from tryptophan and tyrosine	Putative tyrosinase	Cyanophyta	Balskus, Case, and Walsh 2011; Soule et al. 2009

Microalgae as a source of food and nutrition

Microalgae can produce a wide range of bioactive compounds (Wells et al. 2017). It was estimated that microalgal cell contains 8–14% carotene, 12–30% carbohydrates, 4–20% lipids, 40–70% proteins and significant amounts of vitamins A, C, B1, B2, B12, E, K, and D (Becker 2007). Since, now a days people are more concerned about their health and the effect of various kinds of chemicals used during processing of food items that are sold in the markets, different authorities of the world such as the European Food Safety Authority (AESA) and American Food and Drug Administration (FDA) have limited the use of synthetic colors in the food items as these can cause various kinds of cancers and food-related allergies (Vigani et al. 2015). Various multinational companies and health-conscious people have moved towards natural sources of colors (Martelli et al. 2014). Though, microalgae are the vast and diverse class of algae, yet only a few are developed to qualify for human use especially *Chlorella, Spirulina, Nostoc* and *Dunaliella* (Pulz and Gross 2004). Many species of microalgae are grown for their biomass, and some species are exclusively cultivated for various pigment production such as *Haematococcus pluvialis* and *Dunaliella salina* to produce astaxanthin and β-carotene respectively (Markou and Nerantzis 2013).

Spirulina maxima and *Spirulina platensis* are historically known cyanobacterial species which were consumed by humans (Barka and Blecker 2016; Borowitzka 2018). *Spirulina* genus has high protein content especially phycocyanin, a blue pigment with various health benefits. Along with pigment it also has a high content of carbohydrates and fatty acids with different vitamins like B12, C and D2 (Larkum et al. 2012). *Spirulina* and *Chlorella* are directly sold in various countries as rich sources of bioactive compounds. These bioactive compounds include pigments especially β-carotene, astaxanthin and proteins (phycobiliproteins) (Priyadarshani and Rath 2012). Due to the high content of protein, microalgae are competing with traditional sources of protein like soybean, egg, and other plant and animal-based food items. For example, *Dunaliella* can produce 50–80% protein content in comparison of plant product (Ejike et al. 2017) and *Spirulina* has about 50–70% protein content depending upon strain (Plaza et al. 2009). These microalgal proteins have a comparable amino acid profile to soybean, egg and fish, but may have low protein digestibility, protein utilization and biological value which are still required to be addressed and sorted out. The protein content in these microalgae varies and also depends upon light, nutrients, species type and environmental stress.

Phycobiliprotein are exclusively found in cyanobacteria, especially *Nostoc* sp., *Spirulina* sp., *Oscillatoria sp.* and *Anabaena* sp. as colored protein. These species are well studied for the production of different type of phycobiliproteins such as phycocyanin, phycoerythrin, and allophycocyanin due to their health benefits. These protein structure show close resemblance to bilirubin which act as antioxidant and cryoprotectant for tissue-like nerve tissue and myocardium. It acts as scavengers of the free oxygen radical.

Phycocyanin has wide range of pharmaceutical application due to its antioxidant properties. Its antioxidant potential was 16 fold more than Trolox (vitamin E analog) and 20 time higher protective effect than vitamin C against lysis of erythrocytes when induced by peroxyl radicals (Romay and Gonzalez 2000).

Microalgal carotenoids are the most commercially produced natural pigments. These are isoprenoid pigments with color ranging from yellow to red and mostly lipid soluble; which absorb light at a different range. Carotenoids have a wide range of application in human health and pharmaceutical as some of the examples discussed earlier. *Nannochloropsis*, a unicellular microalga produce fatty acids and pigment like astaxanthin, canthaxanthin, and zeaxanthin (Solovchenko et al. 2014). *Chlorella,* a green alga produce lutein, violaxanthin, and zeaxanthin (Cha et al. 2010; Ambati et al. 2014), *Dunaliella salina* a green alga produces a high amount of carotenoids which can be converted into pro-vitamin A by humans (Saini, Pabbi, and Shukla 2018). *D. salina* produced carotenoids are approved safe by USFDA to use as food color (Ambati et al. 2018).

Biosynthesis of different microalgal pigments

Algal species harbor various pigments which are the key to photosynthesis and photoprotection. Most of these pigment share similarities in chemical structure as they are composed of long chain or closed rings of conjugated double bonds. Algal pigments are categorized into two major group *viz.* Tetrapyrroles and isoprenoids depending on their chemical structures. Chlorophyll and phycobiliproteins (PBP) belong to the tetrapyrrole group while carotenoids fall under isoprenoid group (Table 2). For all tetrapyrrole pigments, δ-aminolevulinic acid (ALA) is considered to be the first committed precursor. ALA synthesized from Glutamate (Glu), is converted to Protoporphyrinogen through a series of a chemical reaction. Enzymatic conversion of Protoporphyrinogen IX to Protoporphyrin is the last common step for phycobilins and chlorophyll biosynthesis. Ferrochelatase enzyme inserts the ferrous ion (Fe^{++}) into this protoporphyrin to produce Heme. The macrocycle ring of Heme is opened by heme oxygenase enzyme resulting in biliverdin IXα. Phycocyanobilin and phycoerythrobilin are isomeric and the significant chromophores which in later stages ligated to apoproteins to make the PBP. Phycoerythrobilin is the first wholly reduced form of bilin generated from biliverdin IXα and converted to phycocyanobilin through isomerisation (Chakdar and Pabbi 2016). During the conversion of biliverdin IXα to phycoerythrobilin, a partially reduced intermediate known as 15, 16-dihydrobiliverdinis produced. The phycobilin products may initially be formed in Z configuration containing ethylidine group. Such Z configuration phycobilin is generally reported to be the preferred initial product although the E configuration represents the thermodynamically favored form (Rüdiger et al. 1980; Weller and Gossauer, 1980). Once the phycobilin chromophores are synthesized, those are attached to apoproteins. The PBP apoproteins mainly consist of two subunits (viz. α and β) which are encoded by different genes for different phycobiliproteins *viz.* phycoerythrin (*cpeB, cpeA*), phycocyanin (*cpcB, cpcA*) and allophycocyanin (*apcA, apcB*). The attachment of chromophores to apoproteins may take place spontaneously or with the help of enzymes. For enzymatic attachment of chromophores E/F type lyases or S/U type lyases have been reported in cyanobacteria (Zhou et al. 1992).

Isoprenoid pigments like carotenoids synthesized from isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP) through some enzyme-catalyzed reactions. Understanding carotogenesis in algae is still incomplete and limited and mainly derived from the information available for plants. The detailed pathway for carotenoid biosynthesis is discussed in the following sections.

Apart from tetrapyrroles and isoprenoids, Mycosporine-like Amino Acids (MAA) is also reported in many cyanobacteria having various amino acid substituted aminocyclohexenone or aminocyclohexenimine as the chemical backbone (Garcia-Pichel and Castenholz 1993; Karsten and Garcia-Pichel 1996; Jain et al. 2017). Scytonemin is another class of lipid soluble, hydrophobic photo-protective pigment reported mainly in terrestrial cyanobacteria. Scytonemin is produced through condensation of aromatic amino acids like tryptophan and tyrosine through the production of some essential intermediates like nostodione A. Based on genomic analyses, Soule et al. (2009) has proposed a working model of the subcellular compartmentalization of scytonemin biosynthesis. Upon receiving ultra violet radiation, *trp* and *tyr* genes in cells are expressed to produce tryptophan and p-hydroxyphenylpyruvate (tyrosine). These precursors first processed in the cytoplasm through the proteins encoded by *scyA*, *scyB* and *scyC*. The processed intermediates are transported to the periplasm via some unknown membrane transporters and converted to the reduced form of scytonemin by periplasmic enzymes encoded by genes like *scyD, scyE, scyF, dsbA,* and t*yrP*. When this reduced form of scytonemin is excreted outside, it is auto-oxidized and attains its final yellow-brown appearance.

Carotenoid biosynthesis

Carotenoids which are one of the most widespread and diverse classes of biomolecules belong to a subfamily of isoprenoid compounds. Biosynthesis of carotenoids may vary across the species, but still, it shares a common metabolic pathway where Isopentenyl diphosphate (IPP) which is also the central intermediate in isoprenoid biosynthesis or its isomer dimethylallyl diphosphate (DMAPP) act as the precursor. The five carbon precursor, IPP or DMAPP synthesized from either Acetyl-CoA following mevalonic acid (MVA) pathway or from glyceraldehydes -3-phosphate and pyruvate following methylerythritol 4-phosphate (MEP) pathway. It is reported that the synthesis of IPP or DMAPP in algae and cyanobacteria is achieved mainly through the MEP pathway (Zhao et al. 2013). Deoxyxylulose 5-phosphate (DXP) produced from pyruvate and glyceraldehyde 3-phosphate with the help of DXP Synthase is reduced by DXP

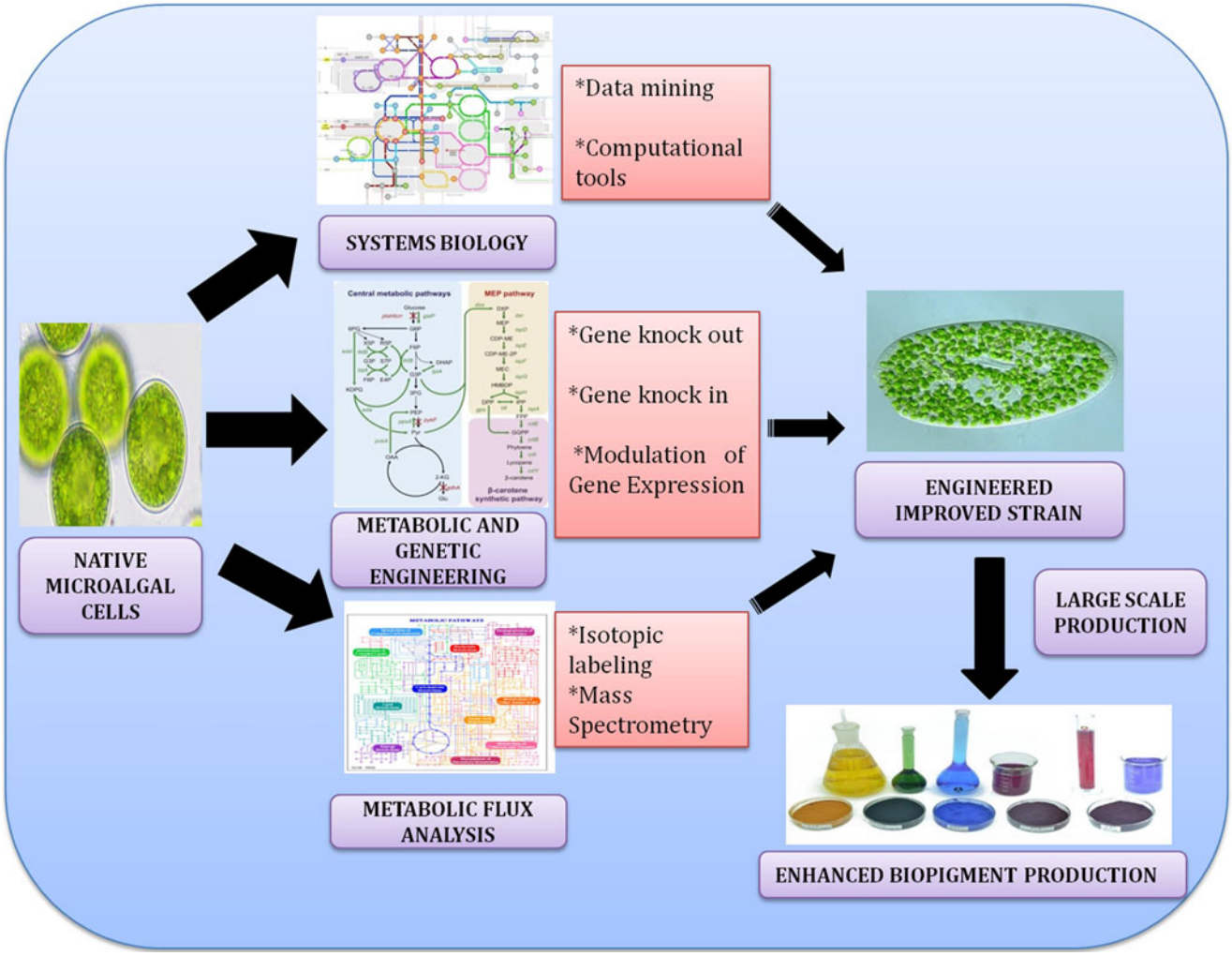

Figure 2. Various types of strategies used to enhance pigment production.

Reductoisomerase to MEP (Paniagua-Michel, Olmos-Soto, and Ruiz 2012).

Once IPP is synthesized, it enzymatically gets isomerized to DMAPP which is condensed into polyprenyl pyrophosphate chains following head to tail condensation with the help of prenyl transferases. Condensation of such C5 units results in various C10, C15, and C20 polyprenyl units of which geranylgeranyl pyrophosphate (GGPP) is an important one (Takaichi 2011). Two molecules of GGPP are condensed by phytoene synthase (PSY) enzyme resulting in the formation of Phytoene (a C40 compound). Conversion of GGPP to phytoene is a rate-limiting step, and hence, PSY is an essential enzyme in carotogenesis. In the presence of Phytoene desaturase (PDS), phytoene converted to ζ-carotene which is further desaturated by ζ-carotene (ZDS) desaturase, to produce pro-lycopene, which is then isomerized to lycopene by specific carotenoid isomerase (CRTISO). From lycopene, the pathway is split into two branches. In one of these branches, lycopene is cyclised into β-carotene through lycopene β-cyclase (LCYB). This β-carotene is further hydroxylated by carotene β-hydroxylase (CHYB) to zeaxanthin which is then epoxidized to violaxanthin by zeaxanthin epoxidase (ZEP). The epoxidation reaction is dependent on oxygen and involves cofactors like NADPH, FAD, and ferredoxin (Varela et al. 2015). Violaxanthin de-epoxidase (VDE) can de-epoxidize violaxanthin to zeaxanthin under higher intensities of light. In the other branch, coordinated catalysis by β-cyclase and ε-cyclases (LCYE) results in α-carotene. The fate of lycopene depends on the relative activities of β-cyclase and ε-cyclases. α-carotene is hydroxylated by carotene β-hydroxylase and carotene ε-hydroxylase to form lutein.

As stated earlier, the basic pathway is common in the majority of the algal species; some of the species can still accumulate unusual carotenoids following a few specific biosynthetic route. Astaxanthin is such an unusual carotenoid which contain oxygen as both oxy- and hydroxyl groups and found in very limited microalgae like *Haematococcus pluvialis, Chlorella zofingiensis, Scendesmus* spp. (Chakdar and Pabbi 2017; Varela et al. 2015). In most of the cases, violaxanthin or zeaxanthin produced form β-carotene is converted into Astaxanthin with the help of β-carotene ketolase (BKT). However, it has been reported that in *H. pluvialis* (the major commercial source of astaxanthin), β-carotene is sequentially converted to astaxanthin through one of the two following routes. One with intermediaries cryptoxanthin, zeaxanthin and adonixanthin while the other produces intermediates like echinenone, canthaxanthin, and

adonirubin (Lemoine and Schoefs 2010; Han, Li, and Hu 2013). However, the later pathway is more predominant in *H. pluvialis* where β-carotene is converted to canthaxanthin by BKT, and then canthaxanthin is converted to astaxanthin with the help of β-carotenoid hydroxylase (CrtRB) (Boussiba 2000).

Genetic regulation of carotenoids biosynthesis

The biochemical and genetic basis of carotenogenesis is well studied for terrestrial plants and various microorganisms (Ruiz-Sola and Rodríguez-Concepción 2012). In algae, most of the research works have focused on microalgae, especially *Chlamydomonas*. During last one decade, some genes involved in carotenoids biosynthesis, have been characterized in *H. pluvialis*, *Chlorella zofingiensis* and *Chlamydomonas reinhardtii* (Gao, Honzatko, and Peters 2012; Kathiresan et al. 2015). Multi-copy genes have been reported to be involved in carotenoid biosynthetis, making the pathway specialized and complicated but at the same time flexible and adaptable. Evolutionary analyses revealed that most of the key genes involved in carotenogenesis in algae had a cyanobacterial origin (Shanshan et al. 2018). The members of Glaucophyta, Rhodophyta, and Chlorophyta acquired their carotenogenic genes from cyanobacteria through primary endosymbiosis mediated gene transfer while the Ochrophyta, Haptophyta, and Cryptophyta obtained their genes via secondary endosymbiosis mediated gene transfer from a rhodophyte-like organism.

A large number of factors like light intensity, nutrient depletion, salinity, application of hormones, etc. influence microalgal carotenoids production. In response to different environmental cues, the genes involved in carotenogenesis are orchestrated to produce a particular type/s of carotenoids. Concerted regulation of some genes in the pathway results in the modulation of carotenogenesis (Fig. 2). Although the regulatory mechanisms that control carotenoid biosynthesis are not completely understood, findings suggested PSY as a key regulatory enzyme in carotenoid biosynthesis. PSY is encoded by the *crtB* gene in bacteria and *psy* gene in algae, cyanobacteria, and plants, but genomic analyses show that they share a common ancestor (Takaichi 2013; Sieiro et al. 2003). Algae like *Chlamydomonas reinhardtii*, *Volvox carteri,* and *Chlorella vulgaris* are known to encode a unique PSY-encoding gene while *D. salina* has two have classes of *psy* gene families (Lohr, Im, and Grossman 2005; Ye, Jiang, and Wu 2008). *psy* has been found to be up-regulated under stress (Couso et al. 2012; Vidhyavathi et al. 2008). Mao et al. (2018) reported up-regulation of *psy* gene in *Chlorella zofengiensis* under nitrogen limitation resulting almost four folds increase in astaxanthin production. Phytoene thus synthesized is sequentially desaturated by PDS- encoded by *Pds* gene in algae and *crtP* gene in cyanobacteria; and ZDS, the product of the *Zds* gene in algae and *crtQ* gene in cyanobacteria. In *Chlamydomonas reinhardtii,* the genes encoding PDS and ZDS are evolutionarily related and probably originated from the same ancestor (Grossman et al. 2004). High light intensity has been reported to up-regulate the expression of *psy* and *pds* genes. Exposure of dark-grown *C. reinhardtii* to high light intensity ($800\ \mu E\ m^{-2}\ s^{-1}$) triggered expression of both *psy* and *pds,* whose mRNA levels were increased by 2 and 4 folds, respectively, after 1 hour of exposure (Couso et al. 2012).

Production of β-carotene and zeaxanthin is catalyzed by the enzymes coded by *Lcyβ* and *chyB*. Genetic and physiological analyses showed that *Lcyβ* mRNA and carotenoid levels were enhanced in *D. salina* under various stress conditions like salinity, high light intensity and nutrient depletion (Ramos et al. 2008). Sun et al. (2010) reported up-regulation of *Lcyβ* in *C. reinhardtii* under high light condition and the expression of *Lcyβ* presented typical circadian pattern as a rhythmic fluctuation of mRNA abundance under both constant light and constant dark observed. During evolution, a duplication followed by functional divergence generated another gene *Lcyε* encoding lycopene ε cyclase. The gene *chyB* responsible for the enzyme converting β-carotene to zeaxanthin in *C. reinhardtii* was also found to be up-regulated by light but did not show any circadian pattern of expression. Under limited light conditions, zeaxanthin is epoxidized to violaxanthin by ZEP encoded by *ZEP1* in *C. renhardtii*, but under high light condition violaxanthin is depoxidized to zeaxanthin with the help of VDE. But in *Chlamydomonas* genome, no gene encoding VDE has yet been reported. Instead, a putative violaxanthin de-epoxidase-related protein (VDR1) encoded by another gene has been found (Merchant et al. 2007). Biosynthesis of lutein from α-carotene is mediated through two cytochrome P450 dependent hydroxylases. It has been suggested that under high light stress, the genes *cyp97a5* and *cyp97c3* encoding the hydroxylases are up-regulated and as a result production of lutein increases in *C. reinhardtii* (Couso et al. 2012).

In *H. pluvialis*, the conversion of β-carotene to astaxanthin needs the introduction of two hydroxy groups at C3 and C30 by CrtRB encoded by the gene *Crt-b* and two keto groups at C4 and C40 positions by BKT encoded by *bkt* gene (Lemoine and Schoef 2010). Different *bkt* genes viz. *bkt*1, *bkt*2 and *bkt*3 have been reported from different strains of *H. pluvialis* (Kajiwara et al. 1995; Huang et al. 2006; Lu et al. 2010). *bkt3* gene reported in *H. pluvialis* WB-1 is highly similar to *bkt*2 with a difference of two amino acids only but was functionally different. Huang et al. (2016) reported that *bkt*1 and *bkt*2 were up-regulated in the presence of glucose for the higher accumulation of astaxanthin in *Chlorella zofingiensis*. At the same time, down-regulation of *Lcyε* and *cyp97c* was reported which suppressed the competing pathway downstream and thus ensuring the supply of precursor (β-carotene) for astaxanthin production. Gao, Honzatko, and Peters (2012) reported up-regulation of *bkt2* gene along with *psy and pds* genes related to high astaxanthin production in *H. pluvialis* strain WB-1 under the influence of gibberelic acid.

Strategies for enhanced pigment production

Biopigments as a product can be significantly improved by metabolic engineering and genetic engineering of suitable

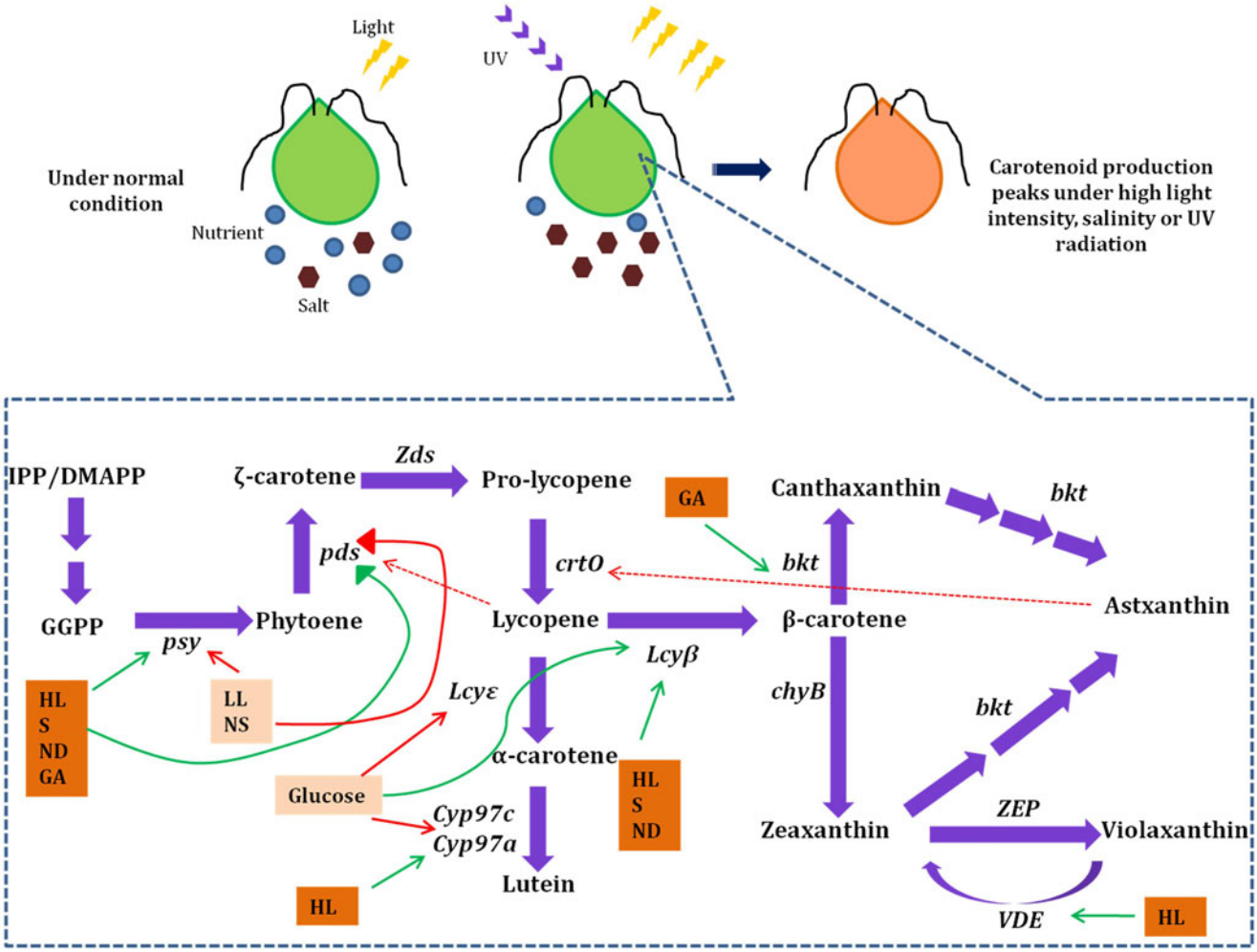

Figure 3. A generalized representation of regulation of microalgal carotogenic genes. Under increased salinity (), light intensity (),) and nutrient (),) depletion, carotenoid production increases. HL: High light intensity; LL: Low light; S: Salinity; ND: Nutrient deficiency; NS: Nutrient sufficiency; GA: Gibberelic acid. Green arrows indicate up-regulation, red arrows indicated own regulation and dotted red arrow indicates feedback inhibition of the gene product. For β-carotene production *psy, pds* and *Lcyβ* genesare up-regulated under high light, salinity and nutrient dieficiency. Additionally, for astaxanthin production from β-carotene, *bkt*gene is up-regulated and *Lcyε*& *Cyp97c* are deficiency down regulated. For lutein production, *Lcyε*& *Cyp97c* are deficiency up-regulated.

microalgae. Conventionally, the product synthesis in microalgal strains used for the industrial purpose can be enhanced by random mutagenesis followed by screening processes. Usually, these techniques are slow and time-consuming. In most cases, it is impossible to get the desired strain through mutagenesis and screening process. Therefore, using rational engineering which includes metabolic and genetic engineering has been used to modify or improve the biochemical capabilities of the microalgal cell. Some advanced technologies which can be used for the higher pigment production and development of microalgal cell as cell factories are shown in Fig. 3.

Conventional methods for strain improvement

For commercial purpose, the microalgal strain is initially selected by different screening processes. The microalgal strain is then subjected to different mutagenesis techniques used to improve the yield and productivity of the strain. Different mutagen agents were applied to obtain the desired phenotype (Fu et al. 2016). In many occasions, different mutagens which include both chemical and physical mutagens are used to develop a desired new strain successfully. Ultraviolet radiation induces the formation of pyrimidine dimers in the DNA strands. It is unpredictable but is reported to be controlled and most successful mutagenesis strategies to improve microalgal strain (Kodym and Afza 2003). For example, *Dunaliella bardawil*, after induction with UV radiation, a mutant strain rich in β-carotene content is developed (Depauw et al. 2012). It has also been reported in the case of *Chlorella* spp. where strain with high lipid content formed after induction (Liu et al. 2015). Gamma radiation, another physical mutagenic agent, has also been successfully used for the development of a mutant strain of *Scenedemus dimorphus* with high lipid accumulation (Choi et al. 2014). Chemical mutagens are also used to improve microbial strain, e.g., N-nitro-N-nitrosoguanidine is successfully used to produce mutant of *Heamatococcus pluvialis* with increased astaxanthin content (Kamath et al. 2008). Irradiance has a direct impact on pigment concentration as we discussed in an earlier section. It was investigated that different light spectra of visible light affect biomass as well as pigment concentration (Straka and Rittmann 2018). It was reported that different dye manipulated solar light to

get the desired wavelength can enhance biomass and pigment in case of species like *C. vulgaris and G. membranacea.* (Mohsenpour, Richards, and Willoughby 2012). Similar results have been recently reported by Ramanna et al. (2018) that use of organic dyes such as Lumogen yellow Diphenyloxazole, Rhodamine 8G, and Diphenylanthracene to manipulate incident irradiance to increase the photon flux density and enhance pigment production. It was shown that algae are grown in Rhodamine 8G increase the pigment concentration up to 45 wt% in the case of chlorophyl and 36 wt%, in carotenoids (Ramanna et al. 2018).

Still, in most cases, the desired phenotype was not obtained as it is an unpredictable, uncontrolled and slow process. These bottlenecks can be overcome through various modern approaches such as genetic and metabolic engineering, and their results are much more predictable and stable.

Metabolic and genetic engineering

Microalgae are considered as a potential candidate for metabolic and genetic engineering since these are unicellular and have a simple genetic organization with rapid reproducing ability as compared to plants and other eukaryotic cells. Microalgae are analogous to plant genetic and cellular structure (Gimpel, Henríquez, and Mayfield 2015; Hlavova, Turoczy, and Bisova 2015). Metabolic and genetic engineering can be effectively used for conversion of natural feedstock into useful metabolite in a more efficient and predictable way. These engineering techniques are used to enhance and modulate the metabolic pathway to bioconversion of substrate into the desired product. Metabolic engineering allows us to get a higher yield of product from a diverse range of carbon sources and provide the ability to withstand environmental stress conditions. It gives the access to manipulate the activity of many rate-limiting enzymes by deletion and over-expression, due to which the productivity increases. By the use of metabolic and genetic engineering new metabolic pathway from different organisms can be fitted into another superior host which has high metabolic rate and survival ability. These together can enhance the productivity of microalgal pigment (Banerjee, Dubey, and Shukla 2016). Advancement of tools for reverse engineering such as selection markers, genome sequence, gene targeting, different promoters, genetic transformation, and molecular biology techniques for example transcription activator-like effectors (TALEs), clustered regularly interspaced short palindromic repeats (CRISPR) and zinc-finger nucleases (ZFN) ease the way to understand the metabolic pathways and synthesize the new biological system (Jagadevan et al. 2018). Many strategies used to enhance carotenoids synthesis are discussed in a further section.

Genetic and metabolic engineering basically involve up-regulation and down-regulation of transcription and translation genes and also knock-out and knock-in of desired genes (Vickers et al. 2014; Stephens et al. 2015). As pigment production involve a complete pathway, with different substrates and enzymes to get a particular pigment. The most critical bottleneck for pigment overproduction is (1) feedback inhibition and (2) space for the accumulation of overproduced pigment droplets. The only pigment for which space is not as much a severe problem is phycobilin as these are present on the cytoplasmic side thylakoids membrane, but due to higher accumulation, their light harvesting efficiency is affected (Mulders et al. 2014; Guedes, Amaro, and Malcata 2011) . Overproduction of carotenoids should simultaneously require transport mechanism which could transport carotenoids molecule from site of synthesis to storage site actively to avoid any feedback inhibition due to overaccumulation of the pigments (Kapoor et al. 2015).

Metabolic engineering of microbial carotenoid production

The basic concepts for algal metabolic engineering for carotenoid production is mostly based on the strategies developed for higher plants. These mainly include interfering with the cellular metabolic processes through modulation of biosynthetic enzymes and formation of a metabolic sink to increase the flux toward the desired metabolite. However, interfering with the cellular metabolism by any means require a thorough understanding of the particular biosynthetic process along with its regulation.

Modulation of biosynthetic enzymes is desired to increase the flux by manipulating the metabolism. Overexpression of the enzymes those control the flux towards the desired product can help to achieve the enhanced production of the desired metabolite without any interference with any other metabolite. However, all enzymes in the pathway need not be overexpressed to the same level; enzymes those catalyze the final reaction needs to be overexpressed to a greater tune than the other enzymes in the biosynthetic pathway. For example, for overproduction of β-carotene, overexpression of LCYB is more desired as compared to the other enzymes in the preceding pathway. Lemoine and Schoefs (2010) suggested that the production of ζ-carotene from phytoene through PDS, is one of the rate-limiting steps in carotogenesis and up-regulation of this enzyme can increase the bioproduction of carotenoids. Nuclear overexpression of endogenous mutated PDS in *C. zofingiensis* resulted in 32.1% increase in total carotenoids while overexpression of endogenous mutated PDS in *H. pluvialis* resulted 26% increase in astaxanthin (Liu et al. 2014; Steinbrenner and Sandmann 2006). Expression of endogenous nuclear *pds* gene in the chloroplast of *H. pluvialis* showed up to 90% higher astaxanthin accumulation per culture volume (Galarza et al. 2018). However, in general, a single enzyme cannot control the flux towards the desired metabolite. Instead, it is controlled through coordinated expression of multiple enzymes. Hence, overexpression of multiple enzymes is required to overproduce specific carotenoids. Likewise, down-regulation of specific enzymes to increase the desired flux by decreasing the flux towards other branches can also be helpful for overproduction of carotenoids (Varela et al. 2015). For example, down-regulation of LCYE is also required for overproduction of β-carotene so that lycopene is not converted to α-carotene. Baek et al. (2018) reported that knockout mutant of the ZEP encoding gene induced by preassembled DNA-free CRISPR-Cas9

ribonucleoproteins in *C. reinhardtii* strain CC-4349 had a significantly higher zeaxanthin content as compared to the wild-type. Overexpression of exogenous genes encoding such enzyme in the target, organism has also been another approach for carotenoid overproduction. Anila et al. (2016) reported metabolic engineering of carotenogenic pathway in *Dunaliella salina* for astaxanthin production through the introduction of an *H.pluvialis* derived *bkt* gene from encoding BKT along with chloroplast targeting. Nuclear overexpression of *D.salina* derived PSY encoding gene in *Chlamydomonas reinhardtii* resulted in a 2.6 fold increase in lutein (Couso et al. 2011).

On the other hand, Cordero et al. (2011) reported that overexpression of PSY encoding gene from *Chlorella zonfingiensis* in the nucleus of *C. reinhardtii* showed 2.2 fold increases in lutein production. However, regulation of enzymes for overproduction of carotenoids may often encounter the problem of feedback inhibition. Such problems can be circumvented by overexpressing feedback resistant flux controlling enzymes.

Formation of a metabolic sink can also increase the flux toward the desired metabolite. In this case, the overproduced metabolite transported away from the site of formation which thus can overcome the problems faced due to feedback inhibition. It has been shown for *Dunaliella salina* and *H. pluvialis* that inhibition of lipid accumulation can stall the biosynthesis of β-carotene and astaxanthin as lipid serves as a metabolic sink for these two carotenoids (Zhekisheva et al. 2005; Rabbani et al. 1998). However, the formation of the metabolic sink may also not work always due to lack of appropriate transporters directing the carotenoids to the sink and absence of specific enzymes required for biosynthesis of the desired carotenoids. In *H. pluvialis*, β-carotene transported out of the photosynthetic apparatus and its subsequent conversion to astaxanthin takes place in the cytoplasm (Lemoine and Schoefs 2010). However, when the additional metabolic sink is created in the cytoplasm, it will be occupied by β-carotene and may not be converted efficiently to astaxanthin if specific enzymes are not targeted to the sink.

Though microalgal group makes up the major source of carotenoids, metabolic engineering in carotenoids biosynthesis pathway in these microorganisms is impeded by the lack of strategies for genetic transformation of commercially important microalgal species (Wichuk, Brynjolfsson, and Fu 2014). Even sometimes, the successful transformation does not guarantee long-term stability. Although some success has been achieved in the metabolic engineering of carotene genes in microalgae, a girth of research is still required for attaining high productivity and stability. RNAi, CRISPR-CAS system, etc. can address such issues. Besides, a better understanding of the modulation of the cartenogenesis and its networking with other metabolic processes is also required for successful engineering of algae.

Flux balance analysis

Flux balance analysis (FBA) is a study of metabolite flow through metabolic pathway and genes present in the organism known by whole genome sequences. This strategy is based upon mass balance (input equals output) and synchronous growth of the organism. FBA is used to find different ways for the optimization of the desired product and how to balance the fluxomics of the organisms (Wu et al. 2015; Maranas et al. 2003). Moreover, FBA has wide-range of application which has been well explained by O'Brien, Monk, and Palsson (2015). It does have few drawbacks, for example, it doesn't tell about concentration and regulation of metabolite production. But, it allows testing gene necessity and effect of various environmental factors speedily. FBA also predicts the utilization of nutrient and carbon sources as well as cell growth and exchange of flux in different ecological conditions (Raman and Chandra 2009).About more than 100 organisms genome-scale models have been constructed, among them *Synechocystis* sp. PCC 6803, was a well-studied cyanobacterium (Mitschke et al. 2011). Microalgae, including cyanobacteria, are the unicellular oxygenic photoautotrophs, which are well recognized as efficient organisms to convert environmental carbon dioxide into complex metabolites and microalgal pigment production is directly linked with biomass accumulation, FBA provides database about biomass composition and effect of various stress factors (light intensity, nutrient stress) on biomass production. Effect of different light intensities on the growth rate of average cells of cyanobacteria *Synechocystis* sp. 6803 and *Emilinia Huxley* was studied by Knoop et al. (2013) and Qian et al. (2017). Qian et al. (2017) have also constructed a genome-scale model of cyanobacterium *Synechococcus* sp. PCC 7002.

Future perspective

Metabolism of a cell defines the potentials of the cell, and metabolic engineering is the key modern technology to develop a cell into cell factories. To increase the concentration of pigment we need to understand the metabolic reaction of primary pigment production. Modern approaches such as 'omics' experiment allows us to understand the regulatory mechanism of pigment production which is responsible for the homeostasis of pigment. For over-production of primary pigment, we should more emphasize on the study of the regulatory mechanism of pigment production. Metabolic engineering relies on to give support or the optimization and assembly of the novel pathway in a microorganism. Many factors effect on the stability of metabolic pathway and also on abundance of mRNA in the cell. Using systems biology along with metabolic approaches can enhance the rate of product formation and also help to understand the effect of various factors on the metabolic rates. Research for pigment formation should more focus on different rate-limiting enzymes and co-factors to enhance the productivity. Different advanced techniques include advances in fluxomics, highly sensitive transcriptomics, metabolomics methods, proteomics, high-throughput sequencing for deciphering genomes (Weber et al. 2015) and computational tools. These emerging technologies along

with metabolic and genetic engineering enhance the potential for the pigment production from microalgae.

Concluding remarks

Microalgae are considered as potential candidates to develop as cell factories, as it has a significant role in the sustainability of the environment. It is used to produce biopigments (carotenoids) and other secondary and bioactive metabolites. Metabolic and genetic engineering approaches are a useful tool for the production of pigments and feedstock in diverse microalgae and cyanobacteria. Compared to random mutagenesis, metabolic engineering is more rapid and useful for strain development. Alternatively, different rate-limiting steps of metabolic pathways can be engineered to optimize for the enhanced metabolite production. Commercialization of pigment requires strains which are of better quality in comparison to native strain and for that matter many genetically engineered microalgal strains are available. Metabolic engineering along with genetic engineering tools allow manipulating microalgae/cyanobacteria to have high growth for biomass as well as pigment production. For commercial production, large-scale nutrient supply and proper light conditions are required, so improvement should be made to minimize the production cost by using cheap nutrient sources. Moreover, the microalgal cell can be designed to use fewer nutrients to accumulate larger biomass and higher pigment production. Detailed study of the microalgal cell, allow us to understand the mechanism of pigment synthesis and production which provides chances to a designed metabolic pathway for the effective pigment production and lower the recovery cost. Bottlenecks of effective pigment production should be understood, and potential genetic and metabolic approaches used to overcome cost-intensive productivity. The recovery cost can also be minimized by using genetic engineering to develop strain having properties like auto-flocculation. Finally, we can conclude that metabolic and genetic engineering have a great impact on the commercialization of pigment production by the microalgal cell.

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