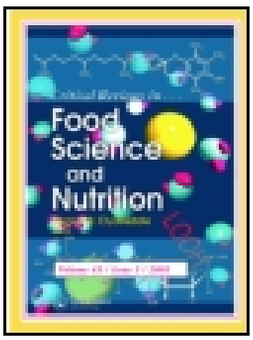

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REVIEW

Transgenic microalgae as bioreactors

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ABSTRACT

Microalgae are unicellular organisms that act as the crucial primary producers all over the world, typically found in marine and freshwater environments. Most of them can live photo-autotrophically, reproduce rapidly, and accumulate biomass in a short period efficiently. To adapt to the uninterrupted change of the environment, they evolve and differentiate continuously. As a result, some of them evolve special abilities such as toleration of extreme environment, generation of sophisticated structure to adapt to the environment, and avoid predators. Microalgae are believed to be promising bioreactors because of their high lipid and pigment contents. Genetic engineering technologies have given revolutions in the microalgal industry, which decoded the secrets of microalgal genes, express recombinant genes in microalgal genomes, and largely soar the accumulation of interested components in transgenic microalgae. However, owing to several obstructions, the industry of transgenic microalgae is still immature. Here, we provide an overview to emphasize the advantage and imperfection of the existing transgenic microalgal bioreactors.

KEYWORDS

Microalgae; genetic modification; recombinant protein; metabolic engineering; bioreactor

Introduction

Beautiful biosphere of the earth is suffering from tremendous perturbation. Globally, biodiversity loss, rising temperature, and climate change are making biosphere stability vulnerable. At the same time, the exploding population and rapid industrialization of human society have increased the demand for environmental resources such as freshwater, arable lands, and biotic resources. However, the predatory exploitation of environmental resources was unsustainable (Smithers 2016; Carpenter et al. 2009). Besides, special or differentiated requirements, such as biopharmaceutical reagents, human nutrition supplements, enzymes, environmentally friendly biofuels, have questioned the traditional biomanufacturing domain. Therefore, it is urgent to exploit more latent bioresource while increasing the capacity of existing biomanufacturing fields.

Thanks to the revolutionary breakthrough in biotechnology, several novel transgenic biomanufacture platforms (or called bioreactors) have been developed. Bacteria, yeast, mammalian cells, transgenic plants, and several *in vitro* systems are widely used to express recombinant genes and to contribute to the economy (Table 1). As an example, the transgene insect-resistant crops planted in the United States have reduced chemical pesticide use and increased crop yields (Carlson 2016). Meanwhile, these transgenic organisms have shown great potential in producing recombinant proteins such as industrial enzymes and therapeutic proteins. In 2012, the revenues from drug produced using genetically modified (GM) organisms in the United States had reached $125 billion (Carlson 2016). Additionally, recombinant enzymes are utilized to reconstruct metabolic pathways in these organisms intending to produce valuable bioactive molecular such as polysaccharides, fatty acids, amino acid derivatives, which do not accumulate in natural organisms.

Owing to several bottlenecks, there is not yet a comprehensive, single-cell, or multicellular platform that can completely respond to human needs for various biological metabolites. In other words, the transgenic platforms are powerful, but it is limited. Eukaryotic microalgae, as developing transgenic bioreactors, have various advantages over the transgenic platforms mentioned above. Characterized for their unicellular lifestyle and autotrophic growth, microalgae are efficient and economic platforms for accumulating organics including proteins, lipids, pigments, and carbohydrates in short period, which are much faster than terrestrial plants (Ravindran et al. 2016; Chen and Jiang 2017). The production of microalgal organics merely requires light, mineral, carbon dioxide, and water (freshwater/saline water/wastewater). Most importantly, the breeding of microalgae will not take up plenty of arable lands and too much water, which are in shortage on the earth (Carpenter et al. 2009). Thus, microalgal products such as animal feeds, human nutrition supplements, and biofuels have shown their great competitiveness to supersede the traditional plant-based agriculture (Liang and Jiang 2013; Liang et al. 2019). The GM microalgae have already utilized in producing recombinant proteins in the chloroplast or cytosol, for instance, the industrial enzymes, vaccines, or other therapeutic

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Table 1. Comparison between different transgenic biomanufacturing platforms.

Platforms	Advantages	Disadvantages	Applications	References
Bacteria	Fast growth; rapid accumulation of target product; simple genetic operation with advanced tools; inexpensive culture conditions	Lack of post-translation modification process which is necessarily to produce eukaryotic protein including eukaryotic enzymes; existence of human pathogens	Industrial proteins: enzymes, therapeutic proteins; small molecular: sugar, amino acid, short chain fatty acid	Hossain et al. (2018); Walsh and Jefferis (2006)
Cyanobacteria	Fast growth; rapid accumulation of target product; photoautotrophic growth	Lack of post-translation modification process which is necessarily to produce eukaryotic protein including eukaryotic enzymes	Fuels: hydrogen, lipids; pigments: carotenoids, phycocyanobilin	Walsh and Jefferis (2006)
Yeasts	Fast growth; rapid accumulation of target product; advanced molecular tools for metabolic regulation and protein modification; inexpensive culture conditions, scalable up to fermentation	Particular glycosylated modification; easily contaminated culture condition; low efficiency production of phytogenic metabolites especially those under complex regulation	Recombinant proteins: for animal feeds or therapeutic applications; lipids; flavoring foods; organic acid; biodegradable bioplastics	Zhang, Nielsen, and Liu (2017); Li, Li, and Smolke (2018); Lebozec et al. (2018); Nevalainen and Peterson (2014)
Plants	Scale-production available; photoautotrophic growth; precursor supplies obtained easily	Protein and metabolite yield are low relative to total biomass; GM pollen and seeds may contaminate environment	Therapeutic proteins; secondary metabolites: phenolics, terpenoids, and alkaloids	Fu, Martin, and Zhang (2018); Xu et al. (2012)
Mammalian (cells)	Suitable to produce complex post-translational modification of humanized protein	Low protein yield; complex cultivation condition and easily contaminated medium; existence of human pathogens	Therapeutic proteins	Jazayeri et al. (2018); Kim, Kim, and Lee (2012)
Cell-free system	On demand, time-saving, simple conditional production; do not need to maintain cell growth; variety of protein modify process	Low protein yield; expensive protein production cost; complex protein modification/folding accurate control not available	Therapeutic proteins	Pardee et al. (2016); Carlson et al. (2012); Villarreal and Tan (2017)
Microalgae	Fast growth; rapid accumulation of target product; photoautotrophic growth	Relatively low recombinant protein accumulation; know less about the metabolism regulation	Therapeutic proteins; pigments; biofuels; fatty acids; lipids	Ravindran et al. (2016); Akbari, Eskandani, and Khosroushahi (2014); Shi et al. (2018); Del Campo et al. (2004); Dyo and Purton (2018)

proteins (Barkan 2011; Akbari, Eskandani, and Khosroushahi 2014). Metabolic engineering, a novel strategy for the production of valuable products, has significantly increased the lipid, pigments, and other commercial molecular content in microalgae (Shi et al. 2018; Del Campo et al. 2004).

However, the current restrictions for the application of microalgal-based bioreactors are obvious. In recent years, the production of microalgal products is still expensive. Taking the *Nannochloropsis* genus as an example, it takes US$2 kg^{-1} for algal biomass production or US$6 L^{-1} for oil production (Chua and Schenk 2017). The exploitation of microalgae source is still in its early stage, with only thousands of species of eukaryotic microalgae that have been successfully cultured in laboratory conditions while only dozens of eukaryotic species have been sequenced. The production yield of target products in GM microalgae is still low. For example, the yield of recombinant protein produced in microalgae ranges from 0.1 to 5% total soluble protein (TSP) (Dyo and Purton 2018; Hempel and Maier 2012). Recently, the production of algae biofuels presents poor performance and demands more energy than the energy that they can deliver (Carneiro et al. 2017). Apparently, it is essential to discover more industrial microalgal strains, strengthen their productivity, and lower the cost. The aim of this review is to give a comprehensive overview of the current applications and limitations of transgene microalgae, includes the well studied or the potential species. Besides, the expression systems of microalgae and their applications are also discussed in this review.

Selecting the ideal microalgae: safety, functions, and strategies

Microalgae are easily contaminated by biological factors such as grazers, fungi, photosynthetic organisms, bacteria, and viruses (Lam et al. 2018) or environmental factors like heavy metals (Matos 2017). These factors may largely increase the production cost, at the same time, lower the yield of end products. The release of microalgae may be inevitable during production (Campbell 2011), which would have potential negative ecological effects. For example, the local extinction and hazardous algal bloom formation, and the algal toxins synthesized in several algae may do harm to

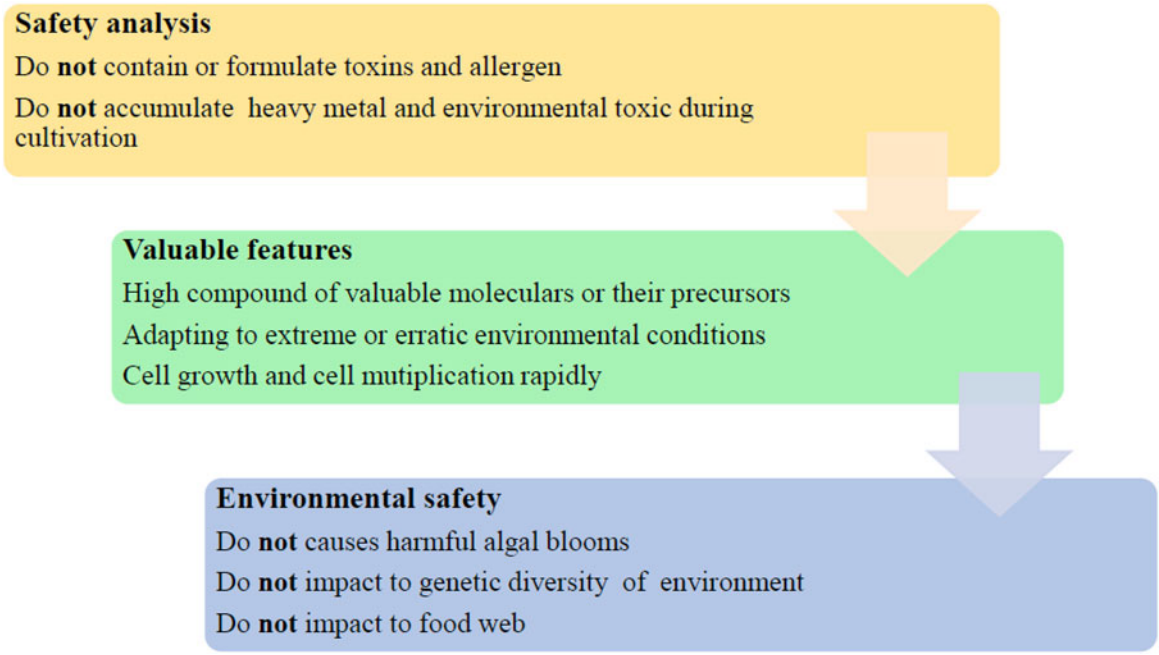

Figure 1. The principles for selecting interested microalgae.

humans, contaminate the marine environment, and poison the aquatic animals (Matos 2017). Thus, the selection of bioreactor, potential microalgae species, and the interactions between microalgal species and environment (the algae production environment and local environment) should be highly assessed. In addition, the development of the microalgae industry cannot always be driven by policy subsidies, and hence it is essential to construct powerful GM microalgae strains that are profitable. In the following sections, several strategies aiming at discovering ideal microalgal strains are discussed in this section (Figure 1).

Nontoxic microalgae

Some microalgal species are generally recognized as safe (GRAS) or no toxins known (NT) (Enzing et al. 2013), which are considered free of harmful viral, prion, or endotoxin contaminants. Potential microalgal bioreactors such as *Dunaliella salina*, *Isochrysis galbana*, *Nannochloropsis* sp., *Phaeodactylum tricornutum,* and *Thalassiosira pseudonana* are believed to be NT. *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Crypthecodinium cohnii*, and *Porphyridium cruentum* are GRAS (Dyo and Purton 2018; Enzing et al. 2013). These merits offer the possibility of the nutritional and biomedical applications in these microalgae, meanwhile, reduce the downstream purification cost. Additionally, the entire frond mentioned above may possibly regard as room-temperature store cabinets to deliver recombinant proteins, including vaccines, enzymes, and hormones after desiccation.

Extreme microalgae

It is hard to avoid the contamination of other algae species, fungi, bacteria, and protozoa in large-scale microalgae cultures, except under the selection environment (Extreme PH and temperature, the existence of herbicides and antibiotics). However, the abuses of herbicides and antibiotics will lead to inevitable environmental risk during the large-scale cultivation of GM microalgae. Several species of microalgae are adapted to the extreme environment on the earth's surface (Table 2). The extreme environment can partly reduce the biological contamination during the cultivation process of microalgae because many microorganisms could not survive or multiply in such extreme conditions. Additionally, the inevitable release of extreme microalgae would not contaminate the local environment owing to the low competitiveness in the altered conditions (Souza et al. 2017; Lukes et al. 2014; Oren 2014). Light and carbon dioxide are main energy and material source of photosynthesis. Hence, microalgae which resist to high levels of light and carbon dioxide show higher photosynthetic potential and higher cell density. Several microalgae such as *Cyanidium caldarium* and *Chlorella* can tolerate up to 100% carbon dioxide (Maeda et al. 1995; Doemel and Brock 1971). *Botryococcus braunii* can grow rapidly in high light intensity ($400–1600\,\mu mol\,m^{-2}\,s^{-1}$), whereas most microalgae suffer from photo-inhibition and oxidative damage with impaired biomass production (Chen et al. 2017; Dacong et al. 2008).

Microalgae based on the high-value compounds

The aim of cultivating microalgae is to obtain valuable products. Thus, it is important to select microalgae species that are rich in valuable compounds such as pigments, fatty acids, and other compounds. Moreover, the adequate precursor from some microalgae is suitable for the production of desired high-value compounds. For example, the metabolic engineering *D. salina* which has high *β*-carotene (the precursor of ketocarotenoids) content can significantly

Table 2. Extreme microalgae and their environmental adaptation.

Extreme conditions	Species	Growth conditions (tolerance)	Reference
Acidic	*Chlamydomonas acidophila*	pH 3.6	Souza et al. (2017)
	Dunaliella acidophila	pH 0–3	Assuncao et al. (2012)
	C. caldarium	(pH = 0.05)	Lukes et al. (2014)
Alkaline	*Chlorococcum alkaliphilus*	(pH = 11)	Xi et al. (2017)
High temperature	*C. caldarium*	45 °C (57 °C)	Eisele et al. (2000)
	Galdieria sulphuraria	45 °C (57 °C)	Schoenknecht et al. (2013)
Low temperature	*Coccomyxa subellipsoidea*	<15 °C (–88 °C)	Blanc et al. (2012)
	Chlamydomonas nivalis	2.5 °C	Lukes et al. (2014)
Hyperhaline	*Dunaliella salina*	(Saturated brine)	Oren (2014)
	Dunaliella viridis	(Saturated brine)	Oren (2014)
High carbon dioxide tolerance	*C. caldarium*	(Pure carbon dioxide)	Maeda et al. (1995)
	Chlorella sp.		Doemel and Brock (1971)
High light intensity tolerance	*B. braunii*	400–1600 μmol m^{-2} s^{-1}	Dacong et al. (2008)

Growth conditions (tolerance) refer to the suitable cultivation condition and the tolerance limit of designated extreme condition.

accumulate ketocarotenoids after the introduction of a beta carotene ketonase (*bkt*) gene from *Haematococcus pluvialis* (Anila et al. 2016; Liang, Zhu, and Jiang 2018). The heterologous expression level of poly-3-hydroxybutyrate (PHB) in *P. tricornutum* are about 100-fold higher than in the cytosol of plants. This might resulting from the high lipid content and the acetyl-CoA pool in *P. tricornutum* that provide the basis for PHB synthesis (Hempel et al. 2011).

Selection of microalgae based on the morphological structure and growth rate

The biological evolution during billions of years has optimized the ability of microalgae to generate specialized components, which makes them fairly adapted to the changeable environment. Frustule, the nanopatterned exoskeleton of diatom, not only protects diatom from predators and environmental damage but also provides high surface area and protein binding site for the biomedical applications (Delalat et al. 2015; Ragni et al. 2018).

Monitoring the growth parameter conditions such as temperature, pH, phosphate and nitrogen contents, and so on, to regulate microalgal cells from growth regime to stress regime, are efficient ways to induce the accumulation of lipids or other value compounds (Paliwal et al. 2017). A two-stage process, in which cells were cultured in nutrient replete conditions first and then transferred to nutrient limitation conditions, would be an efficient way for the production of valuable compounds. Hence, it is essential to screen out fast growth microalgal species.

Fast cell growth rate means rapid biomass accumulation in a short period. The biomass productivity of *Schizochytrium* can reach 1.88 g L^{-1} day^{-1} cultured at sorghum juice (Liang et al. 2010). *Jaagichlorella luteoviridis* can accumulate biomass 0.6 g L^{-1} day^{-1} in continuous flow photo-bioreactor and wastewater treatment (Ramos-Tercero et al. 2014). Obviously, the growth rate of microalgae differs widely from species to species or from different culture conditions. Even under optimized conditions, the biomass productivity of most microalgae is still lower than 0.3 g L^{-1} day^{-1} (Ghosh et al. 2016). As for large-scale diesel production, the appropriate growth rate of microalgae was set at 20 g m^{-2} d^{-1} with an assumed lipid content of 10% (Hoffman et al. 2017). Thus, the selection of fast-growing microalgae is economically important for microalgal industries.

The nuclear and chloroplast expression systems of microalgae

Heterologous genes can be integrated and expressed in the nuclear genome, the chloroplast genome, or the mitochondrion genome of the eukaryotic microalgae (Figure 2). In recent decades, the nuclear expression system and chloroplast expression system of microalgae have been widely studied. However, far too little attention was paid on the mitochondrion transformation system. Recently, heterologous genes have been successfully introduced into the nuclear genome of about 100 microalgae species. Transformation methods such as electroporation, agrobacterium, glass beads method, and biolistic bombardment have been commonly applied in microalgae transformation. Common molecular tools such as promoters, selectable markers, and reporter genes were concluded in the previous reviews (Hempel and Maier 2012; Shamriz and Ofoghi 2016; Jeon et al. 2017; Ghosh et al. 2016; Lior, Na'Ama, and Michal 2016). In the latest decades, the understanding of microalgae genomes has provided various strategies to improve the expression of heterologous genes. The strategies including but not limited: (1) Optimized the function of *cis*-acting elements and interested genes; (2) Utility of functional peptides; (3) Eliminated the negative affection from host genome and metabolism (Table 3). Apparently, the understanding and utilization of nuclear expression machinery in microalgae are staying ahead of that of chloroplast expression machinery.

The advanced nuclear expression system of eukaryotic microalgae is able to achieve post-translational modification (disulfide bond formation, phosphorylation, and glycosylation) of recombinant proteins, which are crucial for the function of these proteins. Additionally, the post-translational modified protein can transfer and localize into the cytoplasm, cytomembrane, cell wall, and subcellular compartments (endoplasmic reticulum, golgi, and chloroplast) of microalgae (Ramos-Martinez, Fimognari, and Sakuragi 2017; Sheppard et al. 2012; Akbari, Eskandani, and Khosroushahi 2014). The bioactive protein expressed from the nuclear expression system can also secrete into the medium, which

reduces the subsequent purification cost, avoids proteotoxicity, and decreases the protein misfolding process.

However, the disadvantages of nuclear expression system are conspicuous, especially the position effect, the exogenous genes silencing, random insertion site or gene dosage of exogenous gene, which may lead to the inefficient expression of recombinant proteins (TSP, 0.05–0.25%) (Matzke and Matzke 1998; Hempel and Maier 2012). In addition, the endogenous proteases in cytosol will degrade the recombinant protein (Doran et al. 2009). The chloroplasts of eukaryotic microalgae originate from the cyanobacterial progenitor, composing of a few introns and 100–200 genes in their small polyploid plastids.

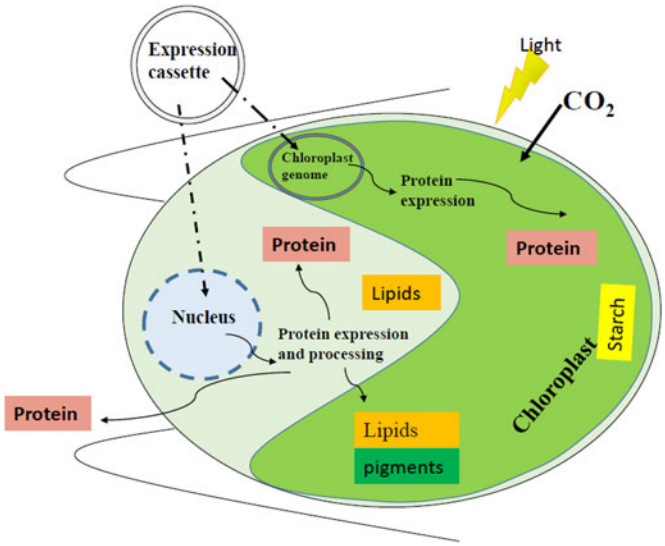

Figure 2. Microalgae as versatile transgenic bioreactors (take green microalgae as examples). Heterologous proteins encoded in the microalgal nucleus could express in the cytosol. They can also transport to chloroplast and secrete outside the cell after post-translational modification. However, heterologous proteins expressing in the chloroplast will only accumulate there. The heterologous proteins such as recombinant enzymes expressed in chloroplast and cytosol could alter the metabolic pathways and increase the accumulation of interested metabolites such as lipids, pigments, and starch.

Most plastid genes encode the essential components of the photosynthetic complexes and the chloroplast's transcription/translation elements (Dyo and Purton 2018). The regulation of gene expression mainly occurs at post-transcriptional steps. Numerous nuclear-encoded protein factors are imported into the chloroplast to mediate RNA processing, splicing, stabilization, and translation initiation (Barkan 2011). In 1988, by using the model microalgae *C. reinhardtii*, DNA transformation of the chloroplast was first reported (Boynton et al. 1988). The stable chloroplast transformation of *P. purpureum* (Lapidot et al. 2002), *D. tertiolecta* (Georgianna et al. 2013), *H. pluvialis* (Gutierrez et al. 2012) was reported. Recently, the development of the microalgae chloroplast platform has almost exclusively focused on *C. reinhardtii*, with more than 100 reports in this species (Dyo and Purton 2018). In contrast to the microalgae nuclear expression system, the chloroplast expression system can accumulate recombinant protein in a higher yield, which ranges from 0.1 to 5% of TSP (Dyo and Purton 2018; Hempel and Maier 2012). The exogenous gene can transfer into the chloroplast genome via particle bombardment transformation or agrobacterium-mediated transformation and integrated into the genome by homologous recombination, which can be inserted into the particular locus (Bock 2007). Chloroplast can be used as a container for protein storage, owning to the lack of endogenous protease.

Table 3. Strategies for stabilizing/increasing the nuclear/chloroplast transgene expression in microalgae.

Hosts	Strategies	Results	References
C. reinhardtii	Screen out the highly heterologous gene expression mutants	Foreign genes express to high levels with higher transformation frequencies	Neupert, Karcher, and Bock (2009)
	Exploit synthetic promoters which express transgene more efficiency than the current best endogenous promoters	Synthetic promoters were capable of driving exogenous gene expression which were better than native promoters	Scranton et al. (2016)
	Promoter-less genes random insert to a region adjacent to endogenous genomic promoter or in frame with a native gene to start expressing	Transformants exhibited self-flocculation abilities between 2- and 3.5-fold higher than the control untransformed strain	Díaz-Santos et al. (2016)
	Codon-optimized synthetic genes with codons that more closely match the host	All transformants showed expression of the allergen with yields between 0.01 and 0.04% of TSP	Hirschl et al. (2017)
	Utilization of Viral 2A peptides to realize the overexpression of interested gene and low expression of marker gene	The 2A-containing dicistronic systems allow differential heterologous gene expression and location	Plucinak et al. (2015)
	The C-terminal of recombinant protein fused with synthetic glycomodule to reduced proteolysis	The secreted yields of the glycosylated recombinant proteins were higher than those without the glycomodule by up to 12-fold	Ramos-Martinez, Fimognari, and Sakuragi (2017)
	Redesign the 5′UTR elements to eliminate nuclear-regulatory elements to improve the transgene expression level in chloroplast	The level of recombinant proteins was increased by using the atpA promoter/5′UTR	Stoffels et al. (2017)
C. cryptica	Separate the recombinant protein production phase from the cell growth phase by using induction condition such as silicon limitation	The yields of recombinant protein have reached 1.8% of TSP in *C. cryptica*.	Shrestha and Hildebrand (2017)
P. tricornutum	Transformation of circular episomes to avoid the disadvantages of genome insertion	These episomes are maintained as closed circles and replicated stably	Karas et al. (2015)

Techniques that use the same methods but apply to other microalgae species will not be repetitive described.

Furthermore, the absence of transcriptional/translational machinery has also avoided the unwanted protein modification process.

Actually, nuclear or chloroplast expression machinery should not be distinguished strictly. The recombinant protein expressed by the nuclear expression system can transfer and localize in the chloroplast (Cordero et al. 2011). Chloroplast-expressed genes are highly regulated by sequence and secondary-structure elements in the 5' untranslated regions (UTRs) of mRNAs. These 5' UTRs of the chloroplast have interacted with nuclear-encoded factors, which regulate mRNA processing, stability, and translation initiation (Specht and Mayfield 2013). In contrast, metabolites, RNA, and hormones may transport into the cytosol and affect the expression of nuclear genes (Boerner 2017).

Microalgae species applied as bioreactors

To date, many microalgae species are considered as potential biomanufacturing platforms (Table 4). Increasing researches currently focus on several genera such as *Chlamydomonas*, *Dunaliella*, *Nannochloropsis*, *Thalassiosira*, and *Phaeodactylum*. Recently, there are numerous studies on the morphology, physiology, and genetics of these species. Besides, the exploitation of gene transformation technologies allows the artificial gene recombination and then expresses recombinant proteins in these species. In this section, potential microalgae species that may apply in GM bio-manufacturing will be discussed.

Chlorophyta

Chlorophyta is a division of green algae, including thousands of microalgae species, which offers incredible biological diversity. Microalgal species of Chlorophyta are widely applied to produce various bioproducts such as pigments, biofuels, and other bioactive compounds (Matos 2017). Several well-studied microalgae genera such as *Chlorella*, *Dunaliella*, *Chlamydomonas*, and *Haematococcus* are economically important and considered as potential transgenic bioreactors.

Chlamydomonas

Chlamydomonas reinhardtii is a eukaryotic green microalga distributed worldwide in soil and freshwater, which characterizes for its cup-shaped chloroplast, a photosensitive eyespot, and two flagella. Several features of *C. reinhardtii*, such as rapid growth as a haploid, can grow in the dark with heterotrophic conditions, which have made it become an excellent laboratory species. In last 60 years, *C. reinhardtii* was well studied and served as a biological and cytobiological model organism to reveal the assembly of eukaryotic flagella (Hoeoeg et al. 2014), photosynthesis (Allorent et al. 2013), plant circadian clocks (Ryo et al. 2016), or cell cycle (Cross and Umen 2015). The nuclear, chloroplast, and mitochondrial genome sequencing of *C. reinhardtii* was completed in 2007 (Merchant et al. 2007). The genetic resource center (https://www.chlamycollection.org/) has already been established to receive, catalog, preserve, and distribute high-quality and reliable wild type and mutant cultures of the green microalgae *C. reinhardtii*, similar to the Arabidopsis Information Resource (https://www.arabidopsis.org/index.jsp), a database of genetic and molecular biology data for the model higher plant *Arabidopsis thaliana*.

In such a way, *C. reinhardtii* is an essential biotechnological hub, which is utilized to identify functional genes of many eukaryotic microalgae (Cordero et al. 2011). Newly developing genome editing technologies (zinc-finger nucleases) or clustered regularly interspaced palindromic sequences (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) (Sizova et al. 2013; Bortesi and Fischer 2015) and bacterial/viral genetic tools were applied in *C. reinhardtii* (Kumar et al. 2004; Plucinak et al. 2015). Moreover, the advanced molecular toolkit and well-annotated genome have facilitated the production of valuable metabolites in *C. reinhardtii*, for example, the hydrogen (Torzillo et al. 2015), acyl-lipids (Li-Beisson, Beisson, and Riekhof 2015) and carotenoids (Cordero et al. 2011). Compared with the metabolic engineering application, more researches are attaching importance to the recombinant protein production, such as the HIV antigen (Barahimipour, Neupert, and Bock 2016) and xylanase (Rasala et al. 2012). The advanced nuclear expression system of *C. reinhardtii* is applied to realize the efficient secrete of recombinant protein by using several secretion signals. For example, the endogenous ars1 secretion signal of *C. reinhardtii* has successfully started the secretion of recombinant xylanase (Rasala et al. 2012). In another report, the exogenous yellow fluorescent protein Venus were C-terminally fused with synthetic glycomodules, which effectively improved the secretion yield than the nonglycosylated Venus (Ramos-Martinez, Fimognari, and Sakuragi 2017).

However, the low expression of heterogenous gene in nuclear expression system of *C. reinhardtii* has hindered the commercial application of recombinant protein production. Hence, the chloroplast of *C. reinhardtii*, a developing transgene expression system, is considered as a potential recombinant protein production platform. More than 100 kinds of recombinant proteins ranging from high-value therapeutic proteins to low-value industrial enzymes were expressed in the chloroplast of *C. reinhardtii* (Tran et al. 2013; Wang et al. 2008; Dreesen, Charpin-El Hamri, and Fussenegger 2010; Yoon et al. 2011). Despite the fact that a host of transcriptomics and proteomics studies have been performed, the genome-scale annotations of *C. reinhardtii* are not completed, which restrict the comprehension of its metabolic network. In the foreseeable future, the available genome-scale annotation might reconstruct the genome-scale metabolic model, allowing the reverse engineering of metabolic models and informing hypothesis-driven metabolic engineering experiments (Reijnders et al. 2014). Above all, considering as the foremost microalgal bioreactor, *C. reinhardtii* has shown its high potential for the commercial application of GM microalgae.

Chlorella

Chlorella are unicellular green algae containing chlorophyll *a* and chlorophyll *b* as photosynthetic pigments. The

Table 4. The introduction of potential microalgae bioreactors and their applications.

Species	Living conditions	Genome sequence availability	Characteristics and advantages	Commercial applications	Transgenic applications
C. reinhardtii	Fresh	Available	A wealth of genetic information already available; Easy of cultivation and manipulation of its genomes		Applications in gene function determination of other microalgal species; production of hydrogen, acyl-lipids and carotenoids; production of recombinant proteins such as therapeutic proteins industrial enzymes
C. zofingiensis	Terrestrial		High resistance against rough conditions and invading organisms; Rapid growth and reproduction	Dietary supplement; Food ingredient	Increase carotenoid content
C. vulgaris	Fresh	Available			Produce recombinant proteins such as hormones
C. sorokiniana	Fresh	Available			Produce recombinant proteins such as hormones
C. ellipsoideum	Fresh				Increase lipid content. Produce recombinant proteins such as therapeutic proteins and hormones
M. homosphaera	Terrestrial				Increase lipid content
D. salina	Fresh/marine	Available	Lack of cell wall, high salinity tolerance	Additive; Vitamin; Colorant	Increase lipid, glycerol, carotenoid content. Produce recombinant proteins such as enzyme inhibitor and therapeutic proteins
D. tertiolecta	Marine				Increase lipid content. Produce recombinant proteins such as industrial enzymes
H. pluvialis	Fresh		Adapt to the fluctuating weather, salinity, temperature, and light radiation	Dietary supplement	Increase carotenoid (especially astaxanthin) content
N. oculata	Brackish		High resistance to environmental pollution; Easy for genetic manipulation	Dietary supplement, food ingredient	Produce recombinant fish hormone proteins and antimicrobial peptides
N. oceanica	Marine	Available			
N. gaditana	Marine	Available			
N. salina	Marine				Increase lipid content
P. carterae	Marine		Extreme PH resistance		
I. galbana	Marine			Aquaculture	
T. pseudonana	Marine	Available	Special siliceous structure for multiple applications	Aquaculture	Increase fatty acids content; produce recombinant therapeutic proteins
P.tricornutum	Marine	Available	A wealth of genetic information already available	Aquaculture	Increase fatty acids content; produce bioplastic materials; produce recombinant therapeutic proteins
F. solaris	Marine		High tolerance to organic pollution		Increase lipid content
C. gracilis	Marine		Special siliceous structure		
C. merolae	Fresh	Available	Extreme environment resistance; Simple cellular architecture; noncell wall		
P. purpureum (cruentum)	Fresh	Available	Noncell wall; Huge plastid genome	Dietary supplement	

Chlorella cells characterize for small spherical shape with no flagella structure and polysaccharide cell wall. In this section, the term "Chlorella" refers to the spherical cell phenotype of *Chlorella sensu lato*, within the class of Chlorophyceae and Trebouxiophyceae (Yang et al. 2016). *Chloroidium ellipsoideum*, *Chromochloris zofingiensis*, and *Mychonastes homosphaera* that were traditionally designated as *Chlorella* genus will be discussed here. As a suitable bioreactor to produce lipids, proteins, and carotenoids, *Chlorella* grow rapidly under autotrophic, heterotrophic, or mixotrophic conditions

(Hsieh, Su, and Chien 2012; Bai et al. 2013; Catarina Guedes et al. 2011; Smithers 2016). They have strong adaptability to high temperatures (up to 42 °C) and high CO_2 concentration (up to 40%) (Soccol, Pandey, and Larroche 2013). The application of biotechnology in *Chlorella* has unlocked the potential of heterologous gene expression. The nuclear genome sequencing of *C. variabilis* (Blanc et al. 2010), chloroplast genome of *C. vulgaris* (Wakasugi et al. 1997), and *C. sorokiniana* (Orsini et al. 2016) are available. In 1991, the transiently expression of firefly luciferase in *C. ellipsoideum* was reported (Jarvis and Brown 1991). Later, *C. sorokiniana* expressing a nitrate reductase gene was published in 1997, which was the first stable transformation of *Chlorella* (Dawson, Burlingame, and Cannons 1997). To date, employing various transformation methods, 12 *Chlorella* species have been genetically engineered. However, all these reports exclusively focused on nuclear transformation (Yang et al. 2016).

The modification of metabolic engineering in *Chlorella* has significantly increased the accumulation of lipid and carotenoid content. Several lipid production-related genes originated from yeast were expressed in *M. homosphaera*, which doubles the lipid content (Hsieh, Su, and Chien 2012). A transcription factor GmDof4 from soybean was expressed in *C. ellipsoideum* and increased the lipid content by up regulating the gene expression and enzyme activity of endogenous acetyl-coenzyme A carboxylase (Zhang et al. 2014). A modified selectable marker norflurazon-resistant phytoene desaturase has been expressed in *C. zofingiensis*, which increased the total carotenoid content and 54.1% more astaxanthin (Liu et al. 2014).

Recently, numerous studies focus on the recombinant proteins produced in *Chlorella*, for instance, the production of human growth hormone (Hawkins and Nakamura 1999), rabbit neutrophil peptide-1 (Chen et al. 2001) and flounder growth hormone (Kim et al. 2002). The yields of recombinant proteins produced by *Chlorella* range from 200 ng/L to 11.42 mg/L (Yang et al. 2016), which are far lower than the *C. reinhardtii*. Moreover, the low efficiency of transformation methods, rigid cell walls, the natural resistance of many widely used antibiotics limit the use of *Chlorella* as a GM bioreactor (Yang et al. 2016). In general, *Chlorella* is the potential GM bioreactor for large-scale production.

Dunaliella

The genus *Dunaliella* contains several halophilic unicellular microalgae, such as *D. salina* and *D. tertiolecta*, which are frequently reported from salt lakes and saltern ponds (Oren 2014). Various studies indicate that some genes in carotenoid metabolic pathway in *Dunaliella* are regulated in response to salt stress partly owing to salt-inducible *cis*-acting elements in their promoters, which provides an explanation as to why *Dunaliella* can adapt to the high salinity environment (Lao et al. 2011, 2014; Liang and Jiang 2017; Liang et al. 2017). The strong adaptability to high salinity (up to 35% salt) has made *Dunaliella* out of the contamination from other microorganisms (Oren 2014).

The commercial production of *β*-carotene and the production potential of biofuel have attracted interests and funding in these organisms (Liang, Qv, et al. 2017). Generally, the species of *Dunaliella* lacks the rigid cell wall and hence they are regarded as convenient transformation platforms. Vegetative gene lauric acid-biased thioesterase and medium-chain-length fatty acid-specific ketoacyl-ACP synthase were expressed in *D. tertiolecta*, which increased the accumulation of lauric acid and myristic acid (Lin and Lee 2017). The transformation of delta-6 desaturase from *T. pseudonana* has notably increased the eicosapntemacnioc acid (EPA) level in *D. salina* (Shi et al. 2018). A Calvin cycle enzyme from *C. reinhardtii* was transformed into *D. bardawil* that enhanced the photosynthesis capability and increased the glycerol production (Fang et al. 2012). The *β*-carotene hydroxylase cloned from *C. reinhardtii* was transferred into the genome of *D. salina* by agrobacterium-mediated genetic transformation that boosted the violaxanthin content under growth conditions or enhanced the zeaxanthin content under stress conditions (Simon et al. 2016). The transformation of a *β*-carotene ketolase (4,4′-beta-oxygenase) gene from *H. pluvialis* has accumulated astaxanthin and canthaxanthin in transformed *D. salina*, which did not naturally accumulate in wild type (Anila et al. 2016).

In 2003, the heterogenous expression of hepatitis B surface antigen in *D. salina* was the first valuable recombinant protein production in *Dunaliella* (Geng et al. 2003). Later, other recombinant proteins, including the soybean Kunitz trypsin inhibitor (Chai et al. 2013), and white spot syndrome virus subunit vaccine (Wang et al. 2014), were stably expressed in *D. salina*. The exploitation of a chloroplast-based transgene expression platform of *Dunaliella is* considerable. For example, *D. salina*, with a single, large, cup-shaped chloroplast, is believed to be a stable, high-effective exogenous expression carrier. Several recombinant enzymes, including xylanase, alpha-galactosidase, phytase, phosphate anhydrolase, and *β*-mannanase, have been successfully expressed in the chloroplast of *D. tertiolecta* in a measurable level, however, it is a rare example (Georgianna et al. 2013). The unclear genome background and lack of gene annotation have hindered the further application of *Dunaliella*, for only a few omics reports about this genus included the draft genome sequence of *D. salina* (Polle et al. 2017) and transcriptome sequencing of *D. tertiolecta* (Yao et al. 2017) and *D. parva* (Shang et al. 2016). In general, *Dunaliella* is a potential GM bioreactor that produces high value-added products in the high salinity environment.

Haematococcus

Haematococcus pluvialis is unicellular freshwater microalgae, which accumulates astaxanthin up to 4% of dry weight under abiotic stress conditions (Ambati et al. 2014). Additionally, *H. pluvialis* falls into categories of GRAS, and thus does not have the risk of viral, prion, or bacterial endotoxin contamination (Enzing et al. 2013). The increasing demand for natural astaxanthin and its stubbornly high price attract interests in applying efficient strategies such as producing astaxanthin from transgenic *H. pluvialis* (Shah

et al. 2016). An endogenous site-directed mutational phytoene desaturase (pds) was transferred in *H. pluvialis*, which exhibited 43-fold higher resistance to the bleaching herbicide norflurazon and increased carotenoid content (Steinbrenner and Sandmann 2006). Recently, the *pds* gene was first transferred into the chloroplast of *H. pluvialis*, which accumulated astaxanthin up to 167% than in wild type (Galarza et al. 2018). However, most studies mainly focus on the astaxanthin synthesis-related gene rather than the whole genome level, and hence the whole genomic data of *H. pluvialis* are still not available. Furthermore, the slow cell growth rate, cost-ineffective cultivation, and the absence of genetically improved/engineered strains and genetic transformation tools are the main challenges for the commercial pigment production of *H. pluvialis* (Shah et al. 2016).

Ochrophyta

Nannochloropsis

The *Nannochloropsis* is the most studied genus in Ochrophyta. The *Nannochloropsis* are found in both marine and fresh water resources that are small (or slightly ovate) unicellular microalgae with a cell diameter of 2–5 µm, with only chlorophyll a as their main pigment. They are appropriate bioreactors for biodiesel production benefits for their rapid growth rate and abundant saturated long-chain fatty acids (up to 47.5% of biomass) (Wang et al. 2014; Chua and Schenk 2017). *Nannochloropsis* can also be used as an animal-feed ingredient or even a high-value human protein supplement, for example, added to foods to produce highly nutritious functional foods. Additionally, *Nannochloropsis* are tolerant to most antibiotics and herbicides, which are considered the "industrial microalgae" (Jeon et al. 2017).

The *Nannochloropsis* genome encoding approximately 6562–9915 genes (Wang et al. 2014). The draft genome map (Radakovits et al. 2012) and the complete genome map (Corteggiani Carpinelli et al. 2014) of *Nannochloropsis gaditana* were published in 2011 and 2014, respectively. The genome of *N. oceanica* was also sequenced in 2012 (Vieler et al. 2012). Various genetic modification tools are available, such as homologous recombination, overexpression of target genes and CRISPR/Cas9 (Kilian et al. 2011; Vieler et al. 2012; Wang et al. 2016; Ajjawi et al. 2017; Kaye et al. 2015). Aiming at increasing lipid content in *Nannochloropsis*, several metabolic engineering strategies were applied. The heterologous expression of a transcription factor AtWRI1 from *Arabidopsis* results in a prominent increase of lipid contents in *N. salina* (Kang et al. 2017). The heterologous expression of *Saccharomyces cerevisiae* type 2 diacylglycerol acyltransferase was transient expressed and increased the lipid contents in the growth phases of *N. salina* (Beacham and Ali 2016). *Nannochloropsis* is also potential bioreactor for recombinant protein production. In 2008, the artemia treated with transgene *N. oculata* containing the fish growth hormone gene was fed to red-tilapia larvae, which largely promoted the growth of red-tilapia larvae (Chen et al. 2008). In 2009, the transgenic *N. oculata* was utilized as a whole-cell vaccine delivery system, which shown a prominent antibacteria effect (Li and Tsai 2009). In conclusion, *Nannochloropsis* are potential GM bioreactors for lipid production and aquaculture; however, further improvements are needed.

Bacillariophyta

Bacillariophyta, commonly known as diatoms, is a robust group of eukaryotic microalgae, inhabiting virtually every photic area on the surface of the earth, contributing 20–25% total surficial biomass production and approximately 40% of marine biomass production on the earth. Diatoms contain a wide range of primary or secondary metabolites such as lipids, proteins, esters, sterols, and acyl lipids (Ren et al. 2013; Gladu et al. 1991), possess great potential to become a bioreactor for biomedical, aquacultural, nutritional, and industrial applications (Matos 2017; Day, Gong, and Hu 2017; Kuppusamy et al. 2017).

Thalassiosira

The marine centric diatom *T. pseudonana* was chosen as the first eukaryotic marine phytoplankton for whole-genome sequencing because it has served as a model for diatom physiology studies (Armbrust et al. 2004). The most special structure of *T. pseudonana* is its frustule, the silicon cell wall. Relatively, the frustule, composed of nanostructures silica, has a barrier for further applications in diatoms; however, the frustule can be disrupted by sonication or detergent treatment easily (Davis et al. 2017). The formation of frustule requires silicon, which controls cell-cycle progression in diatoms. Unlike the nitrogen metabolism pathway, when silicon supplement was limited, the cell-cycle progression and growth in diatoms were blocked, whereas other aspects of cellular metabolism were not negatively affected (Claquin et al. 2002). This characteristic allows the expression of recombinant protein induced by silicon limitation in diatoms (Shrestha and Hildebrand 2017), which separates the cell growth phase and the recombinant protein production phase to achieve two-step production (Werner et al. 2011). Moreover, the GM *T. pseudonana* can serve as a drug-delivery system, whereas bioactive enzymes and GFP can localize on the frustule structure (Delalat et al. 2015; Sheppard et al. 2012; Zulu et al. 2017). The frustule can be isolated easily and shows the same biological activity (Marshall et al. 2012). Lyophilization of cultivated diatoms could eliminate the need for cold temperature storage, and diatom-expressed proteins could provide an all-in-one package of vaccine. This characteristic will significantly reduce the vaccine production cost and do a lot in developing areas of the world (Nazmi et al. 2017).

Gene expression regulation to enhance the production of fatty acids in *T. pseudonana* was reported (Cook and Hildebrand 2016; Trentacoste et al. 2013). In 2016, the genome editing technology CRISPR/Cas9 had successfully deleted a urease gene in *T. pseudonana* (Hopes et al. 2016). Overall, although only a small number of bioreactor applications are reported in *T. pseudonana*, the unique frustule

structure of *T. pseudonana* and the application of new technologies will accelerate the process of biological manufacturing in this species.

Phaeodactylum

Phaeodactylum tricornutum, another mode diatom, has shown a great diversity of its genomic origin, with a great number of genes transfer from prokaryotes (Bowler et al. 2008). These horizontal gene transfer may lead to the environmental adaption of the marine environment, the evolution of the metabolic pathways, and the perception of environmental signals (De Riso et al. 2009). The rapid development of molecular toolkits provides abundant strategies to edit the genome and express heterogenous functional gene in *P. tricornutum*. The gene silencing using antisense or inverted repeat sequences of selected target genes in *P. tricornutum* was first reported in 2009 (De Riso et al. 2009). Several genome editing technologies, such as transcription activator-like effector nucleases (Daboussi et al. 2014) and CRISPR/Cas9 (Nymark et al. 2016), have been reported in *P. tricornutum*. Circular episomes originated from bacteria have been transferred and expressed efficiently in *P. tricornutum*, which avoided the complications of random integration in the nucleus including multiple insertions, position-specific effects on expression, and potential knockout of genomic genes (Karas et al. 2015). Serviceable promoters applied in *P. tricornutum* include inducible promoter photosensitive lhcf1 (Apt, Kroth-Pancic, and Grossman 1996), nitrate-sensitive promoter nr (Chu et al. 2016), constitutive promoter ef2 (Seo et al. 2015), and histone h4 (De Riso et al. 2009). In some cases, selectable marker (antibiotic resistance or herbicide resistance gene) may not be an essential component on account of the newly selectable strategies, such as flow cytometry which separates the transferred cell which successfully expresses the reporter gene GFP (De Riso et al. 2009).

However, the biomanufacturing applications of transgenic *P. tricornutum* are still at the laboratory stage, which shows great potential in improving valuable nutrition content, such as omega-3 long-chain polyunsaturated fatty acid including docosahexaenoic acid (DHA) and EPA (Hamilton et al. 2014). The heterologous coexpression of the Δ5-elongase and acyl-CoA Δ6-desaturase from *Ostreococcus tauri* results in higher accumulation of DHA in *P. tricornutum* (Hamilton et al. 2014).

Since 2011, *P. tricornutum* is applied in recombinant therapeutic protein production, such as human antibody IgG (Hempel, Lau, et al. 2011). In some cases, the recombinant proteins can also secrete into the medium efficiently (Hempel and Maier 2012). The production of bioplastic materials, such as PHB, has been reported in *P. tricornutum* after expressing the heterogenous bacterial enzymes (Hempel, Bozarth, et al. 2011). The coexpression of yeast diacylglycerol acyltransferase and lipid droplet stabilizing oleosin protein have increased the accumulation of triacylglycerol (TAG) in *P. tricornutum* (Zulu et al. 2017). Above all, as a highly investigated diatoms *P. tricornutum* has shown a great capability to become a commercially viable bioreactor.

Fistulifera

Fistulifera solaris is a fast-growing marine pennate diatom with high fatty acid content and high tolerance to organic pollution; therefore, it is considered as a commendable source for biofuel production (Matsumoto et al. 2014). Additionally, the EPA productivity of *F. solaris* is the fastest in microalgae under photoautotrophic conditions (Tanaka et al. 2017). The oleaginous characteristic of *F. solaris* has attracted interest in its lipid metabolic pathways. The genome, chloroplast genome, and mitochondrial genome of *F. solaris* have been sequenced, and preliminarily revealed its oil accumulation mechanism (Tanaka et al. 2011, 2015; Tang and Bi 2016). The latest study focused on the allopolyploid genome of *F. solaris* reveals that the lipid biosynthetic and lipid degradative-related genes showed opposite subgenomic preferences. A feasible explanation for this might be that the high lipid characteristic of *F. solaris* originated from one of its progenitors (Nomaguchi et al. 2018).

The metabolic engineering of *F. solaris* is still in its early stages. In 2013, the transient expression system of *F. solaris* expressed GFP and neomycin phosphotransferase II were constructed (Muto et al. 2013). In 2015, combined with an endogenous chloroplast signal peptide, the nuclear-expressed GFP was transferred into the chloroplast of *F. solaris* (Sunaga et al. 2015).

Cyclotella

Cyclotella cryptica was the first chlorophyll c-containing microalga that undergoes stable nuclear transformation (Dunahay, Jarvis, and Roessler 1995), genome sequenced, and methylome sequenced (Traller et al. 2016). *Cyclotella cryptica* is widely applied in biomanufacturing, such as production of EPA, DHA (Romari, Godart, and Calleja 2012), and fucoxanthin (Guo et al. 2016). By using Si-inducible promoters, the reporter gene *eGFP* has been expressed efficiency in *C. cryptica* under silicon starvation (Shrestha and Hildebrand 2017). However, the understanding and application of *C. cryptica* are still in its early stage.

Chaetoceros

Chaetoceros gracilis, a centric diatom, has been commercially used in aquaculture because of its nutritional properties. The high concentration of lipids in *C. gracilis* has attracted interest as a feedstock for biofuels production. In 2015, by using the electroporation transformation method, exogenous genes such as selectable marker nourseothricin acetyltransferase gene (*nat*) and monomeric Azami–Green protein (mAG) were expressed in *C. gracilis* (Ifuku et al. 2015). However, the understanding and application of this genus are still in its early stage.

Haptophyta

Haptophytes are aquaculturally important; for instance, *Pavlova lutheri* is used as the feed of *Crassostrea gigas* larvae in the aquaculture industry (Ponis et al. 2008). Only a few examples of stable transformation had been reported in Haptophyta, in spite of its status as one of the three kinds of microalgae dominated the contemporary ocean. The main obstacles that have hindered the development of transgenic techniques for haptophytes thus far are their rigid calcified coccoliths and difficulties in controlling the proliferation rate (Endo et al. 2016).

Pleurochrysis

Pleurochrysis carterae is a marine species that contains a high lipid content. *Pleurochrysis carterae* is capable of calcifying and the calcified scales deposited on the surface of the cell result in the formation of a coccosphere. This sophisticated structure allows them to survive under extreme PH conditions and able to avoid predation. These advantages make them quite suitable for scalable outdoor cultivation. However, complicated coccosphere also provides a barrier for efficient gene transfer. Therefore, until 2015, the stable nuclear transformation system of *P. carterae* composing of a hygromycin B-resistance gene and a GFP were successfully constructed and reported (Endo et al. 2016).

Isochrysis

Isochrysis species are heterotroph eukaryotic algae with a high content of EPA and DHA, and widely used as aquacultural feeds. In 2014, a mutated phytoene desaturase from *H. pluvialis* was stably transferred into the genome of *Isochrysis* sp. and *I. galbana* which mediated by Agrobacterium tumefaciens and shows increased resistance to norflurazon (Prasad et al. 2014). Although *Isochrysis* is a promising source for such valuable compounds, the genetic tool for understanding and genetic modification is limited.

Rhodophyta

Rhodophyta, the red algae, is one of the oldest groups of eukaryotic algae using phycobiliproteins as accessory pigments to provide red color. The red algae are characterized by their simple eukaryotic structure with no flagella and centrioles. Several unicellular red algae lack cell walls. Almost 95% of red algae occur in the marine environment. A large proportion of red algae are macroalgae. Only a few genera are microalgae, such as *Porphyridium* and *Cyanidioschyzon*. Unicellular red algae contain various kinds of lipids such as TAG (Sumiya et al. 2015), arachidonic acid, and EPA (Klein et al. 2012). They also accumulate starch, phycoerythrin in their phycobilisomes, which can be utilized as valuable nutritional supplements. Moreover, the plasmid of unicellular red algae may serve as a superior genetic engineering host because the plasmid genome contains many protein-coding and modifiable genes (Tajima et al. 2014), which may be utilized as potential bioreactors.

Cyanidioschyzon

Cyanidioschyzon merolae is extreme unicellular red algae adapting to hot springs with high sulfuric acid content (pH = 1.5, 45 °C) (Matsuzaki et al. 2004). The genome and plastid sequencing of *C. merolae* were completed (Nozaki et al. 2007; Ohta et al. 2003). The cellular architecture of *C. merolae* is extremely simple, containing only a single chloroplast and a single mitochondrion and lacking a vacuole and cell wall, which makes it a model organism to investigate the basic architecture of photosynthetic eukaryotes. Furthermore, only 27 introns in the entire 4775 protein-coding nuclear genes discovered in the small nuclear genome (approximately 16.5 Mbp) of *C. merolae* (Nozaki et al. 2007), which simplifies the procession of genomic manipulation. Owing to these advantages, several recombinant proteins were reported to express in *C. merolae*. By using homologous recombination transformation, cyanobacterial acyl–acyl carrier protein reductase was expressed in the chloroplast of *C. merolae*, resulted in accumulation of TAG (Sumiya et al. 2015). As reported in 2017, a heterogenous chloramphenicol acetyltransferase was also successfully transformed and expressed in the chloroplast of *C. merolae* (Zienkiewicz et al. 2017). The endogenous promoter ApcC has induced the high-efficiency expression of GFP in the plasmid of *C. merolae* (Watanabe et al. 2011).

Porphyridium

Porphyridium purpureum is noncell-wall marine unicellular red microalgae, with phycoerythrin providing the red color to the cells, which was studied for a long time (Fuentes et al. 2000). Owing to the contents of sulfated polysaccharides, phycoerythrin, proteins, and polyunsaturated fatty acids, *P. purpureum* has attracted interest in biotechnology (Klein et al. 2012). The nuclear genome was sequenced in 2013 (Bhattacharya et al. 2013). Later, in 2014, the plastid genome has been sequenced, with more than 200 protein-coding genes, involving many prokaryotic enzymes, introns, and unique nonidentical structure of rRNA operons has been discovered in plastid genome of *P. purpureum* (Tajima et al. 2014). The stable chloroplast transformation of *P. purpureum* was first reported in 2002 (Lapidot et al. 2002); however, no further attempts were reported in recent years. Anyway, characterizes for its unique chloroplast, *P. purpureum* is also a hopeful bioreactor for the commercial production of recombinant proteins.

Prospective

Systematic, comprehensive, and integrated understanding of microalgae in future decades

Although numerous transcriptomics, proteomics, and other system biology studies were reported in dozens of species of eukaryotic microalgae, understanding of the metabolic pathway is still limited (Hildebrand et al. 2013). Thus, it is essential to excavate and convert omics data, construct usable genome-scale models, and provide the basis for effective metabolic engineering (Reijnders et al. 2014). It is

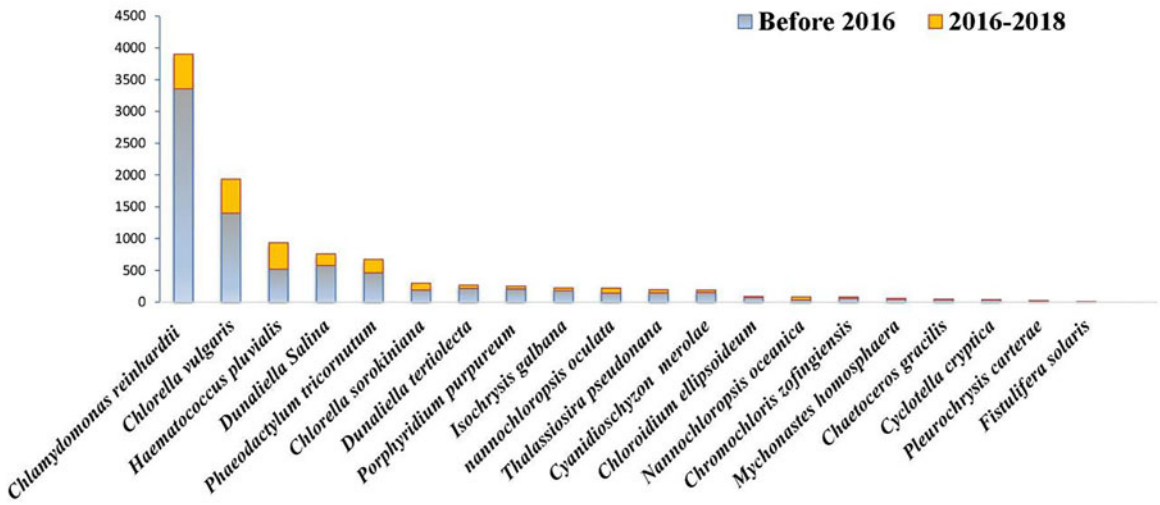

Figure 3. Research hotspot of microalgae. The yellow areas are data that published in recent 3 years. [Data source: all database of Web of Science (http://www.webofscience.com/)].

foreseeable that the application of novel technology such as next-generation sequencing and mass spectrometry will generate abundant data for the construction of the metabolic network in numerous microalgae species at an acceptable cost. Earth Biogenome Project, an ambitious ongoing project to sequence, catalog, and characterize the genomes of all eukaryotic species on earth in 10 years, may cost 4.7 billion USD (Lewin et al. 2018). This amount is less than the cost of creating the first draft human genome sequence in today's dollars. It is foreseeable that the unlocked omics data will help discover microalgae species that have various strengths. The transcriptome sequencing and differential expression analysis of *D. parva* have discovered a transcription factor gene wri1 that expresses differentially between the control and the nitrogen limitation (Shang et al. 2016). This study provides a novel way to improve lipid accumulation in *D. parva* and unlocks its biofuel production potential. The lack of genetic resource center for all microalgae species is also a challenge in the future that limits the data exchange and sharing among scientists. Hence, the construction of a comprehensive database that includes a vast number of omics data and gene annotation (functional, experimental, and locational) data could be of immense benefit to the research field.

Universal, efficient, and infinite possibilities of microalgal-based bioreactors

The development of biological toolkits has greatly promoted the transgenic expression process of microalgae. More and more recombinant genes and regulated elements are expressed in numerous microalgae hosts. The emerging genome editing technology, especially CRISPR/Cas9, has provided a convenient and specific way to modify target genomes (Bortesi and Fischer 2015). Combining the genome editing technology and developing transgenic technology, it is possible to regulate the expression of the endogenous or exogenous genes at will.

However, some restrictions may lead to low-transformation efficiencies, such as the protection of cell wall, immature transformation strategy, limited selection methods, ineffective *cis*-acting elements, and unknown gene regulation pathways (Jeon et al. 2017). For the most part, the transformation strategies, the selection strategies, and the application of *cis*-acting elements are nonuniversal in eukaryotic microalgae species because of the genetic diversity and cell-structure diversity. Therefore, it will be a challenging work to establish a comprehensive toolkit database for all microalgae species. The establishment of the database will be helpful in communicating, classifying and distributing stocks of wild type, useful mutants, available markers and, functional *cis*-acting elements. It will also provide valuable assistance to researchers toward the attainment of a comprehensive understanding of the genetic relationship among dominating factors that determine the level of heterologous protein expressed in microalgae.

Matthew effects, which summarized as the adage of "the rich get richer and the poorer get poorer," are found in every field (Merton 1968), including the funding and research on different fields of microalgae biology, especially the understanding and application of microalgae species. In accordance with the statistics on Web of Science, the data in which header contains "*C. reinhardtii*" are far more than other eukaryotic microalgae species, and the trends are still going on (Figure 3). It is safe to say that *C. reinhardtii* will ultimately develop as a common transgenic platform. However, the utilization of *C. reinhardtii* is deeply restricted, which may not be the best transgenic platform in particular segments such as biofuel production, astaxanthin biosynthesis, or sea farming. Hence, it is important to develop more transgene microalgal platforms that can be cultivated in specific conditions, accumulate various interested molecular in a cost-effective way, and realize different environmental values. For example, fast-growing GM *Chlorolla* are potential source for biofuel production (Hsieh, Su, and Chien 2012; Zhang et al. 2014), the diatom *T. pseudonana* could be modified as a target drug-delivery machine (Delalat

et al. 2015; Sheppard et al. 2012), and marine microalgae *N. oculata* could be used as whole-cell vaccine in mariculture (Li and Tsai 2009). It is foreseeable that the distinctive microalgae species and their products will compete for a market share of transgenic microalgal manufacturing market.

Conclusion

Microalgae have shown great potentials to become transgenic biomanufacturing platforms, with advantages of fast growth rate, low-cost material, and energy input, strong environmental adaptability, and advanced expression machinery. In recent decades, dozens of GM microalgae species have been well studied and shown potentials in biomedical, industrial, biofuel, and agricultural applications, whereas hundreds of microalgae species are on their way. However, even utilizing microalgal chloroplast as the platform, the yield of heterologous protein is still low. The uneconomical production cost in microalgae is still hindering the commercial process of microalgal products (except for the high value-added recombinant protein, pigment, or unsaturated fatty acid production). Innovations and breakthroughs that occur in the exploitation of microalgal resources (species, mutants, and genes), the development of genetic transformation tools and establishment of the systematic and integrated microalgal database will greatly increase the production yield of interested products, lower the production cost, and unlock more application scenarios of microalgae.

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