Adv Biochem Eng Biotechnol (2016) 153: 1–35
DOI 10.1007/10_2014_286

Published Online: 24 December 2014

Biology and Industrial Applications of *Chlorella*: Advances and Prospects

Jin Liu and Feng Chen

Abstract *Chlorella* represents a group of eukaryotic green microalgae that has been receiving increasing scientific and commercial interest. It possesses high photosynthetic ability and is capable of growing robustly under mixotrophic and heterotrophic conditions as well. *Chlorella* has long been considered as a source of protein and is now industrially produced for human food and animal feed. *Chlorella* is also rich in oil, an ideal feedstock for biofuels. The exploration of biofuel production by *Chlorella* is underway. *Chlorella* has the ability to fix carbon dioxide efficiently and to remove nutrients of nitrogen and phosphorous, making it a good candidate for greenhouse gas biomitigation and wastewater bioremediation. In addition, *Chlorella* shows potential as an alternative expression host for recombinant protein production, though challenges remain to be addressed. Currently, omics analyses of certain *Chlorella* strains are being performed, which will help to unravel the biological implications of *Chlorella* and facilitate the future exploration of industrial applications.

Keywords Biofuels · Bioremediation · CO_2 biomitigation · Carotenoids · *Chlorella* · Mass cultivation · Nutritional food

J. Liu · F. Chen (✉)
Institute for Food and Bioresource Engineering, College of Engineering, Peking University, Beijing, China
e-mail: sfchencoe@pku.edu.cn

J. Liu
e-mail: jliu@umces.edu

J. Liu
Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD, USA

J. Liu · F. Chen
Singapore-Peking University Research Centre for a Sustainable Low-Carbon Future, CREATE Tower, Singapore, Singapore

Contents

1 Introduction

Chlorella is a group of eukaryotic green microalgae with high photosynthesis ability. Through efficient photosynthesis, *Chlorella* is able to reproduce itself within several hours, requiring only sunlight, carbon dioxide, water, and a small amount of nutrients. *Chlorella* is easy to grow, has a simple life cycle and metabolic pathways similar to higher plants, and thus has long been employed as a model organism to investigate the mechanisms of photosynthesis and carbon dioxide assimilation (Calvin–Benson cycle). Due to its high protein content and richness in carotenoids, vitamins, and minerals, *Chlorella* has also long been proposed as a food substitute for humans and is now widely produced as health food in Germany, China, Japan, and several other Asian countries. Recently, because of the energy crisis and public interest in green renewable fuels, *Chlorella* has been cited as a promising candidate feedstock for biofuel production and has gained increasing scientific and industrial attention in that it grows fast, has high oil content, and outdoor mass cultures are easy to maintain. This chapter gives an overview of *Chlorella*, the industrially important microalgal genus, covering its taxonomy, growth physiology, cellular chemical composition, mass cultivation, and potential products and applications. An integrated production of biofuels and other bioproducts coupled with the treatment of greenhouse gas and wastewater is proposed, which may offset the *Chlorella*-based production cost while providing significant environmental benefits.

2 Morphology, Ultrastructure, and Taxonomy

Chlorella is a genus of unicellular and nonmobile green algae. *Chlorella vulgaris* is the type species of this genus, which was first described by M.W. Beijerinck in 1890. Commonly, *Chlorella* cells are spherical or ellipsoidal and the cell size may range from 2 to 15 μm in diameter. They are widely distributed in diverse habits such as freshwater, marine water, soil, and are even symbiotic with lichens and protozoa. *Chlorella* has no sexual life cycle and reproduces itself through asexual autospore production. When mature, autospores are simultaneously released via rupture of the mother cell wall. The number of autospores derived from a single mother cell may vary greatly from 2 to 16.

The ultrastructure of *Chlorella* has been studied extensively in past years. Under transmission electron microscopy, the visible structures within the cell include the chloroplast, nucleus, mitochondria, vacuole, starch, lipid bodies, and so on [1–3]. Generally, *Chlorella* has a cup-shaped chloroplast located peripherally in the cytoplasm. The nucleus situates near the cytoplasmic membrane, and the mitochondria are closely associated with the chloroplast. Pyrenoid, a conspicuous and easily recognizable structure, is present in most of the *Chlorella* species [2–4]. Usually, the pyrenoid is centrally located in the chloroplast and surrounded by the starch sheath [3]. In some *Chlorella* species, the pyrenoid contains many lipid-containing globules that are known as pyrenoglobuli and may function as secondary storage products [3, 4]. *Chlorella* has a thick and rigid cell wall, but the cell-wall structure may differ greatly across the species [2, 5]. When transferred to stress conditions (e.g., nitrogen starvation), the cell wall thickens and the chloroplast begins to regress to the proplastid stage with a gradual reduction in thylakoid number, accompanied by the accumulation of lipid bodies in the cytoplasm [3].

To date, there are hundreds of *Chlorella* strains reported in the literature, but the classification of *Chlorella* has been problematic due to the lack of conspicuous morphological characters. Kessler [6] proposed a sound taxonomic method for *Chlorella* based on multiple biochemical and physiological characters, that is, hydrogenase, secondary carotenoids, acid or salt tolerance, lactic acid fermentation, nitrate reduction, thiamine requirement, and the GC content of DNA. By comparing these characters, 77 *Chlorella* strains from the Collection of Algae at Göttingen (SAG, Germany) were assigned to 12 taxa and *Chlorella* was suggested to be an assembly of morphologically similar species of polyphyletic origin. Afterwards Kessler and Huss [7] examined 58 *Chlorella* strains from the Culture Collection of Algae at the University of Texas (UTEX, USA) according to the above-mentioned biochemical and physiological characters and reassigned them to 10 well-established species. The sugar composition of the cell wall (either glucosamine or glucose and mannose) has also been used for *Chlorella* classification [8, 9]. In addition, Huss et al. [10] examined the *Chlorella* genus by using a phylogenic approach based on complete 18S rRNA sequences, and considered it as a polyphyletic assemblage dispersed over two classes of Chlorophyta, that is, Chlorophyceae and Trebouxiophyceae; only four species were suggested to belong to this genus: *Chlorella vulgaris*,

Chlorella sorokiniana, *Chlorella kessleri*, and *Chlorella lobophora*. Recently, based on the sequences of 18S rRNA and ITS2 region, Krienitz et al. [11] further investigated the phylogenesis of *Chlorella* and suggested the exclusion of *Chlorella kessleri* from the *Chlorella* genus. In this chapter, we regard *Chlorella* to be the *Chlorella* sensu lato and include the data of *Chlorella* species that may have been excluded from the genus by the above-mentioned studies.

3 Growth Physiology

Chlorella is able to convert solar light energy to chemical energy through efficient photosynthesis. Similar to C_3 higher plants, photosynthesis in *Chlorella* consists of light-dependent reactions and carbon dioxide fixation. The light-dependent reactions produce high-energy molecules ATP and NADPH, which are utilized in the Calvin–Benson cycle to fix carbon dioxide. *Chlorella* performs photosynthesis efficiently at a relatively low light intensity and becomes saturated when the light intensity reaches a certain value, which may range from 80 to 400 μE $m^{-2}s^{-1}$on a per cell basis [12, 13]. Higher light intensity above the saturation value may inhibit the photosynthesis of the algae and even cause the destruction of chlorophylls (photobleaching) and cell deaths. The incident intensities of solar light can reach up to 2,500 μE $m^{-2}s^{-1}$, much higher than that required for photosynthesis saturation. Most of the sunlight energy is lost as heat and only a small portion can be converted to chemical energy by photosynthesis. Although the theoretical maximum solar energy conversion efficiency of oxygenic photosynthesis is thought to be around 8–10 % [14], the outdoor culture of *Chlorella* can only achieve a low photosynthetic efficiency (PE), for example, 2.7 % in full sunlight [15]. The yet to be enhanced PE remains a big challenge for outdoor mass cultures of algae and presents a promising direction toward the increase of biomass production.

Chlorella growth requires nutrients including carbon, nitrogen, phosphorus, sulfur, and metals. Carbon is the predominant element of *Chlorella* and carbon dioxide is the primary carbon source for photoautotrophic growth of *Chlorella*. *Chlorella* utilizes carbon dioxide principally in the undissociated form of CO_2 or H_2CO_3 [16]. The atmospheric air contains only 0.04 % CO_2, which is not sufficient to maintain rapid growth of *Chlorella* for high cell density. Therefore, a supply of air enriched with CO_2 at the concentration of 1–5 % is usually provided to *Chlorella* cultures [17, 18]. Higher levels of CO_2 may cause a decrease in pH of the medium and thus inhibit or even block the algal growth [19, 20]. Nevertheless, the high-CO_2–tolerant *Chlorella* species as reported by Papazi et al. [21] and Sakai et al. [22] can grow well in the presence of up to 40 % CO_2, although the optimal growth is obtained under lower CO_2 concentrations. Nitrogen is the second most important element in *Chlorella*. Generally, *Chlorella* is able to utilize nitrate, ammonia, and organic sources of nitrogen such as urea, glycine, and amino acids [17, 23, 24]. Both nitrate-N and urea-N cannot be directly incorporated into organic compounds by *Chlorella* and first have to be reduced to ammonia-N. Ammonia and

Table 1 Selected *Chlorella* species reported for mixotrophic and/or heterotrophic growth

Species	Organic carbon sources		References
	Mixotrophic	Heterotrophic	
C. emersonii	–	Glucose	[165]
C. minutissima	Glucose, methanol, glycerin	Glycerin	[37, 166, 167]
C. protothecoides	Glucose, glycerol, proteose peptone	Glucose, glycerol, hydrolyzed carbohydrates, molasses	[34, 35, 61, 135, 168–170]
C. pyrenoidosa	Acetate, glycerol	Glucose	[171, 172]
C. regularis	Acetate	Glucose, acetate	[173, 174]
C. saccharophila	Acetate	Glucose	[175, 176]
C. sorokinianna	Glucose	Glucose	[54, 177]
C. vulgaris	Glucose, glycerol, acetate	Glucose, acetate, glycerol	[36, 178, 179]
C. zofingiensis	Glucose, molasses	Glucose, fructose, mannose, sucrose, molasses	[30–32, 55]

urea are economically more favorable than nitrate as nitrogen sources in that the latter is more expensive per unit N. The uptake of ammonia may result in acidification of the medium, nitrate may cause alkalinization, whereas urea leads to only minor pH changes [25]. In this context, urea is the better choice of nitrogen source, avoiding a large pH shift of unbuffered medium. Different *Chlorella* species may favor different nitrogen sources for growth, for example, *Chlorella pyrenoidosa* prefers urea to nitrate or glycine for boosting biomass production and *Chlorella protothecoides* gives higher biomass yield when using nitrate rather than urea as the nitrogen source [23, 26]. Nitrogen concentration in the culture medium plays an important role in regulating algal growth and metabolism. Nitrogen deficiency/starvation retards the growth of *Chlorella*, causes the decrease of protein levels, and promotes the accumulation of lipids within cells (Illman et al. [27]; Ördög et al. [28, 29]). Phosphorus is the third essential nutrient required for normal growth of *Chlorella*. It is involved in the formation of nucleic acid and cell membrane, as well as of ATP that provides energy for cellular metabolism. The most used phosphorus source for algal cultivation is phosphate, either as $H_2PO_4^{1-}$ or HPO_4^{2-}. Sulfur is an indispensable constituent of some essential amino acids, vitamins, and sulfolipids. Usually, it is provided in the form of sulfate for algal growth. Other inorganic nutrients include K, Ca, Mg, Fe, Cu, Zn, Mn, and Mo, among others, needed in trace amounts.

In addition to photoautotrophy, *Chlorella* is able to utilize organic carbon sources alone or together with CO_2 and light for heterotrophic or mixotrophic growth (Table 1). Sugars are the most conventional organic carbon sources widely used for *Chlorella* fermentation [30–32]. Liu et al. [30] surveyed the utilization of various monosaccharides and disaccharides by *Chlorella zofingiensis*, indicating that glucose, fructose, mannose, and sucrose were able to be efficiently consumed

by *C. zofingiensis* for fast growth whereas lactose and galactose were poorly assimilated. The growth of *C. zofingiensis* was inhibited when the sugar concentration exceeded 20 g L^{-1} as indicated by the decreased specific growth rate [33]. Raw materials rich in carbohydrates such as artichoke tubers and sorghum stems have also been reported as carbon sources to feed heterotrophic *Chlorella* cells [34, 35]. Other organic carbon sources that can be used for heterotrophic *Chlorella* growth include acetate, glycerol, lactate, glutamate, and methanol (Table 1). The high concentrations of these organic carbons, however, may confer an inhibitive effect on *Chlorella* growth; for example, glycerol above 2 % was reported to inhibit the growth of *Chlorella vulgaris* [36] severely, and methanol above 1 % was lethal to *Chlorella minutissima* [37].

4 Mass Cultivation

The outdoor mass cultivation of *Chlorella* started in the late 1940s with the almost concurrent launch in the United States, Germany, and Japan [38]. Afterwards the mass cultivation of algae became one of the hottest topics in algal biotechnology leading to the development of diverse culture systems for mass culture applications [39–42]. Generally, algal cultures can be grown photoautotrophically, heterotrophically, or mixotrophically, in open or closed culture systems.

4.1 Photoautotrophy

Chlorella possesses high PE and is commonly cultured outdoors driven by the sunlight in open ponds or closed photobioreactors (PBRs). The popularly used open ponds include circular ponds and raceway ponds. Circular ponds were first built in Japan and then introduced to China and are now widely employed for mass cultivation of *Chlorella* in Asia. The capacity scale-up of circular ponds can be achieved by increasing the pond diameter, which can reach up to 50 m [40]. The system has several disadvantages including requirement of expensive structures of heavy reinforced concrete, high energy consumption for continuous stirring, inefficiency in land use, and so on. The raceway pond is another popular open culture system in the world. The raceway pond was initially used for commercial production of *Spirulina* and is now also employed for mass culture of *Chlorella*. The raceways are typically made from poured concrete, or they are simply dug into the earth and lined with a plastic liner to prevent the ground from soaking up the liquid. A raceway pond appears to be a single unit or in the form of a meandering channel assembled by individual raceways [39]. Generally, the cultures in open ponds are kept shallow at a depth of no more than 30 cm to facilitate the penetration of sunlight. Overall, the cell density achieved in both circular and raceway ponds is relatively low and commonly less than 1 g dry weight L^{-1}. In contrast, the cascade

system, a newly developed open culture system by Czech researchers, is capable of reaching a high cell density of up to 40 g L^{-1} [41, 43, 44]. This system is characterized by the thin layer of suspension (ca. 6 mm), highly turbulent flow, and high ratio of exposed surface area to total volume. Although the cascade system can achieve high volumetric cell density, its overall areal productivity is just comparable to that of open ponds [43].

Open systems cost less to construct and maintain and are thus regarded more economically favorable than closed PBRs, but they have substantial intrinsic disadvantages including low cell density and biomass productivity, rapid water loss due to evaporation, easy contamination by other microorganisms, and difficulty in managing culture temperature and efficient CO_2 delivery. The ease of contamination is a common problem encountered in outdoor cultures. *Chlorella* culture in open ponds is susceptible to other unwanted algae including diatoms and cyanobacteria, and protozoans such as rotifers, ciliates, and amoebae that feed on the algae, resulting in greatly reduced production of *Chlorella* biomass. Developing a best management practices plan for prevention and treatment of contamination may represent a feasible approach toward increased production economics of *Chlorella* by open systems.

Closed PBRs have the potential to overcome the problems of evaporation, contamination, and low biomass productivity encountered in open systems. PBRs are made of transparent materials with a large ratio of surface area to volume. Tubular PBR is one of the most popular designs. It can be arranged as straight tubes horizontally placed parallel to each other, α-type cross tubes at an angle with the horizon, or coiled tubes helically surrounding a supporting frame [39, 45–47]. Schenk et al. [47] reported a large tubular PBR system installed in Germany, which consists of 500 km of tubes arranged as fences in a north/south direction. This system has a capacity of 700 cm^3 with the annual production of *Chlorella* biomass up to 100 t. Panel PBR is another popular design arranged either vertically or inclined to the ground [48–51]. Compared with tubular PBR, the panel design needs less capital for construction and operation, offers less dark volume, accumulates less dissolved oxygen, and so on. To date, the Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University (United States) has launched a 0.5 acre of a panel PBR system for investigating the biomass production potential of *Chlorella* cultures.

4.2 Mixotrophy

Many *Chlorella* strains are able to grow robustly under mixotrophic conditions utilizing both CO_2 and organic carbons in the presence of light (Table 1). Usually, *Chlorella* grows better under mixotrophic conditions than under autotrophic conditions [32, 36, 52]. Therefore, in some cases, organic carbons are added into open ponds or PBRs to achieve mixotrophic production of *Chlorella* biomass [40, 53]. However, the *Chlorella* cultures in open ponds supplemented with organic carbons,

sugars in particular, are highly susceptible to bacterial contamination. To reduce the chance of contamination, stepwise feeding of acetate but not sugars is employed. Inasmuch as the PBR system is closed, it offers better performance than an open system to maintain a monoculture under mixotrophic conditions. There was a report of successful maintenance of *Chlorella* monoculture mixotrophically grown in outdoor PBRs supplemented with sugars [54]. But the monoculture was achieved only in small-volume PBRs (i.e., 10 L) and it got contaminated and crashed when the culture volume scaled up to 300 L. The fermenter system that was conventionally used for fermentation of nonphotosynthetic organisms such as bacteria, yeasts, and animal cells, has also been proposed to mixotrophically grow *Chlorella* by providing sugars and artificial light sources [55]. The fermenter design, however, is less efficient in light usage and mixotrophic growth, particularly when using large-volume fermenters for high density of cultures.

4.3 Heterotrophy

Heterotrophic growth of *Chlorella* in fermenters has a long history and is now gaining increasing attention [30, 32, 40, 56, 57]. Fermenters are usually placed indoors without provision of light. Almost all *Chlorella* species reported are able to grow robustly under heterotrophic conditions with the addition of organic carbon sources, sugars in particular (Table 1). These organic carbon sources, including sugars, hydrolyzed carbohydrates, acetate, and glycerol, serve as the solo carbon and energy sources to support the growth of *Chlorella*. *Chlorella protothecoides* and *Chlorella zofingiensis* are the most well-studied *Chlorella* species for fermentation. Li et al. [58] reported a scale-up of heterotrophic production of *C. protothecoides* in an 11,000-L fermenter with the cell density reaching up to 13 g L^{-1}, which is comparable to that achieved in a 5-L fermenter. The competitiveness of heterotrophic production of *Chlorella* over photoautotrophic production rests largely with high cell density and great biomass productivity, elimination of the light requirement, ease of control for monocultures, and low-cost biomass harvesting [59]. The high cell density and biomass productivity of heterotrophic *Chlorella* can be achieved by the employment of fed-batch, continuous, and cell-recycle culture strategies that are well developed for the fermentation of bacteria or yeasts [31, 32, 56, 60, 61]. With the optimized fermentation conditions, heterotrophic *Chlorella* was reported to achieve as high as 100 g cell dry weight per liter with an average biomass productivity of 13 g L^{-1} day^{-1} [61]. Although *Chlorella* fermentation has been gaining the increasing attention of industry, it is regarded economically favorable only for high-value products but not for the low-cost commodity products such as biofuels, because of the relatively high production cost.

Each culture strategy mentioned above has its own advantages and disadvantages. The choice of cultivation methods depends on *Chlorella* species/strains, locations of culture systems, production capacities, desired products, and so on. In some cases, hybrid systems (e.g., PBR-pond and fermenter-PBR) instead of a sole

culture system can be employed. In a PBR-pond system, *Chlorella* cells are cultured in PBRs for rapid growth, which serve as the inoculation seed in raceway ponds for large-scale biomass production. In a fermenter-PBR system, *Chlorella* cells are first grown heterotrophically in fermenters for accumulation of high-density biomass, which are then transferred to thin PBRs with high light for induction of desired products, for example, oils or astaxanthin. Regardless of algal strains and culture systems, the key to optimizing a production system lies in the cost balance of output to input.

5 Potential Applications

Chlorella is the sunlight-driven single-cell factory for protein, lipids, carbohydrates, pigments, vitamins, and minerals. It has long been used as health food and additives for human consumption, as well as animal feed in aquaculture. In addition, the green alga proves to be beneficial to environmental cleanup such as bioremediation of industrial flue gases and wastewater. Recently, due to the blooming of renewable energy, *Chlorella* has attracted unprecedented interest as a feedstock for biofuels, biodiesel in particular. Although the microalgal expression system does not reach a stage as mature as bacteria, yeast, mammalian cell, or plant systems, it shows substantial advantages and has been used increasingly for expression of recombinant proteins.

5.1 Chlorella as Human Food and Animal Feed

Chlorella is abundant in protein (up to 68 %) and contains all the essential amino acids. It is also rich in fatty acids, dietary fibers, carotenoids, vitamins, minerals, and other bioactive compounds, enabling the alga an attractive foodstuff of high nutritional quality. The use of *Chlorella* as nutritional food has a long history and can be traced back to food shortage periods during the World Wars. Japan and China are the main *Chlorella*-producing countries, with an annual production of over 3,500 t of biomass in 2005 [40]. Yaeyama Chlorella (Japan), Sun Chlorella (Japan), and Taiwan Chlorella are the most popular companies for *Chlorella* production. The produced *Chlorella* is commercialized mainly in the form of dried powder, tablets, or capsules for human consumption. Other forms of products include *Chlorella* growth factor (CGF), *Chlorella* tea, *Chlorella* noodles, and the like. CGF is a hot-water extract of *Chlorella* and represents a mixture of proteins, nucleic acids, polysaccharides, and a variety of minerals [40]. Administration of *Chlorella* or *Chlorella* extracts has been shown to play positive roles in health care and disease prevention, such as boosting immune functions [62, 63], preventing tumors and cancers [64, 65], enhancing hypoglycemic effects [66, 67], attenuating cognitive decline in age-dependent dementia [68], and lowering blood pressure [69].

Chlorella is also used as a natural color additive for human food because of its high levels of pigments [70, 71]. As stated by [70], the addition of *Chlorella* biomass gave cookies an attractive and innovative appearance and higher textural characteristics.

The use of *Chlorella* as animal feed is more recent. Nutritional *Chlorella* biomass can be used directly as feed or to enrich protozoa such as rotifers that serve as feed in aquaculture [72, 73]. Feeding of *Chlorella* proves beneficial to the growth and nutritional improvement of fish. The level of skin pigmentation is one of the most important quality criteria determining the market value of fish, ornamental fish in particular. They are unable to synthesize carotenoids de novo and have to feed on the carotenoid-containing organisms (e.g., microalgae) to achieve their natural pigmentation. *Chlorella* is rich in pigments and even keto-carotenoids depending on species and therefore is popularly used in aquaculture for coloring ornamental fish [74–76]. In addition, *Chlorella* shows promising applications in poultry, for example, feeding to hens to color their egg yolks [77]. Because *Chlorella* has a tough rigid cell wall, proper pretreatment is commonly necessary to facilitate the digestion and assimilation of nutrients from *Chlorella* [78, 79].

5.2 *Chlorella as a Source of Carotenoids*

Carotenoids commonly found in *Chlorella* include α- and β-carotenes, lutein, zeaxanthin, violaxanthin, and neoxanthin. Some keto-carotenoids such as cantha-xanthin and astaxanthin are also found in certain *Chlorella* species [33]. Figure 1 shows the schematic pathway of carotenoid biosynthesis in *Chlorella*. Generally, hydroxylation of the C-3 and C-3′ positions of β-carotene and α-carotene results in the formation of zeaxanthin and lutein via β-cryptoxanthin and α-cryptoxanthin, respectively. The subsequent epoxidation of zeaxanthin leads to the production of violaxanthin which is further converted to neoxanthin. In *Chlorella zofingiensis* additional keto-carotenoid biosynthetic pathways are present, involving several oxygenation and hydroxylation steps that lead to the formation of astaxanthin from β-carotene [80]. Carotenoids have important applications in food, feed, nutraceutical, and pharmaceutical industries because of their strong coloring ability, powerful antioxidative activity, and beneficial effects on human health [81]. Using *Chlorella* as producers of lutein and astaxanthin has been proposed [31, 32, 82].

Shi et al. [83] analyzed seven *Chlorella* strains for lutein production and the results suggested that *Chlorella protothecoides* CS-41 was a potential producer of lutein inasmuch as it accumulated the highest level of lutein (4.5 mg g^{-1} dry weight). Later, Shi and Chen [82] investigated the growth and lutein production of *C. protothecoides* under both heterotrophic and mixotrophic culture conditions; mixotrophic cultures produced more biomass and higher amounts of lutein than heterotrophic ones. It was revealed that *C. protothecoides* was able to utilize various nitrogen sources including nitrate, ammonia, and urea, but urea proved to be superior to the other two nitrogen sources for growth and lutein production [24].

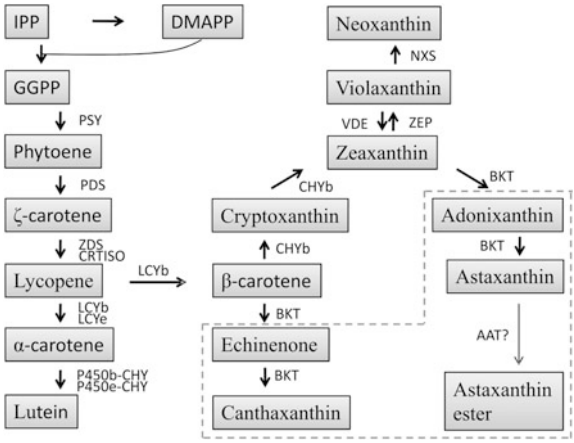

Fig. 1 Schematic diagram of carotenoid biosynthesis in *Chlorella*. In the box is the astaxanthin biosynthetic pathway which is present in *Chlorella zofingiensis*. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *CRTISO* carotene isomerase, *LCYb* lycopene β-cyclase, *LCYe* lycopene ε-cyclase, *P450b-CHY* cytochrome P450 β-hydroxylase, *P450e-CHY* cytochromeP450 ε-hydroxylase, *CHYb* β-carotene hydroxylase, *BKT* β-carotene ketolase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NXS* neoxanthin synthase, *AAT* astaxanthin acyltransferase

In order to increase lutein production, Shi and Chen [84] adopted a fed-batch culture strategy to grow *C. protothecoides* with urea as the nitrogen source, achieving a lutein yield up to 225 mg L^{-1} with the maximal productivity of 48 mg L^{-1} day^{-1}. The comparable lutein productivity was obtained when the fed-batch process was scaled up to a 30-L culture volume [84], indicating the potential of using heterotrophic *C. protothecoides* for scalable production of lutein.

C. zofingiensis is the only *Chlorella* species known to synthesize keto-carotenoids including astaxanthin. The astaxanthin production potential of *C. zofingiensis* has been studied under photoautotrophic, heterotrophic, and mixotrophic conditions [33, 55, 85, 86]. Under photoautotrophic conditions, stresses such as high light intensity and/or nitrogen starvation are needed to induce astaxanthin accumulation in *C. zofinginesis* [87]. These stresses, however, are unfavorable for algal growth and biomass production. In addition, the attenuated light absorption caused by mutual shading of cells severely affects the productivity and quality of algal biomass and products. In contrast, heterotrophic cultivation of *C. zofingiensis* feeding on an organic carbon source can boost algal growth as well as astaxanthin accumulation, eliminating the need for light and light-associated growth issues [33]. It has been reported that heterotrophic cultures of *C. zofingiensis* could achieve a comparable astaxanthin yield to *H. pluvialis* on a volumetric basis, much higher than that under photoautotrophic conditions [87, 88]. In this context, heterotrophic culture mode is regarded to be more feasible than autotrophic mode for astaxanthin production by *C. zofingiensis*. Sugars, glucose in particular, are commonly used for heterotrophic

production of astaxanthin from *C. zofingiensis*, making the production relatively expensive and thus hampering its commercial application to some extent. To reduce the production cost, waste sugars such as cane molasses have been proposed to replace glucose for astaxanthin accumulation [31, 32]. Cane molasses is a by-product of the sugar industry consisting mainly of sucrose, glucose, and fructose, and is much cheaper than glucose. It has been proved that molasses gave a comparable astaxanthin productivity to glucose [32], opening up a possibility of using industrially cheap organic carbons toward large-scale and cost-saving production of astaxanthin. Although *C. zofingiensis* can achieve high cell density under heterotrophic conditions, the intracellular astaxanthin content is relatively low compared to that under phototrophic conditions with high light and nitrogen starvation, offsetting in part the production economics of astaxanthin.

We have developed a heterotrophic–phototrophic two-stage culture strategy to grow *C. zofingiensis* to improve astaxanthin production: *C. zofingiensis* was first cultured in the presence of glucose in the dark for rapid accumulation of biomass, which was then transferred to high light conditions for induction of astaxanthin. The new culture strategy greatly enhanced the intracellular accumulation of astaxanthin to 3.5 mg g^{-1} of dry weight, which is 3.2 times the astaxanthin content obtained under heterotrophic conditions (unpublished data). The drastic increase in astaxanthin content may lie in that the alga needs more astaxanthin to cope with the light-associated adverse effects. Additionally, a record high astaxanthin productivity of 4.7 mg L^{-1} day^{-1} was achieved, which is 2.9- and 2.4-fold higher than that under heterotrophic and phototrophic conditions, respectively (unpublished data). The newly developed heterotrophic–phototrophic two-stage culture strategy combines the advantages of both heterotrophic and phototrophic modes and eliminates the possible contamination associated with mixotrophic growth, which can significantly enhance astaxanthin production and may open up the possibility of substituting the currently used heterotrophic culture method for commercial production of astaxanthin at large scale.

Another strategy to overcome the low astaxanthin content is to manipulate the carotenoid biosynthetic pathway in *C. zofingiensis* through genetic engineering. Aside from astaxanthin, *C. zofingiensis* contains substantial amounts of canthaxanthin and adonixanthin [89], suggesting that CHYb may not accept canthaxanthin as a substrate to produce astaxanthin and BKT might be insufficient to catalyze the formation of astaxanthin from adonixanthin in *C. zofingiensis*. It has been reported that CHYb from *H. pluvialis* can utilize canthaxanthin as the substrate for efficient synthesis of astaxanthin [90] and BKT from *C. reinhardtii* has a high activity of converting adonixanthin to astaxanthin [91]. Therefore, the manipulation of specific astaxanthin biosynthetic steps by introducing these two genes into *C. zofingiensis* may provide a pulling force for astaxanthin synthesis at the cost of both canthaxanthin and adonixanthin, which, when coupled with the pushing force from *PDS* overexpression, may represent a feasible strategy to increase astaxanthin content and purity further. We have developed a sophisticated transformation system for *C. zofingiensis* [80], whereby the genetic engineering for astaxanthin enhancement is possible.

5.3 *Chlorella* for CO_2 Biomitigation and Wastewater Bioremediation

Global warming caused by the increasing greenhouse gases in the atmosphere has attracted great concern by the public and scientific community. Carbon dioxide is the principal greenhouse gas mainly released through burning of fossil fuels. The biomitigation of CO_2 by autotrophs such as microalgae is a promising strategy proposed for fixing CO_2 and attenuating the greenhouse effect. *Chlorella* is one of the most commonly used genera of algae for sequestration of CO_2 due to its high growth rate and strong CO_2 fixation ability [19, 20, 92–94]. *Chlorella* is able to fix CO_2 from different sources, which can be simply classified as air, CO_2-enriched air, and industrial exhaust gases such as flue gas. The CO_2 fixation rate is associated with the CO_2 concentration provided for *Chlorella* growth (Fig. 2). When provided with atmospheric air (ca. 0.04 % CO_2), *Chlorella* grows poorly and shows low fixation abilities due to the mass transfer limitation. Therefore, to facilitate algal growth, the CO_2- enriched air at the concentration of 1–5 % is usually provided, accompanied by the increased CO_2 fixation rate. However, when the CO_2 content is over 5 %, the fixation rate drops down significantly (Fig. 2).This may be mainly attributed to the decreased medium pH at high CO_2 concentration that inhibits the growth of *Chlorella*. The flue gases contain up to 15 % CO_2 and are responsible for more than 7 % of the total world CO_2 emissions [95]. It will be environmentally and cost beneficial if *Chlorella* can directly use flue gases for CO_2 fixation, which requires strains tolerant to high CO_2 concentration as well as to relatively high temperature. High-CO_2 tolerant *Chlorella* strains have been reported, some of which were able to grow in the presence of up to 40 % CO_2 at 42 °C without significant growth inhibitory effects [22, 96].

The tolerance to both high CO_2 and high temperature enables these *Chlorella* strains to be potential cellular reactors for the biomitigation of CO_2 from flue gases. For example, *Chlorella* sp. UK001, one of the CO_2 tolerant strains, showed a CO_2 fixation rate of more than 1 g L^{-1} day^{-1} when aerated with 15 % CO_2 [97], much higher than that of regular *Chlorella* strains. In addition, the choice of culture system affects the capacity of CO_2 fixation. Doucha et al. [98] reported the CO_2 fixation of flue gas from a natural gas-fired boiler by *Chlorella* cultivated in an outdoor open cascade system with a culture area of 55 m^2. The inhibition of algal growth caused by sulphur and nitrogen oxides (SO_x, NO_x) that exists in flue gas [99] was not observed in this study. The biomass productivity and PAR utilization of *Chlorella* cultures saturated with flue gas were 19.4–22.8 g m^{-2} day^{-1} and 5.58–6.94 % respectively, comparable to that with pure CO_2. Later, Douskova and Livansky [100] investigated the CO_2 fixation rate by *Chlorella vulgaris* in aerated columns with flue gas or CO_2-enriched air. Flue gas-aerated *Chlorella* cultures exhibited an even higher CO_2 fixation rate (4.4 g L^{-1} day^{-1}) than that aerated with CO_2-enriched air (3.0 g L^{-1} day^{-1}). Recently, there have been increasing reports of using *Chlorella* wild-type or mutant strains to sequestrate CO_2 from industrial flue gas [92, 101–103].

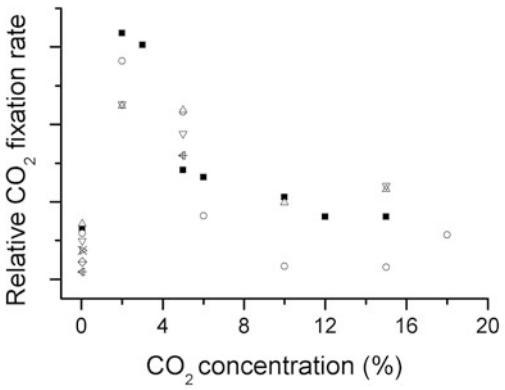

Fig. 2 Relative CO_2 fixation rate of *Chlorella* as affected by CO_2 concentration. Data are based on studies during the past five years

It is noteworthy that a high concentration of CO_2 generally leads to low efficiency of CO_2 removal; for example, the removal efficiency by *Chlorella* sp. in the presence of 15 % CO_2 is only 16 %, much lower than that in the presence of 2 % CO_2 (58 %, [19]), indicating that a major portion of CO_2 is released from the cultures. This can be overcome in part by passing the gases through sequential culture units where CO_2 is resequestrated toward less emission. For example, the fixation efficiency of flue gas CO_2 achieved by *Chlorella* cultures in sequential bioreactors reaches up to 85.6 %, greatly higher than that obtained in a single bioreactor [104]. As suggested by Doucha et al. [98], the daily fixed CO_2 per m^2 is around 34.4 g, with the simultaneous production of 20 g algal biomass. Assuming the culture season lasts for 150 days, one hectare of *Chlorella* cultures is able to sequestrate 21 t CO_2 and produce around 12 t biomass per year.

Bioremediation using microalgae has long been recognized as an environmentally sound approach for wastewater treatment. *Chlorella* is one of the microalgal genera widely used in the biological treatment of wastewater and has proven abilities of removing nutrients (N and P), organic contaminants, and heavy metals (Table 2). Generally, pretreatments of wastewater such as settling, activated sludge process, or dilution are needed before supplying to *Chlorella* for biological treatment [105–108]. Wang et al. [109] intensively investigated the growth of *Chlorella* sp. on wastewater sampled from four different points of the treatment process flow of a municipal wastewater treatment plant for the removal of nitrogen, phosphorus, and chemical oxygen demand (COD) as well as metal ions. The four types of wastewater are classified as wastewater before primary settling (#1), wastewater after primary settling (#2), wastewater after activated sludge tank (#3), and concentrate (#4). The growth rate (0.95 day^{-1}) and COD removal rate (83.0 %) of *Chlorella* for wastewater #4 were much higher than those for wastewater #1 and #2 and the removal rates of nitrogen (78.3 %) and phosphorus (85.6 %) were comparable; *Chlorella* in wastewater #3 showed the lowest growth rate and removal rates of nitrogen, phosphorus, and COD. The efficient removal of nutrients and organic contaminants by *Chlorella* from wastewater was also demonstrated in other studies [107, 110–114]. These results suggest that growing *Chlorella* on wastewater

Table 2 Selected *Chlorella* species reported for wastewater treatment

Chlorella species	Wastewater characteristics	Culture type	Compounds removed[a]	References
C. ellipsoidea	Secondary effluents of wastewater	Suspended	N, P	[114]
C. protothecoides	Urban wastewater	Suspended	N, P	[180]
C. pyrenoidosa	Domestic wastewater; piggery wastewater; palm oil mill wastewater	Suspended	N, P, COD, BOD	[110]
C. pyrenoidosa	Settled and activated sewage filtrates	Suspended	N, P	[107]
C. sp	Municipal wastewater concentrate	Suspended	N, P, COD	[112]
C. sp	Postchlorinated municipal wastewater	Suspended	N, P	[126]
C. sp	Municipal wastewater	Suspended	N, P, COD, metals	[181]
C. sorokiniana	Cattle manure anaerobic digester effluent	Suspended	N, P	[182]
C. vulgaris	Hydroponic wastewater	Suspended	N, P	[183]
C. vulgaris	Textile wastewater	Suspended and immobilized	dyes	[184]
C. vulgaris	Diluted pig slurry	Suspended	N, P, BOD	[105]
C. vulgaris	Piggery wastewater effluent	Suspended	N, P	[111]
C. vulgaris	Textile wastewater	Suspended	N, P, COD	[185]
C. vulgaris	Wastewater	Immobilized	N, P, metals	[115]
C. vulgaris	Artificial wastewater	Immobilized	N, P	[116]
C. vulgaris	Secondary municipal wastewater	Immobilized	N, P	[106]
C. vulgaris	Suspended solids—removed wastewater from steel-making facility	Suspended	N	[108]
C. vulgaris	Primarily treated effluents of domestic wastewater	Immobilized	N, P	[117]
Mixed cultures of *Chlorella* and other species	Wood-based pulp and paper industry wastewater	Suspended	COD, AOX, color	[113]

[a] *COD* chemical oxygen demand, *BOD* biochemical oxygen demand, *AOX* absorbable organic xenobiotics

seems to be a feasible strategy to reduce the released amounts of organic and inorganic nutrients into natural waters, thus preventing the eutrophication problem.

Although the suspended *Chlorella* cultures exhibited their potential use in secondary or tertiary steps for wastewater treatment, one of the major and practical limitations is separation of the algal biomass from the treated wastewater, which requires capital-intensive steps such as flocculation, flotation, filtration, or centrifugation. In this context, using immobilized *Chlorella* cells for wastewater treatment is advantageous in that no harvest step is required [106, 115–117]. The removal efficiency of nutrients by immobilized *Chlorella* is influenced by culture density, pH, and immobilizing matrix [115, 118]. In some cases, *Chlorella* was cocultured with other microalgae for wastewater treatment [113]. In addition to removal of nutrients and organic compounds, *Chlorella* is also able to be used for the biodegradation of toxics [119] and removal of metal ions [109, 115].

The biosorption of *Chlorella* for removing metals from wastewater involves adsorption of metal ions onto the cell surface and binding to the intracellular molecules such as cytoplasmic ligands, phytochelatins, and metallothioneins (for details see the review by Mehta and Gaur [120]). *Chlorella* has been reported to remove a wide range of metals, including Al, Ca, Cd, Cu, Fe, Mg, Mn, Ni, Ur, and Zn [109, 121–125]. Considering the acceptable growth and lipid production of *Chlorella* on wastewater, the integration of biofuel production with wastewater treatment is proposed [109, 111, 112, 114, 126].

5.4 Chlorella as Feedstock for Biofuels

Petroleum fuels are recognized to be unsustainable due to their depleting supplies and release of greenhouse gas [127]. Renewable biofuels are promising alternatives to petroleum, among which biodiesel has attracted unprecedentedly increasing attention in recent years [128]. Compared with traditional fuels, the carbon-neutral biodiesel releases fewer gaseous pollutants and is considered environmentally beneficial. Currently, biodiesel is produced mainly from vegetable oils, animal fats, and waste cooking oils. Plant-oil–derived biodiesel, however, cannot realistically meet the existing need for transport fuels as immense arable lands have to be occupied in cultivating oil crops, causing food–fuels conflicts [39].

Microalgae have been considered as the promising alternative feedstock for biodiesel production because of their rapid growth and high oil content [39, 128, 129]. Furthermore, unlike oil crops, microalgae can be easily cultured in outdoor ponds or bioreactors, making them superior to oil crops in biomass production. *Chlorella* represents a group of green microalgal species that grow fast and are easily able to achieve and maintain mass cultures. There have been many research studies focusing on using *Chlorella* for biodiesel production in the past years, as shown in Table 3. Under optimal conditions *Chlorella* usually synthesizes a relatively low content of lipids (25 % on average) which can be greatly increased up to 66 % by stress

Table 3 Selected *Chlorella* species reported for biodiesel production research

Chlorella species	Lipid content (%)	Culture conditions[a]	References
C. ellipsoidea	32	P	[186]
C. ellipsoidea	15–43	P	[114]
C. minutissima	23.2	M	[166]
C. minutissima	23	H	[187]
C. vulgaris	18.2	P	[183]
C. vulgaris	14.7	P	[188]
C. vulgaris	20–42	P	[189]
C. vulgaris	30.6	H	[178]
C. vulgaris	21–38	P, M, H	[36]
C. vulgaris	11.8–56.6	P	[190]
C. vulgaris	20–23	P	[191]
C. vulgaris	19.2	P	[192]
C. vulgaris	16.5–58.9	P, M, H	[193]
C. vulgaris	25–52	P	[194]
C. vulgaris	35	P	[195]
C. protothecoides	45.2	H	[34]
C. protothecoides	48.1–63.8	H	[56]
C. protothecoides	57	M	[196]
C. protothecoides	49.9	H	[133]
C. protothecoides	42	H	[134]
C. pyrenoidosa	20	P	[197]
C. pyrenoidosa	30	P	[198]
C. pyrenoidosa	10–17.3	M	[171]
C.saccharophila	13–18	P	[199]
C. sorokiniana	19.3	P	[192]
C. sorokiniana	10.9–37	H	[200]
C. sorokiniana	14.5–38.7	H	[201]
C. sp.	11–42	P	[20]
C. sp.	32.6–66.1	P	[17]
C. sp.	35.1[b]	P	[202]
C. sp.	38	P	[203]
C. sp.	28.8	P	[204]
C. sp.	18.7	P	[192]
C. sp.	18.7	P	[181]
C. sp.	25–32	P	[205]
C. zofingiensis	52	H	[30]
C. zofingiensis	25.8–51.1	P, H	[206]
C. zofingiensis	28–45	H	[31]
C. zofingiensis	27.3–54.5	P	[207]

[a] *P* phototrophic, *M* mixotrophic, *H* heterotrophic
[b] Total fatty acids

conditions such as nitrogen starvation [17]. The stress conditions also favor the accumulation of neutral lipids, in particular, triacylglycerols (TAGs) that deposit in the cytosol. TAGs are considered to be superior to polar lipids (phospholipids and glycolipids) for biodiesel production. In this context, *Chlorella* can be first cultured under favorable conditions to maximize biomass production and then exposed to stress conditions to stimulate the accumulation of lipids including TAGs. The important properties of biodiesel such as cetane number, viscosity, cold flow, and oxidative stability are largely determined by the composition and structure of fatty acyl esters which in turn are determined by the characteristics of fatty acids of biodiesel feedstock, for example, carbon chain length and unsaturation degree [130]. The synthesized fatty acids in *Chlorella* are mainly of medium length, ranging from 16 to 18 carbons, despite the great variation in fatty acid composition (Table 4). Generally, saturated fatty esters possess a high cetane number and superior oxidative stability whereas unsaturated, especially polyunsaturated, fatty esters have improved low-temperature properties [131]. It is suggested that the modification of fatty esters, for example, enhancing the proportion of oleic acid (C18:1) ester, can provide a compromise solution between oxidative stability and low-temperature properties and therefore promote the quality of biodiesel [132]. In this regard, *C. protothecoides*, which has the highest proportion of oleic acid (71.6 %), may be better than other *Chlorella* species as biodiesel feedstock [34]. The properties of *C. protothecoides* derived biodiesel were assessed and most of them proved to comply with the limits established by American Society for Testing and Materials (ASTM), including density, viscosity, flash point, cold filter plugging point, and acid value.

Aside from employing photoautotrophic *Chlorella* cells, fermentation by feeding organic carbons has also been proposed by some research groups to enrich heterotrophic biomass as biodiesel feedstock. *C. protothecoides* is the most studied species heterotrophically grown for biodiesel production (Table 5). It could achieve very high cell densities, biomass productivities, and lipid productivities. Glucose is the most widely used carbon source to feed *Chlorella* for boosting biomass production and lipid accumulation. To reduce the production cost, alternative low-cost carbon sources such as hydrolysates of crude carbohydrates, waste molasses, or glycerol were used [34, 35, 61, 133–136]. Molasses proved to be a promising alternative to feed *C. protothecoides* for biodiesel, with the biomass yield, biomass productivity, and lipid productivity being 97.1 g L^{-1}, 12.8, and 7.3 g L^{-1} day^{-1}, respectively [61]. However, the conversion ratio of sugar to biomass in these heterotrophic cultures was restricted to 0.5, which means 2 t of sugar are required for producing 1 t of biomass and 1 t of CO_2 is released during this process. In this regard, fermentation is neither economically viable nor environmentally friendly for the production of biomass for biodiesel as compared with photoautotrophy.

Transesterification is needed to convert *Chlorella* oil to biodiesel. It is a chemical conversion process involving reacting triglycerides catalytically with a short-chain alcohol (typically methanol or ethanol) to form fatty acyl esters (biodiesel) and glycerol. This reaction occurs stepwise with the first conversion of triglycerides to diglycerides and then to monoglycerides and finally to glycerol. Considering the reaction is reversible, a large excess of alcohol is used in industrial

Table 4 Fatty acid profiles of selected *Chlorella* species

Chlorella species	C14:0	C15:0	C16:0	C16:1	C16:2	C16:3	C17:0	C18:0	C18:1	C18:2	C18:3	C20 or above	References
C. ellipsoidea	2		26						4	40	23	5	[186]
C. minutissima	0.7		10.6	2.1				0.6	36.8	43.2	2.6	3.5	[187]
C. sorokiniana	0.1		21.4	3.9	1.5			1.2	32.8	30.3	5.7		[200]
C. sorokiniana			29.2	7.7	4.2	1		3	29.1	24.1	1.6		[201]
C. vulgaris			19.2				4.2	14.6	12.7	3.8	21.1	13.8	[183]
C. vulgaris			63					9	3	11	13		[188]
C. vulgaris	1		32	26			1	5	14	28		3	[178]
C. vulgaris			24	2.1				1.3	24.8	47.8			[208]
C. protothecoides			14.3	1				2.7	71.6	9.7			[34]
C. protothecoides	1.5		9	1.4				4.5	66.1	11.9	1		[133]
C. protothecoides			15.8	1.1				5.2	54.5	21.8	2.3		[134]
C. pyrenoidosa			17.3		7	9.3		1.2	3.3	18.5	41.8		[197]
C. pyrenoidosa			25.2	2	5.3	5.7		1.5	20.9	16.1	22.2		[198]
C. pyrenoidosa	1.1		33.8	4.4	4.3			2.4	30.6	7	13.4		[171]
C. sp.			19.1	1				3.1	25.9	6.8	44.2		[202]
C. sp.	2.8	5.7	39.9				27.8	3.4	10.5	6.2	4		[203]
C. sp.			20.6	6.6	10.4	6	3.4	2.4	12.5	27.2	10.2		[181]
C. sp.	3.3	6.4	49.5					10.1	28.5	1.3			[205]
C. zofingiensis			22.6	2	7.4	2		2.1	35.7	18.5	7.8		[30]
C. zofingiensis			22.6	2.4	7.6	1.9		2.7	33.9	12.3	7.7		[31]

Table 5 Growth and lipid production of *C. protothecoides* feeding on various organic carbon sources

Cell density ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ Day^{-1}$)	Lipid productivity ($g\ L^{-1}\ Day^{-1}$)	Organic carbons	Culture conditions[a]	References
16.5	3.6	1.60	Hydrolysate of Jerusalem artichoke tuber	B, flask, 1 L	[34]
10.8	1.7	0.95	Glucose	B, flask, 1 L	[56]
30	3.3	1.9	Glucose	FB, fermentor, 2 L	
–	–	12.3	Glucose	C, fermentor, 2 L	
6	1.2	0.59	Hydrolysate of sweet sorghum juice	B, flask, 500 mL	[35]
15.5	2.0	0.93	Glucose	FB, fermentor, 5 L	[58]
12.8	1.7	0.81	Glucose	FB, fermentor, 750 L	
14.2	1.7	0.73	Glucose	FB, fermentor, 11,000 L	
14	3.2	1.9	Glycerol	B, flask	[135]
13.1	1.46	0.85	Glucose	B, flask, 250 mL	[26]
14.2	2.2	1.2	Glucose	B, fermentor, 5 L	[216]
51.2	6.6	3.3	Glucose	FB, fermentor, 5 L	[57]
15.5	2.0	1.1	Glucose	FB, fermentor, 5 L	[136]
3.7	0.7	0.36	Corn powder hydrolysate	B, flask, 500 mL	
17.9	3.6	1.45	Hydrolyzed molasses	B, flask, 500 mL	[61]
97.1	12.8	7.3	Hydrolyzed molasses	FB, fermentor, 5 L	
46	6.28	2.06	Glucose	FB, fermentor, 7 L	[60]
64	8.7	4.3	Glycerol	FB, SC, fermentor, 2 L	[133]
9.1 17.2	1.01 1.72	0.42 0.35	Hydrolyzed whey Permeate	B, flask, 250 ml FB, fermentor, 5 L	[134]
7.32	0.88	0.45	Digested chicken manure filtrate	FB, fermentor, 7 L	[209]

[a] *B* batch, *FB* fed-batch, *C* continuous, *SC* semicontinuous

processes to ensure the direction of fatty acid esters. Methanol is the preferred alcohol for industrial use because of its low cost, although other alcohols including ethanol, propanol, and butanol are also commonly used. In addition to heat, a catalyst is needed to facilitate the transesterification. The transesterification of triglycerides can be catalyzed by acids, alkalis, or enzymes [137–139]. Currently, alkali (sodium hydroxide and potassium hydroxide) is the preferred type of catalyst for industrial production of biodiesel.

Chlorella is also capable of producing other biofuels such as hydrogen, ethanol, methane, and biocrude [140–144]. The biohydrogen production from microalgae has long been recognized. This process involves hydrogenase, an enzyme highly sensitive to O_2. During photosynthesis, hydrogen evolution is transient due to the strong inhibition of hydrogenase by photosynthetically evolved O_2 [145]. The sustainable hydrogen production from *Chlorella*, therefore, requires the maintenance of an anaerobic environment, which can be achieved through the inhibition of O_2 evolution by sulfur deficiency [146]. It has been estimated that green algae could theoretically produce a maximum of 20 g hydrogen per m^2 per day [145]. In addition to lipids, *Chlorella* biomass contains a substantial amount of carbohydrates (starch and cellulose) that can be used for ethanol production through technologies such as saccharification and fermentation [142, 147, 148]. *Chlorella* is also capable of producing ethanol through self-fermentation of intracellular starch under dark and anaerobic conditions, although the conversion efficiency is relatively low [142]. Anaerobic digestion of biomass results in the production of methane that can be used as a heat source or for electricity generation. The raw *Chlorella* biomass can be directly subjected to digestion, thus avoiding the biomass-harvest and oil-extraction processes used in algal biodiesel production and significantly bringing down the production cost and energy debt. But the digestion efficiency as stated by Ras et al. [143] was restricted to 50 %, indicating the need of proper pretreatments of the raw biomass for complete digestion. Hydrothermal liquefaction, which requires moderate temperatures as compared to the processes of pyrolysis and gasification, is commonly used for biocrude production from *Chlorella* biomass [141, 149].

The production of biofuels from microalgae is still far from commercialization. There are significant technical challenges yet to be addressed. A promising strategy is to integrate the production of biodiesel with other biofuels and high-value products, as well as the applications of treating flue gas and wastewater (Fig. 3).

5.5 Chlorella as Cell Factories for Recombinant Proteins

Chlorella offers substantial advantages as a promising alternative to currently well-established expression systems of bacteria, yeast, and mammalian cells. In contrast to bacteria, *Chlorella* belongs to eukaryotic organisms and therefore can perform correct posttranscriptional and posttranslational modifications essential for the production of functional eukaryotic proteins. Being easy, rapid, and inexpensive to

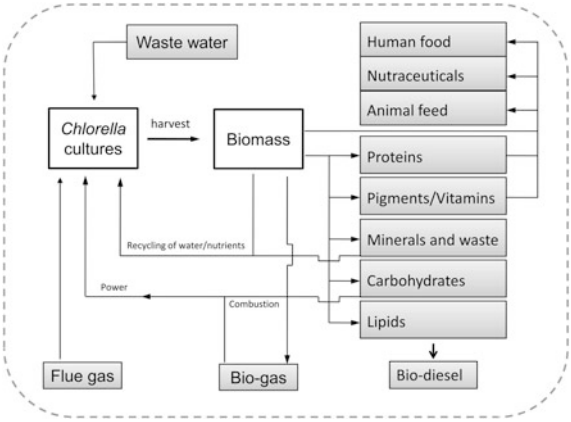

Fig. 3 Schematic illustration of integrated production of biofuels and other products coupled with flue gas and wastewater treatments

grow and maintain on a large scale both indoors and outdoors, *Chlorella* is more cost effective as compared with the capital-intensive and time-consuming expression systems of mammalian cells, large farm animals, and higher plants. In addition, *Chlorella* has long been approved and used as health food for human consumption, suggesting the biological safety of engineered proteins from *Chlorella*. These characteristics enable *Chlorella* to be potentially useful as a bioreactor for synthesizing engineered products of interest such as enzymes, vaccines, monoclonal antibodies, and growth factors.

Nevertheless, the utilization of *Chlorella* for heterologous expression is hampered by the lack of a sophisticated genetic toolbox. The genetic engineering of *Chlorella* has achieved some success during the past 20 years (Table 6). The first report was conducted by Jarvis and Brown [150] who introduced a firefly luciferase gene into *Chlorella ellipsoidea* for heterologous expression; the luciferase activity, however, was not stable and disappeared after a few days. Later, Maruyama et al. [151] performed the transient expression of the β-glucoronidase (GUS) gene in transgenic *Chlorella saccharophila*. The transient expression of GUS was also reported in transgenic *Chlorella ellipsoidea* [152, 153] and *Chlorella* sp. [154]. These results suggested the feasibility of using *Chlorella* as an expression system for recombinant protein production. A dominant selectable marker is essential for easy and reliable selection of target *Chlorella* transformants. The frequently used selectable markers include bleomycin binding protein (Ble, resistant to phleomycin), chloramphenicol acetyltransferase (Cat, resistant to chloramphenicol), hygromycin B phosphotransferase (Hpt, resistant to hygromycin), and neomycin phosphotransferase II (NptII, resistant to kanamycin and geneticin), derived from either *Escherichia coli* or *Streptoalloteichus rimosus* (Table 6). *Chlorella*, however, is not so sensitive to these antibiotics and may need high concentrations to inhibit its growth completely [155]. In addition, *Chlorella* harboring these bacterial genes may be subject to biological safety problems when used as food or pharmaceuticals for human beings. Therefore, endogenous genes are advantageous as selectable markers for *Chlorella* transformation. Dawson et al. [156] reported the use of the

Table 6 Summary of *Chlorella* species for genetic modification

Chlorell species	Reporter or marker[b]	Reporter or marker source	Transformation method	Reference
C. ellipsoidea[a]	Gus	*Escherichia coli*	Bombardment	[152]
C. ellipsoidea	NptII	*Escherichia coli*	Electroporation	[159]
C. ellipsoidea	Nitrate reductase	*Chlorella ellipsoidea*	Electroporation	[157]
C. ellipsoidea[a]	Luciferase	Firefly	PEG	[150]
C. ellipsoidea	Ble	*Streptoalloteichus rimosus*	PEG	[162]
C. ellipsoidea[a]	Gus	*Escherichia coli*	Electroporation	[153]
C. ellipsoidea	Ble	*Streptoalloteichus rimosus*	PEG	[210]
C. kessleri	NptII	*Escherichia coli*	Bombardment	[211]
C. saccharophila[a]	Gus	*Escherichia coli*	Electroporation	[151]
C. sorokiniana	Nitrate reductase	*Chlorella vulgaris*	Bombardment	[156]
C. sp	Hpt	*Escherichia coli*	PEG	[163]
C. sp[a]	Gus	*Escherichia coli*	Electroporation	[154]
C. vulgaris	Hpt-gus	*Escherichia coli*	Electroporation	[212]
C. vulgaris, C.sorokiniana[a]	NptII	*Escherichia coli*	PEG	[155]
C. vulgaris	Cat	*Escherichia coli*	Electroporation	[213]
C. vulgaris	Hpt	*Escherichia coli*	Agrobacterium	[214]
C. vulgaris	NptII	*Escherichia coli*	Bombardment	[215]
C. zofingiensis	Pds	*Chlorella zofingiensis*	Bombardment, electroporation	[80]

[a] Transient expression or unstable transformant

[b] *Ble* bleomycin binding protein, *Cat* chloramphenicol acetyltransferase, *Hpt-gus* fusion of hygromycin B phosphotransferase and β-glucoronidase, *Gus* β-glucoronidase, *NptII* neomycin phosphotransferase II, *Pds* phytoene desaturase (modified with norflurazon resistance)

nitrate reductase (NR) gene from *Chlorella vulgaris* to rescue the NR-deficient *Chlorella sorokiniana* mutants, resulting in stable transformants. Wang et al. [157] also reported the stable transformation of *Chlorella ellipsoidea* using an endogenous nitrate reductase gene to complement the NR-deficient mutant. The limitation of these trials lies in that a NR-deficient mutant is required as the expression host. Very recently, a phytoene desaturase (PDS) from a norflurazon-resistant mutant of *Chlorella zofingiensis* was proposed as a dominant selectable marker for stable transformation of *Chlorella* [80, 89, 158]. The *Chlorella* mutant harbored a point mutation on its PDS, which showed great resistance to norflurazon as well as significantly enhanced desaturation activity. It has been demonstrated that as low as 0.25 μg mL^{-1} of norflurazon is sufficient to select target transformants of *Chlorella zofingiensis* transformed with the mutated *PDS* gene. The transformants retained the

norflurazon resistance after more than 100 times of subculture without selection of norflurazon, suggesting the great potential of using the endogenously derived *PDS* gene mutant as an effective and efficient selectable marker for the stable transformation of *Chlorella zofingiensis* as well as other *Chlorella* species.

Genetic manipulation of *Chlorella* for heterologous gene expression is still in its infant stage. The introduced foreign genes are subject to instability if not lack of expression because of various possible reasons including unstable nuclear integration, position effects, inefficient transcription from heterologous promoters, inaccurate RNA processing, and codon usage bias. Nevertheless, there have been several reports of using *Chlorella* for expressing commercially interesting proteins such as rabbit neutrophil peptide-1 [159–161], human growth hormone [155], flounder growth hormone [162], mercuric reductase from *Bacillus megaterium* [163], and trypsin-modulating oostatic factor from mosquitos [164]. Challenges for getting stable transgene integration and expression in *Chlorella* transformants will require further investigations to develop sophisticated genetic toolboxes with a powerful expression cassette for this small-sized and cell-wall–tough species.

6 Conclusions and Future Prospects

Chlorella is a sunlight-driven single-cell bioreactor that converts carbon dioxide to potential proteins, lipids, carbohydrates, and high-value biocompounds. It is among the most well-studied genera of microalgae for mass cultures. The abundance in protein and other nutritional elements, biological safety, and feasibility of growing and maintaining outdoors on a large scale enable *Chlorella* to be a good source of health food for human consumption. *Chlorella* is also considered as a potential source of microalgal oils for biofuel production. There are still substantial challenges involved in the biofuel production pipeline such as mass cultivation, harvest and drying, biomass disruption for oil extraction and conversion, and recycling of water and nutrients, making the *Chlorella*-derived biofuels currently capital intensive and far from economically viable as compared with fossil fuels. The integrated production of biofuels and other potential high-value products, coupled with the environmentally beneficial applications such as flue gas biomitigation and wastewater treatment represent a promising direction toward a cost-effective production process of *Chlorella*, which requires close collaboration between biologists and engineers. As interest in *Chlorella* increases, comprehensive analyses of certain potential strains are underway via genomic, transcriptomic, proteomic, lipidomic, and metabolomic approaches. The availability of those omics data will uncover the biological implications and facilitate the tailored manipulation of *Chlorella* for broader industrial applications.

Acknowledgments This study was partially supported by a grant from the 985 Project of Peking University, by the State Oceanic Administration of China, and by the National Research Foundation of Singapore.

References

1. Bar E, Rise M, Vishkautsan M, Arad S (1995) Pigments and structural changes in *Chlorella zofingiensis* upon light and nitrogen stress. J Plant Physiol 146:527–534
2. Gerken H, Donohoe B, Knoshaug E (2013) Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta 237:239–253
3. Goncalves E, Johnson J, Rathinasabapathi B (2013) Conversion of membrane lipid acyl groups to triacylglycerol and formation of lipid bodies upon nitrogen starvation in biofuel green algae *Chlorella* UTEX29. Planta 238:895–906
4. Ikeda T, Takeda H (1995) Species-specific differences of pyrenoids in *Chlorella* (Chlorophyta). J Phycol 31:813–818
5. Yamada T, Sakaguchi K (1982) Comparative studies on *Chlorella* cell walls: induction of protoplast formation. Arch Microbiol 132:10–13
6. Kessler E (1976) Comparative physiology, biochemistry, and the taxonomy of *Chlorella* (Chlorophyceae). Plant Syst Evol 125:129–138
7. Kessler E, Huss VAR (1992) Comparative physiology and biochemistry and taxonomic assignment of the *Chlorella* (Chlorophyceae) strains of the culture collection of the University of Texas at Austin. J Phycol 28:550–553
8. Takeda H (1991) Sugar composition of the cell wall and the taxonomy of *chlorella* (Chlorophyceae). J Phycol 27:224–232
9. Takeda H (1993) Chemical-composition of cell-walls as a taxonomical marker. J Plant Res 106:195–200
10. Huss VAR, Frank C, Hartmann EC, Hirmer M, Kloboucek A, Seidel BM, Wenzeler P, Kessler E (1999) Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). J Phycol 35:587–598
11. Krienitz L, Hegewald EH, Hepperle D, Huss VAR, Rohr T, Wolf M (2004) Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia 43:529–542
12. Lloyd NDH, Canvin DT, Culver DA (1977) Photosynthesis and photorespiration in algae. Plant Physiol 59:936–940
13. Winokur M (1948) Photosynthesis relationships of *Chlorella* species. Am J Bot 35:207–214
14. Melis A (2009) Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. Plant Sci 177:272–280
15. Wassink EC, Kok B, van Oorschot P (1964) The efficiency of light-energy conversion in *Chlorella* cultures as compared with higher plants. In: Burlew JS (ed) Algal culture: from laboratory to pilot plant. Carnegie Institution of Washington Publication, Washinton, D.C., pp 55–62
16. Shelp BJ, Canvin DT (1980) Utilization of exogenous inorganic carbon species in photosynthesis by *Chlorella pyrenoidosa*. Plant Physiol 65:774–779
17. Hsieh C-H, Wu W-T (2009) Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour Technol 100:3921–3926
18. Ong S-C, Kao C-Y, Chiu S-Y, Tsai M-T, Lin C-S (2010) Characterization of the thermal-tolerant mutants of *Chlorella* sp. with high growth rate and application in outdoor photobioreactor cultivation. Bioresour Technol 101:2880–2883
19. Chiu SY, Kao CY, Chen CH, Kuan TC, Ong SC, Lin CS (2008) Reduction of CO_2 by a high-density culture of *Chlorella* sp in a semicontinuous photobioreactor. Bioresour Technol 99:3389–3396
20. Fulke AB, Mudliar SN, Yadav R, Shekh A, Srinivasan N, Ramanan R, Krishnamurthi K, Devi SS, Chakrabarti T (2010) Bio-mitigation of CO_2, calcite formation and simultaneous biodiesel precursors production using *Chlorella* sp. Bioresour Technol 101:8473–8476
21. Papazi A, Makridis P, Divanach P, Kotzabasis K (2008) Bioenergetic changes in the microalgal photosynthetic apparatus by extremely high CO_2 concentrations induce an intense biomass production. Physiol Plantarum 132:338–349

22. Sakai N, Sakamoto Y, Kishimoto N, Chihara M, Karube I (1995) *Chlorella* strains from hot springs tolerant to high temperature and high CO_2. Energy Convers Manage 36:693–696
23. Davis EA, Dedrick J, French CS, Milner HW, Myers J, Smith JHC, Spoehr HA (1964) Laboratory experiments on *Chlorella* culture at the Carnegie Institution of Washington department of plant biology. In: Burlew JS (ed) Algal culture: from laboratory to pilot plant. Carnegie Institution of Washington Publication, Washington, D.C., pp 105–153
24. Shi X-M, Zhang X-W, Chen F (2000) Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. Enzyme Microb Tech 27:312–318
25. Goldman JC, Brewer PG (1980) Effect of nitrogen source and growth rate on phytoplankton-mediated changes in alkalinity. Limnol Oceanogr 25:352–357
26. Shen Y, Yuan W, Pei Z, Mao E (2010) Heterotrophic culture of *Chlorella protothecoides* in various nitrogen sources for lipid production. Appl Biochem Biotechnol 160:1674–1684
27. Illman AM, Scragg AH, Shales SW (2000) Increase in *Chlorella strains* calorific values when grown in low nitrogen medium. Enzyme Microb Tech 27:631–635
28. Ördög V, Stirk W, Bálint P, van Staden J, Lovász C (2012) Changes in lipid, protein and pigment concentrations in nitrogen-stressed *Chlorella minutissima* cultures. J Appl Phycol 24:907–914
29. Ramazanov A, Ramazanov Z (2006) Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, and high protein and polyunsaturated fatty acid content. Phycol Res 54:255–259
30. Liu J, Huang J, Fan KW, Jiang Y, Zhong Y, Sun Z, Chen F (2010) Production potential of *Chlorella zofingienesis* as a feedstock for biodiesel. Bioresour Technol 101:8658–8663
31. Liu J, Huang J, Jiang Y, Chen F (2012) Molasses-based growth and production of oil and astaxanthin by *Chlorella zofingiensis*. Bioresour Technol 107:393–398
32. Liu J, Sun Z, Zhong Y, Gerken H, Huang J, Chen F (2013) Utilization of cane molasses towards cost-saving astaxanthin production by a *Chlorella zofingiensis* mutant. J Appl Phycol 25:1447–1456
33. Ip PF, Chen F (2005) Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. Process Biochem 40:733–738
34. Cheng Y, Zhou W, Gao C, Lan K, Gao Y, Wu Q (2009) Biodiesel production from Jerusalem artichoke (*Helianthus Tuberosus L.*) tuber by heterotrophic microalgae *Chlorella protothecoides*. J Chem Technol Biotechnol 84:777–781
35. Gao C, Zhai Y, Ding Y, Wu Q (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl Energy 87:756–761
36. Liang Y, Sarkany N, Cui Y (2009) Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett 31:1043–1049
37. Kotzabasis K, Hatziathanasiou A, Bengoa-Ruigomez MV, Kentouri M, Divanach P (1999) Methanol as alternative carbon source for quicker efficient production of the microalgae *Chlorella minutissima*: role of the concentration and frequence of administration. J Biotechnol 70:357–362
38. Burlew JS (1964) Algal culture: from laboratory to pilot plant, 4th edn. Carnegie Institution of Washington Publication, Washington, D.C.
39. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306
40. Lin L-P (2005) *Chlorella*: its ecology, structure, cultivation, bioprocess and application. Yi Hsien Publishing, Taipei, Taiwan
41. Masojidek J, Kopecky J, Giannelli L, Torzillo G (2011) Productivity correlated to photobiochemical performance of *Chlorella* mass cultures grown outdoors in thin-layer cascades. J Ind Microbiol Biotechnol 38:307–317
42. Moheimani N (2013) Inorganic carbon and pH effect on growth and lipid productivity of Tetraselmis suecica and *Chlorella* sp (Chlorophyta) grown outdoors in bag photobioreactors. J Appl Phycol 25:387–398

43. Doucha J, Lívanský K (2006) Productivity, CO_2/O_2 exchange and hydraulics in outdoor open high density microalgal (*Chlorella* sp.) photobioreactors operated in a Middle and Southern European climate. J Appl Phycol 18:811–826
44. Douskova I, Doucha J, Livansky K, Machat J, Novak P, Umysova D, Zachleder V, Vitova M (2009) Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. Appl Microbiol Biotechnol 82:179–185
45. Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70:313–321
46. Lee Y-K, Ding S-Y, Low C-S, Chang Y-C, Forday W, Chew P-C (1995) Design and performance of an α-type tubular photobioreactor for mass cultivation of microalgae. J Appl Phycol 7:47–51
47. Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. BioEnergy Res 1:20–43
48. Hu Q, Kurano N, Kawachi M, Iwasaki I, Miyachi S (1998) Ultrahigh-cell-density culture of a marine green alga *Chlorococcum littorale* in a flat-plate photobioreactor. Appl Microbiol Biotechnol 49:655–662
49. Liu J, Sommerfeld M, Hu Q (2013) Screening and characterization of *Isochrysis* strains and optimization of culture conditions for docosahexaenoic acid production. Appl Microbiol Biotechnol 97:4785–4798
50. Zemke P, Sommerfeld M, Hu Q (2013) Assessment of key biological and engineering design parameters for production of *Chlorella zofingiensis* (Chlorophyceae) in outdoor photobioreactors. Appl Microbiol Biotechnol 97:5645–5655
51. Zhang CW, Richmond A (2003) Sustainable, high-yielding outdoor mass cultures of *Chaetoceros muelleri* var. subsalsum and *Isochrysis galban* in vertical plate reactors. Mar Biotechnol 5:302–310
52. Lee Y-K (2001) Microalgal mass culture systems and methods: their limitation and potential. J Appl Phycol 13:307–315
53. Lee Y-K (1997) Commercial production of microalgae in the Asia-Pacific rim. J Appl Phycol 9:403–411
54. Lee Y-K, Ding S-Y, Hoe C-H, Low C-S (1996) Mixotrophic growth of *Chlorella sorokiniana* in outdoor enclosed photobioreactor. J Appl Phycol 8:163–169
55. Ip PF, Wong KH, Chen F (2004) Enhanced production of astaxanthin by the green microalga *Chlorella zofingiensis* in mixotrophic culture. Process Biochem 39:1761–1766
56. De la Hoz Siegler H, Ben-Zvi A, Burrell RE, McCaffrey WC (2011) The dynamics of heterotrophic algal cultures. Bioresour Technol 102:5764–5774
57. Xiong W, Li X, Xiang J, Wu Q (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Appl Microbiol Biotechnol 78:29–36
58. Li X, Xu H, Wu Q (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnol Bioeng 98:764–771
59. Chen F (1996) High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol 14:421–426
60. Chen Y-H, Walker TH (2012) Fed-batch fermentation and supercritical fluid extraction of heterotrophic microalgal *Chlorella protothecoides* lipids. Bioresour Technol 114:512–517
61. Yan D, Lu Y, Chen Y-F, Wu Q (2011) Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. Bioresour Technol 102:6487–6493
62. Cheng FC, Lin A, Feng JJ, Mizoguchi T, Takekoshi H, Kubota H, Kato Y, Naoki Y (2004) Effects of *chlorella* on activities of protein tyrosine phosphatases, matrix metalloproteinases, caspases, cytokine release, B and T cell proliferations, and phorbol ester receptor binding. J Med Food 7:146–152

63. Suárez ER, Kralovec JA, Noseda MD, Ewart HS, Barrow CJ, Lumsden MD, Grindley TB (2005) Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of *Chlorella pyrenoidosa*. Carbohydrate Res 340:1489–1498
64. Morimoto T, Nagatsu A, Murakami N, Sakakibara J, Tokuda H, Nishino H, Iwashima A (1995) Anti-tumour-promoting glyceroglycolipids from the green alga, *Chlorella vulgaris*. Phytochem 40:1433–1437
65. Sheng J, Yu F, Xin Z, Zhao L, Zhu X, Hu Q (2007) Preparation, identification and their antitumor activities in vitro of polysaccharides from *Chlorella pyrenoidosa*. Food Chem 105:533–539
66. Cherng J-Y, Shih M-F (2005) Potential hypoglycemic effects of *Chlorella* in streptozotocin-induced diabetic mice. Life Sci 77:980–990
67. Cherng J-Y, Shih M-F (2006) Improving glycogenesis in Streptozocin (STZ) diabetic mice after administration of green algae *Chlorella*. Life Sci 78:1181–1186
68. Nakashima Y, Ohsawa I, Konishi F, Hasegawa T, Kumamoto S, Suzuki Y, Ohta S (2009) Preventive effects of *Chlorella* on cognitive decline in age-dependent dementia model mice. Neurosci Lett 464:193–198
69. Merchant RE, Andre CA (2001) A review of recent clinical trials of the nutritional supplement *Chlorella pyrenoidosa* in the treatment of fibromyalgia, hypertension, and ulcerative colitis. Altern Ther Health Med 7:79–91
70. Gouveia L, Batista AP, Miranda A, Empis J, Raymundo A (2007) *Chlorella vulgaris* biomass used as colouring source in traditional butter cookies. Innov Food Sci Emerg 8:433–436
71. Gouveia L, Raymundo A, Batista A, Sousa I, Empis J (2006) *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. Euro Food Res Technol 222:362–367
72. Hirayama K, Nakamura K (1976) Fundamental studies on the physiology of rotifers in mass culture—V. Dry *Chlorella* powder as a food for rotifers. Aquaculture 8:301–307
73. Işik O, Sarihan E, Kuşvuran E, Gül Ö, Erbatur O (1999) Comparison of the fatty acid composition of the freshwater fish larvae *Tilapia zillii*, the rotifer *Brachionus calyciflorus*, and the microalgae *Scenedesmus abundans*, *Monoraphidium minitum* and *Chlorella vulgaris* in the algae-rotifer-fish larvae food chains. Aquaculture 174:299–311
74. Gouveia L, Choubert G, Pereira N, Santinha J, Empis J, Gomes E (2002) Pigmentation of gilthead seabream, *Sparus aurata* (L. 1875), using *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga. Aquac Res 33:987–993
75. Gouveia L, Gomes E, Empis J (1996) Potential use of a microalga (*Chlorella vulgaris*) in the pigmentation of rainbow trout (*Oncorhynchus mykiss*) muscle. Z Lebensm Unters For A 202:75–79
76. Gouveia L, Rema P, Pereira O, Empis J (2003) Colouring ornamental fish (*Cyprinus carpio* and *Carassius auratus*) with microalgal biomass. Aquacult Nutr 9:123–129
77. Gouveia L, Veloso V, Reis A, Fernandes H, Novais J, Empis J (1996) *Chlorella vulgaris* used to colour egg yolk. J Sci Food Agri 70:167–172
78. Janczyk P, Franke H, Souffrant WB (2007) Nutritional value of *Chlorella vulgaris*: effects of ultrasonication and electroporation on digestibility in rats. Anim Feed Sci Tech 132:163–169
79. Komaki H, Yamashita M, Niwa Y, Tanaka Y, Kamiya N, Ando Y, Furuse M (1998) The effect of processing of *Chlorella vulgaris*: K-5 on in vitro and in vivo digestibility in rats. Anim Feed Sci Tech 70:363–366
80. Liu J, Sun Z, Gerken H, Huang J, Jiang Y, Chen F (2014) Genetic engineering of the green alga *Chlorella zofingiensis*: a modified norflurazon-resistant phytoene desaturase gene as a dominant selectable marker. Appl Microbiol Biotechnol 98:5069–5079
81. Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265
82. Shi XM, Chen F (1999) Production and rapid extraction of lutein and the other lipid-soluble pigments from *Chlorella protothecoides* grown under heterotrophic and mixotrophic conditions. Food Nahrung 43:109–113

83. Shi X-M, Chen F, Yuan J-P, Chen H (1997) Heterotrophic production of lutein by selected *Chlorella* strains. J Appl Phycol 9:445–450
84. Shi X-M, Chen F (2002) High-yield production of lutein by the green microalga *Chlorella protothecoides* in heterotrophic fed-batch culture. Biotechnol Prog 18:723–727
85. Del Campo JA, Moreno J, Rodriguez H, Vargas MA, Rivas J, Guerrero MG (2000) Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). J Biotechnol 76:51–59
86. Del Campo JA, Rodriguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2004) Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 64:848–854
87. Orosa M, Valero JF, Herrero C, Abalde J (2001) Comparison of the accumulation of astaxanthin in *Haematococcus pluvialis* and other green microalgae under N-starvation and high light conditions. Biotechnol Lett 23:1079–1085
88. Sun N, Wang Y, Li Y-T, Huang J-C, Chen F (2008) Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). Process Biochem 43:1288–1292
89. Liu J, Zhong Y, Sun Z, Huang J, Sandmann G, Chen F (2010) One amino acid substitution in phytoene desaturase makes *Chlorella zofingiensis* resistant to norflurazon and enhances the biosynthesis of astaxanthin. Planta 232:61–67
90. Linden H (1999) Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. Biochim Biophys Acta 1446:203–212
91. Huang J, Zhong Y, Sandmann G, Liu J, Chen F (2012) Cloning and selection of carotenoid ketolase genes for the engineering of high-yield astaxanthin in plants. Planta 236:691–699
92. Borkenstein CG, Knoblechner J, Fruhwirth H, Schagerl M (2011) Cultivation of *Chlorella emersonii* with flue gas derived from a cement plant. J Appl Phycol 23:131–135
93. de Morais M, Costa J (2007) Carbon dioxide fixation by *Chlorella kessleri, C. vulgaris*, *Scenedesmus obliquus*, *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors. Biotechnol Lett 29:1349–1352
94. Yewalkar S, Li B, Posarac D, Duff S (2011) Potential for CO_2 fixation by *Chlorella pyrenoidosa* grown in oil sands tailings water. Energy Fuel 25:1900–1905
95. Wang B, Li Y, Wu N, Lan C (2008) CO_2 bio-mitigation using microalgae. Appl Microbiol Biotechnol 79:707–718
96. Michiki H (1995) Biological CO2 fixation and utilization project. Energy Convers Manage 36:701–705
97. Murakami M, Ikenouchi M (1997) The biological CO_2 fixation and utilization project by rite (2) - Screening and breeding of microalgae with high capability in fixing CO_2. Energy Convers Manage 38(Supplement):S493–S497
98. Doucha J, Straka F, Lívanský K (2005) Utilization of flue gas for cultivation of microalgae *Chlorella* sp. in an outdoor open thin-layer photobioreactor. J Appl Phycol 17:403–412
99. Negoro M, Shioji N, Miyamoto K, Micira Y (1991) Growth of microalgae in high CO_2 gas and effects of SO_x and NO_x. Appl Biochem Biotechnol 28–29:877–886
100. Doucha J, Lívanský K (2009) Outdoor open thin-layer microalgal photobioreactor: potential productivity. J Appl Phycol 21:111–117
101. Cheng J, Huang Y, Feng J, Sun J, Zhou J, Cen K (2013) Mutate *Chlorella* sp. by nuclear irradiation to fix high concentrations of CO_2. Bioresour Technol 136:496–501
102. He L, Subramanian VR, Tang YJ (2012) Experimental analysis and model-based optimization of microalgae growth in photo-bioreactors using flue gas. Biomass Bioenerg 41:131–138
103. Kumar K, Banerjee D, Das D (2014) Carbon dioxide sequestration from industrial flue gas by *Chlorella sorokiniana*. Bioresour Technol 152:225–233
104. Cheng J, Huang Y, Feng J, Sun J, Zhou J, Cen K (2013) Improving CO_2 fixation efficiency by optimizing *Chlorella* PY-ZU1 culture conditions in sequential bioreactors. Bioresour Technol 144:321–327

105. Fallowfield HJ, Garrett MK (1985) The photosynthetic treatment of pig slurry in temperate climatic conditions: a pilot-plant study. Agr Wastes 12:111–136
106. Shi J, Podola B, Melkonian M (2007) Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J Appl Phycol 19:417–423
107. Tam NFY, Wong YS (1989) Wastewater nutrient removal by *Chlorella pyrenoidosa* and *Scenedesmus* sp. Environ Pollut 58:19–34
108. Yun Y-S, Lee SB, Park JM, Lee C-I, Yang J-W (1997) Carbon dioxide fixation by algal cultivation using wastewater nutrients. J Chem Technol Biotechnol 69:451–455
109. Wang L, Min M, Li YC, Chen P, Chen YF, Liu YH, Wang YK, Ruan R (2010) Cultivation of breen algae *Chlorella* sp in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 162:1174–1186
110. Aziz MA, Ng WJ (1992) Feasibility of wastewater treatment using the activated-algae process. Bioresour Technol 40:205–208
111. Ji M-K, Kim H-C, Sapireddy V, Yun H-S, Abou-Shanab RI, Choi J, Lee W, Timmes T, Jeon Inamuddin B-H (2013) Simultaneous nutrient removal and lipid production from pretreated piggery wastewater by *Chlorella vulgaris* YSW-04. Appl Microbiol Biotechnol 97:2701–2710
112. Li Y, Chen Y-F, Chen P, Min M, Zhou W, Martinez B, Zhu J, Ruan R (2011) Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour Technol 102:5138–5144
113. Tarlan E, Dilek FB, Yetis U (2002) Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresour Technol 84:1–5
114. Yang J, Rasa E, Tantayotai P, Scow KM, Yuan H, Hristova KR (2011) Mathematical model of *Chlorella minutissima* UTEX2341 growth and lipid production under photoheterotrophic fermentation conditions. Bioresour Technol 102:3077–3082
115. Mallick N, Rai LC (1994) Removal of inorganic ions from wastewaters by immobilized microalgae. W J Microbiol Biotechnol 10:439–443
116. Megharaj M, Pearson HW, Venkateswarlu K (1992) Removal of nitrogen and phosphorus by immobilized cells of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. Enzyme Microb Technol 14:656–658
117. Tam NFY, Lau PS, Wong YS (1994) Wastewater inorganic N and P removal by immobilized *Chlorella vulgaris*. Water Sci Technol 30:369–374
118. Mallick N, Rai LC (1993) Influence of culture density, pH, organic acids and divalent cations on the removal of nutrients and metals by immobilized *Anabaena doliolum* and *Chlorella vulgaris*. W J Microbiol Biotechnol 9:196–201
119. Jin J, Yang LH, Chan SMN, Luan TG, Li Y, Tam NFY (2011) Effect of nutrients on the biodegradation of tributyltin (TBT) by alginate immobilized microalga, *Chlorella vulgaris*, in natural river water. J Hazard Mater 185:1582–1586
120. Mehta SK, Gaur JP (2005) Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit Rev Biotechnol 25:113–152
121. Chong AMY, Wong YS, Tam NFY (2000) Performance of different microalgal species in removing nickel and zinc from industrial wastewater. Chemosphere 41:251–257
122. Kalin M, Wheeler WN, Meinrath G (2004) The removal of uranium from mining waste water using algal/microbial biomass. J Environ Radioact 78:151–177
123. Lau PS, Lee HY, Tsang CCK, Tam NFY, Wong YS (1999) Effect of metal interference, pH and temperature on Cu and Ni biosorption by *Chlorella vulgaris* and *Chlorella miniata*. Environ Technol 20:953–961
124. Mehta SK, Gaur JP (2001) Removal of Ni and Cu from single and binary metalsolutions by free and immobilized *Chlorella vulgaris*. Euro J Protistol 37:261–271
125. Sandau E, Sandau P, Pulz O (1996) Heavy metal sorption by microalgae. Acta Biotechnol 16:227–235
126. Mutanda T, Karthikeyan S, Bux F (2011) The utilization of post-chlorinated municipal domestic wastewater for biomass and lipid production by *Chlorella* spp. under batch conditions. Appl Biochem Biotechnol 164:1126–1138

127. Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26:126–131
128. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–639
129. Liu J, Huang J, Chen F (2011a) Microalgae as feedstocks for biodiesel production. Biodiesel—Feedstocks and processing technologies. InTech, Available from http://www.intechopen.com/articles/show/title/microalgae-as-feedstocks-for-biodiesel-production
130. Knothe G (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070
131. Knothe G (2008) "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuel 22:1358–1364
132. Knothe G (2009) Improving biodiesel fuel properties by modifying fatty ester composition. Energy Environ Sci 2:759–766
133. Cerón-García MC, Macías-Sánchez MD, Sánchez-Mirón A, García-Camacho F, Molina-Grima E (2013) A process for biodiesel production involving the heterotrophic fermentation of *Chlorella protothecoides* with glycerol as the carbon source. Appl Energy 103:341–349
134. Espinosa-Gonzalez I, Parashar A, Bressler DC (2014) Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production. Bioresour Technol 155:170–176
135. O'Grady J, Morgan JA (2011) Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Bioprocess Biosyst Eng 34:121–125
136. Xu H, Miao X, Wu Q (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J Biotechnol 126:499–507
137. Fukuda H, Kondo A, Noda H (2001) Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 92:405–416
138. Gerpen JV (2005) Biodiesel processing and production. Fuel Process Technol 86:1097–1107
139. Ranganathan SV, Narasimhan SL, Muthukumar K (2008) An overview of enzymatic production of biodiesel. Bioresour Technol 99:3975–3981
140. Bala Amutha K, Murugesan AG (2011) Biological hydrogen production by the algal biomass *Chlorella vulgaris* MSU 01 strain isolated from pond sediment. Bioresour Technol 102:194–199
141. Duan P, Jin B, Xu Y, Yang Y, Bai X, Wang F, Zhang L, Miao J (2013) Thermo-chemical conversion of *Chlorella pyrenoidosa* to liquid biofuels. Bioresour Technol 133:197–205
142. Hirano A, Ueda R, Hirayama S, Ogushi Y (1997) CO_2 fixation and ethanol production with microalgal photosynthesis and intracellular anaerobic fermentation. Energy 22:137–142
143. Ras M, Lardon L, Bruno S, Bernet N, Steyer J-P (2011) Experimental study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris*. Bioresour Technol 102:200–206
144. Wang HY, Fan XL, Zhang YT, Yang DW, Guo RB (2011) Sustained photo-hydrogen production by *Chlorella pyrenoidosa* without sulfur depletion. Biotechnol Lett 33:1345–1350
145. Melis A, Happe T (2001) Hydrogen production. Green algae as a source of energy. Plant Physiol 127:740–748
146. Rashid N, Lee K, Mahmood Q (2011) Bio-hydrogen production by *Chlorella vulgaris* under diverse photoperiods. Bioresour Technol 102:2101–2104
147. Cheng Y-S, Zheng Y, Labavitch JM, Vander Gheynst JS (2013) Virus infection of *Chlorella variabilis* and enzymatic saccharification of algal biomass for bioethanol production. Bioresour Technol 137:326–331
148. Zhou N, Zhang Y, Wu X, Gong X, Wang Q (2011) Hydrolysis of *Chlorella biomass* for fermentable sugars in the presence of HCl and $MgCl_2$. Bioresour Technol 102:10158–10161
149. Zhang J, Chen W-T, Zhang P, Luo Z, Zhang Y (2013) Hydrothermal liquefaction of *Chlorella pyrenoidosa* in sub- and supercritical ethanol with heterogeneous catalysts. Bioresour Technol 133:389–397

150. Jarvis EE, Brown LM (1991) Transient expression of firefly luciferase in protoplasts of the green alga *Chlorella ellipsoidea*. Curr Genet 19:317–321
151. Maruyama M, Horakova I, Honda H, Xing XH, Shiragami N, Unno H (1994) Introduction of foreign DNA into *Chlorella saccharophila* by electroporation. Biotechnol Tech 8:821–826
152. Chen Y, Li W, Bai Q, Sun Y (1998) Study on transient expression of gus gene in *Chlorelia ellipsoidea* (Chlorophyta) by using biolistic particle delivery system. Chin J Oceanol Limn 16:47–49
153. Wang P, Sun YR, Li X, Zhang LM, Li WB, Wang YQ (2004) Rapid isolation and functional analysis of promoter sequences of the nitrate reductase gene from *Chlorella ellipsoidea*. J Appl Phycol 16:11–16
154. Wang C, Wang Y, Su Q, Gao X (2007) Transient expression of the GUS gene in a unicellular marine green alga *Chlorella* sp. MACC/C95, via electroporation. Biotechnol Bioprocess Eng 12:180–183
155. Hawkins RL, Nakamura M (1999) Expression of human growth hormone by the eukaryotic alga, *Chlorella*. Curr Microbiol 38:335–341
156. Dawson HN, Burlingame R, Cannons AC (1997) Stable transformation of *Chlorella*: rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. Curr Microbiol 35:356–362
157. Wang YQ, Chen Y, Zhang XY, Wang P, Geng DG, Zhao SM, Zhang LM, Sun YR (2005) Isolation and characterization of a nitrate reductase deficient mutant of *Chlorella ellipsoidea* (Chlorophyta). J Appl Phycol 17:281–286
158. Huang JC, Liu J, Li YT, Chen F (2008) Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 44:684–690
159. Chen Y, Wang YQ, Sun YR, Zhang LM, Li WB (2001) Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. Curr Genet 39:365–370
160. Han XM, Li YG, Sun XZ, Wei XD, Sun YR, Wang YQ (2005) Studies on the heterotrophic cultivation of transgenic *Chlorella* containing the rabbit defensin gene. Process Biochem 40:3055–3060
161. Wang Y, Chen Y, Bai Q, Zhao S, Li W, Sun Y (2001) Using transgenic *Chlorella ellipsoidea* as bio-reactor to produce rabbit defensin. High Technol Lett 9:1–5
162. Kim DH, Kim YT, Cho JJ, Bae JH, Hur SB, Hwang I, Choi TJ (2002) Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*. Mar Biotechnol 4:63–73
163. Huang CC, Chen MW, Hsieh JL, Lin WH, Chen PC, Chien LF (2006) Expression of mercuric reductase from *Bacillus megaterium* MB1 in eukaryotic microalga *Chlorella* sp DT: an approach for mercury phytoremediation. Appl Microbiol Biotechnol 72:197–205
164. Borovsky D (2003) Trypsin-modulating oostatic factor: a potential new larvicide for mosquito control. J Exp Biol 206:3869–3875
165. Avery SV, Codd GA, Gadd GM (1992) Replacement of cellular potassium by caesium in *Chlorella emersonii*: differential sensitivity of photoautotrophic and chemoheterotrophic growth. J Gen Microbiol 138:69–76
166. Oh SH, Kwon MC, Choi WY, Seo YC, Kim GB, Kang DH, Lee SY, Lee HY (2010) Long-term outdoor cultivation by perfusing spent medium for biodiesel production from *Chlorella minutissima*. J Biosci Bioeng 110:194–200
167. Yang J, Li X, Hu H, Zhang X, Yu Y, Chen Y (2011) Growth and lipid accumulation properties of a freshwater microalga, *Chlorella ellipsoidea* YJ1, in domestic secondary effluents. Appl Energy 88:3295–3299
168. Li T, Zheng Y, Yu L, Chen S (2013) High productivity cultivation of a heat-resistant microalga *Chlorella sorokiniana* for biofuel production. Bioresour Technol 131:60–67
169. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 97:841–846

170. Ruiz NJ, García MDCC, Mirón AS, Haftalaui EHB, Camacho FG, Grima EM (2009) Lipids accumulation in *Chlorella protothecoides* through mixotrophic and heterotrophic cultures for biodiesel production. New Biotechnol 25:S266–S266
171. Rai MP, Nigam S, Sharma R (2013) Response of growth and fatty acid compositions of *Chlorella pyrenoidosa* under mixotrophic cultivation with acetate and glycerol for bioenergy application. Biomass Bioenergy 58:251–257
172. Running J, Huss R, Olson P (1994) Heterotrophic production of ascorbic acid by microalgae. J Appl Phycol 6:99–104
173. Endo H, Sansawa H, Nakajima K (1977) Studies on *Chlorella regularis*, heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetate concentration. Plant Cell Physiol 18:199–205
174. Sansawa H, Endo H (2004) Production of intracellular phytochemicals in *Chlorella* under heterotrophic conditions. J Biosci Bioenergy 98:437–444
175. Herrera-Valencia V, Contreras-Pool P, López-Adrián S, Peraza-Echeverría S, Barahona-Pérez L (2011) The green microalga *Chlorella saccharophila* as a suitable source of oil for biodiesel production. Curr Microbiol 63:151–157
176. Tan C, Johns M (1991) Fatty acid production by heterotrophic *Chlorella saccharophila*. Hydrobiologia 215:13–19
177. Chen F, Johns M (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*. J Appl Phycol 3:203–209
178. Heredia-Arroyo T, Wei W, Ruan R, Hu B (2011) Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials. Biomass Bioenergy 35:2245–2253
179. Zhang K, Sun B, She X, Zhao F, Cao Y, Ren D, Lu J (2014) Lipid production and composition of fatty acids in Chlorella vulgaris cultured using different methods: photoautotrophic, heterotrophic, and pure and mixed conditions. Ann Microbiol 64:1239–1246
180. Ramos Tercero EA, Sforza E, Morandini M, Bertucco A (2014) Cultivation of *Chlorella protothecoides* with urban wastewater in continuous photobioreactor: Biomass productivity and nutrient removal. Appl Biochem Biotechnol 172:1470–1485
181. Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan RR (2010) Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol 101:2623–2628
182. Kobayashi N, Noel EA, Barnes A, Watson A, Rosenberg JN, Erickson G, Oyler GA (2013) Characterization of three *Chlorella sorokiniana* strains in anaerobic digested effluent from cattle manure. Bioresour Technol 150:377–386
183. Bertoldi FC, Sant'Anna E, da Costa Braga MV, Oliveira JLB (2006) Lipids, fatty acids composition and carotenoids of *Chlorella vulgaris* cultivated in hydroponic wastewater. Grasas Y Aceites 57:270–274
184. Chu W-L, See Y-C, Phang S-M (2009) Use of immobilised *Chlorella vulgaris* for the removal of colour from textile dyes. J Appl Phycol 21:641–648
185. Lim S-L, Chu W-L, Phang S-M (2010) Use of *Chlorella vulgaris* for bioremediation of textile wastewater. Bioresour Technol 101:7314–7322
186. Abou-Shanab RAI, Hwang J-H, Cho Y, Min B, Jeon B-H (2011) Characterization of microalgal species isolated from fresh water bodies as a potential source for biodiesel production. Appl Energy 88:3300–3306
187. Cao J, Yuan H, Li B, Yang J (2014) Significance evaluation of the effects of environmental factors on the lipid accumulation of *Chlorella minutissima* UTEX 2341 under low-nutrition heterotrophic condition. Bioresour Technol 152:177–184
188. Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process 48:1146–1151
189. Feng P, Deng Z, Hu Z, Fan L (2011) Lipid accumulation and growth of *Chlorella zofingiensis* in flat plate photobioreactors outdoors. Bioresour Technol 102:10577–10584

190. Liu Z-Y, Wang G-C, Zhou B-C (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour Technol 99:4717–4722
191. Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A, Legrand J (2011) Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. Bioresour Technol 102:150–158
192. Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009) Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioenergy 102:100–112
193. Scarsella M, Parisi MP, D'Urso A, De Filippis P, Opoka J, Bravi M (2009) Achievements and perspectives in hetero- and mixotrophic culturing of microalgae. In: Pierucci S (ed) Icheap-9: 9th International Conference on Chemical and Process Engineering, Pts 1-3, vol 17., Chemical Engineering TransactionsAidic Servizi Srl, Milano, pp 1065–1070
194. Widjaja A, Chien C-C, Ju Y-H (2009) Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem E 40:13–20
195. Yeh KL, Chang JS, Chen WM (2010) Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga *Chlorella vulgaris* ESP-31. Eng Life Sci 10:201–208
196. Wang Y, Rischer H, Eriksen NT, Wiebe MG (2013) Mixotrophic continuous flow cultivation of *Chlorella protothecoides* for lipids. Bioresour Technol 144:608–614
197. D'Oca MGM, Viêgas CV, Lemões JS, Miyasaki EK, Morón-Villarreyes JA, Primel EG, Abreu PC (2011) Production of FAMEs from several microalgal lipidic extracts and direct transesterification of the *Chlorella pyrenoidosa*. Biomass Bioenerg 35:1533–1538
198. Shekh AY, Shrivastava P, Krishnamurthi K, Mudliar SN, Devi SS, Kanade GS, Lokhande SK, Chakrabarti T (2013) Stress-induced lipids are unsuitable as a direct biodiesel feedstock: a case study with *Chlorella pyrenoidosa*. Bioresour Technol 138:382–386
199. Chinnasamy S, Bhatnagar A, Hunt RW, Das KC (2010) Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol 101:3097–3105
200. Li Y, Yuan Z, Mu J, Chen D, Feng B (2013) Proteomic analysis of lipid accumulation in *Chlorella protothecoides* cells by heterotrophic N deprivation coupling cultivation. Energy Fuel 27:4031–4040
201. Zheng Y, Li T, Yu X, Bates PD, Dong T, Chen S (2013) High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production. Appl Energy 108:281–287
202. Matsumoto M, Sugiyama H, Maeda Y, Sato R, Tanaka T, Matsunaga T (2010) Marine diatom, *Navicula* sp. strain JPCC DA0580 and marine green alga, *Chlorella* sp. strain NKG400014 as potential sources for biodiesel production. Appl Biochem Biotechnol 161:483–490
203. Moazami N, Ranjbar R, Ashori A, Tangestani M, Nejad AS (2011) Biomass and lipid productivities of marine microalgae isolated from the Persian Gulf and the Qeshm Island. Biomass Bioenerg 35:1935–1939
204. Phukan MM, Chutia RS, Konwar BK, Kataki R (2011) Microalgae *Chlorella* as a potential bio-energy feedstock. Appl Energy 88:3307–3312
205. Yeesang C, Cheirsilp B (2011) Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour Technol 102:3034–3040
206. Liu J, Huang J, Sun Z, Zhong Y, Jiang Y, Chen F (2011) Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*: assessment of algal oils for biodiesel production. Bioresour Technol 102:106–110
207. Feng Y, Li C, Zhang D (2011) Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. Bioresour Technol 102:101–105
208. Yoo C, Jun S-Y, Lee J-Y, Ahn C-Y, Oh H-M (2010) Selection of microalgae for lipid production under high levels carbon dioxide. Bioresour Technol 101:S71–S74

209. Liang G, Mo Y, Zhou Q (2013) Optimization of digested chicken manure filtrate supplementation for lipid overproduction in heterotrophic culture *Chlorella protothecoides*. Fuel 108:159–165
210. Liu L, Wang Y, Zhang Y, Chen X, Zhang P, Ma S (2013) Development of a new method for genetic transformation of the green alga *Chlorella ellipsoidea*. Mol Biotechnol 54:211–219
211. El-sheekh MM (1999) Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. Biol Plantarum 42:209–216
212. Chow KC, Tung WL (1999) Electrotransformation of *Chlorella vulgaris*. Plant Cell Rep 18:778–780
213. Niu Y, Zhang M, Xie W, Li J, Gao Y, Yang W, Liu J, Li H (2011) A new inducible expression system in a transformed green alga, *Chlorella vulgaris*. Genet Mol Res 10:3427–3434
214. Cha T, Yee W, Aziz A (2012) Assessment of factors affecting *Agrobacterium*-mediated genetic transformation of the unicellular green alga, *Chlorella vulgaris*. World J Microbiol Biotechnol 28:1771–1779
215. Talebi A, Tohidfar M, Tabatabaei M, Bagheri A, Mohsenpor M, Mohtashami S (2013) Genetic manipulation, a feasible tool to enhance unique characteristic of Chlorella vulgaris as a feedstock for biodiesel production. Mol Biol Rep 40:4421–4428
216. Xiong W, Gao C, Yan D, Wu C, Wu Q (2010) Double CO_2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. Bioresour Technol 101:2287–2293