Rakesh Bajpai · Aleš Prokop
Mark Zappi *Editors*

# Algal Biorefineries

## Volume 1: Cultivation of Cells and Products

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# Preface and Editorial Plan

There is a strong correlation between per capita energy consumption and standard of living as envisaged by per capita gross domestic production (GDP). As a result, global energy consumption is going up rapidly. Over the past century, a majority of chemical and energy needs of our industrial society has been met by fossilized carbon sources (coal, crude oil, natural gas). Increasingly, there is a realization that utilization of the fossilized carbon sources has adverse environmental consequences in the form of increasing concentration of greenhouse gases. We are also becoming aware of the limited nature of these resources. As a result, considerable efforts are being made to produce chemicals and fuels from renewable carbon resources.

The renewable carbon is basically produced in two systems—terrestrial and aquatic. Both of these capture solar energy and atmospheric carbon dioxide as a part of natural carbon cycle. The production of terrestrial biomass is highly developed and it is an important component of our food chain. Significant amounts of terrestrial biomass such as forest products, agricultural residues, and plant products can be, and are being, made available for transformation into fuels and chemicals. The amounts of terrestrial biomass that can be spared for these activities are large yet limited and these can support only a fraction of renewable carbon needs. Aquatic biomass production, on the other hand, is less developed but it has a huge potential for delivering renewable carbon. Serious efforts are, therefore, underway targeting cultivation of photosynthetic autotrophic microbes for the production of biomass and lipids. In this category, algae appears to offer the most potential for capturing solar energy and atmospheric carbon dioxide, and delivering sufficient quantities of biomass/lipids that can offset the fossilized carbon utilization in a meaningful manner without impacting food supplies adversely. But several advances both technologically as well policy-wise are needed before algae can realize its full potential. It is also clear that a biorefinery approach must be undertaken in order to get renewable energy and chemicals from algae economically.

In a refinery, multiple products are produced to take advantage of all the components in the raw materials thus making economic production feasible. This allows one to take advantage also of market shifts, seasonal or otherwise. A classic example of a biorefinery is the wetting milling of corn where seasonal variations of market demands is accommodated by producing shifting production between corn ethanol

and high fructose corn syrup, while producing corn oil, gluten, corn steep liquor, corn protein hydrolyzate, etc. A successful biorefinery operation requires not only a set of efficient and productive processing technologies, it also requires an effective system of collection and transportation of raw materials, economic analysis of integrated systems to establish the optimal plant sizes based on local conditions, public education, and favorable policy environment.

Microalgae have been explored as sources of fuels and chemicals for the past forty years. Because of the very large scale of demands of fuels and chemicals, the production systems being sought are those that can be easily scaled-up and those that result in very large scale production of biomass. Different algal platforms (photoautotrophic vs. heterotrophic, even mixotrophic, oleaginous vs. starch producers) are being considered. Similarly, production reactors ranging from outdoor open raceways utilizing natural sunlight to indoor photobioreactors with artificial lights are being investigated. In this volume, several different aspects of algal production systems are reviewed. These provide a background of the state of the art that can form a basis for discussing the advances relating to the algal platforms for production of fuels and chemicals in multi-authored multi-volume edited-series which will document new advances involving algae-based technology. The following topics are planned to be covered in the subsequent volumes on Algal Biorefineries:

- Algal selection and metabolism
  - Classical selection methods with aim to produce useful products
  - Metabolic engineering to improve photosynthetic efficiency and carbon dioxide capture
  - Metabolic engineering to direct carbon flow to carbohydrates or to lipids
  - Genetic approaches of monitoring cultures in open bioreactors
- Cultivation of algae and algal substrates
  - Algal strains—sources, characterization, selection, preservation
  - Nutritional and environmental requirements
  - Closed bioreactors—photosynthetic efficiency, volumetric yields and products formed
  - Open bioreactors—volumetric and surface area productivity, stability of operation, start-up issues, strategies for monoculture operation, contamination control, seasonal effects on composition of algae
  - Cultivation in cold climates
  - Heterotrophic and mixotrophic cultivation of algae
  - Carbon dioxide delivery and pH control
  - Light attenuation and shading effects in bioreactors
  - Temperature control in algal bioreactors
  - Concentration, harvesting and processing of cells
  - Ecological control of contamination in open bioreactors
  - Strategies of cultivation

- Products from algae
  - Algal lipids and their uses
  - Carbohydrates from algae and their utilization
  - Extraction of lipids and carbohydrates from algae
  - Algal cake utilization
  - High value products from algae and their fractionation
- Environmental and social issues in algal biorefineries
  - Water needs, conservation, and reutilization
  - Solvents used in extraction and air quality
  - Land quality and use
  - Impact of local and migratory birds
- Process improvement and economics
  - Process optimization and increase of efficiency
  - Systems simulation of multiproduct batch/continuous facility
  - Systems analysis (systems biology models of algae metabolism and their exploration at optimization)
  - Life-cycle assessment
  - Costing and economic analysis

The following volumes may include other biomass resources, such as short rotation forestry (willow, poplar, eucalyptus), wood wastes (forest residues, sawmill and construction/industrial residues, etc.), sugar crops (sugar beet, sweet sorghum, jerusalem artichoke), starch crops (maize, wheat), herbaceous lignocellulosic crops (miscanthus), oil crops (rapeseed, sunflower), agricultural wastes (straw, slurry), municipal solid waste and refuse, and industrial wastes (residues from the food industry). It will also discuss other processing technologies such as pretreatments (physical/chemical/microbial and enzyme) and other conversion technologies: Fermentation of Sugar/Starch Crops, Fermentation of Lignocellulosic Biomass, Transesterification of Triglycerides, Gasification: Formation of Syngas, Fast Pyrolysis, Fischer-Tropsch Synthesis, Hydrogenation, Conversion of Syngas to Methane and Anaerobic Digestion.

The following individuals contributed with reviewing in this volume: internal reviewers (those who are authors of this volume): Rakesh K. Bajpai, Larry E. Erickson, Aleš Prokop, and Vilem Zachleder; external reviewers: F. Gabriel Acien, Tomas Branyik, John R Benemann, Oliver Lenz, Dusan Lazar, and Rodrigo E. Teixera. Their contributions are gracefully acknowledged.

Finally, we invite contributions from different researchers to this Series.

Rakesh K. Bajpai
Aleš Prokop
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# Part I
# Bioreactors for Cultivation of Algae

# Status of Algae as Vehicles for Commercial Production of Fuels and Chemicals

**Rakesh Bajpai, Mark Zappi, Stephen Dufreche, Ramalingam Subramaniam and Aleš Prokop**

**Abstract** This chapter provides a brief overview of role of algae for the production of fuels and chemicals. Characteristics of algae and its production in open raceway ponds have been covered to identify the critical areas that require further exploration and development.

**Keywords** Microalgae • Photobioreactors • Raceway ponds • Fracking • Harvesting • Extraction • Technoeconomics • Life-cycle analysis • Algae cake

## 1 Introduction

Microalgae have come into prominence in the past several decades due to their ability to utilize solar energy to fix atmospheric carbon dioxide, and produce biomass and lipids at productivities much higher than those possible with terrestrial plants (Dragone et al. 2010). Indeed, their ability to grow on non-arable lands and thus potential for producing fuels and chemicals without competing with food production has been commented extensively (Zhou et al. 2013). Growing concerns about the limited reserves of crude petroleum, energy security, and adverse impacts of increasing greenhouse gases have also prompted many to explore the cultivation of algae, both autotrophically as well as heterotrophically. Even though the advances in fracking technology have resulted in recent years into increased production of natural gas and crude oil (USEIA 2011), and thus some respite from the immediate worries of energy supply and energy security, sources of liquid hydrocarbons for the increasing global needs of transportation sector remain a major cause for concern. Still, few examples exist of commercial use of algal system for production of biomass and lipids (Day et al. 2012; Borowitzka and Moheimani 2013) for reasons adequately pointed out by Richmond (2004).

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A seminal paper on mass cultivation technologies was published by Tamiya (1957) describing the state of the art of cultivation of algae. This report focused on cultivation of algae for the purposes of production of value-added compounds, for the purposes of nitrogen fixation, for treatment of wastewater, and outlined the characteristics of algae for different tasks and their production. Another USDOE report (Sheehan et al. 1998) summarized the results of a two-decade long experience of algae program. This program focused on production of biofuels using algae and concluded that algal technologies were way too costly for making a bulk chemical such as fuel. Both the reports concluded that outdoor open systems would be most economical for cultivation purposes, and that remains true even today (Greenwell et al. 2010), even though considerable progress has been made in terms of understanding the metabolic processes in algae and in cultivating pure algal cultures in closed photobioreactors. Unfortunately, most of the economic analyses still conclude that biofuels produced using photoautotrophic algae cannot compete with fuels derived from crude oil in spite of the spike in oil prices over the past decade (ANL, NREL, PNNL 2012; Lundquist et al. 2010; Sun et al. 2011). In all the cases, a running theme is need for improved strains (those with characteristics of fast growth while still sporting high neutral lipid content, auto-flocculation thereby facilitating easy recovery of cells from broth, capability to withstand the onslaught of viruses, contaminants, and predators), recycle and reuse of spent broth, and economic recovery of lipids from cells. Further research and developments in the areas of cell harvesting, product recovery, product fractionation into high value products, and research and market development for algal cake (residue) are also deemed necessary in order to make biofuels from algae economical, since cell harvesting and lipid recovery are themselves reported to account for 50 % of the cost of lipid produced (Greenwell et al. 2010).

Considerable efforts have recently been devoted to developing algal strains, systems for cultivation and harvesting, and processing of algae produced for fuels and chemicals (Adarme-Vega et al. 2012; Afify et al. 2010; Agwa et al. 2012; Beer et al. 2009; Chen et al. 2011; Faria et al. 2012, Khola and Ghazala 2012; Miranda et al. 2012; Rajvanshi and Sharma 2012; Rosenberg et al. 2011; Rulong et al. 2012). Most of these stem from the results of Aquatic Species Program (ASP) of US Department of Energy (USDOE) that created a significant database of potential algal species and reactor designs for their cultivation (Sheehan et al. 1998). The results of ASP efforts established that ‘open-pond’ cultivation systems represent the most promising pathways for large-scale bulk production of algal biomass. Recent analysis by Davis et al. (2011) reaffirmed this observation. A renewed effort starting in 2008 under the auspices of National Algal Biofuels Technology Roadmap Work (USDOE-EERE 2012) organized by USDOE identified the algal cultivation, cell harvesting and processing to produce lipids and other desirable valuable byproducts, and conversion technologies as major challenges to economic production of biofuels from algae. This chapter will focus on providing a summary of recent developments relating to economics of autotrophic cultivation of algae, especially as it relates to raceway-type outdoor open cultivation, economic models, and identify areas that need radical improvements and demonstrations for commercialization

of algal biofuel production. A similar report involving high density incline surface thin-film outdoor open cultivators is presented separately by Doucha and Livansky in a later chapter in this book.

## 2 Advances in Algal Strains

Algae have commercially been produced in various parts of the world and their annual sales in 1997 already exceeded US $ 30 billion (Spolaore et al. 2006). However, most of this production was for nutraceuticals and aquaculture. Typical algal strains used for these purpose are *Chlorella*, *Arthrospira*, *Dunaliella*, and *Haematococcus*. Other species such as *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema*, *Thalassiosira*, *Porphyridium*, *Phaeodactylum*, *Isochrysis*, and *Nitzschia*. These are utilized for their protein content and polyunsaturated fatty acids which are typical high value products. From the perspective of commodity items such as biofuels, the strains selected for production must show the following attributes: (a) high growth rate and sustainable production in an open culture (Dragone et al. 2010; USDOE-EERE 2012), (b) high photosynthetic efficiency (Brennan and Owende 2010), (c) reusability of filtrate from harvesting operations, (d) capability of withstanding toxic components in gases used to deliver carbon dioxide, and (e) ease of harvesting and extraction of intracellular components. Sustainable outdoor production in open systems involves cells that can withstand onslaught of contaminants/predators as well as weather conditions (to keep cultivation systems from crashing), and those that have low internal metabolism during conditions of low photon irradiation, including night time (to keep cells from reutilizing the chemicals being targeted for production).

It has been suggested that native algal strains isolated from local environments stand the best chance for meeting the above mentioned criteria of sustainability (Sheehan et al. 1998; Chen et al. 2009). Since there are around ten million algal species on our planet and only a few thousand have been identified (Norton et al. 1996), there is considerable potential for finding natural algal strains capable of producing biofuels. Using this approach, Ravikumar (2013) was able to maintain open raceway cultivation systems predominantly monoculture by using locally isolated *Scenedesmus* strains. Stephens et al. (2010) have reported that several outdoor open pond systems have been operated without significant contamination for as long as six months. Still, cultivating desirable algal strain in open ponds remains very challenging (NRC 2012). While selecting algal strains for production of lipids, it is obvious that the focus be on cells that can generate a high fraction of their dry weight as lipids (Hussain et al. 2010). However, the conditions that result in high lipid content in the cells (environmental stresses or nutritional limitations) are generally also the conditions that cause the lipid productivity to drop, and vice versa (Chen et al. 2009). As a result, strain selection strategies such as those based on lipid productivity (the amount of neutral lipids produced per unit time per unit lighted area or volume), ease of harvesting of cells and recovery of lipids from cells,

ability of cells to overcome the environmental and nutritional shocks, and adaptability of cells to new environments, need to be emphasized (Griffiths and Harrison 2009; Mata et al. 2010; Day et al. 2012). Mutanda et al. (2011) have provided a summary of techniques for isolation of algae from natural samples and concluded that micromanipulation methods coupled with fast screening methods such as those based on dye fluorescence (either lipophilic dye BODIPY 505/525 or lipid-soluble dye Nile Red) can be used to rapidly identify desirable algal strains. In doing so, it is necessary to note that the goal is to cultivate cells with high neutral-lipid productivity, rather than cells that can accumulate just high concentration of lipids in them (Zhou et al. 2013; Pareira et al. 2011). Such fluorescence-based methods are especially attractive since lipid accumulation in cells normally takes place during the stationary phase of cell growth caused by the nutrient limitations in cell broth; in other words, cell growth and lipid production is generally decoupled. It would be interesting to identify / develop cell lines in which lipid production is coupled with cell growth.

Courchesne et al. (2009) have summarized the status of genetic and transcriptional factor approaches being explored to enhance lipid production in algae. These authors reported that multiple bottlenecks as well as competing pathways to those for lipid synthesis exist in cells; although successful overexpression of some of rate-limiting enzymes has been reported, no successful enhancement of lipid production in cells has been demonstrated as yet. On the other hand, genetic engineering approaches have demonstrated enhanced production of hydrogen by algal cells (Beer et al. 2012). Since multi-enzyme systems are generally involved in production of lipids, it may be more appropriate to utilize a transcription factor approach to enhance the production of lipids in algae (Courchesne et al. 2009). In such a case, efforts need to focus on characterizing the transcription factors that participate in lipid synthesis in algae (Nguyen et al. 2008).

Considering that heterotrophic cultivation of selected algal cells result in higher lipid production (Chen et al. 2009), several researchers have explored isolating strains capable of growing on organics in waste water. Agwa et al. (2012) optimized biomass production using a *Chlorella* sp. growing on different animal wastes. The strategy involving heterotrophic growth of algal cells on lignocellulosic hydrolyzates is being pursued by a San Francisco based algal-lipid producer, the Solazyme Inc., at semi-commercial scale. At the same time, other groups are focusing on naturally selected proprietary strains in raceway reactors for commercial cultivation of algae for biofuels. The most prominent of these is Saffire Energy based in San Diego, CA. As suggested by Luque (2010), the basic productivity of lipids by many of these companies may be placed too high resulting in unrealistic expectations. It is, therefore, not surprising that many companies fold their doors within a few years after generating considerable investor interest.

Selection of algae need not be based solely on lipid production by the cells (Rodolfi et al. 2009). Since production of lipids in microorganisms is influenced by environmental stress factors (Brennen and Owende 2010), accumulation of large quantities of lipids in cells is often accompanied by reduced growth rates of cells which can be a major disadvantage for cells growing in open culture systems.

Hence, strategies for selection of algal strains must account for utilization of high-value byproducts such as PUFA, as well as residual cake that may serve as source of carbon skeletons for making additional biofuels or of nutrition. Another consideration may also be the ease of recovery of lipids and the quality of biofuels that can be made from the extracted lipids. Towards this end, Afify et al. (2010) evaluated growth conditions and extraction solvents for eight algal species for lipid recovery and biodiesel production. Recently Liu and Curtiss (2012) have explored genetically modified algal cells that release free fatty acids when subjected to $CO_2$ limitation followed by exposure to increased temperatures. Although this strategy was time consuming and cumbersome, further developments in this direction can result in significantly reduced cost of lipid recovery.

Other major considerations in making algal systems economical include the sources of nutrients and their reutilization from spent media after harvesting of cells. Although algae are characterized by simple nutrient requirements (Brennan and Owende 2010; Chisti 2007), residual nutrients in spent media represent a major cost of production both in terms of their procurement as well as need to meet the regulatory requirements in any discharge waters. Nitrogen can be efficiently taken-up by the cells in the form of ammonium ions, urea, or even as nitrates, and its limitations have been reported to influence the production of neutral lipids in the cells (Rodolfi et al. 2009). As a result, almost all the nitrogen in broth is available to cells for uptake. On the other hand, phosphorous is required in the form of phosphates which complex with several cations present in broth and thus are not available to the cells completely. Proper optimization of N and P needs in the culture broth is an on-going research area that needs to be vigorously pursued. In this respect, the statistical techniques of medium optimization based on response-surface methodology may come very handy (Ponnusamy and Subramaniam 2013). In order to address these nutritional needs, several authors have considered use of wastewaters for cultivation of algae (Batten et al. 2013; Olguin 2012; Park et al. 2011; Pittman et al. 2011). In such cases, selection of algal strains capable of photoheterotrophic growth would result not only in reducing N and P content of waste water, but also much higher efficiencies of cell growth and lipid production. Doucha and Livansky (2006) have reported that algal cells may be grown to cell densities up to 100 times more in thin-film photobioreactors than in raceway ponds. This can be particularly useful since harvesting of cells is a major cost item in production of biofuels using algal systems.

## 3 Advances in Cultivation Technologies

Phototrophic cultivation of algal cells is carried out in laboratory in lighted environment in clear shake flasks, culture vessels, bottles, tanks, or specially-designed photobioreactors. In order to take advantage of solar energy, outdoor cultivation is carried in tubular or cylindrical photobioreactors or in open ponds/raceways. Due

to the attenuation of light in liquid media (Daultani 2010; Benson 2003), the depth of fluid in these reactors is limited to no more than 30 cm (Murphy et al. 2010). Based on the production capacities needed for biofuels, outdoor production facilities, mainly the raceways, are the only viable options although some very large capacity closed tubular photobioreactors are in use in Germany (Spolaore et al. 2006). Among open configurations, raceway design is the reactor of choice due to ease of creating circulation patterns and potential for addition of carbon dioxide and nutrients. These can be constructed on non-arable land also.

Recent advances in open outdoor photobioreactor designs have taken place in delivery of carbon dioxide to the cells, mixing of cells, and in control of contaminants/grazers. Since, the depth of culture medium is limited (approximately 30 cm or so, and no more than 50 cm), considerable amount of sparged carbon dioxide would escape into atmosphere unless an appropriate delivery system is designed and utilized. One such system is a floating $CO_2$ injector which consists of a floating compartment with a hollow enclosure. Gas under the cover filled with $CO_2$ permits the device to float. As the gas gets consumed, the floating device gets submerged in the medium causing a float valve to supply more gas under the cover. As the float emerges from the medium, the valve closes the supply of gas. Gas is introduced in the medium through a gas sparger due to pressure in the cover. Such systems result in very low (as little as 4%) losses of $CO_2$ (NRC 2012). Based on carbon content of the cells, a minimum of 1.8 kg of carbon dioxide would be required to produce 1 kg of dry cell mass. In practice, up to 5 kg carbon dioxide may be needed per kg of dry algae due to low efficiency of transfer of $CO_2$ from the gas phase (Seambiotic Ltd. 2010; Murphy et al. 2010; Doucha et al. 2005). In order to deliver $CO_2$ to cells, sparging of $CO_2$-laden gases may be conducted using either perforated tubes or diffusers. Since diffusers result in formation of much smaller bubbles compared to perforations in tubes, these have a significantly higher efficiency of mass transfer of $CO_2$ from gas to broth (Weiss and Lezion 2008). The efficiency of gas-liquid mass transfer can be enhanced further by increasing the height of medium at the sparging locations through creation of sparging (sump) wells (Murphy et al. 2010). Air, with its 395 ppm $CO_2$levels (NOAA 2013), cannot deliver enough carbon dioxide to achieve reasonable production rates (20 g DW algae/$m^2$ day) and a concentrated source of carbon dioxide is required. Such sources are available in the form of power plant stack gases (carbon dioxide concentrations between 9–14% v/v), monoethanol amine (MEA) scrubbers for $CO_2$ in natural gas, or even exhaust gases from alcoholic fermentations. Of these, the power plant stack gases represent the most obvious sources of plentiful carbon dioxide available in disperse locations where the energy from the hot gases may be utilized also for controlling temperatures in algal ponds. Power plant stack gases have been successfully used as $CO_2$ sources by Seambiotic (2010) in outdoor open ponds as well as by Olaizola (2000) in a large outdoor photobioreactor. The power plant stack gases contain, in addition to carbon dioxide, other components as well, such as particulates, oxygen, nitrogen, carbon monoxide, nitrogen and sulfur oxides, and trace metals depending on the source of carbon and operation of combustor. Of these, sulfur oxides are most harmful to the algal cells and needs to be reduced to concentrations below 60 ppm. On the other

**Table 1** Effect of fluid velocity and open channel length on liquid circulation time and the fluid head needed to overcome flow resistance

| Velocity, m/s | Channel length, m | Liquid head, mm | Circulation time, min |
|---|---|---|---|
| 0.3 | 500 | 10.0 | 27.8 |
| | 1000 | 20.0 | 55.6 |
| | 2000 | 39.9 | 111.1 |
| 0.4 | 500 | 18.2 | 20.8 |
| | 1000 | 36.4 | 41.7 |
| | 2000 | 72.5 | 83.3 |

hand, many of the trace metals in stack gases may even promote algal cell growth, as was found by Seambiotic (2010).

Another area in which significant progress has been made is in the designs of fluid mixing and propulsion systems. The central part of these systems in raceway systems consists of paddles mounted on an axial shaft connected to a motor either directly or through a pulley. The purpose of mixing is to keep the algal cells suspended in the growth medium (decaying cells in the sedimented material are good food for contaminating bacteria) and to ensure that the cells rotate routinely between the darker interior of the broth and the lighted air-liquid interface (light in these systems rarely penetrates beyond 15 cm liquid depth; Daultani 2010). Another important function of the paddle system is to circulate the suspended cells through the $CO_2$-rich zone near the spargers. In the pilot cultivation system of Seambiotic (2010), the paddles propel fluid forward with an average velocity of 20 cm/s and a single sparger zone is used in raceways such that a full circulation of fluid through the raceway takes place every 3–3.5 min. Such circulation times between sparged zones are similar to the circulation times experienced by cells in traditional agitated bioreactors. Murphy et al. (2010) suggest 30 cm/s linear fluid velocity as minimum to keep the cells in suspension.

Murphy et al. (2010) projected using raceways of 245 m length and 18 m width (aspect ratio of around 12). This ratio is in the range of 10:1–20:1 for aspect ratio suggested by Benemann and Oswald (1996) whereas the aspect ratio (length to width of each arm or raceway) used in the pilot plant raceways of Seambiotic (2010) appears to be around 4 (based on the pilot plant photographs). The proposed length of raceways by Benemann and Oswald (1996) is as large as 1000 m. With increasing length of the raceway, liquid head needed for flow of fluid will also increase. Using correlations and governing equations for subcritical flow in open channels (Tilton 1997), the liquid heads and circulation times between different channel lengths can be calculated for specified fluid velocities and these are listed in Table 1. Here channel length is the total distance between paddle wheels; the calculations are based on horizontal channels of zero slope and identical to natural streams with clean straight bank full stage.

Thus in a raceway of 1000 m equivalent length (2000 m channel length), the liquid head to be generated by a single paddle for 30 cm/s liquid velocity is modest (around 1/7th to 1/8th of the liquid depth), but the circulation times are of the order

of 110 min. This implies that several carbon dioxide spargers will need to be placed every 80–100 ms in the raceway channel in order to ensure a mean residence time around 4 min for cells between sparged zones. Doucha et al. (2005) recommend using carbon dioxide spargers every 50 m in order to prevent $CO_2$ limitation for the cells which in their case corresponds to around 30 s residence time between spargers.

For most of the algal cells, the fluid velocity of 40 cm/s would keep the cells in suspension (Murphy et al. 2010). Fast settling cells such as *scenedesmus* may require higher velocities to keep them suspended. In such a case, higher fluid velocities up to 1 m/s may be employed by reducing the length of raceways to 100–125 ms. This would keep the liquid head modest around 7–8 cm and require only two sparging stations in the raceway.

In deciding the flow rate of fluid in the raceways, shear sensitivity of algal cells being cultivated must be considered. For shear sensitive cells, however, such high velocities may be detrimental (Michaels et al. 2010). The shear sensitivity of algal cells follows the order below of sensitivity to shear stresses: *green algae* < *bluegreen algae* < *diatoms* < *dinoflagellates* (Thomas and Gibson 1990). In a study of shear stress on algal cells, Contreras et al. (1998) reported that cells of *Phaeodactylum tricornutum* grew fastest at a shear rate of 7000 $s^{-1}$ whereas the dinoflagellate *Protoceratium reticulatum* cells showed damage already at a shear rate of 0.12 $s^{-1}$ (García Camacho et al. 2007). *Chlorella* and *Scenedesmus* are relatively shear tolerant (Dragone et al. 2010; Setlik et al. 1970). Michaels et al. (2010) recently showed that cells of microalgae *Chaetoceros muelleri* experience an abrupt but definite loss of cell viability between shear rate of 750 and 975 $s^{-1}$ without showing any external sign of cell damage, but increasing shear rate further to 14500 $s^{-1}$ did not show any further loss of cell viability. Several algal varieties such as *Dunaliella* show sensitivity to turbulence created by sparging of gases (Barbosa et al. 2004).

Although dissolved oxygen and temperature control are not major issues in raceways, evaporation of water in dry season and flooding of raceway during rains are major issues. pH in the broth also must be controlled within the parameters of the strains being cultivated. When sparging $CO_2$-enriched gases, broth pH may drop and appropriate measures should be taken to control it back to the desired range. Another major issue in these reactors is the potential for contamination by viruses, bacteria, and grazers. The issues of contamination have been addressed either by use of extremophiles (high pH, high salinity) or small quantities (1–3 ppm) of antibiotics in broth (Brennan and Owende 2010). Protozoa population in the medium can be controlled by deliberately lowering broth pH for a short period and then raising it again. Chemical agents such as chlorine or UV-treatment may also be used to control contaminant population, especially in the reclaimed broth after it is made-up for losses due to evaporation and during cell harvesting and before it is recycled. In any case, regular cleaning of channels for any sediment is strongly advised to control bacterial contamination. Seambiotic (2010) reports restarting the raceway frequently with fresh culture inoculated with as much as 30% v/v inoculums of good algal cell suspension. In some cases, judicious use of local strains of algae

along with carefully designed operational techniques has also been used to keep the raceway systems running for several weeks in a row (Ravikumar 2013, Stephens et al. 2010). Still, lack of appreciable authenticated data for long-term algae production (of the order of a year or more) in outdoor raceways is hindering scale-up of production facilities (Murphy et al. 2010).

## 4 Advances in Harvesting and Extraction Technologies:

Recovering cells from broth and extraction of lipids from algal cells is critical for economic production of biofuels using open algal ponds. Recovering cells from broth involves several operations (Sing et al. 2013) including bulk harvesting (increasing cell concentration to ~0.5–1 % w/v, dry weight basis), thickening (final cell concentration ~5–10 %), dewatering (final concentration to 15–25 %), and drying (if required). Cell recovery itself costs as much as 20–30 % and more towards the cost of algal lipids (Brennan and Owende 2010). The costs occur due to difficulties in separation of cells from broth (due to small cell size as well as low cell concentrations), need for processing of large volumes of liquids, tendency of cells to reutilize the lipids as energy source during stationary phase, and intracellular nature of product(s). One of the biggest difficulties faced in harvesting is the low concentration of cells which in open raceway bioreactors ranges between 300 to 500 g $m^{-3}$. This means that almost a million $m^3$ (or 264.2 million US gallons) water must be separated in order to obtain 300–500 ton dry algae. Considering an average 20 % extractable neutral lipids in dry algae, it amounts to 34–57 $m^3$ (or 9000 to 15000 gal) broth that must be processed to make a gallon of lipids from algae. In this sense, some cell lines such as cyanobacterium *Spirulina* that are filamentous, may permit considerable cost savings in harvesting (Benemann and Oswald 1996). Most others such as *Chlorella*, *Nannochloropsis*, and *Scenedesmus* are unicellular. High density algal cell cultivations in thin-film open outdoor systems, as proposed by Doucha and coworkers (Doucha et al. 2005; Doucha and Livansky 2006), are highly desirable as these reduce not only the liquid volumes but also the frequency of liquid handling resulting in considerable savings in operating costs. Unfortunately, the inclined bed systems suggested by these authors increase capital costs several fold.

Initial separation of algal cells from broth in raceway ponds will depend strongly on the size and shape of cells, density of wet cells and their agglomerates, and surface charge. Sizes of algal cells range from 2 to >200 μm. Shapes of cells can be spherical, rod-like, or filamentous (Henderson et al. 2008). Some cells are buoyant due to gas-vacoules while others are denser due to heavy presence of minerals (Greenwell et al. 2010). Presence of excessive lipids may also make some cells lighter than the broth, but these effects are generally not significant as the wet algal cells are over 80 % water. Generally algal cells carry negative charge under physiological conditions (zeta potential in the range of −5 to −40 mV). In light of these cell characteristics, different combinations of flocculation, floatation, fil-

tration, sedimentation, centrifugation, and electrically assisted cell separations are commonly employed for harvesting algal cells from broth.

## *4.1 Flocculation*

Flocculation of cells is based on modifying cellular charge by changing culture conditions, culture pH, addition of chemical flocculants, or exposing the cells to electric field. Several algal species tend to autoflocculate as their metabolism slows down (Uduman et al. 2010). Sirin et al. (2012) found that the microalgae *Phaeodactylum tricornutum* efficiently self-coagulates at pH 9.75 which is just a tad over its cultivation pH of 9.12; at cultivation pH, the natural flocculation as well as sedimentation rate is very poor. In cases like this, autoflocculation may be the most efficient method for initial concentration of cell. The most common chemical flocculants are inorganic (aluminum, ferric, zinc sulfates or chlorides) or organic (cationic polymers, starches, chitosans, etc) in nature. Papazi et al. (2010) found aluminum salts to be most efficient among inorganic salts but caused some lysis of *Chlorella minutissima* cells. Chlorides caused an almost immediate aggregation of cells upon addition of requisite quantities, whereas with sulfates it took 2–5 h to cause aggregation. The concentrations of the salts ranged from 0.5 to 0.75 kg $m^{-3}$ which though effective may make use of these salts cost prohibitive. Using these coagulants may also interfere with reuse of residual water in cultivation reactors. Chitosan is a natural cationic polysaccharide that is non-toxic, produces large flocs, and results in high sedimentation rate (Sirin et al. 2012). These authors found that addition of 20 mg/L chitosan to *Phaeodactylum tricornutum* broths at pH 9.75 enhanced flocculation as well as sedimentation rates, but not at pH 9.12. Polymeric coagulants such as nonionic polymer Magnafloc LT25 and cationic polymer Praestol are very effective in inducing coagulation in many algal systems at concentrations as low as 0.5 ppm and have no adverse effect on cell growth in recycled media (Milledge and Heaven 2012). Saline systems require higher doses of electrolytes and polymers in causing coagulation, even though a combination of polyelectrolytes and chemical coagulants has been reported to be highly effective (Knuckey et al. 2006). Zeng et al. (2012) reported concentration factor of greater than 20 and over 90 % cell recovery with the addition of around 20 mg/L poly (γ)-glutamic acid as organic flocculent for several marine and freshwater algae (*Chlorella vulgaris*, *Chlorella protothecoides*, *Nannochloropsis oculata* LICME 002, *Phaeodactylum tricornutum*, *Botryococcus braunii* LICME 003). At times, the efficiency of flocculation can be enhanced by pretreatment of the cells by ozonation (Greenwell et al. 2010).

Bioflocculation is the term associated with formation of flocs in an otherwise nonflocculating culture in presence of another organism that flocculates easily. In several waste treatment systems utilizing algae, presence of contaminating bacteria have been shown to cause considerable flocculation (Lee et al. 2009; Medina and Neis 2007). Recently, Salim et al. (2011) reported mixing of two microalgae as potential means of causing easy flocculation of algal mass from cultures. Nonfloc-

culating freshwater microalga *Chlorella vulgaris* was flocculated with the help of flocculating freshwater algae *Ankistrodesmus falcatus* and *Scenedesmus obliquus*, and nonflocculating marine alga *Neochloris oleoabundans* was harvested with the aid of flocculating marine alga *Tetraselmis suecica*. In all the cases, addition of flocculating coagulating cells to non-flocculating cells increased sedimentation rates of all cells in the mixture; bridging and entrapment were proposed as possible mechanism of enhanced recovery of the nonconforming cells.

Electrically-induced flocculation has also been proposed as a means of concentrating algal cells from broth (Vandamme et al. 2011). Electro-coagulation-flocculation utilizes a sacrificing anode (either iron or aluminum) in causing flocculation of algal cells; Vandamme et al. (2011) found that aluminum anodes were superior to iron anodes for *Chlorella* and *Phaedactylum* broths and power consumption was of the order of 0.3–2 kWh/kg cells harvested. Electroflocculation, on the other hand, does not require sacrificing anodes and flocculation takes place at the anode where cells give-up their charge. Power consumption in these systems is also of the order of 0.3 kWh/kg cells harvested, but significant fouling of anodes has been reported (Uduman et al. 2010). These methods have been shown to be effective at bench scale, but performance of any scaled-up unit has not been reported as yet.

## *4.2 Floatation and Sedimentation*

These gravity-based technologies have been proposed for separation of algal flocs from broth. A review of principles of these processes is provided by Milledge and Heaven (2011) and it clearly shows the role of particle size in the separation. As a result, floatation/sedimentation are generally used after flocculation of cells. Due to small density differences between cells and culture medium, settling of cells and flocs is easily disturbed by convection currents and sedimentation is normally not used as a unit-operation for separation of algal cells (Uduman et al. 2010). Floatation, on the other hand, is relatively fast as it can be assisted by addition of small air-bubbles in the mix. In dispersed or suspended-air-floatation (SAF), micro-bubbles are generated chemically in the broth using surfactant and depressurization from 2 atm (absolute) to atmospheric pressure. In dissolved air floatation (DSF), 10–100 micron size bubbles are generated by releasing high pressure fluid (~5 atm absolute) directly in the culture medium (Wiley et al. 2009). With the use of surfactants (~2.5 mL/L of floatation water equivalent to ~21 mL surfactant/$m^3$ medium) and relatively modest pressures to form microbubbles, the SAF process resulted in significant cost savings compared to DAF which is generally regarded as one of the most promising mode of separation of cells from medium. At the bench scale of 100 L, the energy requirements were calculated to be 7.6 kWh/$m^3$ sample treated using DAF compared to 3 Wh/$m^3$ sample treated using SAF for identical solid capture. Recently, Hanotu et al. (2012) have reported production of micron-sized bubbles from porous spargers using a fluidic oscillator based on Coanda effect with 2–3 orders of magnitude reduction in energy usage. Although a recent review by Brennan and Owende (2010) concluded that flocculation and floatation are not likely to be

cost effective in view of material (flocculant) and energy (microbubble generation) requirements, the advances in more effective polymeric flocculants coupled with algal surface modifications (via mild ozonation) and new techniques in formation of microbubbles make this a promising alternative to other methods of cell harvesting.

### *4.3 Filtration*

Filtration is a commonly used unit operation for separation of solids from liquids. The conventional devices include pressure filters (plate and frame press, chamber filter press, belt press, pressure suction filter, cylindrical sieve filter, filter basket) and vacuum filters (vacuum leaf or Moore filter, vacuum Nutsche or batch bed filter, rotary drum filter). For the case of algae, two major issues need to be confronted in order to successfully use filtration for the desired separation. Firstly, algal cells are rather small (3 to 100 μm in size) and these are suspended in culture medium in an extremely dilute manner. Secondly, the algal cells often release extracellular organic materials (EOM or EPS—extracellular polymeric substances) depending on the cultivation conditions (Drews et al. 2006). EOMs have a high potential of fouling the filtration media. Generally cultivation under high light intensities and high temperatures results in stunted cell growth and production of larger amounts of EOMs (Round 1981).

Shelef et al. (1984) provided an excellent review of these devices for algal systems. Direct filtration, using conventional filtering devices, is a possibility for some algal cells (*Coelastrum* and *Spirulina*) that are large in size (>70 μm). As reported by Shelef et al. (1984), filtration of *Coelastrum* broths could produce algae cake containing as much as 27 % solids at energy cost ranging from 0.1–6 kWh/m$^3$ fluid processed; such cakes would be ready for extraction either as such or after drying. For algal cells of smaller size (such as those of *Dunaliealla*, *Scenedesmus*, *Chlorella*), these methods suffer from incomplete separation and rapid drops in filtration rates. In such cases, due to small cell size and low cell density, filtration alone is not a feasible operation for harvesting algal cells.

Microfiber membranes, esp. when used with cross-flow filtration to reduce the buildup of algae cake on filter have been proposed for use after process volumes have been reduced by a factor of 100 (Greenwell et al. 2010). Babel and Takizawa (2010) recently explored filtration of *Chlorella* cells using cellulose ester and polyvinylidine difluoride (PVDF) microfiber membranes and found that the extracellular organic material (EOM) released by the cells causes considerable fouling of both types of membranes; the *Chlorella* cells themselves did not foul the membranes. The cell cake was found to be compressible with a compressibility index of 0.44. Satone et al. (2011) demonstrated a novel tangential-flow filtration device in which shear rate around the filtration membranes (of 1.5 μm pore size) was increased by use of a concentric spiral guide within the ceramic membranes. In such a system it was possible to concentrated algal suspensions from 3 to 11 kg m$^{-3}$ rapidly under a pressure of 6 bar with a relatively constant flux of 0.5 L m$^{-2}$ min$^{-1}$ and no

deposits were observed on the spiral guide rod; during batch filtration using this device, the filtrate flux dropped rapidly in the first few minutes from around >4 to 1 L $m^{-2}$ $min^{-1}$ and settled to around 0.5 L $m^{-2}$ $min^{-1}$ after several hours. Since it is the EOMs that interfere with filtration membranes, it is likely that surface treatments of membrane surface to reduce fouling would enable significant improvements in filtration rates. Milledge and Heaven (2012) have reported an ultrafiltration membrane (0.03 μm pore diameter) from Avanti Membrane Technology Inc. that resulted in filtration rate of ~1.1 L $m^{-1}$ $min^{-1}$ with 95% recovery of microalgae but only 20 fold concentration factor. No data were provided for the starting of final concentration of cells in the retentate. The energy consumption was between 1 and 3 kWh $m^{-3}$ fluid processed.

## 4.4 Centrifugation

For any slowly settling system, such as the one with algal suspension, centrifugation is definitely desirable from the viewpoint of accelerating the separation process as it increases the driving forces for separation by several orders of magnitude. This is the reason several researchers (Rosch and Posten 2012; Seambiotic Ltd 2010; NRC 2012) have concluded that centrifugation is an essential part of final thickening of algal slurries, following at least flocculation and sedimentation/floatation. It is a highly energy intensive process, consuming around 1.4 kWh $m^{-3}$ fluid processed. The centrifuges can be either disk stack type or decanter type. Disk stack centrifuges can be used for very dilute algal slurries (concentration 0.02%) and produce >20% solid slurry. Decanter centrifuges require higher feed cell concentrations (10% or more) but these can produce >40% solids in the cake; these centrifuges consume even more power than the disk stack centrifuges. Milledge and Heaven (2012) have reported on a new type of spiral-plate centrifuge in which the centrifugal force was used to force solids on the outer bottom edge of the bowl vanes and a solid paste (31.5% dry solids) was obtained from 0.025% suspension of *Nannochloropsis* for energy usage of 1.9 kWh $kg^{-1}$ dry algae. This energy consumption is considerably lower than that for disk stack centrifuges. The operation was semi-batch and the capacity was only 4 $m^3$ $h^{-1}$. Based on the information available on Evodus Web site (http://www.evodos.eu/market-specific-solutions/algae.html), the energy picture improves even further as the feed stream cell concentration increases (1 kWh $g^{-1}$ dry wt at 0.05% feed, 0.53 kWh $kg^{-1}$ dry wt at 0.1% feed, and 0.45 kWh $kg^{-1}$ dry wt at 0.15% feed).

## 4.5 Drying

The wet algae paste is highly perishable and it needs immediate processing to get the valuable products out of it. Alternatively, it can be frozen (for short term) or dried. Drying of cells may be necessary also depending on the process of extraction

of lipids from algal cells. This can be done by several means which include using energy directly from the sun (solar drying) or indirectly through hot gases (spray drying), hot surfaces (roller drying), or drying under vacuum at low temperature (freeze drying). In all of these cases, minimum energy to be removed per kg of water removed is established by thermodynamic considerations (i.e. 2.6 MJ $kg^{-1}$, Milledge and Heaven 2012). As a result, the cells must be dewatered as much as possible before drying. While using solar energy the only requirements are space, but it could be very slow depending on the global location. Freeze drying is also very slow and it is a very energy intensive process. Both roller and spray drying are quite fast, although they may incur loss of lipids as well as other valuable constituents in the cells. Drying temperature and duration together determine the lipid content in the cells (Brennan and Owende 2010). Drying at or below 80 °C retains most of the lipids (Graham 2011).

## *4.6 Extraction of Lipids*

Lipids in algal cells are intracellular in the form of lipid bodies and require extraction utilizing solvents. The extractions can be conducted either from dry cells or from the wet cake. An excellent review of extraction principles and procedures has recently been published by Halim et al. (2012).

Common solvents used for extraction are hexane, chloroform, methanol, ethanol, isopropanol, butanol, ethyl acetate, petroleum ether (NRC 2012). The non-polar solvents such as hexane and chloroform are necessary to extract neutral lipids (triacylglycerols, TAGs) from the cells. Polar solvents such as methanol and isopropanol in solvent mixture generally serve the role of disruptors of hydrogen bonds and electrostatic forces between membrane-bound lipids and proteins, thus making the membrane porous and allowing the non-polar solvents better access to the non-polar lipids within the cell (Cooney et al. 2009). The use of polar solvents may even result in higher lipid extraction as these solvents are able to remove the polar lipids also from the cells. Extraction of polar lipids such as phosphoglycerides may even be problematic during subsequent processing of lipids into biofuels (Dufreche 2008; Halim et al. 2012).

Before lipid extraction, the cells need to be disrupted to ensure an intimate contact between the solvent and the intracellular lipid bodies. The cell disruption can be achieved using either mechanical devices (bead milling, sonication, French press, etc.) or using physical-chemical means (enzymatic digestion of cell walls, acid/alkali based hydrolysis of cell walls, or even osmotic shock if the cell structure permits, Lee et al. 2010). French presses disrupt cells by first pressurizing the suspension to 500–850 bar and then passing it through a small opening to lower pressure (Halim et al. 2012). The efficiency of single pass cell disruption is very high (~74 %) and it causes a quick solvent extraction of lipids from cells. This method is commonly utilized for cell disruptions at industrial scale. Bead mills and sonication are more laboratory scale operations.

The extraction methods commonly employed are variations of the classical Bligh and Dyer (1959) methods or its many variations. In this method, the cells are first disrupted in presence of solvent at room temperature. Water is then added forming a multi-phase system consisting of a heavy lipid-rich chloroform phase, a light aqueous phase containing methanol, and a solid phase that forms a layer between the light and heavy phases. Although absence of water in the beginning permits better access of the non-polar solvent to the cells, it is not necessary to completely dry the cells before using Bligh and Dyer extraction. This extraction utilizes chloroform and methanol in the ratio of 2:1 as solvent system. After removal of the solvent phase, the remaining phases are then extracted repeatedly with the solvent phase. The solvent phase is evaporated to recover the solvents for reuse and the lipids are retained for further processing. Unless already removed by special processing, the photosynthetic pigments also extract out with the lipids during extraction of algae and impart a greenish color to the algal oil. This method is mostly used at bench scale. Two other extraction methods used at bench scale are accelerated solvent extraction (ASE) and supercritical extraction (SCE). Both involve high pressures and temperatures, except subcritical conditions are maintained during ASE. These are highly efficient methods and do not generally involve any prior cell disruption. Both can utilize any of the solvent combinations that can be thought of for facilitating extractions.

Since both chloroform and methanol are toxic chemicals, alternative solvent system have been explored. In many situations, hexane/isopropanol solvent system does as good a job of extracting neutral lipids from the cells as the chloroform-methanol system. In a comparative study of four different solvent systems (chloroform-methanol, chloroform, hexane, hexane-isopropanol, methy tert-butyl ether MTBE), Subramaniam et al. (2011) compared the extent of extraction of lipids from freeze-dried as well as wet cells of oleaginous yeast *Lipomyces starkeyi* using Bligh and Dyer procedure; these authors found that the hexane-isopropanol (3:2) solvent system, the chloroform-methanol (2:1) solvent system, and MTBE performed almost identically in terms of extracting lipids from the yeast cells, hexane was the worst, and chloroform alone was in between. On the basis of cost of extraction, the hexane-isopropanol system was least costly. Use of wet cell cake in the extractions resulted in 50 % loss of extraction efficiency, although this may not be as bad economically considering the high costs of drying the cells and the fact that lipid-bearing residual algae cake will have higher energy content due to presence of lipids as well as better nutritional value.

Graham (2011) explored the effect of drying temperature on the extent of extraction and composition of lipids from a local strain of *Scenedesmus* cultivated in open raceway ponds. The algae cells were freshly obtained and dried at three different drying temperatures (80, 109, and 180 °C) followed by extractions in an accelerated solvent extractor. The data show that gravimetric yields of lipids extracted from samples dried at lower temperatures were significantly higher than those dried at 180 °C samples. Furthermore, the yields of overall esterifiable lipids from the samples dried at 109 and 80 °C were nearly two orders of magnitude higher than those from the sample dried at 180 °C. ASE procedure was evaluated also by

Rulong et al. (2012) who found the ASE to have 'wide applicability'. These authors found that ethanol and acetone were the best solvents for extraction of lipids from marine microalga *Nannochloropsis oculata*. Increasing temperature and pressure greatly facilitated solvent access to the lipids and increased extraction rate as well as efficiency of extraction.

Ionic liquids (IL) have also been suggested as solvents for fast extraction of lipids from algae. Kim et al. (2012) used (Bmin)-based hydrophilic as well as hydropobic ionic liquids in a mixture with methanol to extract lipids from *Chlorella vulgaris* cells and found that ionic lipids were able to extract more lipids from cells than the Chloroform-methanol solvent mixture. The extraction efficiency of ionic liquids was dependent on the anionic nature of ionic lipids and addition of water to the mixture increased the efficiency of separation of lipids.

Another unique solution to recovery of intracellular lipids from algal cells has been proposed by Liu and Curtiss (2012). This method involves utilizing genetically engineered cells in which lipases are induced by keeping cells limited in $CO_2$ for a day, followed by incubating the cells at increased temperature (46 °C) for two days. Such a treatment resulted in production of extracellular free fatty acids. This technique, however, is more suitable for algae cultivated in closed photobioreactors than in open raceway ponds.

## 5 Techno-economic and Life-cycle Analyses

Techno-economic analysis of photoautotrophic algal cultivation for production of lipids has been conducted by several researchers recently (Davis et al. 2011). These studies are based on experimental data that are at best only partially confirmed at the scale at which the analyses are conducted. The base-level production data are generally obtained from a considerably smaller scale and even those are from studies conducted over relatively short periods (several weeks to months at most). Moreover, the analyses of larger scale operations necessarily involves considering longer distances over which production occurs and making informed assumptions regarding when and how harvesting and processing of algal biomass is to be conducted. The difficulties posed in deciding the harvesting and processing sequences can be deemed from the algal biofuel life-cycle-analysis report of Murphy et al. (2010). Depending on the selection of unit operations in an algae cultivation- and processing-facility, and the assumptions made regarding their efficiencies and operational costs, a number of economic scenarios emerge for algal biofuels. Realistic guidelines for choosing one operation over the other do not exist since little experience with actual large scale systems exists. This is in spite of the fact that various unit operations under consideration have been in use in other industries for a long time, and considerable research activity has been undergoing to develop basic biological systems.

A number of researchers have attempted to put together process flow sheets for algal biofuel production and conducted techno-economic analyses for different hy-

pothetical production scenarios. In one such analysis, Darzins et al. (2010) considered three different production scenarios for a plant with 10 million gallon per year lipid production capacity. These were (a) algal raceway pond productivity of 10 g $m^{-2}$ $day^{-1}$ with 10% lipids in cells based on the experience of Roswell pond cultivation conducted under DOE's Aquatic Species Program, (b) a hypothetical case in which cellular productivity remains same, but cells accumulate 40% lipid intracellularly, and (c) a third case where the oleaginous algae of case (b) can be cultivated with cell productivity of 50 g $m^{-2}$ $day^{-1}$. In each case, an efficient $CO_2$ transfer is hypothesized such that 2 kg $CO_2$ is needed per kg dry cell production. Only in case (c), a preliminary estimate of biodiesel cost get under $ 1 per liter. Such biomass productivities have been shown to take place under the most optimized conditions but not on a consistent basis under field conditions. Moreover, this case envisages a high lipid content as well which is also presently not feasible. All the techno-economic studies conducted so far confirm that the cost of production of algal biofuels under realistic present-day technology far exceed the cost of petroleum-derived fuels (Ribeiro and da Silva 2012; Davies et al. 2011; Andersson et al. 2011; Lundquist et al. 2010; Quinn et al. 2011; Williams and Laurens 2010). A very detailed life cycle analysis of large-scale algae cultivation in outdoor raceway ponds by Murphy et al. (2010) demonstrated the difficulties that will be faced in the area of materials handling and the real bottleneck that one can expect during harvesting of cells. The biggest problems occur because several of the steps in harvesting are likely to be too slow and energy intensive. The problem is exacerbated by the fact that the algal biomass is fragile in nature and it is rapidly spoiled once it is taken out of its natural environment in the cultivation system. Moreover, biofuels are a bulk commodity for which it is difficult to draw a significant premium. Other components in the residual algal cake are potentially high value items and it will be necessary to develop efficient fractionation processes for such compounds.

## 6 Conclusions

In summary, algae have potential to be carbon sources for future needs of fuels and chemicals. But there are major technical roadblocks in their utilization. Future of biofuels from algae depends on the following technical developments:

1. For each kg of neutral lipids produced by the cells, four or more kgs of residual cake is produced. Given the large amounts of biofuels that are targeted for production, it will be necessary to find useful avenues for utilization of the residual cake.
2. The cake residue has potentially high value components that need to be identified and for which efficient fractionation methods need to be developed.
3. There is a need for techniques (a) that can separate and concentrate algal cells from broths rapidly with ultralow energy consumption (of the order of 0.01 kWh per $m^3$ fluid processed), and (b) that can be easily automated and

scaled-up for local use so that transport of dilute cell suspensions even over short distances can be avoided.
4. Ultimately, sustained cultivation of dominant algal species in septic open systems with reuse of a substantial part of spent medium needs to be demonstrated and documented.

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# Algal Reactor Design Based on Comprehensive Modeling of Light and Mixing

**Alexandra D. Holland and Joseph M. Dragavon**

**Abstract** The prospect of autotrophic (or light-driven) algal biomass production as a sustainable substitute for fossil feedstocks has yet to fulfill its potential. As a likely cause, the inability to robustly account for algal biomass production rates has prevented the derivation of satisfactory mass balances for the simple parameterization of bioreactors. The methodology presented here aims at resolving this shortcoming. Treating photons as a substrate continuously fed to algae provides the grounds to define an autotrophic yield $\Phi^{DW}$, in grams of dry weight per mole of photons absorbed, as an operating parameter. Under low irradiances, the rate of algal biomass synthesis is the product of the yield $\Phi^{DW}$ and the flux of photons absorbed by the culture, modeled using a scatter-corrected polychromatic Beer-Lambert law. This work addresses the broad misconception that Photosynthesis-Irradiance curves, or the equivalent use of specific growth rate expressions independent of the biomass concentration, can be extended to adequately model biomass production under light-limitation. Since low photon fluxes per cell maximize $\Phi^{DW}$, the photosynthetic units mechanistic model was adapted to determine a corresponding maximum residence time under high light. Such high speeds in the photic zone, which call for fundamental changes in bioreactor design, enable the use of $\Phi^{DW}$ to describe biomass productivity under otherwise inhibitory irradiances. Nitrogen limitation-induced lipid accumulation corresponds to a photon flux excess with respect to the rate of nitrogen uptake, such that continuous lipid production can be achieved using the $\Phi^{DW}$ and nitrogen quotient parameters. Additionally, energy to photon-counts conversion factors are derived.

**Keywords** Algal chemostat parameterization · Algal growth autotrophic yield · Continuous algal lipid production · Photic zone target speed · Photosynthetic units mechanistic model · Scatter-corrected polychromatic Beer-Lambert law

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## Abbreviations and Nomeclature

### A. Abbreviations

| | |
|---|---|
| AM | Air-mass |
| AU | Absorbance unit |
| CARPT | Computer-automated radioactive particle tracking |
| Chl *a* | Chlorophyll *a* |
| DW | Dry weight |
| ELT | Exponential-to-linear |
| LHS | Left hand side |
| NPQ | Non-photochemical quenching |
| NREL | National Renewable Energy Laboratory |
| PAR | Photosynthetically active radiation (400-700 nm) |
| PI | Photosynthesis-irradiance |
| PPFD | Photosynthesis photon flux density |
| PQ | Plastoquinone |
| PSI | Photosystem I |
| PSII | Photosystem II |
| PSU | Photosynthetic unit |
| REC | Reduced carrier |
| $Q_A$ | Quinone A |
| SC | Scatter-corrected |

### B. Variables and Corresponding Units

| | |
|---|---|
| $a$ [$mol_{PSII}$] | Number of open of PSII centers (or oxidized) |
| $a^*$ [$mol_{PSII}$] | Number of closed of PSII centers (or reduced) |
| $a_0$ [$mol_{PSII}$] | Total number of PSII centers |
| $Abs_{RAW}(\lambda)$ [AU] | Raw algal absorption at wavelength λ |
| $Abs_{SC}(\lambda)$ [AU] | Scatter-corrected algal absorption at wavelength λ |
| $Abs_{SCATTER}(\lambda)$ [AU] | Scatter contribution to algal absorption at wavelength λ |
| $A_C$ [$m^2$] | Area of the culture perpendicular to the light source |
| $C$ [$g_{DW}\ m^{-3}$] | Algal culture biomass concentration in the bioreactor |
| $c$ [$m\ s^{-1}$] | Celerity of light |
| $C_0$ [$g_{DW}\ m^{-3}$] | Algal culture biomass concentration at inoculation time $t_0$ |
| $C_E$, [$g_{DW}\ m^{-3}$] | Culture biomass concentration during spectrum acquisition |
| $c_{EJ}$ [$E\ J^{-1}$] | Einstein-to-Joules conversion factor |
| $C_{PI}$ [$g_{DW}\ m^{-3}$] | Algal biomass concentration in the PI chamber |
| $d$ [m] | Depth of the photic zone, where light is $>99\%\ I_0$ |
| $E_P(\lambda)$ [$W\ m^{-2}\ nm^{-1}$] | Photon energy reported for each wavelength increment $d\lambda$ |
| $EF(x)$ μE $g_{DW}^{-1}\ h^{-1}$ | Specific energy flux at depth $x$ |
| $EF_T$ μE $g_{DW}^{-1}\ h^{-1}$ | Threshold specific energy flux at onset of light limitation |
| $E_{LIGHT}(\lambda)$ [counts $nm^{-1}$] | Light source emission spectrum at λ |
| $F_{CHEM}$ [$m^3\ h^{-1}$] | Chemostat volumetric flow rate (bioreactor) |

| | |
|---|---|
| $F$ [$mol_{PSII}\ g_{DW}^{-1}$] | Weight fraction of PSII |
| $F_{IN}$ [$m^3\ h^{-1}$] | Inlet stream volumetric flow rate (bioreactor) |
| $F_{OUT}$ [$m^3\ h^{-1}$] | Outlet stream volumetric flow rate (bioreactor) |
| $F_{PAR}$ [-] | Fraction of energy in the PAR region |
| $h$ [SI Units] | Planck's constant |
| $I(x)$ [$\mu E\ m^{-2}\ h^{-1}$] | Local PPFD at a given depth $x$ |
| $I_0$ [$\mu E\ m^{-2}\ h^{-1}$] or [$\mu E\ m^{-2}\ s^{-1}$] | Incident photosynthesis photon flux density (PPFD) |
| $I_{ABS}$ [$\mu E\ m^{-2}\ h^{-1}$] | Absorbed PPFD by the algal culture |
| $I_H$ [$\mu E\ m^{-2}\ s^{-1}$] | Highest possible direct normal solar irradiance |
| $I_{OUT}$ [$\mu E\ m^{-2}\ h^{-1}$] | PPFD transmitted through the algal culture |
| $I_T$ [$\mu E\ m^{-2}\ s^{-1}$] | Threshold irradiance at which NPQ becomes significant |
| $k_1$ [$s^{-1}$] | Rate of PSII excitation |
| $k_2$ [$s^{-1}$] | Rate of PSII relaxation |
| $L$ [m] | Depth of the culture |
| $L_E$ [m] | Pathlength of the light through the spectrophotometer |
| $L_{PI}$ [m] | Depth of the PI chamber |
| $m_P$ [$\mu E\ g_{DW}^{-1}\ h^{-1}$] | Maintenance parameter |
| $\dot{n}(\lambda)$ [$E\ s^{-1}\ m^{-2}\ nm^{-1}$] | Photon flux reported for each wavelength increment $d\lambda$ at $\lambda$ |
| $Na$ [$mol^{-1}$] | Avogadro's constant |
| $OD$ [AU] | Algae culture absorbance at 680 nm |
| $P$ [$g_{DW}\ m^{-2}\ h^{-1}$] | Algal biomass area productivity |
| $P(\lambda)$ [cps] | Spectrometer reading (in counts per second) |
| $P_{BIOREACTOR}$ [$g_{DW}\ m^{-3}\ h^{-1}$] | Bioreactor productivity |
| $P_i$ [$g_{DW}\ h^{-1}$] | Zone i contribution to the algal biomass productivity |
| $P_i^V$ [$g_{DW}\ m^{-3}\ h^{-1}$] | Local volumetric biomass production rate in zone i |
| $P_{LIGHT}(\lambda)$ [$nm^{-1}$] | Normalized light-source photon fraction at $\lambda$ |
| $P_{LIPIDS}$ [$g_{LIPIDS}\ h^{-1}$] | Lipid productivity |
| $P_{MAX}$ [$g_{DW}\ m^{-2}\ h^{-1}$] | Maximum algal biomass area productivity (light-limited) |
| $P_{SUN}(\lambda)$ [$nm^{-1}$] | Normalized solar spectrum photon fraction at $\lambda$ |
| $qL$ [-] | Fraction of open PSII centers |
| $qN$ [-] | Fraction of closed PSII centers |
| $Q_N$ [$g_N\ g_{DW}^{-1}$] | Nitrogen weight fraction (or nitrogen quotient) |
| $S$ [$g_S\ m^{-3}$] | Substrate S concentration in the bioreactor |
| $S_0$ [$g_S\ m^{-3}$] | Inlet stream substrate S concentration |
| $t$ [h] | Time in the light phase, truncated for duration in the dark |
| $t$ [s] | Time scale for the PSU model |
| $t_0$ [h] | Reference inoculation time |

| | |
|---|---|
| $u$ [-] | PSU model integrating factor (L or S subscript indicates linear or sinusoidal trajectory submodel respectively) |
| $V_C$ [$m^3$] | Culture volume in bioreactor |
| $v_T$ [$m\ s^{-1}$] | Target velocity in the photic zone for near maximum $\Phi^{PSII}$ (additional L or S subscript indicates linear or sinusoidal trajectory submodel respectively) |
| $x$ [m] | Distance from the light incidence surface |
| $x_T$ [m] | Threshold depth (onset of light limitation in poorly-mixed reactor) |
| $Y_{C/S}$ [$g_{DW}\ g_S^{-1}$] | Biomass yield on the substrate S |
| $Y_{C/N}$ [$g_{DW}\ g_N^{-1}$] | Biomass yield on nitrogen substrate |
| $\beta$ [-] | Proportionality constant between the spectrometer count reading and the incident photon flux |
| $\lambda$ [nm] | Wavelength |
| $\mu$ [$h^{-1}$] | Specific growth rate |
| $\mu_{MAX}$ [$h^{-1}$] | Maximum specific growth rate |
| $\sigma$ [$m^2\ g^{DW}$–1] | Monochromatic absorption cross section |
| $\sigma^{DW}$ [$m^2\ g_{DW}^{-1}$] | Scatter-corrected algae-specific light source-dependent absorption cross section |
| $\tau$ [s] | Time for the incident light to excite half the threshold PSII fraction |
| $\psi(\lambda)$ [$m^2\ g_{DW}\ m^{-2}$] | Hyperbolic model parameter |
| $\omega(\lambda)$ [$m^2\ g_{DW}^{-1}$] | Hyperbolic model parameter |
| $\Phi^{APP}$ [$mol_{CO2}\ E^{-1}$] | Apparent efficiency parameter in mole $CO_2$ fixed per mole incident photons |
| $\Phi^{CO2}$ [$mol_{CO2}\ E^{-1}$] | Quantum yield |
| $\Phi^{DW}$ [$g_{DW}\ \mu E^{-1}$] | Autotrophic yield |
| $\Phi^{C2}$ [$mol_{OZ}\ E^{-1}$] | Quantum yield |
| $\Phi^{PSII}$ [-] | Photon fraction used to excite the $Q_A$ pool, or PSII operating efficiency |

## 1 Introduction

The prospect of autotrophic (or light-driven) algal biomass production as a sustainable substitute for fossil feedstocks holds promise, but has yet to fulfill its potential. Arguably, the discrepancy between theoretical and achieved productivities in the field results from the lack of a working comprehensive algal growth model to guide bioreactor design. Akin to the petroleum industry in the early 50's, distillation of crude oil heavily relied on trial-and-error and was as a result very wasteful. In the mid-50's, scientific contributors such as John Prausnitz pioneered molecular thermodynamics to model the behavior of such complex chemicals mixtures (Sanders 2005). The resulting ability to predict these separation properties has revolutionized

the petroleum industry, and is the cornerstone of all petrochemical processes. The approaches introduced by Holland et al. (Holland et al. 2011; Holland and Wheeler 2011), further detailed in this chapter, hold the potential to provide such model for industrial algal biomass production processes, guiding bioreactor design and parameterization to maximize biomass and lipid productivity.

The inherent particle nature of light as a growth substrate has been broadly overlooked. Treating photons as a substrate continuously fed to algae provides the grounds to define an autotrophic yield, which is key for comparing productivities as well as parameterizing bioreactors. Indeed, within the Photosynthetically Active Radiation (PAR) region, regardless of its energy, an absorbed photon exciting the photosynthetic apparatus drives carbon fixation and therefore biomass synthesis. As such, the concept of biomass yield, reported for heterotrophic growth as biomass produced per mass of input sugar substrate, translates to its autotrophic counterpart by normalizing the biomass produced per number of input photons. The unit of choice for photon counting is the Einstein (E), or mole of photons in the PAR region.

Importantly, the goal of algal bioreactor designs is to maximize yield—not solely productivity. Sun-lit outdoor ponds require land area while artificially lit bioreactors require a primary energy source (wind power or other). Hence light is an expensive substrate that should not be wasted. Biomass productivity is the product of the autotrophic yield per absorbed photon flux. Notably, under conditions of complete absorption of the photons by the algal culture, maximum yield leads to maximum productivity (Sect. 2.1). However, the converse does not hold (Sect. 3.2). Most often, algal productivities are reported (in mass per time per volume or area) with omitted incident light levels or incomplete reactor geometries. This, in turn, precludes yield-based performance comparisons between the various characterized systems. The work presented here introduces routine determination of the algal autotrophic yield as the key parameter for setup evaluation.

Current efforts toward modeling light as a nutrient treat the algal population as a whole system, whose growth rate follows saturation kinetics (Sect. 3.2). For chemical substrates, the Monod saturation kinetics reflect that the microbial population growth rate increases with increasing concentration, and saturates when the substrate reaches a concentration greater than its uptake affinity. In chemostat bioreactors, such microbial populations reach highest productivities at high substrate concentrations supporting near maximum growth rates. For light as a substrate, Photosynthesis-Irradiance (PI) curves describe the saturation behavior of the algal population growth rate (or specific rate of biomass increase) as a function of incident light. In a given bioreactor, while productivity increases with incident light levels until light excess is reached, the biomass yield decreases. As proof, at a given biomass concentration with known cell geometry, PI curve data can be used to calculate the biomass yield (from the ratio of specific growth rate to irradiance), which shows a maximum at low irradiance. As further evidence, fluorescence response studies show highest quantum yields at low light levels (Sect. 3.1). In the authors' opinion, an apparent analogy between PI curves and Monod saturation kinetics has laid the ground for widespread misleading analyses for biomass productivity calculations as well as optimization.

Once established that highest yields are reached at low light intensity, inhibitory light levels reached at the surface of most outdoor systems can be treated using two distinct methodologies. The more widespread approach is to model cell damage and energy losses as unavoidable consequences of growth. As a contrast, the work presented here uses the same mechanistic model to derive bioreactor characteristics enabling highest yields under high irradiance. Indeed, in a dense algal culture, high speeds across the photic zone allows for high frequency light-dark fluctuations, which therefore reduce photon flux per cell to levels conducive to high yield biomass production. Through deriving target bioreactor properties from strain attributes, this new paradigm provides a reliable framework to estimate outdoor productivities from yields determined experimentally under low light.

Provided adequate agitation to sustain high yield biomass production, steady-state biomass production can be easily parameterized using the autotrophic yield. Achieving such a steady-state is key to maximizing productivity. The set of simple equations presented in this work, the validity of which hinges on vigorous mixing conditions under high irradiance, averts the complex control strategies detailed in the literature.

Algal lipid accumulation has been broadly documented under nitrogen limitation in growth arrested cultures. However, growth arrest lowers overall lipid productivity and can lead to erroneous productivity projections (Wilhelm and Jakob 2011; Rodolfi et al. 2009). The concept of light as a continuously fed substrate brings about a different understanding of such lipid accumulation. Namely, lipid accumulation corresponds to a photon flux excess with respect to the flux of nitrogen molecules taken up by the culture, which can be parameterized under steady-state (Sect. 5.3). Upon determination of the culture autotrophic and nitrogen yields under nutrient-replete conditions, the nitrogen flux is lowered gradually until lipid production is achieved—at the cost of a lowered overall dry-weight productivity. This chapter details the methodology to achieve continuous autotrophic lipid production.

## 2 Sustainable Algal Lipid Production: Current Achievements and Upcoming Prospects

### *2.1 Biomass and Lipid Production Estimates*

Algal lipids have been widely promulgated as a precursor to renewable transportation biofuels. Stress-induced autotrophic lipid accumulation has been documented in many algal species (Rodolfi et al. 2009; Griffiths and Harrison 2009), including phosphate limitation in *Ankistrodesmus falcatus* (Kilham et al. 1997), silicon and nitrogen deficiency (Tornabene 1983; Sheehan et al. 1998; Shifrin and Chisholm 1981) and alkaline pH stress in *Chlorella* sp. (Guckert and Cooksey 1990). However, lipid accumulation under these stress conditions—on the order of 20–40 % on a dry weight (DW) basis—have invariably been associated with prolonged growth arrest or severe growth rate reduction (Reitan et al. 1994; Gressel 2008).

**Table 1** Example increase in dry weight specific energy in nitrogen-limited algal cells

| | Mass fraction[b] | |
|---|---|---|
| | N-rich | N-limited |
| Carbohydrates (15.7 kJ $g_{DW}^{-1}$)[a] | 0.52 | 0.35 |
| Lipids (37.6 kJ $g_{DW}^{-1}$)[a] | 0.08 | 0.3 |
| Proteins (16.7 kJ $g_{DW}^{-1}$)[a] | 0.4 | 0.35 |
| DW specific energy (kJ $g_{DW}^{-1}$)[c] | 17.9 | 22.6 |

[a] Values from (Rebolloso-Fuentes et al. 2001)
[b] Representatitve values ((Holland et al. 2011) and unpublished data)
[c] DW specific energy ratio of N-rich to N-limited is 0.79

Reported outdoor algal biomass productivities are on the order of 20–40 $g_{DW}$ $m^{-2}$ $d^{-1}$ (Capo et al. 1999; Lundquist et al. 2010) under nutrient-replete conditions for average yearly irradiances of 390 μE $m^{-2}$ $s^{-1}$ (such as in southern US latitudes). As an oft-neglected consequence, the corresponding upper bound of lipid productivity (16 $g_{LIPIDS}$ $m^{-2}$ $d^{-1}$ or 7,400 gal $acre^{-1}yr^{-1}$ at a lipid density of 850 g $L^{-1}$ and 40 % lipids) needs to be lowered to account for the duration of the culture maturation and growth arrest. Indeed, the sole requirement of a one-day nitrogen starvation period to achieve high lipid content in a culture growing at 40 $g_{DW}$ $m^{-2}$ $d^{-1}$ would half the above lipid productivity upper bound estimate to 8 $g_{LIPIDS}$ $m^{-2}$ $d^{-1}$.

Upon nitrogen limitation and subsequent lipid accumulation, the cell specific energy increases due to the higher specific energy of lipids, which is illustrated in Table 1 with representative values of algal cell compositions. Assuming a constant photosynthetic efficiency despite mild stress, nitrogen-limited lipid productivity estimates from nitrogen-replete productivity data should reflect the difference in DW specific energy, and should therefore be multiplied by 0.79 for the example given in Table 1.

Measured quantum efficiencies of 0.102 $g_C$/mole photons in algae (Cleveland et al. 1989) correspond to an achievable productivity of 82 $g_{DW}$ $m^{-2}$ $d^{-1}$, assuming 50 % C on a dry weight basis (Kroon and Thoms 2006) and average yearly irradiances of 390 μE $m^{-2}$ $s^{-1}$. Such two- to four- fold increase in large-scale algal biomass productivity may be achievable using the methodology and insights provided in this work. Furthermore, understanding algal metabolism in a way to achieve continuous lipid production at high biomass productivities would permit lipid productions on the order of 16 $g_{LIPIDS}$ $m^{-2}$ $d^{-1}$ (25 % harvestable lipids from cells containing 30 % on a DW basis, at 390 μE $m^{-2}$ $s^{-1}$, and corrected for higher specific energy content of lipid-rich cells as in Table 1) or 7,500 gal $acre^{-1}yr^{-1}$ (at a lipid density of 850 g $L^{-1}$). For comparison with crop-based agriculture, Malaysia palm oil productivity was 473 gal $acre^{-1}$ $yr^{-1}$ in 2008 (Malaysian Palm Oil Industry Performance 2008 (Anon 2009), with a density of 890 g $L^{-1}$), which is 16-fold less than the projected algal lipid productivity.

## 2.2 Irradiance Unit Conversions

Energy calculations of achievable productivity depend on the measurement of incident light as a Photosynthesis Photon Flux Density (PPFD, in μE $m^{-2}$ $s^{-1}$) in the PAR region between 400 nm and 700 nm. While quantum meters readily provide such measurements, outdoor light levels are commonly reported in W $m^{-2}$ using a pyranometer. Unit conversions between photon flux and energy are derived using corresponding light source spectra.

Two geometries of sensors are commonly used in the field to measure incident light, either as energy per area or photon flux per area. The more common 2π half-sphere sensors measure light incident onto a surface, and the 4π full-sphere sensors measure light incident from all directions. The use of 4π sensors, which can give readings up to twice those of 2π sensors, is more relevant for bioreactors at an angle from the ground, whereas 2π sensors are more relevant for pond configurations. For complex reactor geometries, (Sánchez Mirón et al. 2000) used chemical actinometry to measure the precise incident PPFD. In our analysis below, we assume the use of 2π sensors to quantify direct normal-incident PPFD.

Solar radiation spectra are typically reported as a plot of photon energy $E_p(\lambda)$ (in W $m^{-2}$ $nm^{-1}$) measured for each wavelength increment $d\lambda$ (ASTM 2003; Thuillier et al. 2003). The photon energy $E_p(\lambda)$ is proportional to the photon flux $\dot{n}(\lambda)$:

$$E_P(\lambda)d\lambda = \dot{n}(\lambda) \cdot Na \cdot \frac{hc}{\lambda} d\lambda \tag{1}$$

where $h$ is Planck's constant in S.I. units; $c$ the celerity of light in m $s^{-1}$; $Na$ is Avogadro's constant in $mol^{-1}$; $E_p(\lambda)$ is the energy reported for each wavelength increment $d\lambda$ at λ in W $m^{-2}$ $nm^{-1}$; $\dot{n}(\lambda)$ is the photon flux reported for each wavelength increment $d\lambda$ at λ in Einstein $s^{-1}$ $m^{-2}$ $nm^{-1}$. These solar spectra can thus be used to convert units of Einstein and Joules ($c_{EJ}$ in units of E $J^{-1}$), with Einstein as the photon flux in the PAR region, and total energy measured in the wavelength range $\lambda_1$- $\lambda_2$:

$$c_{EJ} = \frac{\int_{400}^{700} \dot{n}(\lambda)d\lambda}{\int_{\lambda 1}^{\lambda 2} E_P(\lambda)d\lambda} = \frac{10^{-9}}{h \cdot c \cdot Na} \frac{\int_{400}^{700} \lambda \cdot E_P(\lambda)d\lambda}{\int_{\lambda 1}^{\lambda 2} E_P(\lambda)d\lambda} \tag{2}$$

Analogously, spectra measured in W $m^{-2}$ $nm^{-1}$ can be converted to a normalized photon flux frequency $P_{SUN}(\lambda)$ in $nm^{-1}$ in the PAR region (Fig. 1):

$$P_{SUN}(\lambda) = \frac{\lambda \cdot E_P(\lambda)}{\int_{400}^{700} \lambda \cdot E_P(\lambda)d\lambda} \tag{3}$$

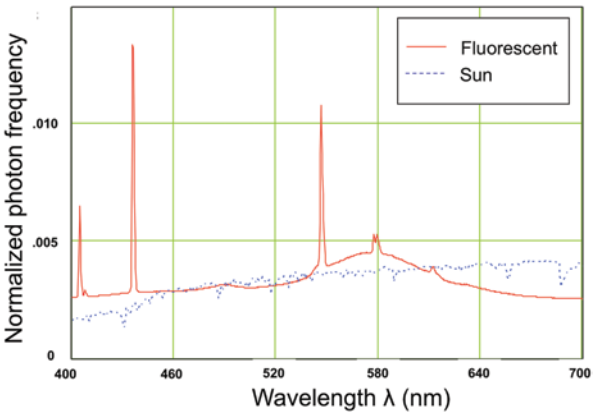


**Fig. 1** Normalized photon frequency in the PAR region for the acquired fluorescent light spectrum and calculated for the ASTM ground direct-normal spectrum (Eq. 3)

The fraction of energy in the PAR region, given a total energy measured in the wavelength range $\lambda_1$–$\lambda_2$, is:

$$F_{PAR} = \frac{\int_{400}^{700} E_P(\lambda)d\lambda}{\int_{\lambda 1}^{\lambda 2} E_P(\lambda)d\lambda} \tag{4}$$

Percent energy in the PAR region as well as Einstein-to-Joules conversion factors are reported in Table 2. Outer space (Space) spectrum data was kindly provided by Dr. Thuillier (Thuillier et al. 2003). For ground irradiance, ASTM spectra (ASTM 2003) were used as reference ground spectra, for a 37° tilted surface (Tilted) and a direct-normal surface (Flat). The ASTM spectra are reported for an air-mass (AM) coefficient of 1.5, which provides a description on the relative light attenuation due to atmospheric water vapor concentration (Mecherikunnel et al. 1983), at conditions still conducive to photovoltaic applications (ASTM 2003). The wavelength ranges were chosen to reflect apparatus available commercially, such as a Li-Cor pyranometer, usually 400–1100 nm range, (Kania and Giacomelli 2001) or a Precision Spectral Pyranometer (PSP, 285–2800 nm range), used by the NREL (http://rredc.nrel.gov/solar/pubs/redbook/) for its solar radiation measurements.

Despite marked variations in the overall sun spectrum due to an air-mass coefficient AM of 1.5, the conversion factors and percent energy calculations do not vary significantly between ground and outer-space data (Table 2). This is likely due to the fact that the ground solar spectrum in the PAR region changes drastically in shape for AM >1.5 but not below (Mecherikunnel et al. 1983). These tabulated values provide an updated tool which should help prevent the use of erroneous conversion factors (Kania and Giacomelli 2001).

**Table 2** Einstein-to-Joules conversion factors $c_{EJ}$ and Percent energy in the PAR region. An outer space spectrum (Space), an ASTM 37° tilted ground spectrum (Tilted) and an ASTM ground direct-normal spectrum (Flat) were used

| | $c_{EJ}$ in μE J$^{-1}$ | | | % Energy in the PAR region | | |
|---|---|---|---|---|---|---|
| Ref. spectrum range | 200–2400 nm | 280–4000 nm | | 200–2400 nm | 280–4000 nm | |
| Ref. spectrum | Space | Flat | Tilted | Space | Flat | Tilted |
| PAR (400–700 nm) | 4.55 | 4.63 | 4.60 | 100 | 100 | 100 |
| Li-Cor (400–1100 nm) | 2.67 | 2.55 | 2.61 | 58.7 | 55.1 | 56.7 |
| PSP (285–2800 nm) | 1.84 | 1.95 | 1.99 | 40.5 | 42.0 | 43.3 |
| Overall sun spectrum | 1.83 | 1.93 | 1.98 | 40.2 | 41.6 | 43.0 |

As photosynthesis is known to occur in the near-UV range between 350 and 400 nm (Sakshaug and Johnsen 2006), the various reference spectra were used to calculate the % photon flux in the near-UV range compared to the flux in the 350–700 nm range (near UV+PAR). These values were 5.76% (Space), 3.94% (Flat) and 4.89% (Tilted), such that the near-UV contribution can be mostly neglected for outdoor level estimates.

Spectrometers allow for the acquisition of light source spectra in the PAR region, where the count reading $P(\lambda)$ in a given increment $d\lambda$ is proportional to the photon flux $\dot{n}(\lambda)$ by a constant β:

$$P(\lambda) = \beta \cdot \dot{n}(\lambda) = \frac{\beta}{h \cdot c \cdot Na} \cdot \frac{E_P(\lambda)}{\lambda} \tag{5}$$

These relative photon-count spectra can therefore be used to calculate $c_{EJ}$ (in units of E J$^{-1}$) the conversion from Einstein to Joules (or E s$^{-1}$ to W), as shown above, where both PPFD and energy are measured in the PAR region:

$$c_{EJ} = \frac{10^{-9}}{h \cdot c \cdot Na} \frac{\int_{400}^{700} P(\lambda) d\lambda}{\int_{400}^{700} \frac{P(\lambda)}{\lambda} d\lambda} \tag{6}$$

Two $P(\lambda)$ spectra for fluorescent light sources of different intensities were acquired using an Ocean Optics spectrometer (in the 400–700 nm range), in both cases resulting in calculated conversion factor $c_{EJ}$=4.49 μE J$^{-1}$. An incident PPFD of 50 μE m$^{-2}$ s$^{-1}$, for example, provides a culture with an energy of 11.1 W m$^{-2}$.

## 2.3 Sustainability Considerations

Achieving sustainable biomass production from algae entails a comprehensive analysis of the overall process, for which all feedstocks and energy sources should be renewable. Hence, providing flue gas from coal fired plants as a $CO_2$ source

(Vunjak-Novakovic et al. 2005), chemical fertilizers as nutrient sources, or nuclear energy to power algal bioreactors represent examples of unsustainable processes. Since sugar feedstocks are currently plant-derived and therefore require fossil-based pesticides, fertilizers and processing, algal lipid production under heterotrophic conditions (Xu et al. 2006; Liang et al. 2009) does not constitute a long term transportation fuel energy solution.

Large-scale production relies on the supply of tremendous volumes of freshwater. Indeed, while the prospect of using seawater algae seems attractive, algal biomass processing requires mechanical steps sensitive to corrosion by seawater. In addition, evaporation leads to an inhibitory increase in the bioreactor salinity unless fresh make-up water is added or unless the operation is periodically shut down and restarted. Efficient water recycling provides a partial solution to the environmental impact associated with such water management. Unlike plants, algae do not rely on evaporative cellular processes to avert overheating under high light, such that the net area water consumption of algae is comparatively lower than crops provided water recycling (Table 1 in Yang et al. (2010) and Table 2 in Gerbens-Leenes et al. (2009)). Processing algal biomass at a concentration of 2 $g_{DW}/L$ and 25 % harvestable lipids (density of 850 g $L^{-1}$) contributes 1700 $L_{WATER}/L_{LIPIDS}^{-1}$, but only 85 $L_{WATER}/L_{LIPIDS}^{-1}$ with 95 % recycling. Evaporation rates estimated from US "pan" evaporation data (Farnsworth and Thompson 1982) are on the order of 55–120 cm $d^{-1}$, which represent 285–520 $L_{WATER}/L_{LIPIDS}^{-1}$ for an algal lipid production of 16 $g_{LIPIDS}$ $m^{-2}$ $d^{-1}$. These values are much lower than rapeseed (14,200 $L_{WATER}/L_{LIPIDS}^{-1}$) or Jatropha (19,900 $L_{WATER}/L_{LIPIDS}^{-1}$) (Gerbens-Leenes et al. 2009). Nevertheless, rainwater collection, desalination and/or wastewater supply are key to reduce adverse environmental effects associated with freshwater consumption.

The sustainable supply of nutrients can be achieved through integration of Anaerobic Digesters (AD) in various configurations. AD microbial populations metabolize residual high energy carbon from the fed biomass into biogas, which is a mixture of about 55 % methane and 45 % $CO_2$, and a concentrated NP-rich effluent (Lansche and Müller 2009; Möller and Müller 2012; Nasir et al. 2012). Oswald and co-workers realized visionary designs integrating AD and algal biomass production as early as the 50's (Oswald and Golueke 1960; Golueke et al. 1957; Golueke and Oswald 1959; Bailey Green et al. 1996). In the open-loop configuration (Fig. 2), light energy is used to convert organic waste streams (such as manure) into lipids, clean water and a residual biomass rich in protein and carbohydrates. The high oxygen content of the algal pond reduces the pathogen count of the waste stream (Mata-Alvarez et al. 2000), such that the residual biomass can be used directly as fertilizer (Mulbry et al. 2005), animal feed (Wilkie and Mulbry 2002), or further processed into sugars. Once primed with nutrients, the closed configuration (Fig. 3) results in the net conversion of light energy and water into lipids. Valorization of the biogas into energy produces a $CO_2$ stream which is combined with the air stream and bubbled into the algae photobioreactor. Achieving sustained water and nutrients recycling necessitates the use of biodegradable flocculating agents such as bacterial cultures (Kurane et al. 1986; Oh et al. 2001), cationic starches (Pal et al. 2005) or biopolymers such as chitosan (Divakaran and Sivasankara Pillai 2002).

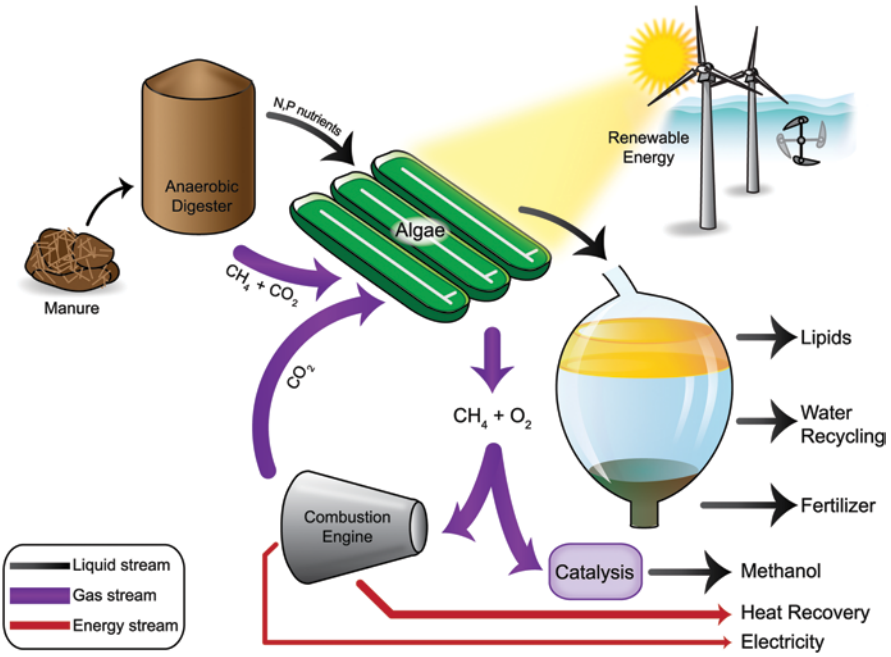


**Fig. 2** Open-loop configuration (wastewater to biofuel, clean water and fertilizer)

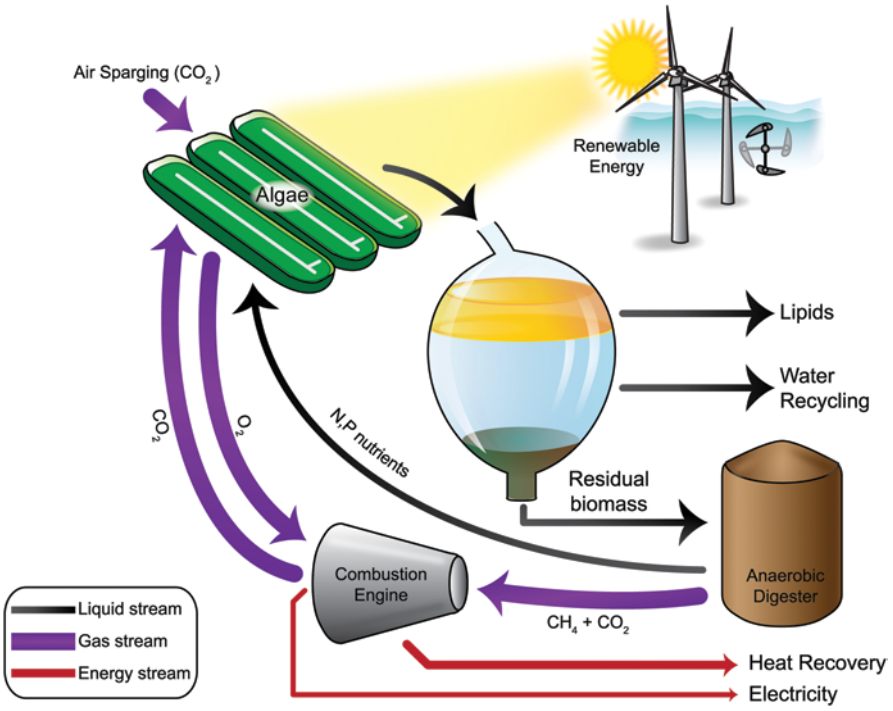


**Fig. 3** Closed-loop configuration (atmospheric $CO_2$ and water to lipids and methane)

# 3 Autotrophic Biomass Yield $\Phi^{DW}$ and Scatter-corrected Extinction Coefficient $\sigma^{DW}$

## *3.1 Algal Biomass Yield $\Phi^{DW}$*

Autotrophic batch algal cultures receive a continuous supply of photons as their source of energy. At low light regimes, most absorbed photons (>80%) are used for photochemical reactions (Baker 2008), which is reflected by an elevated quantum efficiency (in mole $CO_2$ absorbed per Einstein). Assuming a nutrient-replete environment and low light, algal autotrophic growth in a batch reactor (such as a flask) is analogous to heterotrophic bacterial growth in fed-batch, for which energy is provided by a continuously fed organic carbon substrate (such as glucose). For heterotrophs, vigorous mixing ensures that the fed substrate is homogeneously distributed and taken-up within the culture, which enables the determination of a yield ($g_{DW}\ g_{SUBSTRATE}^{-1}$) to predict the culture growth behavior (Blanch and Clark 1997; Yamanè and Shimizu 1984). In a dense illuminated culture, the photon flux per cell is inherently inhomogeneous, due to the exponential decrease of flux as a function of depth (Yun and Park 2003). However, under conditions of low photon flux per cell, the rate of biomass production occurs at its maximum quantum efficiency everywhere in the culture, such that, on average (spatial and temporal), the rate of biomass production is proportional to the rate of light absorption by the algal culture. As discussed in Sect. 4, low photon flux per cell can be achieved under low irradiance, or under elevated light with vigorous mixing.

The fed-batch analogy guides the establishment of the algal growth behavior descriptive equations and the existence of an intrinsic autotrophic yield $\Phi^{DW}$, expressed in $g_{DW}\ \mu E^{-1}$.

Nutrient-replete algal growth under excess low light follows an exponential behavior in batch cultures, independent of the light input and is described by:

$$\frac{d(V_C \cdot C)}{dt} = \mu \cdot V_C \cdot C \tag{7}$$

where $t$ is the duration (in h) in the light phase; $C$ is the algal culture biomass concentration (in $g_{DW}\ m^{-3}$) at time $t$; $V_C$ is the batch culture constant volume (in $m^3$); $\mu$ is the algal culture specific growth rate (in $h^{-1}$). In the dark phase, the supply of energy to the algal culture is effectively interrupted. Hence, the time $t$, as used in this work, represents the cultivation time in the light phase, which is the total growth duration reduced by the duration in the dark.

As the algal culture density increases, the light input becomes limiting. The culture biomass production rate transitions to a non-exponential behavior, and the following equation describes the system behavior, as based on derivations for fed-batch heterotrophic cultures (Yamanè and Shimizu 1984):

$$\frac{d(V_C \cdot C)}{dt} = \Phi^{DW}\left[I_0 \cdot A_C - I_{OUT} \cdot A_C - m_P \cdot V_C \cdot C\right] \tag{8}$$

where $I_0$ is the incident Photosynthesis Photon Flux Density (PPFD, in μE $m^{-2}$ $h^{-1}$); $A_C$ is the area of the culture perpendicular to the light source (in $m^2$); $I_{OUT}$ is the transmitted/scattered PPFD (in μE$m^{-2}$ $h^{-1}$); $\Phi^{DW}$ is the autotrophic yield (in $g_{DW}$ $\mu E_{absorbed}^{-1}$); $m_P$ is the maintenance energy to sustain biomass (in μE $g_{DW}^{-1}$ $h^{-1}$). $I_0$ can be routinely measured at the algal culture-incident light interface using a quantum meter.

Measurements of cellular parameters have shown that housekeeping metabolism in the dark is minimal (G. Finazzi, personal communication and Finazzi and Rappaport (1998)). In addition, biomass loss was consistently not observed during the dark phase (Holland et al. 2011). Thus, the algal biomass maintenance parameter is considered negligible, setting: $m_P = 0$.

As a consequence, at all times of growth, the rate of biomass production is proportional to the amount of light absorbed by the culture:

$$\frac{d(V_C \cdot C)}{dt} = \Phi^{DW} A_C I_{ABS} \tag{9}$$

in which $I_{ABS}$, the absorbed PPFD (in μE $m^{-2}$ $h^{-1}$) is:

$$I_{ABS} = I_0 - I_{OUT} \tag{10}$$

$I_{ABS}$ is the absorbed PPFD (in μE $m^{-2}$ $h^{-1}$)

Assuming the light becomes limiting, the fraction of the incident light which is not absorbed by the algae becomes negligible and the known incident PPFD $I_0$ is fully absorbed by the culture, such that:

$$I_0 = I_{ABS} \tag{11}$$

Therefore, under light limitation, growth becomes linear:

$$\frac{d(V_c \cdot C)}{dt} = \Phi^{DW} \cdot I_0 \cdot A_C \tag{12}$$

In the heterotrophic case, the biomass yield can be used in the linear growth region (limiting substrate) to infer volumetric productivity of biomass, if given the culture maintenance parameter $m_P$, the substrate feeding rate, and the bioreactor volume (Yamanè and Shimizu 1984).

Since unabsorbed photons cannot accumulate within the batch culture volume, at the onset of light-limitation, both Eqs. 7 and 12 hold true, thereby defining an exponential-to-linear transition (ELT). The ELT occurs at the point of maximum biomass productivity along the exponential phase, after which a constant productivity is reached in the linear phase. At the ELT, Eqs. 7 and 12 simplify to, at constant volume $V_C$:

$$\left.\frac{dC}{dt}\right|_{transition} = \Phi^{DW} \cdot I_0 \cdot \frac{A_C}{V_c} = \mu \cdot C \Big|_{transition} \tag{13}$$

These equations allow for the determination of $\Phi^{DW}$ as $\Phi^{DW,\,ELT}$ from the region of maximum productivity during batch growth, either as the maximum productivity in the exponential phase or the constant productivity during the linear phase, as detailed in Holland et al. (2011). As an example, the transition from exponential to linear growth under nutrient-replete conditions has been documented in Van Wagenen et al. (2012) and in Huesemann et al. (2013).

Under conditions of low PPFD per cell, full incident light absorption and a planar geometry, the experimentally determined autotrophic yield $\Phi^{DW}$ allows for the estimate of a maximum area productivity $P^{MAX}$ (in $g_{DW}\ m^{-2}\ d^{-1}$) as:

$$P^{MAX}(I_0) = \Phi^{DW} \cdot I_0 \tag{14}$$

$I_0$ is the average incident PPFD (in $\mu E\ m^{-2}\ d^{-1}$) at the site of interest.

## 3.2 Scatter-corrected Polychromatic Beer-Lambert Law

In order to model the flux of light absorbed by algal suspensions, measurements of absorbance using a spectrophotometer or photon fluxes using a quantum meter are routinely performed at varying culture depth and concentration, and the data is subsequently fitted (Yun and Park 2001, 2003; Barbosa et al. 2003a, b; Ragonese and Williams 1968). These planar geometry detection apparatus count scattered photons as effectively absorbed by the algal suspension. However, elastic scattering on whole algal cells does not incur energy loss, such that the scattered photons can be used by the algal culture for photosynthesis (Welschmeyer and Lorenzen 1981). In other words, a scattered photon which does not reach the detector at a depth $x$ from the light incidence surface can still be used by algal cells at a depth $x$-d$x$. Therefore, models to estimate photon fluxes as a function of culture depth or concentration should be based on scatter-corrected absorbance or PPFD data.

Scatter-corrected absorbance data was first acquired using an integrating sphere (Welschmeyer and Lorenzen 1981). Alternatively, pigment discoloration may be performed by using sodium hypochlorite (NaClO) as described by Ferrari and Tassan (1999). Since after pigments discoloration, the algal culture absorbance $Abs_{SCATTER}(\lambda)$ solely reflects scatter, the scatter-corrected (SC) absorbance spectrum $Abs_{SC}(\lambda)$ is obtained from the raw absorbance spectrum $Abs_{RAW}(\lambda)$ as shown in Fig. 4:

$$Abs_{SC}(\lambda) = Abs_{RAW}(\lambda) - Abs_{SCATTER}(\lambda) \tag{15}$$

Below, the Beer-Lambert model is adapted to account for the polychromatic nature of the light source. The wavelength $\lambda$ spans the Photosynthetically Active Radiation (PAR) region, between 400 and 700 nm. At each wavelength $\lambda$, the light absorbed between the depth $z$ and $z+\Delta z$ is proportional to the incident light flux at depth $z$, the concentration of algae cells $C$, the absorption cross-section $\sigma$, and the liquid depth $\Delta z$ through which the light travels:

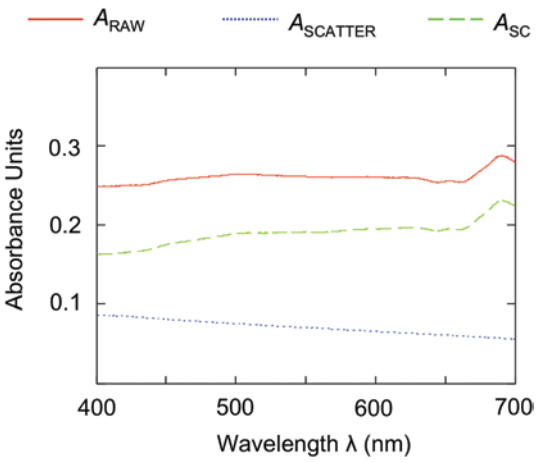


**Fig. 4** Example absorbance spectra for *Chlorella vulgaris* in nutrient-replete medium

$$I(z+\Delta z,\lambda) - I(z,\lambda) = -C \cdot \sigma(\lambda) \cdot I(z,\lambda) \cdot \Delta z \tag{16}$$

$z$ is the distance (in m) from the surface of light incidence; λ is the light wavelength (in nm); $I(z, \lambda)$ is the photon flux density (in μE $m^{-2}$ $s^{-1}$) at depth $z$ and wavelength λ; $C$ is the algal culture biomass concentration (in $g_{DW}$ $m^{-3}$); σ(λ) is the algal culture absorption cross section (in $m^2$ $g_{DW}^{-1}$) at a given λ; $\Delta z$ depth (in m) over which the photon-flux balance is performed.

Performing the summation of Eq. 16 over the PAR spectrum wavelengths:

$$\sum_{\lambda=400}^{700} I(z+\Delta z,\lambda) - I(z,\lambda) = -C \cdot \Delta z \cdot \sum_{\lambda=400}^{700} I(z,\lambda) \cdot \sigma(\lambda) \tag{17}$$

The photon flux at depth $z$ at each wavelength can be decomposed as follows:

$$I(z,\lambda) = P_{LIGHT}(\lambda) \cdot I(z) \tag{18}$$

where $P_{LIGHT}$ is the wavelength-dependent photon fraction (in $nm^{-1}$) of the light source, determined from the light source emission spectrum $E_{LIGHT}(\lambda)$ acquired using a spectrometer:

$$P_{LIGHT}(\lambda) = \frac{E_{LIGHT}(\lambda)}{\int_{400}^{700} E_{LIGHT}(\lambda) d\lambda} \tag{19}$$

Combining Eqs. 17 and 18, and taking the limit $\Delta z \to 0$:

$$\sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot \frac{\partial I(z)}{\partial z} dz = -C \cdot dz \cdot \sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot I(z) \cdot \sigma(\lambda) \tag{20}$$

From the definition of $P_{\mathrm{LIGHT}}$ (Eq. 19), the following relation holds:

$$\sum_{\lambda}^{spectrum} P_{LIGHT}(\lambda) = 1 \tag{21}$$

Equation 20 becomes:

$$\frac{dI(z)}{I(z)} = -C \cdot dz \cdot \sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot \sigma(\lambda) \tag{22}$$

Integration of the Eq. 22 between depths $z=0$ and $z=L$ yields:

$$-\ln\left(\frac{I_L}{I_0}\right) = C \cdot L \cdot \sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot \sigma(\lambda) \tag{23}$$

where $I_0$ is the incident photon flux density (in μE $m^{-2}$ $s^{-1}$) at depth $z=0$; $I_{\mathrm{L}}$ is the incident photon flux density (in μE $m^{-2}$ $s^{-1}$) at depth $z=L$; $L$ is the culture depth (in m) over which the photon flux balance is performed.

The scatter-corrected absorption spectrum $Abs_{SC}^{E}(\lambda)$ is determined in a cuvette of thickness $L_{\mathrm{E}}$ (in m) at an arbitrary cell concentration $C_{\mathrm{E}}$ (in $g_{DW}$ $m^{-3}$). At a single wavelength λ, the Beer-Lambert law states that:

$$\sigma(\lambda) = \frac{\ln 10 \cdot Abs_{SC}^{E}(\lambda)}{C_E \cdot L_E} \tag{24}$$

Combining Eqs. 23 and 24:

$$-\ln\left(\frac{I_L}{I_0}\right) = C \cdot L \cdot \sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot \frac{\ln 10 \cdot Abs_{SC}^{E}(\lambda)}{C_E \cdot L_E} \tag{25}$$

Hence the absorbed PPFD $I_{\mathrm{ABS}}$ (in μE $m^{-2}$ $s^{-1}$) by a culture of concentration $C$ and depth $L$ is

$$I_{ABS}(C,L) = I_0 - I_L = I_0\left[1 - \exp(-\sigma^{DW} CL)\right] \tag{26}$$

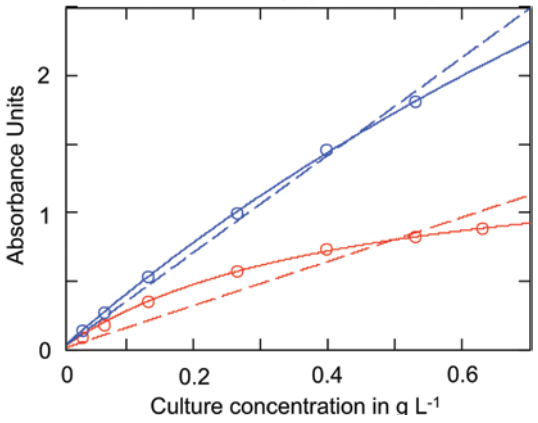


**Fig. 5** *Chlorella vulgaris* culture UTEX 2714 monochromatic scatter-corrected absorbance as a function of concentration in g $L^{-1}$. Absorbance measurements at 450 nm (*blue*) and 550 nm (*red*). Hyperbolic fit (*solid lines*) and Beer-Lambert linear fit (*dashed lines*)

where the scatter-corrected light source-dependent extinction coefficient $\sigma^{DW}$ (in $m^2\ g_{DW}^{-1}$) is:

$$\sigma^{DW} = \frac{\ln 10}{C_E \cdot L_E} \cdot \sum_{400}^{700} P_{LIGHT}(\lambda) \cdot Abs_{SC}^{E}(\lambda) \cdot d\lambda \tag{27}$$

Yun and Park found that the hyperbolic model fitted raw absorbance measurements as a function of concentration better than the Beer-Lambert law or the Cornet model (Yun and Park 2001). After scatter correction, as shown in Fig. 5, this observation still holds true. However, the Beer-Lambert approximation offers a mathematical simplicity which allows for full parameterization of the PPFD from a single scatter-corrected absorbance spectrum (Eqs. 26–27). As a contrast, using the hyperbolic model would require fitting $Abs_{SC}(\lambda, C)$ at each wavelength increment using two parameters $\omega(\lambda)$ in $m^2\ g_{DW}^{-1}$ and $\psi(\lambda)$ in $m^2\ g_{DW}\ m^{-2}$:

$$Abs_{SC}(\lambda, C_E) = \frac{1}{\ln 10} \frac{\omega(\lambda) CL}{\psi(\lambda) + CL} \tag{28}$$

The corresponding hyperbolic parameters can subsequently be used to estimate the absorbed PPFD $I_{ABS}^{HYPER}$ (in µE $m^{-2}\ s^{-1}$) by a culture of concentration $C$ and depth $L$ as:

$$I_{ABS}^{HYPER}(C, L) = I_0 \left[ 1 - \exp\left( -\sum_{\lambda=400}^{700} P_{LIGHT}(\lambda) \cdot \frac{\omega(\lambda) CL}{\psi(\lambda) + CL} \right) \right] \tag{29}$$

Thus, the Beer-Lambert approximation affords a much needed simplicity for the determination of the autotrophic yield, as described in Sect. 2.3.

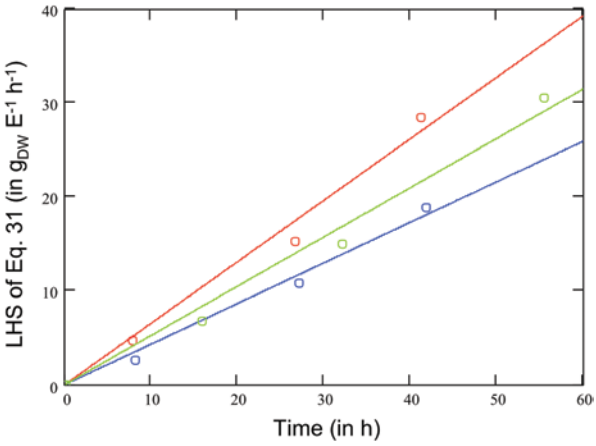


**Fig. 6** LHS of Eq. 31 (in $g_{DW}$ $E^{-1}$ $h^{-1}$) as a function of time in h for algal cultures grown in sealed nutrient-replete medium with carbonate added. Cultures of environmental sample (*red*), *Monoraphidium* sp. (*blue*) and *Dunaliella primolecta* (*green*) grown with 3 mM nitrate as described in Holland et al. (2011). Linear fit forced to the origin (*solid lines*)

## 3.3 The Ragonese and Williams Model

As shown above and as stated by Ragonese and Williams (1968), algal biomass production is proportional to the amount of light absorbed by the culture. Therefore, using the Beer-Lambert model and combining Eqs. 9 and 26 to account for scatter correction:

$$\frac{dC}{dt} = \frac{\Phi^{DW} \cdot I_0}{L}\left[1 - \exp(-\sigma^{DW} \cdot L \cdot C)\right] \tag{30}$$

This differential equation can be solved explicitly to allow for the determination of the autotrophic yield $\Phi^{DW}$ from batch growth data $C(t)$:

$$\frac{L}{I_0} \cdot \left\{ C - C_0 + \frac{1}{\sigma^{DW} \cdot L} \cdot \ln\left[\frac{1 - \exp\left[-\sigma^{DW} \cdot L \cdot C(t)\right]}{1 - \exp\left[-\sigma^{DW} \cdot L \cdot C_0\right]}\right]\right\} = \Phi^{DW} \cdot t \tag{31}$$

where the variables are defined above. A linear least-squares fit forced to the origin of the Eq. 31 left hand side (LHS) vs. time $t$ allows for determination of the autotrophic yield $\Phi^{DW}$, as shown in Fig. 6. As for bacterial cultures, monitoring the algal biomass concentration is easily done by correlating dry weight and optical density at 680 nm (or other wavelength in the 500–700 nm range) using a spectrophotometer without correcting for scatter (Holland et al. 2011).

# 4 Photosynthetic Efficiency Is Highest At Lower Irradiances

## 4.1 Fluorescence Response

In an algal culture, the absorbed photons are either processed into biomass through the generation of an electron flow or dissipated (as chlorophyll fluorescence or heat). Photosynthetic electron transport can be modeled as a two-phase process. First, the photosystem II (PSII) is excited by light which results in reduction of the first quinone PSII electron acceptor, $Q_A$. PSII centers with oxidized $Q_A$ are referred to as 'open' while those with reduced $Q_A$ as 'closed' (Baker 2008). The PSII operating efficiency $\Phi^{PSII}$, which is the product of the fraction of open PSII centers and the quantum yield of photochemistry in these open PSII centers, is a measure of the photon fraction channeled into $Q_A$ reduction. Second, the high energy electron from $Q_A$ is transferred to a series of carriers down an electrochemical gradient, which generates ATP and reducing equivalents for biosynthesis. An increase in the fraction of closed PSII leads to a decrease in $\Phi^{PSII}$ and a concurrent increase in non-photochemical quenching (NPQ), which dissipates the absorbed photons as heat.

Algal physiologists routinely quantify quantum yields as $\Phi^{CO2}$ (or $\Phi^{O2}$) in mole $CO_2$ fixed (or mole $O_2$ evolved) per mole photons absorbed, for which gas exchange probes are used to monitor $O_2$ or $CO_2$ levels. The saturation pulse method analysis of chlorophyll fluorescence (or chlorophyll fluorescence quenching analysis) has been developed as a noninvasive tool to monitor photosynthetic performance in algae and plants (Baker 2008; Schreiber 2004; Schreiber et al. 1986). This technique allows for the measurement of the operating efficiency $\Phi^{PSII}$, which provides an estimate of the linear electron flux through PSII, as well as the fraction of open PSII centers $q_L$. Over a range of light intensities and $CO_2$ concentrations, good correlations between $\Phi^{PSII}$ and quantum yields $\Phi^{CO2}$ have been shown (Baker 2008; Holmes et al. 1989; Campbell et al. 1998; Oberhuber and Edwards 1993; Genty et al. 1989). As can be seen in Fig. 7, $\Phi^{PSII}$ decreases with increasing irradiance, which is indicative of a reduction in quantum yield $\Phi^{CO2}$. Indeed, at high photon flux per cell, the decreased fraction of open PSII centers, measured as $q_L$, leads to an increase in NPQ. Hence, fluorescence response data provide evidence that the autotrophic yield is highest at lower photon flux per cell.

## 4.2 PI Curves

Photosynthesis-irradiance (PI) curves are obtained by subjecting an algal culture, at a given biomass density, to various levels of incident light and measuring $O_2$ evolution (Grobbelaar 2006; Macedo et al. 1998). Since the rate of $O_2$ evolved reflects the rate of biomass production, rates of photosynthesis, reported in $g_{O2}\ g_{DW}^{-1}\ h^{-1}$,

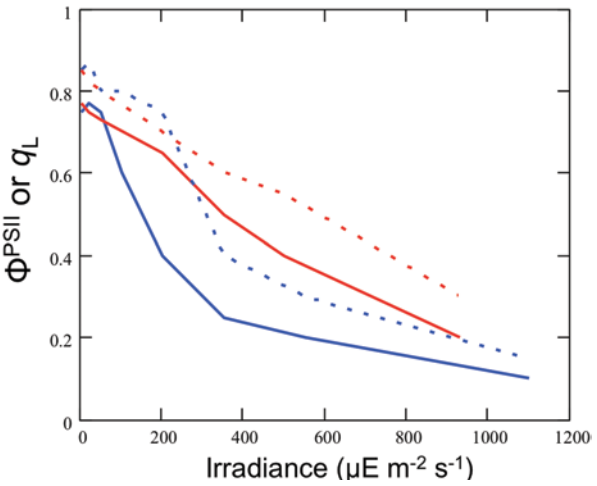


**Fig. 7** Representative trends of PSII operating efficiency $\Phi^{PSII}$ (*solid lines*) and fraction of open PSII centers $q_L$ (*dashed lines*) as a function of irradiance under high $CO_2$ (*red lines*) and low $CO_2$ (*blue lines*). Data trend reproduced from Kramer et al. (2004)

are analogous to biomass specific production rate $\mu$ (in $g_{DW}\ h^{-1}\ g_{DW}^{-1}$, or $h^{-1}$). In his extensive review, Aiba (1982) duly notes that the PI curves reported as $\mu(I_0)$ are calculated from short-term $O_2$ evolution data, and do not represent growth rates calculated from an exponentially growing algal culture. As stated by Koizumi and Aiba (1980), and as can be readily derived from Eqs. 7 and 9–11, the specific growth rate $\mu$ becomes, under light-limitation, a function of biomass concentration:

$$\mu = \frac{1}{C}\frac{dC}{dt} = \frac{I_0}{C}\cdot\Phi^{DW}\cdot\frac{A_C}{V_C} \tag{32}$$

Photosynthesis-irradiance (PI) curves (Grobbelaar 2006; Grobbelaar et al. 1996; Huesemann et al. 2009; Macedo et al. 1998) can be used to determine the maximum photosynthetic efficiency. Given the culture volume and area exposed to light and the mass of chlorophyll *a* (Chl *a*) in the tested culture, the ratio of the reported rate of photosynthesis (in µmol of $O_2$ evolved $mg_{Chl\ a}^{-1}\ h^{-1}$) to the corresponding incident irradiance (in µE $m^{-2}\ s^{-1}$) can be normalized to yield an apparent efficiency parameter ($\Phi^{APP}$ in mole $CO_2$ fixed per mole incident photons) as a function of irradiance, with a maximum in the tested range of irradiances. Hence, PI curves can be converted to a $\Phi^{APP}(I)$ curve by plotting the ratio $P/I$ as a function of $I$, as shown in Fig. 8. As expected, the apparent $\Phi^{APP}(I)$ displays a high initial value at low irradiance (at which all incident photons are absorbed and $\Phi^{APP} = \Phi^{CO2}$), a decrease due to non-photochemical quenching and, at even higher irradiance, deactivation of the photosystems. Such $\Phi^{APP}(I)$ can be used to determine the range of incident light levels at which the autotrophic yield $\Phi^{DW}$ is maximum or near its maximum for a given algal culture. PI curves, however, are often mistaken for an intrinsic parameter of algal cultures, and inherently depend on culture concentration (Grobbelaar et al. 1996), physiological state and growth cell geometry. The effective use of PI

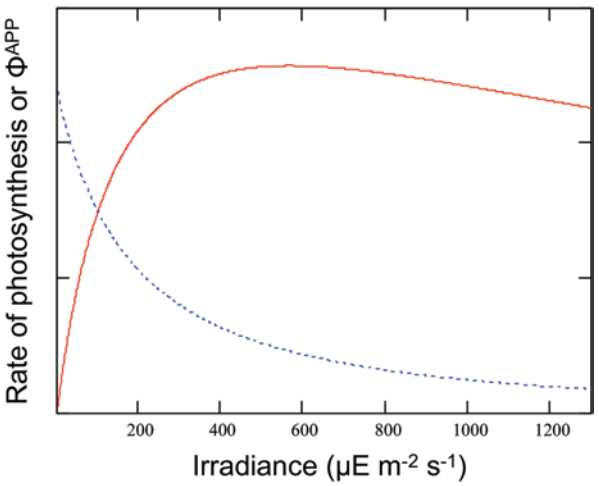


**Fig. 8** PI curve example: specific rate of photosynthesis (*red*). Corresponding $\Phi^{APP}(I)$ curve (*blue*)

curves has proven limited by the incomplete report of such parameters. Nevertheless, the general trends displayed in PI curves corroborate that the autotrophic yield is highest at lower irradiance.

## *4.3 Growth Rate μ and Biomass Yield Φ Do Not Correlate*

Algal cultures growth rates are widely used as a strain selection criterion in the field (Shifrin and Chisholm 1981; Sheehan et al. 1998). However, there is no correlation between the autotrophic biomass yield $\Phi^{DW}$ and the culture maximum growth rate, such that the use of growth rate as a productivity indicator is an erroneous approach. Experimental evidence is shown in Fig. 9. As further support, Wong et al. (2009) theoretically derived that the yield provides an upper bound for growth rate, but does not correlate with it. Consistently, heterotrophs display a trade-off between growth rate and yield, since bacterial metabolism optimizes for both adaptation in the event of sudden stress and biomass formation (Fischer and Sauer 2005).

The following analogy may provide a more intuitive understanding. The algal growth rate is defined in the exponential phase under a condition of excess light. This would correspond to feeding fish pellets in excess. Fish A can take a maximum of 4 g pellets per hour, and turn 2 g into biomass, while fish B can take a maximum of 1 g pellets per hour, and turn 1 g into biomass. Cell division occurs when 10 pellets have been processed into biomass. Thus, fish A has a division time of 5 hours, and fish B has a division time of 10 hours. While fish A grows faster than fish B, the biomass yield of fish A (50% g biomass/ g pellet) is lower than that of fish B (100%).

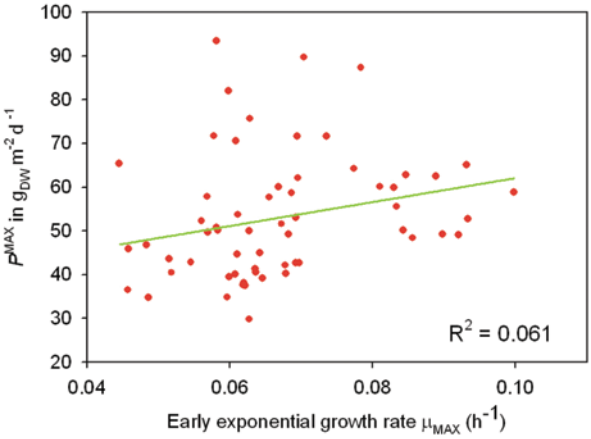


**Fig. 9** Autotrophic yield $\Phi^{DW}$ reported as $P^{MAX}$ (in $g_{DW}$ $m^{-2}$ $d^{-1}$, according to Eq. 14 at a daily incident PPFD average of 500 μE $m^{-2}$ $s^{-1}$) as a function of maximum growth rate $\mu_{MAX}$ in $h^{-1}$ calculated in the early exponential phase. Algal cultures grown in sealed nutrient-replete medium supplemented with carbonate and 3 mM nitrate (Holland et al. 2011)

# 5 Target Mixing Conditions and Bioreactor Design

## *5.1 The PSU Model and Target Photic Zone Velocity*

Maximum algal biomass productivity under high outdoor irradiances (1000–2500 μE $m^{-2}$ $s^{-1}$) can only be achieved if the autotrophic yield $\Phi^{DW}$ is near its maximum value. In their development of the PSU model, Camacho-Rubio et al. (2003) integrated kinetics of photoinhibition to more accurately model photosynthetic rates under inhibitory irradiances. As a contrast, the PSU model basis (Eqs. 33–36) is used below to investigate whether, under very high irradiance, mixing conditions can be achieved in order to avoid a decrease in PSII operating efficiency $\Phi^{PSII}$, which in turn induces a decrease in autotrophic yield $\Phi^{DW}$ [2]. The goal of our model is to determine a target velocity $v_T$ for the alga particle in the photic zone in order to maintain a $\Phi^{PSII}$ near its maximum value. We use two different trajectory models, linear and sinusoidal, which lead to two distinct estimates of $v_T$: $v_{T,L}$ and $v_{T,S}$ respectively.

Photosynthetic electron transport can be modeled as a two-step kinetic process. First, PSII is excited by light which results in the reduction of the first quinone PSII electron acceptor, $Q_A$. PSII centers with oxidized $Q_A$ are referred to as 'open' while those with reduced $Q_A$ as 'closed' (Baker 2008). The PSII operating efficiency $\Phi^{PSII}$ is a measure of the photon fraction channeled into $Q_A$ reduction. Second, at variable rates on the order of milliseconds (Kroon and Thoms 2006), the high energy electron from $Q_A$ is transferred to a series of carriers down an electrochemical gradient, which generates ATP and reducing equivalents for biosynthesis. While electron transport in PSI is faster than in PSII, PSI electron transport can be grouped with the slow steps of photosynthesis since PSI is downstream of PSII. An increase in the fraction of closed PSII, which is caused by bottlenecks in the photosynthetic electron transport chain, leads to a decrease in $\Phi^{PSII}$. Employing a using a simplified

PSU kinetic model, and using an experimentally determined incident PPFD at which $\Phi^{DW}$ or $\Phi^{PSII}$ starts to decrease, we derive an expression for the threshold fraction of closed PSII above which NPQ is considered significant. Alternatively, this threshold fraction of closed PSII can be directly measured by fluorescence response. With these simplifying assumptions, this threshold fraction is used to determine the alga speed across the photic zone, as an indicator of agitation conditions, which can maintain a fraction of open PSII conducive to a maximum $\Phi^{PSII}$.

The PSII centers exist as closed PSII ($A^-$) and open PSII (A):

$$A + h\nu \rightarrow A^- \left(\text{ratek } k_1 \text{ in } s^{-1}\right) \tag{33}$$

$$A^- \rightarrow REC + A \left(\text{rate} k_2 \text{ in } s^{-1}\right) \tag{34}$$

where REC designates the pool of reduced carriers downstream of $Q_A$.

$$a_0 = a + a^- \tag{35}$$

where $a_0$, $a$, $a^-$ are respectively total, open and closed PSII concentration in the culture (in $mol_{PSII}$).

Under low light, we assume that the fraction of closed PSII remains low enough not to induce saturation kinetics in the slow step, and that the concentration of $A^-$ is quasi-steady:

$$\frac{da^-}{dt} = 0 = k_1(x) \cdot (a_0 - a^-) - k_2 \cdot a^- \tag{36}$$

The following expression for $k_1$ which is analogous to that given in Camacho Rubio et al. (2003), was modified to display Beer-Lambert's law and $\Phi^{PSII}$, and account for the photon flux splitting between the PSI and PSII. Assuming that an equal number of PSI and PSII absorb the incident light at an equal rate (factor ½):

$$k_1(x) = \Phi^{PSII} \cdot \frac{I_0 \cdot 10^{-6}}{2} \cdot \frac{\sigma^{DW}}{F} \cdot \exp(-\sigma^{DW} \cdot C \cdot x) \tag{37}$$

where $x$ is the distance (in m) from the light incidence surface; $\Phi^{PSII}$ is in moles excited PSII per Einstein absorbed; $I_0$ is the absorbed PPFD in µE $m^{-2}$ $s^{-1}$; $\sigma^{DW}$ is the scatter-corrected algal cross section in $m^2$ $g_{DW}^{-1}$ (Sect. 2.2); $C$ is the biomass concentration in $g_{DW}$ $m^{-3}$; $F$ is the fraction of PSII in $mol_{PSII}$ $g_{DW}^{-1}$, determined experimentally (Falkowski et al. 1981; Cunningham et al. 1990). While the time constant of $Q_A$ reduction is on the order of nanoseconds, the rate of exciton formation $k_1$ depends on the incident light intensity as shown by Eq. 37. Under the high irradiance of 3400 µmol $m^{-2}$ $s^{-1}$, Lazar and Pospisil (1999) estimated this rate to

**Table 3** Example parameters for calculation of the threshold closed reaction fraction (last row) for *Dunaliella tertiolecta*

| Variable | Value | Unit | Organism | Reference |
|---|---|---|---|---|
| PSII/cell | $54\times10^{-19}$ | $mol_{PSII}$ $cell^{-1}$ | *Dunaliella tertiolecta* | (Falkowski et al. 1981) low light |
| $g_{DW}$/cell | $70\times10^{-12}$ | $g_{DW}$ $cell^{-1}$ | *Dunaliella tertiolecta* | (Shifrin and Chisholm 1981) |
| $k_2$ | 153 | $s^{-1}$ | (model) | This work, (Kroon and Thoms 2006) |
| $\Phi^{PSII}$ | 0.80 | – | (common value) | (Kramer et al. 2004) |
| $\sigma^{DW}$ | 0.2 | $m^2$ $g_{DW}^{-1}$ | *Dunaliella tertiolecta* | (Barbosa et al. 2003a) |
| $I_T$ | $50\times10^{-6}$ | E $m^{-2}$ $s^{-1}$ | (common value) | (Macedo et al. 1998) |
| $(1-q_L)_T$ | 0.25 | – | | |

be 5500 $s^{-1}$. Using the example values in Table 3, Eq. 37 evaluates $k_1$ as 2900 $s^{-1}$, which is on the same order.

At a threshold irradiance $I_T$ (μE $m^{-2}$ $s^{-1}$), $\Phi^{PSII}$ (and therefore $\Phi^{DW}$) starts to decrease significantly due to NPQ. This arbitrarily defined $I_T$ threshold value corresponds to the maximum acceptable loss in productivity. $I_T$ can be determined from PI curve data (Sect. 3.2). Alternatively, fluorescence response can be used to measure the decrease in $\Phi^{PSII}$ with increasing incident PPFD (Baker 2008; Kramer et al. 2004; Campbell et al. 1998; Schreiber 2004). Combining Eqs. 36–37, and taking $x=0$ (point of maximum irradiance), the corresponding threshold fraction of excited centers $(1\text{-}q_L)_T$ is given by:

$$(1-q_L)_T = \left.\frac{a^-}{a_0}\right|_T = \frac{1}{1+\dfrac{k_2}{k_1(0)}} = \frac{1}{1+\dfrac{2F\cdot k_2}{\Phi^{PSII}\cdot I_T\cdot 10^{-6}\cdot\sigma^{DW}}} \tag{38}$$

The determination of this threshold value is highly sensitive to the choice of $k_2$. Due to the complexity of the electron transport mechanisms involved in channeling PSII electrons, we choose the rate of the slowest PSII step, as the slowest step will be the first responsible for an increase in $a-$. Using the rates published by Kroon and Thoms (Kroon and Thoms 2006), and noting that these values highly underestimate the rate of PSII charge recombination (de Wijn and van Gorkom 2002), we evaluate $k_2$ as the slower average electron transfer rate between $Q_A$ and $Q_B$ ($k_2=153$ $s^{-1}$). This rate can also be estimated experimentally from determination of the 'turnover time' (Dubinsky et al. 1986). An example calculation is presented for *Dunaliella tertiolecta* (Table 3). The calculated threshold (maximum desired excited fraction) is 25 % for *D. tertiolecta* (Table 3), consistent with published fluorescence response trends. Fluorescence response, which can directly measure the fraction of closed PSII ($1\text{-}q_L$) along with $\Phi^{PSII}$ under increasing irradiance (Baker 2008; Kramer et al. 2004; Campbell et al. 1998), can alternatively

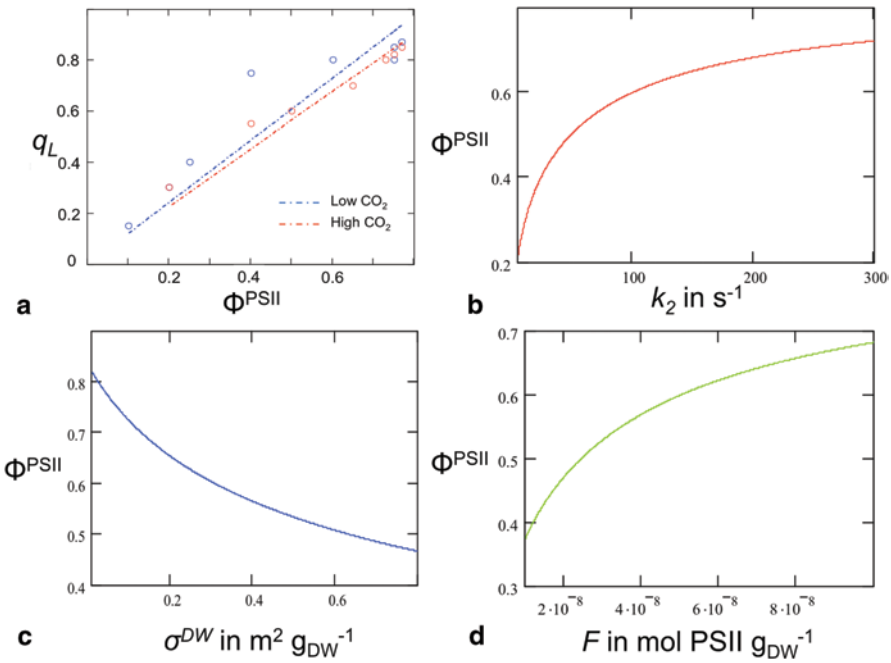


**Fig. 10** Variation of $\Phi^{PSII}$ as a function of various parameters. **a** Rough correlation between the fraction of open centers $q_L$ and $\Phi^{PSII}$. **b** Increase of $\Phi^{PSII}$ as a function of the $k_2$ kinetic parameter. **c** Decrease of $\Phi^{PSII}$ as a function of the scatter-corrected algal cross-section $\sigma^{DW}$. **d** Increase of $\Phi^{PSII}$ as a function of the concentration of PSII centers $F$

be used to determine experimentally both $I_T$ and the threshold (1-$q_L$) value. An added benefit of this method is the ability to estimate $k_2$ from Eq. 38 for further use in the estimation of target mixing velocities.

Qualitative insights can be derived from the simplified PSU model as follows. Subjecting the algal culture to a given irradiance $I_T$ constrains the fraction of open centers $q_L$ as well as the PSII operating efficiency $\Phi^{PSII}$, as seen in Fig. 7, such that a resulting correlation between $\Phi^{PSII}$ and $q_L$ can be established (Fig. 10a). This correlation can be used in Eq. 38 to solve for $\Phi^{PSII}$ as a function of the limiting kinetic rate $k_2$, the scatter-corrected algal cross-section $\sigma^{DW}$, or the concentration of excitable PSII centers $F$ (Fig 10b, c and d). As expected, the PSII operating efficiency $\Phi^{PSII}$ increases with $k_2$, and $F$, and decreases with $\sigma^{DW}$. Regarding the latter, a reduced number of Chl *a* per PSII (or antenna size) leads to a decrease in $\sigma^{DW}$, which in turn reduces the flux of absorbed photons per cell. Accordingly, genetic mutants with a reduced antenna size (reduced number of Chlorophyll per PSII) have been shown to enable greater biomass productivity under high irradiance (Huesemann et al. 2009; Beckmann et al. 2009).

Under elevated outdoor light levels, the $A^-$ pool is no longer quasi-steady. Adequate agitation can render the system essentially bi-phasic (Merchuk et al. 2007), in which the concentration of $A^-$ oscillates between low and high levels as the cell

moves between dark and light zones. In the general case, for which an alga trajectory $x(t)$ is known, Eq. 36 becomes

$$\frac{da^{-}}{dt} + \left[k_1\left[x(t)\right] + k_2\right] \cdot a^{-} = k_1\left[x(t)\right] \cdot a_0 \tag{39}$$

Solving this first order ODE for $a^{-}(t)$ in the general case requires integrating factor $u(t)$:

$$u(t) = \exp\left\{\int_0^t \left[k_1\left[x(t')\right] + k_2\right] dt'\right\} \tag{40}$$

Assuming the known trajectory $x(t)$ starts in the dark zone, such that $a^{-}(0) = 0$, the fraction of closed PSII centers after time $t$ in the photic zone is:

$$\frac{a^{-}(t)}{a_0} = \frac{1}{u(t)} \cdot \int_0^t u(t') \cdot k_1\left[x(t')\right] dt' \tag{41}$$

The height of the photic zone is arbitrarily defined as the depth at which 99 % of the incident light is absorbed by the biomass of concentration $C$:

$$d = \frac{\ln(100)}{\sigma^{DW} \cdot C} \tag{42}$$

As an example, a *Dunaliella* culture (Table 3) at a concentration of 2 g $L^{-1}$ has a penetration depth of 1.2 cm.

In a simple linear-trajectory case, the target speed $v_{T,L}$ can be calculated as follows. We assume that the alga particle travels at a constant speed $v_{T,L}$ from the dark zone ($x = d$) to the surface ($x = 0$), and back to the dark zone. For each cycle the alga particle spends time $2\tau$ in the photic zone such that

$$\tau = \frac{d}{v_{T,L}} \tag{43}$$

An implicit equation for the target speed $v_{T,L}$ can be obtained by substituting a linear trajectory into Eq. 41, with a constraint that half the threshold PSII fraction is excited during one half cycle ($0 \le x \le d$) of the photic zone trajectory:

$$\frac{1}{2}\frac{a^{-}}{a_0}\bigg|_T = \frac{1}{u_L(\tau)} \cdot \int_0^{\tau} u_L(t') \cdot k_1\left(v_{T,L} \cdot t'\right) dt' \tag{44}$$

where

$$u_L(t) = \exp\left\{\int_0^t \left[k_1\left[v_{T,L}\cdot t'\right] + k_2\right] dt'\right\} \tag{45}$$

One numerically adjusts $v_{T,L}$ until Eq. 44 is satisfied.

A more realistic approximation is that the alga particle follows a sinusoidal trajectory with a target speed $v_{T,S}$. For purposes of discussion we assume the dark zone has the same thickness $d$ as the photic zone, and that the period in which the alga occupies each zone is $2\tau$. At $t=0$, the particle enters the photic zone from the dark zone and $a^-(0)=0$. The sinusoidal trajectory is

$$x_S(t,\tau) = d\left[1-\sin\left(\frac{\pi}{2\tau}t\right)\right] \tag{46}$$

As with the linear case above, time $\tau$ can be calculated from an implicit equation:

$$\left.\frac{a^-}{a_0}\right|_T = \frac{1}{u_S(2\tau,\tau)}\cdot\int_0^{2\tau} u_S(t',\tau)\cdot k_1\left[x_S(t',\tau)\right]dt' \tag{47}$$

where

$$u_S(t,\tau) = \exp\left\{\int_0^t \left[k_1\left[x_S(t',\tau)\right] + k_2\right] dt'\right\} \tag{48}$$

With $\tau$ in hand, the corresponding target speed $v_{T,S}$ can be estimated as the root-mean-square velocity of the alga particle in the photic zone:

$$v_{T,S} = \frac{\pi d}{2\sqrt{2\tau}} \approx 1.11\frac{d}{\tau} \tag{49}$$

The Mathcad program used to calculate $v_T$ (linear and sinusoidal models) is provided in Holland and Wheeler (2011). The dependence of the target velocity $v_T$ on the biomass concentration $C$ and the culture extinction coefficient $\sigma^{DW}$ is due to that of $d$ (Eq. 42) and $k_1$ (Eq. 37).

Calculated values of $v_{T,L}$ and $v_{T,S}$ represent upper and lower bounds, respectively, for the desired average algal particle speed, as the linear model does not account for the 'turn-around' time at the light incidence surface, while the sinusoidal model likely overestimates it. Given a velocity $v_T$ on the order of $v_{T,L}$ to $v_{T,S}$ across the photic zone, an alga particle effectively avoids over-excitation of the PSII system and a resulting decrease in quantum efficiency $\Phi^{PSII}$. Subsequently, a dark phase on the order of 50–100 ms suffices to relax the PSII to a mostly open state (Nedbal et al. 1999).

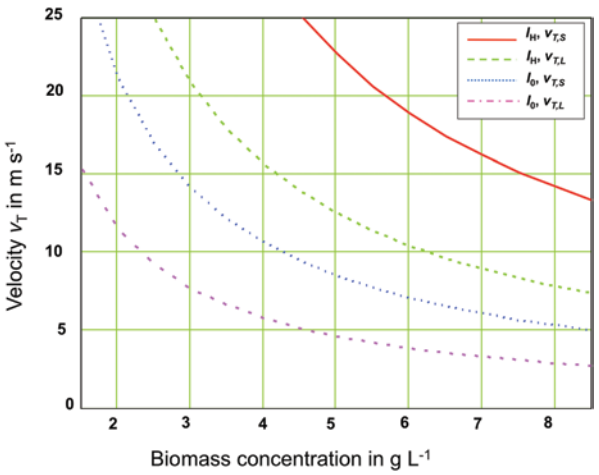


**Fig. 11** Calculation of target velocities $v_T$ (m s$^{-1}$) using the linear model ($v_{T,L}$) and the sinusoidal model ($v_{T,S}$). Velocities were calculated as a function of culture concentration $C$ (g L$^{-1}$) for the high solar irradiance $I_0$ (1000 µE m$^{-2}$ s$^{-1}$) and the highest possible direct normal solar irradiance $I_H$ (2500 µE m$^{-2}$ s$^{-1}$) for *Dunaliella tertiolecta* (parameter values from Table 3)

Target velocities $v_{T,L}$ and $v_{T,S}$ were calculated for *Dunaliella tertiolecta* as a function of biomass concentration (Fig. 11), using the values listed in Table 3. Two incident irradiance values are used: a high value $I_0$ of 1000 µE m$^{-2}$ s$^{-1}$ and the highest possible solar irradiance $I_H$. Incident solar energy before atmospheric scattering is 1387 W m$^{-2}$ (Thuillier et al., 2003), which converts to $I_H$=2538 µE m$^{-2}$ s$^{-1}$ using the conversion factor 1.83 µE J$^{-1}$ (Table 2).

## 5.2 Target Velocity and Bioreactor Design

The high biomass densities required under high illumination (Fig. 11, 5–10 $g_{DW}$ L$^{-1}$) also set constraints on the nutrients feed concentration (Sect. 5.2). The very high calculated speeds (Fig. 11) in the photic zone in order to avoid a decrease in $\Phi^{PSII}$ are consistent with the well-documented 'flashing light' effect, which shows an increase in photosynthesis when light is pulsed at high frequency (Grobbelaar et al. 1996). Indeed, the calculated high target speeds correspond to residence times on the order of 0.6–1 ms at $I_0$ and 0.23–0.37 ms at $I_H$, using the sinusoidal and linear models for the lower and upper bounds, respectively. In effect, such agitation, equivalent to LD cycles on the order of milliseconds, should extend the linear range of PI-curves to very high incident PPFD, which is what Nedbal et al. (1996) observed experimentally. While Nedbal et al. (1996) linked the flash-induced growth enhancement to a pool exhaustion along the electron transport chain (namely the PQ pool), the present work additionally provides a simple mathematical model to estimate a target speed as a key design parameter.

Actual fluid velocity perpendicular to the light incidence surface, which can be measured using radioactive particle tracking (Luo et al. 2003) or a conductivity probe (impulse-response technique (Gluz and Merchuk 1996)), usually range in the 5–40 cm s$^{-1}$ for tubular reactors. While tubular reactors present the advantage

of a closed axenic environment, desirable for the synthesis of high value added products, and lower mixing velocities, better suited for culturing shear-sensitive diatoms (such as *Porphyridium* sp. and *P. tricornutum*) (Thomas and Gibson 1990), they are not able to achieve mixing velocities capable of averting photon dissipation under high outdoor irradiances. The above calculations call for a fundamental change in reactor design for algal cultivation under high irradiance, in order to reach velocities on the order of 5–20 m $s^{-1}$ in the photic zone (~2 cm or less). Green algae (Chlorophyceae) have been shown to display highest resistance to shear (Thomas and Gibson 1990), but additional selection strategies may need to be designed given such high estimated speeds. As an encouraging result, Barbosa et al. (2004) showed that sparger maximum bubble velocities on the order of 1–20 m $s^{-1}$ did not cause lethal shear to the green-algae *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii*.

In this work, simple linear and sinusoidal functions were used to simulate the light/dark cycles which the algal cells are exposed to. At the high modeled target speeds, the turbulent fluid flow would be more accurately modeled using computational fluid dynamics (Luo and Al-Dahhan 2011). Alternatively, the random character of light/dark cycles can be captured experimentally using computer-automated radioactive particle tracking (CARPT) techniques (Luo et al. 2003; Luo and Al-Dahhan 2004). CARPT provides a invaluable means to fully characterize novel turbulent algal bioreactor designs. In addition, the proposed model can be further refined by incorporating a more sophisticated mechanistic description of photosynthesis (Lazar and Pospisil 1999; Lazar 2003; Lazar 2006), with identification and estimation of key kinetic parameters.

Microalgae, macroalgae and plants all can achieve quantum yields close to the theoretical maximum of 0.125 mol $CO_2$ fixed (or mol $O_2$ evolved) per mol photons absorbed. Plants such as *Flaveria* spp. can achieve a $\Phi^{O2}$ on the order of 0.108 (Lal and Edwards 1995), macroalgae on the order of 0.08 (Frost-Christensen and Sand-Jensen 1992), and microalgae a $\Phi^{CO2}$ on the order of 0.106 (Welschmeyer and Lorenzen 1981). However, only microalgae can achieve high frequency turnover in the photic zone, which is crucial to maximize utilization of a continuous source of incident light. Despite the relative ease of harvest of plants and macroalgae, their static nature prevents dark relaxation under high irradiance outdoor conditions. Therefore, under appropriate mixing conditions, microalgae are best suited to achieve area productivity reflecting these measured maximum quantum yields (or autotrophic yields).

## *5.3 Proposed Bioreactor Design*

The following algal bioreactor design (Fig. 12) fulfills the various criteria described above. At steady-state, reactor depth and biomass concentration allow for full absorption of the incident light (Sect. 2.1) as well as a dark zone for Light Dark cycling (Sect. 4.1). Sparging high velocity air a few centimeters below the surface creates adequate mixing in the photic zone and averts photoinhibition in an open

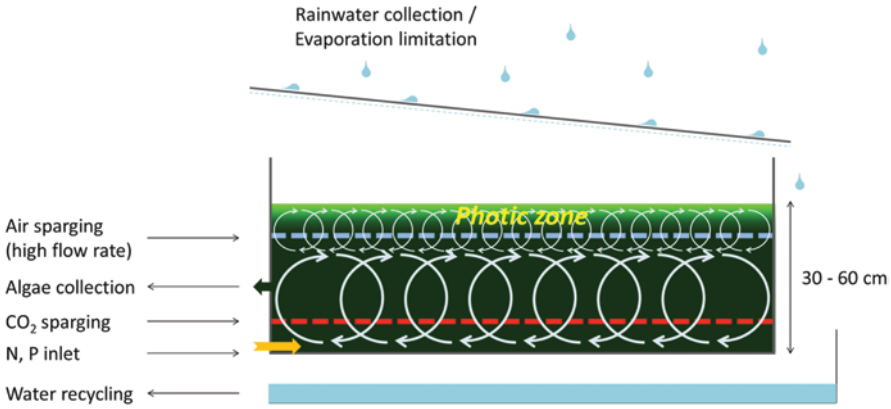


**Fig. 12** Proposed bioreactor design

pond configuration under high irradiance. The shallow water column between air sparging and the free surface of the pond would allow to reduce power consumption and yet effect high Light Dark frequencies. This sparging would provide a fraction of the $CO_2$ required for growth. Under high irradiance, $CO_2$ could be supplemented as a concentrated gas stream (coming from AD biogas combustion) at or near the bottom of the pond, sufficiently lower than the air sparging zone to avoid $CO_2$ stripping and maximize $CO_2$ dissolution. This is consistent with the fact that, by design, the light-activated cells would carry out the slow steps of photosynthesis and carbon fixation in the dark zone. N and P nutrients should be supplemented as a liquid stream at a depth which should be optimized: the effective local nutrient concentration, which depends on the feed characteristics and reactor mixing, will affect the algal physiology.

Since the high rate air sparging occurs at a fixed depth, bioreactor level management becomes crucial, such that make-up water and feed flow rates need to match the flow rate of the continuously harvested algal biomass (see Sect. 5 for details). The presence of a shield may help reduce water evaporation by creating a high humidity air zone above the pond, and allow for rainwater collection for storage and recycling. In addition, the sparged air may need to be pre-humidified to reduce evaporative losses.

The much debated addition of a temperature control system depends on the pond geographic location and the degree of processing achieved on-site. Combined Heat and Power generation from the AD biogas would contribute heat, while underground water storage would provide cooling. Pond depth helps provide an additional buffering mechanism for temperature regulation. Importantly, efficient conversion of the incident light into biomass (through photochemical quenching) reduces the temperature increase due to radiation seen in otherwise poorly mixed ponds under elevated irradiance.

Design optimization entails increasing high rate air sparging in order to restore maximum biomass yield under increasing light levels. Such optimization could be

facilitated by the use of chlorophyll fluorescence quenching analysis (Sect. 3.1) by sampling algae at different pond locations. Under continuous operation, air sparging rates would then become dependent upon the measured incident light levels.

# 6 Bioreactor Parameterization and Strategy to Achieve High Lipid Production

## *6.1 Poor Mixing Conditions: Fish Tank Analogy*

Under condition of poor mixing and elevated irradiance (saturating and/or inhibitory), the simple model presented in this work (Sect. 2.1 and Eq. 30) no longer fully describes algal biomass production, such as for tubular reactors or raceway ponds subjected to irradiances over 1000 μE $m^{-2}$ $s^{-1}$, and as can be inferred from the velocity modeling results in Sect. 4. Provided well-defined geometries for the PI experimental chamber and the modeled reactor, PI curves provide critical information which can be used as described below.

As a possible approach, poorly-mixed reactors can be theoretically divided into 4 zones, the location of which depends on the reactor geometry, the incident PPFD $I_0$, the scatter-corrected culture extinction coefficient $\sigma^{DW}$ (in $m^2$ $g_{DW}^{-1}$), the distance $x$ from the light incidence surface, the area perpendicular to the light source $A_C$ in $m^2$ and the algal biomass concentration $C$. In order to help understanding, a fish-tank analogy is provided (Fig. 13): the fish are swimming horizontally and are circumscribed to a given depth $x$; the constant and elevated PPFD is represented by a high rate supply of fish food; in zone 1, the overfed fish divide more slowly than their well-fed counterpart in zone 2; all the food entering zone 3 is taken-up by the fish such that the fish biomass production is proportional to the food intake; all pellets have been utilized in zones 1-3 such that zone 4 does not support fish growth.

Correspondingly, for the sake of simplicity in the discussion below, the zones are taken to be 1D strata. Zone 1 is closest to the light incidence surface ($x=0$), with Zones 2 and onward corresponding to increasing values of $x$. The location of these zones is well defined when mixing is poor, allowing for a quasi steady-state approximation which circumscribes an algal particle to an infinitesimal volume with a defined PPFD. The upper-most region (zone 1) undergoes photoinhibition, which corresponds to a specific growth rate lower than its maximum values; in zone 2, the light-excess region supports exponential growth at $\mu_{MAX}$; in zone 3, the light-limited region supports a linear biomass increase; zone 4 light levels are too low to support biomass production. At a specified dilute biomass concentration $C_{PI}$ and PI chamber geometry (assumed planar), PI curves (Macedo et al. 1998) can be parameterized to satisfactorily describe growth behavior in zones 1 and 2, as well as the transition point between zones 2 and 3. This transition between light excess and light limitation occurs at the threshold depth $x_T$ (in m).

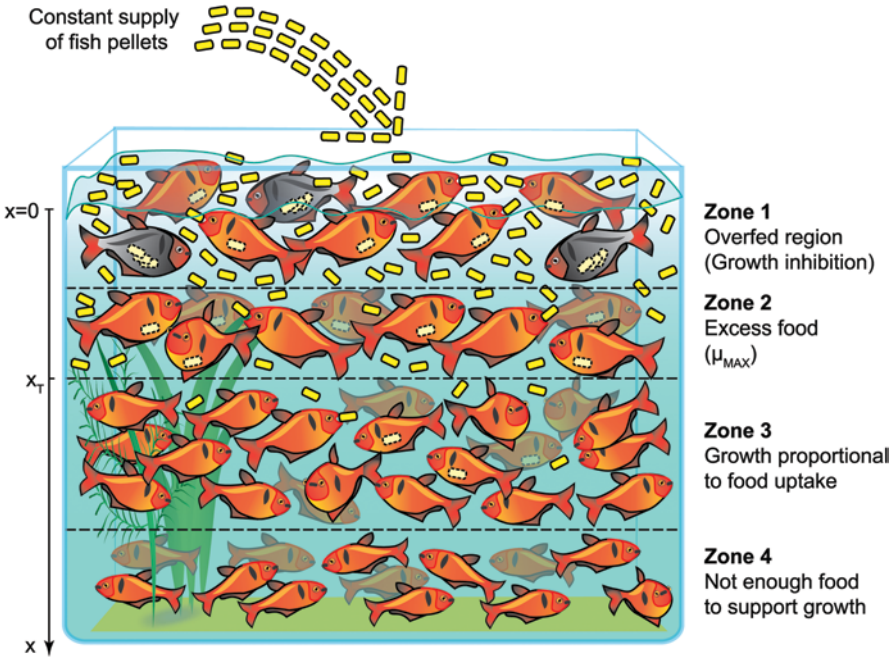


**Fig. 13** Fish-tank analogy describing poorly mixed algal bioreactors under high irradiance (no vertical mixing). $Q_A$ reduction is represented by a pellet in the fish mouth; the slow steps of photosynthesis, which correspond to an energy transfer from $Q_A$ down the electrochemical gradient, are represented by a pellet in the fish stomach. Photoinhibited algal cells are represented by grey fish with two pellets in their stomach

As currently modeled in the literature (Yun and Park 2003), the local volumetric production rate $P_{1-2}^{V}$ (in $g_{DW}\ m^{-3}\ h^{-1}$) in zones 1 and 2 ($0<x<x_T$) is

$$P_{1-2}^{V}(x) = \mu_{PI}(x) \cdot C \tag{50}$$

where, $\mu_{PI}$ depends on the depth-dependent PPFD $I(x)$, which follows the Beer-Lambert law provided correction for scatter (Sect. 2.2):

$$I(x) = I_0 \cdot \exp(-\sigma^{DW} \cdot C \cdot x) \tag{51}$$

The algal biomass production $P_{1\text{-}2}$ (in $g_{DW}\ h^{-1}$) in zones 1-2 is:

$$P_{1-2}(x) = C \cdot A_C \int_0^{x_T} \mu_{PI}(x) dx \tag{52}$$

At a threshold irradiance $I_T$, the ratio of the specific growth rate over the irradiance starts to decrease with increasing incident PPFD, reflecting a decrease in

autotrophic yield (discussed in Sect. 4.1). This corresponds to a threshold specific energy flux ($EF_T$, in μE $g_{DW}^{-1}$ $h^{-1}$):

$$EF_T = \frac{I_{ABS,T}}{C_{PI} \cdot L_{PI}} = \frac{I_T \cdot \left[1 - \exp(-\sigma^{DW} \cdot C_{PI} \cdot L_{PI})\right]}{C_{PI} \cdot L_{PI}} \tag{53}$$

where $C_{PI}$ is the algal biomass concentration (in $g_{DW}$ $m^{-3}$) in the PI chamber, $L_{PI}$ is the depth of the chamber (in m). At low biomass concentration and chamber thickness,

$$EF_T \to I_T \cdot \sigma^{DW} \tag{54}$$

The $EF_T$ can also be solved from the batch growth concentration at which the ELT occurs.

In the reactor, the specific energy flux $EF(x)$ (in μE $g_{DW}^{-1}$ $h^{-1}$) is related to the decrease in transmitted radiation:

$$EF(x) = \frac{dI(x)}{dx} \cdot \frac{1}{C} = \sigma^{DW} \cdot I_0 \cdot \exp(-\sigma^{DW} \cdot C \cdot x) \tag{55}$$

such that $x_T$ can be solved algebraically using Eqs. 53 and 55 by setting

$$EF_T = EF(x_T) \tag{56}$$

In the event of negligible biomass maintenance in zone 4, integration over depth yields the following productivity in zones 3–4 $P_{3\text{-}4}$(in $g_{DW}$ $h^{-1}$):

$$P_{3-4} = \int_{x_T}^{\infty} \Phi^{DW} \cdot A_C \cdot C \cdot EF(x) dx \tag{57}$$

Assuming a constant autotrophic yield $\Phi^{DW}$ in zone 3 yields:

$$P_{3-4} = A_C \cdot \Phi^{DW} I_0 \cdot \exp(-\sigma^{DW} \cdot C \cdot x_T) = A_C \cdot \Phi^{DW} \cdot I(x_T) \tag{58}$$

In the event of non-negligible maintenance energy in the dark, a threshold depth between zones 3 and 4 can be derived analogously to $x_T$.

Importantly, NPQ photon dissipation in zones 1 and 2 limits maximization of the bioreactor productivity. Additionally, photoinhibition necessitates additional recovery time (Wu and Merchuk 2001) which in turn may impair the autotrophic yield $\Phi^{DW}$ in zones 1-3. As discussed in Sect. 4, vigorous mixing allows one to bypass such complex analysis and maximize productivity. The corresponding fish tank analysis is shown in Fig. 14.

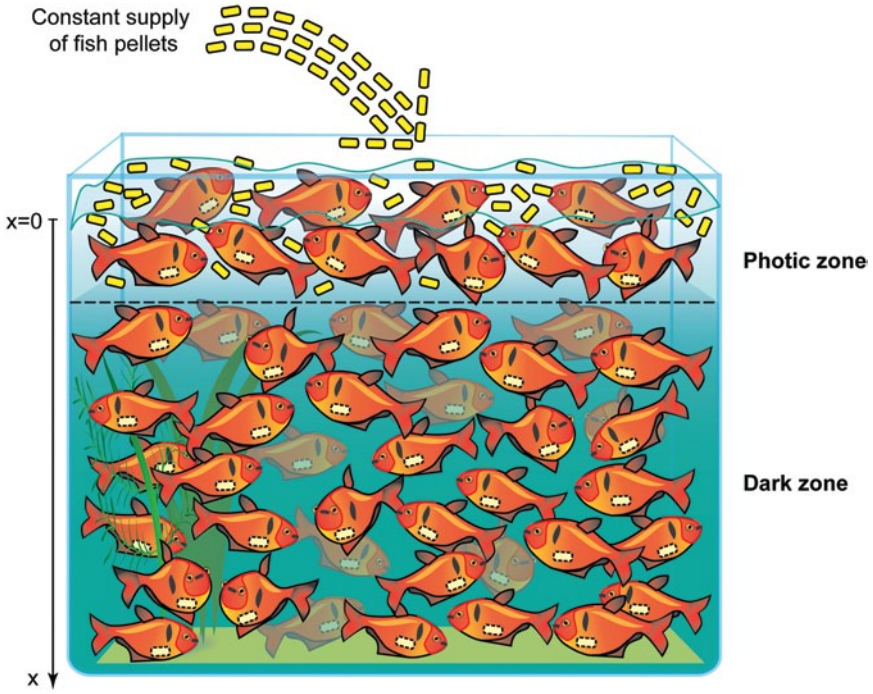


**Fig. 14** Fish-tank analogy describing the well-mixed counterpart to Fig. 13 (excellent vertical mixing)

## 6.2 Bioreactor Parameterization Under Vigorous Mixing

As an example of simple geometry (Fig. 15), we consider a planar bioreactor (such as an outdoor pond) illuminated from one side with an incident PPFD $I_0$, of culture volume $V_C$ ($m^3$) and area $A_C$ ($m^2$) normal to the light source. A nutrient feed stream (such a N or P) is supplied to the reactor at a concentration $S_0$ ($g_S\ m^{-3}$) and a volumetric flow rate $F_{IN}$ (in $m^3\ h^{-1}$), with a concentration $S$ ($g_S\ m^{-3}$) in the reactor such that $S \ll S_0$. The algal culture at a concentration $C$ is drawn out of the reactor at volumetric flow rate $F_{OUT}$ (in $m^3\ h^{-1}$). The biomass yield on the substrate $Y_{C/S}$ (in $g_{DW}\ g_S^{-1}$) is constant (Blanch and Clark 1997). Assuming that $I_0$ is low enough to support maximum yield photosynthesis at the surface of light incidence, Eq. 30 can be used to estimate the rate of photosynthesis $P(I_0,C,L)$ in $g_{DW}\ m^{-2}\ h^{-1}$ as:

$$P(C, I_0, L) = \Phi^{DW} \cdot I_0 \cdot \left[1 - \exp(\sigma^{DW} \cdot L \cdot C)\right] \tag{59}$$

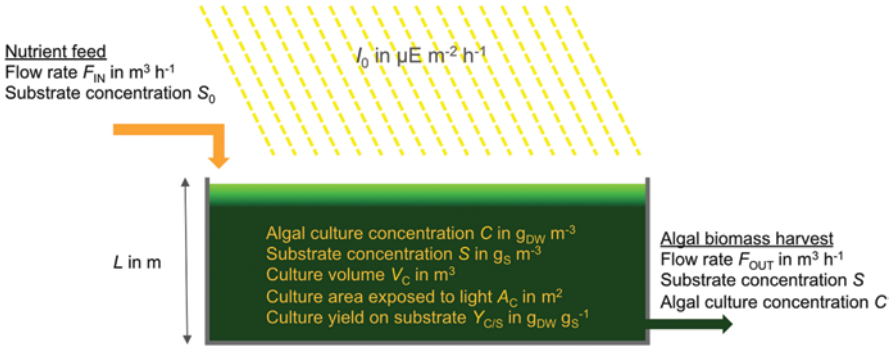


**Fig. 15** Bioreactor parameters

In this case, the following general conservation equations (Blanch and Clark 1997) hold for any type of reactor (batch, fed-batch in the substrate S, or chemostat) and whether the light is partially or fully absorbed by the algal biomass:

$$\frac{d(S \cdot V_C)}{dt} = S_0 \cdot F_{IN} - S \cdot F_{OUT} - \frac{A_C}{Y_{C/S}} P(C, I_0, L) \tag{60}$$

$$\frac{d(C \cdot V_C)}{dt} = -C \cdot F_{OUT} + A_C \cdot P(C, I_0, L) \tag{61}$$

where $t$ is the duration (in h) of growth under light.

In the example of a chemostat ($F_{CHEM} = F_{IN} = F_{OUT}$) of initial biomass concentration $C_0$, solving Eqs. 59–60 at constant $S$, $V_C$ and $C$, and taking $S \ll S_0$ constrains the nutrients feed concentration to:

$$S_0 = \frac{C_0}{Y_{C/S}} \tag{62}$$

and, solving Eq. 61, the corresponding volumetric flow rate $F_{CHEM}$ is,

$$F_{CHEM} = A_C \frac{P(C_0, I_0, L)}{C_0} \tag{63}$$

For variable irradiance $I_0(t)$, the volumetric flow rate $F_{CHEM}(t)$ can be adjusted so as to maintain a constant $S$, $V_C$ and $C$. In the event of an inhibitory light level $I_0$ for which the autotrophic yield is not maximum, the biomass concentration $C$ and culture depth $L$ can be increased to ensure not only full absorption of the incident light, but also the presence of a dark zone. Under such condition, as detailed in Sect. 4,

vigorous mixing conditions enable the use of Eqs. 59–61 to parameterize the reactor, and Eq. 59 to estimate the rate of biomass production $P$.

## 6.3 Lipid Accumulation Strategy

Growth arrest in batch culture has been the only reported means of achieving a nitrogen starvation conducive to lipid accumulation (discussed in Sect. 1.1). The parameterization presented above provides a method to accomplish it under steady-state conditions. Algal lipid accumulation in batch culture under nitrogen limitation effectively corresponds to an excess flux of light quanta compared to the rate of nitrogen taken-up. Under nitrogen excess, the algal biomass exhibits a steady-state nitrogen weight fraction (or nitrogen quotient) $Q_N$ (in $g_N\ g_{DW}^{-1}$), which is the inverse of the yield on nitrogen $Y_{C/N}$ (in $g_{DW}\ g_N^{-1}$), where:

$$Q_N = \frac{1}{Y_{C/N}} \tag{64}$$

At a given irradiance, lowering the nitrogen feed concentration $S_0$ effectively lowers the nitrogen quotient $Q_N$ due to the constraint in Eq. 62, or:

$$Q_N = \frac{S_0}{C_0} \tag{65}$$

In turn, lowering the nitrogen quotient of the biomass will lead to a lowering of the autotrophic yield $\Phi^{DW}$. As discussed in Sect. 1.1, the sole augmentation in the biomass lipid fraction increases the specific energy of the biomass, and hence decreases the autotrophic yield assuming that the metabolic energy efficiency remains unchanged under N-limitation. In all likelihood, a trade-off between lipid content and metabolic efficiency will further lower the autotrophic yield upon nitrogen limitation. The lowered autotrophic yield needs to be evaluated using Eq. 60 from a transient decrease of biomass concentration $C$ over time, assuming all other parameters are maintained at their nutrient replete value. Alternatively, the $\Phi^{DW}(S_0)$ can be established from $C(t)$ by running the reactor in fed-batch mode, by shutting the effluent. Assuming all incident light is absorbed, Eq. 60 becomes:

$$V_C \frac{dC}{dt} + C \cdot F_{IN} = A_c \cdot \Phi^{DW}(S_0) \cdot I_0 \tag{66}$$

Nevertheless, the operating condition $\Phi^{DW}(S_0)$ can be optimized for each algal culture so as to reach the greatest continuous lipid productivity $P_{LIPIDS}$ (in $g_{LIPIDS}\ h^{-1}$) as:

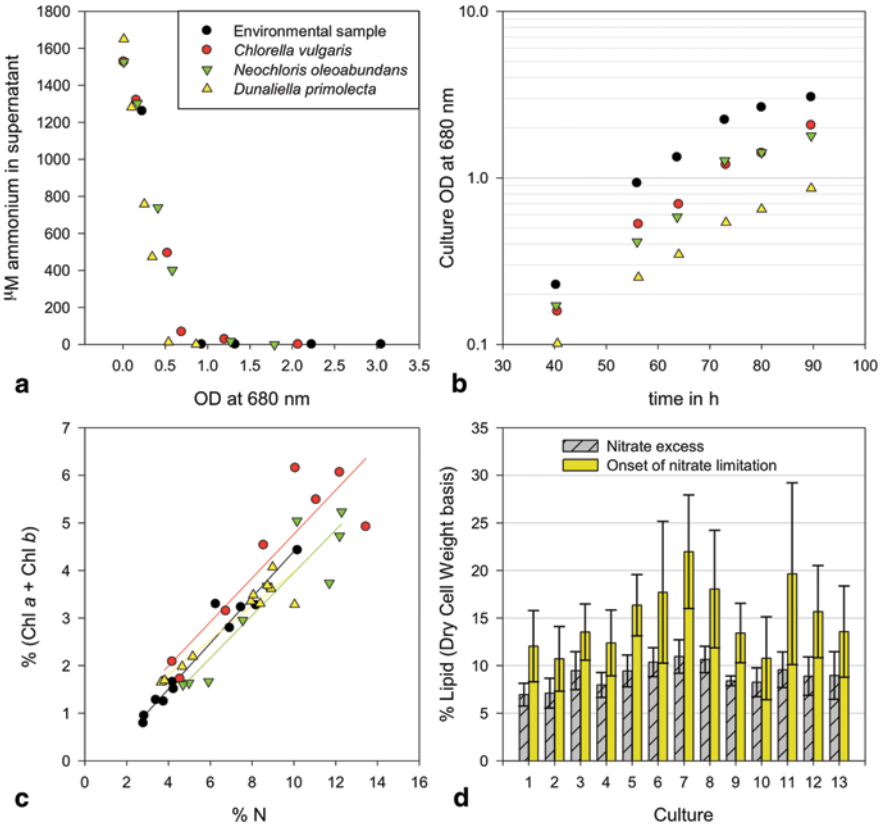


**Fig. 16** Onset of nitrogen limitation. **a** Ammonium depletion in the culture medium upon algal growth in batch. **b** Growth curves corresponding to the ammonium uptake shown in a. **c** Correlation between chlorophyll content and nitrogen quotient upon ammonium depletion for the cultures shown in a. **d** Increase in lipid content at the onset of nitrate limitation. Growth conditions using sealed carbonate addition and culture identity (1–13 correspond to Cs, Ds, Es, C, E, SE, MON, Pr5, FRA, E1, Pr8, Pr9, Pr10 respectively) are detailed in Holland and Wheeler (2011)

$$P_{LIPIDS}(S_0) = A_c \cdot \Phi^{DW}(S_0) \cdot I_0 \cdot F_{LIPIDS}(S_0) \tag{67}$$

The ability to achieve continuous lipid production under fed-batch or chemostat mode remains to be demonstrated. The following batch results (Fig. 16) provide encouraging evidence to this possibility. Ammonium depletion (Fig. 16a) occurring at an early stage during growth did not show a significant slowdown in growth rate (Fig. 16b). Hence, the nitrogen quotient can be significantly lowered from its nutrient replete value and still support good growth. The chlorophyll content correlated with the nitrogen quotient (Fig. 16c), such that biomass with a lower $Q_N$ displays a reduced antenna size, which is associated with a lower $\sigma^{DW}$ and a higher $\Phi^{DW}$ (discussed in Sect. 4.1). Finally, lipid content at the onset of nitrate starvation in batch cultures showed a significant increase in lipid content (Fig. 16d).

## 7 Concluding Remarks on Modeling Bioreactors in the Literature

Advanced control algorithms have been developed in view of controlling algal growth in bioreactors. The growth models usually followed Monod saturation kinetics, developed by Droop (1973) for algae, which assumed a limiting nutrient but did not account for light. In these complex models, light energy was either assumed in excess or not mentioned (Bernard and Gouzé 1999; Surisetty 2009; Mailleret et al. 2005; Rusch and Malone 1998; Takache et al. 2009).

Generally, characterization of algal bioreactor productivity hinges on the determination and modeling of the culture specific growth rate $\mu$. The stated or underlying assumption is that the bioreactor productivity $P_{\text{BIOREACTOR}}$ (in $g_{DW}\ m^{-3}\ h^{-1}$) follows a law of the form (Hu et al. 2012; Mailleret et al. 2005; Barbosa et al. 2003a; Vunjak-Novakovic et al. 2005; Hall et al. 2003; Molina et al. 2001; Bernard and Gouzé 1999):

$$P_{BIOREACTOR} = \frac{dC}{dt} = \mu \cdot C \tag{68}$$

where $\mu(I)$ follows a saturation kinetics described by Photosynthesis-Irradiance (PI) curves (Macedo et al. 1998; Hu et al. 2012; Molina et al. 2001).

Photosynthesis-Irradiance (PI) curves are obtained by using an algal culture of fixed concentration, such that the biomass-concentration dependency discussed Sect. 3.2 does not lead to any noticeable model discrepancies when parameters are fitted (Macedo et al. 1998). However, the resulting $\mu(I)$ derived from a PI-curve does not describe biomass growth in a bioreactor over an extended period of time, assuming light-limitation is reached, as remarked by Yun and Park (2003). The mechanistic PSU model, thoroughly reviewed and further developed by Camacho-Rubio et al. (2003), was used to account for photoinhibition processes and 'flashing light effects' in predicting rates of photosynthesis as a function of irradiance. As expected from this analysis, the PI-curve approach was satisfactory to predict specific growth rates at high rates (under high light) but deviated significantly from experimental data at low rates (Fig. 13 in Camacho Rubio et al. (2003)). Hence, the direct use of PI curves should only be limited to regions of light excess, in which the algal growth follows an exponential behavior.

As discussed in Sect. 2.1 and Sect. 5.1, biomass production follows such a law (Eq. 68) only under conditions of light excess, since under light limitation the bioreactor productivity becomes independent of the algal biomass concentration $C$. Equivalently, under conditions of complete light absorption by the bioreactor, the specific growth rate $\mu$ becomes dependent upon the biomass concentration $C$ (Eq. 32). As shown in Sect. 3, bioreactors in which light excess is stated (Rusch and Malone 1998) or implied through the use of Eq. 68 do not function optimally, since photons are lost as either passing through the culture or dissipated through non-photochemical quenching. In the case of poorly mixed bioreactors which display both a light excess and a light-limited region, Eq. 68 does not describe biomass production (Sect. 5.1). Hence, parameterization and determination of algal culture

specific growth rates $\mu$ either provide an erroneous description of biomass productivity, or an accurate description of a bioreactor displaying sub-optimal productivity.

Hence, to-date, the inability to robustly account for algal biomass production rate under light-limitation as well as light-excess has prevented the derivation of a satisfactory mass balance for the simple parameterization of bioreactors. The methodology presented here fully resolves this shortcoming.

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# Low Cost Nutrients for Algae Cultivation

**Manjinder Singh and K. C. Das**

**Abstract** Microalgae are aquatic microorganisms growing phototrophically using sunlight and inorganic nutrients viz. carbon, nitrogen, phosphorus and other micro-nutrients. Sustainable production of microalgae biomass as feedstock for renewable biofuels is facing important bottlenecks in nutrient and water requirements that may hinder commercial scale development of algal systems. Fertilizer nutrients and fresh water contribute up to 50% of the total biomass production cost that eventually impact the economical feasibility of algal fuels. In the algae-biofuels industry, nutrients must be found in lower-value sources like wastewaters and other waste streams and for sustainable production, those nutrients be recycled within the system. Integration of algal wastewater treatment with biofuel production has been strongly promoted recently. Utilizing nutrient rich wastewaters and animal wastes like poultry litter can greatly reduce the water and fertilizer demands for alga culture. Additionally, producing algal feedstock from low-cost waste based nutrient media has multiple benefits including improved water quality, N and P recycling from animal waste, reduced environmental footprints, and economic efficiency. This approach appears very attractive, since the impacts of releasing N and P and greenhouse gases into the environment could be mitigated, while conserving nutrients and simultaneously producing a material that can replace crude oil as a fuel feedstock.

**Keywords** Algae · Wastewater · Nutrient recycling · Poultry litter · Anaerobic digestion

**List of abbreviations**

| | |
|---|---|
| AD | Anaerobic Digestion |
| ADE | Anaerobic Digestion effluent |
| C | Carbon |
| CC | Carbonation Column |

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CI Carpet Industry
GHG Green house gases
N Nitrogen
P Phosphorus
PL Poultry Litter
PLE Poultry litter extract
R&D Research and Development
VTR Vertical Tank Reactor

## 1 Introduction

Two of the most pressing current global issues are environmental sustainability and the energy crisis. About 87% of global energy consumption is satisfied by fossil fuels (BP 2012). The availability of fossil-based energy may not be a threat for a considerable period of time; however, the more immediate concern is the potential threat of global climate change due to greenhouse gas (GHG) emissions from fossil fuel usage. Producing energy from renewable biological sources is critical to improved energy security and to establish an environmentally sustainable future. Significant self-reliance on alternative sources of energy protects the economy by eliminating uncertainties caused by fluctuations in fossil fuel prices. Reducing the buildup of GHG can be accomplished by using renewable and $CO_2$ neutral biofuels produced from biomass. Conventional biomass includes cultivation of energy crops, harvesting forestry residues and agricultural plant residues. Another emerging class of biomass source is microalgae, which have higher photosynthetic efficiency compared to terrestrial plants and grow rapidly. Efficient recovery of biofuel from microalgae can reduce our dependence on fossil fuels.

Microalgae use solar energy; consume nutrients such as nitrogen, phosphorus and other micronutrients from water, and $CO_2$ from the atmosphere to grow rapidly accumulating renewable biomass. Some species of microalgae produce high (>50% of their dry weight) quantity of lipids that can be converted into biodiesel and jet fuels (Sivakumar et al. 2010). The unique potential of microalgae has therefore generated interest in the potential use of algae as a new source of renewable energy. Algae as a source of biomass for energy production are particularly attractive because of their higher photosynthetic efficiency (~5%) than terrestrial plants (<1%) resulting in higher growth rates (Posten and Schaub 2009). Microalgae can produce 15–300 times more oil for biodiesel production than land-based crops (Schenk et al. 2008). As microalgae can potentially be grown on non-arable land and using wastewater and seawater resources, they do not compete with food crops for land or water. Although algal production has been tested at large scale for several years, recent evaluations (e.g. Brennan and Owende 2010) indicate potential bottlenecks to wide-scale production in areas of nutrient and water requirements, algal productivity, and energy needed for downstream processing. According to Davis et al. (2011) under current open pond and photobioreactor technologies for

commercial scale algae cultivation, the minimum selling price of algal biodiesel would be $ 9.84 and $ 20.53/gal respectively to achieve 10% rate to return. This is however, 3–7 times more expensive than petroleum diesel given its current production cost of $ 3.1/gal (EIA 2012). Therefore, the current economics of microalgal biofuels production is not competitive with traditional fossil fuels. Increasing the algae biomass productivity will provide economy-of-scale and reduce costs. Utilization of spent algal biomass after oil extraction for more valuable co-products using algae biorefinary technology could lead the algae industry into age of bio-based energy and economy (Das et al. 2010).

Algae require large quantities of carbon (C), nitrogen (N) and phosphorus (P) in addition to micronutrients such as iron (Fe). Additionally, raceway cultivation typically produces algal cell densities of 1 g/L (Pulz 2001). The biomass productivity in open raceways and closed photobioreactors is generally expressed as per unit area (g $m^{-2}d^{-1}$) and volumetrically (g $L^{-1}d^{-1}$) respectively. Most of the data assumptions reported in literature are based on extrapolations from laboratory experiments and are therefore misleading. The techno-economic analysis reported by Davis et al. (2011) assumed that currently achievable productivity in open ponds is 25 g $m^{-2}$ $d^{-1}$. This can be translated into ~80 T $ha^{-1}$ $year^{-1}$ with 330 days of operation. However, the freshwater requirements for algae cultivation in open ponds would be ~1.5 million L $ha^{-1}$ $year^{-1}$ and the evaporation losses would be ~7–11 million L $ha^{-1}$ $year^{-1}$ (Chinnasamy et al. 2010). The present state of the algal production industry (which predominantly targets high-value products such as protein supplements, nutraceuticals, and pigments) uses fertilizer grade nutrient inputs and fresh water for cultivation, a practice that cannot be carried over to the algae-biofuels industry. In the algae-biofuels industry, the sheer size of fuel demand requires that nutrients be found in lower-value sources like wastewaters (and other waste streams) and that those nutrients be recycled to the best of our abilities. In this chapter we first discuss important algal nutrients (presently derived from chemical fertilizers) and their contribution to the overall biomass production process, and thereafter we present the concept of utilizing low cost nutrients from sources such as municipal wastewater, animal wastes, and flue gases.

## 2 Algae Cultivation

Microalgae have long been used as a source of biomass and the most recent advances in systems biology, genetic engineering, and biorefining techniques suggest that microalgal biofuels could emerge as an economical and sustainable fuel source in the next 1–2 decades (Singh and Gu 2010). However, even with the newfound interest in microalgal biofuels, the technology has a long way to go to achieve cost competitiveness. As microalgal biofuel R&D moves forward, it will confront several key challenges including: finding suitable algal strains; acquiring sufficient low cost nutrients; developing better downstream processing to produce a variety of biofuels and value-added products, etc.

For high algal growth rate environmental conditions such as light, salinity (for marine microalgae), pH, and nutrient levels must fall within a preferred range (Pate et al. 2011). However, particular optimizations of physical factors that affect algal growth are strain specific. The most practical and economically feasible method of commercial scale algal cultivation is the photoautotrophic production in raceways under natural growth conditions using sun light as the energy source (Davis et al. 2011). Competing systems used for algal cultivation include outdoor raceways and outdoor closed photobioreactors. Both systems of cultivation have certain advantages and limitations. Raceways are a lower cost method of commercial scale algal cultivation with lower energy needs and maintenance costs and easy cleaning, resulting in large net energy production (Rodolfi et al. 2009). The inherent limitation of open raceway systems is the potential of contamination from other algae species and protozoa (common algal grazers). Open systems are less efficient than closed photobioreactors in terms of biomass productivity due to uncontrolled physical factors such as temperature fluctuations, evaporation losses and $CO_2$ deficiencies (Harun et al. 2010). Closed photobioreactors include tubular, flat panel and column reactors designed for advanced photoautotrophic algal cultivation. They are designed to provide greater control over temperature and nutrient delivery, prevent predators, enhance light penetration, and maintain an optimal growth environment thereby improving biomass productivities per unit area (Carvalho et al. 2006). However, the relatively high capital cost and limitations with ease of scaling up of closed photobioreactors put them under the less-preferred category for commercial scale algae production for biofuels (Benemann 2009; Davis et al. 2011).

## 3 Nutrients Requirements for Algae Cultivation

As aquatic organisms, microalgae need water for growth along with inorganic salts and $CO_2$. The major essential inorganic elements are nitrogen (N) and phosphorus (P) and for diatoms silicon (Si). Other nutrients required for favorable algal growth are iron, sulfur, potassium, magnesium and other micronutrients and cofactors (vitamins).

### *3.1 Water*

Algal biomass production utilizes large quantities of water that not only provide a growth environment for algae to live and multiply, but also serves as a medium for nutrients delivery, waste removal and temperature regulation. To generate a dry gram of algae biomass, more than a kilogram of non-cellular water is required (Murphy and Allen 2011). The volume of water required for algal cultivation depends on system type and geometry, natural losses from the system, and most importantly, the ability to retain, reclaim and reuse water within the system. This way, the volume of water necessary to grow algae is estimated from two key factors;

amount of water needed to be retained in the system to maintain target biomass productivity and amount required for replacing water losses due to evaporation and downstream processing. One approach to minimize freshwater footprints of algal cultivation is efficient water recycling. Life cycle analysis by (Yang et al. 2011) indicates that 3,276 kg of water is required to generate 1 kg of biodiesel if freshwater is used without recycling, and that a 55 % reduction potential exists if water is recycled. Microalgae have the potential to generate $220 \times 10^9$ L year$^{-1}$ of oil which is equivalent to 48 % of current U.S. petroleum imports for transportation. However, the water footprints (312,079 GL year$^{-1}$) for this level of production would be nearly equivalent to three times of the 113,135 GL year$^{-1}$ of fresh water used for irrigated agriculture in USA in 1995 (Wigmosta et al. 2011; Roy et al. 2005).

## *3.2 Carbon*

Carbon (C) is the most abundant element in algae, contributing around 50 % of algal biomass by weight (Grobbelaar 2004). Under photoautotrophic conditions algae utilize atmospheric $CO_2$ as carbon source to synthesize organic compounds. Once dissolved in water, there are three principle interconvertible chemical forms of dissolved inorganic carbon viz. $CO_2$ (aq), $HCO_3^-$ and $CO_3^{2-}$ whose concentrations vary based on the pH of the aqueous environment (Goldman et al. 1981; Becker 1994). Although $HCO_3^-$ is easily absorbed by algal cells, $CO_2$ is reported to be the most preferred source of inorganic carbon (Goldman et al. 1981). However, at pH > 10.3 the $CO_3^{2-}$ form dominates which is generally an unusable form and not available for algal uptake (Knud-Hansen 2006).

$CO_2$ is often the limiting substrate for photosynthetic cultivation of algae in high rate algal ponds growing at specific productivity of 20 g m$^{-2}$ d$^{-1}$. Atmospheric air provides to the pond surface only 5 % of the $CO_2$ required for photosynthesis (Stepan et al. 2002). Hence, $CO_2$ is usually provided via bubbling of concentrated $CO_2$-air mixture into the algal pond. Analyses indicate that $CO_2$ procurement is a significant cost and accounts for about 40 % of energy consumption and 30 % of GHG emissions in algal cultivation (Clarens et al. 2010). Therefore, targeting industrial C emissions as a source is attractive and can increase the overall sustainability of algal cultivation.

There are a number of algae that are facultatively heterotrophic and prefer, where available, an organic carbon substrate over fixing $CO_2$ (Shi et al. 2000). Some algae are mixotrophic and can simultaneously drive phototrophy and heterotrophy to utilize both inorganic ($CO_2$) and organic carbon substrates (Sun et al. 2008; Bhatnagar et al. 2011), thus leading to an additive or synergistic effect of the two processes that enhances the productivity and in turn capability of microalgae to grow in wastewaters. Under phototrophic growth, algae harvest radiant energy from sun and convert into valuable biomass at the expense of inorganic nutrients and natural resources (Carvalho et al. 2006). However, microalgae biomass production via

this mode cannot reach maximum cell density since light penetration is inversely proportional to the cell concentration (Chen and John 1995). Light requirements increase as cell concentrations increase because mutual shading blocks the penetration of light to algae further in the culture vessel (Posten 2009). As a result deeper regions within algae cultivation system (e.g. ponds) will get deprived of light and net photosynthesis cannot occur (Richmond 2004). Shallow ponds are therefore preferred mode of phototrophic algae cultivation though it brings along several drawbacks such as, large footprints, high evaporation rate (Harun et al 2010). The light penetration limitation becomes more prominent when goal is autotrophic algae cultivation using dark colored industrial wastewater as light penetration inhibition effect become even more prominent. Mixotrophy could overcome problems associated with phototrophic algal growth, such as, light limitation at high cell densities and when using dark colored (opaque) wastewaters.

## *3.3 Nitrogen*

Nitrogen is a major component of cellular proteins and amino acids and comprises around 5–10% of algal biomass by weight. Despite atmospheric abundance of nitrogen (78% by volume), algae cannot directly utilize nitrogen gas. The primary N molecules that can be utilized by all algae are ammonia and nitrate, between which the former is the preferred form for algal growth. The biological nitrogen fixation by diazotrophic microbes such as *Rhizobia*, *Azospirillum*, *Anabaena* and *Nostoc,* via reduction of dinitrogen to ammonium was the only route where atmospheric nitrogen entered into living systems until 1909 when the Haber–Bosch process, which chemically converts nitrogen gas into ammonia (Smil 2001), was invented. Currently, algal cultivation predominantly uses nitrogen fertilizer produced from the Haber–Bosch process. Production of 227 billion L (60 billion gal) of algal-biodiesel, which is equivalent to 30% of the U.S. transportation fuel consumption in 2010, requires 36 million T of nitrogen fertilizer (Huo et al. 2011). However, to produce this volume of biofuels 26% of the energy in the final fuel product is spent in fertilizer production, adding to the overall cost of algal-biofuels production (Huo et al. 2011). Fertilizer-grade nutrient inputs and freshwater accounts for 50% of energy inputs associated with algal cultivation (Clarens et al. 2010). Requirements of fertilizer nitrogen input can be minimized either by recycling algal-biomass nutrients via anaerobic digestion and/or thermochemical conversion techniques (Rösch et al. 2012) or by utilizing nutrient rich wastewater as culture medium (Clarens et al. 2010).

## *3.4 Phosphorus*

Of the three primary nutrients (N, P and C) necessary for algal growth, phosphorus (P) is the scarcest nutrient in natural environments. Typically, microalgae contain 1% P by weight in their biomass (Borchardt and Azad 1968). In some cases algae have higher concentrations resulting from luxury uptake of P, which is performed

when this element is available is excess in the medium (Powell et al. 2008). The major source of P in natural environment is phosphate rock obtained through mining. World P reserves are being depleted and some believe that the reality of "peak phosphorus" could be reached in the next few decades (Cordell et al. 2009). Clearly, for sustainable implementation of algal biofuels on a large scale, P requirement has to be obtained from sources other than mineral rock phosphate. The only practical means of sustainable P supply for agriculture and algaculture is through recycling P from manure and other kinds of plant and animal waste. In principle, to close the P cycle, the P content of the algal-waste left after the oil-extraction or conversion process must be recycled into growing the next batch of algae. (Rösch et al. 2012) reported in a material flow modeling study that nutrient recycling rates in the range from 30 to 90% for nitrogen and from 48 to 93% for phosphorus can be achieved via anaerobic digestion (AD) and hydrothermal gasification of oil-extracted algal biomass. Biogas and biocrude oil would be the biofuel products from these processes respectively thereby adding economy to algae biofuels. The nutrient rich AD effluent can be used as growth medium for growing second batch of algae (Singh et al. 2011).

## 4 Integration of Algal Technology with Waste Recycling for Bioremediation and Biofuels

Open pond cultivation of microalgae require large quantities of water, which subjects algal cultivation to controversy, as world water resources are already depleting against exponentially increasing demand for agricultural and industrial use (IWMI 2009). Additionally, the global target for feedstock crops for biofuels production for 2030 itself would demand a staggering 180 $km^3$ of water (IWMI 2008). Consuming fresh water and fertilizers for algal cultivation will therefore not be environmentally sustainable or economically viable. To reduce this impact, nutrient rich wastewater should be used to offset such environmental and cost burdens associated with algal cultivation. Variations in the composition of wastewater and presence of several unknown constituents, however, could limit the growing of monoculture algal strains in wastewater. Therefore it is essential to select robust, mixotrophic algal consortia that are capable of growing in a variety of wastewaters (municipal, industrial, agricultural and aquaculture wastewaters), improving water quality and simultaneously producing feedstock for biofuels. Integration of biomass production with wastewater treatment will improve the economic feasibility of commercial scale algal cultivation.

### *4.1 Wastewater as Algae Cultivation Medium*

Based on nutrients available in the wastewaters from municipal sources, piggery, and dairy cattle worldwide, Harmelen and Oonk 2006 estimated that the production of 90 million T of algae $year^{-1}$ will be technically feasible in 2020 using

municipal wastewater (~40 million T), dairy wastes (~30 million T) and pig wastes (20 million T). Also it was estimated that wastewaters from about 30,000 people or about 5,000 pigs or 1,200 dairy cattle are required for a minimum economically viable scale of about 10 ha of algal ponds. This study shows the biomass production potential of various waste streams (Harmelen and Oonk 2006).

In addition to municipal, agriculture and aquaculture wastewaters, industrial effluents also can be used for algal cultivation to produce bioenergy. In previous research in our group, we evaluated different cultivation systems, namely, raceways, vertical tank reactors (VTRs) and polybags for mass production of algal consortia using carpet industry (CI) untreated wastewaters (Chinnasamy et al. 2010). Overall areal biomass productivity of polybags (21.1 g $m^{-2}$ $d^{-1}$) was found to be the highest, followed by VTRs (8.1 g $m^{-2}$ $d^{-1}$) and raceways (5.9 g $m^{-2}$ $d^{-1}$). We estimated biomass productivity of 51 and 77 Tons $ha^{-1}$ $year^{-1}$ can be achieved using 20 and 30 L capacity polybags, respectively (Chinnasamy et al. 2010). Though the lipid content of the wastewater grown algae is low, the energy stored in other constituents of the biomass could also be recovered through thermochemical liquefaction where the algal biomass with less lipids and 80–85 % moisture could be converted directly to a biocrude with yield in the range of 30–44 % and a heating value of 34.7 kJ $g^{-1}$ (Amin 2009) or into biogas through anaerobic digestion.

We also assessed the potential of carpet wastewater grown algae as energy crop for biomethane production and found that bioenergy recovery from algal consortia cultivated using the wastewater was better than yields estimated for cereals and sunflower (Chinnasamy et al. 2010). That study estimated that the consortium of algae cultivated in polybags using carpet industry untreated wastewater has the potential to produce ~134,144 kWh of renewable power $ha^{-1}$ $year^{-1}$ compared to the estimated value of 97891, 42585 and 39543 kWh for maize, cereals and sunflower, respectively.

Our research group has been operating duplicate 100 $m^2$ raceways at a carpet wastewater treatment facility where treated wastewater is fed to raceways for algae cultivation. Over a one-year period of continuous operation, on average 200 mg/L biomass production was achieved in raceways. The most interesting result obtained was the 70–80 % removal of P from the wastewater which might be due to luxury uptake of P by algae as the algal biomass harvested was found to have around 3 % P by weight in contrast to the more typical 1 % P content in algae. Raceways were initially inoculated with a consortium of *Chlorella minutissima, Scenedesmus bijuga* and *Chlorella sorokiniana* strains. However, as was expected in open raceways system using wastewater, these strains could not maintain their dominance and were superseded by other locally dominant algae. Weekly samples collected from the two raceways were analyzed for microalgal diversity and biovolume ratio of various species that dominated in different seasons. The results revealed that cyanobacteria (blue green algae) dominated during summer, representing 95 % of the total microalgal population identified in the raceways. *Synechococcus elongates* was the most dominant cyanobacteria during summer and was on average 41 % of the total microalgal population throughout the duration of a batch run. Another dominant strain was *Synechocystis* spp. contributing up to 39 % of the total population. *Leptolyngbya breviarticulata* was also among

the dominant cyanobacterial spp. In contrast, during winter green and blue green algae were almost in equal proportions, representing 47–53 % of the total microalgal populations, respectively. Among green microalgae, *Paulinella* was the most dominant with average biovolume of 53 % of the total green algae identified. *Chlorella vulgaris* (15 %) and *Trebouxia gelatinosa* (8 %) were also among the dominant green algal species seen. *Euhalothece sp.,* a salt tolerant cyanobacterium, was the most dominant blue green representing 14 % of the total microalgal population. We conclude from these experiences that for sustainable algal cultivation using wastewater, indigenous algae that are already adapted to the local environment will grow dominantly and is preferred. Further, the seasonal variation in environmental conditions will affect microalgal diversity and biomass quality and quantity from cultivation systems.

## *4.2 Nutrient Delivery From Animal Waste*

Poultry production is the number one agricultural business in the state of Georgia (USA). With more than 5,000 poultry farms in the state, approximately two million tons of poultry litter (PL) is generated annually in Georgia which is ~20 % of the national annual PL generation. The PL from broilers contains approximately 11.3 kg-P and 32.6 kg-N per ton of litter. An estimated amount of nutrients equivalent to 108 T of urea and 85 T of Di-ammonium phosphate can be recovered from PL (Lory and Fulhage 1999). Authors earlier reported isolation of several mixotrophic microalgae belonging to *Chlorella* and *Scenedesmus* genera capable of preferably growing on wastewater and poultry litter extract (PLE) and producing biomass as much as typically achieved from freshwater-based enriched growth media (Bhatnagar et al. 2011). In comparative open pond algal cultivation studies, it was observed that PLE media promoted better growth than standard synthetic fertilizer media. The average growth rate of algae over 18 days operation of ponds was >200 mg $L^{-1}$ and the biomass productivity was 7 g $m^{-2}$ $d^{-1}$ which represents approximately 21 T $ha^{-1}$ $year^{-1}$ with 300 days of cultivation in raceways. Further, an efficient method was developed for extracting nutrients from PL and used those nutrients as growth medium for algal cultivation. This in-situ extraction technique was further optimized in pre-pilot raceways. The insoluble residue from extracted PL was anaerobically digested and was found to have no significant difference in biogas yield when compared to un-extracted PL.

## *4.3 Nutrient Recycling via Anaerobic Digestion of Algae Biomass*

Recently, anaerobic digestion (AD) has seen a resurgence of interest due to its potential for biogas production using high moisture substrates like algae. Methane ($CH_4$, the principal component of biogas) is one of the cleanest and most energy efficient transportation fuel. Interest is growing in use of $CH_4$ as a transportation

fuel in Europe and Asia where natural gas vehicles are widely driven. Also, biogas generated biopower can be used in electric vehicles. Campbell et al. (2009) noted that biopower pathways deliver more transportation GHG offsets than liquid biofuels. A key advantage of AD is the mineralization of organic N and P into ammonium and phosphate, which can then be recycled to satisfy N and P requirements of algal cultivation making algal production environmentally and economically sustainable.

Although AD of algal biomass has not been reported widely, AD as a technology is well established and commercial scale operations are economically viable. Major challenges in AD of algal biomass include its low C/N ratio due to high protein content of algae and the inability of AD bacteria to degrade intact algal cells, because of cell wall recalcitrance, resulting in low conversion efficiencies (Ehimen et al. 2010). A literature review has shown that there are several researchers who have mentioned the potential for effluent recycling, but we could not find any that have provided experimental data. Our laboratory has had a 1,000 L digester consistently generating biogas with average $CH_4$ content of 50–80 %(v/v) for the last several months. Effluent from the digester typically is dark in color, which can be centrifuged to obtain a supernatant rich in dissolved nutrients. A representative composition of the AD effluent (ADE) supernatant from our 1000 L digester is given in Table 1.

The reactor was fed an algal slurry at 2 % solids and since algae have about 0.76 % P (Rösch et al. 2012), a total of 152 mg-P/L was fed to reactors. Of this, 100 mg/L (66 % of input P) was recovered as soluble *o*-phosphate suitable for algal cultivation. The ADE supernatant has almost all the nutrients required in a balanced algal growth medium at about tenfold the required concentration. In previous work, we have demonstrated significant algal growth using diluted (6 %) effluents from poultry litter anaerobic digesters without supplementation of any nutrients (Singh et al. 2011). Results showed that all organisms evaluated reached cell densities greater than 0.55 g/L, with *Scenedesmus bijuga* reaching the highest concentration of just below 0.70 g/L in eight days. Biomass productivity of 76 mg $L^{-1}$ $d^{-1}$ was recorded for microalgae grown in PLDE with concomitant nitrogen (60 %) and phosphorus (80 %) removal from effluent in 8 days. The algal biomass was rich in proteins and low in lipids and could be used as an animal feed supplement. Although wastewater grown algae may not have a high lipid content, AD of algal biomass can produce as much energy as can be recovered from extraction of lipids (Sialve et al. 2009). Several researchers have concluded that bioenergy pathways that include wastewater use and biogas production may be the most practical processes for converting algae into energy (Costa et al. 2008; Campbell et al. 2009; Wiley et al. 2011).

## *4.4 Carbon Supplementation*

Algal biomass contains about 50 % C and it has long been known that supplementing C in algal cultivation will increase productivity significantly. Growing algae at high productivities (>20 g $m^{-2}$ $d^{-1}$) is typically done by bubbling air (or 5 % $CO_2$)

**Table 1** Elemental composition of clear Algae-AD Effluent (ADE)

| Elemental analysis | ADE (mg/L) |
|---|---|
| Total Phosphorus (TP) | 103 |
| *o*-phosphate P ($PO_4$-P) | 100 |
| Potassium (K) | 100 |
| Calcium (Ca) | 94.8 |
| Sodium (Na) | 160 |
| Magnesium (Mg) | 53.7 |
| Sulfur (S) | 13.3 |
| Iron (Fe) | 4.78 |
| Total Nitrogen (TN) | 322 |
| Nitrate-N ($NO_3$-N) | 0.6 |
| Ammonium-N ($NH_3$-N) | 281 |
| Total organic carbon (TOC) | 300 |
| COD | 973 |

through the culture liquid. Targeting industrial C emissions as a source is attractive and can increase the overall sustainability of algal cultivation.

An important challenge with using exhaust gases from fixed sources is the geographical disconnect between the source and algal farms. Pumping $CO_2$ gas distances over a mile is cost prohibitive (Sheehan et al. 1998), and such pumped gas is only usable during daylight hours for photosynthesis, thus requiring a capture and storage method. Typically, $CO_2$ is separated from a mixed gas stream (e.g. flue gas), compressed to 150 atm and transported off site in containers, which are energy intensive processes. Kadam (1997) estimated delivered $CO_2$ cost for the standard process with monoethanloamine (MEA) extraction as US$40.5/MT for a 500 MW power plant. This cost included $ 28.72 for $CO_2$ capture via MEA extraction, $ 8.48/MT $CO_2$ for compression and drying, and $ 3.30/MT $CO_2$ for transportation.

It is known that bubbling a gas stream through the algal culture medium results in only a fraction of the $CO_2$ taken up by the algae and as much as 80–90 % of the $CO_2$ is simply lost to the atmosphere (Becker 1994; Richmond and Becker 1986). We have developed and used a carbonation column (CC) system to increase the interfacial area of contact available for gas exchange to liquid and propose it as an efficient alternative (Putt et al. 2011). The CC performance recorded was 83 % $CO_2$ transfer efficiency. This CC design is an example of a hybrid system combining a column bioreactor and open pond. The proposed device can be used with any exhaust gas stream with higher concentrations of $CO_2$ in conjunction with raceways for optimizing algal production. The use of the CC for $CO_2$ mass transfer into microalgal culture ponds not only offers enhanced efficiency of gas transfer but also meets the $CO_2$ demand of high-rate algae outdoor ponds. The CC performance is twice the transfer rate compared to direct bubbling, and thus offers the opportunity to significantly reduce the cost of algae pond carbonation. For less than a 1 % increase in the installed cost of the farm, the cost of the carbon dioxide when a CC pit is used is nearly half that of the deepest in-pond carbonation well reported in the literature (Putt et al. 2011). The ease to design and construct the CC makes it an economical

device for carbon recycling and can be used with various $CO_2$ rich air streams for optimizing algae production.

Overall, microalgae have the potential to curb emerging environmental problems, by fixing $CO_2$ released from industries and treating industrial wastewaters. Such technology meets the priorities of developing countries handling wastewater from different sources in a sustainable, cost-effective, and environmentally sound manner. Future work should focus on evaluating the economics of integrated waste treatment processes for commercial-scale production of algae biodiesel, biomethane, bioethanol and biocrude through biochemical and thermal conversion processes.

## 5 Conclusion

Production of bioenergy from waste streams conserves natural resources. Integrated waste management coupled with bioenergy production, can be a near term solution. Apart from treating wastes, this microalgae technology also produces renewable algal biomass for conversion into value added products such as biomethane, biocrude, biodiesel, bioethanol and protein supplements. Coupling microalgae cultivation with nutrient removal from animal wastes (e.g. poultry litter) will be an attractive option for minimizing fertilizer requirements and eventually the energy costs of biofuel generation from microalgae. Successful implementation of microalgae technologies for wastewater treatment and $CO_2$ cycling would help to establish advanced integrated waste management facilities for production of bioenergy and bioproducts in the future. Producing energy locally from renewable biomass sources is critical for achieving energy independence and these pathways described here show great promise. Future work should focus on large-scale microalgae technologies for waste treatment coupled with bioenergy production.

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# Microalgae Bioreactors

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**Abstract** Photobioreactor design and operation mode are essential steps to ensure a high overall microalgae yield and cell productivities, making viable the commercial production. For this reason, there are trends of research in the field of microalgae that encompass design and development of reactor systems towards maximum productivity with minimum operation costs. In the literature, various photobioreactor designs have been employed such as open ponds, bubble column, flat plate, and tubular (conical, helical, etc.). Open ponds are the most commonly applied photobioreactor design in industrial processes. On the other hand, studies have been focused on tubular photobioreactors due to the possibility of achieving high volumetric productivity and better biomass quality. Therefore, in this chapter, some photobioreactor designs and their characteristics such as geometrical configuration, building material, and cell circulation systems will be discussed. Moreover, the operation mode, such as temperature and pH control, nutrient feeding, $CO_2$ addition systems, flow rate, light supply, mixing, cultivation process and cleanness will also be considered to be important parameters in this field.

**Keywords** Photobioreactors · Microalgae · Photobioreactors operation · Microalgae culture circulation · Microalgae cultivation processes

**Acronyms**

| | |
|---|---|
| DHA | Docosahexaenoic acid |
| DO | Dissolved oxygen |
| DW | Dry weight |
| H/D | Height to diameter ratio |
| HDPE | High Density Polyethylene |
| LDPE | Low Density Polyethylene |
| NER | Net Energy Ratio |
| PBR | Photobioreactor |
| PBRs | Photobioreactors |
| PEP | Photosynthetic efficiency |
| PMMA | Rigid acrylic |

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PVC Poly Vinyl Chloride
TRC Transparent rectangular chamber
UV Ultraviolet
VAP Vertical alveolar panels
VFPP Vertical flat-plate photobioreactor

## 1 Introduction

Microalgal production has been studied for decades, given the wide variety of potential metabolic products, such as protein, lipids, starch, vitamin, enzymes, polymers, and pigments that can be used as food supplements, feed, biodiesel, and "green energy" products.

Different reactor configurations and cultivation strategies are still targets of intense research to improve biomass growth, since all have some inherent problems, such as control of light intensity, $CO_2$ introduction, pH control, inoculum size, proper flue gas rate, optimal nutrition level, oxygen concentration control, culture mixing, water loss, contamination, temperature control, nutrients addition, huge land area, algae collection and algae aggregation and adhesion.

Most of problems are either unresolvable or may require a lot of costly modifications that are not very much favored by microalgae producers. Among main designs there are: open ponds, bubble column, flat plate, and tubular photobioreactors. Each design has its own problems which place the efficiency far behind theoretical calculations and wishful expectations.

Commercial-scale culture of microalgae generally requires the ability to economically produce ton quantities of algal biomass. Acién et al. (2012) showed that the cost of photobioreactors is the major factor in the production cost for large scale and that the reduction of the photobioreactor cost dramatically decreases the biomass unit production cost.

Mode of culture circulation of culture, providing light availability, $CO_2$, $O_2$ removal, pH control, temperature and nutrient feeding, materials utilized for constructing photobioreactor and mode of cleaning are several basic features that must be considered when building a photobioreactor. In this chapter the main characteristics of different configurations of photobioreactors will be considered.

## 2 Photobioreactors

Different photobioreactor configurations have been designed, built, and described depending on the ultimate goal. Important design aspects include lighting, mixing, water consumption, $CO_2$ consumption, $O_2$ removal, nutrient supply, temperature maintenance, material for construction, and cleaning. In general, sophisticated photobioreactors are more versatile but more expensive to construct and more

complicated to operate. The choice depends on several factors, including the microalgal growth mode, the culture medium composition and the value of the final product. The photobioreactor geometry must maximize production of microalgal bioproducts while minimizing the land surface occupied.

## *2.1 Types of Photobioreactors*

Several types of photobioreactors have been constructed to support the demands of microalgal products such as open ponds, illuminated stirred tank reactors, tubular or flat plate photobioreactors. Although there are several types of photobioreactors described in the literature, there are still several researchers proposing new types of photobioreactors in order to increase productivity and reduce costs of industrial production. Many challenges include: (i) minimizing contamination (ii) efficient supply of carbon dioxide and light; (iii) controlling cultivation conditions; (iv) reducing capital and production costs; and (v) minimizing space requirements (Marxen et al. 2005; Molina Grima et al. 1999). This item is going to address some of the main types found in the literature.

In general, we can classify the several types of photobioreactors as:

1. Indoor or outdoor systems
2. Open or closed systems
3. Photobioreactor configurations: Open ponds, flat plate, vertical-column, tubular and their variations such as horizontal, inclined, vertical, spiral, conical, helical; manifold and serpentine.

### 2.1.1 Indoor or Outdoor Systems

Several types of lighting have been used to produce large quantities of phototrophic algae such as sunning, artificial light or both. Outdoor systems are where light is supplied as sunlight and the cells are subject to environmental variations. Sun light is free-cost, the cheapest light source available, whereas artificial light sources may be very expensive. Solar energy contains the full spectrum of light energy, and, through a specific UV filter, it can provide a suitable absorption wavelength for both microalgae cell growth and target product production (Chen et al. 2008). Efficient utilization of solar energy can simultaneously solve the problems of a high operating cost, electricity consumption and environmental pollution. Outdoor system has been utilized in industrial scale to Chlorella and *Arthrospira* (*Spirulina*) production for health food, *Dunaliella salina* for β-carotene, *Haematococcus pluvialis* for astaxanthin, *Crypthecodinium cohniia* and *Schizochytrium* sp. for docosahexaenoic acid (DHA). It is worth mentioning that although according to Silva et al. (1996) the correct scientific designation for *Spirulina platensis* is *Arthrospira platensis,* in this chapter, the denomination given by the authors of the referenced articles was maintained.

Generally microalgae cultures in photobioreactors are suitable for outdoor cultures. Most outdoor photobioreactors are characterized by largely exposed illumination surfaces and are subject to changes in time of day, weather, season, and geography. Commercial outdoor cultivation is generally restricted to tropical and subtropical zones in regions of low temperature change, rainfall and low cloud cover. Unfortunately, all outdoor reactors using natural light are subject to the absence of light during night time, what suggest that studies focusing the night light supplying with solar energy accumulated during the day could be useful for the development of this technology. In fact, According to Chisti (2007), biomass losses might reach as high as 25 % during the night, depending on the light intensity during the day, the temperature during the day, and the temperature at night.

Several indoor microalgal cultivations have been extensively studied in laboratory-scale photobioreactors. However, scientific information about outdoor cultivations is still scarce, even though those are so important for industries.

Some care need to be taken into account when attempting to change the type from indoor to outdoor. Scoma et al. (2012) related that the $H_2$ production from *Chlamydomonas reinhardtii* is possible after a previous cell acclimatizing to sunlight, a condition that caused a number of physiological changes, namely: (i) a decrease in the chlorophyll content per unit of dry weight; (ii) an increase in the photosynthesis and respiration rates, and (iii) a higher induction of the xanthophylls cycle pigments as compared to non-acclimated cultures.

In the indoor systems, light is supplied by electric lights and it allows control over illumination and temperature in the photobioreactor cultivation. Laboratory-scale photobioreactors are usually artificially illuminated with fluorescent or other types of lamps, which can require high power consumption and high operating cost. This system is more used in laboratory scale since energy used to keep the light on can endear to industrial production. On the other hand, artificial lighting techniques can provide to researchers an insight into how algae respond to varying light conditions and other parameters. In addition, it is also possible to transmit solar energy from outside to illuminate indoor photobioreactors, such as solar-energy-excited optical fiber systems. The major obstacle to their practical application is the high power consumption and operating cost due to the need for artificial light sources.

Lopez-Elias et al. (2005) related that outdoor cultivation of diatom *Chaetoceros muelleri* growing may be as safe and reliable as indoor one, and that the savings caused by the lower power consumption may mean a reduction of more than 40 % of the annual operating cost of the area of microalgae production.

### 2.1.2 Open or Closed Systems

Open systems are mostly uncovered ponds and tanks or natural lakes. These systems have almost always been located outdoor and rely on natural light for illumination. Although they are inexpensive to install and run, open systems suffer from many problems: cultures are not axenic so contaminants may outcompete the desired algal species; predators like rotifers can decimate the algal culture; weather

variations can hinder proper control of nutrients, light intensity, and $CO_2$; poor light utilization by the cells; evaporative losses of water and others volatile compounds to the atmospheres; and requirement of large areas of land (Carvalho et al. 2006).

As open system is more readily contaminated than closed culture vessels such as tubes, flasks, carboys or bags, it is limited to a relatively small number of algae species that control contamination by using highly alkaline, saline selective environments or other strategies. The open raceway pond reactor can be used to strains cultivation that, by virtue of their weed-like behavior (e.g. *Chlorella* sp.) or by their ability to withstand adverse growing conditions as *Spirulina* (*Arthrospira*) sp. or *Dunaliella* sp., can outcompete other microorganisms (Del Campo et al. 2007).

Open ponds systems are made of leveled raceways 2–10 m wide and 15–30 cm deep, running as simple loops or as meandering systems. Each unit covers an area of several hundred to a few thousand square meters. Turbulence is usually provided by rotating paddle wheels, which create a flow of the algal suspensions along the channels at a rate of 0.2–0.5 m $s^{-1}$ (Del Campo et al. 2007).

Cultivation of microalgae using natural and man-made open-ponds is technologically simple, but not necessarily cheap due to the high downstream processing cost and low cell productivity. Products of microalgae cultured in open-ponds could only be marketed as value-added health food supplements, specially feed and reagents for research (Lee 2001) and energy source.

An alternative to open ponds are closed photobioreactors which provide better options to grow every microalgae strain, protecting the culture from invasion of contaminating organisms and allowing exhaustive control of operation conditions. Moreover, in closed photobioreactors, the higher ratio between illuminated area and culture volume leads to higher final biomass concentration compared to open-pond culture.

The technical difficulty in sterilizing closed photobioreactors has hindered their application for the production of high value pharmaceutical products. Cultivation of microalgae in heterotrophic mode in sterilizable fermentors has achieved some commercial success (Lee 2001).

Closed photobioreactors can adopt a variety of designs and operation modes. But only a few attempts have been made for scale-up photobioreactors to commercial size. They offer higher productivity and better quality of the generated biomass (or product), although they are certainly more expensive to build and operate than the open systems.

Closed photobioreactors are considered the most productive systems but to be competitive their cost must be reduced below the cost of the current open raceways reactors. In addition, large facilities capable of producing more than 150 t/ha·year must be operated with low labor costs, using flue gases as carbon source and wastewater as growth medium in the largest possible extent (Acién et al. 2012). But, even aiming to diminish the cost, it is necessary to keep in mind the application of the product to be produced.

New design has been done by improving shaping of the photobioreactor (PBR), controlling environmental parameters during cultivation, aseptic designs, cleanness mode and operational approaches to overcome rate-limiting of growth, such as pH,

temperature, light supply, nutrient addition, mixing, and gas diffusion. The main final goal of any PBR is reduction in biomass production costs and/or allowing mixotrophic or heterotrophic cultivation of microalgae. A feasible alternative for phototrophic cultures in PBRs, but restricted to a few microalgae species, is the use of their mixotrophic or heterotrophic growth.

Algae have also been cultivated in bag type reactors. However, according to Borowitzka (1999), the big bag system suffers from the need to be operated indoors for adequate temperature control. In addition, if installed indoors, the large bags cannot be sufficiently illuminated by artificial lighting, and mixing is generally insufficient (Kunjapur and Eldrigde 2010). Thus, it is necessary additional studies considering this type of photobioreactor, including temperature maintenance and mixing systems.

### 2.1.3 Photobioreactors Configurations

Many configurations of photobioreactors have been devised and built. They range from ponds to tubular and cylindrical systems to conical systems to flat-sided vessels. The most natural method of growing algae is through open-pond. For convenience, the closed photobioreators are described herein by categories:

1. Vertical column photobioreactor
2. Tubular photobioreactor
3. Flat panel photobioreactor

There are some works on high performance photobioreactors of closed type. Flat-plate, horizontal and inclined tubular photobioreactors are among the most suitable types for mass cultivation of algae because they have large illumination surface (Chisti 2006), but there are difficulties in scaling them up. Though large-scale designs are the focus, the majority of experiments comparing the designs were performed at laboratory scale.

For any type of photobioreactor, the mixing is an essential parameter to be considered. Some preliminary comments are presented here. More detailed study will be presented in posterior sections.

The mixing systems most described in the literature are those mechanically agitated with paddle wheels found mainly in open ponds and agitation with air as found in bubble column and air-lift systems. Choice of mixing system depends on the bioreactor configuration, type of cell and medium composition properties. Liquid flow velocity must be sufficiently high to ensure a turbulent flow so that cells do not stagnate in the interior darker portion of the tube for long. However, excessive turbulence can damage cells and this poses an upper limit on the culture velocity. The trichomes of *Arthrospira platensis* are sensitive to shear when mechanical agitation systems are used, so pneumatic system is most suitable for this type of cultivation. Markl et al. (1991) showed that hydrodynamic stress reduced the size of the cyanobacterial trichomes of *Spirulina platensis.* Sánchez Pérez et al. (2006) related

that the average shear rate is related with the superficial aeration velocity and the rheological properties of the fluid.

The stirred reactors have paddle wheels which consist of simple partial-depth blades or high speed rotors whereas in bubble column and airlift photobioreactors, the agitation occurs due to gas added that causes agitation in the culture. One of the costs of growing algal cultures in airlift and bubble column reactors is that of the added gases. Merchuk et al. (1998) compared *Porphyridium* culture in an airlift photobioreactor with that in a bubble column reactor. By adding a helical flow promoter to the airlift system, the cost of gases for the production per kg microalgal biomass was 50 % of that in the bubble column to achieve the same specific growth rate.

Photobioreactors such as bubble-column, and stirred-tank have good scalability though their use in outdoor cultures is limited since they have low illumination surface areas (Ugwu et al. 2008), but they have great potential for industrial bioprocesses, because of the low level and homogeneous distribution of hydrodynamic shear. One growing field of application is the flue-gas treatment using algae for the absorption of $CO_2$.

Degen et al. (2001) proposes airlift photobioreactor with baffle to induce a regular light cycling of microalgae and increase the cell productivity. The reactor is based on the airlift principle and baffles to induce a regular light cycling of microalgae. The use of baffles can contribute to productivity enhancement in several possible ways as (i) the baffles increase the residence time of gas bubbles in the reactor and this can affect the mass transfer rates of carbon dioxide and oxygen. However, because oxygen did not accumulate and $CO_2$ was supplied on demand so that it was never a limiting factor, the mass transfer effects do not explain the better performance of the baffled reactor; (ii) the baffles affect mixing; however, both reactors were always mixed sufficiently to supply the cells with dissolved nutrients. Thus, it seems that the baffles provided the better light cycling of the microalgae, thus improving the performance of the cultivation system.

## *2.2 Characteristics of Photobioreactors*

There are a number of different implementations of photobioreactors. Some characteristics of main photobioreactors cited in the literature such as open ponds, vertical column and tubular photobioreactors will be described in this section.

*Open ponds* Several types of open ponds have been developed for large-scale outdoor algal culture. The most common is the “raceway pond”, an oval for resembling a car-racing circuit (Lee 2001; Pulz 2001; Chisti 2007) (Fig. 1). These cultivation ponds present relatively low construction and operating costs and can be constructed on degraded and non agricultural lands, avoiding use of high-value lands and crop producing areas (Chen 1996; Tredici 2004).

Two other types of open ponds more common are circular ponds with a rotating arm or long channel ponds, single or connected to each other that are mixed with a paddle wheel.

**Fig. 1** Laboratory scale open tanks

Open ponds are the simplest algae growing systems, which consist of open vessels using outdoor sunlight. Individual ponds are up to 1 ha in area with an average depth of about 20–30 cm and are mixed via a paddle wheel that circulates water with nutrients and microalgae. Paddle wheels seem to be the most efficient device for mixing the algal cultures and are the easiest to maintain. The design of the paddle wheel also affects flow rate and energy requirements. Pond size affects water circulation, which in turn affects the design and operating cost of the circulation/mixing system (Borowitzka 2005).

Besides paddle wheels, earlier designs also used air lifts, propellers, and drag boards (Becker 1994). Drag board is a wooden board that closes the pond in cross-section except for a slot of only some centimeters above the bottom. It is dragged through the culture pond to create turbulence (Valderrama et al. 1987).

Open ponds may have several drawbacks: (i) Growth parameters depend on local weather conditions, which may not be controlled, causing seasonality to the production; rainfall may also be a problem, significantly diluting the medium in the pond and favoring rapid invasion by predators; (ii) Monoculture of the desired microalgae is difficult to maintain for most microalgae species because of constant contamination by the air, except for extremophile species; (iii) reduced light diffusion inside the pond, decreasing with depth and causing self-shading; consequently shallow depth is required for ponds and they have a low volume to area ratio; (iv) $CO_2$ is not used efficiently; (v) a large area of land is required, so only unproductive or waste land can be used; (vi) biomass productivity is lower than that in closed cultivation systems; (vii) harvesting is laborious, costly, and sometimes limited for low cell densities; (viii) continuous and clean water is needed; and (ix) production of food

or pharmaceutical ingredients is very limited or even not viable (Vonshak 1997b; Perez-Garcia et al. 2011; Xu et al. 2009).

The few commercial species that are currently being successfully cultured in large open ponds are extremophiles growing in a highly selective environment (high pH, salinity, or temperature). These conditions preclude the growth of most other algae and even many bacteria (Xu et al. 2009). Single, rectangular ponds with a paddle wheel (raceway ponds) are the most widely used for the production of *Arthrospira* due to high alkalinity, *D. salina* due to high salinity, and *Haematococcus* sp., and represent the most efficient design for the large-scale culture of most microalgae. Large inoculum amounts is another approach that can be used for fast-growing species such as *Chlorella* (Borowitzka 1999).

When installing open ponds, source and quality of water, and availability and cost of land are important factors to be considered, as well as near-optimal climatic conditions. If the land costs are high, other types of reactors, such as tubular photobioreactor, may be more convenient. The larger the pond area, the greater the amount of water lost by evaporation and when cultivating marine or hypersaline algae, natural source of seawater or saltwater is desirable for the cost of salt is prohibitive. Moreover, water sources may also contain heavy metals or other contaminants, and a final product contamination makes the microalgae culture inappropriate (Borowitzka 2005).

The final product is certainly an important factor to take into account when choosing a photobioreactor. Depending on the purpose of the use of the biomass, a "cleaner" production may be required and open ponds may become inadequate. On the other hand, if the biomass is used for animal feed or if a specific bio-product is extracted from the algae and contamination is not a concern, open ponds can be utilized.

*Vertical-column photobioreactor* Vertical tubular photobioreactors were among the first enclosed algal mass culture systems described in the literature, but their high cost discouraged their use (Miyamoto et al. 1988).

Vertical columns are frequently used especially in larger laboratory scale for indoor experiments. Diameters of 20 cm and more are necessary to work with sufficient volume. This leads to considerable high dark fraction in the middle of the cylinder. This part does not contribute to productivity or has even detrimental effects on growth (Posten 2009). To leave this part out of the internal reactor space the so-called annular column has been developed.

Various designs and scales of vertical-column photobioreactors have been tested for cultivation of algae (Choi et al. 2003; Vega-Estrada et al. 2005; García-Malea et al. 2006; Kaewpintong et al. 2007). Vertical-column photobioreactors are compact, low-cost, and easy to operate monoseptically (Sanchez Miron et al. 2002). Furthermore, they are very promising for large-scale cultivation of algae. It was reported that bubble-column and airlift photobioreactors (up to 0.19 m in diameter) can attain a final biomass concentration and specific growth rate that are comparable to values typically reported for narrow tubular photobioreactors (Sanchez Miron et al. 2002). Some bubble column photobioreactors are equipped with draft tubes

to improve the circulation. In this case, mixing occurs between the riser and the downcomer zones of the photobioreactor through the walls of the draft tube (Ugwu et al. 2008).

Vertical tubular reactors have the inherent advantage, over horizontal or serpentine reactor, of allowing continuous gassing of the cultures, resulting in efficient gas transfer ($CO_2$ in and $O_2$ out) and mixing of the cultures (Miyamoto et al. 1988). These reactors are mainly used because of their simple construction; excellent heat and mass transfer properties (Lau et al. 2010; Kaidi et al. 2012), better handling of solids, low operating costs and easier to operate, high height to diameter ratio (H/D) that minimize the effect of gas distributors where small bubbles are generated. It was found that the gas distributor design depended on the system properties (Haque et al. 1986). In bubble columns, the source of agitation is the pneumatic power input.

Extensive research on mixing has been carried out in bubble column reactors and empirical correlations have been given for gas holdup, however the flow inside these reactors remains complex. Often three flow regimes have been assumed in bubble columns (homogeneous, transition and heterogeneous) with the increase of gas velocities (Chen et al. 1994). Comprehension of the hydrodynamics inside the vertical column reactors is important for modeling and optimization of gas-liquid reactors.

The bubble size was the most important parameter for better understanding the dispersion of the gas inside the bubble column reactor. The variation in the average bubbles diameter depends on the type of sparger (Shah et al. 1982) and increases slightly with the increase of superficial gas velocity (Kaidi et al. 2012).

Gas hold up is an important parameters characterizing the gas–liquid systems. It is necessary to the hydrodynamic design in different industrial processes because it governs gas phase residence time and gas–liquid mass transfer. It depends mainly on the superficial gas velocity and the type of sparger.

It is worth mentioning that vertical tubular reactors can be efficiently mixed distributing Rushton turbines along the photobioreactor (Ávila-Leon et al. 2012), thus improving both the light cycling and the mass transfer.

*Tubular photobioreactors (PBR)* Tubular photobioreactors are the most popular closed systems, being basically constituted by tubes arranged in multiple possible orientations such as vertical, horizontal, inclined, spiral, helicoidal and their variations, but all orientations have basically the same work way. Besides the arrangement of tubes, tubular photobioreactors differ in the tube length, flow velocity, circulation system, and geometric configuration of the light receiver.

Tubular photobioreactor is a good option to obtain large microalgae biomass. It is increasing the number of facilities that produce microalgae using tubular photobioreactors aiming to get the advantages of this system, like high productivity and minor area required to cell growth if compared with open ponds. Tubular reactors are made of transparent tubes (rigid or flexible) arranged in parallel lines, inclined or not, at the ground or disposed at a difference of height, coupled by manifolds.

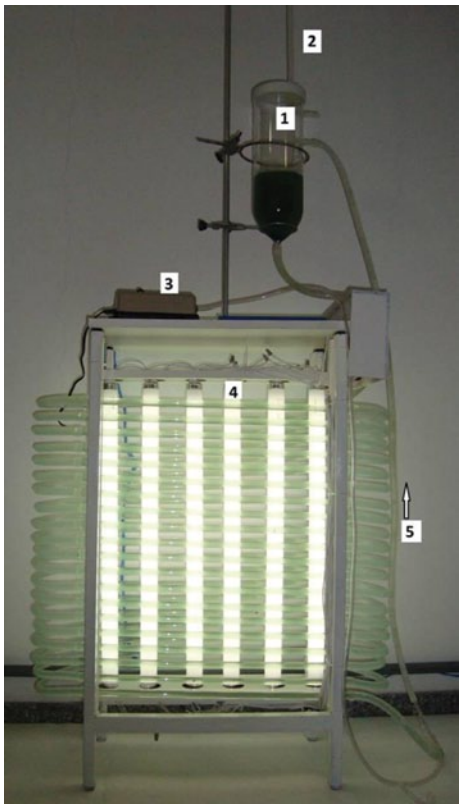


**Fig. 2** Tubular photobioreactor. (*1*) Degasser; (*2*) Condenser tube; (*3*) Air pump; (*4*) 20 W Fluorescent lamps; (*5*) Airlift system (Carvalho et al. 2013). (With kind permission from Springer Science+Business Media)

The single tubes can be straight, they can follow a meandering course either flat on the ground or ordered in panels or coils, also called helical reactor. The tubes have diameters of 10 to maximum 60 mm, and lengths of up to several hundred meters. The employment of tubes leads to a quite high surface to volume ratio over 100 $m^{-1}$, which is one of the main advantages of this design (Posten 2009). Increasing tube diameter results in a decrease in the surface/volume ratio, and this factor has a strong impact on the culture.

Horizontal tubular photobioreactor (Fig. 2) allow a better use of light (relative to vertical orientation) requiring however a large area installed. The horizontal arrangement of the tubes also raises difficulties in introducing $CO_2$ and removing $O_2$.

Culture circulation through the tubes can be done by various methods; use of airlift circulators is especially common. High levels of mixing are necessary to reach a turbulent flow of the culture, in order to optimize the light regime, nutrients availability, and to avoid biofouling. Mechanical pumping and/or high culture velocity can produce some cell damage due to hydrodynamic stress, limiting the species possible to cultivate in this type of system.

The tubular design allowed the possibility of overcoming some of the problems, such as contamination, pH control, harvesting and introduction of nutrients, but light intensity, mixing, aggregation and temperature control are not resolved yet.

Large-scale PBRs have some disadvantages that make them uneconomical for low-cost end-products: At operational volumes of 50–100 L or higher, it is no longer possible to disperse light efficiently and evenly inside the PBR (Chen 1996; Pulz 2001); development of algal biofilm fouls PBR surfaces and thereby limiting light penetration into the culture. The algae may stick to the inside surface of the tubes and block sunlight, and tubes may get too hot. Photoinhibition, difficulty to control temperature, and accumulation of dissolved oxygen along the tubes are common occurrence. When scaled up, mass transfer becomes a problem and light distribution is not very effective.

*Flat-plate Photobioreactor* The flat plate reactors are surely the most robust design in which algae are cultivated. They are made of two sheets that have to be glued together and with any desired light path length in the range from a few mm up to 70 mm (Posten 2009). Compared to tubular bioreactors, however, there are some advantages with respect to compactness: their narrow U-turns may use less space than coiled tubes, and their wall thickness can be thinner than tubular bioreactor (Pulz and Scheibenbogen 1998).

The panels/plates are made of transparent materials for maximum utilization of solar light energy. It has been reported that high photosynthetic efficiencies can be achieved with flat-plate photobioreactors. Flat-plate photobioreactors are very suitable for mass cultures of algae. Accumulation of dissolved oxygen concentrations in flat-plate photobioreactors is relatively low compared to horizontal tubular photobioreactors. It has been reported that with flat-plate photobioreactors, high photosynthetic efficiencies can be achieved due to large illumination surface area (Hu et al. 1996; Richmond 2000). It is relatively cheap, easy to clean; suitable for outdoor cultures and due to their modular design convenient for scale-up. Flat Plate reactors also consume less power than tubular reactors to achieve similar or greater mass transfer capacity. In fact, power consumption is another important criterion for comparisons among reactor types (Kunjapur and Eldridge 2010).

Although high biomass concentrations (up to 80 g $L^{-1}$) can be reached in narrow light path flat panels (Hu et al. 1998), there are some limitations. Scale-up requires many compartments and support materials, shows difficulty in controlling culture temperature, some degree of wall growth, and exhibits possibility of hydrodynamic stress to some algal strains resulting from aeration, a problem that has never been reported in tubular reactors (Raehtz 2009).

*Thin-layer photobioreactor* This kind of photobioreator has been designed and operated for a long time, with the aim of increasing productivity and reducing production cost. In a thin-layer photobioreactor well-mixed microalgal suspension flows continuously in a very thin layer (6–8 mm) on inclined lanes, construed by transparent material, and arranged in meandering way (Doucha and Lívanský 2006, 2009). This configuration allows to decrease algal layer exposed to the light to as low values as technologically possible and, consequently, increase the light/dark

periods of single cells, thus increasing the efficiency of light utilization and decreasing the photoinhibitory effect up to very high solar light intensities (Doucha and Lívanský 2006). In addition, thin-layer photobioreactor reaches a very high algal density at harvest (Doucha and Lívanský 2006) up to 40–50 g (DW) $L^{-1}$, corresponding to an areal density of 240–300 g DW $m^{-2}$ (Doucha and Lívanský 2009). These authors related that thin-layer culture technology is suitable for the production of biomass as a feedstock for bioethanol and, under climate conditions, yields of 80–100 t DW *Chlorella* biomass per 1 ha area for a 300-day culture season can be reached.

In thin-layer photobioreactor the high production rates and possibility of achieving high cell density decrease the presence of undesirable algal species and operational costs. Moreover besides providing better distribution of light among the cell, control of cultivation parameters like temperature, for instance, is also favored (Doucha and Lívanský 2006). Thin cell layer is also easily heated up by high light intensity, but on the other hand it is also spontaneously cooled by water evaporation at higher temperatures (Masojídek et al. 2011).

## *2.3 Material of Photobioreactors*

The materials for the construction of photobioreactors represent a significant practical issue both from the standpoint of the investment cost and biological performance. The materials used for the reactor include glass, concrete, bricks, compacted earth, plexiglass, Low Density Polyethylene (LDPE), acrylic, High Density Polyethylene (HDPE), Rigid acrylic (PMMA) and Poly Vinyl Chloride (PVC).

The choice of materials for pond construction depends on many factors such as temperature, composition, pH, and salinity of the medium culture and high light resistant material. Several coating materials are available, and the most appropriate choice depends on its cost, durability, and effect on algal quality and growth (Borowitzka 2005).

Light is the most important parameter in the design and construction of a photobioreactor. Wherever possible, the surface of the photobioreactor should be designed to minimize reflection of light to increase the light capture by the cell. Photobioreactors made with tightly curved surfaces like tubes will have less light available than those made with flat surfaces (Tredici and Zittelli 1998). Some designs have incorporated sophisticated parabolic light collection devices, fiberoptics, or light guides (Ogbonna et al. 1999; Janssen et al. 2002).

Large open ponds can be built of glass, plastic, concrete, polyethylene, PVC bricks, or compacted earth in a variety of shapes and sizes and have been used successfully for algal production. They are the most cheaply constructed ponds.

Ponds can be constructed on ground with walls of bricks, concrete blocks or other resistant material and floors of either concrete or some other suitable line. In addition, pond reactor can be excavated and lined with impermeable material. Considering cost of construction and operation, concrete blocks appear to be the

most effective material for building ponds. On a leveled ground, the pond may be constructed by building the external walls and the central divider of concrete blocks and then installing a suitable impermeable coating material (Borowitzka 2005).

A well-manufactured polyvinyl chloride (PVC) liner of 0.75 mm lasts about 5 years in temperate desert climates, and has been used for microalgae cultivation. But flexible PVC tubing can be damaged by UV rays and it tends to break down. PVC when attacked by UV rays will discolor the surface of the pipe limiting the light availability in the medium. Besides, PVC can lead to decrease in microalgae growth (Dyer and Richardson 1962; Blankley 1973).

PVC linings are frequently stabilized by lead, and it can be accumulated by the microalgae cells as already evidenced for some studies. An alternative to PVC is chlorinated polyethylene (CPE). CPE has little effect on algal growth (Bernhard et al. 1966; Blankley 1973) and does not appear to present any human health hazard.

Photobioreactors must be built with fully translucent material without any loss in transparency over time. Glass and acrylic are widely used in the construction of photobioreactors. Ultraviolet (UV)-stabilized acrylic is more appropriate because it is lighter, more flexible, stronger, and easier to machine, cut, bond, and so on.

Glass can be a suitable material to build photobioreactors. It requires a supporting structure and many more connection fittings as lengths of more than a few meters. In addition, this structure is difficult to transport and assemble. Among the types of glasses, borosilicate (Pyrex) glass is commonly used in the solar hot water collector of PBR. For a tubular photobioreactor, glass has considerably higher NER (Net Energy Ratio) than rigid polymers like acrylic material (polymethyl methylacrylate). Clear acrylic tubing with outer diameter in the range of 30 to 60 mm and wall thickness of 3 to 5 mm has been used in a number of prototype photobioreactor systems, (Tredici and Zittelli 1998). Studies indicate that the acrylic sheet transmits 95 % of the incident light from 390 to 800 nm. Based on its excellent transmittance in the Photosynthetic Activity Radiation, acrylic is suitable construction material for photobioreactors (Berberoglu et al. 2008).

Metals and metal alloys are used primarily as structure of the photobioreactors (support of the photobioreactors). The connections between the different materials can be made by various techniques such as bolted joints, bonded joints and welding. It is important to choose materials that are suitable to seal joints and adhesives with chemical compositions which are compatible with the environment to which they will be subjected, and do not react with the environment or release harmful substances to the culture.

The photobioreactors can also be made of large bags made of low density polyethylene. They are cheaper and generally used in the production of inoculum for larger volumes of culture or for the production of microalgae for feeding bivalves and crustaceans in aquaculture

As described above, a variety of materials is available for photobioreactor construction which not only have considerable benefits but also show a few drawbacks. Therefore it is important to select the best material that satisfies all the criteria for designing an efficient PBR.

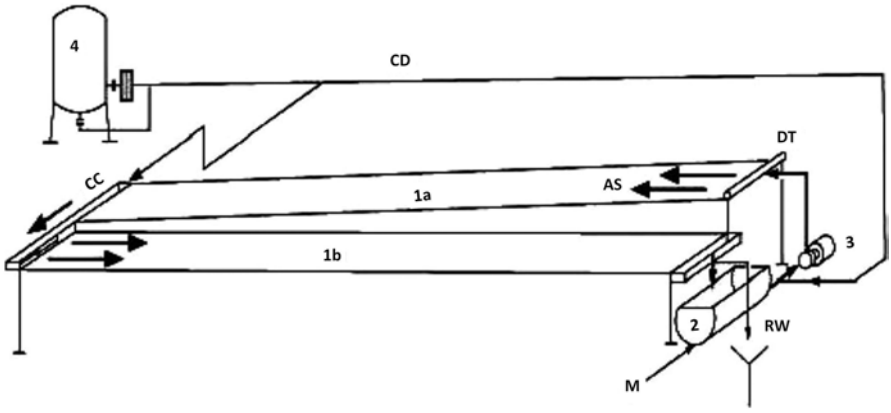


**Fig. 3** (*1a* and *1b*) culture area; (*2*) retention tank; (*3*) pump; (*4*) $CO_2$ storage tank; (M) aeration air; (*RW*) rain water; (*DT*) distribution tube; (*CD*) carbon dioxide; (*CC*) connecting channel (Doucha and Lívanský 2006). (With kind permission from Springer Science+Business Media)

## *2.4 Circulation Devices*

The success of growing photosynthetic microorganisms inside photobioreactors also depends on the applied circulation device, which will be different for each reactor type or configuration. It is important to consider that there is a small variety of devices used to circulate the cells in open artificial ponds. In these kinds of photobioreactors the culture is usually driven by paddle wheels to furnish the necessary turbulence that will put the cells in contact with the light and nutrients (Pulz 2001; Rosa et al. 2011). Doucha and Lívanský (2006) proposed the use of a pump, as circulation device, and inclined cultivation sheets, which is composed by two glass sheets inclined in opposite directions; these sheets are connected by a channel, through which the culture flows from the upper to the lower area; at the end of the lower sheet there is a retention tank, where a pump, to connect the upper edge of the culture area, is located (Fig. 3). This circulation device is applied to an outdoor open photobioreactor for a 2000 L culture cultivation of a termophilic strain of *Chlorella* sp. that was well grown using a flow rate of 60 cm $s^{-1}$ furnished by the pump.

Closed photobioreactors are more versatile than the open ones when talking about different configurations, which give the possibility of using various types of circulation devices. One of the most common circulation devices, mainly for tubular reactors, are the air pumps, which circulate the culture through the so called airlift system. In this kind of system, the air is injected inside the reactor to drive the culture in a given path, differently of a bubble column reactor where the culture flows randomly, through the air injection at the bottom of the photobioreactor (Siegel and Robinson 1992). There are a lot of authors that use the airlift mechanism to cultivate microalgae in tubular photobioreactors, as Soletto et al. (2008) who granted the circulation of *Spirulina platensis* culture by mixing the air from an air pump to uplift this culture using a flow rate of 1 L $min^{-1}$ in helical photobioreactor;

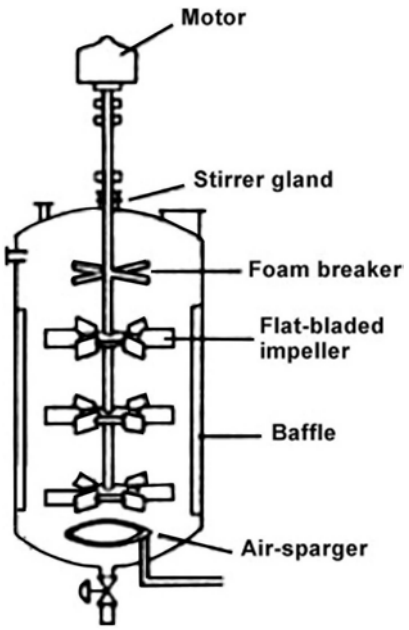


**Fig. 4** Stirred tank photobioreactor (Reprinted from Renew Sustain Energy Rev; Singh and Sharma 2012, with permission from Elsevier)

they used different light intensity from fluorescent lamps and different carbon dioxide concentrations from cylinder and obtained the highest cell productivity value of about 0.25 g $L^{-1}$ $d^{-1}$. There are other researchers which equally, through air pumps by the airlift system, cultivated: *Arthrospira platensis* using sunlight as the energy source (Carlozzi 2003); *Phaeodactylum tricornutum* in a tubular outdoor photobioreactor and, also using the sunlight (Fernández et al. 2001); *Chlamydomonas reinhardtii* in a closed and novel geometric configuration of airlift photobioreactor, using fluorescent lamps (Loubiere 2011); and, *Dunaliella salina* cultivated in a 250 L airlift loop photobioreactor using artificial light (Zimmerman et al. 2011). All these microorganisms achieved maximum cell concentration of about 0.3–2.5 g $L^{-1}$ using air pumps as circulation device. Although the source of air can be air pumps, in large scale conditions, the compressed air could be used for culture circulation.

Other mechanism for cell circulation inside closed reactor is mechanical agitation, in which impellers and baffles are used. Sassano et al. (2010) verified the influence of nitrogen source supply rate on protein and lipid contents of *Arthrospira platensis* biomass. The cultivations were carried out in a vertical, cylindrical glass tank, with 20.7 cm internal diameter (V = 9.0 $dm^3$), four turbines, and four 2.00 cm-wide chicanes, which ensured a great homogeneity and consequently, efficient mixing. This vertical cylindrical glass tank is one of the most conventional reactors also named stirred tank, where the agitation is the mechanical one. This reactor type contains impellers that are responsible for circulating cells inside the photobioreactor, and baffles to avoid vortex formation (Singh and Sharma 2012) (Fig. 4) or even break up the gas bubbles that increase $k_L a$ values (volumetric mass transfer coefficient).

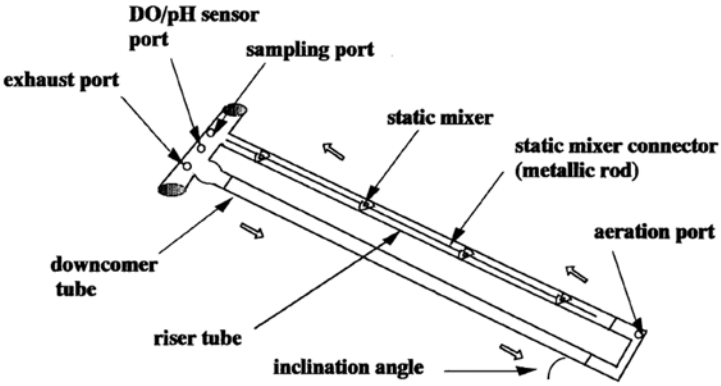


**Fig. 5** Scheme of the inclined tubular photobioreactor with internal static mixers (Ugwu et al. 2002). (With kind permission from Springer Science+Business Media)

Ugwu et al. (2002) used static mixers inside an inclined tubular photobioreactor to cultivate *Chlorella sorokiniana*. The culture was circulated by the injection of air through an orifice at the bottom of the riser tube, where the static mixers were located, and then the culture went to the downcomer tube (Fig. 5). The static mixers have a v-cut and an orifice to permit, respectively, gas dispersion and liquid circulation, which resulted in better mixing when compared to not using the static mixers, and a maximum biomass productivity of 1.47 g $L^{-1}$ $d^{-1}$.

Masojídek et al. (2003) applied a peristaltic pump as circulation apparatus to mix *Spirulina platensis* culture inside a tubular inclined photobioreactor using a working volume of 65 L and sunlight as the energy source. The peristaltic circulation pump takes the culture from the degasser flask, which is located at the lowest position of the cultivation system and, it is connected to the inlet tube of the highest position of the reactor. In this highest position a frequency inverter, to vary the flow speed of suspension, is placed. Finally, the culture goes down through the tubes to reach again the degasser flask (Fig. 6). The pump was applied to flow the culture at 20 cm $s^{-1}$ and they obtained a cell productivity of 0.5 g $L^{-1}$ $d^{-1}$, which was considered a relatively high value by the authors.

Other different circulation devices were tested by Ferreira et al. (2012a) who compared three different cell circulation systems: motor driven pumping, airlift and pressurized ones (Fig. 7). In the first system, a motor pump was used to circulate the cells in the photobioreactor, as in the pump, submersed into the degasser flask (located at the top of the reactor), propels the culture to move downwards through the external silicon tube and, upwards through the glass tubes. In the second system, an air pump was used to move the culture oppositely to the previous system. In the pressurized one, the cells were moved down or upwards depending on the activation of a solenoid valve. The authors evaluated various parameters and concluded that the traditional airlift one could be substituted by the others to cultivate *Arthrospira platensis* in a tubular photobioreactor.

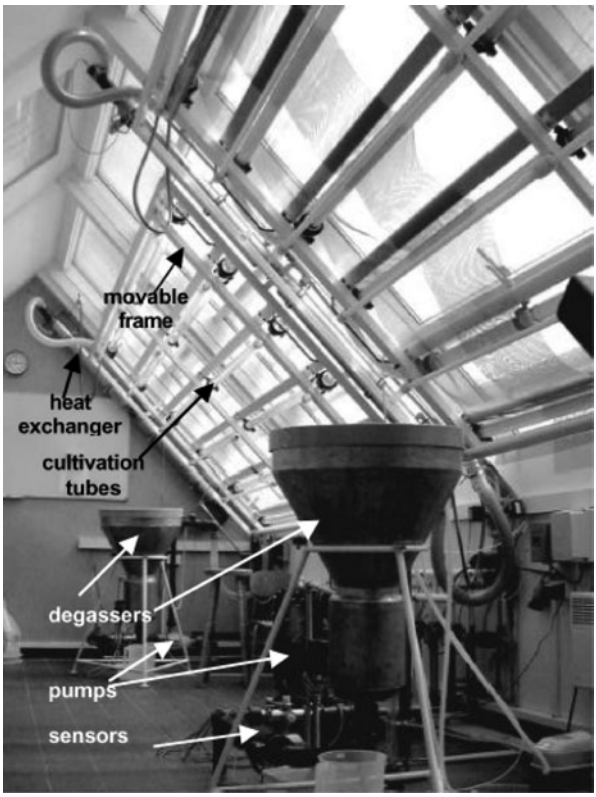


**Fig. 6** The main parts of tubular inclined photobioreactor: movable frame, heat exchanger, cultivation tubes, degasser, pump, sensors (Masojídek et al. 2003). (With kind permission from Springer Science+Business Media)

## 3 Photobioreactor Operation

For several decades, numerous efforts have been addressed to make possible the exploitation of microalgae on a technological scale as a source of food, feed, pharmaceuticals, fertilizers and several other compounds like vitamins, pigments, and lipids (Becker 1994).

As in conventional heterotrophic cultivations, in microalgal biotechnology high volumetric productivities are required to reduce the size of cultivation system and consequently reduce production and downstream processing costs (Barbosa 2003).

Cultures with high-cell density can be achieved in microalgae biotechnology with a proper reactor design and process optimization (Richmond 2000). It is important to improve the knowledge in this field, emphasizing the most critical scale-up and operational parameters: light, mass transfer, shear and mixing rates, etc. These parameters are intimately interrelated and highly influence the productivity and efficiency of the system.

Microalgal cultivations require continuous monitoring. One kind of monitoring is regular microscopic examination to detect any unusual morphological changes and the presence of contaminants, such as undesirable algae and protozoa. Regular

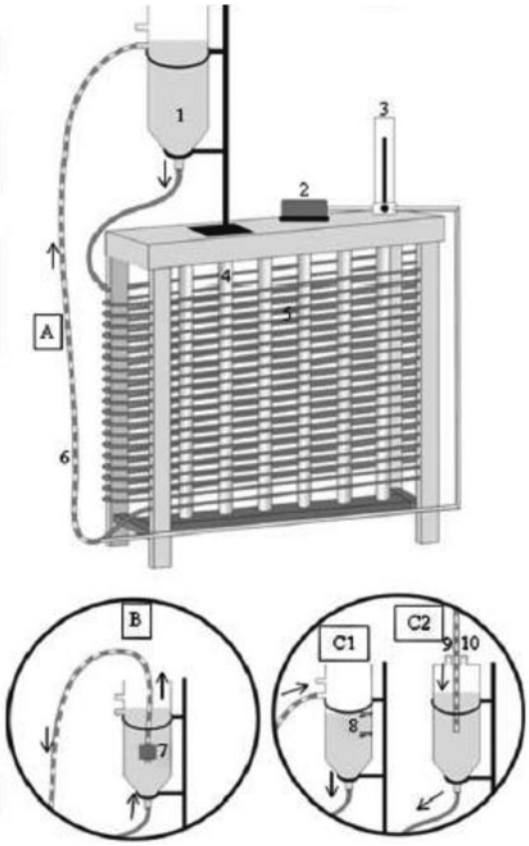


**Fig. 7** Scheme of the photobioreactor and the three cell circulation systems. (*1*): degasser; (*2*): air pump; (*3*): rotameter; (*4*): 20 W fluorescent lamps; (*5*): glass tubes; (*6*): external silicon tube; (*7*): motor pump; (*8*): level sensors; (*9*): connected air pump; (*10*): connected solenoid valve. **a** airlift system; **b** motor-driven pumping system; **c1** pressurized system (glass flask on the top); **c2** pressurized system (glass flask on the bottom). (Ferreira et al. 2012a)

verification on the nutrient concentration must also be carried out to avoid unexpected nutrient deficiencies. Nowadays, there are different ways of automatically controlling cultivation conditions like pH, temperature and nutrients, that can be applied for photosynthetic microorganisms reactors, even with devices for on-line data acquisitions (Barbosa 2003).

Many reports in the literature point out that changes in nutrient levels, pH, temperature, and light intensity can cause alterations in the growth and secondary metabolism of algae. For instance, many algae can channel carbon into storage biomolecules, either lipid or carbohydrate, when nitrogen is limited but carbon is still available (Behrens 2005).

For the microalgae cultivation four basic requirements are very important: carbon, water, light, and space. By maximizing the quality and quantity of these requirements, it is possible to maximize the quantity of biomass and the return on investment (Demirbas and Demirbas 2010).

Investigations in the field of applied phycology have led to the design and construction of several types of cultivation plants capable of producing quantities of algae ranging from kilograms to tons. But, it seems that, at least until now, only

raceway ponds and tubular photobioreactors are practicable methods for large scale production (Molina Grima 1999; Molina Grima et al. 1999; Sánchez Mirón et al. 1999).

The production of algal biomass can also be distinguished in three different systems, depending on the raw materials and the destination of the products (Becker 1994):

I. Open system in which a specific algal strain is cultivated using fresh water, mineral nutrients and additional carbon sources, in a so-called clean process. The resultant biomass is suitable to be utilized mainly as food supplement.
II. System in which sewage or industrial waste waters are used as culture medium, without addition of mineral and external carbon. Here, it's difficult to maintain a unialgal population and the biomass will probably have more than one species in the presence of high amounts of bacteria. It's also possible that certain algae adapt their metabolism from autotrophy to heterotrophy, so that they are able to utilize both inorganic and organic compounds from the medium, in a mixotrophic culture.
III. Enclosed systems to cultivate microalgae, using sunlight or artificial light, growing preferably under autotrophic metabolism. It can be considered to employ more sophisticated biotechnologies with a high input of equipment and energy for the growth of specific microalgal strains for the production of specific biochemicals such as enzymes, pigments and therapeutic agents, and the large initial investment costs are justified by the product quality.

Large-scale outdoor culture of microalgae and cyanobacteria in open ponds, raceways, and lagoons is well established. It is commercially used in the USA, Japan, Australia, India, Thailand, China, Israel, and elsewhere to produce algae for food, feed, and extraction of metabolites (Becker 1994).

The use of open ponds requires wider cultivation areas and may hamper an appropriate control of some culture conditions like temperature, light intensity, gases, etc. In fact, according to Brennan and Owende (2010), fluctuations in temperature and light availability due to diurnal cycles and seasonal variations are a major problem for open systems. This aspect is possibly responsible for a lower productivity.

Closed photobioreactors are certainly a very good alternative for microalgae cultivation. In this case, the algae fluid remains in a closed environment to enable accelerated growth and better control of environmental conditions. These glass or plastic enclosures, frequently operated under modest pressure, can be mounted in a variety of horizontal or vertical configurations and can take many different shapes and sizes (Demirbas and Demirbas 2010).

In closed photobioreactors it is possible to achieve up to fivefold higher productivity with respect to reactor volume. Besides saving water, energy, and chemicals, closed bioreactors have many other advantages that are increasingly making them the reactor of choice for microalgal biomass production, as their costs are lower (Schenk et al. 2008).

Closed photobioreactors may be required for special purposes. The high value of certain algal products and the need for good manufacturing practices as well as

the necessity for sterile reactors, or controlled unialgal reactors, justify the operation of such installations (Becker 1994). One of the main advantages of such closed systems is that they are not as subject to contamination with whatever organism happens to be carried in the wind (Demirbas and Demirbas 2010) as it would happen in the case of open ponds.

Enclosed photobioreactors have been employed to overcome problems with contamination and evaporation encountered in open ponds (Molina Grima et al. 1999; Matsudo et al. 2012). Nevertheless, as expected, there are some limitations for closed systems, particularly for the tubular ones: The long pathways of some of these reactors result in significant hydraulic problems as well as depletion of $CO_2$ in the culture. Another major limiting factor is the accumulation of photosynthetically produced oxygen in the tube (Torzillo 1997).

Schenk et al. (2008) have also proposed a hybrid system, in which both open ponds as well as closed bioreactor system are used in combination for better results. Open ponds are lucrative and skilled method for algae cultivation, but they become contaminated with unwanted species quickly. Therefore, a combination of both systems may be the most coherent choice for cost-effective cultivation of high productive strains. In this sense, open ponds are inoculated with a specific strain that had invariably been cultivated in a closed bioreactor, whether it is as simple as a plastic bag or a high-tech fiber-optic bioreactor. It is important to note here that the size of the inoculums has to be large enough for the desired species to establish in the open system before undesired species. Anyway, for minimizing contamination, cleaning or flushing the ponds routinely is very important.

It is also worth mentioning that production plants for algal biomass cultivation have to be preferably located in areas with suitable climatic conditions. Nevertheless, unfortunately, it seems to be difficult to find places where all the involved parameters are optimal.

When a photobioreactor is going to be designed, parameters like reactor size, light requirements, flow rate, culture condition, algae species, economic value and reproducibility have to be considered. Depending on the reactor size, installation locality, and local climate, these parameters can determine the type of cultivation system needed (open or closed). This reactor design should allow good mixing properties, efficiency, and reproducibility and also be easy to maintain and sterilize. An efficient photobioreactor has to provide good productivity and the possibility of cultivating multiple strains of algae. The performance of this photobioreactor can be measured by volumetric productivity, areal productivity, and productivity per unit of illuminated surface. Volumetric productivity is defined as a concentration of biomass per unit volume of bioreactor per unit of time. Areal productivity is a function of biomass concentration per unit of occupied land per unit of time. Productivity per unit of illuminated surface is measured as biomass concentration per area per unit of time (Demirbas and Demirbas 2010). The operation of the photobioreactors, irrespective to their configurations must be accomplished during the whole period of the cell growth, and, in order to obtain the best performance of the cultivation process, some physico-chemical controls of the culture in the photobioreactors are imperative. Such controls are discussed below.

### 3.1 Temperature Control

Temperature is one of the most important factors affecting metabolic rate in living organisms. It is worth to test different temperature values in laboratory bench scale for each specific microalgae, and the starting point may be the temperature of the environment where the strain was isolated from. It seems that for the many tropical/subtropical algae, a temperature range of 20–25 °C is the most suitable for growth.

For temperature monitoring, classical mercury thermometers are useful but, unfortunately, easily breakable. Recently, indoor-outdoor, water-resistant, max-min, digital thermometers are proving very useful, durable, and economical (West 2005).

Constant temperatures throughout cultivations are desired. In a laboratory, it is possible to have thermal stability in a temperature-controlled room, even using air conditioner equipment or a thermostatically controlled heater for higher temperatures (Morocho-Jácome et al. 2012). And it is also important to remember that in air-conditioning units, often the temperature near the light will be 1–2 °C above the room ambient level. Another way of controlling temperature in bench scale is using a water jacket connected to a water bath operated at a desirable temperature (Barbosa 2003).

Nevertheless, for larger scales, the control of the temperature can be an important challenge, and a suitable system for temperature control may have a high impact on operational cost. For instance, Santos et al. (2012) describes the use of flat-plated reactors, which they considered inexpensive and easy to construct and maintain, but the large surface area presented scale-up problems, including difficulties in controlling temperature, besides carbon dioxide diffusion rate and the tendency of algae adhering to the walls.

Tubular photobioreactors can be advantageous in areas with moderate temperatures but become problematic in warm climates because the temperature in the tubes may reach values 10 ~ 15 °C higher than the ambient air temperature during the day. Therefore it is necessary to set up a cooling system. Several different possibilities have been tested to prevent overheating of the algal suspension:

I. shading of the tubes with dark-colored sheets. However, to achieve a significant shading effect, up to 80 % of the surface area has to be covered, which causes a large reduction of the illumination and consequently in the yield of biomass (Torzillo 1997);
II. cooling of the culture by spraying water on the surface. Experiments performed in Italy maintaining the temperature of the algal suspension between 33 and 35 °C demonstrated that the amount of water lost by evaporation ranges between 1 and 2 L $d^{-1}$ $m^{-2}$ (Torzillo 1997; Becker 1994);
III. Floating the tubular system on a large body of water. This solution ensures sufficient heat exchange besides providing agitation through wave action and reduction of support structures. In a French bioreactor, thermal regulation is ensured by flexible tubes mounted beyond the cultivation tubes, which may be inflated with air so that the culture tubes are lifted during periods of tolerable temperatures or are immersed when the temperature in the culture tubes becomes too warm or too cold (Becker 1994);

IV. use of Aluminum or stainless steel water jackets to the external surfaces (usually the bottom to not interfere with illumination) of the reactor (Barbosa et al. 2003);
V. use of stainless steel coiling coils submerged in the culture medium. Employing a refrigerated water source, a temperature probe, connected to a temperature controller, operates a solenoid valve to regulate cooling water flow. It is recommended to acquire a "normally open" solenoid valve for granting an overcool instead of an overheating, in the case of a controller failure. The only problem here is that cooling coil may interfere with the circulation within the reactor (Barbosa 2003).

In contrast, at night, temperature can become a limiting factor of algae production at some locations, reducing the extension of the cultivation period during seasons with low ambient temperatures. In this case, there is a possibility of building a larger covered system for maintaining relatively higher temperatures at night. However, there is evidence that the maximum increase in algal biomass by providing increase night temperatures seems to be less than 20%, a questionable benefit when compared with the costs for that cover (Becker 1994).

Also for larger scale in colder weather, Becker (1994) describes a method of adjusting temperature of a tubular photobioreactor (40 mm inner diameter and 79 m length), with a water-jacketed 2 m long tube. In this heat exchanger, it was possible to achieve maximum of 38 °C temperatures inside the reactor with a water bath temperature of 60 °C.

Another possibility of heat exchanger is a design in which to improve the unfavourable geometry of the tower for illumination, a thinner glass tube ("finger") has been inserted from above, eliminating the central space, which is poorly illuminated in dense cultures, but can be filled by water, connected to a thermostat, for controlling the temperature of the culture (Becker 1994).

## *3.2 pH Control*

The pH of the culture medium is another important factor in algal cultivation. Fortunately, it is a very simple parameter to control using existing technology (Sonnleitner 1999). Nowadays, there are several commercially available pH controllers.

The value of pH determines the solubility of minerals and carbon dioxide and influences the metabolism of the microalgae. Algae exhibit a clear dependency on the pH of the growth medium and different species vary greatly in their response to pH. *Cyanidium,* for instance, has its optimum growth at pH 2.0 (Becker 1994), whereas *A. platensis* grows well at pH 9.5 (Sanchez-Luna et al. 2007).

Careful control of pH and other physical conditions for introducing $CO_2$ into the ponds allow for more than 90% utilization of injected $CO_2$ (Demirbas and Demirbas 2010), and it is also important for optimizing the uptake of ammoniacal nitrogen sources, like ammonium salts and urea, when they are employed (Ávila-Leon et al. 2012; Rodrigues et al. 2011; Carvalho et al. 2013).

The pH of algal cultures can be influenced by various factors such as composition and buffering capacity of the medium, amount of dissolved $CO_2$, temperature (which controls $CO_2$ solubility) and metabolic activity of the algal cells. It was found that in actively growing *A.platensis* cultures, the pH continuously increased to values up to 11, mainly because of the depletion of the anions $NO_3^-$ and $HCO_3^-$ from the medium (Vieira et al. 2012).

A more sophisticated method for maintaining the pH at a desired value is the introduction of pH controllers (pH states), which measure the actual pH of the medium and, as soon as a preselected pH is exceeded, open a solenoid valve that releases $CO_2$ (Matsudo et al. 2011) or turn on a peristaltic pump to add an inorganic acid, normally HCl solution, to the medium until the desired pH is reached. One should note though that in the case of inorganic acid addition, it led to loss of the carbon source in the form of $CO_2$, which points out the importance of addition of $CO_2$ instead of acid solution to control pH.

Employing a tubular photobioreactor, Matsudo et al. (2011) evaluated the use of $CO_2$ released from alcoholic fermentation, without any prior treatment, for pH control and carbon source replacement, in continuous cultivation of *A. platensis*. They used a continuously operating pH controller coupled to a solenoid valve to release $CO_2$ when necessary for maintaining constant pH value ($9.5 \pm 0.2$). Irrespective of the carbon source used (pure $CO_2$ or from alcoholic fermentation), similar behavior in cell growth was observed, and no difference was observed in the protein content of the dry biomass.

When an automated pH controller is not available, pH maintenance may be done by adding pulses of $CO_2$ or even in a constant flow, employing a small ratio with the air. This ratio or the duration and number of pulses in a day may be predetermined by preliminary tests.

Moreover, in continuous cultivation, it is possible to modify the proportion of nutrients for adjusting the initial pH in the feeding medium (Sassano et al. 2007). For instance, Ávila-Leon et al. (2012) employed Schlösser (1982) culture medium, which has high concentration of carbonate and bicarbonate, for continuous cultivation of *A. platensis* with urea as nitrogen source. Since the optimum pH for its cultivation is around 9.5, they adjusted the proportion of carbonate and bicarbonate salts to achieve a pH of 9.3, but keeping the same overall carbon amount. With this procedure, the pH inside the bioreactor kept constant between 9.5 and 10.3, depending on the dilution rate and urea concentration.

On the other hand, it should be kept in mind that, in the case of outdoor large scale open reactors, high pH could prevent night-time losses of respiratory $CO_2$ allowing the maintenance of reserves of this nutrient.

### *3.3 Nutrients*

Once the composition of a culture medium is optimized for a specific or a group of microalgae, another challenge is to maintain adequate levels of main nutrients throughout a cultivation.

For this reason, in commercial scale production, it is possible to set up instrumentation for measuring selected nutrients and controlling by automated compensation of them. Nevertheless, it leads to an elevated initial investment and operational cost. Alternatively it is possible to estimate the required quantity of nutrient addition during the cultivation, taking into account a standard cultivation.

The major absolute requirements include carbon, phosphorus, nitrogen, sulfur potassium and magnesium. Elements like iron and manganese are required in small amounts. Various other elements like cobalt, zinc, boron, copper and molybdenum are essential trace elements. In addition to these basic minerals, several algae may require additional organic substrates (vitamins, nucleic acids, growth factors) for their growth (Becker 1994).

**Carbon source** About 50 % of the algal biomass consists of carbon (Cornet et al. 1998), therefore an adequate supply of carbon is of utmost importance for its cultivation, what is justified by the low solubility of $CO_2$ in the medium and its high demand in cultivations with high final cell concentrations. Even in systems in which it is possible to have higher reserves of $CO_2$ (alkaline media), it is important to consider that the higher the carbon uptake, the higher the pH of the cultivation medium, what certainly leads to deficient production of the microalgae. Carbon can be supplied as an inorganic substrate in the form of gaseous $CO_2$, as in the case for most photoautotrophic forms (organisms that obtain the energy for their metabolism from light and all the elements from inorganic compounds), or in the form of bicarbonate. Concerning $CO_2$ sources, pure $CO_2$ is commonly used, although there are works that have been focused on use of $CO_2$ from burn of organic materials and/or from industrial wastes (Carvalho et al. 2009; Matsudo et al. 2011; Ferreira et al. 2012b; Demirbas and Demirbas 2010).

There is also the possibility of exploring the mixotrophic metabolism, nutritional mode in which the energy is derived either through photosynthesis or by chemical oxidation (of sugars, for example), and both organic and mineral carbon sources are required.

In several algal species, the mode of carbon nutrition can be shifted from autotrophy to heterotrophy by modifying the carbon source, based on the ability of the naturally photoautotrophic algae to utilize both inorganic and organic carbon substrates. Becker (1994) describes that different sugars are able to promote the growth of *Scenedesmus obliquus*. The highest increase in biomass was obtained with glucose, followed closely by mannose, whereas sucrose, fructose and galactose had much lower effect, even allowing higher biomass concentrations, in comparison with control cultures, with no sugar addition.

The cultivation of *Spirulina* was studied by Márquez et al. (1993) who showed that it is able to grow heterotrophically in a glucose containing medium in aerobic and dark conditions but also mixotrophically if light is provided. Chen et al. (2006) also showed that acetate may be employed as carbon source for mixotrophic cultivation of *Spirulina,* even for the production of several photosynthetic pigments.

It is very common to alter culture growth conditions, like deprivation of essential nutrients (mainly nitrogen) for enhancing lipid content in microalgae, but starving

the cell to enhance lipid content requires time, and will in the end result in lowered biomass productivity. In this sense, the employment of an organic carbon source could accelerate cell growth, and moreover it seems that mixotrophic growth for several species of microalgae, when grown in the presence of an appropriate carbon source, has been shown to result in higher intracellular lipid levels than those grown only under photoautotrophic conditions (Das et al. 2011).

On the other hand, it seems that *Dunaliella* is unable to grow heterotrophically. Using acetate or glucose as the sole source of carbon (Ben-Amotz and Avron 1989), it was impossible to grow this alga in the night, allowing the conclusion that this microorganism is an obligate photoautotrophic.

Especially in developing countries, for developing an economic algal mass cultivation, where the use of pure $CO_2$ is unfeasible due to the high cost, it is necessary to explore the possibilities of utilizing low cost carbon sources such as industrial or agricultural by-products (Becker 1994). As it is going to be explored in a later section, there are some studies about the use of $CO_2$ from alcoholic fermentation for the cultivation of *Arthrospira* (*Spirulina*) *platensis* (Carvalho et al. 2009; Matsudo et al. 2011; Ferreira et al. 2012b). In these cases, the authors had success in using this exhaust gas directly from the fermenter, but for other photosynthetic microorganisms, it is important to consider that this $CO_2$ can include some volatile organic compounds that, depending on the concentration, can serve as organic carbon source or even inhibit the microalgal growth.

Lodi et al. (2005) tested different organic carbon sources for *A. platensis* in fed-batch mixotrophic process, under continuous illumination. For avoiding carbon source accumulation in the medium, acetate and propionate were added by pulse feeding equimolar amounts about 12 h after their complete depletion. In this sense, acetate was added every 3.4 days, propionate every 4.0 days, and glucose once a day. The results indicated that glucose was metabolized faster than the other 2 carbon sources for algal growth.

**Nitrogen source** Besides carbon, nitrogen is the most important element in algal growth, since approximately 10 % of the algal biomass may consist of this element (Cornet et al. 1998). It is a key element for cell growth and reproduction, since it is an essential building block of nucleic acids and proteins. Therefore, it is important to choose a suitable and economic nitrogen source. Nitrate, ammonia and urea are widely used, dependent on the species and the optimum pH. Certain cyanobacteria are also capable of assimilating nitrogen in its elemental form from the atmosphere. Changes in the nitrogen supply essentially influence the metabolic pathways in the alga and subsequently the overall composition of the organism (Becker 1994).

Xu et al. (2012) observed that nitrogen significantly affected the algal growth and oil production of a microalga in batch culture, and they found out that it is possible to improve algal oil production from *Brotryococcus braunii* by feeding nitrate up to its initial concentration 15 days after the beginning of the cultivation. They also observed that low nitrate concentration limited microalgal growth and high nitrate concentration inhibited hydrocarbon accumulation, which justify the need of controlling nutrients concentration throughout a cultivation. In fact Li et al.

(2008) observed that sodium nitrate in a concentration of 15 mM or higher seemed to be inhibitive for *Neochloris oleoabundans* cell growth, and around 10 mM was the optimum concentration, but the nitrogen sources was depleted after 3 days of cultivation.

It is known that the preferred nitrogen supply is in the form of ammonia or urea, either of which is economically more favorable than nitrate, which is more expensive and requires considerable metabolic energy for its assimilation (Sassano et al. 2004; Carvalho et al. 2013). It is generally assumed that, before assimilation, nitrate-nitrogen is reduced to ammonium-N in two reducing steps catalyzed by the enzymes nitrate reductase and nitrite reductase (Hattori and Myers 1966).

Excessive ammonium may be deleterious for microalgae. Diatoms were found to be damaged when ammonium-N exceeded 5 mmol $L^{-1}$, particularly when the pH was above 8.0 (Azov and Goldman 1982).

Several studies are available in the literature dealing with the use of alternative nitrogen sources, such as urea and ammonium salts, for reducing production cost by replacing the traditionally used sodium or potassium nitrate. It is a particular kind of cultivation because of the alkalinity. In high pH medium, ammonia is easily assimilated by the microorganism, since ammonia uptake involves simple diffusion followed by trapping through protonation (Boussiba 1989). It is worth remembering that urea is hydrolyzed to ammonia in alkaline conditions (Danesi et al. 2002) and/or by urease (Shimamatsu 2004).

However, it has been reported that ammonia concentrations are acceptable only in a certain limit, depending on the microorganism, for its toxicity, especially in combination with pH. For instance, 10 mM of ammonia can be toxic for the *Spirulina platensis* (Belkin and Boussiba 1991). Therefore, if ammonium salts or urea are added in large amount at the beginning of the process, the excessive ammonia released may provoke cell death.

Notwithstanding, the addition of these nitrogen-containing compounds by fed-batch or continuous process can be a very promising for low-cost microalgal biomass (Danesi et al. 2002; Sánchez-Luna et al. 2004; Sassano et al. 2004; Bezerra et al. 2008; Matsudo et al. 2012; Ferreira et al. 2010; Matsudo et al. 2011). Moreover the simultaneous use of nitrate and urea or nitrate and ammonium showed to be even more advantageous (Rodrigues et al. 2010; Vieira et al. 2012).

Considering the efficiency of microalgae for uptaking nitrogen and phosphorus from culture medium, algae cultivation can be coupled to another type of environmental remediation that will enhance productivity while mitigating pollution. High nutrient wastewater from domestic or industrial sources, which may already contain nitrogen and phosphate salts, can be added to the algal growth medium directly (Schneider 2006). This allows for inexpensive improvement in algae production along with simultaneous treatment of wastewater.

By evaluating the annual productivity of *Spirulina* and its ability to remove nutrients in outdoor raceways treating anaerobic effluents from pig farm wastewater, Olguín et al. (2003) also showed that this photosynthetic microorganism can be used for ammonia removal from wastewater in processes in which the wastewater is fed periodically, diluting it in a mixture of sea and fresh water.

**Trace elements** Trace elements are found to be essential and are mostly supplied in very small quantities from stock solutions. For this reason, they are difficult to be chemically analyzed and monitored in a cultivation.

Fresh water could be a valuable natural resource of the salts and minerals needed, but it is common to have stock solution of minerals which is added in the culture medium in very little amount. Alternatively, salt water can be used, either from a saline aquifer or seawater. This means that competition for water will be low (Demirbas and Demirbas 2010).

It is worth mentioning that phosphates may complex with metal ions, even in trace concentrations, and not all the added phosphorus is bio-available. Therefore, phosphorus must be supplied in significant excess in the culture medium (Belay 1997).

## *3.4 $CO_2$ Addition Systems*

Culture media containing salts as bicarbonate and carbonate (carbon sources) are frequently used in laboratory cultivations (Danesi et al. 2002; Rodríguez-Maroto et al. 2005; Bezerra et al. 2008; Rodrigues et al. 2010; Vasumathi et al. 2012). $CO_2$ gas is other carbon source form used by photosynthetic microorganisms, and it has been applied to cultivations in order to reduce costs by using carbon dioxide gas from cylinders or from industrial waste gases. It is interesting to highlight the importance of using $CO_2$ gas from wastes, since it will contribute to greenhouse gases and global warming mitigation. There are some examples, as the successful use of $CO_2$ from ethanol fermentations in *Arthrospira platensis* cultivations in tubular photobioreactors when compared to the use of $CO_2$ from cylinders (Ferreira et al. 2012b; Matsudo et al. 2011); or, the use of flue gas into microalgae (*Chlorella vulgaris*, *Scenedesmus* sp., *Botryococcus braunii*) cultivations by Yoo et al. (2010) who obtained great results of biomass, productivity and a better C-fixation ability for *Scenedesmus* sp. when using ambient air enriched with 10 % of flue gas.

The amount of $CO_2$ to be added to an algal culture depends on the efficiency of gas sparging, $CO_2$ loss from microalgae culture and $CO_2$ consumption by algal cells (Doucha and Lívanský 2006). These variables are related to $CO_2$ addition systems and, consequently, to photobioreactor type and its configurations.

Open photobioreactors present disadvantages as example, a short way to the gas-liquid transfer, which results in higher gas loss if compared to closed photobioreactors. Some researchers reported that about 13–20 % of supplied carbon dioxide was absorbed in open tanks (Richmond and Becker 1986; Becker 1994). However, Doucha and Lívanský (2006) cultivated *Chlorella* sp. in open reactors and they concluded that, from all $CO_2$ supplied to the culture, about 70 % of $CO_2$ was utilized for photosynthesis. These authors cultivated the microalga using a gaseous $CO_2$ from a storage tank, where liquid $CO_2$ was kept under pressure; this gas was supplied into the suction pipe of the suspension circulating pump, and when the $CO_2$ content was detected in excess, the magnetic valve for $CO_2$ delivery was switched off.

Binaghi et al. (2003) have also cultivated a photosynthetic microorganism (*Spirulina platensis*) inside an open photobioreactor, where pure $CO_2$ from cylinder was bubbled into open tanks resulting in maximum cell concentration of 1.5 g/L as the higher value obtained in this work, but still lower than that obtained by cultivations in closed photobioreactors (Ferreira et al. 2010; Molina et al. 2001).

Closed photobioreactors are more efficient in the case of $CO_2$ addition due to the higher time of the gas in contact with the culture medium. There are a lot of researchers that use $CO_2$ gas to maintain the pH at an optimum value and also as carbon source for photosynthetic microorganisms cultivations. Masojídek et al. (2003), when cultivating *Spirulina platensis* in tubular photobioreactor, fed pure carbon dioxide into the cyanobacterium suspension before the inlet to the circulation pump according to the pH value, and they obtained biomass productivity of about 0.5 g $L^{-1}$ $d^{-1}$.

Sobczuk et al. (2000), when using the same kind of photobioreactor, injected $CO_2$-air mixtures directly into the culture, and studied the $CO_2$ uptake efficiency by *Phaeodactylum tricornutum*. They found that the $CO_2$ uptake efficiency was 63 % when the $CO_2$ concentration in the gas inlet was 60 % v/v. The $CO_2$ loss during the photosynthetic period was about 10–20 % of the added amount, and the highest biomass productivity value was 2.47 g $L^{-1}$ $d^{-1}$.

It is interesting to highlight that the success of $CO_2$ addition system also depends on other parameters as the flow rate, since the higher the flow rate the lower the coalescence of $CO_2$ bubbles (Doucha and Livanský 2006). Zhang et al. (2002) observed that the amount of $CO_2$ added depends on $k_La$, i.e., on mass transfer and mixing. Accordingly, mixing methods and devices have to be considered for high $CO_2$ utilization efficiency.

## *3.5 Cultivation Processes*

Process optimization is a very important aspect in the development of technologies for microalgal production, overcoming the obstacles and making it economically feasible. The improvement of the bioprocess is very important to link the discovery and the commercialization and should take place after the identification of a strain and the product of interest.

Microorganism cultivations may be carried out under different processes, according to the microorganism, kinds of nutrients, the desired product, etc. In both closed or open systems, microalgae can be cultivated by batch (Watanabe and Hall 1995), fed-batch (Danesi et al. 2002; Torre et al. 2003; Carvalho et al. 2004; Bezerra et al. 2008; Matsudo et al. 2009), semi-continuous (Lee and Low 1992) or continuous processes (Matsudo et al. 2011; Ávila-Leon et al. 2012; Matsudo et al. 2012).

In batch process, neither nutrient is added after the initial charge nor the product is removed until the end of the process. Conventional batch operation can provoke inhibitory concentration of substrate or even formation of undesired products through direct metabolic pathway of the organism but, on the other hand, represents

the most safety one when facing problems with asepsis. Besides, it shows flexibility of operation, better control of genetic stability of the strain and permit the traceability of all materials related to a specific lot, which is of utmost importance in pharmaceutical industries, for example (Carvalho and Sato 2001). A variant of this process is fed-batch process.

Fed-batch process is defined as a technique applicable in microbial processes in which one or more nutrients are added to the reactor throughout the cultivation, while cells and products remain until the end of the operation. The culture volume may vary, depending on the nutrient concentration and evaporation of the system (Carvalho et al. 2013).

The advantages of this process include: deviation of cell metabolism for the synthesis of the desired product; prevention of release of toxic substances in microbial metabolism or catabolite repression; and control of the specific growth rate (Yamane and Shimizu 1984).

Fed-batch process was particularly important in the studies of *Arthrospira* cultivations. In this case, it was used to prevent or reduce substrate-associated growth inhibition by controlling nutrient supply. Since both overfeeding and underfeeding of nutrient is detrimental to cell growth and/or product formation, the development of a suitable feeding strategy is critical in fed-batch cultivation. Fed-batch operations can be the best option for some systems in which the nutrients or any other substrates are only sparingly soluble or are too toxic to add the whole requirement for a batch process at the start (Carvalho et al. 2013).

In the fed-batch mode of operation, nutrient feeding during a fed-batch process can be done utilizing either constant of variable mass flow rate (Danesi et al. 2002; Bezerra et al. 2008), and by pulses or continuous mode (Sánchez-Luna et al. 2004).

More recently, Ferreira et al. (2010) observed that parabolic protocol for ammonium sulfate addition appeared to be the best one for *A. platensis* biomass production in tubular photobioreactor. Additionally, due to elevated cell growth in the cultivations in such photobioreactor, the nitrogen amount required was extremely high, reaching values around 12 mM per day. Considering that the inhibitory levels of ammonia is around 6 mM (Carvalho et al. 2004), in this case, the addition of 12 mM of ammonium sulfate per day in a single daily addition would probably lead to cell death. Thus, the daily addition was divided into eight separate additions, providing very promising results.

Still, the fed-batch cultivation can be carried out as a repeated fed-batch process, that can avoid unproductive times of the photobioreactors. Once the cultivation reaches a certain stage, where cell concentration is almost stable or processes the end of the logarithmic growth, a portion of the culture medium is removed from the reactor and replaced by fresh nutrient medium. As it happens in semi-continuous process, it is possible to keep part of the medium in the reactor at the end of cultivation, reusing the exponentially growing cells for the following runs, granting high starting cell levels, and avoiding long stopping of the process (Carvalho et al. 2013). In this sense, Matsudo et al. (2009) showed that this mode of operation can be successfully exploited for long-term *A. platensis* cultivation with urea as nitrogen source, in open ponds, obtaining high cell productivity and high protein content biomass.

In semi-continuous process (repeated batch process), a batch process is initiated, and once the cell concentration is stabilizing, part of the culture medium is removed, and the remaining part is used for a subsequent cultivation, by the addition of fresh culture medium, replacing the withdrawn volume. In this sense, the cell suspension remaining in the reactor serves as inoculum for the following cultivation (Borzani 2001). This process, as the repeated fed-batch, can be used to avoid any unproductive time of the photobioreactor as well, mainly in conditions in which it is not necessary to feed any nutrient to the photobioreactor during the cell growth. Continuous process is carried out by continuous feeding of fresh medium to the reactor, in a specific flow rate, and continuous withdrawal of exhausted medium in the same flow rate for maintaining a constant volume and aiming to achieve a "steady-state" condition (Pamboukian 2003). In this case, the cultivation may last long-term, having several advantages in comparison with batch process.

Cultures with high cell concentration of photosynthetic microorganisms may have the shadowing effect (Vonshak et al. 2000), which reduces the light available for each cell and, consequently, negatively affects carbon fixation. This drawback can be overcome withdrawing, periodically, part of the medium from the reactor at the end of cultivation, reusing the cells for subsequent runs, in a semi-continuous or repeated fed-batch process (Matsudo et al. 2009) or maintaining constant optimum cell concentration range, by continuous process (Matsudo et al. 2011; Ávila-Leon et al. 2012; Matsudo et al. 2012).

According to Maxon (1954), a disadvantage of continuous process could be the possibility of contamination by undesired microorganisms. In fact, this kind of process is more recommended for extremophiles organisms, like *Arthrospira*, which is cultivated in high pH and salinity. Besides these characteristics, not only the use of inorganic medium but also the use of ammoniacal salts and closed photobioreactors may help to avoid contamination.

In general, mixotrophic culture experiments for microalgae have been conducted under sterile conditions in closed laboratory systems, mainly because addition of organic substrates in a open system is likely to induce growth of undesired heterotrophic bacteria, resulting in a low microalgal biomass yield.

## *3.6 Light Supply*

Microalgae obtain their metabolic energy by photosynthesis process, which shows the great importance of light supply for their growth. There are a lot of photobioreactor configurations with internal or external illumination used for microalgae cultivation. Tank reactors, plate or tubular are examples of photobioreactors with different illumination which will influence on the way the cells, inside the reactor, will receive the provided light (Perner-Nochta and Posten 2007).

Open photobioreactors are the most commercially used for microalgae cultivations, because of their advantages, as example, the ease of construction and low cost. Hsieh and Wu (2009) developed an effective system for enhancing light utili-

zation in an open photobioreactor. In this proposed system, transparent rectangular chamber (TRC), made of transparent acrylic, was placed inside an open rectangular reactor to provide a larger area of illumination, which conduct the light deep into the culture. The authors obtained, as the main result, higher biomass value (56 % higher) for *Chlorella* sp. cultivations in open photobioreactor with TCR when compared to that without the chamber.

The open reactors resemble the microalgae natural habitat and the water depths are, usually, 15–20 cm (Pulz 2001). This measure is the length that the light has to go through to reach the photosynthetic cells, and this is the major difference when compared to the closed photobioreactors.

Notwithstanding, Doucha and Lívanský (2006, 2009) proposed the employment of a thin-layer culture technology for the production of microalgal biomass. In this outdoor photobioreactor, with a total volume of 2,000 L, the microorganisms are grown in a 6–8 mm thick layer, allowing a higher efficiency of light utilization. Indeed, Doucha and Livanský (2009) showed that it was possible to achieve a cell density higher than 40 g DW $L^{-1}$ in less than 10 days.

There are many closed photobioreactor configurations used for laboratory experiments. They are composed of thin structures (tubes or flat plates, as examples) where the culture flows. Converti et al. (2006) cultivated *Arthrospira platensis* in tubular reactors, whose tubes have 1.2 cm of internal diameter. In this photobioreactor, the light had to go through 1.2 cm as a maximum to reach the cells, and the result was a high photosynthetic efficiency (PE), i.e., a high efficiency of light conversion by the cells, (PE = 8.1 % for the best experiment) when compared to a cultivation in an open tank using the same strain and the same light intensity (water depth of 5 cm) (PE = 7.1 % for the best experiment).

It is important to notice that there are techniques and apparatus used to increase the better use of light by the cells, both in open or closed photobioreactors. Ugwu et al. (2002), as mentioned before, have studied the use of static mixers, which were able to improve mixing and, probably, result in better capture of light by *Chlorella sorokiniana*. As this last paper, all the researches previously mentioned, in the section named “Circulation Devices”, were about the use of apparatus, which can change circulation inside the photobioreactor and, consequently, can improve capture of light energy by photosynthetic cells. Another applied technique was the success of changing the angle illumination at the surface of a conical photobioreactor (photo-redistribution), which resulted in efficient light use and high microalgae productivity (Morita et al. 2000).

It is important to remark that low light intensities can result in photolimitation, that is, lack of light energy for photosynthetic cell growth; and on the other hand, high light intensities can lead to photoinhibition, i.e., a reduction in the photosynthesis rate of cells when the light is provided in excess (Vonshak 1997a). Through this last knowledge, Cuaresma et al. (2011) studied microalgae cultivation in an effort to improve light capture by cells, and also to avoid the photoinhibition effect. For this purpose they cultivated *Chlorella sorokiniana* in a vertical panel photobioreactor with 14 mm of light path and, by an outdoor condition simulation, different light intensities were tested during the day (higher light intensity was provided at midday).

When cultivating photosynthetic microorganisms, it is important to note that the use of narrow transparent tubes can result in high microalgae productivities, whereas the excess light intensity on these tubes could result in photoinhibition and low light conversion efficiencies. Photoinhibition can be decreased if at a constant incidence light intensity, light absorbed per unit cell will be very low. Ugwu et al. (2003) observed that even at relatively low aeration rate, the use of static mixers modify the broth flow from plug flow to turbulent circulation, which results in a good culture homogenization inside the closed photobioreactor. Therefore, static mixers utilization will ensure that the light will reach more the cells resulting in better light utilization efficiency.

The increase in photosynthetic rates and light utilization efficiencies was already found by Grobbelaar et al. (1996) who applied light/dark periods and observed that the higher the light/dark frequencies the higher the photosynthetic rates. According to Merchuk et al. (1998), the introduction of dark period can avoid long exposure to high light intensities and also reduce photoinhibition effect; however, if the dark cycle is higher than 50 % of the cycle time, the light utilization efficiency is reduced (Janssen et al. 2001). This influence of light/dark cycles shows the importance of controlling periods of light both in outdoor (sunlight) and indoor (artificial light) cultivations.

Finally, independently on the reactor configuration (open or closed), apparatus, structures, and techniques utilization, the efforts have to be made to provide the required light energy to the microalgae growth.

## *3.7 Mixing*

Mixing is the most important requisite to obtain constant high yields of microalgae biomass, when considering that the environmental conditions are not limiting. The most important reason to mix cells is to maintain a good suspension. The high turbulence around the cells results in high nutrients and gas gradients, which are responsible for the achievement of a dense algal culture (Torzillo 1997). Mixing inside any kind of photobioreactor can influence the oxygen tension, hydrodynamic stress, light cycling and gaseous transfer in the culture medium.

Microalgae are photosynthetic microorganisms, i.e., they obtain energy from light and release oxygen gas. The amount of dissolved oxygen (DO) in microalgae culture medium may vary depending on the photobioreactor type and mixing. It is known that high dissolved oxygen levels are toxic to most algae and may lead to photooxidative culture death (Richmond 1986). This problem could be solved by using vigorous mixing, which, according to Richmond and Grobbelaar (1986), will result in less oxygen build-up in the culture. For *Spirulina*, for example, Vonshak (1997b) observed that in small ponds, in which high flow rates can be employed, oxygen concentration can reach levels not higher than 200 % of air saturation ($12 \sim 14$ mg. $L^{-1}$), but in larger ponds, where water flow is limited, oxygen concentration as high as 500 % of air saturation can be observed.

Inside open photobioreactors the accumulation of oxygen generated by photosynthesis leads to a severe cell growth inhibition. Vonshak (1997a) observed in their studies that when DO concentrations were higher than 20–22 mg $L^{-1}$ the cyanobacterium photosynthetic activity was reduced. It is known that the paddle wheels, used to circulate cell cultures inside tanks (open reactors), are more efficient on removing oxygen gas from reactors when compared to air bubbling applied in closed ones (Fontes et al. 1989), which suggests the ability of mixing and circulation devices on gases removal.

The problem of dissolved oxygen becomes dramatic when the microalgae cultivation is carried out in closed photobioreactors, in which oxygen gas accumulates during the loop cycle and is removed only at the degassing flasks. Among closed reactors, tubular ones are still disadvantageous due to the long way the gas has to go through the tubes until exit the system. However, there are a lot of methods used for oxygen removal in tubular photobioreactors, for example: cell circulation systems, like air bubbling by a system called airlift (Converti et al. 2006) or the utilization of static mixers inside the reactor tubes (Ugwu et al. 2002). Moreover, Torzillo (1997) constructed a tubular photobioreactor with several tubes laid side by side on a white polyethylene sheet and joined by PVC bends to form a loop, and in each bend, a narrow tube was incorporated for allowing the release oxygen.

*P. tricornutum* was cultivated in outdoor tubular photobioreactor and its results showed that DO percentage in the culture medium changed according to the hour of the day and season when the cultivation was carried out, i.e., it is related to light intensity. This paper also showed that the photosynthetic activity, i.e., cell growth, clearly was reduced at dissolved oxygen concentrations higher than 100 % of saturation, and at dissolved oxygen of 475 %, the photosynthesis rate was reduced by 55 % (Molina et al. 2001).

There are great variety of closed photobioreactor configurations and one of them is the vertical alveolar panels (VAP) used by Tredici et al. (1991) to carry out *Anabaena azollae* cultivations. They reported that microalgae yield decreased when the dissolved oxygen accumulated to 400 % of saturation. This reactor configuration (VAP) cannot be considered a typical closed system since it presents the inherent advantage of allowing a better degas action of air.

It is worth mentioning that studies on mixing have to be done to avoid hydrodynamic stress, which may be affected by geometry of the bioreactor involved, type of pump utilized, morphology of algal cells, physiological conditions of microalgae. Some authors have already mentioned that circulation devices can damage photosynthetic cells, for example, utilization of screw-pumps for *Porphyridium* cultivation (Gudin and Chaumont 1991), and even air pumps for airlift systems used to cultivate *Dunaliella* sp., whose cells were increasingly sensitive to high specific bubble rates (Silva et al. 1987).

Another relevant point to be considered about hydrodynamic stress is the applied flow rate. According to Torzillo (1997), the cell stress depends mostly on the rate at which cells flow, since the higher the flow rate the greater the damage to cells. In fact, a culture flow rate of about 0.3 m/s was not enough to cause damage to *Arthrospira platensis* cells, but when it was increased to 0.8 m $s^{-1}$, the biomass concentration was reduced by 16 %.

The hydrodynamic, mass and gaseous transfers are related to vigorous mixing, which can: increase flashing-light effect, remove excess dissolved oxygen, improve $CO_2$ supply, and, consequently, result in higher cell productivity. In order to study hydrodynamic and mass transfer in a flat-panel photobioreactor with high light path (0.15 m) for *Spirulina* sp. cultivation, Reyna-Velarde et al. (2010) evaluated the volumetric mass transfer coefficient ($k_L a$), the gas hold-up and the mixing time as a function of superficial gas velocity. The results suggested that this photobioreactor is more efficient than the tubular and flat-plate configurations, thus indicating the possible use of photobioreactors with higher light paths than yet proposed.

Zhang et al. (2002) described the effect of $CO_2$ flow rate on microalgal productivity and the ability of gaseous transfer in a vertical flat-plate photobioreactor (VFPP) for *Synechocystis aquatilis* cultivation. According to these authors, increasing the height of the VFPP, both the $CO_2$ mass transfer and the illumination conditions could be improved, which suggests the possibility of scaling up. They also tested different $CO_2$ aeration rate, and observed that a rate lower than 0.005 vvm resulted in mass transfer rate from the aerated $CO_2$ as the main limiting factor for microalgae growth. If low concentration of $CO_2$ is supplied to a culture, a high critical $k_L a$ value is necessary to reach the $CO_2$ requirement for microalgae growth.

Ugwu et al. (2003), also studied gaseous transfer and verified that the use of static mixers inside a closed photobioreactor could increase mass transfer, which is proved by the increase of gas hold up and $k_L a$ values.

Lastly, an efficient mixing system in microalgae photobioreactors require methods or apparatus to move both the culture and the gases, between the upper and lower parts of the reactor. One of the variables for microalgae cultivation success is a well mixing, which will contribute to good gaseous transfer, besides avoiding hydrodynamic stress.

## *3.8 Cleanness*

To develop a culture of microalgae in monoculture, it is necessary to prevent the contamination by other organisms, as this can compromise the productivity of the cultivation and the safety of the product, mainly in the case in which the whole biomass will be used as food supplement. The main types of contaminants in clean algal cultures are, bacteria, other algal forms, zooplankton, viruses, fungi and insects, depending on the local conditions, the algal species cultivated, and the particular cultivation system (Becker 1994).

For many phototrophs, gross contamination by bacteria, fungi and protozoa is not a significant problem because there is generally very little free organic carbon to support their growth. A higher concern is to prevent contamination of the photobioreactor by other phototrophs.

To avoid the contamination of a microalgal cultivation with other phototrophs, the best strategies are high concentration of the inoculum, periodic cleaning of the ponds, and creating specific environmental conditions that favor the growth of the desired algal species, for example cultivation with high salt concentration in

*Dunaliella* sp. cultures, high pH to *Arthrospira* sp. cultivation, absence of nitrogen source to cultivation of nitrogen-fixing cyanobacteria. These types of photosynthetic microorganisms are common in open ponds cultivation which does not require expensive cleaning or sterilization processes.

Photobioreactors are usually made of optically clear materials (e.g., glass or acrylic) which do not allow themselves to sterilization by steam, and the size of most photobioreactors go over what can be settled in an autoclave. It could be possible to use systems with ozone for sterilization, but they are expensive and difficult to use. Therefore sanitization rather than sterilization is recommended, which can be easily performed with bleach. Air pumps, analyzers, and other equipment can be kept free of other microorganisms by the use of the proper prefilters. (Behrens 2005).

In the Large-scale photobioreactor, the disinfection and sterilization can be done with solutions containing peroxyacetic acid (5%, 20 min) or chlorine which is an oxidizing agent (sodium hypochlorite). The choice of sodium hypochlorite is due to the fact that it is cheap, effective, and it is also possible to neutralize the residual chlorite solution with sodium thiosulfate ($Na_2S_2O_3$), if necessary. According to De Beer et al. (1994), sterilization should be performed after assembly of all components of the reactor through the circulation of chlorine-based solutions. Finally, the PBR was washed with distilled water several times to remove organic and inorganic residues. At the end, the system was emptied and left to dry.

In mixotrophic and heterotrophic cultivation, mainly, the culture medium must also be sterilized. Two general methods are filter sterilization and steaming. Filter sterilization is effective for small volumes or for heat-sensitive components such as vitamins. Steam sterilization of culture medium is more common. The culture medium can be either sterilized and aseptically transferred to the vessel or sterilized in the vessel (Quesnel 1987; Demain and Davies 1999).

## 4 Scaling-up and Final Considerations

When thinking about developing a microalgal production, the starting point may be the selection of an appropriate and commercially suitable algal strain. It can be performed in small volumes laboratory cultures. The emphasis of this step have to be addressed for optimization of medium composition and culture conditions, analyses of biomass chemical composition, development of product analysis methods and harvesting process, and finally, extraction procedures. Particular interesting photosynthetic micro-organisms are those that grow under particular or stress conditions, which helps in the cultivation in large scale.

When the optimization in the laboratory scale is successfully concluded and the microalgal strain is suitable for being produced in a large scale, the next step is the scale up, either in open ponds or in closed photobioreactor. If preferred, it can be done in small outdoor units, before going to larger ones. Moreover, it should be mentioned that if the desired product is a high value compound produced by the microalgae and it is released to the medium, it is worth evaluating the possibility

of cultivating this microorganism indoor, in small units, for better control of cultivations parameters and mainly the asepsis.

When scaling-up microalgal cultivation, environmental factors like temperature, contamination, agitation, and aeration deserve much attention but it seems that light is one of the most common problems encountered in large-scale microalgae cultivations. Light is rapidly attenuated inside the photobioreactor leading to light intensities heterogeneity inside the photobioreactor. When scaling-up, illumination surface area per unit volume is often used as a photobioreactor design criterion (Ogbonna and Tanaka 1997). In this sense, an efficient photobioreactor has a high surface area-to-volume ratio and, therefore, a pond, for example, should be as shallow as possible. This must also be the reason for the existence of several studies about tubular and panels-like photobioreactors, which allow high surface area-to-volume ratios.

Large scale microalgal production may require great capital and operating costs. Open ponds, mainly mixed raceway ones, are cheaper to build and operate and the most common method of choice for commercial microalgae production, but may require large land areas. But considering the drawbacks such as high susceptibility for contamination, temperature limitations, and light availability, it is worth to intensify the efforts in developing outdoor photobioreactors for effectively producing high amounts of good quality microalgal biomass, meeting the demand that must gradually increase in coming years.

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# Micro Algae in Open Raceways

**Ramanujam Ravikumar**

**Abstract** The quest for renewable and sustainable energy source is necessary to meet the growing global demand, and to substitute the fast depleting nonrenewable fossil fuels. Biofuel is a viable and proven alternative to petroleum based fuel. Algae are appearing to have the greatest potential to replace the fossil fuel. Continuous research on growing algae, harvesting, oil extraction and conversion to biofuel in large scale is indispensable to identify the practical difficulties involved, and find solutions to manipulate the complications, to operate the system farmer friendly and sustainable. This chapter discusses about the influence of various factors such as light, temperature, nutrients and culture mixing in mass cultivation of microalgal species in open raceway clay ponds with the results on average growth and oil content over a period of five years.

**Keywords** Microalgae • Raceway pond • Mixing • Scenedesmus • Scale up • Light • Temperature • Carbon Dioxide • Nutrients • Harvest • Extraction • Algal oil • Contaminants

**Acronyms**

| | |
|---|---|
| Å | Angstrom |
| °C | Celsius |
| c/ml | Cells per milliliter |
| $CO_2$ | Carbon Dioxide |
| cm/sec. | Centimeter Per Second |
| DAF | Dissolved Air Floatation |
| Fig. | Figure |
| $gm/m^2$ | Grams per square meter |
| K | Potassium |
| m | Meter |
| M | Million |
| Mg | Magnesium |
| mM | Millimolar |

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| | |
|---|---|
| µm | Micromolar |
| mg/l | Milligram per Liter |
| nm | Nanometer |
| pH | Decimal logarithm of the reciprocal of the Hydrogen ion Concentration |
| ppm | Parts per Million |
| PUFA | Poly Unsaturated Fatty Acids. |
| UV | Ultra violet |
| WHO | World Health Organization |
| < | less than |
| $10^{-2}$ | 0.01 |

**List of Extended Keywords**

| | |
|---|---|
| Algal oil | An oil extracted from algae using solvents. |
| Algal Powder | Dried and powdered algae. |
| Algal slurry | A mixture of algal biomass and water. |
| Anaerobic dead zones | Areas with no oxygen. |
| Bio fertilizers | A substance that contain living microorganisms used to improve soil fertility. |
| Carbon dioxide | A colorless, odorless, incombustible gas. |
| Chloroform | An organic compound with the molecular formula $CHCl_3$. |
| Circulation | The movement of water and culture in circular direction. |
| Clumping | Algal cells stick together as a mass. |
| Competitors | Undesired organisms compete for food, light and space. |
| Contaminants | Microorganisms infect the algal cells and/or affect the culture quality. |
| Culture | Growing a single or group of organisms in a given area. |
| Dispensers | Device used to supply $CO_2$ in minimum doses to diffuse in to the liquid. |
| Fluorometer | Equipment used to measure lipid content. |
| Hexane | A hydrocarbon with chemical formula $C_6H_{14}$, used as solvent in oil extraction. |
| Light | Electromagnetic radiation that has a wavelength in the range from 4000 to 7700 Å. |
| Mixing | To combine or blend into one. |
| Nitrogen | A colorless, odorless and almost inert diatomic gas. |
| Nitrate | Polyatomic ion, used as fertilizers. |
| Nutrients residue | Quantities of unused nutrients present in the culture. |
| Paddle wheel | Blades attached to a central frame and move in circular motion in the pond to push the liquid culture. |

| | |
|---|---|
| Phosphate | A fertilizer containing phosphorus compounds. |
| Photobioreactors | Devices that used to grow micro algae in a controlled environment. |
| Photo shading | Shades caused by a high density of algae that prevent light penetration. |
| Raceway pond | A pond track resembles the horse raceway track. |
| Scale up | Increase proportionally. |
| Temperature | A physical property of matter that quantitatively expresses the common notions of hot or cold. |
| Tolerance | The maximum capacity to endure. |
| *Botryococcus braunii* | A green microalga widely grown to extract hydrocarbons. |
| *Chaetoceros* sp. | Microalga belonging to diatom group widely used as aquatic feed. |
| *Chlorella* sp. | Single celled, green micro algae grown for high food values. |
| *Crypthecodinium cohnii* | A dinoflagellate alga, with high docosahexaenoic fatty acid content. |
| *Cylindrotheca* sp. | Microalga belonging to the diatom group. |
| Chytridium sp. | A fungal parasite infects and kills the microalgae. |
| *Dunaliella primolecta* | An oval shaped green alga grown for beta carotene. |
| *Isochrysis* sp. | Microalgae used in the aquaculture industry. |
| *Nannochloris* sp. | A marine unicellular green alga. |
| *Nannochloropsis* sp. | A green alga with high PUFA content. |
| *Neochloris oleoabundans* | A green alga used for biofuel production. |
| *Nitzschia* sp. | A diatom genus found common in aquatic habitats. |
| *Phaeodactylum tricornutum* | A diatom with high lipid content. |
| *Phlyctidium scenedesmi* | Fungal parasite infects and destroys the mass culture of algae. |
| *Scenedesmus* sp. | Unicellular green alga. |
| *Skeletonema* sp. | A diatom cultured as food for marine shrimp and fish larvae. |
| *Schizochytrium* sp. | Heterotrophic micro alga with high fatty acid content. |
| *Tetraselmis suceica* | A marine green alga, used in aquaculture. |

## 1 Introduction

Increasing global demand and environmental concerns have led to a search for alternative and greener sources of fuel and other products. Algae shine as an attractive source with their ability to grow in areas unsuitable for agricultural purposes and thereby do not compete with arable land for food production. However,

the production of various products from algae presents several obstacles like the selection of suitable algae, developing suitable growth conditions for optimal lipid yield and prevention of contamination from undesired algal species and other organisms. These obstacles multiply to an even greater magnitude when algal growth is pursued on large-scale outdoor settings where weather and contamination pose a constant threat. Therefore, there exists an exigency for new algal production technologies.

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure. They can potentially be employed for the production of biofuels in an economically effective and environmentally sustainable manner. Microalgae have been investigated for the production of a number of different biofuels including biodiesel, bio-syngas, and bio-hydrogen. Production of these biofuels can be coupled with flue gas $CO_2$ mitigation, wastewater treatment, and the production of high-value chemicals (Li et al. 2008). Microalgal farming can also be carried out with seawater using marine microalgal species as producers. Developments in microalgal cultivation and downstream processing (e.g., harvesting, drying, and thermochemical processing) are expected to further enhance the cost-effectiveness of the biofuel from microalgae strategy (Table 1).

Microalgae are grown commercially in photo-bioreactors and open ponds. Benemann in 2008 compared the economies of open ponds with closed bioreactors. Growing microalgae in bioreactors is a high yielding technology (0.5–8 g/l), low risk of contamination, but expensive ($ 250,000–1,000,000/ha) with an expected approximate life span of 10–15 years for the bioreactors. The raceways are perceived to be less expensive than photobioreactors, because they cost less to build and operate. Although raceways are low-cost ($ 100,000/ha), they have a low biomass productivity (0.1–0.5 g/l) when compared with photobioreactors (Pulz 2001; Alabi et al. 2009). Richardson et al. 2012 estimated the total cost of lipid production is $ 12.74/gal and $ 32.57/gal for the open pond and PBR, respectively.

The first attempt to overcome difficulty in managing culture growth in open ponds was by the use of simple plastic covers or greenhouses. This allows for an extension of the growing period, facilitates the provision of carbon dioxide and the maintenance of high temperature at nights result an improvement of the biomass productivities (Richmond 1992). The open ponds are highly scalable; some commercial pond culture systems such as Earthrise, Cyanotech have been operating profitably for more than 30 years. Culturing species of *Chlorella*, *Scenedesmus* sp., *Haematococcus* sp., *Spirogyra* sp., *Dunaliella* sp., *Nannochloropsis* sp., *Botryococcus* sp., in open ponds at commercial scale targeting fuel and food resources have also been successful (Robert Henrikson 1989; Kay 1991; Pauline et al. 2006; Gouveia and Oliveira 2009). Biomass of *Skeletonema* sp., *Chaetoceros* sp., *Tetraselmis* sp., *Isochrysis* sp., *Crypthecodinium* sp., is popular as feed for juvenile shrimp and fish species (Hoff and Snell 2008). Algae also play great roles in absorbing hazardous materials from wastewater and convert them as useful bio fertilizers (Oswald 1988; Kaushik 1998).

**Table 1** provides an estimate of microalgae oil content in a few of the prominent algal species. (Helm et al. 2004; Chisti 2007; Brennan and Owende 2010; Gouveia 2011)

| Microalgal species | Oil content (% dwt.) |
|---|---|
| *Botryococcus braunii* | 25–75 |
| *Chaetoceros calcitrans* | 17 |
| *Chaetoceros gracilis* | 19 |
| *Chaetoceros muelleri* | 21 |
| *Chlorella emersonii* | 63 |
| *Chlorella minutissima* | 57 |
| *Chlorella sorokiana* | 22–44 |
| *Chlorella protothecoides* | 50–58 |
| *Chlorella vulgaris* | 14–56 |
| *Chlorococcum* sp. | 53 |
| *Crypthecodinium cohnii* | 20 |
| *Cylindrotheca* sp. | 16–37 |
| *Dunaliella bioculata* | 8 |
| *Dunaliella primolecta* | 23 |
| *Dunaliella salina* | 14–20 |
| *Isochrysis galbana* | 20–24 |
| *Microcystis aeruginosa* | 50–58 |
| *Monallanthus salina* | >20 |
| *Monodus subterraneus* | 30 |
| *Nannochloris* sp. | 20–35 |
| *Nannochloropsis* sp. | 31–68 |
| *Neochloris oleoabundans* | 35–54 |
| *Nitzschia* sp. | 45–47 |
| *Pavlova lutheri* | 50 |
| *Pavlova salina* | 49 |
| *Phaeodactylum tricornutum* | 20–30 |
| *Porphyridium cruentum* | 9 |
| *Scenedesmus dimorphus* | 6–40 |
| *Scenedesmus obliquus* | 11–55 |
| *Schizochytrium* sp. | 50–77 |
| *Skeletonema costatum* | 12 |
| *Spirulina maxima* | 4–9 |
| *Tetraselmis sueica* | 15–23 |
| *Thalassiosira pseudonana* | 24 |

## 2 Strain Selection

As mentioned earlier, microalgae have been a potential source of biofuels, and there is an increasing focus for finding appropriate species and exploiting them for various applications. The promising algal species are distributed in fresh, brackish and marine water habitats (Metzger and Largeau 2005; Natrah et al. 2007; Deng et al. 2009). Many microalgal strains are collected from local freshwaters and are grown in modified Bold Basal and or BG11 medium (Allen and Stanier 1968; Stein

1973; Watanabe et al. 2000), incubated at 25 ± 1 °C under 100–200 μmoles photon $m^{-2}$ $s^{-1}$ irradiance with 12:12 light-dark cycle. Sforza et al. 2012 observed the growth rate of *Nannochloropsis* culture was optimum at 150 μE/$m^2$/s and declined at 350–1000 μE/$m^2$/s. Solovchenko et al. 2008 reported the optimum growth rate of *Parietochloris incisa*, a green unicellular micro alga at 400 μE/$m^2$/s on a complete medium. Hence, the growth media and light intensities are adjusted to find an increase in algal productivity. The emerging, faster growing species are isolated; purified by serial dilution, repeated agar plating and antibiotic treatment. The pure culture is subsequently scaled up and transferred to 20 L glass carboys, recurrently subcultured for 10–12 times. Then the cultures are domesticated by treating in higher (~33 °C) and lower (~10 °C) temperatures, various light intensities (30–1000 μmoles photon/$m^2$/s), and different nutrient formulations to pretend the outdoor weather conditions. The culture is then inoculated into 50 L open mini raceway ponds with inbuilt paddle wheels made of acrylic sheet, are exposed to ambient conditions and subsequently subcultured several times for a period of 10–12 weeks. These cultures are monitored closely for growth, polymorphism, contamination, light and temperature tolerance, nutritional requirement, and oil content. All selected strains undergo this process simultaneously. The effective, robust strains are selected based on faster growth rate (g/$m^2$), higher oil content (% dwt.) and simple to harvest and extraction process. These outdoor experiments enable the researcher to determine the highest productivity peak of a particular strain corresponding to its nutrient composition, light, temperature and mixing velocity requirements.

## 3 Cultivation in Open Raceways

The mass cultivation ponds are of various shapes from circular, square to rectangular raceways. Raceway ponds for mass culture of microalgae have been used since the 1950s (Burlew 1953) for food production and waste management (Benemann et al. 1987). Extensive experience exists in the operation and engineering of raceways (Dodd 1986). The largest raceway-based biomass production facility occupies an area of 440,000 $m^2$ (Pauline et al. 2006). A raceway pond is a closed loop recirculation channel that is typically about 0.3 m deep, comprises parallel rectangular channels with semi-circular or sufficiently curved channels on either end joining neighboring ends of the parallel rectangular channels to form a continuous channel (Lee 2001). Ponds involve evenly divided lanes by the width of each lane staying constant throughout the course of the pond (Douglas and Blanca 2012). The pond bottom is compacted, smooth and even. A small dip, approximately one foot deep is constructed in one corner of the pond to support pumping operations. A foot raise of pond sidewalls prevents the culture from rainwater runoff into the pond and a continuous running paddle wheel circulates the growing culture (Vonshak 1997; Chisti 2007; Chen et al. 2009; James and Boriah 2010). The smaller ponds are constructed with concrete or fiberglass materials to minimize stress to the freshly inoculated culture from soil borne contaminants and to enable adaptation to the new conditions at the initial stages of the culture cycle. The clay ponds in the pilot


**Fig. 1** Open raceway ponds **a** 3 × 1, **b** 15 × 1.6, **c** 122 × 14 m (Aquatic Energy LLC)

site, at Aquatic Energy LLC, range from 30 × 5 to 122 × 14 m to hold a total volume of 2,000,000 L of culture. The depth of the pond culture is maintained maximally up to 25 cm to allow sunlight to pass through the entire water column of the pond. Sheehan et al. 1998 extensively studied the production of microalgal biomass for making biodiesel in raceway ponds.

Several nontoxic acrylic liners may also be used on the sidewalls of the clay pond to avoid soil erosion on the course of rainfall and frequent culture operations. The edges of the lines must be fixed in order to protect from wind (Fig. 1).

The pond size decides the paddle wheel size and number. The smaller ponds are designed with single paddle wheels with four or six paddles and the larger ponds are constructed with two paddle wheels of six paddles each on either side of the pond. The paddle wheel is positioned so that it straddles the median divide and outside wall of the pond, and is able to push the culture to a great distance before the lane curves. Experience has shown that the ground clearance between the paddle and pond bottom less than 0.3 in. is effective to provide maximum contact with the culture. The mixing speed should ensure 90% of the algae are suspended. Lesser mixing would affect the productivity by ways of biomass sedimentation and decay. The microscopic cell counting, optical density analysis and chlorophyll estimation before and after mixing the culture is employed to detect the suitable mixing velocity. The normal mixing velocity ranges between 12 cm/sec and 18 cm/sec, but the exact value could slightly vary for different strains (Borowitzka 2005). Generally,

for the heavier or larger strains stronger mixing is preferred than the lighter ones. Ugwu and Aoyagi 2012 discussed various designs, operation and application methods in microalgal culture systems.

A shallow depth of < 5 in. in a clay based raceway pond results in large volumetric productivity but it leads to sudden pH rise or fall, $CO_2$ depletion and other negative effects in the culture such as cell stress in hot summer & winter seasons and upwelling of silt affecting light penetration. Thus, a depth of 8 to 10 in. would increase the efficiency of the paddle wheel and minimize dead zones in the pond (Boyd and Tucker 1998).

## *3.1 Scale Up and Culture Management*

The domesticated pure *Scenedesmus* cultures are inoculated into smaller outdoor raceway ponds (3 × 1 m and 60 L) with clean water. A nutrient composition is supplied to the raceway pond at about the same time as the diluting step, wherein the nutrient composition comprises urea, phosphate and minerals. The denser cultures are subsequently transferred from smaller to larger ponds (122 × 14 m and 50,000 L). At each stage, the ponds are closely monitored for cell abnormalities and contaminants.

A cooling liquid is added to the raceway pond if a temperature of 33˚C or higher is reached. The pH is maintained < 9 using $CO_2$ dispensers. The paddle wheels are in continuous operation to keep the culture in suspension and to circulate the nutrients throughout the pond area. The culture depth is maintained between 8 and 10 in. in each pond. Samples are collected three times a day to estimate culture density and nutrient residue. The depleting nutrients are added to the culture near the paddle wheels to ensure that the algae are not starving. The cell density is maintained at 5 M cells/ml and harvest or dilution is done when the cell density reaches between 5 M and 7 M cells/ml. Culture density above 7 M cells/ml affects light delivery. The highly efficient chlorophyll antenna systems of microalgae cause mutual shading as cell concentration increases. The chlorophyll absorbs excess light even though they cannot process all the photons absorbed. This affects the light penetration depth and thus the depth of the photic zone (Park and Lee 2001). As a result, photosynthetic efficiency will be decreased and photoshading results in colony clumping and settling affecting gas exchanges in the pond.

## *3.2 Light and Water Temperature*

Light is a fundamental factor determining the growth of algae. Open cultivation of microalgae is challenging, where the light intensity widely fluctuates from time to time. An increase in respiration rate occurred (Molina Grima et al. 2001) at low light conditions and higher intensities lead to photoinhibition (Lu and Vonshak 1999; Gouveia 2011) affecting productivity. Water temperature is another factor

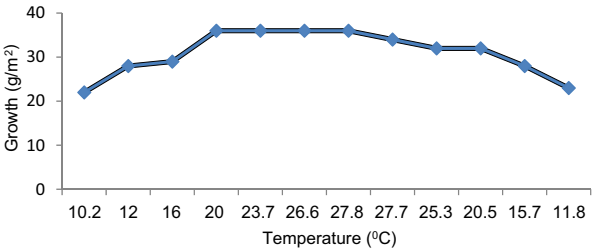


**Fig. 2** Growth vs. time with monthly average temperatures shown for each month

that greatly influences the microalgal growth. Especially in an open pond situation, the temperature fluctuation is inevitable. The high ambient temperatures extremely affect the cell metabolism by interacting with nutrients, cell membrane transport system, enzymes, and production of useful metabolites (Quinn and Williams 1983; Wheeler 1983). A reduction in cell size at higher temperatures was observed by Rijssel and Gieskes 2002; a significant decrease in protein content and an increase in lipids and carbohydrates in *Spirulina* cultures was noticed (Tomaselli et al. 1988; Oliveira et al. 1999). Hancke et al. 2008 studied the temperature effects on photosynthesis activities of a few species of *diatoms*, and observed the photo synthetic rate was strongly stimulated by temperature and the optimum growth occurred between 20 and 25 °C. Chen et al. 2012 evaluated four species of marine microalgae growing at different temperatures for their ability to remove ammonia from intensive marine fish and shrimp culture systems. The growth of *Chlorella vulgaris* was affected at temperatures above 30 °C and a 17 % decrease in its growth rate at 35 °C. Further increase in temperature led to an abrupt interruption of microalgal growth and resulted in cell death (Converti et al. 2009). Temperature fluctuation can also bring changes in pond water ionic equilibrium, pH, $O_2$, $CO_2$ solubility; although different species are influenced to differing degrees by this effect (Bouterfas et al. 2002).

In Aquatic Energy LLC culture facility, as Fig. 2 indicates, the growth reached its maximum of 34 g/m$^2$ when the monthly average temperature ranges between 21 and 27 °C. There was a decline in growth when the temperature was either above or below the optimum. The highest productivity was achieved in midsummer months and low growth during the winter months, to 22 g/m$^2$ at 10 C.

## *3.3 Nutrients*

Microalgae use $CO_2$ as a basic carbon source for growth. The absence or insufficient availability of $CO_2$ seriously affects productivity in raceway ponds. Based on the average chemical composition of algal biomass, approximately 1.8 t of $CO_2$ are needed to grow 1 t of biomass (Rodolfi et al. 2008). Natural dissolution of $CO_2$ from the air into the water is not enough. This could be improved by bubbling air through the water but, since air contains $CO_2$ as a trace gas at a concentration of about 0.0383 % per volume, all of the $CO_2$ in about 37,000 m$^3$ air is needed for 1 t

**Table 2** summarizes the $CO_2$ tolerance of various species. Note that some species may tolerate even higher carbon dioxide concentrations than listed in the table. Overall, a number of high $CO_2$ tolerant species have been identified. (Ono et al. 2003)

| Species | Known Maximum $CO_2$ Concentration (%) | References |
|---|---|---|
| *Cyanidium caldarium* | 100 | Seckbach et al. 1971 |
| *Scenedesmus* sp. | 80 | Hanagata et al. 1992 |
| *Chlorococcum littorale* | 60 | Kodama et al. 1993 |
| *Synechococcus elongatus* | 60 | Miyairi 1995 |
| *Euglena gracilis* | 45 | Nakano et al. 1996 |
| *Chlorella* sp. | 40 | Hanagata et al. 1992 |
| *Eudorina spp* | 20 | Hanagata et al. 1992 |
| *Dunaliella tertiolecta* | 15 | Nagase et al. 1998 |
| *Nannochloris* sp. | 15 | Yoshihara et al. 1996 |
| *Chlamydomonas* sp. | 15 | Miura et al. 1993 |
| *Tetraselmis* sp. | 14 | Matsumoto et al. 1995 |

of dry algae. Thus an additional supply of $CO_2$ is required to maintain the pond algal productivity. The assimilation and tolerance rate of $CO_2$ in raceway culture is complicated to understand because of the impact of other factors such as light, temperature and nutrients. Maraskolhe et al. 2012 observed a maximum growth of *Scenedemus* species when the $CO_2$ concentration in the medium was 36%.

Several species have been tested with flue gas for $CO_2$ tolerance, Maeda et al. 1995 found a strain of *Chlorella* sp. T-1 which could grow under 100% $CO_2$, despite the maximum growth rate occurred under a 10% concentration. *Scenedesmus* sp. could grow under 80% $CO_2$ tolerance conditions but the maximum cell mass was observed in 10–20% $CO_2$ concentrations (Hanagata et al. 1992). *Cyanidium caldarium* (Seckbach et al. 1971) and some other species of *Cyanidium* can grow in pure $CO_2$ (Graham and Wilcox 2000, Table 2).

The carbon content in $CO_2$ is around 27% and the shortage of carbon in the medium is overcome by supplying carbon source from external $CO_2$ cylinders using micro bubble diffusers (Ben-Amotz 2007; Yun et al. 1997; Sawayama et al. 1995). Though, higher $CO_2$ dosages result in rapid cell flooding, the dosage between 60 and 63 g/m$^2$ showed a marginal increase in productivity (Fig. 3) but unstable during the course of growth. The $CO_2$ requirement varies between species and the dosage is decided in accordance with other parameters that favors growth (Ono and Cuello 2003).

It was observed that nitrogen and phosphate fertilizers added in tiny amounts, help to maintain the culture quality and to ward off the competitors. The nitrogen metabolism is affected by environmental changes (Thomas and Krauss 1955; Dohler 1998); similar results are observed in open pond cultures at Aquatic Energy LLC pilot facility. For even more addition of nitrogen and phosphate nutrients, during high (>30 °C) and low (<15 °C) temperature conditions, no increase in growth is detected. It is suggested that; the nutrients are to be dosed matching the growth rate, temperature and light conditions. Excess or overdosing of nutrients is not necessary. The surplus nutrients in the culture ponds either evaporate by volatilization into the

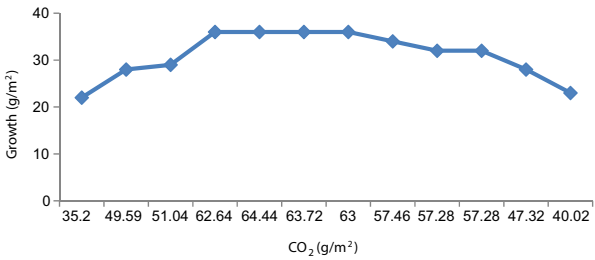


**Fig. 3** Growth vs. time with average monthly dosage of $CO_2$ per day shown for each month

atmosphere or precipitate at higher pH values, increasing the cost of production, further attract undesirable species that contaminate the culture.

## *3.4 Biomass Harvest*

The selection of harvesting technology is crucial to the economic production of microalgal biomass (Schenk et al. 2008). A factor such as strain selection is an important consideration since certain species are much easier to harvest. *Spirulina*'s long spiral shape (20–100 mm long) naturally lends itself to the relatively cost-efficient and energy-efficient microscreen harvesting method (Benemann and Oswald 1996). Thus the selection of harvesting technique is dependent on the characteristics of microalgae e.g. size, density, and value of the target products (Olaizola 2003). Generally, microalgae bulk harvesting is focused on separation of biomass from the bulk suspension. This will depend on the initial biomass concentration and technologies employed, including flocculation, flotation or gravity sedimentation. Another method is to concentrate the slurry through techniques such as centrifugation, filtration and ultrasonic aggregation, hence, is generally a more energy intensive step than bulk harvesting (Brennan and Owende 2010).

At the onset of harvest, 1 or 2 in. of the culture is pumped from the growth ponds into a wet well and the mixture is allowed to settle for about 2 h and then pumped into a Dissolved Air Flotation System (Fig. 4a). At this time, nontoxic polymers are added to separate the algal biomass from water. The algal slurry (Fig. 4b) is stored in a holding tank (Fig. 4c) and pressed onto a belt press (Fig. 4d) until it becomes 30 % dry (Fig. 4e). This is further dried in a propane-fueled oven to obtain a 90–95 % dry biomass. The harvested water is evaluated for nutrient residue with an analyzer (Fig. 4f), and pumped back to the culture pond after filtration and UV treatment, replenished with necessary nutrients.

## *3.5 Extraction of Algal Oil*

Microalgae compared with traditional crops, they have a high areal productivity, a relatively high oil and protein content, and do not depend on arable land. Microalgae are theoretically capable of producing much more lipids than any conventional


**Fig. 4** **a** DAF unit. **b** Algal slurry collection. **c** Holding tank. **d** Belt press. **e** Scrapped algae. **f** Nutrient analyzer. **g** Extractor

crop, approximately 20 times higher than soybean (Chen et al. 2009; Darzins et al. 2010) and are, therefore, attractive as a potential source of biodiesel. Because lipids from algae are often rich in the long-chain omega-3 fatty acids EPA and DHA, algae may also be a more sustainable source of these fatty acids for use as food or feed compared with fish oil (Lee et al. 2010).

Lipids have been recovered from microalgae via a multitude of extraction methods (Dunahay et al. 1996; Xu et al. 2004; Hossain et al. 2008; Ryckebosch et al. 2011). Because of the nature of microalgae, regular extraction methods (used for example as food) may not be applicable. First of all, microalgae are either single celled or colonial, but each cell is surrounded by an individual cell wall. Furthermore, they often contain 'unusual' lipid classes and fatty acids differing from those in higher animal and plant organisms (Guschina and Harwood 2006).

Lipid analysis is performed using both fluorescence and total lipid extraction. Fluorescence test is a quick method using a fluorometer to measure the lipid content in the cells. The harvest process from the larger raceway pond is performed once the lipid concentration of about 25 % of the cell mass is reached. The dye Nile Red is highly fluorescent in the presence of lipids and used to achieve readings (Castro et al. 2005). A Turner model 1–10 fluorometer with emission filters (420–470 nm) and excitation filters (>520 nm) are employed. For this procedure, the culture is diluted to 3 mg/l. The dye is then added at a concentration of 1 mg/l. This solution is mixed using a vortex mixer for 5 min and results are read at 5-minute intervals for one hour, and are compared against a standard solution of 1 mg/l triolein with 1 mg/l Nile red.

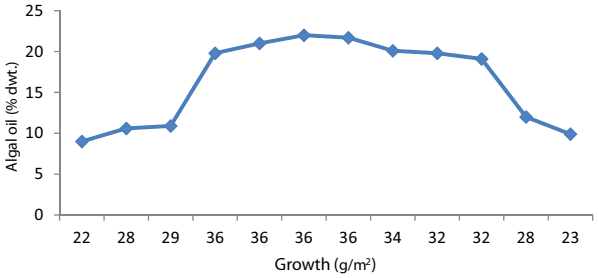


**Fig. 5** Algal oil vs. growth with average monthly oil content shown for each month

In a laboratory set up, the total lipid extraction is performed using a modified Bligh and Dyer 1959 method. The algal slurry is dried overnight using a bench-top dehydration unit. The dried algal flakes are ground well using a grinder and then weighed. Chloroform and methanol solvents are used to extract algal oil. The algal power is mixed with the solvents and water using a vortex. After 30 min, the mixture is poured into a separating apparatus, and left for a few hours undisturbed. After the appearance of three distinct layers (Methanol, Chloroform and Water) in the separating flask, the green algal oil layer is collected and filtered. Later evaporating the solvents, the algal oil residue is measured and computed with initial weight to determine the oil content. Testing samples with ethanol as a solvent (Fajardo et al. 2007) for total lipid extraction resulted a low yield (< 10 dwt. %) against Bligh and Dyer method (> 20 dwt. %). A Dionex Accelerated Solvent Extraction unit is also used (Fig. 4g) to extract the oil from algal biomass using different solvents and the lipid results are more or less similar to Bligh and Dyer method.

In a large-scale extraction, the algal sample is placed inside a tubular column with a very fine mesh that separates the algal powder to prevent flowing into the collection apparatus. The hot hexane (70 °C) is poured onto the algal powder and the dripping liquid is collected and drizzled on to the algal powder repeatedly for five times. After this process, the mixture is finally washed with fresh hot hexane. The collected liquid is filtered to remove any foreign material, and heated to evaporate the hexane. The percentage of algal oil is calculated by the initial weight of the sample.

A proportionate relationship is observed (Fig. 5) between the growth and oil content in the cultures of *Scenedesmus*. In some instances, few of the culture samples are manipulated with low dosages of nitrogen (< 2 mg/l/m$^2$) and phosphate (< 0.5 µg/l/m$^2$), and some culture ponds are left with no nitrogen source, also showed 2–5 (% dwt.) increase in lipid content. In some cases, when the ambient temperature is high, the algal cells are unable to cope with nitrogen starvation leads to photo-oxidative damage. Several research papers supported that the nitrogen starvation results to an increase in lipid content, but in the open raceway culture facilities, the nitrate and phosphate starvation showed both positive and negative effects in biomass yield and lipid content as well, possibly due to the influence of other environmental factors. The sustained growth of *Scenedesmus* biomass after the exhaustion of phosphate in phosphorus starvation mode led to a significant increase in biomass yield and was nearly six times more than that nutrient feeding (Yin et al. 2012).

High temperature and different light intensities also impact on the lipid content in microalgae (Pohl and Zurheide 1979; Mayzaud et al. 1989). Therefore one or a combination of many factors plays a significant role in an open culture system, either increasing or decreasing the lipid content besides nutrient manipulation (Lewin 1962; Tedesco and Duerr 2006).

After the extraction processes, the resulting microalgal oil is converted into biodiesel through transesterification process. This reaction consists of transforming triglycerides into fatty acid alkyl esters, in the presence of an alcohol, such as ethanol or methanol and a catalyst, such as an alkali or acid with glycerol as a byproduct (Vasudevan and Briggs 2008; Dragone et al. 2010; Khola and Ghazala 2012).

The algal meal of Aquatic energy's *Scenedesmus* strain dry weight comprises 3% crude fiber, 0.1% calcium and 39% protein. The omega-3 fatty acids are obtained as a byproduct during the lipid extraction process by treating the lipids under different temperature processes. The alga yields around 22% of *omega 3*, 29% of PUFAs, 20% of monounsaturated fat and 27% of saturated fat. Carbon chains include, but are not limited to, C12–C24 chains in different percentages. Actual lipid profile varied with an increase or decrease in one or more components of the culture system.

## 4 Management of Contaminants

It is impossible to avoid contamination in open ponds. Bacteria, viruses, fungi, zooplankton, insects, leaves and airborne materials are the common constituents of contaminants. It is important to keep the contamination under acceptable limits, as this should not affect the health of the main culture. In large ponds, the floating contaminants are removed by scooping nets or fixing screens along the water flow.

Close monitoring of pH, dosing the right nutrients at the right time, maintaining the culture at high density and following strict sanitation practices control the contaminants in *Scenedesmus* culture ponds. Drastic lowering of pH to about 3 by surging $CO_2$ for 2 h is a common practice to control *Brachionus* infestation (Becker 1994). In a clay pond, pH above 9 favors the growth of blue green algae (Van der Westhusian and Eloff 1983) and a low pH invite other competing green algae. Availability of nitrogen and phosphate sources in excess may flourish toxic cyanobacteria (Hughes et al. 1958). Some fungal contaminants such as *Phlyctidium scenedesmi*, *Phlyctidium* sp. are identified infecting the *Scenedesmus* leading to cell death in a short span of time. Fungicides, such as *Triton-N* (Benderliev et al. 1993) and *Funginex* are used at different concentrations depending upon the infection rate as a control measure. The infection of *Chytridium* species is not uncommon in open clay ponds; it is identified by microscopic observation, the parasite harbors between the outer and the inner part of the thallus and produces a residual structure that absorbs the cellular contents of the host. Addition of $Mg^{2+}$ or $K^{+}$ at concentrations of $10^{-2}$ moles or higher inhibited their growth (Abeliovich and Dikbuck 1977) (Fig. 6).

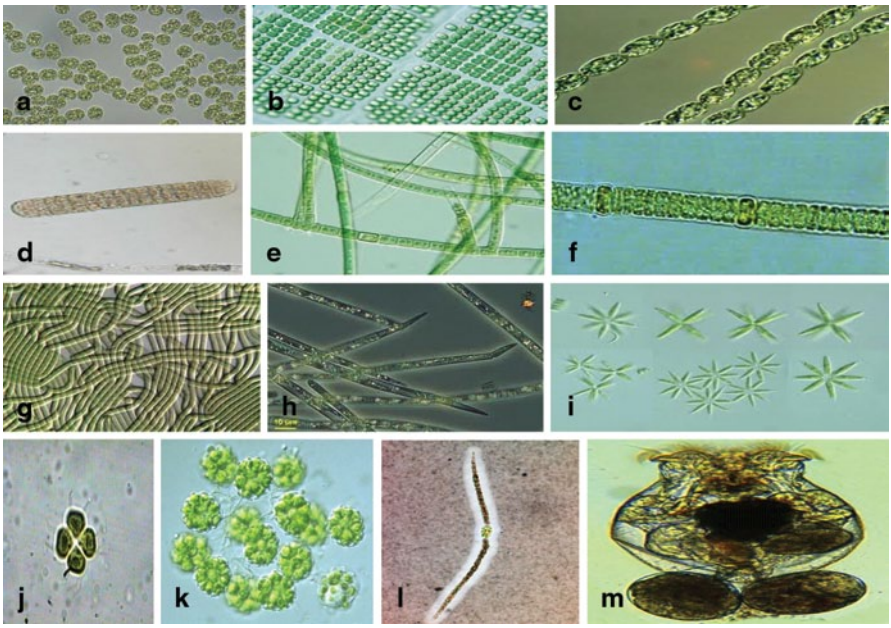


**Fig. 6** Contaminants observed in outdoor cultures: **a** *Microcystis* sp. (×450). **b** *Merismopedia* sp. (×450). **c** *Anabaena* sp. (×450). **d** *Oscillatoria* sp. (×450). **e** *Haplosiphon* sp. (×100). **f** *Nodularia* sp. (×450). **g** *Lyngbya* sp. (×100). **h** *Cylindrospermopsis* sp. (×450). **i** *Actinastrum* sp. (×450). **j** *Tetrastrum sp.* (×450). **k** *Coelastrum* sp. (×450). **l** *Umizakia* sp. (×450). **m** *Brachionus* sp. (×450)

Clean contaminant free water is one of the best ways to avoid most of the contaminant sources. Underground bore well water is allowed to pass through a pre-filter followed by ultraviolet exposure to deactivate most of the aquatic contaminants (Whitby and Palmateer 1993). The UV treated water was filtered before pumping into the pond. The motor and hoses are thoroughly cleaned; chlorine disinfection is done on a weekly basis to ensure cleanliness of the equipment. The poor pond construction and improper mixing may cause many anaerobic zones in the pond affecting productivity.

## 5 Conclusions

Mass cultivation of microalgae in open raceway pond is economical but limits for a few species only. The effort in successfully growing, harvesting microalgae and extracting for biodiesel and other products have been in practice for decades. Although significant literature exists on microalgal growth and biochemistry, more work needs to be undertaken to understand and potentially manipulate algal lipid metabolism to determine the viability of the various options for large-scale culture. The greatest potential for cost reduction and increased yields most probably lies

within open production systems. Knowledge on various contaminants, their sources and controlling techniques are utmost important for a sustainable operation. Apart from bestowing useful products, the large-scale cultivation of microalgae plays an important role in saving the planet earth.

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# High Density Outdoor Microalgal Culture

**Jiří Doucha and Karel Lívanský**

**Abstract** Despite the common use of open raceway pond technology for algae production, the system has many serious drawbacks resulting in low productivities and relatively high production costs. The key to higher yields and a cheaper product rests with the lowering of culture volume by decreasing the thickness of the algal layer exposed to the light. The higher the culture surface-to-volume ratio (S/V), the higher the culture density and the lower the cost of handling and harvesting. Basic parameters (light, temperature, mixing, carbon dioxide, oxygen, nutrition) affecting algal productivity in thin-layer (TL) photobioreactor have been assessed. In a low volume of vigorously mixed culture, utilization of light energy and algal yields are increased. Production costs are reduced to about one fifth (20%) compared to raceways ponds.

**Keywords** Microalgae · *Chlorella* · Culture parameters · High density culture · Open ponds · Thin layer photobioreactor · Productivity · Photosynthetic efficiency · Economic consideration

**List of Acronyms**

| | |
|---|---|
| DO | dissolved oxygen |
| dw | dry weight |
| EPDM | ethyl propylene dimer |
| PAR | photosynthetic active radiation |
| PE | photosynthetic efficiency |
| PUFA | polyunsaturated fatty acid |
| S/V | surface to volume ratio |
| TL | thin layer |

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## 1 Introduction

Microalgae have high potential for use in many fields of human activities. Due to their biological quality, (high protein content, nearly all essential vitamins, pigments, carotenoids, unsaturated fatty acids, organically bound minerals) most of algal products have been used in human and animal nutrition, pharmacy and cosmetics.

Lately, increasing attention has been focused on microalgae as a feedstock for the production of biofuels, currently produced from crop plants containing starch (corn, wheat) or sucrose (sugarcane, sugar beet), for the production of bioethanol, and lipids (rapeseed, soya, palm oil) for the production of biodiesel (Chisti 2007, 2006; Chen et al. 2011; Lam et al. 2012).

One of the factors limiting the extent of algal utilization is their production economics. While the market price of dried *Chlorella* biomass used for the production of health food supplements ranges from 40 to 60 USD per 1 kg, which makes it feasible to grow the algae even under extensive culture conditions, the price of the biomass for economically viable biofuel production should be about two orders of magnitude lower. Thus, cheap and high yielding culture technology is essential.

## 2 Open Ponds

In the 1960s, large-scale cultivation of unicellular *Chlorella* was established in Japan and Taiwan, and some years later, *Spirulina* and *Dunaliella*, using open circular or oblong ("raceway") ponds (Fig. 1). Nowadays, these ponds are also located in the USA, China, India, Thailand, Indonesia, and other countries (Belay 1997; Lee 1997; Borowitzka 1999; Tredici 2004; Spolaore et al. 2006).With the exception of a large tubular photobioreactor producing *Chlorella* biomass in Klötze, Germany (Pulz 2001), and *Haematococcus* for production of astaxanthin in Israel (Algatechnologies, Ltd.), this open pond technology is the only system used commercially for the production of algae. Global annual biomass production is estimated to be up to 6,000 t of *Spirulina* and about 5,000 t of *Chlorella*.

### *2.1 Circular Ponds*

Circular ponds are still used for production of *Chlorella* biomass in Japan and Taiwan. The 20–40 cm thick algal layer is mixed by means of a centrally anchored rotating arm. The diameter of the pond, made of concrete, can be up to 45 m. The main disadvantages of the system are too high thickness of the algal layer requiring very low culture densities, low turbulence in the poor mixed culture especially in the middle of the pond, insufficient supply of $CO_2$ by nozzles located on the arm, high energy consumption for mixing and downstream processing of biomass and low productivity.

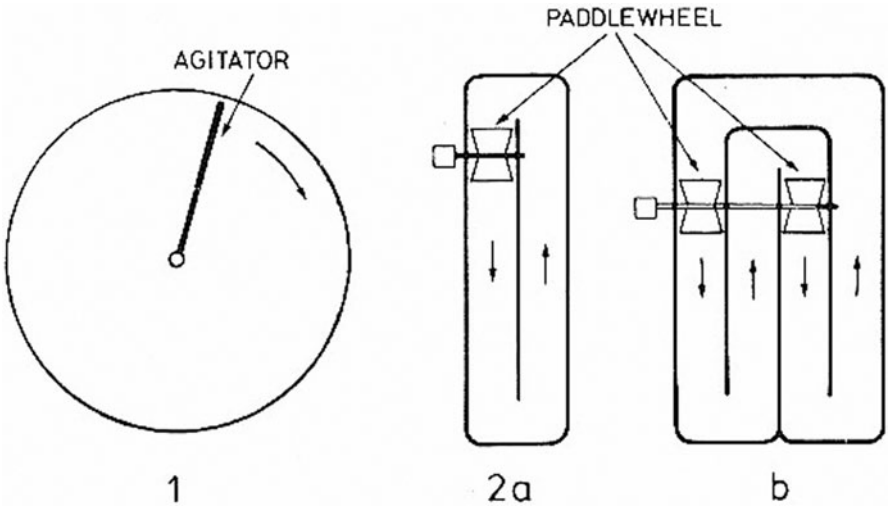


**Fig. 1** Scheme of a circular and raceway pond (courtesy E. W. Becker). *1* algal layer is mixed by means of a centrally anchored rotating arm; *2* **a**, **b** oblong ponds with a central dividing wall, around which the algal suspension is circulated by means of paddle wheels

## *2.2 Raceway Ponds*

Raceway ponds are mostly oblong basins equipped with a central dividing wall, around which an algal suspension circulates by means of paddle wheels. They are characterized by simple construction and relatively low building costs. The bottom is often sand upon which a high quality UV-resistant plastic lining is laid. The walls of the pond are constructed from bricks or concrete blocks covered with plastic sheets. The lining must be carefully laid to prevent wrinkling at the bottom or water accumulation beneath the lining.

For higher productivity of *Chlorella*, mixotrophic culture technology has been used by Taiwanese producers, utilizing acetic acid or glucose as an organic carbon source. To prevent excessive bacterial growth, addition of organic carbon ceases during the night. Despite this, the use of these substrates increases contamination of algal cultures by heterotrophic microorganisms. Removal of bacterial cells by size segregation or by heat and radiation treatments decreases the quality of the algal product and increases production costs.

Despite the common use of raceway technology, the system has many serious drawbacks resulting in low productivities (Pulz and Scheibenbogen 1998; Tredici 2004) and relatively high production costs:

- due to high thickness of the culture layer the concentration of algae should not be higher than 500 mg algal dry weight (dw) per 1 l. With increasing densities the productivity sharply decreases (Richmond 1988; Grobbelaar et al. 1990; Vonshak 1997). Low algal densities increases the danger of

contamination by bacteria and undesirable algal species (Apt and Behrens 1999; Doucha and Lívanský 2006).

- low velocity flow (10–30 cm $s^{-1}$) of poorly mixed algal suspension may result in photoinhibition of the upper overlighted algae and in the accumulation of oxygen, thus increasing photorespiration and decreasing photosynthetic efficiency and productivity (Demirbas 2010; Park et al. 2011).
- circulation of a large volume of algal culture is a continuous (day and night) and thus costly process. Separation of algae at harvest is also energy demanding and requires high capital expenditure. To obtain the desired concentration of algae for further processing, the culture requires at least two stages of thickening using expensive plate separators.
- a large volume algal culture accumulates heat energy from the sun during the course of cultivation, thus increasing the night respiration loss of biomass.
- supply of the algal culture with $CO_2$ is performed by its bubbling through the perforated tubes laying on the bottom of the pond. $CO_2$ utilization is only 13–20 % (Richmond and Becker 1986; Becker 1994).

## *2.3 Thin-Layer Culture Technology*

The key to higher yields and lower production costs rests with a low volume culture by decreasing algal layer exposed to light to as low value as is technologically possible. The higher the surface-to-volume ratio (S/V), the higher the culture density and the lower the cost of handling and harvesting. In a well-mixed culture, several millimeters thick, it is possible to optimize the frequency of light/dark periods of single cells, thus increasing the efficiency of light utilization (photosynthetic efficiency, PE) and decreasing the photoinhibitory effect up to very high solar light intensities.

The first pilot-scale open photobioreactor for microalgae cultivation in a thin-layer (TL) was built at Třeboň's division of the Institute of Microbiology (Czech Republic) and started operation in 1963 (Šetlík et al. 1970). A 50 mm layer of algal suspension flowed down an inclined surface of 3 % slope, with 4 cm high slanted baffles placed at 15 cm distance perpendicularly to the flow. The baffles maintained the required thickness of algal layer over the whole culture area. The vigorously mixed suspension flowing down the 30 m × 30 m inclined area was returned by a collection channel to a tank, from which it was continuously pumped to the upper edge of the incline. A culture of *Scenedesmus quadricauda* started at a density of about 0.5 g (dw) per liter and was harvested at a density of about 3 g (dw) per liter. Productivity was relatively low (10–14 g algal dw per $m^2$ per day for 150 days of cultivation season). The main disadvantage of this system was the high energy consumption for circulating 50,000 l of algal suspension during the day and for night aeration of the suspension in the tank (Doucha and Lívanský 2006). Nevertheless, for 15 years this technology was used successfully for the commercial production

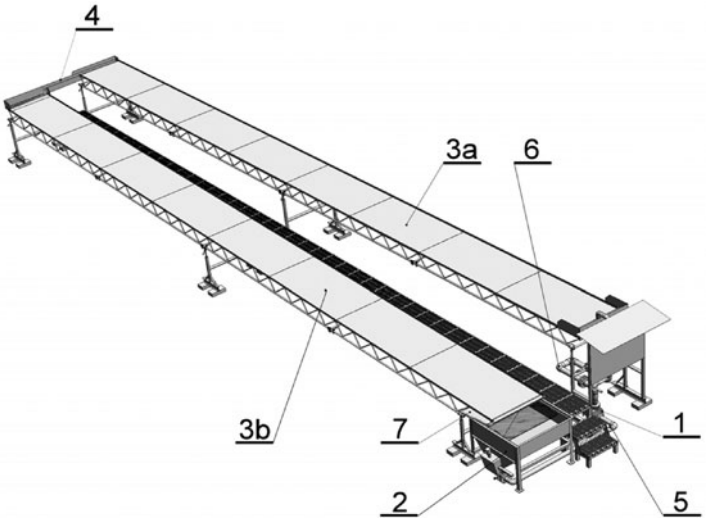


**Fig. 2** Scheme of an open solar pilot plant photobioreactor for growth of microalgae in thin layer. The culture area consists of two lanes, inclined in the opposite directions and connected by a channel, *4*. The algal culture is supplied by means of a centrifugal pump, *1*, equipped with a frequency converter and flows from the retention tank, *2*, onto the upper edge of the culture area and along the inclined glass lanes, *3a* and *3b*. An optimal flow velocity is 50–60 cm $s^{-1}$ at an inclination of 1.6–1.7%, and a culture layer thickness of 6–10 mm; the inclination can be changed. At the end of the lower lane, *3b*, the culture falls through the sieve to the tank, *2*. Scattering of the culture due to flow through the sieve decreases concentration of the DO and prevents impurities falling into the open culture. At night, the culture is kept in the tank and aerated by means of a pump, *1*, set on a night circulation regime. Supply of carbon dioxide: if pure $CO_2$ is used, it is supplied to the pipe, *5*, that enters the pump, *1*. If flue gas (containing 10–15% vol $CO_2$) is used, it is supplied to the porous ethylpropylendimer (EPDM) tubes placed in the pipe loop, *6*. A sliding plate, *7*, covers the suspension in the tank at night or on rainy days. The tank is equipped with a sensor that controls the addition of water lost by evaporation during the course of cultivation and keeps the darkened culture flowing through the tank at the required low level. The optimal $CO_2$ concentration is monitored by a $pCO_2$ electrode and maintained by means of a pH-stat or infraanalyzer (Doucha and Lívanský, 2006). Oxygen concentration in the culture which indicates the rate of photosynthesis during algal growth is measured by means of the Clark electrodes located at the beginning and end of the culture lane

of algal biomass in Rupite, Southern Bulgaria, where a photobioreactor of 3,000$m^2$ culture area was built (Fournadzieva and Pilarski 1993).

A modified version of an inclined-area photobioreactor whose culture parameters were in course of years in details analyzed and further optimized (Lívanský and Doucha 1996, 1997, 1998, 1999; Doucha 1998; Doucha and Lívanský 1995a, b, 1998, 1999, 2006, 2009; Doucha et al. 1993, 2005) started operation at Třeboň in 1991. Modifications consist of removing the baffles, reducing the culture layer to 6–8 mm, changing the inclination of the culture area to 1.6–1.7% and arranging the culture area into two lanes inclined in opposing direction (Figs. 2, 3). At night the algal culture is maintained in an aerated tank.

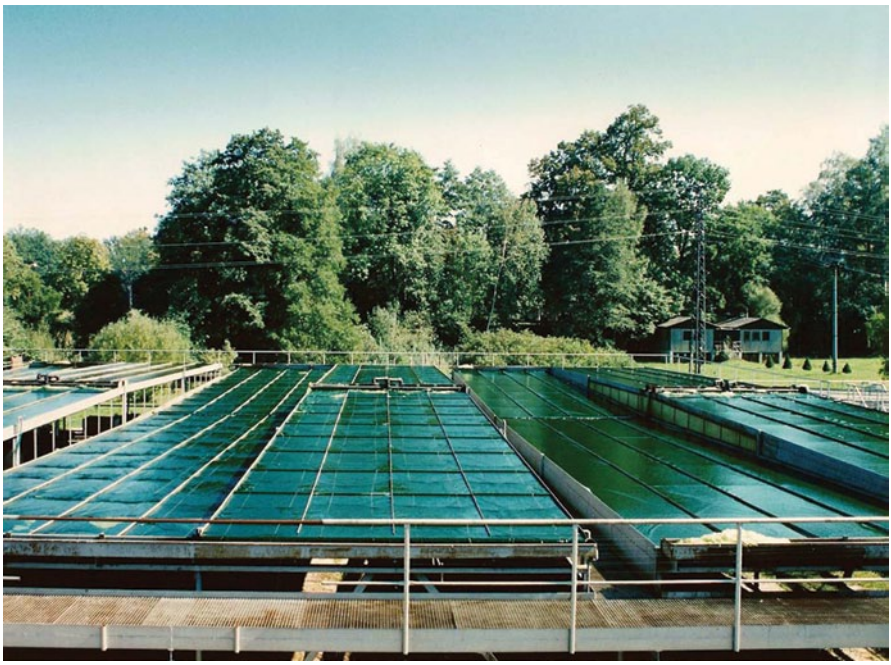

**Fig. 3** Open thin-layer photobioreactor of 700 $m^2$ culture area in Třeboň's Institute. The photobioreactor serves for both growth experiments and production of high-quality biomass used in human and animal nutrition. (Photograph by J. Doucha)

## Basic Characteristics of Thin-Layer Culture Technology

- high turbulence of the 6–8 mm layer of algal suspension flowing at a velocity of 50–60 cm $s^{-1}$ along the inclined surface results in a high frequency of light/dark periods for single cells thus increasing light utilization and reducing the photoinhibition effect even at high sunlight intensities. This results in elevated PE and productivity.
- the culture volume per unit of cultivation area is 50x lower and the algal density at harvest is 100x higher than in raceways significantly reducing the cultivation and downstream processing costs; low culture volume permits better control of growth parameters. High algal density minimizes culture contamination.
- growth rate of the algal culture does not decrease with culture density up to high harvesting densities thus making the downstream processing cheaper.
- supply of the culture with $CO_2$ is achieved by (1) its supply into the suction pipe of the culture circulating pump (in case of pure $CO_2$) or (2) in case of flue gas, by $CO_2$ delivery to the EPDM tubes placed in the pipe loop between the circulation pump and the upper edge of the cultivation lane. In both cases, the utilization of $CO_2$ by algal cultures is about 70 % and reaches values achieved with closed culture systems.

- due to high cooling effect by evaporation of water from the thin algal layer, growth is possible even under high air temperatures and high solar light intensities (Doucha and Lívanský 2006, 2009).

## 3 Closed Photobioreactors

In the last decade, increasing attention has been focused on closed culture systems. Basic principles have been reviewed by many authors (Grima et al. 1999; Janssen et al. 2003; Carvalho et al. 2006; Chisti 2007; Xu et al. 2009; Posten 2009). The following parameters are considered to advantageous in closed bioreactors in comparison with open culture technology: prevention of, or minimizing contamination, growth temperature can be maintained higher and better controlled, prolonged cultivation season in climate with lower ambient temperature, better control of basic culture parameters (pH, $pO_2$, $pCO_2$), lower water and $CO_2$ losses, smaller area requirements in the case of vertically arranged bioreactors (tubes, plates).

However, there are some serious drawbacks to closed culture technology, which arise from its large-scale use: high building costs, diminished PE due to age-related decreasing transparency of the material from which walls of the reactor are made and due to loss of light incident under angle on the culture surface, and excessive accumulation of oxygen which inhibits photosynthesis. Removal of oxygen must be carried out in non-illuminated degassing columns (Chisti 2007). Attachment of algae to the inside surface of the culture area and fouling of the culture at higher algal densities decreases the absorbance of light energy and reduces PE. High energy demand: the culture must be forced through the tubes or in meandering way arranged channels of the flat-plates continuously (day and night), the density of growing algae is low (mostly less than 1.5 g dw $l^{-1}$) increasing thus further energy demands for algal separation at the harvest.

Many types of closed bioreactors have been developed (Tredici 2004; Carvalho et al. 2006; Posten 2009): tubular systems of various length, volume and diameter constructed from glass or transparent plastic tubes arranged either vertically, horizontally or in the form of coils; flat plates in which the suspension is exposed directly to the sunlight and mixed by air bubbling; panels arranged vertically where the algal suspension moves through horizontal channels in a meandering way by means of a pump.

The closed cultivation units often equipped with artificial lights were widely used in experimental work in laboratories and in the preparation of the high valuable products requiring special culture technology (e.g. production of labeled compounds, microalgae in aquacultures, preparation of PUFA's for pharmaceutical use). Only very few bioreactors have been practically operated on a large scale. The largest plant for *Chlorella* production is running in Klötze, Northern Germany (Pulz 2001). This plant, installed in a greenhouse, consists of 500 km of 50 mm diameter glass tubes arranged horizontally in 20 modules that form 3 m high vertical walls.

Nevertheless, the volumetric productivity is relatively low. A considerable increase of algal yield is achieved by use of mixotrophic culture technology (light and an organic carbon source). The product, spray-dried algal biomass is mostly used in the food and cosmetic industries.

## 4 Parameters Affecting Algal Productivity in Dense Open Cultures

### *4.1 Light*

Solar radiation covers a broad wavelengths spectrum, of which only a part, photosynthetically active radiation, (PAR, wavelengths 400–700 nm) is useful for photosynthesis. For most applications, efficient capture of light energy represents one of the most important factors controlling culture productivity. Microalgal growth is mostly saturated at a PAR of about 200 μmol $m^{-2}$ $s^{-1}$, which is about 1/10 of the maximum light intensity outdoors in summer (Torzillo et al. 2003). For solar light a conversion factor of 1 W $m^{-2}$ = 4.94 μmol $m^{-2}$ $s^{-1}$ (μE $m^{-2}$ $s^{-1}$) was found for PAR (Doucha and Lívanský 2006). Chlorophyll fluorescence measurements in high-density mass cultures exposed to strong light showed a decrease in photoinhibition with increasing biomass concentration (Richmond 2000). A high column of water in the pond absorbs much higher portion of light which could not be used for the cells, compared to a thin-layer culture. This factor alone justifies the use of low areal densities in ponds.

The occurrence of the "light saturation effect" is one of the most serious limitations of high solar irradiance utilization. Three types of approaches have been proposed to alleviate the saturation effect: (1) increase culture density and the mixing rate; (2) use of special designs of photobioreactors to dilute light intensity incident upon the culture (Mori 1986; Carlozzi 2003); (3) search for strains having small antenna size (Nakajima and Ueda 1997; Melis et al. 1999).

However, in certain cases, saturating light is a prerequisite for stimulating the synthesis of valuable products such as secondary carotenoids (e.g. by the alga *Haematococcus*).

Algal productivity is influenced mainly by light intensity available to cells inside the thin culture layer. The PAR intensity $I$ (W $m^{-2}$) at a distance $z$ (m) from the algal culture surface can be expressed, in accordance with the Lambert-Beer law, as: $I = I_0 \exp(-\varepsilon X z)$, where: $I_0$ (W $m^{-2}$) is PAR irradiance of the algal culture, ε($m^2$ g $dw^{-1}$) is the extinction coefficient, $X$ (g dw $m^{-3}$) is the cell concentration. At an algal cell concentration higher than ca 5 g dw $l^{-1}$, almost all incident light is absorbed in the layer (Doucha and Lívanský 2009).

The mean light intensity in the layer of thickness $h$ is:

$$I_{mean} = \frac{1}{h}\int_0^h I\, dz = \frac{I_0 - I_h}{\varepsilon_{mean} X\, h} \tag{1}$$

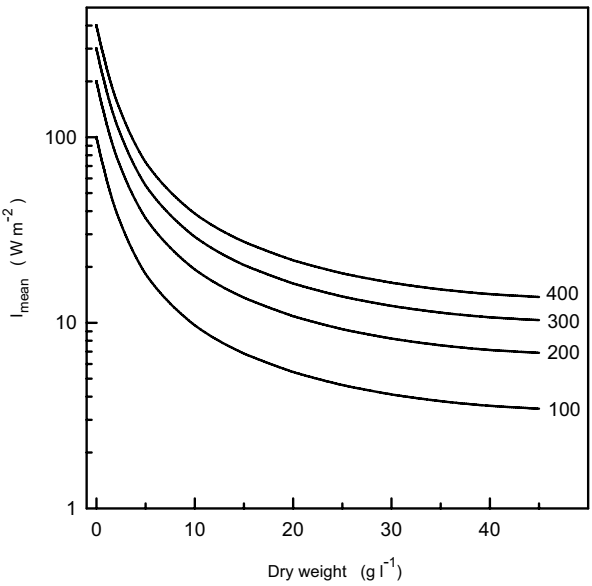


**Fig. 4** Dependence of the mean light intensity inside an 8 mm thick culture layer on *Chlorella* sp. dry weight. Numbers at curves: PAR irradiance ($Wm^{-2}$)

where $I_h = I_0 \exp(-\varepsilon_{mean} X h)$ is the unabsorbed light intensity in the layer, and $\varepsilon_{mean}$ is the mean extinction coefficient. The following correlation was found for *Chlorella* culture: $\varepsilon_{mean} = \varepsilon_0 (1 - a_1 h/2)(1 - a_2 X)$, with values of empirical coefficients: $\varepsilon_0$ ($m^2$ g $dw^{-1}$) = 0.175; $a_1 = 46.165$; $a_2 = 9.664.10^{-6}$ (Doucha and Lívanský 2009). The mean light intensity inside the layer decreases with increasing cell concentration (Fig. 4). At high cell concentrations, this decrease is slow. This may partly explain why algal productivity in high density thin-layer cultures is influenced only slightly by cell concentration (Doucha and Lívanský 2006, 2009).

## *4.2 Temperature*

Temperature significantly affects biomass yield. Due to a very thin algal layer and a low culture volume in the TL photobioreactor, the optimum growth temperature is reached very quickly after beginning of the cultivation day. The rapid rise in temperature may prevent photoinhibition of algal growth, which occurs in raceway ponds, where the temperature rises more slowly due to the large volume of suspension that must be heated. On the other hand, even on very hot and sunny days, cooling of the culture in the TL photobioreactor due to water evaporation is very effective: the maximum temperature of a 6 mm thick algal layer does not exceed 38.5 °C at noon hours. The evaporated water is replaced by means of a controller (Fig. 2). Loss of the evaporated water (5–6 l $m^{-2}$ $d^{-1}$ in clear summer days) is comparable, at the same solar energy input, to the consumption of water for cooling of closed bioreactors by spraying from the outside (Doucha and Lívanský 2006, 2009).

In addition, the relatively low evening culture temperature in TL bioreactor causes smaller loss of algal biomass by its night respiration in the tank.

### *4.3 Mixing*

Mixing of algal culture in photobioreactors avoids thermal stratification, distributes the nutrients and improves gas ($CO_2$, $O_2$) transfer and mass transfer between the cells and the liquid. Adequate mixing can counter the negative effect of a light gradient. Turbulence exerts the following major effects: it prevents culture stratification which could result in photoinhibition of cells exposed to high irradiance, decreases the cell boundary layer and prevents sedimentation of the cells.

Photobioreactor design should ensure an optimum light-dark cycling of the cells to enhance PE (Chisti 2006). Algal growth can be influenced by three types of intermittent illumination (light/dark periods): high frequency fluctuations of 100 ms (10 Hz) or less ("flashing light effect") (Kok 1953; Nedbal et al. 1996), medium frequency fluctuations of seconds to minutes and low frequency cycles of hours to days (Grobbelaar 1991). Open raceway ponds, mixed mostly by paddle wheels at flow rates of 10–30 cm $s^{-1}$, operate with random light/dark frequencies (seconds to minutes). No photosynthetic or growth rate enhancement occurred at these frequencies (Weissman et al. 1988; Grobbelaar 1989). Slow light/dark changes, in the range of seconds, diminishes the specific growth rate below the value expected for the same net irradiance applied as continuous light (Morweiser et al. 2010). On the other hand, Ogbonna et al. (1995) reported an increase in productivity caused by random mixing of a dense culture of *Chlorella pyrenoidosa*. This effect was most pronounced in a photobioreactor with a shallow suspension layer. From these findings we may expect that even random mixing (microeddies) caused by turbulence in an optically dense culture, in outdoor sloped photobioreactors, could lead to better light utilization. Nonrandom mixing designs, using foils (Laws et al. 1983) or baffles (Doucha and Lívanský 1995a, b) immersed in a dense shallow algal culture, producing vortices with rotation rates of ca. 0.5–1.0 Hz, taking advantage of the flashing light effect on productivity.

Mixing rate and layer thickness determine frequencies of light/dark periods. Quantitative estimation of turbulence parameters were derived for algae flowing in a 6–8 mm layer down a inclined culture area. The flow is turbulent (Reynolds number 3,600–6,000). A mean transfer time for a turbulent vortice from the irradiated culture surface to the layer bottom is in the range of $T = 0.38–0.45$ s, and the transverse motion frequency of the algal cells is $1/T = 2.2–2.6$ Hz. Thus, a layer of well mixed dense algal culture several millimeters thick increases the frequency of dark/light periods of the single cells thus increasing PE and decreasing photoinhibition up to very high solar light intensities.(Doucha and Lívanský 2006).

The Manning equation was used (Oswald 1988; Weissman et al. 1988) to calculate algal culture velocity $u$ (m $s^{-1}$) in deep-layer ponds where the culture is mixed by paddle-wheels: $u = (1/n)\ h^{2/3}\ I^{1/2}$, where $n$ ($m^{1/3}\ s^{-1/2}$) is the roughness coeffi-

cient, $h$ (m) is the layer thickness, and $I$ (−) is the inclination of the culture area. This equation was verified for a small (24 $m^2$) outdoor TL photobioreactor made of glass sheets with $n = 7.945 \cdot 10^{-3}$ m $^{1/3}$ $s^{-1/2}$ (Doucha and Lívanský 2006). Oswald (1988) reported $n = 10 \cdot 10^{-3}$ $m^{1/3}$ $s^{-1/2}$ for smooth concrete. Turbulent flow, efficient transverse mixing of the culture layer on an inclined surface and a very low surface roughness coefficient prevent sedimentation and sticking of the cells.

Hydraulic power $E$ required for pumping an algal suspension onto a culture area is the product of volumetric flow rate $Q$, specific weight $\gamma = \rho g$ and head loss (Weissman et al. 1988). In the case of an inclined area of length $L$, and inclination $I$, the hydraulic power needed for lifting the suspension from the end to the beginning of the culture area is: $E = \rho g L Q I$. Volumetric flow rate $Q$ is proportional to the product of suspension velocity $u$, layer thickness $h$ and culture area width $b$: $Q = u h b$. Thus, hydraulic power for 1 $m^2$ of culture area will be: $E_A = E/(bL) = \rho g u h I$. For a layer thickness $h = 0.006$ m, it follows from the Manning equation that $u = 0.54$ m $s^{-1}$. For $\rho = 1{,}000$ kg $m^{-3}$, $g = 9.81$ m $s^{-2}$, $I = 0.017$ we obtain $E_A = 0.54$ kg $s^{-3} = 0.54$ W $m^{-2}$. Similarly, for a layer thickness of 0.008 m, we obtain $u = 0.66$ m $s^{-1}$ and $E_A = 0.88$ kg $s^{-3} = 0.88$ W $m^{-2}$. The total hydraulic power needed for pumping the suspension onto a culture area will however, be higher than the above values due to pressure drop within the tubes and less than 100 % efficiency of the circulation pump.

Substituting for velocity $u$ from the Manning equation into the formula for $E_A$, the following proportionality can be found: $E_A \approx h^{5/3} I^{1.5}$. Hence, to save pumping energy, the suspension layer thickness and culture area inclination should be as low as possible. On the other hand, a thicker layer may have some beneficial effects in summer cultivation in regions with high solar energy inputs: a greater capacity of the culture layer for dissolved $CO_2$ and $O_2$, thus diminishing concentration and pH gradients along the flow path of the suspension. A greater culture volume on the area diminishes overheating. The optimum thickness of the algal layer must be a compromise between energy cost for pumping and the influence of the above factors on cultivation conditions in the TL bioreactor. From practical reasons, a 6–8 mm thick algal layer in a sloped culture system seems to be optimal (Doucha and Lívanský 1995b, 2006).

## *4.4 Carbon dioxide*

Carbon comprises 45–50 % of algal dry weight (dw), so that 1.65–1.83 g $CO_2$ is theoretically required for the biosynthesis of 1 g dw. Carbon is stored in nutrient solution as a free carbon dioxide, bicarbonate or carbonate. The relative amount of each species is pH-dependent. In autotrophic microalgal mass cultures, such as *Chlorella*, $CO_2$ is used as the main carbon source. Carbon dioxide is responsible for the physiological processes and influences the buffering capacity and electrolyte balance of the nutrient solution. Although $HCO_3^-$ is easily absorbed by the chlorococcal cells it is a poor source of carbon compared to $CO_2$ (Goldman et al. 1981). Alkaline bicarbonate alone cannot provide sufficient carbon to optimize biomass

yields due the formation of chemical precipitates and, therefore, $CO_2$ must be added to the cultures. *Arthrospira* (*Spirulina*) is the only alga produced in large scale that can use carbonate or bicarbonate. In *Chlorella* sp. grown outdoors, the maximum rate of $CO_2$ consumption per 1 $m^2$ of TL bioreactor area was about 10 g $CO_2$ $m^{-2}$ $h^{-1}$ at noon hours on clear summer days (Doucha and Lívanský 2006).

The cost of pure $CO_2$ for large-scale algal cultures is of prime importance to total production economics. In our experience, the cost of $CO_2$ represents 60–65 % of the total cost of nutrients needed for algal growth. This proportion can be lowered considerably by use of waste $CO_2$.

Large-scale algal cultures are subjected to change in the $CO_2$ concentration regardless of the culture system design. The $CO_2$ supply depends on: (1) the efficiency of mass transfer; (2) loss by escape into the atmosphere; (3) consumption by algal cells. To minimize $CO_2$ loss, the partial pressure ($pCO_2$) of dissolved $CO_2$ must be as low as possible. The minimum value for non-limiting *Chlorella* growth was found to be $pCO_2 = 0.1–0.2$ kPa (Weissman et al. 1988; Lívanský and Doucha 1996, 1998).

The question of $CO_2$ supply to algal cultures is a key engineering problem in autotrophic production of algae (Xu et al. 2009). Several techniques have been developed for raceway ponds to distribute $CO_2$ in the culture medium ranging from plastic dome exchangers, air stones and commonly used perforated pipes placed at the bottom of the pond. However, only 13–20 % of the $CO_2$ supplied is absorbed (Richmond and Becker 1986; Becker 1994). In the open TL bioreactor, pure $CO_2$ is supplied into the suction pipe of the suspension circulating pump (Lívanský and Doucha 2005). Alternatively, $CO_2$ containing flue gas is supplied via porous ethylpropylene dimer (EPDM) membrane tubes inserted between the circulation pump and the culture area. 80 % of the $CO_2$ is absorbed and about 70 % is utilized for photosynthesis. This compares well with the utilization of $CO_2$ in closed systems (Sobczuk et al. 2000).

Along the algal flow path, the $pCO_2$, which is buffered by formation of $CO_2$ from bicarbonate ions, decreases approximately exponentially along length of the flow while the culture pH correspondingly rises (Lívanský and Doucha 2005, Fig. 5). The decrease in $pCO_2$ is less at a more alkaline pH and higher at high irradiance of the culture. The log $pCO_2$ decreased linearly with increasing pH of the culture (Lívanský and Kajan 1994; Lívanský and Doucha 1998). For a prediction of $pCO_2$ changes in outdoor open TL photobioreactors, models that can be applied to rational design and scale-up of the photobioreactors were developed (Lívanský et al. 1993, 2006).

Experiments with laboratory *Chlorella vulgaris* culture showed that use of $KNO_3$ as a nitrogen source for growth caused a considerable shift in the log $pCO_2$-pH relationship to higher pH values. This behavior can be attributed mainly to the accumulation of $K^+$ ions during cultivation. Buffering of culture pH by addition of bicarbonate (1 g $KHCO_3$ $l^{-1}$) was not much efficient. With urea as the nitrogen source, the log $pCO_2$-pH shift was much less (Lívanský and Kajan 1994). An example of the $pCO_2$-pH relationship in a *Chlorella* culture in a TL photobioreactor is shown in Fig. 6.

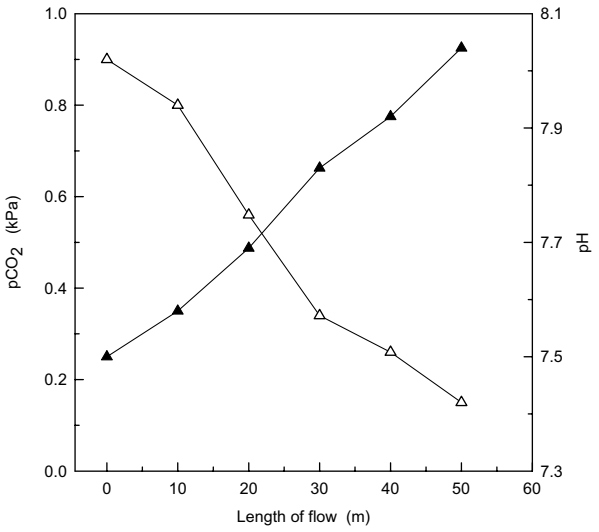


**Fig. 5** The course of $pCO_2$ (Δ) and pH (▲) along the flow path of a *Chlorella* culture (layer thickness 8 mm). Sunny day, July, 14:00 h

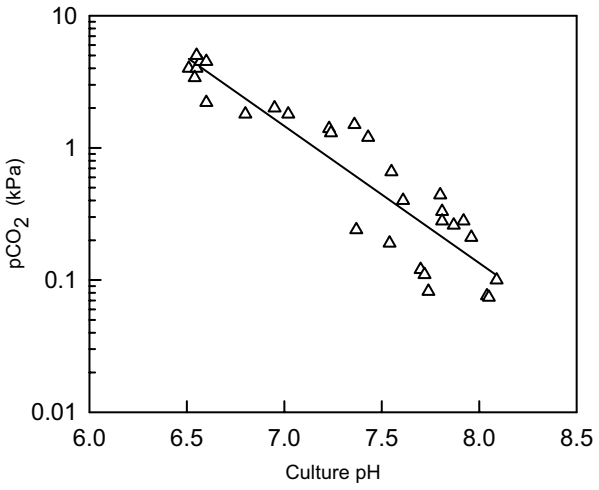


**Fig. 6** Relationship between $pCO_2$ and culture pH in the TL photobioreactor. (Doucha et al. 2005)

To minimize $CO_2$ loss and ensure sufficient $CO_2$ for growth, the rate of $CO_2$ supply must be controlled. One way is to use a pH-stat system: $CO_2$ is supplied to balance the production of $OH^-$ ions and pH control is by means of an on-off valve and standard controllers, without taking plant dynamics into account. As an example, in the pilot TL photobioreactor containing 290 l of *Chlorella* suspension, the response of $pCO_2$ (and hence culture pH) to changes in light intensity lasted several minutes to reach a new quasi steady-state (unpublished results). The pH sensor, connected to a regulation pH-meter, was placed in the pipe that enters to circulation pump of the bioreactor. The pH meter was set at values in the range pH = 7.9–8.0. In this

**Fig. 7** Pilot-scale photobioreactor for thin-layer cultivation of *Chlorella* algae in a dairy farm (1,200 pcs of dairy cattle) at Dublovice, Czech Republic. After combustion of anaerobically generated $CH_4$, flue gas is used as a source of $CO_2$ for algal photosynthesis. A modified liquid fraction of anaerobically digested manure can serve as an additional source of nutrients. (Photograph by J. Langová, BCS Engineering)

way, the $pCO_2$ at the end of the culture flow is held approximately constant during cultivation. Another method using an infraanalyzer was described by Doucha and Lívanský (2006) for $pCO_2$ control in an outdoor open thin-layer photobioreactor. About 70 % of supplied $CO_2$ is utilized by the algae and, for the production of 1 kg (dw) of *Chlorella,* about 2.6 kg of $CO_2$ is required.

In contrast, the system dynamics in raceway ponds do not permit a rapid response to disturbances in culture parameters (light intensity, culture temperature) due to the large volume of culture and the buffering capacity of $CO_2$-$HCO_3^-$ in the nutrient solution.

Using a microalgal photobioreactor as the $CO_2$ mitigation system is a practical approach for eliminating $CO_2$ emission and for decreasing biomass production costs. Commercially produced pure carbon dioxide can be replaced with $CO_2$-containing flue gas generated by e.g. combustion of the biogas formed by anaerobic digestion of organic wastes, (Eureka project OE 09025 for 2009–2012) by combustion of municipal wastes (Doušková et al. 2009; Eureka project OE 221 for 2006–2009), and other sources (limekiln and cement industry, power stations, fermentation processes etc).

Flue gas containing 13 % vol. $CO_2$ after combustion of anaerobically generated $CH_4$ from cattle manure was used successfully for outdoor production of *Chlorella* in a pilot plant TL photobioreactor (Fig. 7, Doucha 2012).

## *4.5 Oxygen*

Oxygen accumulating in the nutrient solution (dissolved oxygen, DO) during the course of algal growth is one of the most important factors influencing algae productivity. Its high concentration lowers the photosynthetic rate due to inhibition of ribulose-1,5 bisphosphate carboxylase (RuBisCO), which catalyses the incorporation of $CO_2$ to the first organic product - phosphoglycerate. Most investigations of the oxygen influence on algal growth were carried out on laboratory cultures using constant cultivation parameters: temperature, irradiance, gas mixture composition, etc. (Ogawa et al. 1980; Torzillo et al. 1984; Akyev and Tsoglin 1992; Lívanský 1996). These studies may not accurately reflect the adaptation of algae to changing culture conditions outdoors.

Due to poor mixing of a 30 cm thick culture, oxygen removal in raceways is insufficient. Its concentration does not vary much along the flow path of the culture and the cells may be constantly exposed to inhibitory oxygen concentration. This is not the case in TL bioreactor, where the culture is getting rid off oxygen by flow through the scattering sieve placed between the lowest part of the culture lane and the pump which pushes the culture up to the upper edge of the culture area. Therefore the concentration of DO is lowest at the beginning of algal flow along the culture lane and is highest at the end of it. Thus, growth inhibiting concentration of DO can occur mainly at the end of the culture area.

A combination of high DO, high temperature and high irradiance of a low density raceway culture may result in photooxidative damage to algal cells (Richmond 1986). In a TL photobioreactor, the photooxidative effect is reduced due to the high flow velocity (50–60 cm $s^{-1}$) and strong turbulence, causing a high frequency of light/dark periods for single cells (Grobbelaar et al. 1995; Tichý et al. 1995).

For non-limited growth, oxygen concentrations should be maintained below 400 % of air saturation value (Kunjapur and Eldridge 2010)which corresponds to 30 mg $l^{-1}$ of DO, assuming that the equilibrium solubility of $O_2$ in air is 7.5 mg $l^{-1}$ (21 % $O_2$ v/v) at 30 °C. The maximum DO found in ponds is typically 25–40 mg $l^{-1}$ (Weissman et al. 1988). In dense *Chlorella* cultures, the DO at the end of the inclined culture area should not be greater than 30–35 mg $l^{-1}$. The typical course of DO concentration in *Chlorella* culture grown in an open TL photobioreactor in Southern Greece is shown in Fig. 8.

Oxygen produced in the culture is removed by mass transfer to the atmosphere. The extent of transfer depends on the rate of oxygen diffusion and on intensity of turbulence, both of which influence the overall mass transfer coefficient for oxygen $K_L$ (m $h^{-1}$). Additional removal of oxygen takes place outside the culture area trough the following processes: (1) flow of suspension from the culture area into the retention tank of the bioreactor through the scattering sieve; (2) contact of algae with carbon dioxide (or by $CO_2$ containing flue gas) where dissolved oxygen is transferred from the culture to the gas phase; (3) depletion of oxygen by dark respiration of algal cells in non-illuminated regions of the culture, i.e. in the retention tank, in the circulation pump and in pipes that supply and distribute the culture throughout the

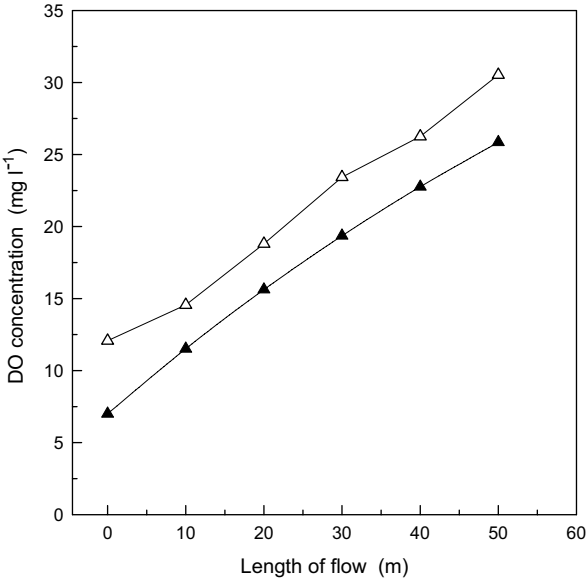


**Fig. 8** DO concentration along the flow path of a *Chlorella* culture (layer thickness 8 mm). Sunny day, July, 14:00 h. Δ pure $CO_2$ was supplied; ▲ estimated from the model (Lívanský and Doucha 1999) for supply of flue gas after combustion of anaerobically generated $CH_4$

bioreactor. Generally, under a quasistationary state, the mass of oxygen produced by algae is equal to that which is removed from the culture as described above.

Knowledge of DO concentration in a photobioreactor can provide useful and rapid information about the state of the culture. DO is proportional to the rate of algal growth (Ben-Yaakov et al. 1985; Fournadzieva et al. 1993). A mathematical model concerning the hydraulics of culture flow, oxygen transfer to the atmosphere and the rate of oxygen evolution during the course of algal photosynthesis was described by Lívanský and Doucha (2003). The model was verified for a *Chlorella* culture, several millimeters thick, grown outdoors up to a density of 40 g dw $l^{-1}$. The model enables to estimate the net rate of oxygen evolution $R$ (g $O_2$ per 1 $m^2$ of culture area) and the mass transfer coefficient $K_L$. This value for a 6–8 mm thick *Chlorella* culture was in the range of $K_L = 0.18–0.24$ m $h^{-1}$. The mean net rate of oxygen evolution in the culture was correlated with PAR irradiance $I_0$ (W $m^{-2}$) as: $R$ (g $O_2$ $m^{-2}$ $h^{-1}$) $= k\, I_0/(K_I + I_0)$, where $k = 12.82$ g $O_2$ $m^{-2}$ $h^{-1}$, $K_I = 193.4$ W (PAR) $m^{-2}$ (Fig. 9, Doucha and Lívanský 2006).

The efficiency of all techniques used to date to provide oxygen removal from microalgal cultures are still not at a satisfactory level. There are a few solutions open to the reactor designer for lowering oxygen concentration: increasing turbulence; $O_2$ stripping with air (e.g. in the airlift zone of a tubular bioreactor); increasing the velocity of the culture flow, accompanied however by higher energy consumption for pumping and higher shear stress that may damage the cells. Algal strains that can tolerate high oxygen concentrations have not yet been isolated.

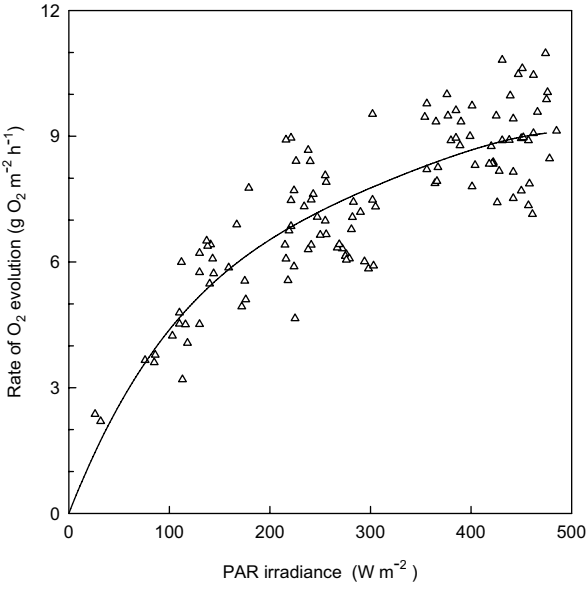


**Fig. 9** Relationship between the net rate of oxygen evolution by *Chlorela* sp. per 1 $m^2$ of culture area and PAR irradiance

## *4.6 Nutrition*

Carbon, nitrogen and phosphorus are the most important nutrients for algae production. Usually, macronutrients are supplied at concentrations in g $l^{-1}$ and the micronutrients in mg $l^{-1}$.

The nitrogen content of algal biomass may be up to 10 % dw. Nitrogen is usually supplied as urea, nitrate ($NO_3^-$), ammonium salts ($NH_4^+$) or their combination. Ammonium nitrogen is often the preferred N source. Assimilation of nitrate N leads to an increased pH of the nutrient solution whereas assimilation of ammonium N causes a pH decrease. In an outdoor *Chlorella* culture with $KNO_3$ as a sole nitrogen source, an increased concentration of DO was observed, compared to when urea was used as a N source. We have found (unpublished results), for both laboratory and outdoor *Chlorella* cultures, that up to 50 % of urea nitrogen can be replaced by $NH_4HCO_3$ or $(NH_4)_2CO_3$ without any negative effect on algal productivity or pH of the medium. Ammonium nitrogen is utilized well by cells, with a minimum of $NH_4$-N remaining at the end of several days culture cycle. From a practical point of view, the best N source for growing *Chlorella* is urea - it is cheap, well soluble, well utilized by the cells and has little influence on culture pH.

The preferred form of phosphorus for algae is phosphate ($PO_4^{3-}$). Algal biomass usually contains less than 1 % P. Based on our results, to reduce the cost of nutrients, $H_3PO_4$ and $K_2CO_3$ can replace commonly used $KH_2PO_4$ for *Chlorella* growth.

The composition of nutrient solution is designed to reflect the mean content of basic chemical elements (P, N, K, Mg, S) in algal biomass. Nutrients are supplied daily at the beginning of cultivation (a variant of fed-batch culture technology) in

quantities based on a determination of urea-N and phosphate-P consumption. In this way, a balanced nutrient composition is maintained throughout the fed-batch growth cycle. To economize nutrients utilization, their addition is stopped two days before harvest.

## 5 Productivity and Photosynthetic Efficiency

Basic factors that influence the productivity of autotrophically grown algae are irradiance, temperature, biomass density, mixing and nutrients. These factors are in a close relationship with culture requirements and the type of culture system.

Net productivity is defined as gross daylight productivity minus night biomass loss caused by dark respiration of energetically rich cell reserve materials (starch, glycogen). This process is tightly temperature dependent. Net areal productivity is related to volumetric productivity $P_V$ (g dw $l^{-1}$ $d^{-1}$) as: $P_{24}$ (g dw $m^{-2}$ $d^{-1}$) = $P_V$ $V/A$, where $V$ is the volume (l) of the culture in the bioreactor, $A$ is the culture area ($m^2$). Volumetric productivity can be expressed as a product of mean daily specific growth rate $\mu_{mean}$ ($d^{-1}$) and cell dry weight $X$ (g $l^{-1}$). By evaluating $\mu_{mean}$ using several mathematical models found in the literature, and applying this to the areal rate of oxygen evolution (g $O_2$ $m^{-2}$ $h^{-1}$) in a thin-layer *Chlorella* culture, we concluded (unpublished results) that $\mu_{mean}$ yielded a better fit to local light intensity (at a distance z from the irradiated culture surface) than to the mean light intensity in the layer. Thus, specific growth rate of the cells at a distance $z$ can be expressed for light-limited growth of the cells as: $\mu = \mu_{max}\, I/(K_I + I)$. Here $\mu_{max}$ is the maximum specific growth rate, $K_I$ is the light saturation constant, $I$ is the intensity of PAR at distance $z$. The above formula is analogous to the Monod equation for the growth of microorganisms, with light considered as a specific substrate for growth of algae. From this equation and $I$, expressed by the Lambert-Beer law, for the mean specific growth rate in an algal culture layer of thickness $h$, we have (Simmer 1979):

$$\mu_{mean} = \frac{1}{h}\int_0^h \mu \quad dz = \frac{\mu_{max}}{\varepsilon_{mean} X h} \ln\left(\frac{I_0 + K_I}{I_h + K_I}\right) \tag{2}$$

Taking into account Eq. (1), this equation can be modified:

$$\mu_{mean} = \frac{\mu_{max} I_{mean}}{I_0 - I_h} \ln\left(\frac{I_0 + K_I}{I_h + K_I}\right) \tag{3}$$

It can be seen that mean specific growth rate is proportional to mean light intensity in a layer of algal culture. This was verified for a dense *Chlorella* culture in the pilot TL bioreactor (Doucha and Lívanský 2009). The following values were found: $\mu_{max} = 2.15$ $d^{-1}$, $K_I = 102.7$ W $m^{-2}$.

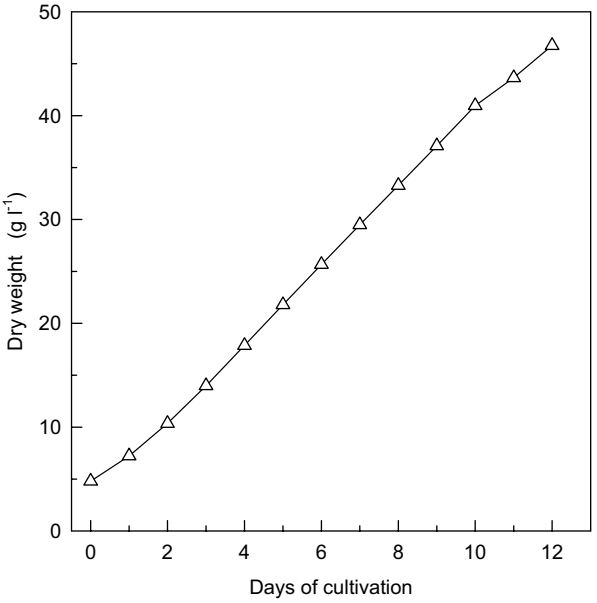


**Fig. 10** Time course of *Chlorella* cell concentration in a TL photobioreactor. Daily solar PAR input is 3,400 Wh $m^{-2}$

Net photosynthetic efficiency, PE, expressed in % of solar PAR energy utilization, can be estimated from the net areal $P_{24}$ productivity (g dw $m^{-2}$ $d^{-1}$) and daily solar PAR energy input $E_0$ (Wh $m^{-2}$ $d^{-1}$) incident on 1 $m^2$ of culture area, as: $PE = 6.4 \times 100 \times P_{24}$ divided by the energy $E_0$. The conversion factor 6.4 represents the energy (Wh) of chemical bonds in 1 g (dw) of algae (Kubín 1983; Morita et al. 2001). Generally, PE decreases with increasing irradiance in photosynthetic systems. This can also be derived from Eq. (2), for a dense algal culture $I_h << K_I$, $PE \approx (1/I_0) \cdot \ln [1 + (I_0/K_I]$. Thus, the PE decreases with increasing $I_0$.

Productivity of a dense *Chlorella* culture grown in thin layer is practically independent of algal dry weight. This is in sharp contrast with the frequently published data describing algal productivity in raceway ponds as being strongly density dependent. While an optimal areal density for maximal productivity was found to be 38–41 g (dw) $m^{-2}$ (Grobbelaar et al. 1990) and 40–45 g (dw) $m^{-2}$ for *Scenedesmus obliquus* (Hartig et al. 1988), we have not observed any decrease in productivity of *Chlorella* up to a cell concentration of 40–50 g (dw) $l^{-1}$ (areal biomass densities 240–300 g (dw) $m^{-2}$) commonly processed for harvest (Fig. 10).

The mean seasonal net productivity of *Chlorella* cultures in TL bioreactors, depending on climatic conditions, ranges from about 22–25 g dw $m^{-2}$ $d^{-1}$ (Pulz and Scheibenbogen 1998; Borowitzka 1999; Doucha and Lívanský, unpublished resul ts), with a peak of about 38 g for short summer culture periods (Doucha and Lívanský 2009). This corresponds to a volumetric productivity of 4.3 g $l^{-1}$ $d^{-1}$ and a PE of 7.05 %. In contrast, long-term productivity in commercial raceways ranges mostly between 12 and 15 g dw $m^{-2}$ $d^{-1}$(Tredici 2004) corresponding to a volumetric productivity of 0.05 g $l^{-1}$ $d^{-1}$. Productivities of about 20–25 g $m^{-2}$ $d^{-1}$ are also reported

for short growth periods in tropical regions (Pulz and Scheibenbogen 1998; Lee 2001; Tredici 2004).

However, published algal productivities obtained using different culture systems are hard to compare, because they involve different climatic conditions, algal strains and operating systems.

## 6 Economic Considerations and Concluding Remarks

The main advantage of raceway ponds is their simple and, therefore, relatively cheap construction. From our unpublished calculations, the building and material costs should not be greater than 25–40 € per 1 $m^2$ of culture area. In contrast, based on detailed analyses provided by the stock company BCS Engineering, a.s., Brno, Czech Republic, who have designed and built up to now several thin-layer photobioreactors, building cost per 1 $m^2$ culture area for a 1,000 $m^2$ production module ranges from 200 to 230 €, i.e. 5–6 times higher than raceways. Nevertheless, the areal productivity in TL bioreactors is roughly double that of ponds (for production of an equal amount of biomass we need only half the operating staff) and the volumetric productivity, one of the deciding factors on the economics of algal production, is about 50–100 times higher. Extremely low culture volumes, higher algal yields and high harvest densities reduce the production costs to about one fifth (20%) compared with raceways. For the economics of the whole process, the deciding parameter is the cost of long-term production, not investment costs.

**Raceway vs thin-layer technology - comparison of parameters that differ between systems.**

*Power input for circulating and mixing the culture ( per 24 hrs and 100 $m^2$ culture area)*

**Raceway:** paddle wheels, 600 W $h^{-1}$100 $m^{-2}$ (Richmond and Becker 1986), per 24 h = 14.4 kWh. For production of 1 kg of algal dw, (mean productivity 15 g dw $m^{-2}d^{-1}$), 9.6 kWh is needed.

**Thin-layer:** circulation pump, 288 W $h^{-1}$100 $m^{-2}$, per 12 h of growth (light) period = 3.46 kWh; night circulation: the culture is kept in a tank, aeration requires 40% of the power input necessary for culture circulation during the course of algal growth,115 W × 12 h = 1.38 kWh; mixing and aeration: 3.46 + 1.38 = 4.84 kWh. For production of 1 kg of algal dw, (mean productivity 25 g $m^{-2}d^{-1}$), 1.94 kWh is needed.

For both culture systems, cooling of $CO_2$ stored in liquid form in a 10 $m^3$ tank requires about 1.3 kWh per 1 kg of biomass produced.

*Supply of pure $CO_2$* **Raceway:** utilization of the pure $CO_2$ added to the algal suspension by means of tubes laid on the bottom of the ponds ranges from 13–20% (Richmond and Becker 1986; Becker 1994). Thus, for an average net productivity of 15 g dw $m^{-2}$ $d^{-1}$, about 13.7–21.0 kg $CO_2$ per 100 $m^2$ culture area is required and for production of 1 kg dried biomass, 9–14 kg of $CO_2$ must be supplied.

**Thin-layer:** pure $CO_2$ is supplied via the pump (see Fig. 2). Its utilization by algae is about 70 % (Doucha and Lívanský 2006). Thus, for average net productivity 25 g dw.$m^{-2}$.$d^{-1}$, 6.5 kg $CO_2$ per 100 $m^2$ culture area is needed and for production of 1 kg of dry biomass, 2.6 kg $CO_2$ must be supplied.

*Supply of a flue-gas $CO_2$* **Raceway:** in the case of flue gas containing about 13 % vol. $CO_2$, the amount required, is 7–8 times greater than pure $CO_2$. Thus, for an average net productivity of 15 g dw $m^{-2}$ $d^{-1}$, 57–88 $Nm^3$ must be supplied and for production of 1 kg algal dry mass, the amount of flue gas required is 38–59 $Nm^3$ (1 $Nm^3$ is considered as flue gas volume at normal pressure and at temperature 20 °C. Thus, 1 $Nm^3$ $CO_2$ = 1.842 kg $CO_2$). To supply this gas at 0.5 bar to the algal culture, an additional 0.23–0.36 kW of power per 100 $m^2$ is required for a compressor, i.e. 2.76–4.32 kWh per 100 $m^2$ for a 12 h growth period. Therefore, the total energy consumption (mixing and flue gas supply) is 17.2–18.7 kWh per 100 $m^2$ $d^{-1}$ and for the production of 1 kg algal dw, 11.5–12.5 kWh is required.

**Thin-layer:** flue-gas $CO_2$ is supplied to the EPDM tubes in the pipe loop between the circulation pump and the upper edge of the cultivation lane. At a net productivity 25 g dw $m^{-2}$ $d^{-1}$, 10.9 $Nm^3$ must be supplied for the production of 1 kg dw algal biomass. To supply this gas at 0.5 bar to the algal culture, an additional 0.044 kW $kg^{-1}$dw of power is required for a compressor, i.e. 0.53 kWh $kg^{-1}$ dw for a 12 h growth period. Therefore, the total energy consumption (mixing and flue gas supply) is 6.15 kWh per 100 $m^2$ $d^{-1}$ and for the production of 1 kg algal dw, 2.46 kWh is needed.

*Consumption of water* According to Becker and Venkataraman (1980), water consumption for a 20 cm layer large-scale raceway pond, where *Scenedesmus* was grown at a net productivity of 15 g dw $m^{-2}$ $d^{-1}$, was estimated to be about 0.8 $m^3$ per 1 kg algal dw (evaporation was not included). For a similar culture located in Peru, Castillo et al. (1980) calculated the liquid discharge at about 22,000 $m^3$ per hectare yearly (not considering evaporation). At a mean productivity of 15 g dw $m^{-2}$ $d^{-1}$, the calculated specific water consumption is 0.4 $m^3$ of water per 1 kg dw of algal dw.

In the Třeboň laboratory, 2,000 l of *Chlorella* culture in each of three 224 $m^2$ TL photobioreactors are grown and successively harvested. At a net productivity of 25 g dw $m^{-2}$ $d^{-1}$, after 14 days fed-batch growth, a harvest density of about 40 g dw $l^{-1}$ and 80 kg dw of biomass is produced in each of the bioreactors. After harvest, 2,000 l of fresh water is supplied to prepare a nutrient solution for a next cultivation cycle. The corresponding specific water consumption, including water for evening washing of the culture area, (not considering evaporation, that is similar in both systems) is about 0.025 $m^3$ of water per 1 kg of algal dw. This is about one order of magnitude less than for raceways.

*Downstream processing costs* After harvesting *Chlorella* cultures, the common steps of downstream processing technology are as follows: (1) thickening of the biomass, mostly by means of plate-separators, to the density of about 150 g dw $l^{-1}$, (2) disintegration of algal cells by bead-mills (Doucha and Lívanský, 2008), (3) drying of the biomass using spray driers.

**Table 1** Raceway vs thin-layer technology - parameters that differ between both systems

| Items | Raceway | Thin-layer | Thin layer, % of raceway |
|---|---|---|---|
| Circulation and mixing (kWh per1kg dw) | 9.6 | 1.9 | 19.8 |
| Supply of $CO_2$ | | | |
| Pure $CO_2$ (kg $CO_2$ kg dw) | 9–14 | 2.6 | 18.6–28.9 |
| Flue gas $CO_2$ ($Nm^3$ per 1 kg dw) | 37.5–58.5 | 10.9 | 18.6–29.1 |
| flue gas compression (kWh per 1 kg dw) | 1.8–2.9 | 0.53 | 18.3–29.4 |
| Water consumption (without evaporation) ($m^3$ per 1 kg dw) | 0.4 | 0.025 | 6.3 |
| Biomass thickening at the harvest (kWh per 1 kg dw) | 2–4 | 0.05 | 1.3–2.5 |

Because the steps of disintegration and drying are similar in both raceway and TL technology, the calculations of downstream processing costs are only focused on the thickening the biomass after harvest. While the harvest density of algae in raceway ranges from 0.5 to 1 g dw $l^{-1}$, and for processing, must be increased 150–300 times, the density of algae at harvest in the TL system is about 40–50 g dw $l^{-1}$ and, for processing, needs to be increased only 3–4 times.

For thickening of a 10 $m^3$ $h^{-1}$ algal culture to a density of 150–200 g dw $l^{-1}$ (nozzle separator, materials of GEA Westfalia Separator Group), about 2 kWh $m^{-3}$ (including the energy input for the feed pump), i.e. 2–4 kWh per 1 kg of dry algal biomass, is needed. For TL technology, only 0.05 kWh per 1 kg of dry algal biomass is needed.

The data from these analyses are summarized in Table 1.

*Labour* Becker and Venkataraman (1980) provided model cost calculations for a 5 ha production plant (50 raceway ponds, each pond consisting of two 100 × 5 m joined units, yielding a 1,000 $m^2$ size pond). About 50 workers were needed for operation of the plant, i.e. 10 workers for 1 ha culture area.

TL production photobioreactors are equipped with automatic refilling of evaporated water and with automatic maintenance of the optimal $CO_2$ concentration in the growing culture. The formation of oxygen in a growing culture, as a result of algal photosynthesis, is continuously monitored by means of a Clark electrode.

Manpower requirements are focused on simple activities covering start and closing of the culture day, analysis and addition of nutrients, determination of algal productivity, control of the biological state of the culture and, work connected with processing of the harvested biomass (thickening, disintegration of the cells, drying and packing).

Based on the above data, we estimate that about 4–5 workers are needed for operation of a 1 ha plant.

**Scale up Cultivation** Raceway *Chlorella* or *Spirulina* production plants consist mainly of 1,000–5,000 $m^2$ single modules forming a production area of up to several hectares (e.g. Belay 1997).

On the contrary, for commercial production of fast growing microalgae, thin-layer culture modules of 1,000 $m^2$ area are proposed (Fig. 11).

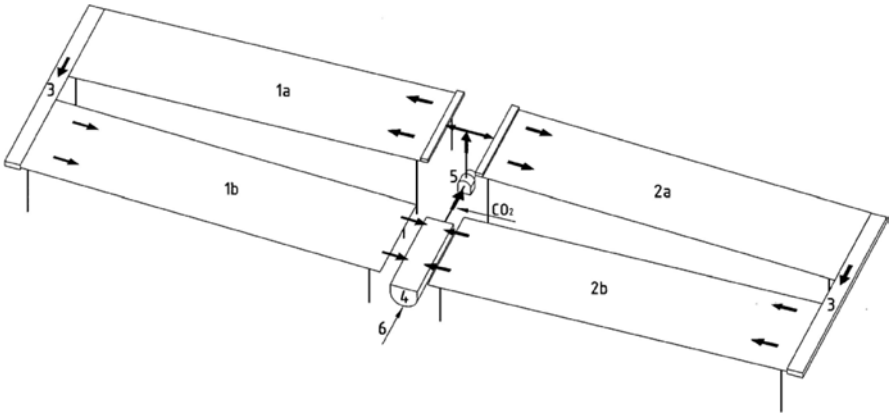


**Fig. 11** Scheme of a 1,000 $m^2$ area production module: *1a–2b*, culture areas; *3*, connecting channels; *4*, retention tank; *5*, circulation pump; *6*, night aeration air. Flow of algae is depicted by *arrows*

The length of the inclined cultivation area is an important parameter for efficiency and economy of algal biomass production: the longer the inclined culture lane the larger the proportion of total algal volume that is exposed to sunlight. The length of the culture area is limited by DO concentration and by carbon dioxide dissolved in the algal suspension at the end of the inclined culture lane. Whilst the oxygen concentration should not exceed 30–35 mg $l^{-1}$ (higher concentration can inhibit photosynthesis) the $pCO_2$ value at the end of the culture lane should be at least 0.1–0.2 kPa, and lower values limit algal growth. (Lívanský and Doucha 1998; Doucha and Lívanský, 2006).

At a volumetric productivity of 3–4 g algal dw per liter per day, a fed-batch culture cycle that starts in an algal density of about 5 g dw $l^{-1}$, lasts 12–14 days; 15 modules form a culture area of 1.5 ha.

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# Part II
# Algae Products

# Mixotrophic Algae Cultivation for Energy Production and Other Applications

**Amarjeet Bassi, Priyanka Saxena and Ana-Maria Aguirre**

**Abstract** Micro-algae offer potentially significant advantages over other approaches to overcome the current challenges of energy shortages and for pollution control. In addition major new directions in the genetic manipulation of algae and in new bioreactor design have been initiated in an effort to design new systems and approaches for this purpose. However, significant barriers for the commercialization of microalgae still exist including economic barriers, the level of technology readiness and lack of established co-products to make the overall process of energy from algae attractive. Mixotrophic algae cultivation offers the benefits of high biomass productivity and allows for integrated approaches which combine both photosynthetic and heterotrophic components during the diurnal cycle. This chapter focuses mainly on mixotrophic algae, the different methods of cultivation and different roles of algae in energy production. The large number of algal species and their versatile habitats and adaptability make mixotrophic microalgae serious candidates for applied research and development and for commercialization of new technologies. Approaches based on mixotrophic cultivation of algae will continue to play a role in efforts to mitigate the above mentioned challenges.

**Keywords** Mixotrophic algae • Microalgae • Heterotrophic algae • Bioenergy • Algal cultivation systems • Photobioreactors • Renewable resources • Autotrophs • Chlorella • Algal bioproducts • Biochemical processes • Algal ponds • Microbes • Phototrophs

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**Abbreviations**

| | |
|---|---|
| ATP | Adenosine triphosphate |
| DOC | Dissolved Organic Carbon |
| DW | Dry weight |
| FPR | Flat Panel or Plate Reactor |
| HTR | Horizontal tube Reactor |
| TAG | Triaclyglycerol |
| VTR | Vertical tube Reactor |

## 1 Introduction

Among the several major challenges facing human kind in the 21st century and beyond are the critical issues of energy shortfall as population rises and the pressure on depleting resources increases. It is interesting to note that in 2010 alone, the energy consumption grew by 5.6%, the highest rate in almost 40 years. A study by Andres et al. (2011) correlated the combustion of fossil fuels with increased carbon dioxide ($CO_2$) released into the atmosphere. Some research results based on the carbon cycle model simulations have estimated that if the $CO_2$ levels continue to increase at current rates, there will be significant changes in ocean levels and air temperature which will impact human life on earth. The World Health Organization reports that every year around 160,000 people died from the side-effects of climate change caused mainly by greenhouse gases emissions (Asif and Muneer 2007). Thus, an urgent need for energy alternatives has arisen. Recently microalgae are being touted as a transformational alternative to the world energy crisis. While the claims may be exaggerated and even though many scale-up and commercialization strategies are unproven or unrealized, the production of bio-fuels from microalgae does seem to offer a potentially attractive alternative.

The study of microalgae systems for energy production possibly began in 1955, when the production of methane from microalgae biomass was proposed by Meier (DOE 2010; Saxena and Bassi 2012). Since that time, especially in last decade, intensive research has been carried out on microalgae cultivation and down streaming processing. Apparently, hundreds of laboratories around the world are working on basic and applied microalgae biotechnology, from gene manipulation to photobioreactor scale-up, only with the purpose of commercially viable production of biofuels from microalgae.

Micro-algal technologies offer some advantages over other commercial or potential energy sources. These include: (1) a potential for genetic manipulation of metabolic pathways for increased production of specific metabolites (lipids, starch, polymers, and high-value compounds) (Garcia et al. 2005; Rosenberg et al. 2008);) (2) a wide array of configurations for algae cultivation such as low cost production in open ponds or closed bioreactor systems (of interest especially for cold climate countries) (Shang et al. 2010). (3) less pressure on water

resources compared to land crops; (4) and utilization of wastewater as a resource with the simultaneous removal of nutrients (nitrates, ammonia and phosphates or salt water) and toxic metal (Christenson and Sims 2011; Bhatnagar et al. 2011; Wang et al. 2012). Significant challenges for the commercialization of microalgae still exist and these include (i) high cost of downstream processing operations, (ii) few advances in the level of technology readiness of algae processing, and (iii) lack of established co-products to make the overall process of energy from algae economically viable.

Thus improved and better strategies are needed, specifically, selection of high-oil-yielding algae strains, well designed large-scale bioreactors and use of different modes of cultivation for high biomass and established scale-up strategies. Mixotrophic algae cultivation offers an advantage of high biomass productivity since this allows the integration of both photosynthetic and heterotrophic components during the diurnal cycle. This chapter focuses mainly on mixotrophic algae, different methods of cultivation and different roles of algae in energy production.

## 2 Microalgae

Algae consist of a group of eukaryotic protists whose members share the properties of oxygenic photosynthesis. Algae species can vary from micron sized single cells to multicellular colonies or even more complex leafy structures. Also, algae can vary in size from less than 2 mm (picoplankton) to 50–60 m in length (giant kelp).

Algae classifications have been developed by Bold and Wynne (1978), and more recently by Graham and Wilcox (2000), to name only a few sources. Of interest to the bio-fuel and environmental industry are the microalgae. The Cyanophyta and Prochlorophyta comprise the prokaryotic membership of this group while eukaryotic microalgae are placed into nine groups or divisions, namely, (1) Glaucophyta (2) Rhodophyta (3) Heterokontophyta (4) Haptophyta (5) Cryptophyta (6) Dinophyta (7) Euglenophyta (8) Chlorarachniophyta (9) Chlorophyta (Williams and Laurens 2010). In general, biologists have classified algae on the basis of pigmentation, product storage structures, cell wall composition, cycle life (for eukaryotes) and basic cellular structure (Hoek et al. 1995).

Algae have a diverse habitat and occur in both aqueous (freshwater or saline waters) and moist soil environments. Aquatic algal species are also able to tolerate a range of environmental conditions such as differing oxygen or carbon dioxide levels, pH or temperature. The unicellular planktonic algae mentioned earlier may survive as suspended cells in water bodies. These phytoplanktons are at the bottom of the food chain in the marine environment. They also produce a large amount of oxygen on the earth by carrying out the fixation of atmospheric carbon dioxide, and almost accounts for 50 % of the photosynthesis that takes place on the Earth. Other types of algae such as benthic algae live in sediments or shallow waters. Benthic algae can form attachments to rock surfaces.

Under unfavourable environmental conditions for growth, many microalgae may preferably synthesize neutral lipids especially triacylglycerols (TAG) and hydrocarbons (Guschina and Harwood 2006) which is a raw material for biofuels. In recent years, microalgae have been commercially exploited in other applications such as production of nutraceuticals, Vitamin A, or as nutritional supplements, for drug-screening and also for wastewater treatment. Thus, algae represent an important commercial interest. For example, for all aforementioned activities, more than $7.5 \times 10^6$ t of algae are harvested every year representing a world market of US \$ $6 \times 10^9$/year.

## 2.1 Mixotrophic Algae

In most cases, algae are photo-autotrophs and use light and carbon dioxide to generate ATP and carbohydrates. However, several algae can also act as heterotrophs utilizing only organic compounds as the carbon energy source. In fact, several algal species are mixotrophic and may range from predominantly photoautotrophs to those which are heterotrophs. Mixotrophic cultivation of algae may be advantageous for certain applications in industrial microbiology for the production of organic acids, antibiotics, and a wide variety of other products. Biomass density is theoretically limited only by the solubility of nutrients (most notably oxygen), and dry weights of over 100 g $L^{-1}$ have been reported for some microorganisms (Yee and Blanch 1992). Heterotrophic algal biomass densities of up to 40 g $L^{-1}$ have been reported by Gladue (1991) and Soong (1980).

In mixotrophs, the relative contribution of photosynthesis and organic carbon assimilation is influenced by the light regime, organic and inorganic nutrient concentration, food size, food abundance, and other physical or chemical factors (Legrand et al. 1998). Mixotrophy is a prominent mode of nutrition for phytoplankton in oligotrophic habitats. Mixotrophy encompasses several processes, including osmotrophy, (nutrition by direct absorption and uptake of organic molecules), and phagotrophy, (ingestion of prey or other food particles).

On the basis of nutritional strategies, mixotrophs may be classified into both facultative and obligate types. Facultative mixotrophs are defined as those which can utilize both types of metabolism: photoautotrophy and heterotrophy (*Dinophyta* are examples of this type). Obligate mixotrophs are phototrophs but also utilize autophagy and/or osmoregulatory processes to provide essential nutrients for growth (e.g. *Euglena gracilis*).

## 2.2 Assimilation of Nutrients

The mode of nutrition adopted by mixotrophic algae is between photosynthetic and heterotrophic activities, and it is characterized by low light and/or nutrient deficiency (Stoecker 1998; Stoecker et al. 2006; Legrand et al. 1998). Algae with a primary

**Table 1** Commercially important microalgae that could be cultured mixotrophically

| Species | Carbon Source | Strain |
|---|---|---|
| *Anabaena variabilis* | *Fructose, Glucose* | Pearce and Carr 1969; Valiente et al. 1992 |
| *Brachiomonas submarina* | *Acetate* | Tsavalos and Day 1994 |
| *Chlorella minutisima* | *Methanol* | Kotzabasis et al. 1999 |
| *Chlorella regularis* | *Acetate* | Endo et al. 1977 |
| *Chlorella sorokiniana* | *Glucose* | Lee et al. 1996 |
| *Chlorella vulgaris* | *Glucose* | Ogawa and Aiba 1981 |
| *Chlorococcum* sp | *Acetate, Fructose, Glucose, Maltose, Manose* | Non-published data |
| *Cyclotella cryptica* | *Glycerol, glucose* | Wood et al. 1999 |
| *Euglena gracilis* | Glucose, glycerol, galactose, ethanol | Tani and Tsumura 1989 |
| *Haematococcus pluvialia s.p* | *Acetate* | Kobayashi et al. 1992 |
| *Nannochloropsis CCAP879/5* | *Glycerol glucose* | Wood et al. 1999 |
| *Nanochloropsis CCAP211/78* | *Glycerol* | Wood et al. 1999 |
| *Navicula saprophila* | *Acetate* | Kitano et al. 1997 |
| *Nitzschia* sp | *Acetate* | Kitano et al. 1997 |
| *Phaeodactylum tricornutum* | *Glycerol* | Garcia et al. 2005a |
| *Rhodomonas reticulate* | *Glycerol glucose* | Wood et al. 1999 |
| *Rhodomonas salina* | Acetate | Kitano et al. 1997 |
| *Scenedesmus acutus* | Glucose, | Ogawa and Aiba 1981 |
| *Scenedesmus obliquus* | *Methanol* | Kotzabasis et al. 1999 |

photoautotrophic strategy grow at the expense of inorganic nutrients via the process of photosynthesis without uptake of organic substrates; these algae only assimilate organic substrates under deprivation of inorganic nutrients and sunlight. Algae with a primary heterotrophic strategy utilize oxygenic photosynthesis only when organic substrates are depleted in the growing environment (Caron et al. 1990; Stoecker 1998; Brennan and Owende 2010).

In addition to inorganic carbon assimilation, mixotrophs utilize dissolved organic carbon (DOC), which, in general, can supply the same metabolic requirements for growth as photosynthetic processes. Previous studies indicate that numerous algal species can utilize DOC (Wood et al. 1999; Vonshak et al. 2000). Similar observations were found in field experiments in diversified environments; for example, marine microbial mats (Paerl and Goldman 1972), estuarine waters (Lewitus and Kana 1994), and wastewaters (Kirkwood et al. 2003). Recently, Tuchman et al. (2006) reported that cultivation of diatoms can exploit a variety of organic compounds including carbohydrates, amino acids, amines, alcohols and other compounds. Amino acids and peptides constitute an important source of DOC (Zotina et al. 2003; Mulholland and Lee 2009) and the ability to utilize individual amino acids is species-specific, or even strain-specific (Flynn and Butler 1986). Table 1 depicts some examples of commercially important microalgae that could be cul-

tured mixotrophically. Common carbon sources such as glucose, acetate or glycerol are used for cultivation of mixotrophs.

Mixotrophic algae acquire organic carbon substrates from the environment by a process of active transport. In some species, this energy dependent mechanism appears to be regulated by the irradiance process. For example, *Cyclotella cryptica* minimized the uptake of glucose when irradiance is high enough to activate photosynthesis; while, glucose uptake was maximum in the dark (Hellebust 1971). In the natural environment, when the concentration of DOC is too low in surroundings to sustain efficient passive uptake, algae prefer uptake of exogenous organic carbon via a passive process. Sometimes algae incorporate a selective range of organic carbon and nitrogenous compounds at low irradiance for storage products as a means of subsidizing their photosynthetic metabolism (Allison et al. 1954; Zotina et al. 2003). In certain species, nevertheless, heterotrophic metabolic activity may contribute to a significant portion of carbon and nitrogen budgets of algae growing under low irradiance conditions (Zotina et al. 2003).

## 3 Production Systems for Mixotrophs

### *3.1 Open Pond Systems*

Interestingly microalgae were considered as a potential source of biodiesel during the period of the two world wars. Emphasis on the mass cultivation of microalgae led to the concept of open pond systems in 1950s, and it is still in practice for almost all commercial production of algae (Borowitzka 1999). Open pond systems can be categorized as man-made (circular, raceway and open cascade) or natural waters (lakes, lagoons, and ponds) sources. Circular ponds are shallow (depth less than 5 cm) with rotating scrapers for mixing of algae. These are still widely used in Japan and Indonesia. Raceway ponds are usually constructed of concrete in Israel, the United States of America, China and other countries. Open cascading pond systems of depth of less than 1 cm have also been reported with a cell density of 10 g/L (Setlik et al. 1970).

However, open pond systems have certain limitations. Firstly, they require more "extreme" culture conditions such as high salinity, alkalinity and high nutritional status and restrictive environmental conditions to restrain the threat of pollution from other aggressive algae species and protozoa (Lee 1986; Pulz and Scheinbenbogan 1998). To date, only mono-culture cultivation of *Dunaliella, Spirulina* and *Chlorella* has been found to be successful for commercial cultivation. In general, mixotrophic cultivation has conventionally and commercially not been applied in open pond system due to the competition with bacteria for the organic carbon. However in some open pond cultivation systems, the controlled addition of small amount of the organic substrates such as acetate may be possible to achieve high biomass. Important design considerations will include the growth rate of the algal species, size of the inoculums and control of environmental parameters.

**Table 2** Considerations and requirements for algal cultivation in different modes

| Cultivation mode | Energy source | Carbon source | Cell density | Reactor scale-up | Cost | Issues associated with scale-up |
|---|---|---|---|---|---|---|
| Phototrophic | Light | Inorganic | Low | Open pond or photobioreactor | Low | Low cell density<br>High condensation cost |
| Heterotrophic | Organic | Organic | High | Conventional bioreactor | Medium | Contamination<br>High substrate cost |
| Mixotrophic | Light and organic | Inorganic and organic | Medium | Closed photobioreactor | High | Contamination<br>High equipment cost<br>High substrate cost |

## *3.2 Photobioreactors*

Open ponds are successful models for commercial production of photosynthetic algae, but not a choice for mixotrophic cultivation. For industrial production of mixotrophs, another approach to overcome the major problems associated with open pond system is to use photobioreactors. Bioreactor technology offers the advantages of cultivation of algae under controlled conditions, prevents the invasion of undesirable or aggressive microorganisms, is independent of location or weather, and allows for much higher areal productivities. The addition of organic carbon nutrients can be carried out in a controlled fashion, allowing algal metabolism to be mixotrophic. The merits and demerits of different modes of cultivation have been listed in Table 2. Different designs and most appropriate models for mixotrophic cultivation are considered below.

### 3.2.1 Tubular Photobioreactors

The tubular photobioreactor is one of the most successful models for outdoor mass cultures of algae, considering that the light source required is free and readily available. Most tubular photobioreactors are constructed of transparent, economical material like polyethylene or glass tubes, which allows sufficient light penetration, and their cultures are recirculated either by a pump or an air lift system (Ugwu et al. 2008). Most of the current configurations of tubular bioreactor have been compared in Table 3 with other configurations and are discussed below.

Vertical Tubular Photobioreactor

The airlift and bubble column reactors are examples of vertical tubular photobioreactors, undoubtedly the most popular choice for outdoor and indoor cultivation of algae. The airlift reactor consists of two interconnected chambers or concentric

**Table 3** Main Design Features of Closed Photobioreactors

| Reactor design | Light harvesting efficiency | Degree of control | Land area required | Scale-up | Productivity ($g\ L^{-1}\ d$) | Species | References |
|---|---|---|---|---|---|---|---|
| Vertical tubular | Medium | Medium | Medium | Possible | 0.5 | *P. cruentum* | Alias et al. 2004 |
| Horizontal tubular | Good | Medium | Poor | Possible | 0.25–0.7 | *S. platensis; Nannochloropsis* sp | Barbosa et al. 2004; Trotta 1981 |
| Helical | Medium | Good | Excellent | Easy | 0.4 | *S. platensis* | Markl 1977 |
| A-shaped | Excellent | Good | Poor | Very difficult | | | Chrismada and Borowitzka 1994 |
| Flat- plate | Excellent | Medium | Good | Possible | 0.85–2.15 | *Nannochloropsis* sp. *platensis* | Miron et al. 1999; Richmond and Cheng 2001 |
| Fermenter type | Poor | Excellent | Excellent | Difficult | 0.03−0.05 | Several | Tredici and Zittelli 1998 |

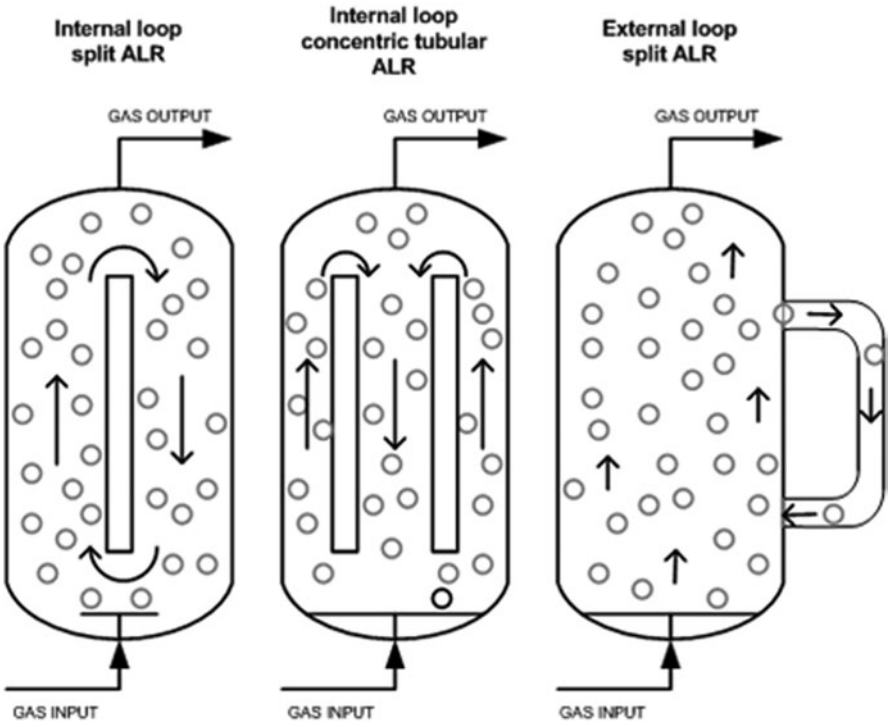


**Fig. 1** Different types of air lift bioreactors

tubes as shown in Fig. 1. The inner tube or riser or one side of the reactor is sparged with air or gas mixture whereas the other chamber, which does not receive gas, is called a downcorner (Fig. 1). The sparger is usually located at the bottom of the riser tube, which allows upward random movement of air in the riser tube. This upward movement of gases allows distribution of the gases in fluid. The airlift reactor has the advantage of creating circular mixing pattern. This mixing pattern allows the liquid culture to pass through a dark and light phase continuously, therefore, providing a flashing light effect to algal cells (Barbosa et al. 2003a).

Bubble column reactors are cylindrical vessel with a height: diameter ratio greater than two. Mixing and $CO_2$ mass transfer is done by bubbling the gas mixture from a sparger (Fig. 2). Scaled up or tall bubble columns have perforated plates to break and redistribute coalesced bubbles (Doran 1995). A dark zone may exist at the center of the bioreactor thus affecting the photosynthetic efficiency in column bioreactors. The circulation flow pattern does not exist at gas superficial velocity less than 0.01 $ms^{-1}$ (Janssen et al. 2003), therefore, high gas velocity (>0.05 $ms^{-1}$) is recommended for increasing the photosynthetic efficiency. Some examples in the VTR have been discussed in Table 4.

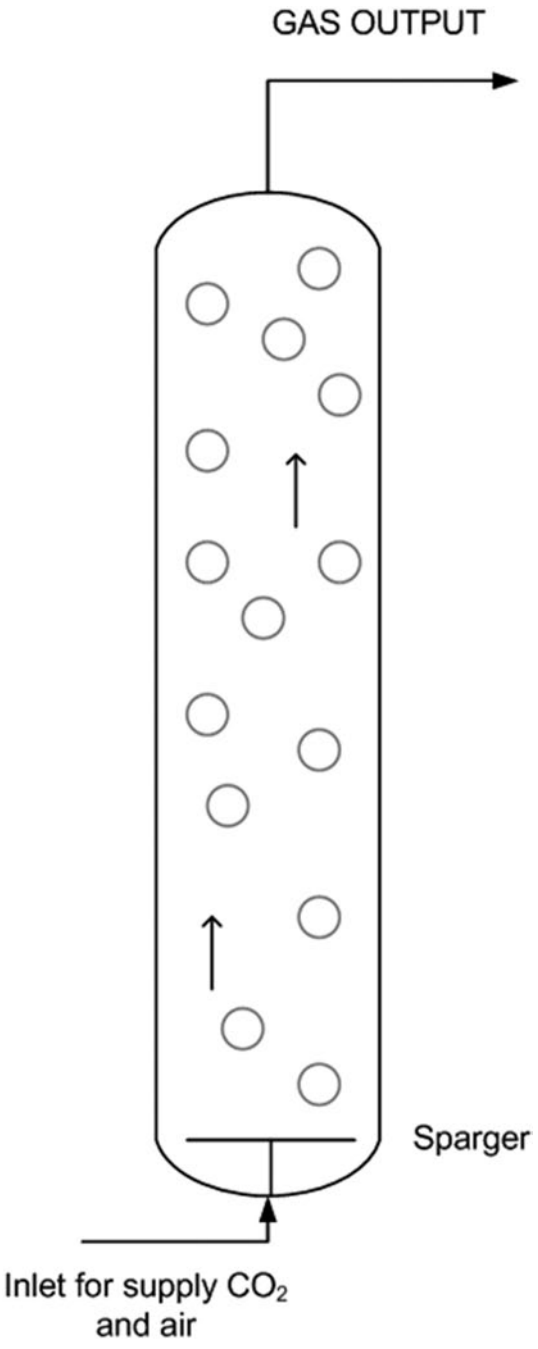


**Fig. 2** Scheme of bubble column bioreactor

**Table 4** Examples of Vertical Tubular and Horizontal bioreactors

| Vertical tubular reactors | References |
|---|---|
| Several 30 cm × 180 cm (ca. 50 L) polyethylene bag reactors for continuous monoxenic culture system for marine phytoplankters. Low-cost and easily available materials and complemented with various closing devices, automated air and medium supplies. This system was intended to produce adequate algae inoculum for large-volume batch cultures in hatcheries | Trotta 1981 |
| 40 L polyethylene turbidostat for the cultivation of The marine unicellular lagellates *Tetraselmis suecica* (Kylin) and *Isochrysis* aff. *Galbana*. Six 80 W fluorescent lamps used as light source. Average production of $0{\cdot}56 \times 10^{11}$ *T. suecica* cells day$^{-1}$ for 33 days and $5{\cdot}18 \times 10^{11}$ *I. galbana* cells day$^{-1}$ for 21 days was obtained | Laing and Jones 1988 |
| Flexible transparent plastic film contained in a rigid metal framework, so as to form a vertical panel of reduced width | Tredici and Rodolfi 2004 |
| Pilot-scale photo-bioreactor that uses sunlight and flue gas for the cultivation of *Euglena gracilis* and consisted of a vertical tubular part (kept in the dark) and a horizontal tubular (subject to sunlight) protein content of 47% and an AME value of 4700 kcal/kg. | Chae et al. 2006 |
| *Horizontal tubular bioreactor* | |
| Long tubular reactors of 14 cm in diameter for cultivation of *S. platensis*. The maximum working volume of 8000 L in a land area of 80 m$^2$. The bioreactor was fitted with diaphragm pump designed to drive the culture to a feeding tank, and gas transfer was allowed in the tube connections. The maximum productivity of bioreactor was ca. 0.25 g L$^{-1}$ d$^{-1}$. The major problem encountered was control of temperature | Torzillo et al. 1986 |
| Reactor system with capacity of 7000 L in all, for which a productivity of 36 g m$^{-2}$ d$^{-1}$ when using *Phorphyridium cruentum* (*44*). This reactor system consisted of several 70-L glass VTR, all connected to a gas exchange unit, and the culture was mechanically pumped between the light harvesting and the gas exchange units-whereas the temperature was controlled by submerging the culture in a water pool. The major drawbacks of this reactor were its relatively high cost and intrinsic fragility of its constitutive glass | Gudin and Chaumont 1983 |
| The HTR consisted of several plexiglass tubes having 3.4 cm internal diameter, Tubes were aligned side by side without any space between on a wooden framework facing south with an angle of 5°. Surface area to volume ratio was maintained 70 m−1, however gas holdup was kept 10.3% of total volume occupied by the gas bubbles. For controlling temperature, automatic evaporative cooling system was used for maintaining the temperature control. Volumetric productivity and photosynthetic efficiency was higher than flat reactor | Tredici and Zittelli 1998 |

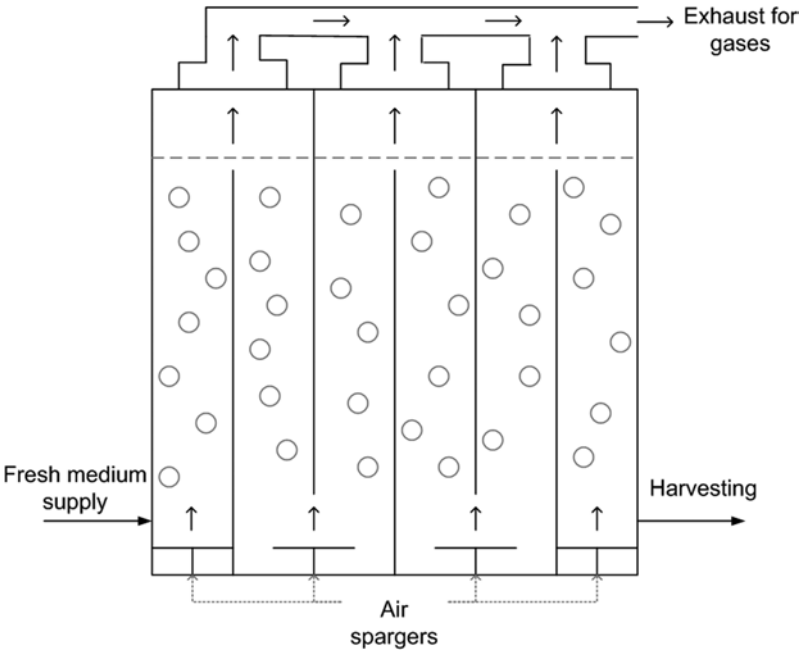


**Fig. 3** Schematic diagram of pump-driven FP-PBR

## *3.3 Horizontal Tubular Photobioreactor*

Horizontal tubular reactors (HTRs) consist of an array of parallel transparent tubes, generally diameter less than 0.1 m; and gas transfer takes place in the tube connection via gas exchange units. HTRs are usually oriented in north-south direction to capture maximum sunlight, which contributes in generation of considerable amounts of heat (Table 4). This system usually requires a dedicated cooling system for regular operations. Spraying water on the surface of the tubes, overlapping of tubes, placing the light-harvesting unit inside a pool of temperature controlled water, and regulating the temperature of feed or recirculation stream are some methods for controlling the temperature of HTR.

## *3.4 Flat Plate Photobioreactor*

Flat plate (panel) reactors (FPR) are conceptually designed to utilize maximum sunlight, therefore, FPR are characterized by high surface area to volume ratio with minimal light path. FPR panels are constructed from transparent materials like glass, plexiglass, polycarbonate etc, and agitation is provided by either bubbling of compressed air from the bottom, through perforated tubes or by rotating the reactor configuration itself mechanically through a motor (Fig. 3). The FPR has the provi-

sion of an open gas transfer unit, which prevents the problem of oxygen build up as compared to other closed reactors.

Barbosa et al. (2003b) described a flat plate bioreactor for cultivation of *Dunaliella salina*. Plates were built up by from lexan (polycarbonate) held together in a stainless steel frame. A mixture of $CO_2$ and air was sparged through 17 needles of 0.8 mm in diameter pinched through a piece of silicon placed at the bottom of the reactor. Further this model was modified by Zhang et al. (2001) by inclusion of baffles to improve agitation. Iqbal et al. (1993) designed a V-shaped flat-sided photobioreactor (working volume: 2 L) to achieve high surface-to-volume, efficient mixing, eliminating escape corners, which minimizes shear stress, and cell adhesion to the walls of the reactor. A transparent cooling jacket was also attached on the front (illuminated) side of the reactor.

## *3.5 Current Status and Proposed Models for Mixotrophic Cultivation*

Efficient mixotrophic cultivation requires axenic culture, diurnal cycle and good sources of organic/inorganic carbon. These requirements are only possible in controlled and closed systems, which can be operated under sterile conditions (to avoid contamination), have a good light supply coefficient (for efficient photoautotrophic metabolism) and good mass transfer characteristics (for efficient heterotrophic metabolism). Photo bioreactors are the only configuration able to fulfill these requirements, but at present, it is difficult and/or expensive to construct a photo-bioreactor, which meets the above criteria.

Lee et al. (1996) successfully cultivated mixotrophic culture of *Chlorella* in a 10 L outdoor tubular bioreactor. They compared the heterotrophic or mixotrophic growth of *Chlorella* in outdoor enclosed tubular photobioreactors, and concluded that if axenic algal culture could be maintained, such as adopting an enclosed photobioreactor system, mixotrophy is the better mode of growth for outdoor mass production of algal biomass. However, axenic algal culture in a scaled-up tubular bioreactor (300 L) could not be maintained in the presence of sugar substrates.

Fernandez et al. (2004) investigated a pilot-scale (0.19 m column diameter, 2 m tall, and 60 L working volume) outdoor vertical bubble column and airlift photobioreactors (a split-cylinder and a draft-tube airlift device) to compare mixotrophic cultivation of cultures of *Phaeodactylum tricornutum*. Initially, the cultures were started photoautotrophically to acquire high cell concentration; after this, the cultures were supplemented with glycerol (0.1 M) and a low nitrogen source which showed an immediate boost in growth rate. The maximum biomass yield in this work was comparable with commercial photoautotrophic cultivation of *P.tricornutum* in closed continuous-run tubular loop bioreactors with tubes (diameter less than 0.08 m). The vertical airlift and bubble-column bioreactors were found to be quite productive in comparison to open ponds. Therefore, bubble-column bioreactors seem to be suitable candidates for mixotrophic cultures, since it is easy to maintain completely

sterile conditions. For efficient productivity of bioreactors, the illumination surface to volume ratio should be high (very narrow columns), and spacing between columns should be large enough to avoid mutual shading of the columns.

The bioreactor design for such a mixotrophic culture system would probably be based on the presumption that the organic substrate is the main carbon/energy source, whereas light and carbon dioxide are supplementary, as it is difficult to optimize the utilization of the varying solar irradiance in outdoor cultures. This represents a major shift in the mindset from designing bioreactors for optimizing photosynthesis to ones that favor mixotrophic growth, with light as the supplementary source of energy.

A promising solution for the economical production of high algal biomass (enriched with contents of useful metabolites) is sequential heterotrophic-photoautotrophic cultivation. In this process, photosynthetic cells are first cultivated in the presence of organic carbon source i.e. heterotrophically to achieve high cell concentrations and then transferred to photo-autotrophic conditions for accumulation of the photosynthetic products. Ogbonna et al. (1999) and Ogbonna and Tanaka (1997) have demonstrated this process for cultivation of *Chlorella* and *Euglena.* In this process, microalgae were initially cultivated in a conventional heterotrophic bioreactor to achieve high cell density, after that culture was passed into the conventional large scale photobioreactor for accumulation of the desired metabolites. Thus, the sequential heterotrophic-mixotrophic cultivation may be a suitable approach which has applications for existing commercially used heterotrophic and photoautotrophic bioreactors.

## 4 Energy from Mixotrophic Algae

Microalgae cultures can simultaneously produce different products such as lipids, starch and high-value compounds, increasing the process profitability (Brennan and Owende 2010; DOE 2010). As in any other new process, some challenges still need to be overcome to make mixotrophic algal technology feasible at a commercial scale. For example, in the case of biofuels from microalgae, the strain selected should have high performance (Pruvost et al. 2011); the source of nutrients should be sustainable (Pittman et al. 2011), and process parameters must be the optimal leading to the highest product productivity (Barbosa et al. 2003a), but a considerable effort is needed in research to satisfy all this requirements. Many investigators have focused their interest on improving the energy balances for the whole process by taking into account all the energy required by pumping, harvesting, extraction and other steps in the process (Khoo et al. 2011; Yang et al. 2011), since a potential for negative balance is likely.

As mentioned before, several energy products can be obtained from microalgae (See Fig. 4). A source of carbon, water, light and energy is required for all of them. As in all bioprocess, biomass is a reactant and a catalyst of its own reaction and the products obtained produce energy and also the biomass as itself. Transesterifica-

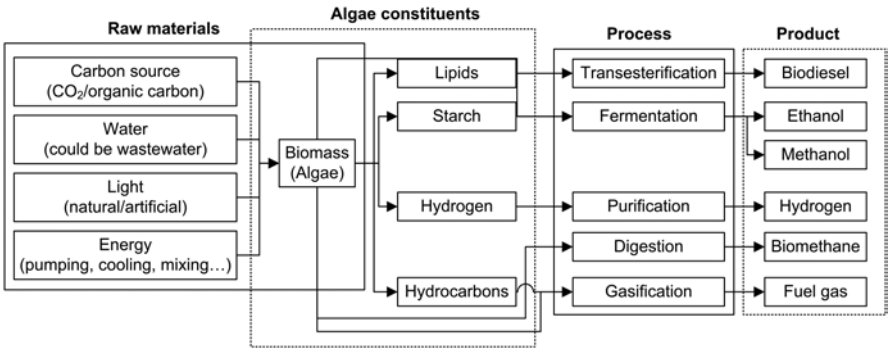


**Fig. 4** Energy products from algae

tion, fermentation, digestion and gasification are the most common ways to obtain energy products from algae (Leung et al. 2010; John et al. 2011; Ehimen et al. 2011; Biller et al. 2011). In this chapter, some of these processes will be discussed.

## *4.1 Biodiesel*

There is a growing interest all around the world on research and production of biodiesel as diesel substitute(Korbitz 1999; Demirbas 2007). Biodiesel can be made from vegetables oils (Jatropha, Mahua, Linseed, Neem, Pongamia/Karanja, palm, coconut, others), animal fats and, microalgae (Singh and Singh 2010; Encinar et al. 2011; Satyanarayana et al. 2011). This biofuel has several advantages over petrodiesel as it is non-toxic, biodegradable, and renewable. Biodiesel also produces fewer pollutant compounds than diesel and can be used in current diesel engines with few or non-modifications (Bozbas 2008). In general biodiesel is an alkyl ester, and a product of the transesterification reaction between a triglyceride (lipid or oil) and an alcohol in the presence of a catalyst. Table 5 shows the properties for traditional biodiesel and for biodiesel from microalgae (Mata et al. 2010); research results have shown that the chemical composition of biodiesel changes according to its source (Albuquerque et al. 2009).

Table 6 compares the oil content and biodiesel productivity for different feedstocks. Microalgae and seed plants have similar oil content but the higher biomass productivity of microalgae is reflected in significantly higher lipid productivity. Microalgae have different lipid content according to strains. For example, *Chlorella vulgaris* growing under mixotrophic conditions has a biomass productivity of 0.09–0.25 $gL^{-1}d^{-1}$, a lipid content of 21–34 % DW and lipid productivity of 22–54 $mgL^{-1}d^{-1}$ (Liang et al. 2009), and *Scenedesmus obliquus* has a biomass productivity of 0.10–0.51 $gL^{-1}d^{-1}$, a lipid content of 6.6–11.8 % DW and lipid productivity of 11.6–58.6 $mgL^{-1}d^{-1}$ (Mandal and Mallick 2009).

**Table 5** Properties comparison of conventional diesel and biodiesel from microalgae. (Mata et al. 2010)

| Properties | Biodiesel from microalgae oil | Diesel fuel | ASTM biodiesel standard |
|---|---|---|---|
| Density (kg $L^{-1}$) | 0.864 | 0.838 | 0.86–0.90 |
| Viscosity ($mm^2$ $s^{-1}$, cSt at 40 °C) | 5.2 | 1.9–4.1 | 3.5–5.0 |
| Flash point (°C) | 115 | 75 | Min 100 |
| Solidifying point (°C) | −12 | −50 to −10 | – |
| Cold filter plugging point (°C) | −11 | −3.0 (max −6.7) | Summer max 0<br>Winter max <−15 |
| Acid value (mg $KOHg^{-1}$) | 0.374 | Max 0.5 | Max 0.5 |
| Heating value (MJ $kg^{-1}$) | 41 | 40–45 | – |
| H/C ratio | 1.81 | 1.81 | – |

**Table 6** Comparison between different oil feedstock for biodiesel production. (Mata et al. 2010)

| Plant source content | Oil content (% w/w) | Oil yield (L oil/ha year) | Land use ($m^2$ year/kg biodiesel) | Biodiesel productivity (kg biodiesel/ha year) |
|---|---|---|---|---|
| Corn/Maize (*Zea mays L.*) | 44 | 172 | 66 | 152 |
| Hemp (*Cannabis sativa L.*) | 33 | 363 | 31 | 321 |
| Camelina (*Camelina sativa L.*) | 42 | 915 | 12 | 809 |
| Canola (*Brassica napus L.*) | 41 | 974 | 12 | 862 |
| Sunflower (*Helianthus annuus L.*) | 40 | 1070 | 11 | 946 |
| Palm oil (*Elaeis guineensis*) | 36 | 5366 | 2 | 4747 |
| Microalgae (low oil content) | 30 | 58,700 | 0.2 | 51,927 |

Several parameters affect microalgae growth and lipid production. Algae productivity depends on the mode of cultivation including photoautotrophic, heterotrophic and mixotrophic production, types of culture (open and closed systems), culture strategies (batch, fed-batch or continuous culture) (Gallardo et al. 2010), inhibitors concentration, mixing, dilution rate, temperature, pH, depth and harvests frequency

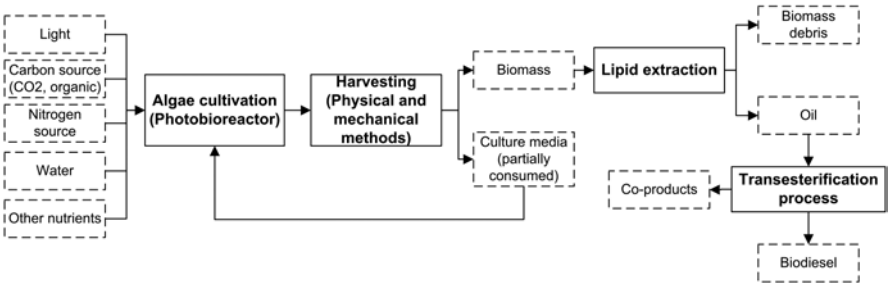


**Fig. 5** Process for biodiesel production from microalgae

(Bold and Wynne 1978; Renaud et al. 2002; Azov and Goldman 1982). Numerous researches have shown that light exposure, $CO_2$ and nitrate concentration have a remarkably strong effect on biomass and lipid production (Yeh et al. 2010). Figure 5 summarizes the general process for biodiesel production from microalgae.

## *4.2 Bioethanol*

The term bioalcohol is usually used to denominate ethanol obtained by fermentative process using a wide range of feedstock as substrate and it is known to be one of the most antiques technologies used by humans. Ethanol is a volatile, flammable and colorless liquid with many different applications as a solvent and fuel. Microalgae are known for storage energy in the form of starch (in some cases 50 % of algae weight corresponds to starch) that can be fermented to produce bioalcohol. Starch from microalgae is not the only component that can be used as substrate for bioalcohol production. Total carbohydrates from microalgae like mono, poly, and oligosaccharides (Ng et al. 2010) can also be used. Microalgae can produce these carbohydrates by solar conversion of $CO_2$ (Matsumoto et al. 2003) which is also an alternative for mitigation of $CO_2$ in the atmosphere. Cellulose in the cell walls can be converted into ethanol with the main advantage that microalgae cell wall do not have or have extremely low amount of lignin, and therefore, is more easily degraded in comparison with biomass from land crops.

A main advantage of ethanol production from microalgae is that microalgae can still produce lipids as a prior step; so many biofuels can be obtained from the same process (Mata et al. 2010; Kuhad et al. 2011). Among all microalgae specie some of them are promising candidates for bioethanol production due to their high carbohydrate content e.i. *Sargassum* (multicellular, 48 % DW carbohydrate content), *Glacilaria* (multicellular, 45 % DW carbohydrate content), *Prymnesium parvum* (unicellular, 25–33 % DW carbohydrate content), *Euglena gracilis* (unicellular, 14–18 % DW carbohydrate content).

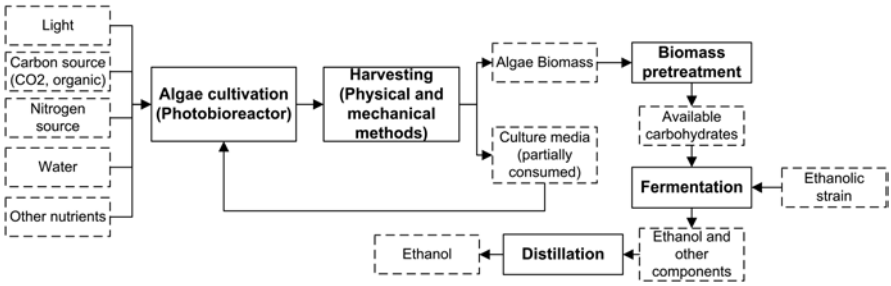


**Fig. 6** Process for the production of bioalcohol from microalgae

It is estimated that algae can produce around 14000 gallons of ethanol per year just in one acre of culture, for strains with high carbohydrate content, which is almost 30 times higher than ethanol from land crops (Amin 2009). The production of bioethanol from microalgae is affected by many parameters. The accumulation of starch in biomass depends of photoperiod, light intensity, temperature, media composition among others (Varfolomeev and Wasserman 2011).

For the production of bioethanol from microalgae, the biomass is harvested (usually by filtration or centrifugation), then the carbohydrates must be available for microorganism conducting the fermentation, therefore, microalgae cells are subjected to mechanical and/or biochemical treatments (enzymes) in order to release starch. Once the starch is available, microorganisms (i.e, *Sascharomices cerevisiae*) are added to transform sugars into alcohol. The fermented broth is then treated by distillation where the ethanol is obtained (see Fig. 6) (Amin 2009).

## *4.3 Biogas*

The gas produced by the biological treatment of organic matter under anaerobic conditions is called biogas. There are many organic materials that could work as substrate for gas production as land crops biomass, manure, wastewater, and microalgae biomass. The biogas is mainly composed of methane and carbon dioxide, but other gases may be present in lower concentrations i.e. hydrogen. These gases can be combusted releasing energy, and therefore, they are used as a fuel. If the biogas is subjected to cleaning and upgrading processes it reaches the standards of natural gas and then is called biomethane. This biomethane comprises about 60–70 % of initial biogas. This process has the advantage of using wet algae (no need for expensive drying) (Harun et al. 2011), but the establishment of this process at industrial scale still has many drawbacks due to production costs. Probably the best way to overcome this problem is by cultivation of algae in a multi-purpose facility, where other fuels and high-value products are also produced and extracted (Kerner et al. 1991; Sialve et al. 2009; Ehimen et al. 2011).

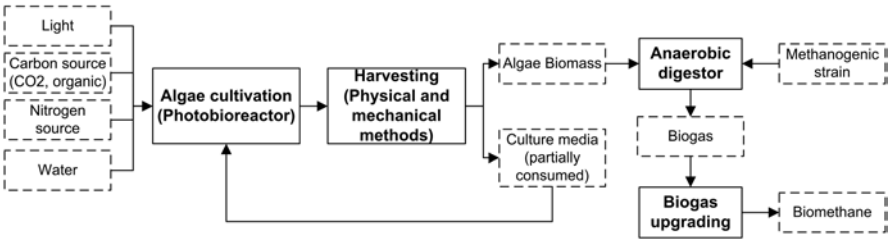


**Fig. 7** Steps for biogas production from microalgae

From 1968 to 1990 a research on the technical feasibility of biomethane production from different biomass sources was conducted. The study compared biomethane production from algae, wood and grass, and the results indicated to algae biomass as being the most potential feedstock. The bioavailability of nutrients is the main parameter affecting the growth of algae for biogas production. Some solutions for this problem include the external addition of nutrients normally present in marine water or by the culture of algae near shore (Chynoweth 2005). Figure 7 shows the general steps for biogas production from microalgae.

## 5 Other Applications

More than 350000 species of microalgae have been reported. Therefore, it is normal to find an unlimited number of products that can be obtained from them, and the product`s quantity and quality may change according to culture condition, providing a largest number of potential products. However, there are few established processes using microalgae at industrial scale and some of them make use of naturally growing biomass rather than that biomass cultivated in controlled environments.

The most prominent industries for the production of non-energy based products are in China, India, Japan, USA, Israel, Australia and Taiwan. These countries have implemented processes for the production of human and animal nutrition, cosmetics, polymers, and high-value oils. It is estimated that about 7000 t of dried algae biomass is annually produced for non-energy based products (Pulz and Gross 2004). A big advantage of the production of these compounds is that they can be produced simultaneously. Following are some aspects of non-energy products derived from microalgae.

## 5.1 Human and Animal Food from Mixotrophic Microalgae

Microalgae have many reasons to be considered as a healthy source of nutrient for humans and animals. The most salient characteristic is it high protein content (40–70 % DW), with an appropriate balance of amino acids, especially those that are essential for humans. The carbohydrates are present in many forms in microalgae, but they are mainly starch, glucose, polymers like agar, and cellulose. The digestibility of almost all of them is high increasing their potential as an energy source for animals. As mentioned earlier the lipid content is also significant and it is composed of glycerol and saturated or unsaturated fatty acids (omega 3 and omega 6 are of particular interest) (Spolaore et al. 2006; Christaki et al. 2011; Varfolomeev and Wasserman 2011). Microalgae. Microalgae are a very attractive source of vitamins for humans, as they produce vitamins A, $B_1$, $B_2$, $B_6$, $B_{12}$, C, E, among others (Spolaore et al. 2006).

Even though, some microalgae can be used as human food, the majority of them have high cellulose content, which cannot be digested by humans. *Spirulina* is the best known and more studied microalgae used for human nutrition. Production of this microalgae is labor intensive and its production does not generate co-products since the cell is consumed entirely; culturing is easy since cells grow at high pH reducing the risk of contamination. Cultures have high conversion efficiencies also, and there is no need for harvesting since animals can consume the product directly (Benemann 1992). The biggest advantage of *Spirulina* as part of humans' diet is its high protein content (around 60 %). This strain is not only used for human consumption but also for animal feed and as a fertilizer. In the case of other microalgae, these are used for feeding larvae in aquaculture, finfish and zooplankton. It is calculated than more than 40 microalgae strains are used for feeding purposes around the world, including the genera *Skeletonema, Chaetoceros, Phaeodactylum, Nitzschia, Thalassiosira, Isochrysis, Pavlova, etraselmis, Chlorella, Scenedesmus, Dunaliella, and Spirulina* (Pulz and Gross 2004).

Just for fish and shrimp feeding more than 100 tons per year are produced (Benemann 1992). Another microalgae species that has captured attention is *Haematococcus* which is responsible for the pinkish color in salmon and birdbaths. This strain produces the photopigment astaxanthin, the presence of that high-value product increases the cost of *Haematococcus* to of $ 60–150 per kilogram. *Chlorella* and *Dunaliella* are also potential strains for animal feeds, but they require harvesting before they are consumed by animals making them expensive (Benemann 1992). Xanthophylls from green algae can also be used as chicken feed with an estimated cost of $ 200–500 per kilogram. According to research results, algae can be used up to a level of 5–10 % in poultry nutrition (Spolaore et al. 2006), can also be consumed by animals with a cost around $ 0.3–0.5 per kilogram (Benemann et al. 1987).

Despite all the potential uses of microalgae as human and animal feed, production still is low or undeveloped. Improvement of this issue could be done by integration of microalgae technologies in hatcheries or by centralized production of microalgae biomass in specialized facilities (Hemaiswarya et al. 2011).

**Table 7** Cost of different algae products. (McHugh 2003)

| Product | Production (tons/year) | Value (Mio US $) |
|---|---|---|
| Agar | 7630 | 137 |
| Carreegenan | 33000 | 240 |
| Alginate | 30000 | 213 |

### *5.2 Polymers*

Perhaps the most well established and economically profitable products from microalgae are polysaccharides, also known as phycocolloids that are used to modify the rheological properties of liquids. Among them are agar, carrageenan, and alginate. In the cell, they are used as structural compounds and/or for energy storage.

Agar is polymer comprised of agarose (made up of monomeric agarobiose units) and agaropectin (made up D-galactose and 3,6-anhydro-L-galactopyranose). Its applications cover a wide range of uses including food, paper, coating and adhesives, molecular biology, and medical industries (Cardozo et al. 2007). After algae are cultivated they are pretreated, and the agar is extracted, the agar obtained must be purified, dehydrated and desiccated (Sukhoverkhov et al. 2000). Rhodopyhceae algae are the principal producers (polysaccharide) and these polymers are mainly used as an emulsifier in the food industry, especially in mixtures with milk, chocolate, jellies, and salad dressings. It also has several potential uses in the pharmaceutical industry e.g. antitumor and anticoagulant properties (Cardozo et al. 2007). Alginate, as the previously mentioned polymers, is used to modify rheological properties in products, but also in biotechnological industries as one of the most used matrices for biomass and enzyme immobilization (Cardozo et al. 2007). Table 7 presents the global market of algae derived polymers.

## 6 Conclusions

Microalgae offer great potential to produce a variety of industrial bioproducts or in the biofuel sector. Microalgae have a specific advantage in that they may be cultured in a mixotrophic mode which offers great flexibility to the biochemical engineer or biotechnologists. The large number of species, their versatile habitats and adaptability make mixotrophic microalgae serious candidates for applied research and development. There will continue to be new breakthroughs in mixotrophic microalgae technologies in the years ahead.

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# Engineering Photobiological $H_2$-Production

**Linda Vuorijoki, Pauli Kallio and Patrik R. Jones**

**Abstract** Our dominant current fuel sources are consumed at a far greater rate than they are replenished, prompting a search for economically sustainable alternatives for the conversion of solar energy into infrastructure-compatible fuel. Di-hydrogen is an appealing energy carrier due to its chemical versatility and benign impact on the environment upon combustion. Here, we discuss current issues for utilization of photobiological organisms for the direct conversion of solar energy and water into $H_2$. The focus is narrow, centering on cyanobacteria as the catalytic host, with a discussion on the (1) $O_2$-sensitivity of proteins participating in $H_2$-pathways, (2) incomplete oxidation of stored sugars under anoxic fermentative conditions in the dark, (3) thermodynamic limitations associated with NAD(P)H:$H_2$-pathways, and (4) competition for electrons between $H_2$-pathways and host metabolism. It is concluded that several possible scenarios exist without any clear 'winner' at this premature stage and that a broad spread of current research targets is most appropriate as opposed to any one single dominant path.

**Keywords** $H_2$ • Photobiology • Renewable fuel • Metabolic engineering

**Acronyms**

| | |
|---|---|
| [FeFe] | iron-iron |
| [NiFe] | nickel-iron |
| MBH | membrane bound hydrogenase |
| FQR | ferredoxin:quinone reductase |
| FNR | ferredoxin:NAD(P)H oxidoreductase |
| NDH | NADPH dehydrogenase |
| SDH | succinate dehydrogenase |
| CET | cyclic electron transfer |
| PMF | proton motive force |

There is an urgent need for the development of sustainable renewable energy production technologies in order to reduce greenhouse gas emissions, replace and conserve fossil fuels, enhance our energy self-sufficiency and limit costs associated

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with fossil fuel imports (approx. 1 billion euro per day in EU in 2011, based on the average oil price February 2011). It is essential that any production technology that provides a replacement for fossil fuel is economically and environmentally sustainable and that it has a minimal environmental footprint in order to contribute to a conservation of existing resources, including water, phosphorus and land quality (Gerbens-Leenes et al. 2009; Cordell et al. 2009; Kim et al. 2009). Biological processes can contribute towards sustainable energy production on a large scale as has already been demonstrated by commercial production of ethanol from sugarcane and corn and biodiesel from various biomass-sources. The approach to implement biological processes for renewable fuel production, however, needs to be carefully considered in order to avoid both direct and indirect negative effects on the environment (Chu and Majumdar 2012).

Di-hydrogen ($H_2$) is a versatile fuel for the future as it links electricity to fuel via electrolysis and fuel cells, and does not directly involve carbon dioxide. There are many bottlenecks on the way to a 'Hydrogen Economy', however. Storage of $H_2$ is one of these, and a large amount of research and development has and will continue to be invested in identifying suitable methods to store and distribute $H_2$ (Hull et al. 2012; Yang et al. 2010). Natural gas may here serve as a link between liquid fuels and $H_2$ by providing a source of $H_2$ through reforming and materials- and knowledge-infrastructure to enable gaseous fuels to be more widely used in the future (Ball and Wietschel 2009).

Biological fuel-production processes are currently not major contributors to renewable energy supply. Several direct photobiological fuel-production concepts have been proposed, however, none is at present in commercial large-scale practice for a number of reasons including a lack of economically sustainable production processes. There is no lack of vision and concepts however (Stephens et al. 2010; Ghirardi et al. 2009), with expected continued development as we gain further collective insight.

To achieve economically sustainable and entirely renewable conversion of solar energy and $H_2O$ into fuel is a challenge. The conversion process can be carried out using many renewable and non-renewable non-biological conversion systems that have electricity as the main output coupled with hydrogenenesis (e.g. photovoltaic panel + electrohydrogenesis). Alternatively, biological systems can convert water into $H_2$ and $O_2$ using sunlight as the sole driver, either indirectly via terrestrial biomass, or directly using photobiological prokaryotes or eukaryotes (Hallenbeck and Ghosh 2012). The intuitive advantage with biological methods of conversion is that the catalyst is self-reproducing, does not depend on rare metals and is capable of some degree of self-repair. Direct photobiological processes have the added advantage that, at least in theory, there are far less strict constraints regarding land quality and therefore no competition for agriculturally valuable land (Boddiger 2007). If economically sustainable fuel production systems can be developed, this should enhance our chances to reach a globally sustainable system that does not compromise on our ability to generate fuel for human metabolism.

Oxygenic photosynthetic microbes, green algae (e.g. *Chlamydomonas reinhardtii*) and cyanobacteria (e.g. *Synechocystis spp.*PCC 6803), are promising hosts for

long term $H_2$ production as they only depend on sunlight, $H_2O$ and $CO_2$ as principal substrates. In these systems, the energy from the sun is ultimately used to generate reducing equivalents which, directly or indirectly via NADP(H) and/or ferredoxin, may be used to produce $H_2$ by the committed enzymes hydrogenases and nitrogenases. Hydrogenases catalyse the reversible oxidation of molecular hydrogen. They can be categorized based on the metal cofactors in their active site as iron-iron [FeFe] hydrogenases, nickel-iron [NiFe] hydrogenases, and iron [Fe] hydrogenases (Kim and Kim 2011). An alternative classification is based on the co-substrates (e.g. NAD(P)H, ferredoxin or cytochromes) that the hydrogenases utilize as electron donors/or acceptors. Nitrogenases on the other hand are hetero-complexes composed of two enzymes, a [4Fe-4S] protein dinitrogen reductase and a metalloprotein [MoFe] dinitrogenase, which fix atmospheric $N_2$ and produce $H_2$ as a by-product. All hydrogenases and nitrogenases require multiple auxiliary maturation factors to obtain the active quaternary conformation. Whilst the complement of maturation factors appears to be host-specific for each [NiFe] hydrogenase (Weyman et al. 2011), the [FeFe] hydrogenase maturation factors HydF, HydE and HydG are sufficiently promiscuous to mature [FeFe] hydrogenases from other species (Posewitz et al. 2004). Due to their involvement in $H_2$ production, both hydrogenases and nitrogenases have been subjected to intense study and reviewed for their structure and function (Nicolet and Fontecilla-Camps 2012; Ogata et al. 2009) as well as their potential biotechnological applications (Bothe et al. 2010; Ghirardi et al. 2009).

Many aquatic photosynthetic prokaryotes, algae and cyanobacteria, have been reported to possess the inherent capability to convert $H_2O$ into $H_2$ and $O_2$ (Kruse et al. 2005a). With the exception of nitrogen-fixing microorganisms, however, it is not a dominant feature of their primary metabolism. This leads to the interesting fundamental question: Why those photobiological organisms that do not fix nitrogen contain such metabolic pathways, particularly when they often also are highly $O_2$-sensitive? In the case of cyanobacteria, $H_2$-production in native non-nitrogenase systems has in most cases only been detected with highly sensitive equipment that allows the analysis of trace amounts of $H_2$ in liquid media (Cournac et al. 2004), or after addition of selective inhibitors (Burrows et al. 2011) or nutrient starvation (Burrows et al. 2009; Antal and Lindblad 2005). Similarly, algal production systems depend on precisely defined environmental conditions, and even then it is still unclear what proportion of $H_2O$-derived electrons ultimately are channeled to $H_2$ (Kruse et al. 2005a), as current systems depend on the reduction of $O_2$ back to $H_2O$ in order to maintain an $O_2$-free environment. Nitrogenase-dependent organisms appear to be more robust in their ability to produce $H_2$ under temporally nitrogen-deficient conditions, even in the presence of $O_2$ (Masukawa et al. 2002; Leino et al. 2012). Consequently, the most productive photobiological $H_2$ production platforms are currently based on nitrogenases (Prince and Kheshgi 2005) despite the fact that they are, at least in theory, energetically less efficient than the corresponding hydrogenase systems (Ghirardi et al. 2009).

There have been many comprehensive reviews on the topic of photobiological $H_2$-production (Barber 2009; Ghirardi et al. 2009; Kruse et al. 2005b; Prince and Kheshgi 2005; Rupprecht et al. 2006; Hallenbeck and Ghosh 2012; Lee et al. 2010;

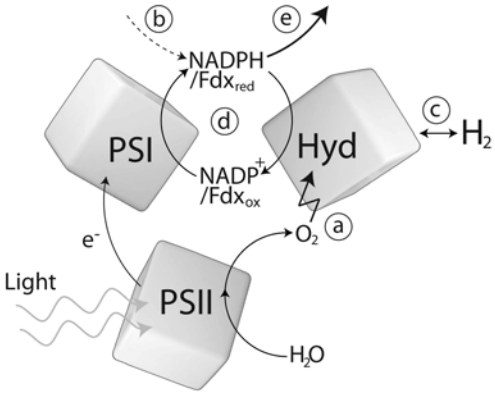


**Fig. 1** Some of the factors influencing the performance of $H_2$-producing hydrogenase systems in vivo. (**a**) Sensitivity of the hydrogenase (Hyd) -reaction towards oxygen from the photosynthetic water-splitting reaction in photosystem II (PSII). (**b**) Relative inefficiency of fermentative systems for providing reducing equivalents (NADPH/ reduced ferredoxin) under anoxic conditions due to incomplete oxidation of glycogen. (**c**) Thermodynamic limitations to $H_2$ production linked with (**d**) intracellular NADPH-homeostasis, and (**e**) host metabolism reactions competing for electrons (e.g. production of biomass)

McKinlay and Harwood 2010). The present chapter narrows the focus on a selection of issues for engineering photobiological $H_2$-production using cyanobacteria as the catalytic host (Fig. 1).

**$O_2$-sensitivity of $H_2$ producing enzymes** In an ideal photosynthetic $H_2$ production system, electrons from the water splitting reaction would be transferred directly from PSI to the hydrogenase (Waschewski et al. 2010). The principal bottleneck with this concept is the inherent sensitivity of the hydrogenases (Erbes et al. 1979) and nitrogenases (Rippka and Stanier 1978) to $O_2$ and $O_2$-derived species originating from the splitting of $H_2O$ by PSII (Fig. 1a). Mechanistically, the sensitivity of hydrogenases to $O_2$ is caused by either the rapid oxidative damage inflicted on the enzyme-bound iron-sulfur clusters involved in electron transfer or by the binding of $O_2$ to the catalytic bi-metal center. This results in the loss of hydrogenase activity or the inability to resume activity after exposure to oxygen. The [FeFe] hydrogenases are irreversibly inactivated by $O_2$ (Stripp et al. 2009a) through the destruction of the [4Fe4S] domain of the catalytic H-cluster (Stripp et al. 2009a, 2009b). In contrast, most [NiFe] hydrogenases are reversibly inactivated and an active conformation can be re-established after removal of $O_2$ and reduction of the Ni-Fe center (Schlesier and Friedrich 1981; Lukey et al. 2011).

*Enzyme structural features affecting $O_2$ sensitivity/tolerance* Some bacterial [NiFe] hydrogenases, such as those found in *Rubrivivax gelatinosus* (Maness et al. 2002), the hyperthermophilic bacterium *Aquifex aeolicus* (Guiral et al. 2005) and the chemolithotroph *Ralstonia eutropha* (Bernhard et al. 2001), are naturally more robust against $O_2$ than most other hydrogenases. Even though the mechanism(s) for enhan-

ced oxygen tolerance are still not comprehensively understood, these enzymes may serve as templates for engineering enhanced $O_2$-tolerance in established or native hydrogenases. It has been shown that the molecular structure of the Fe-S cluster (Fritsch et al. 2011) as well as specific cysteine's in the vicinity of the cluster proximal to the active site (Lukey et al. 2011) play important roles in the observed oxygen tolerance by influencing the electron relay towards the active site. In the membrane bound hydrogenase (MBH) of *Ralstonia eutropha* H16 the oxygen tolerance has been associated with the cofactor environment of the stable, high potential [3Fe4S] and [4Fe4S] clusters. Besides the unusual redox potential of these clusters, it has also been shown that the MBH is missing the oxygen inhibited $Ni_u$-A state during oxidation (Saggu et al. 2009). Also the amino acid composition of the gas channel between the active site and the solvent may restrict the diffusion rate of $O_2$ into the catalytic pocket, and/or modulate the kinetics associated with the reactivation of the enzyme (Wang and Blumberger 2012; Liebgott et al. 2011). The existing knowledge has been applied in rational protein engineering to improve hydrogenase $O_2$ tolerance by, for example, amino acid substitutions around the Fe-S cluster (Liebgott et al. 2011) or the gas channel (Dementin et al. 2009).

Despite the progress, all $H_2$-producing enzymes are sensitive to oxygen, although the degree of catalytic efficiency and the rate of inactivation in the presence of $O_2$ may vary considerably. The practical question is whether it is possible to obtain an enzyme which can catalyze $H_2$ evolution at a reasonable rate and remain active for a sufficient time to allow economically sustainable production to be implemented. Although different structural and mechanistic features related to $O_2$ tolerance have already been identified, the possibility of utilizing these solutions for biotechnological application still remain to be evaluated *in vivo*.

*Isolation of $H_2$-evolving enzymes from oxygen* Nitrogen-fixing cyanobacteria have evolved at least two additional strategies to protect nitrogenases from oxygen, thereby allowing them to split water and fix nitrogen within the same organism. The first strategy is based around spatial separation of the $O_2$-sensitive nitrogenases in partially anoxic heterocysts while the water splitting PSII complex is restricted to vegetative cells (Rippka and Stanier 1978). The electrons derived from photosynthetic water splitting are then transferred from vegetative cells to the heterocysts (Kumar et al. 2010) to drive $N_2$ fixation (and $H_2$ synthesis) in an environment containing less oxygen. In order to allow the $O_2$-sensitive nitrogenase to retain activity, heterocysts have several structural and metabolic features that enable the intracellular $O_2$ content to be maintained at low level. These include a thick, oxygen impermeable and hydrophobic cell wall as well as a relatively high respiration rate. (Murry and Wolk 1989). Similarly, in the case of unicellular *Cyanothece* 51142, differential sub-cellular localization with differing $O_2$ partial pressure was suggested as a potential explanation for the observed ability to catalyze $H_2$-synthesis at an atmospheric $O_2$ partial pressure (Bandyopadhyay et al. 2010). A second naturally evolved strategy is temporal separation between oxygenic photosynthesis and anoxic $N_2$-fixation in the different phases of the diurnal cycle. In the night, the lack of oxygenic photosynthesis in combination with respiration could potentially allow

sufficiently anoxic conditions for nitrogen fixation to be driven by fermentative catabolism of stored polysaccharides (Skizim et al. 2012; Bandyopadhyay et al. 2010; McNeely et al. 2010).

*Potential engineering solutions* Hydrogenases are more efficient in converting solar energy to $H_2$ than nitrogenases as they do not require ATP and exhibit far higher catalytic rates (Ghirardi et al. 2009). It would therefore be favorable to re-direct electrons targeted for nitrogenase to a native or heterologous hydrogenase that is spatially or temporally separated from the oxygen-evolving reaction. Recently this strategy was attempted by the heterologous expression of an [FeFe] hydrogenase in heterocysts of *Anabaena sp.* PCC 7120 (Gärtner et al. 2012). Although the enzyme synthesized under oxic conditions displayed activity *in vitro*, $H_2$-evolution was not obtained *in vivo* without external supply of electron donors. This suggests that the electron-flux to the heterologous hydrogenase was restricted, an issue that is likely to surface also with other $O_2$-tolerant hydrogenases if the *in vivo* specificity towards the non-native electron acceptor/donor substrate(s) do not match.

Nitrogen-fixing cyanobacteria also express a so-called "uptake hydrogenase" in heterocysts alongside nitrogenases. Their role is most likely to oxidize $H_2$ produced by the nitrogenase with the objective to minimize the loss of electrons (otherwise escaping as $H_2$) and/or to drive the nitrogenase-reaction forward (Bothe et al. 2010). The directionality of the flux through any hydrogenase will be determined by the redox-potential and concentrations of its electron-acceptor/donors. When heterocyst-specific hydrogenase has been removed by targeted deletion, the nitrogenase-dependent $H_2$-yield has been observed to increase substantially (Happe et al. 2000). It may also be possible to enhance $H_2$-production by increasing the heterocyst frequency as was recently demonstrated for $N_2$-fixation (Chaurasia and Apte 2011). Aside from metabolic engineering strategies there is considerable potential to improve $H_2$-productivity by optimization of the cellular environment, for example, by fixing cyanobacteria to a solid support for prolonged $H_2$ production (Leino et al. 2012). In addition, as indicated by the recent discovery that unicellular *Cyanothece sp.* ATCC 51142 can synthesize $H_2$ under aerobic daylight conditions (Bandyopadhyay et al. 2010), there are alternative hosts that could be used as platforms for future engineering and others that still remain to be discovered.

**Incomplete oxidation of sugars under anoxic conditions** As hydrogenases are $O_2$-sensitive, the breakdown of stored glucose could be used to drive hydrogen production under anoxic fermentative conditions even in oxygenic phototrophic organisms (McNeely et al. 2010; Bandyopadhyay et al. 2010; Skizim et al. 2012) (Fig. 1b). This strategy, however, is relatively energy-inefficient as the complete oxidation of glucose in heterotrophic metabolism requires $O_2$ as the final electron-acceptor (Glucose + 6 $O_2$ → 6 $CO_2$, ignoring biomass formation); in the absence of oxygen, stored sugars cannot be oxidized to completion, and electrons are lost as partially reduced fermentation-products. The redirection of available electrons towards hydrogen production is thermodynamically restricted by the large difference in redox-potential between the $O_2/H_2O$ and $H_2/H^+$redox-couples which represent the alternative final electron acceptors (Bar-Even et al. 2012; Stal and

Moezelaar 1997). As a result, anoxic fermentation of sugars can only produce 33 % of the maximal theoretical $H_2$ yield, with acetate being the most optimal fermentation-product (Glucose + 4 $H_2O$ → 2 Acetate + 2 $CO_2$ (total) + 4 $H_2$). To optimize overall productivity, it could be advantageous for the microbial host to be able to recycle the excreted fermentation products during the light periods, especially if the growth is limited by carbon availability or accumulation of acids or alcohols.

As a prerequisite for fermentative $H_2$ production, the host organism needs to carry out sufficient metabolic flux both to and from glycogen during the diurnal cycle. Most cyanobacteria are capable of fermentative metabolism (Stal and Moezelaar 1997) but with the exception of recent studies in *Synechococcus sp.* PCC 7002 (McNeely et al. 2010), there is limited information regarding the extent to which this could be used for $H_2$-synthesis. *Cyanothece* species (e.g. *Cyanothece* sp. strain ATCC 51142) are promising in this aspect as they display dynamic cyclic changes in glycogen metabolism during day and night (Schneegurt et al. 1994). Further progress will come from (1) in depth understanding of electron-metabolism during cyclic day/night conditions (Bandyopadhyay et al. 2010; Skizim et al. 2012) and (2) the development of molecular tools and knowledge for comprehensive metabolic engineering of non-model organisms such as *Cyanothece* species (Min and Sherman 2010).

**Thermodynamic limitations for photobiological $H_2$-production** Fermentative $H_2$-production has been demonstrated to be thermodynamically limited (van Niel et al. 2003; Veit et al. 2008), a concept which also needs to be evaluated in photobiological $H_2$-production systems (Fig. 1c). In fermentative species, the thermodynamic limitation is typically observed as a shift in metabolism at high partial $H_2$ pressure, resulting in increased excretion of reduced carbon-containing fermentation products and a lower rate of $H_2$-synthesis (van Niel et al. 2003). This limitation is directly influenced by NAD(P)(H) homeostasis inside the cell (Fig. 1d). In photobiological systems, NADP(H)-homeostasis is linked to $H_2$-metabolism by reversible NADP(H):$H_2$-pathways (multimeric [NiFe] hydrogenase such as HOX in *Synechocystis*, (Germer et al. 2009) and/or ferredoxin/flavodoxin-dependent hydrogenase coupled to a ferredoxin/flavodoxin:NADP(H)-oxidoreductase such as HydA1/FNR in *Chlamydomonas*, (Kruse et al. 2005a; Ghirardi et al. 2009)). Consequently, if the 'biochemically effective' intracellular NADPH/$NADP^+$-ratio is regulated in photobiological organisms (and particularly under dark fermentative conditions), it is possible that net accumulation of $H_2$ will be limited by an NADP(H):$H_2$-pathway that is operating very close to equilibrium. As a result, the $H_2$-pathway is likely to be highly unfavorable in the direction of interest ($H_2$-synthesis) at ratios of NADPH/$NADP^+$reported in most organisms (in the range of 1:10–10:1) (Andersen and von Meyenburg 1977; Veit et al. 2008; Brumaghim et al. 2003; Takahashi et al. 2008; Korn et al. 2009). The direction of any one reaction, however, is influenced by multiple interconnected reactions and regulators in the cell (Henry et al. 2007), and consequently, the outcome may be difficult to predict based on *in vitro* data and incomplete understanding of the overlapping metabolic networks. Importantly, natural nitrogenase systems should not be restrained by possible thermodynamic

limitations associated with NAD(P)(H) homeostasis, as they are driven by ATP-hydrolysis.

**Competition for electrons between native host metabolism and $H_2$-pathways** Outside of defined laboratory conditions, the photosynthetic apparatus of photobiological organisms needs to be sufficiently robust to withstand rapid and large dynamic changes in the natural environment. Several different safety mechanisms are coordinated to ensure that the flow of electrons from water splitting can be continually diverted away from the photosynthetic metabolons. This is presumably of greatest importance under conditions when the rate of photon capture and water splitting exceeds the rate of $CO_2$ fixation. Together, these response actions minimize the chance for an over-reduced state that in turn can cause the formation of harmful $O_2$-derived radical species (Muramatsu et al. 2009). In cyanobacteria there are several different "escape" mechanisms for electrons, including Mehler-like water-water cycle(s) and one or more types of cyclic electron transport involving either PGR5-like proteins (Yeremenko et al. 2005) together with FQR (ferredoxin:quinone reductase) and FNR (ferredoxin:NADP(+)-oxidoreductase) in direct recycling of electrons, or SDH (succinate dehydrogenase) or NDH (NADPH dehydrogenase) complexes (Mi et al. 1995), which indirectly cycle electrons to the plastoquinone pool. Although these mechanisms are important for photobiological organisms to survive under dynamic light and $CO_2$ conditions in nature, they are theoretically also competing for electrons that in an optimal production system could be diverted to $H_2$ (Waschewski et al. 2010) (Fig. 1e).

At the end of the photosynthetic electron transport chain, $NADP^+$is reduced to NADPH, which is the direct electron donor for both $CO_2$-fixation and NDH-1 mediated cyclic electron transfer (CET) around PSI in cyanobacteria. If NADPH starts to over-accumulate due to a poor $CO_2$ fixation rate, cyanobacteria will enhance CET around PSI and over-produce ATP without concomitant reduction of $NADP^+$(Okegawa et al. 2007). If $H_2$-synthesis was active under such conditions and constituted a stronger sink for electrons than other NADPH-oxidizing routes it is tempting to speculate that that this would automatically down-regulate CET, while still keeping up the proton motive force (PMF) across the thylakoid membrane (by using hydrogenase as an electron valve). If this would be true, there would not be any apparent need to engineer electron-metabolism, rather it should be sufficient to make sure that the electron-sink of preference ($H_2$-synthesis) is installed and as active as possible.

A practical example of the potential to modify the competition for electrons is given by the *Synechocystis sp.* PCC 6803 *ndhB* mutant M55. This strain is defective in the type I NADPH dehydrogenase complex (NDH-1) and exhibits a lowered rate of CET and respiration (Cournac et al. 2004; Mi et al. 1995). As a consequence, more electrons are directed toward hydrogenase resulting in an extension of continuous $H_2$ production, after the onset of light. Also oxidation of stored carbohydrates contributes to the increased electron flux to the hydrogenase via PSII-independent reduction of the PQ-pool in this mutant (Cournac et al. 2004). In green algae, the *stm6* mutant in *Chlamydomonas reinhardtii* represents another example of how the disruption of electron transfer mechanisms may have a positive influence on

**Table 1** The calculated changes in Gibbs free energy (from eQuilibrator (Flamholz et al. 2012))

| |
|---|
| **2 ferredoxin(red) + 2 H+ → H2 + 2 ferredoxin(ox)** |
| **Estimated $\Delta_r G'^{\circ}$** **21.3 kJ/mol** [$K'_{eq}$ = 0.00018] |
| **4 ATP + 2 ferredoxin(red) + 2 H+ + 4 H2O → H2 + 2 ferredoxin(ox) + 4 ADP + 4 Pi** |
| **Estimated $\Delta_r G'^{\circ}$** **-129.0 kJ/mol** [$K'_{eq}$ = 4.2e+22] |
| **NADPH + 2 H+ → NADP+ + H2** |
| **Estimated $\Delta_r G'^{\circ}$** **32.1 kJ/mol** [$K'_{eq}$ = 2.4e-06] |
| **4 ATP + NADPH + 2 H + 4 H2O → NADP + H2 + 4 ADP + 4 Pi** |
| **Estimated $\Delta_r G'^{\circ}$** **-118.3 kJ/mol** [$K'_{eq}$ = 5.5e+20] |

$H_2$-production under defined conditions (Kruse et al. 2005a). The *stm6* mutant was unable to perform state transitions, was impaired in cyclic electron transfer, and had 40 % lower oxygen levels than in the wild type cells. This strain also had large starch reserves, which theoretically increases the amount of available substrate (NADPH) for the hydrogenase. Together these properties contributed to 5-13 times more efficient hydrogen production than in wild type strains (Kruse et al. 2005a). However, in experiments where both the availability of electrons for hydrogenases and the concentration of $O_2$ are influenced by the same genetic or environmental factors it remains difficult to differentiate between cause-and-effect.

**Concepts for realization of photobiological $H_2$-production—where to next?** The theoretically most optimal system for photobiological $H_2$-production would be to introduce an $O_2$-tolerant hydrogenase that directly accepts electrons from PSI, or alternatively via ferredoxin (Lubner et al. 2011). Below, we describe two alternative concepts for practical production systems that do not depend on the construction of a hydrogenase that is active in the presence of $O_2$. It is likely, though, that the availability of such a fully functional enzyme would possibly end the search for an alternative "best" concept.

*(1) Nitrogenase-dependent $H_2$-production—is it really so bad?* The excessive demand for ATP in the nitrogenase-catalyzed reduction of protons has been argued to be one of the major bottlenecks that limits its utility (Ghirardi et al. 2009). Yet, nitrogenase-catalyzed $H_2$-production is one of few current methodologies where long-term high-yielding direct conversion of sunlight energy into $H_2$ has already been demonstrated (Leino et al. 2012; Bandyopadhyay et al. 2010). Why, then, is there such a great demand for ATP and how does that influence our ability to produce $H_2$?

A comparison between the $H_2$-forming reactions carried out by both hydrogenase and nitrogenase in eQuilibrator (Flamholz et al. 2012) shows that the addition of ATP hydrolysis to the reaction (not surprisingly) converts an endogenous and close to thermodynamically unfeasible reaction into an irreversible and highly exergonic reaction (Table 1).

Even though the high demand for ATP will limit the maximum potential stoichiometric efficiency of the $H_2$-production process, ATP-hydrolysis will drive the nitrogenase reaction in the direction of $H_2$-synthesis and therefore be important from a biotechnological process perspective. The question then is whether ATP-dependent $H_2$-production is an issue, or rather a benefit, for effective photo-biotechnological $H_2$-production?

*(2) Dark anoxic $H_2$-synthesis with stored intracellular carbohydrate feedstock* If cyanobacteria can store electrons harvested in the light period in the form of glycogen and later utilize them for anoxic fermentation in the dark period, it would be possible to utilize $O_2$-sensitive hydrogenases for photobiological $H_2$-production, as already demonstrated (McNeely et al. 2010). The issues of (1) incomplete oxidation of stored sugars in the absence of oxygen, resulting in the loss of most harvested electrons in the form of excreted waste-products, and (2) distribution of electron flux between biomass and stored sugars remain to be solved. These challenges could to some degree potentially be addressed by metabolic engineering.

**Conclusions** The development of strains that are capable of economically sustainable photobiological $H_2$-production is still in pursuit. A selection of bottlenecks, and potential solutions to circumvent them, have been discussed. Three main concepts stand out:

1. Direct water-splitting coupled to $H_2$-synthesis with an $O_2$-tolerant hydrogenase is theoretically the most optimal solution. It can be questioned, however, whether this can ever be sufficiently efficient for sustainable production.
2. Direct water-splitting and $H_2$-synthesis with nitrogenase, in both uni- and multicellular $N_2$-fixing organisms, is currently the best working system. The economic feasibility of such a production system warrants comprehensive evaluation.
3. Cyclic day-time water-splitting and night-time $H_2$-synthesis using either nitrogenase or hydrogenase and glycogen as a metabolic intermediate. Research towards this direction have only recently commenced.

Once such concepts have been demonstrated, the next challenge is to optimize the system. Here, the metabolic control of electron flux becomes the central focus as it is heavily influenced by the complexity of evolved regulation with the objective to produce biomass, not $H_2$.

It appears that no one solution will solve all the interlinked problems, but the on-going fundamental research, and combination of different individual approaches could lead towards more efficient and robust production systems in the future.

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# Starch Overproduction by Means of Algae

**Vilém Zachleder and Irena Brányiková**

**Abstract** This chapter provides an overview of the state of knowledge of starch production as an ultimate energy reserve in algae. It includes a survey of recent discoveries on controls that direct the metabolism of algal cells towards starch hyper-accumulation with the aim of providing starch-enriched biomass for the production of bioethanol as a biofuel of the future. We also outline basic research from the 1960s, from which the recent starch research stems, although the use of algal starch for biofuel production was not considered at that time. The principles of, and basic approaches to a directed synthesis of starch are described in both laboratory experiments and large scale-up outdoor photobioreactors.

**Keywords** Algae · Bioethanol · Cell cycle · Carbon dioxide · Inhibitors · Light intensity · Nutrient limitation · Starch · Temperature

**List of Abbreviations**

| | |
|---|---|
| CHX | Cycloheximide |
| DW | dry weight |
| FdUrd | 5-fluorodeoxyuridine |
| PBR | photobioreactor |

## 1 Introduction

Algae represent a vast variety of photosynthetic species growing photoautotrophically using light as a source of energy for photosynthesis and fixing inorganic carbon, from which a reserve material such as starch and/or lipid is synthesized

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and then serve as an energy and carbon source for future cellular growth and reproduction.

Despite the pioneering papers from the 1960–1980s, not much attention was paid to starch research for more than twenty years. The main motivation for the recent boom in starch research is that algae can be a high-yielding source of energy-rich reserve components, which can be used for the production of biofuels (third generation biofuels) without compromising food supply chains and be a renewable alternative to fossil fuels (Nigam and Singh 2010). In general, the algae from different taxons can produce either lipids (*Nannochloropsis, Trachydiscus* and other members of Eustigmaceae) or starch (most chlorococcal and volvocean algae) as their energy and carbon reserves. In general, starch-producing algae can also accumulate small amounts of lipid under standard conditions, but only some of them, under specific growth conditions, can be induced to overproduce lipids instead of starch (Zhukova et al. 1969).

In this review, we will focus exclusively on those algal species that accumulate polysaccharides (starch, cellulose), which can be used as a feedstock for the production of bioethanol.

In these species, starch is the final energy storage molecule formed in the photosynthetic fixation of carbon dioxide. Located in chloroplasts, it is utilized to provide energy for the cell's vital metabolic processes, and is independent of varying external energy and carbon supplies.

Many microalgae grow rapidly and are considerably more productive than land plants and macroalgae (seaweed) (Chisti 2007). There are several main groups of microalgae, which differ primarily in pigment composition, biochemical constituents, ultrastructure, and life cycle. In this chapter, we will pay attention to those representatives of the green algae (Chlorophyta) that may be a potential source of starch for future biotechnological applications in the production of bioethanol.

The green algae are a large group from which the Embryophytes (higher plants) emerged. The group, including both green algae and embryophytes, is monophyletic (and often just known as the Kingdom Plantae). There are about 6,000 species of green algae; many live most of their lives as single cells, while others form colonies or long filaments.

Almost all forms have chloroplasts. These contain chlorophylls *a* and *b*, giving them a bright green color (as well as the accessory pigments β-carotene and xanthophylls), and have stacked thylakoids (van den Hoek et al. 1995). All green algae have mitochondria with flat cristae. The storage product for members of this group is true starch, found inside the chloroplasts and consisting of amylose and amylopectin. The starch (seen as whitish granules under the electron microcopy) can often be observed surrounding the pyrenoid, a distinct spherical structure embedded in the chloroplast. There may be more than one pyrenoid, the pyrenoid may not always be present (e.g., *Ankistrodesmus* and *Tetraedron*), or the pyrenoid may be lacking completely. In most representative taxa, the cells are surrounded by a cellulosic wall. Some taxa may also have sporopollenin deposited on the wall. This gives added strength and is thought to help prevent desiccation.

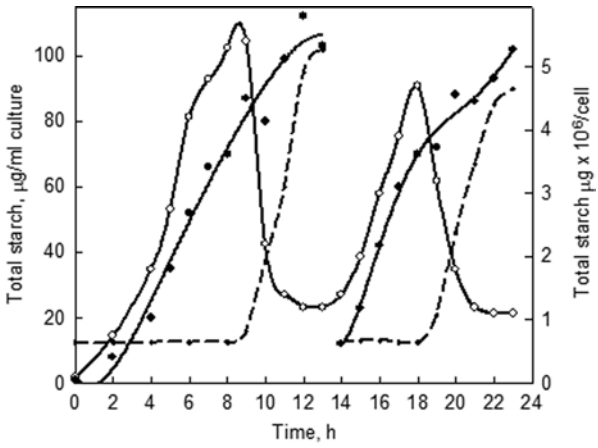


**Fig. 1** Total cellular starch per ml of culture and per cell during two cycles of synchronized culture of *Chlorella pyrenoidosa*. *Full circles*: starch/ml; *empty circles*: starch/cell; crosses, *dashed line*: cell number/ml of culture. (modified to Duynstee and Schmidt 1967)

Some microalgae (*Chlorella*, *Scenedesmus, Chlamydomonas*, *Tetraselmis* etc.) are known to contain a large amount of starch and cellulose, providing the raw materials for bioethanol production (Matsumoto et al. 2003). Many of the properties of starches, which determine their suitability for particular end-uses, depend on their amylose/amylopectin ratios. These properties include gelatinisation characteristics, solubility, and the formation of resistant starch. The structure of algal starch (*Chlorella*) resembles that of cereals (ca. 34 % of amylose content) and its gelatinisation temperature (ca. 65 °C), as determined by viscosity measurements, also suggests a structural similarity (Maršálková et al. 2010).

## 2 Starch Synthesis During the Cell Cycle

As early as 1967, Duynstee and Schmidt (1967) monitored the net content of starch within the cell cycle in synchronous cultures of a high-temperature strain of *Chlorella pyrenoidosa*. They found that in light, starch accumulates continuously nearly to the end of the cell cycle, followed by cessation immediately prior to and during the period of nuclear division and cytokinesis (Fig. 1).

These results were confirmed in synchronized cultures of the chlorococcal alga *Scenedesmus quadricauda* (Šetlík et al. 1972) where the starch content, promoted mainly by light energy input, was also dependent on the stage of cell development. Starch content rose in the early growing phase and decreased thereafter, even under continuous light (Hirokawa et al. 1982). Net starch accumulation can be an indicator of the balance between material and energy supply (provided by photosynthesis) and their use in synthetic processes. During nuclear and cellular divisions, starch is dramatically degraded, as indicated by cytological (Murakami et al. 1963) and biochemical (Baker and Schmidt 1964a; Baker and Schmidt 1964b; Curnutt and Schmidt 1964; Herrmann and Schmidt 1965; Johnson and Schmidt 1966; Cole et al.

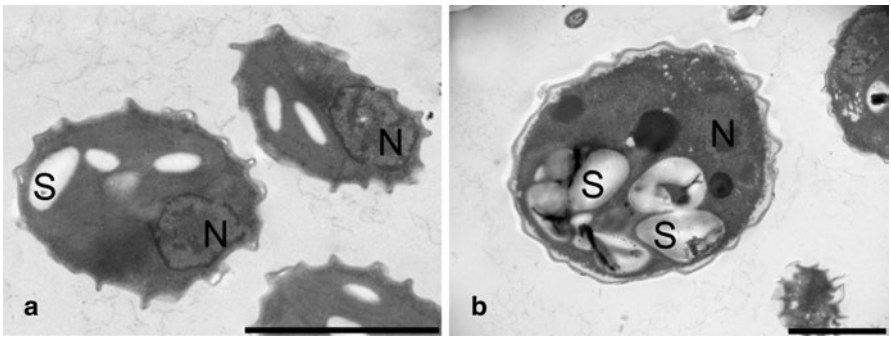


**Fig. 2** Electron microscopic photographs of daughter (**a**) and mother (**b**) cells of *Chlorella vulgaris* grown in complete mineral medium, *N*, nucleus; *S*, starch granules. Bars: 2 μm. (modified according to Branyiková et al. 2011)

1998; Schmidt 1966) changes and increased activity of starch degrading enzymes (Levi and Gibbs 1984; Wanka et al. 1970). Starch degradation products fulfill demands for both carbon building blocks for organic synthesis and increased energy demands during nuclear and cellular division. Because the photosynthetic rate was approaching its lowest level of the cell cycle during this stage of cellular development (Sorokin 1957), accumulated carbon and energy reserve starch was mobilized and utilized during this stage of cellular development. The net content of starch is comprised of starch that was synthesized minus that which was utilized. It is apparent therefore that net accumulation data underestimates the value of actual starch synthesis during the cell cycle. Accumulation of starch during the cell cycle and its phases was also studied in synchronized batch cultures of *Chlorella vulgaris* (Fig. 2). Culture proliferation and changes in starch content were monitored under continuous illumination. The relative starch content reached a maximum of 46% of DW just prior to cell division, which commenced at about the 10$^{th}$ hour of growth in light (Brányiková et al. 2011).

During cell division, the relative starch content decreased, even in cells grown in light, to 13% of DW. If the cells were transferred to the dark, they exhausted the stored starch nearly completely (4% of DW). Hence, significant cellular energy and material reserves are maintained to ensure that at any point in development, vital processes can run to completion even if the cells are placed in the dark.

## *2.1 Starch Production as Affected by Light Intensity*

As the ultimate storage form of photosynthetically fixed carbon, starch plays a central role in algal metabolism. Chloroplasts convert carbon dioxide into glucose, which, with the action of starch synthases and branching enzymes, is converted to a mixture of linear and branched glucans (amylose and amylopectin) that determine the properties of the starch. The extent of starch synthesis and degradation in the

chloroplast is tightly regulated by the available light (Sundberg and Nilshammar-Holmvall 1975; Brányiková et al. 2011) and by a number of environmental factors.

Synchronized cultures of *Scenedesmus quadricauda* were grown at two different light intensities and thus differing in specific growth rate. Populations characterized by low growth rates accumulated starch to somewhat more than one third of the value (per cell) obtained in cells with a high specific growth rate. Hence even in light-limited growth, significant cellular energy and material reserves are maintained. As described above for *Chlorella*, this is, perhaps, to ensure that at any point in development, vital processes can run to completion even if the cells are placed in the dark. Starch reserves were consumed nearly completely if cultures were transferred to the dark (Šetlík et al. 1972).

In synchronized cultures of *Chlorella vulgaris* grown under continuous illumination, the relative content of starch was substantially reduced (maximum 26 % of DW) in response to an increase in biomass concentration and corresponding decreased mean light intensity (Sundberg and Nilshammar-Holmvall 1975; Brányiková et al. 2011). The important role of light intensity on starch content was confirmed in experiments with asynchronous cultures grown at different starting biomass concentrations and consequently, at different mean light intensities, which were continuously decreasing with increasing biomass (Fig. 3).

The higher the mean light intensity, the higher was the starch content. A decrease in mean light intensity below a certain level (275 μmol/($m^2$ $s^1$))caused a gradual decline in the rate of starch synthesis, leading finally, to no net increase in starch content or even to its decrease in the case of very low mean light intensities (Fig. 3) (Zhang et al. 2002; Eriksen et al. 2007; Thu et al. 2009).

Beside light quantity, its quality can also play a role in starch synthesis and its degradation. Kamiya and Kowallik (1986) studied starch synthesis and degradation in *Chlorella vulgaris* under autotrophic conditions in blue and red light and found that in blue light, increased starch degradation occurred, causing a decrease in total starch content when compared with cells grown in red light (Miyachi et al. 1978; Kamiya and Kowallik 1986).

## *2.2 Starch Production as Affected by Temperature*

Semenenko and his collaborators published a series of papers in which they described and analyzed restrictive effects of high temperatures on the reproductive and synthetic processes in different strains and species of *Chlorella* (Semenenko et al. 1967, 1969; Semenenko and Zvereva 1972a, b). Unfortunately, their work was published in Russian more than 40 years ago and was neglected by researchers both then and now. They had, however, discovered the basic principles and rules, which control the production of energy reserves in algae, information, which only recently has been rediscovered.

The authors found that for the high-temperature strain of *Chlorella* sp. K (growth optimum at 36–38 °C) the effective restrictive temperature was 43–44 °C. With a mesophilic strain of *C. pyrenoidosa* (growth optimum 26–28 °C) the restrictive

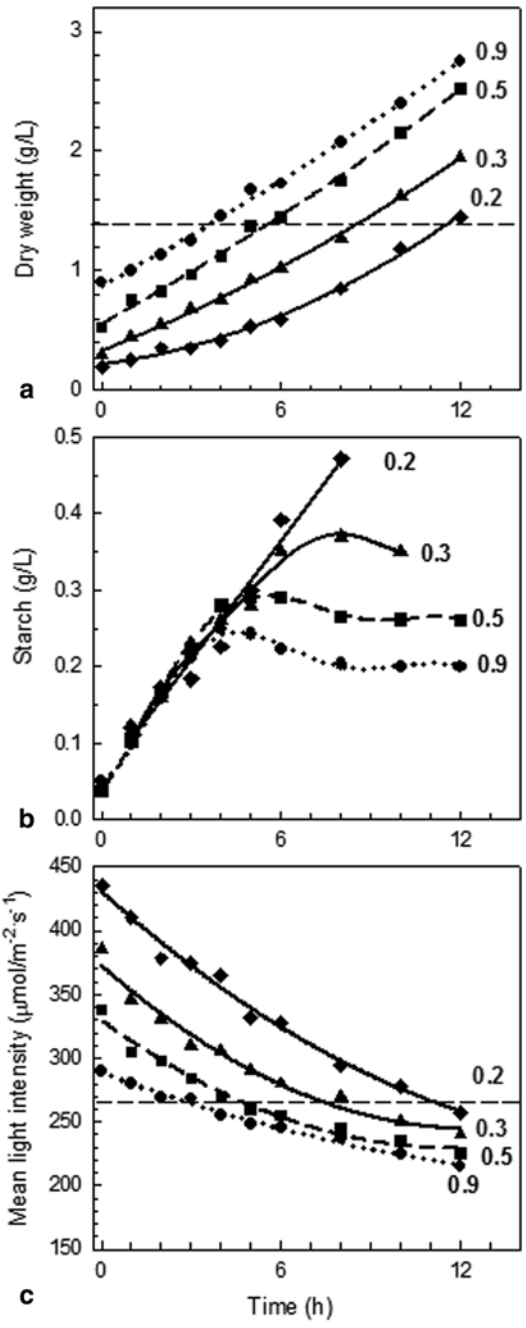


**Fig. 3** The effect of mean light intensity (**c**) on changes in biomass concentration (DW in g/L) (**a**), starch content in g/L (**b**) in asynchronous cultures of *Chlorella vulgaris*. The cultures were grown in a laboratory photobioreactor continuously illuminated by a constant incident light intensity of 780 µmol/($m^2$ $s^1$) and at various biomass concentrations (numerals on curves indicate initial dry weight in g/L). *Dashed horizontal lines* indicate the biomass concentration (panel **a**) and the mean light irradiance (panel **c**) under which the net starch content begins to decrease. (modified according to Brányiková et al. 2011)

temperature for cellular division is 35–36 °C. At the time when cells ceased dividing, giant cells with a diameter up to 5–6 times that of the daughter cells formed a significant part of the population. The higher the average irradiation received by the cells, the greater were the number of giant cells ultimately obtained, and also the faster was attainment of the final stage. Very large quantities of starch were found in the giant cells of *Chlorella* sp. K, while extensive lipid inclusions appeared in *C. pyrenoidosa*. To explain the behavior of cell populations at the restrictive temperature, Semenenko et al. assumed that the extreme temperatures effectively blocked reproductive events while other synthetic processes were mainly unaffected or were even stimulated by the high temperature, as was the case with photosynthesis. However, with reproduction processes inhibited, the demand for cell construction material gradually decreased, photosynthates accumulated in the later phases and by a feedback type of inhibition, they finally stopped photosynthesis.

The effect of temperature on $CO_2$ fixation in photosynthesis and on starch conversion into sucrose was studied in a series of papers by Nakamura and Miyachi (Nakamura and Miyachi 1982a, b, 1980; Nakamura 1983; Nakamura and Imamura 1983). They found that in *Chlorella vulgaris*, a temperature higher than 30 °C caused acceleration of the conversion of starch to sucrose. This temperature-induced degradation of starch also occurred in the dark, proving that photosynthetic electron transport is not involved (Nakamura and Miyachi 1982a; Nakamura and Miyachi b).

## 3 Treatments for Overproduction of Starch

Algal cells grown in light synthesize starch in chloroplasts, in the form of starch bodies (see, chapter *Starch synthesis during the cell cycle*). However, these starch reserves only represent the net production of starch, comprising the difference between gross synthesis of starch and that used to drive many cellular processes and events, particularly for the highest consumers of energy and carbon such as DNA replication, nuclear division and cytokinesis.

The final cellular content of starch is determined by two main factors: (1) the mean light intensity, which determines the rate of starch synthesis and its final content, and (2) processes (mainly cell division) consuming starch for their energy demands. Therefore to attain a maximum yield of starch, processes that utilize starch as energy and carbon source must be stopped or minimized, while conditions enabling production of starch should be optimized (Brányiková et al. 2011).

### *3.1 Applying Specific Inhibitors*

#### 3.1.1 Inhibition of Nuclear DNA Replication

In the chlorococcal alga *Scenedesmus quadricauda*, specific inhibition of nuclear DNA synthesis by 5-fluorodeoxyuridine (FdUrd) was used to study cell cycle regulatory processes (Zachleder 1994; Zachleder 1995). FdUrd interrupts the

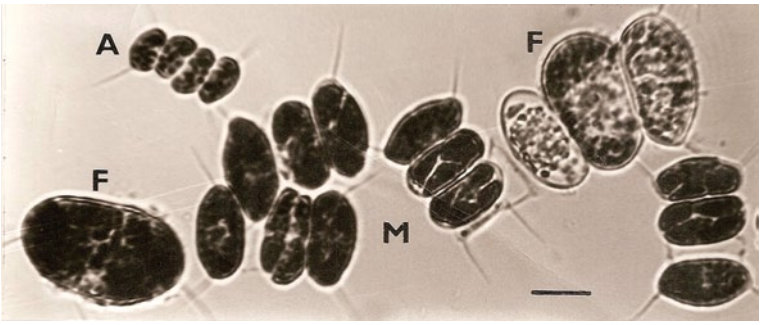


**Fig. 4** Microphotographs of the mixture of daughter coenobia (*A*), dividing mother cells (*M*) in a control culture of *Scenedesmus quadricauda* (after 14 h of light), and "giant" cells (*F*) from an FdUrd-treated culture (36 h of growth in the presence of FdUrd 25 mg/L). Scale bar = 10 μm. (modified according to Zachleder 1995)

deoxyribonucleotide metabolic pathway just before the last step by inhibiting the enzyme thymidylate synthase (EC 2.1.1.45), which catalyzes the reductive methylation of 2′-deoxyuridylate to form deoxythymidylate (Cisneros et al. 1993; Follmann 1983; Bachmann et al. 1983). As a consequence, subsequent events such as nuclear division and cytokinesis were also blocked (Zachleder et al. 1996). If FdUrd was added to a synchronized culture of *Scenedesmus quadricauda* at the beginning of the cell cycle, the cells remained uninuclear with no nuclear DNA replication, and no nuclear or cellular division occurred during experiments (Zachleder 1995). The growth of cells and processes involved in their growth, like RNA and protein accumulation, were not affected for an interval corresponding to at least the duration of two or three cell cycles, attaining six doublings in cell volume by the end of this period. While FdUrd effectively blocked DNA replication in *Scenedesmus quadricauda*, growth processes were not affected by the presence of the inhibitor. Because activity of the chloroplast was not affected, cells grew to a giant size, producing large numbers of starch grains in their chloroplasts (Fig. 4).

### 3.1.2 Inhibition of Cytoplasmic Proteosynthesis

Cycloheximide, an antibiotic inhibiting eukaryotic cytoplasmic protein synthesis, prevented cultures of *Scenedesmus quadricauda* (Zachleder et al. 2002) or *Chlorella vulgaris* (Brányiková et al. 2011) from undergoing nuclear division and consequently no cell division occurred. Starch, however, was intensively synthesized and in the case of *Chlorella vulgaris,* attained (within 4–5 h) a level of at least 60% of DW (Figs. 5 and 6).

After 5 h of growth in the presence of cycloheximide, the rate of starch synthesis equilibrated with biomass growth, and a high relative content of starch remained constant (Fig. 5). This shows that starch synthesis in the chloroplast is independent of cytoplasmic protein synthesis as well as DNA replication and other cell reproductive processes.

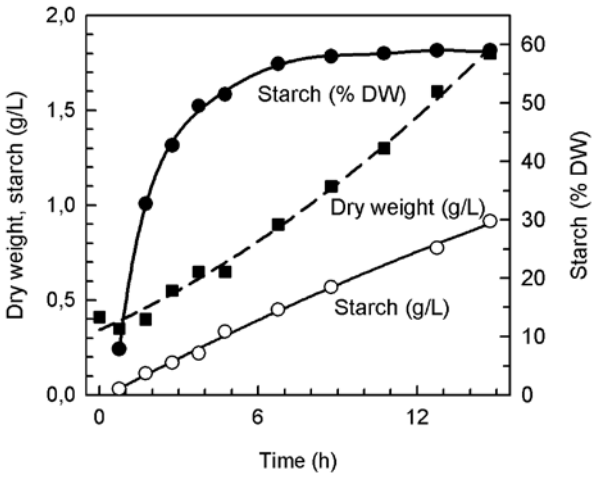


**Fig. 5** Effect of (1 mg/L) treatment on changes in biomass concentration (DW in g/L), starch content in g/L, and relative starch content (% of DW) in asynchronous cultures of *Chlorella vulgaris*. The cultures were grown in a laboratory photobioreactor at a constant incident light intensity of 780 µmol/($m^2$ $s^1$). (modified according to Brányiková et al. 2011)

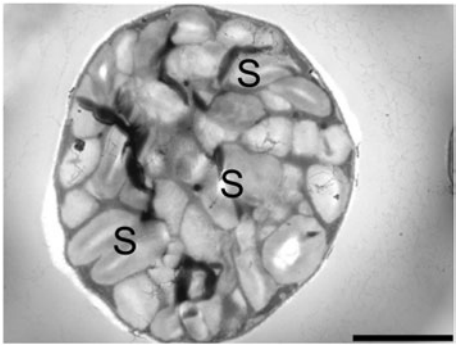


**Fig. 6** Electron microscopic photographs of cells of *Chlorella vulgaris* grown in the presence of cycloheximide (1 mg/L). *S*, starch granules. Bar: 5 µm. (modified according to Brányiková et al. 2011)

## *3.2 Nutrient Limitation*

While treatments with inhibitors are useful and important from the viewpoint of basic research, their industrial utilization is problematic for reasons of economics and possible environmental contamination. Suppression of cell cycle events requiring energy and carbon from starch reserves can however, be attained by limitation of macroelements in mineral media used for culturing autotrophically grown algae. An increase of starch content in algal cells upon limitation of some elements (nitrogen, sulfur, or phosphorus) was described a long time before the utilization of algal starch for production of biofuels was considered (Klein 1987; Ball et al. 1990; Zachleder et al. 1988; Šetlík et al. 1988; Ballin et al. 1988). Although in contrast to the use of specific inhibitors, the exact mechanism of element limitation-induced starch synthesis is neither well known nor specific, their application for scale up culturing is advantageous both from economic (saving fertilizers) and environmental points of view. Nevertheless, the principles of this approach are similar to that of inhibitors;

the absence of different elements caused a cessation of processes consuming starch while starch production was unaffected.

### 3.2.1 Effect of Nitrogen Starvation

When microalgae are starved for nitrogen, the main element of protein and nucleic acids, cell division ceases and photosynthetic carbon partitioning is switched towards starch synthesis. In *Chlamydomonas reinhardtii* cultivated in 1.0 mM $KNO_3$, protein and chlorophyll *a* content decreased when nitrate was exhausted, while carbohydrate content increased nearly six-fold (Klein 1987). Similarly in *Scenedesmus quadricauda,* macromolecular syntheses of RNA, protein and DNA were arrested when a nitrogen source was lacking (Fig. 7). This diverted the photosynthetic carbon flow into starch without it being consumed as a carbon and energy source (Ballin et al. 1988).

More than 20 years later, the effect of nitrogen starvation was verified in a production strain of *Chlorella vulgaris* p12 that accumulated starch from 35 to 58 % of dry weight (DW) in nitrogen-limited medium (Brányiková et al. 2011; Behrens et al. 1989; Dragone et al. 2011).

Starch accumulation has been studied mostly in freshwater microalgal strains including *Chlamydomonas reinhardtii* (Zhang et al. 2002; Eriksen et al. 2007; Thu et al. 2009), *Chlorella vulgaris* (Brányiková et al. 2011; Behrens et al. 1989; Dragone et al. 2011) and *Scenedesmus obliquus* (Rodjaroen et al. 2007). Less attention, however, has been paid to marine microalgae other than *Dunaliella tertiolecta* (Ike et al. 1997), and *Chlamydomonas perigranulata* (Hon-Nami 2006).

The marine green microalga, *Tetraselmis subcordiformis*, accumulates starch autotrophically (Zheng et al. 2011) or mixotrophically (Ji et al. 2011). The maximum starch content was 35 % DW and starch productivity was 0.26 g $L^{-1}$ $d^{-1}$. Using sulfur starvation to direct starch overproduction, *T. subcordiformis* could achieve a starch productivity of 0.62 g $L^{-1}$ $d^{-1}$ and a starch content of 62.1 % DW, thus endowing it with great potential for starch production (Yao et al. 2012).

### 3.2.2 Effect of Phosphorus Starvation

Phosphorus is indispensable for the basic processes of energy flow in cells and is one of the essential macromolecules. In *Scenedesmus quadricauda,* the first process affected by the absence of phosphorus was RNA synthesis, which leveled off relatively early in the cell cycle, *i.e.* around the 5th hour. Protein synthesis showed the first signs of restriction much later and ceased at the time when protoplast fissions were taking place and the release of daughter cells started (Zachleder et al. 1988). Accumulation of starch, however, indicated that the rate of photosynthesis was nearly normal during the entire cycle in which RNA synthesis stopped as well as in the next cycle, in which no macromolecular syntheses took place (Fig. 8). There were sufficient cellular phosphate reserves in diverse forms at the stage when RNA synthesis leveled off (Vořišek and Zachleder 1984).

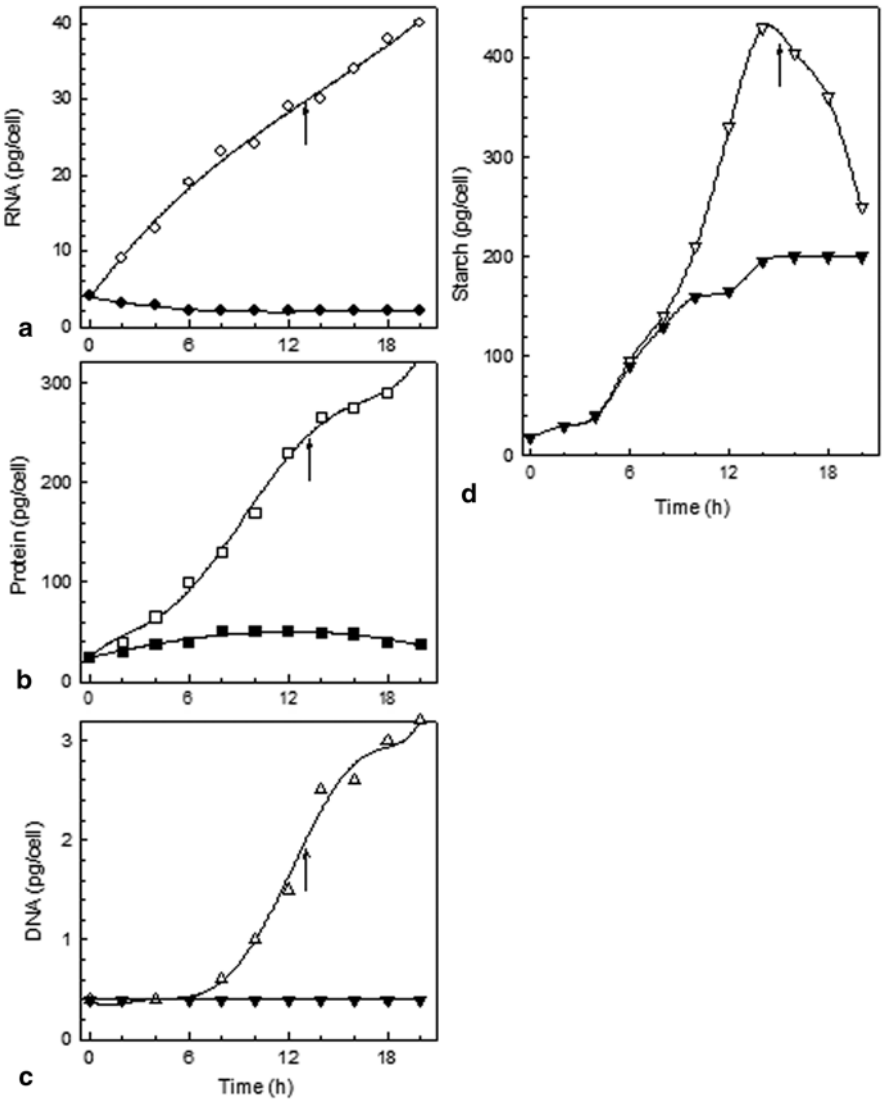


**Fig. 7** Variation in RNA (**a**), protein (**b**), DNA (**c**), starch (**d**) content during the cell cycle in control (*open symbols*) and nitrogen-starved (*closed symbols*) synchronous populations of *Scenedesmus quadricauda*. *Arrows* indicate the beginning of the dark period in control cultures. The nitrogen-starved culture was kept in continuous light. (modified according to Ballin et al. 1988)

Daughter cells generated under phosphate deficiency and exposed to light in a phosphate-free medium were unable to develop further. The synthesis of nucleic acids was completely suppressed. Protein synthesis was also severely restricted, and only a slight increase in the amount of protein was observed (Fig. 8). The photosynthetic apparatus continued to work at a moderate but sustained rate and starch accumulated, characteristic of a phosphorus-fed cell.

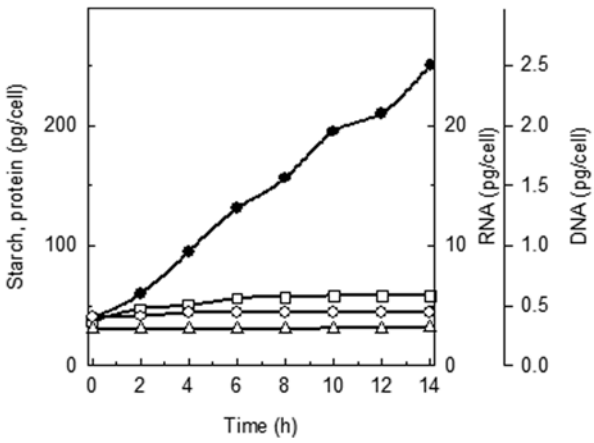


**Fig. 8** Variation in RNA (*circles*), protein (*squares*), DNA (*triangles*) and starch (*solid circles*) content during the light period of the second cell cycle in a phosphate-starved synchronous population of *Scenedesmus quadricauda*. (modified according to Zachleder et al. 1988)

In complete medium, the phosphorus-starved daughter cells resumed macromolecular syntheses with a lag of about 5 h. Due to the large amount of starch accumulated in these cells, they were able to progress through an entire cell cycle in the dark after being supplied with phosphorus (Zachleder et al. 1988).

Phosphorus limitation in *Chlorella vulgaris* caused cell division to be blocked and was severe enough to reduce biomass synthesis from the very beginning of the experiment and to stop it completely after 12 h in the absence of phosphorus in the medium, or after 20 h of growth in medium with a five fold decreased concentration of phosphate. Thereafter, starch began to degrade, and its concentration (in g/L) and relative content (% of DW) decreased (Fig. 9, Brányiková et al. 2011).

### 3.2.3 Effect of Sulfur Starvation

Sulfur, which is necessary for the metabolism of proteins, lipids, and molecules associated with electron transport chains, is another essential element that regulates starch accumulation in microalgae.

If synchronized cultures of *Scenedesmus quadricauda* were incubated under photosynthesizing conditions in a sulfur-free medium, inhibition of RNA synthesis occurred close to the end of the first cell cycle and protein synthesis ceased two hours later (practically at the time of protoplast fission). If the daughter cells derived from the starved populations were kept in sulfur-free medium, macromolecular syntheses were dramatically restricted (Fig. 10).

Only photosynthesis continued to produce starch at a similar rate to that of normally grown cells. Thus, a very large amount of starch accumulated (Figs. 10 and 11).

Supported by these reserves, when starved cells were refed with sulfur, they progressed through an entire cell cycle in the dark and divided into eight daughter cells. In sulfur-supplied cells, both in the dark and in light, RNA protein and DNA synthesis started without any delay in a similar way to the control culture. If competition for sulfur reserves occurred between growth and division processes, the former were preferred in the light and the latter in the dark (Šetlík et al. 1988).

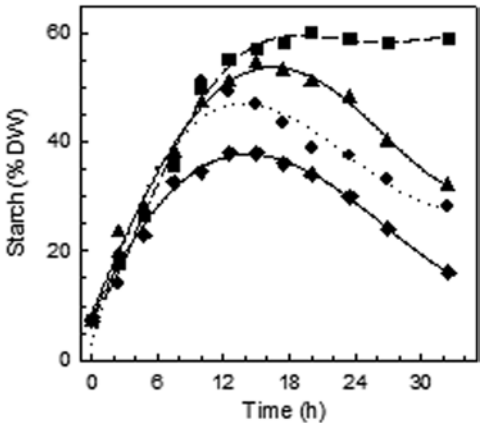


**Fig. 9** Effect of nitrogen, phosphorus, or sulfur limitation on changes to relative starch content (% of DW) in asynchronous cultures of *Chlorella vulgaris*. The cultures were grown in a laboratory photobioreactor with continuous illumination at an incident light intensity of 780 μmol/($m^2$ $s^1$) in a complete mineral medium (*diamonds, dotted line*) or in nitrogen (*diamonds, solid line*), phosphorus (*triangles*), or sulfur (*squares*) limited media. (modified according to Brányiková et al. 2011)

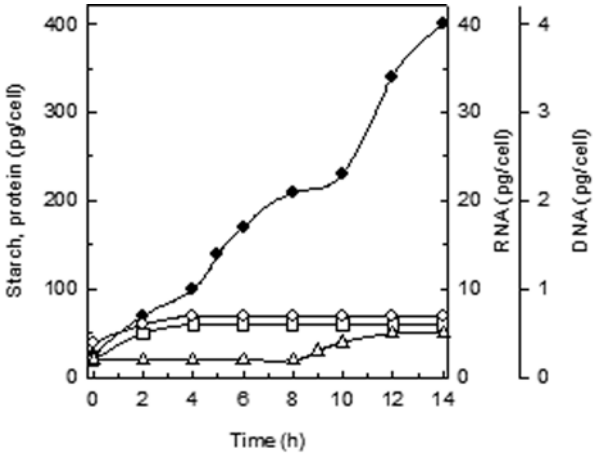


**Fig. 10** Variation in RNA (*circles*), protein (*squares*), DNA (*triangles*) and starch (*solid circles*) content during the light period of the second cell cycle in a sulfur-starved synchronous population of *Scenedesmus quadricauda*. (modified according to Šetlík et al. 1988)

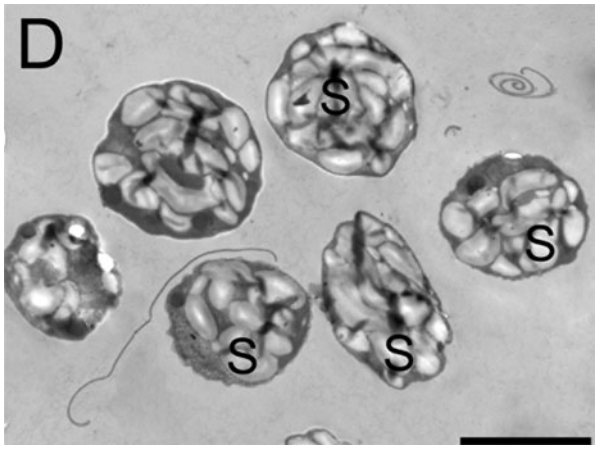


**Fig. 11** Electron microscopic photographs of cells of *Chlorella vulgaris* grown in sulfur-limited medium. *S*, starch granules. Bar: 5 μm. (modified according to Brányiková et al. 2011)

In *Chlamydomonas reinhardtii,* deprivation of sulfur caused changes in cellular metabolism resulting in cell expansion, regulated protein degradation and a significant increase (nearly ten times) in the starch/protein ratio (Ball et al. 1990; Zhang et al. 2002). Similarly, the alga *Dunaliella salina* altered the partitioning of photosynthate between starch and protein and, under sulfur-deprived conditions, induced a ten-fold increase in the starch/protein ratio (Cao et al. 2001).

Similarly, it was recently demonstrated that a sulfur-limited culture of *Chlorella vulgaris* maintained a high starch content (60%) and a starch productivity of 0.8 g $l^{-1}$ $d^{-1}$ (Brányiková et al. 2011), levels significantly higher than those calculated under nitrogen limiting conditions (0.119–0.55 g $l^{-1}$ $d^{-1}$) (Dragone et al. 2011; Fernandes et al. 2010; Behrens et al. 1989). This suggests that compared to nitrogen and phosphorus, sulfur limitation is more effective in enhancing starch productivity. For practical and industrial applications, it was also important that, in the case of sulfur limitation, the high level of starch was maintained for more than 24 h, whereas limitation of either nitrogen or phosphorus caused a decay in starch content and cell death within a few hours (Fig. 9, Brányiková et al. 2011).

Comparing cultures grown in complete medium and sulfur-starved cultures under conditions simulating alternating days and nights, degradation of starch was substantially lower in sulfur-starved cultures than in cultures grown in complete medium, while net synthesis during the light period was substantially higher (Fig. 12).

The effect of sulfur limitation was apparently due to inhibition of processes using energy and carbon derived from starch reserves (Brányiková et al. 2011) as is the case for other treatments.

## *3.3 Utilization of Waste $CO_2$*

To attain high productivity, microalgae require $CO_2$ as a photosynthetic carbon source at the same time the cost of $CO_2$ represents one of the major cost factors for algal biomass. Microalgae can tolerate and utilize substantial levels of $CO_2$; thus, photosynthetic carbon dioxide can be derived from cheap sources such as combustion of organic waste, fermentation processes or other sources (Mann et al. 2009; Douskova et al. 2009; Doucha et al. 2005). Some cheap sources of $CO_2$, such as flue gas from incineration of domestic waste (Douskova et al. 2009), biogas produced from distillery stillage (Doušková et al. 2010; Douskova et al. 2009; Kaštánek et al. 2010) or flue gas from cogeneration unit combusting biogas from anaerobic digestion of swine manure (Doušková et al. 2010; Douskova et al. 2009; Kaštánek et al. 2010) have been used successfully.

This provides an additional benefit of algal biomass production because carbon dioxide from different sources including waste incinerators, power stations, limekilns, cogeneration units, etc. *in situ* could be bioremeditated (Doušková et al. 2010; Douskova et al. 2009; Kaštánek et al. 2010; Nigam and Singh 2010).

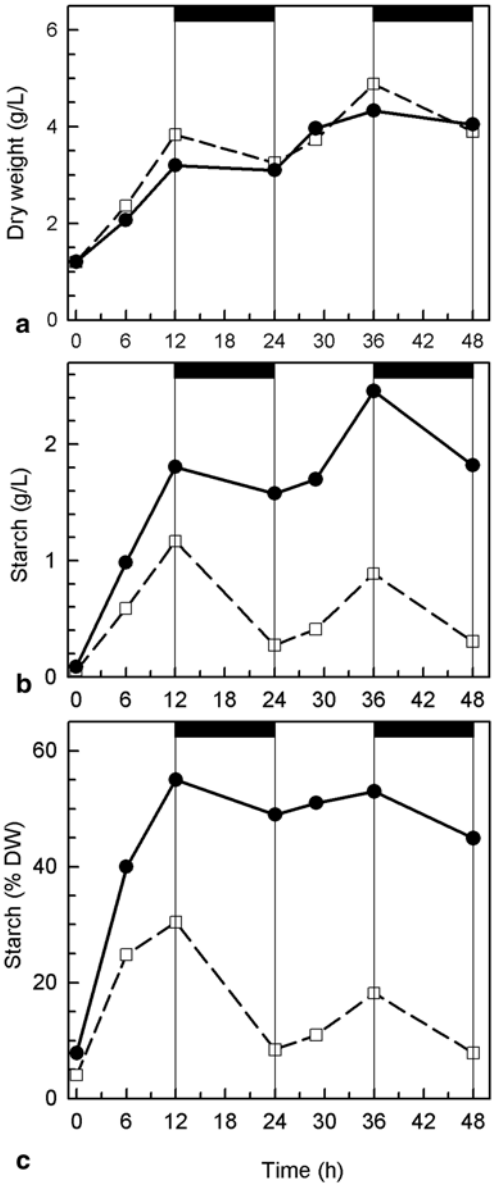


**Fig. 12** Effect of alternating light and dark periods on changes in biomass concentration (DW in g/L) (**a**), starch content (g/L) (**b**) and relative starch content (% of DW) (**c**) in untreated (*empty symbols*) and in sulfur limited (*full symbols*) cultures of *Chlorella*. (modified according to Brányiková et al. 2011)

## *3.4 Scale Up Culturing*

To verify laboratory findings, experimentation using large-scale production units is the only way to prove real outcomes of any proposed approach. Unfortunately, to date, with the exception of the paper by Brányiková et al. (2011), large-scale production studies giving relevant data on the production of lipid-enriched algal biomass have not been carried out.

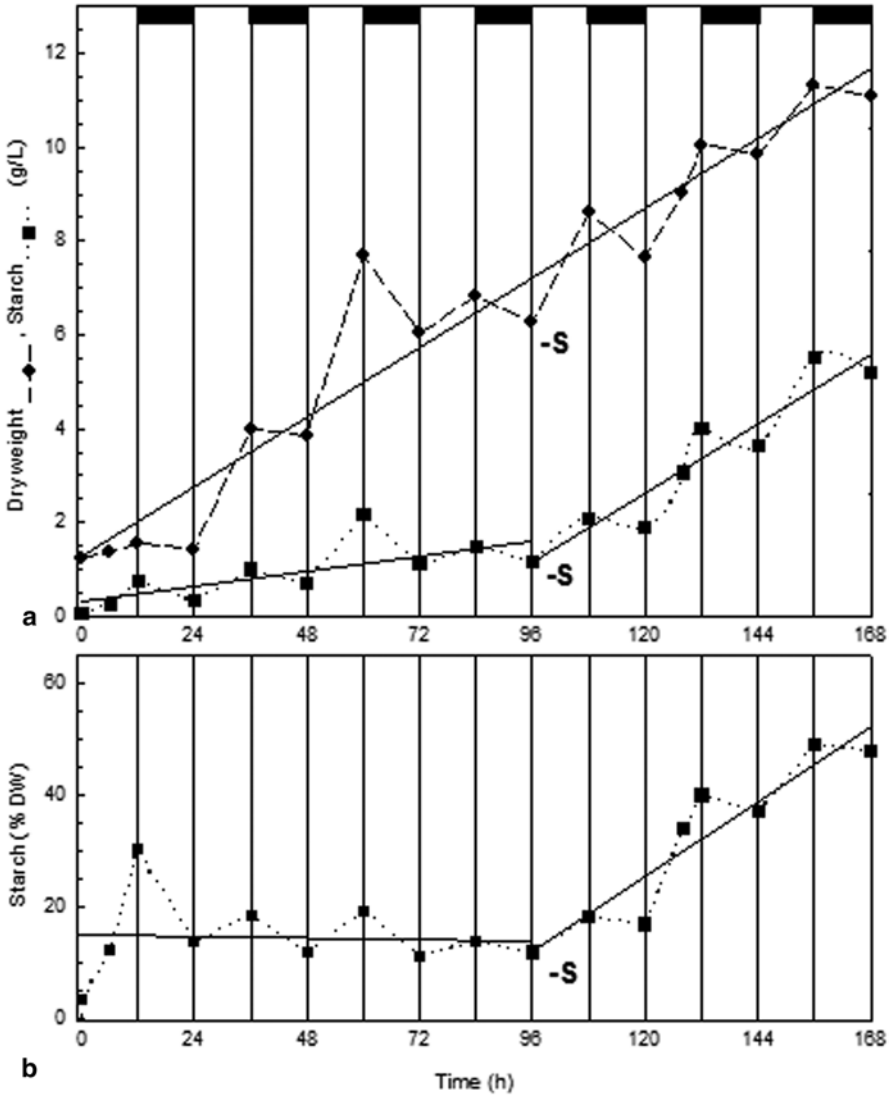


**Fig. 13** Changes in biomass concentration (DW in g/L), starch content (g/L) (**a**), and relative starch content (% of DW) (**b**) in cultures of *Chlorella vulgaris*. The cultures were grown in an outdoor scale-up thin-layer photobioreactor in complete mineral medium for 96 h and then in sulfur-limited medium (-S). *Dark periods* (nights) are marked by black stripes and separated by vertical solid lines. Linear regression curves of the 1st order (*solid lines*) were fitted to the experimental data separately for the periods of culture grown in complete medium and sulfur-limited cultivation. (after Brányiková et al. 2011)

An increase in starch production in sulfur-limited culture, up to a maximum of 50% of algal biomass (DW) was demonstrated under field conditions using the outdoor scale up, thin-layer solar photobioreactor (Fig. 13).

Despite the relatively unfavorable climatic conditions of Trebon (Czech Republic), a total yield of starch, calculated per ha over a season of 150 days, was 7 t (Brányiková et al. 2011). In optimum locations for photoautotrophic production, such as Greece, with a season lasting approximately 250 days, the overall harvest might be increased by a factor of 5.

Based on the findings described above cheap starch-enriched biomass could be produced from highly productive *Chlorella* cultures if grown in suitable outdoor photobioreactors in which photosynthetic carbon dioxide is derived from combustion of organic waste, fermentation processes or other sources (Mann et al. 2009; Douskova et al. 2009; Doucha et al. 2005). Furthermore, after starch extraction, the remaining cell residue, comprising mostly protein, can be used as an animal feed supplement to further decrease the cost of starch production. This characteristic enhances the ecological and economic impact of the proposed technology because of its potential to bioremediate carbon dioxide emissions from different $CO_2$ sources including waste incinerators, power stations, limekilns, cogeneration units, etc. *in situ*.

# 4 Bioethanol

## *4.1 Principles of Bioethanol Production from Algae*

Even though some algae can act as mini factories for the production of ethanol during dark fermentation, and attempts have been made to create genetic engineered microalgae for the direct production of ethanol (Ueda et al. 1996; Hirano et al. 1997), these approaches are still not applicable in large-scale biotechnology. The general principle of bioethanol production from microalgal biomass is similar to its production from higher plants. Algal polysaccharides (starch, eventually cellulose and hemicellulosis) are enzymatically hydrolyzed to fermentable sugars, which are subsequently fermented to ethanol by a suitable ethanol producer (yeast, bacteria) (Kelsall and Lyons 1999). Ethanol is then concentrated by distillation or membrane filtration and dehydrated. Despite the fact that the overall procedure is quite simple, and for different raw materials, well managed even in large scale, each step poses specific difficulties in the case of microalgal biomass. The microalgae store starch inside the cell, which is why the cells must be disintegrated prior to hydrolysis in order to liberate starch for the action of enzymes. Disintegration can be by mechanical means (e.g., ultrasonic, explosive disintegration, mechanical shear, etc.) or by dissolution of cell walls using enzymes (Percival Zhang et al. 2006). The starch-rich biomass is then processed using technologies similar to other starch-based feedstocks, involving processes of (1) gelatinization (heating in water) which improves the availability of starch for amylase hydrolysis (2) liquefaction i.e. breaking of starch via α-amylases into soluble, short-chain dextrins and oligosaccharides resulting in significant reduction of viscosity, (3) saccharification of the starch-hydrolysate to glucose syrup catalyzed by glucoamy-

lase and (4) fermentation of simple sugars to ethanol by a suitable strain of yeast, (e.g. *Saccharomyces cerevisiae,* for review, see Singh et al. 2010) or bacteria, e.g. *Zymomonas mobilis* (Matsumoto et al. 2003; Rubin 2008). Using special enzymes and yeast strains, the whole process can be designed so that no gelatinisation is necessary and liquefaction, saccharification and fermentation are carried out simultaneously (Doran-Peterson et al. 2009).

Finally, the ethanol is separated from the fermentation broth by distillation or membrane filtration and the concentrated ethanol (95%) can be directly used in special types of engines as so called E100, or dehydrated and blended with gasoline (Demirbas 2001; Nigam and Singh 2010; Brennan and Owende 2010). The solid residue from the process can be used as animal feed (McKendry 2002), agricultural fertilizer or as a feedstock for biogas production (for more details, see review John et al. 2011).

The recent extensive research effort to develop algal biomass with a high starch/cellulose content is aimed at providing a product that could serve as a substrate for ethanol production. As mentioned in the preceding chapters there are algal species that can be directed to produce large amounts (>50% of the dry weight) of starch, suitable for ethanol production after hydrolysis (Brányiková et al. 2011; Yao et al. 2012).

## *4.2 Approaches to Cheapen Bioethanol Production*

### 4.2.1 Carbon Dioxide Supply from Flue Gases

Since biomass accumulation by feedstock crops can utilize carbon dioxide from flue gases, their growth for bioethanol production can reduce green house gas levels as well as the price of bioethanol production (Borowitzka 2008; Douskova et al. 2009).

### 4.2.2 Hydrolysis of Algal Biomass for Yeast Nutrition

In order to achieve the highest possible fermentation yield (theoretically 0.5L g of ethanol/L g of glucose) appropriate nutrition for yeast has to be ensured (source of N, P, S etc.). The cost of nutrients can be cut by adding proteases during polysaccharide hydrolysis, which liberates free amino acids (a source of nitrogen for yeast) from the protein fraction of the algal biomass. The processes of enzymatic hydrolysis and fermentation can also be carried out simultaneously (Doran-Peterson et al. 2009).

### 4.2.3 Production of Ethanol from Residual Algal Biomass

The residual biomass obtained e.g. after oil extraction can also be used as a substrate for ethanol production, as was shown when *Chlorococcum* sp. was used as a substrate for bioethanol production through fermentation by *Saccharomyces bayanus* (Harun et al. 2010; Harun et al. 2011). The lipid-extracted microalgal debris was used for fermentation and the yield of bioethanol was about 3.8 g/L from 10 g/L of the substrate. Enzymatic hydrolysis of *Chlorococcum* sp. by cellulase obtained from *Trichoderma reesei* was examined. Hydrolysis was conducted under varying conditions of temperature, pH and substrate concentration, with constant enzyme dosage. The highest glucose yield of 64.2 % (w/w) was obtained at a temperature of 40 °C, pH 4.8, and a substrate concentration of 10 g/L of microalgal biomass (Ueno et al. 1998). Overall, the enzymatic hydrolysis process proved to be an effective mechanism to enhance the saccharification process of microalgal biomass.

### 4.2.4 Hydrolysis of Cellulose from Cell Walls

Besides starch, green algae can accumulate cellulose as the cell wall carbohydrate, which can also be used for ethanol production. The cellulosic biomass from other plant sources that from algae can be enzymatically or chemically hydrolyzed to simple sugars, which can then be fermented to ethanol (Kelsall and Lyons 1999; Doran-Peterson et al. 2009).

The advantage of microalgal cellulose is that it is not linked to lignin (unlike cellulose in wood, straw, etc.), which makes hydrolysis easier comparing to the lignocellulosic materials.

### 4.2.5 Dark Fermentation—Direct Production of Ethanol

Some algae can even serve as a self-biorefinery for ethanol production during anaerobic dark conditions by utilizing their photosynthates. Microalgae fix $CO_2$ during photosynthesis and accumulate starch in their cells. Some microalgae can also grow under dark conditions in the presence of organic nutrients such as sugars and thereby accumulate starch (heterotrophic nutrition) (Chen et al. 2009). If dark and anaerobic conditions are established, the oxidative degradation of starch becomes incomplete and, depending on the type of the microalga, hydrogen gas, carbon dioxide, ethanol, lactic acid, formic acid, acetic acid and other products can be produced in varying proportions (John et al. 2011). Algal cells contain large amounts of polysaccharides composed of glucose, which can be catabolized rapidly under dark and anaerobic conditions to ethanol. Conversion from intracellular starch to ethanol under dark and anaerobic conditions was observed in several algal strains (Hirano et al. 1997). However, the levels of conversion to ethanol were significantly different from each other. Relatively high conversion rates of 30–40 % (vs. a theoretical yield of 0.56 g of ethanol/L g of starch) were observed in two strains, *Chlamydomonas reinhardtii*

(UTEX2247) and Sak-1. Importantly, there was no need for nitrogen flushing due to the complete utilization of oxygen and there was no need for agitation for ethanol production. The optimal pH for ethanol production from *Chlamydomonas* was 7–8 and a temperature of 25–30 °C. Ethanol was also produced via dark fermentation of cellular starch from *Chlorococum littorale*, where an increase in the incubation temperature affected the mode of cellular starch decomposition and brought about an increase in ethanol productivity. If methyl viologen was added to the reaction, hydrogen formation drastically decreased while ethanol productivity increased (Ueno et al. 1998). Exploiting microalgal strains to accumulate starch/cellulose and directly utilize their enzymatic or anaerobic digestion systems to produce ethanol can provide a cost-effective bioethanol production process. (For detail review of these alternative ways of ethanol production, see John et al. 2011).

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# Oil Overproduction by Means of Microalgae

**Pavel Přibyl, Vladislav Cepák and Vilém Zachleder**

**Abstract** Microalgae have been recognized as promising microorganisms potentially capable of meeting enhanced demands of alternative fuel production due to their high growth rate. Under certain conditions, some species and strains are known to store considerable amounts of intracellular oils (neutral lipids), whose composition shows potential to be used similarly to crop oils. In spite of the fact that high growth rate and oil accumulation are mutually exclusive characteristics, rapid progress has been made in algal production systems. This review summarizes recent achievements in microalgal oil productivity, and approaches and methods to its enhancement. Future research directions and obstacles in the algal oil field are discussed.

**Keywords** Biodiesel • Fatty acid • Lipid • Lipid bodies • Microalga • Oil • Overproduction • Productivity • Triacylglycerol

**Abbreviations**

| | |
|---|---|
| ACC | Acetyl CoA carboxylase |
| ACP | Acyl carrier protein |
| ASP | Aquatic Species Program |
| BC | Biotin carboxylase |
| CCALA | Culture Collection of Autotrophic Organisms |
| DAG | Diacylglycerol |
| DAGAT | Diacylglycerol acyltransferase |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| FA | Fatty acid |
| FAS | Fatty acid synthase |

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|---|---|
| LHC | Light harvesting complex |
| MLDP | Major lipid droplet protein |
| MUFA | Monounsaturated fatty acid |
| PA | Phosphatidic acid |
| PBR | Photobioreactor |
| PDH | Pyruvatedehydrogenase |
| PEP | Phosphoenolpyruvate |
| PK | Pyruvate kinase |
| POS | Primary oleaginous species |
| PUFA | Polyunsaturated fatty acid |
| SCO | Single cell oil |
| SFA | Saturated fatty acid |
| SOS | Secondary oleaginous species |
| TAG | Triacylglycerol |
| TEM | Transmission electron microscopy |
| UFA | Unsaturated fatty acid |

## 1 Introduction

An enormous boom in algal biotechnology has been evident in recent years. Increasing energy consumption, accompanied by natural resource depletion, has led to the search for novel renewable energy resources, especially those replacing petrodiesel, as well as for reduction in carbon dioxide emissions. Feedstocks such as rapeseed, soybean or palm oil are considered to be first generation biodiesel feedstocks because they were the first crops to be used to produce biodiesel. To reduce the dependency on edible oil, alternative biofuel resources, such as non-food feedstocks, have been developed to produce biodiesel. Energy crops such as jatropha, mahua, jojoba oil or waste cooking oil represent some of these second-generation biodiesel feedstocks. Nowadays, third generation biodiesel feedstocks, which are derived from microorganisms, have emerged as one of the most promising alternative sources of oils for use in biodiesel production. More specifically, no other potential sources of third-generation biodiesel come close to microalgae in realistic production for biodiesel (Ahmad et al. 2011). In future, crude algal oil may be an important renewable feedstock not only for energy but also for the chemical or food industries. Several start-up companies are already attempting to commercialize algal oil, mostly in the United States (Chisti and Yan 2011).

However, there are also critical perspectives on microalgal oil-based biofuels. It has been shown that the total cost of nutritional salts and electrical energy needed for microalgal biodiesel production is still too high, and that the economic potential for algal biodiesel production has been overestimated regarding the balance of investments and expected returns. The calculated energy expense during production of biodiesel from microalgae is fivefold higher than the income

(Petkov et al. 2012). Some biological constraints for biodiesel production from microalgae were also formulated, most importantly, low photosynthetic efficiency and lipid productivity. Thus, screening and selection of appropriate production strain(s) must address these key criteria for the future success of algal biofuels (Day et al. 2012).

The idea of production of oils from microalgae is not new. The potential for microalgal oil as a feedstock for renewable liquid fuel production was first proposed decades ago (Meier 1955). From 1978 to 1996, the U.S. Department of Energy's Office of Fuels Development funded a program to develop renewable transportation fuels from algae. The main focus of the program, known as the Aquatic Species Program (ASP) was the production of biodiesel from oleaginous algae grown in raceway ponds, utilizing waste $CO_2$ from coal fired power plants (Sheehan et al. 1998). However, the newly developed production systems were confronted with several bottlenecks that limited the cost-competitiveness of microalgal oils compared with classical oil mining. In spite of many recent advances in microalgal production systems, no energy from microalgae is being commercially exploited at present because production of microalgal oils is still more expensive than oils from crops.

Recently, many complex reviews and studies concerning the potential to produce biodiesel from algal oil have emerged (e.g., Chisti 2007, 2008; Smith et al. 2010; Cheng and Timilsina 2011; Rosenberg et al. 2011; Singh et al. 2011), covering a large spectrum of topics such as strain selection, cultivation conditions, bioreactor construction and design, productivity assessment, biomass processing, oil extraction and transesterification, economic feasibility studies etc. Rather than to duplicate these biodiesel topics, we intend to highlight microalgal oils as a primary prerequisite for biotechnological exploitation. We believe that any further progress can only be achieved using an optimised oil production system. Thus, this review specifically deals with one of the most important characteristics of a microalgal production system, i.e. oil production.

## 2 Single Cell Oil

The term "single cell oil" (SCO) was originally referred to as a triacylglycerol type of oil from yeasts, analogous to plant and animal edible oils and fats (Ratledge 1988). However, later this term was also adopted for oils from other oleaginous microorganisms such as fungi (Ratledge 2004), chytrids or microalgae (Gouveia et al. 2009; Makri et al. 2011). Attempts to replace the production of vegetable oils by SCO from microalgae were based on two main ideas: the high growth and production rates of microalgae, and the non-competition for arable land compared to oil crops (Chisti 2007). However, there are also drawbacks of microalgal oils, the most severe being economic feasibility associated with high costs for bioreactors and downstream processing of microalgal biomass.

## 2.1 Oil Composition

Chemically, algal storage lipids (oils) are triacylglycerols (TAG) composed of glycerol and fatty acids (FA). FAs are carboxylic acids with an aliphatic chain consisting of 4–26 carbons, joined by either saturated or unsaturated bonds. In most of the algal species/strains examined, TAGs are composed primarily of saturated or monounsaturated C14-C18 FAs (Roessler 1990). As an exception, long-chain (>C20) polyunsaturated fatty acid (PUFA) synthesis and partitioning of such FAs into TAGs have been observed in the green microalga *Parietochloris incisa* (Bigogno et al. 2002a, b), red microalga *Porphyridium cruentum* (Cohen et al. 2000), marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae), *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bacillariophyceae) and the thraustochytrid *Thraustochytrium aureum* (Iida et al. 1996; Gupta et al. 2012).

Manipulation of oil composition by cultivation conditions seems to be an interesting and easy option for enhancing the proportion of desired FAs (Tang et al. 2011). Growth rate is one of the important factors influencing FA composition in *Isochrysis galbana*. At low growth rates, higher accumulation of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) into neutral lipids occurred, in contrast to high growth rates when PUFAs accumulated (Saoudihelis et al. 1994). *Nannochloropsis salina*, growing at high light intensities, had elevated levels of higher saturated FAs (palmitic and palmitoleic acids). On the other hand, low irradiances caused unsaturated FA accumulation, mostly palmitoleic acid and eicosapentaenoic acid (EPA) (Van Wagenen et al. 2012). In microalgae capable of utilizing different carbon and energy sources, the cultivation mode can influence FA composition as well. *Chlorella zofingiensis* grown heterotrophically had a higher saturated FA content than growing photoautotrophically (Liu et al. 2011). The effects of temperature on growth, lipid and FA composition were also examined. Relative amounts of EPA and the PUFA docosahexaenoic acid (DHA) increased at low temperatures in the haptophyte microalga *Pavlova lutheri* (Tatsuzawa and Takizawa 1995). Similarly, in *Nannochloropsis salina*, the unsaturated FA content was inversely proportional to temperature (Van Wagenen et al. 2012). The effect of nitrogen starvation was studied in *Parietochloris incisa* accumulating high levels of the PUFA arachidonic acid (ARA)-rich TAGs. In nitrogen starved cells a highly elevated proportion of ARA in total FAs was recorded (Solovchenko et al. 2008). In *Phaeodactylum tricornutum*, with a decreasing nitrogen concentration in the growth medium, an increased proportion of SFAs and MUFAs in neutral lipids was reported (Alonso et al. 2000). A decreased concentration of phosphorus in the growth medium caused a decreasing EPA proportion in *Monodus subterraneus* (Khozin-Goldberg and Cohen 2006). Polyunsaturated FA (EPA and DHA) concentrations declined in the diatom *Thalassiosira oceanica*, cryptophyte *Rhodomonas salina* and *Isochrysis galbana* growing in Fe-deficient media (Chen et al. 2011c).

**Table 1** List of the most common fatty acids present in microalgae. After Roessler 1990; Alonso et al. 2000; Bigogno et al. 2002a; Khozin-Goldberg and Cohen 2006; Abou-Shanab et al. 2011; Doan and Obbard 2011; Liu et al. 2011; Řezanka et al. 2011; Tang et al. 2011; Davidi et al. 2012; Přibyl et al. 2012a; Van Wagenen et al. 2012.

| Saturated and monounsaturated (SFA, MUFA) | | Polyunsaturated (PUFA) | |
|---|---|---|---|
| Lauric | C12:0 | Hexadecatrienoic (HTA) | C16:3n3 |
| Myristic | C14:0 | Linoleic (LA) | C18:2n6 |
| Palmitic | C16:0 | α-linolenic (ALA) | C18:3n3 |
| Palmitoleic | C16:1n9 | γ-linolenic (GLA) | C18:3n6 |
| Margaric | C17:0 | Stearidonic (SDA) | C18:4n3 |
| Stearic | C18:0 | Eicosatrienoic (ETE) | C20:3n6 |
| Oleic | C18:1n9 | Arachidonic (AA) | C20:4n6 |
| Erucic | C22:1n9 | Eicosapentaeonic (EPA) | C20:5n3 |
| | | Docosahexaenoic (DHA) | C22:6n3 |

### 2.1.1 Saturated and Monounsaturated Fatty Acids

Saturated fatty acids (SFA) present in microalgal oils are long-chain carboxylic acids, usually with 12–24 carbon atoms without double bonds. Some strains of thraustochytrids accumulate large amounts of SFA. *Aurantiochytrium* strains produce high levels of pentadecanoic acid and the total SFA varied up to 51 % (Chang et al. 2012). Unsaturated fatty acids (UFA) contain one (monounsaturated fatty acids, MUFA) or more (polyunsaturated fatty acids, PUFA) double bonds between carbon atoms. For most microalgae the presence of oils containing a substantial proportion of FAs, either MUFAs or PUFAs, is typical. Their quantity and composition depends on algal species (strains), cultivation techniques and growth conditions. The most common species of SFAs and MUFAs are listed in Table 1.

### 2.1.2 Polyunsaturated Fatty Acids

It is largely believed that polyunsaturated fatty acids (PUFA) are present mainly in polar lipids and are important membrane constituents in microalgae. However, PUFAs were found only rarely in microalgal TAGs; larger amounts were reported in *Porphyridium cruentum* (Cohen et al. 1988), in *Parietochloris incisa* (Bigogno et al. 2002b) or in the eustigmatophyte *Trachydiscus minutus* (Řezanka et al. 2011).

PUFAs indisputably have a favorable impact on human health; the most important are 3-omega PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Their impact is multilateral; medical studies have proven that these PUFAs prevent or lessen cardiovascular diseases, asthma, arthritis, kidney and skin injuries, diabetes II, schizophrenia etc. (Simopoulos 2008). Therefore, they are often used as supplements in human and animal diets. The most common species of PUFA are listed in Table 1.

## 2.2 Triacylglycerol Biosynthesis

As shown above, triacylglycerols (TAG) consist of glycerol with 3 esterified fatty acids (FA), which are quantitatively the main components of TAGs. In algae, *de novo* synthesis of fatty acids (FA) occurs primarily in the chloroplast, where photosynthesis provides an endogenous source of acetyl CoA, the essential precursor for FA biosynthesis. Acetyl CoA can be produced from pyruvate by pyruvate dehydrogenase (PDH), however, more than one pathway may contribute to maintaining the acetyl CoA pool. In green algae, both glycolysis and pyruvate kinase (PK), which catalyzes the irreversible synthesis of pyruvate from phosphoenolpyruvate (PEP), occurs in the chloroplast in addition to the cytosol (Andre et al. 2007). Acetate can be another precursor of acetyl CoA in chloroplasts; it may be directly activated to acetyl-CoA by a one-step reaction catalyzed by acetyl CoA synthetase. Sufficient concentrations of acetate were found in the stroma of isolated chloroplasts (Roughan 1995) and light dependent channeling of acetate into long chain FAs was described in various plants (Roughan and Ohlrogge 1996). Acetate was successfully used as a carbon source for heterotrophic DHA production in the dinoflagellate, *Crypthecodinium cohnii* (Ratledge et al. 2001; De Swaaf et al. 2003). Occurrence of plastidial ATP:citrate lyase generating acetyl CoA for fatty acid synthesis from citrate was found in some plants (Rangasamy and Ratledge 2000), however this pathway has not yet been proven in microalgae. The general overview of TAG biosynthesis is shown in Fig. 1.

The first step in FA biosynthesis, carboxylation of acetyl CoA, is initially catalyzed by acetyl CoA carboxylase (ACC) (Fig. 1, step 1). The gene transcript of biotin carboxylase (BC), the subunit of ACC, was found to be up-regulated by sugars in the growth medium in *Chlorella zofingiensis* (Liu et al. 2010). A relatively conservative multienzyme complex of fatty acid synthase (FAS) is responsible for the subsequent elongation of the FA chain in many organisms, including microalgae. Malonyl CoA, the product of acetyl CoA carboxylation, is the central carbon donor for fatty acid synthesis. The malonyl group is transferred from CoA to a protein co-factor on the acyl carrier protein (ACP; Fig. 1, step 2). All subsequent reactions of the pathway involve ACP until the finished products are ready for export from the chloroplast. The malonyl group of malonyl ACP participates in a series of condensation reactions with acyl ACP (or acetyl CoA) acceptors. The elongation of fatty acids is terminated when the acyl group is removed from ACP by an acyl-ACP thioesterase (Fig. 1, step 3) and releases free fatty acid or FA CoA is formed by acyl-CoA synthetases (Ohlrogge and Browse 1995). Excess ATP and NADPH necessary for energy-consuming FA synthesis is provided by primary photosynthetic processes. In heterotrophically cultured *Chlorella protothecoides*, glycolysis and the pentose phosphate pathway were documented, producing excess NADPH for FA biosynthesis under N-limitation conditions (Xiong et al. 2010a).

The immediate precursor of the three-carbon “glycerol backbone” of TAG is glycerol-3-phosphate, originating either from photosynthesis in the chloroplast or via glycolysis in the cytosol. TAG biosynthesis in algae has been proposed to occur

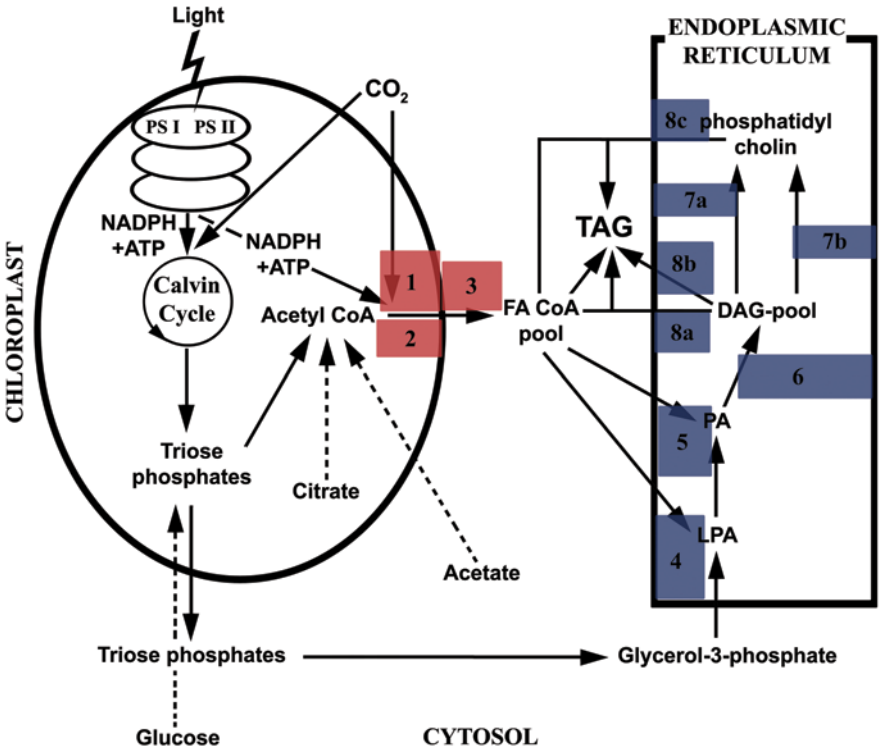


**Fig. 1** Basic scheme of triacylglycerol (TAG) biosynthesis in plants and green algae; dashed lines are unknown pathways in algae. Main enzymes involved are numbered as follows: *1* acetyl CoA carboxylase, *2* fatty acid synthase, *3* fatty acid thioesterases and acyl-CoA synthetases *4* acyl CoA:glycerol 3-phosphate acyltransferase, *5* acyl CoA:LPA acyltransferase, *6* PA phosphatase, *7a* posphatidylcholine:DAG cholinephosphotransferase, *7b* DAG:cholinephosphotransferase, *8a* acyl CoA:DAG acyltransferase, *8b* DAG:DAG:transacylase, *8c* phospholipid:DAG acyltransferase. *DAG* diacylglycerol, *LPA* lysophosphatidic acid, *PA* phosphatidic acid. (For details see: Ohlrogge and Browse 1995; Ståhl et al. 2004; Lung and Weslake 2006; Durrett et al. 2008; Hu et al. 2008; Scott et al. 2010; Merchant et al. 2011)

via the direct glycerol pathway (Ratledge 1988). Fatty acids produced in the chloroplast and transported to the cytosol as fatty acyl CoA are sequentially transferred from CoA to positions 1 and 2 of glycerol-3-phosphate by specific acyltransferases (Fig. 1, enzymatic steps 4, 5), resulting in formation of the central metabolite, phosphatidic acid (PA) (Ohlrogge and Browse 1995). Dephosphorylation of PA catalyzed by a specific phosphatase (Fig. 1, step 6) releases diacylglycerol (DAG). Moreover, in *Chlamydomonas reinhardtii*, both PA and DAG can be supplied from membrane lipids by the action of lipases (Merchant et al. 2011). In the final step of TAG synthesis, a third fatty acid is transferred to the 3 position of DAG and this step can be accomplished in three different ways. The first pathway involves acyl CoA transfer catalyzed by acyl CoA:diacylglycerol acyltransferase (DAGAT)

(Fig. 1, step 8a), an enzymatic reaction that is unique to TAG biosynthesis (Lung and Weselake 2006; Hu et al. 2008). TAGs may also be synthesized by a reversible DAG:DAG transacylase (Fig. 1, step 8b; Ståhl et al. 2004), but the corresponding genes are not yet known (Merchant et al. 2011). Finally, trans-esterification by a phospholipid:DAG acyltransferase (Fig. 1, step 8c), transferring a fatty acid from a membrane phospholipid to DAG was described in *Arabidopsis* (Ståhl et al. 2004), but this pathway is not yet confirmed in microalgae (Merchant et al. 2011).

## *2.3 Biogenesis of Lipid Bodies*

Triacylglycerols (TAGs, oils), synthesized as described above, need to be stored inside the microalgal cell, where they are mostly present in the form of cytoplasmic lipid (oil) bodies or droplets. These are variable in both size and number and mostly spherical; such a pattern of lipid bodies was found across microalgal taxons, namely in the chlorophytes *Chlamydomonas reinhardtii* (Wang et al. 2009; Li et al. 2010a; Work et al. 2010; Goodson et al. 2011; Siaut et al. 2011), *Pseudochlorococcum* sp. (Li et al. 2011b), in the trebouxiophytes *Chlorella pyrenoidosa* (Ramazanov and Ramazanov 2006), *Chlorella vulgaris* (Přibyl et al. 2012a) and *Parietochloris incisa* (Merzlyak et al. 2007), in the haptophyte *Isochrysis* (Liu and Lin 2001; Eltgroth et al. 2005), or in the eustigmatophytes *Monodopsis subterranea* (Liu and Lin 2005) and *Nannochlopsis* sp. (Yu et al. 2007). In contrast, hydrocarbons in the trebouxiophyte *Botryococcus braunii* are stored in the outer cell walls associated with their external matrix (Metzger and Largeau 2005) and under specific conditions, form huge droplets excreted outside the cell (Cepák and Lukavský 1994).

In the model green microalga *Chlamydomonas reinhardtii*, various types of lipid inclusions were described in detail using deep etch electron microscopy. (i) Plastoglobules, small lipid bodies in the chloroplast with connections to thylakoid lamellae, and (ii) small α-cytoplasmic lipid bodies, were present in nitrogen-replete cells in log growth phase. In nitrogen-starved cells, after 15 h of transfer from log growth phase, they appeared larger (iii) β-cytoplasmic lipid bodies, often connected with the endoplasmic reticulum and outer chloroplast membrane envelope. These probably arise from α-cytoplasmic lipid bodies and enlarge during prolonged nitrogen starvation. Moreover, in starchless mutants, (iv) chloroplast lipid bodies were present when nitrogen-starved for at least 12 h. Those probably arise from plastoglobuli and enlarge during prolonged nitrogen starvation. All lipid bodies were surrounded by a membrane monolayer. Light microscopy revealed a large number of lipid bodies, even when cells were filled with them (Goodson et al. 2011), suggesting that a fusion of lipid bodies does not play a substantial role in *Chlamydomonas reinhardtii*.

Using transmission electron microscopy (TEM), lipid body formation in the haptophyte *Isochrysis* sp was shown to occur in the thylakoid space of plastids. Lipid body size varied with growth phase and they finally formed spherical droplets; after their release into the cytosol they formed large lipid globules. In stationary phase cells, lipid bodies from 0.5 to 3.0 μm were detected (Liu and Lin 2001). Biogenesis

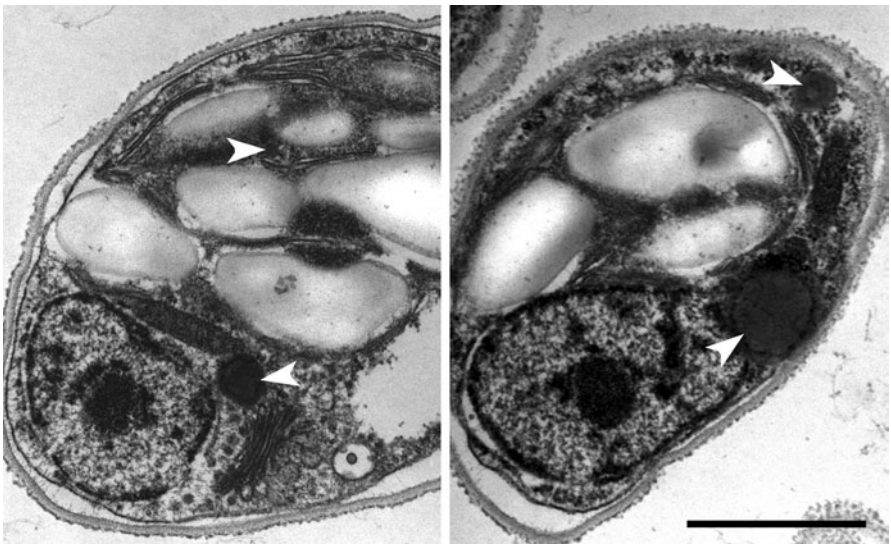

**Fig. 2** TEM images of *Chlorella vulgaris* CCALA 256 cells in the exponential-log growth phase under conditions inducing the start of oil production. *Arrowheads* depict first recognizable lipid bodies. Scale bar = 1 μm. (Original work, previously unpublished)

of lipid bodies was most recently described in detail in the eustigmatophyceaen microalga *Trachydiscus minutus*. It was found that tiny lipid droplets arose in plastidial stroma between thylakoid membranes (Přibyl et al. 2012b), remaining as plastoglobuli as described earlier (Antia et al. 1975; Liu and Lin 2005; Yamaguchi et al. 2008). These small droplets were released from the plastid but remained attached to its surface, where they fused forming larger lipid bodies. Usually one large central lipid body is formed, which can be observed by fluorescence microscopy owing its autofluorescence properties, and sometimes, especially in older cells, also by light microscopy (Přibyl et al. 2012b). Unlike *Chlamydomonas reinhardtii* (Goodson et al. 2011) a membrane surrounding the lipid bodies was not observable, corresponding to the fusion of smaller lipid bodies into larger ones.

Contrary to the biogenesis of lipid bodies as described above, in *Chlorella vulgaris*, small oil droplets were present mostly in the cytoplasm and only rarely inside the chloroplast, which was usually occupied by large starch granules (Fig. 2). Under conditions inducing energy storage in the form of oil instead of starch, the lipid bodies grew rapidly and were present solely in the cytoplasm, finally filling-up most of the cell volume (Fig. 3). Similarly, in the trebouxiophyte *Parietochloris incisa* grown in N-deficient media, cytoplasmic lipid globuli (oil bodies) occupied most of the cell volume (Merzlyak et al. 2007). Contrary to *Chlorella vulgaris*, numerous lipid bodies were present in stationary phase cultures (Merzlyak et al. 2007).

A detailed insight into molecular mechanisms of lipid body formation was presented recently. The major lipid droplet protein (MLDP) was isolated from nitrogen-deprived *Dunaliella salina* cells and sequenced. Using immunoelectron microscopy, MLDP was detected and localized at the surface of lipid globules. The induction of

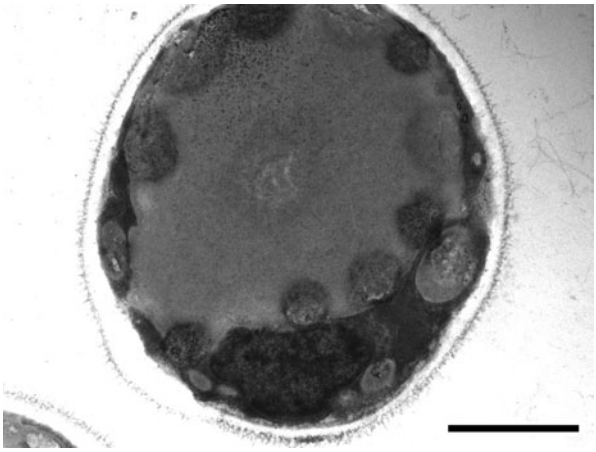

**Fig. 3** TEM image of *Chlorella vulgaris* CCALA 256 cell in the stationary growth phase, strongly nutrient-limited. A huge lipid body oppressing the nucleus towards the cell wall; minor lipid droplets are also seen. Scale bar = 1 µm. (Original work, previously unpublished)

MLDP by nitrogen deprivation was kinetically correlated with TAG accumulation, and inhibition of TAG biosynthesis impaired MLDP accumulation, suggesting that MLDP induction is co-regulated with TAG accumulation (Davidi et al. 2012). This MLDP showed high sequence similarity to both MLDP isolated from *Chlamydomonas reinhardtii* (Moellering and Benning 2010) and to the "Haematococcus oil globule protein", detectable after oil-inducing conditions (nitrogen depletion/high light) in *Haematococcus pluvialis* (Peled et al. 2011).

## 3 Overproduction of Oils

Numerous data indicate that microalgal oils are more than just a reservoir of energy-rich molecules; they are actively involved in the processes of adaptation to environmental conditions (Solovchenko 2012). PUFAs stored in TAG can be utilized for membrane rearrangements during adaptation to harsh environmental conditions (Khozin-Goldberg et al. 2005). Biosynthesis of TAG can serve as an efficient way to consume excessive solar energy, protecting against production of reactive oxygen species and photo-damage of microalgal cells (Hu et al. 2008). Parallel synthesis and storage of secondary carotenoids in TAG provides protection against stressful conditions inducing oil biosynthesis, such as high light and nutrient deficiency (Solovchenko et al. 2011; Solovchenko 2012).

From the biotechnological point of view, it is important to use algal strains that not only produce high biomass concentrations within relatively short cultivation periods, but that are also capable of achieving a high lipid content (Li et al. 2007). However, the accumulation of lipids as an energy-demanding process and the accumulation of biomass at a high growth rate are mutually exclusive characteristics (Přibyl et al. 2012a). Efforts and approaches leading to increased oil productivity therefore deserve the greatest attention in current microalgal oil biotechnology.

## *3.1 Microalgal Strains for Oil Overproduction*

According to current taxonomical knowledge, algae cover more than 30,000 species worldwide, most of them being microalgae (Guiry and Guiry 2012). Only a negligible number of them are traditionally used in experimental research or for biotechnological utilization. Our knowledge of the vast microalgal potential is therefore fundamentally limited. Therefore, any screening effort to efficiently expand information about chemical composition, specific microalgal compounds or their production potential is of great importance. Using the basic elemental content and degree of reduction, extraordinary high intracellular lipid contents (70–84 % of DW) were calculated in early publications on *Chlorella pyrenoidosa* (Milner 1948; Spoehr and Milner 1949) or *Chlorella ellipsoidea* (Iwamoto et al. 1955). Later, the actual measured maximal values were always lower; some microalgal species and strains known to produce high levels of lipids are listed in Table 2. It is remarkable that these "lipid hyperaccumulators" are mostly members of either green algae or heterokontae and comprise only four microalgal classes, namely Chlorophyceae, Trebouxiophyceae, Bacillariophyceae and Eustigmatophyceae, with the exception of some marine haptophyte or dinophyte microalgae. It is very likely that there are many more prospective species still unrevealed. Therefore, it is very important to focus relevant screening and bioprospecting concepts on microalgal lipid hyperproducers (Rodolfi et al. 2009; Abou-Shanab et al. 2011; Mutanda et al. 2011; Sydney et al. 2011).

Traditionally, microalgal species able to accumulate large amounts of oil have been described as "oleaginous" species. As the oil content in individual species or strains can substantially vary depending on the cultivation conditions, we prefer to concentrate on the inherent abilities of microalgae to synthesize a specific storage compound. The main storage compounds in the microalgal classes above are starch and oil. Here we propose to draw a line between "primary" and "secondary" oleaginous species.

### 3.1.1 Primary Oleaginous Species

Primary oleaginous species (POS) are defined here as "microalgal species using lipids (oils) as a primary and main storage compound and storing them in large amounts". Mostly members of Chromophyta are included in this group, for example microalgae from classes Eustigmatophyceae, Bacillariophyceae (diatoms), Xanthophyceae (=Tribophyceae), Chrysophyceae or some haptophytes, as *Emiliania huxleyi* or *Isochrysis* sp. As a result of extensive screening for oil-rich species in the Culture Collection of Autotrophic Organisms (CCALA), Třeboň, Czech Republic (http://www.butbn.cas.cz/ccala/index.php), some examples are shown (Fig. 4).

The amount of oil in POS can be substantially affected by cultivation conditions and also depends on growth phase; higher levels can usually be found in stationary phase (Eltgroth et al. 2005; Přibyl et al. 2012b). Microalgal strains shown in Fig. 4

**Table 2** Some microalgal species able to hyperaccumulate lipids. Only reports of at least 50% of total lipids per dry weight are presented

| Species | Class | Lipid content (% of DW) | References |
|---|---|---|---|
| *Chlorella vulgaris* | Trebouxiophyceae | 56.6 | Liu et al. (2008) |
| | | up to 60 | Přibyl et al. (2012a) |
| *Chlorella protothecoides* | Trebouxiophyceae | 50.5 | Shen et al. (2010) |
| | | 55 | Miao and Wu (2006) |
| | | 57.8 | Xiong et al. (2008) |
| *Chlorella sorokiniana* | Trebouxiophyceae | 52 | Wan et al. (2011) |
| *Chlorella pyrenoidosa* | Trebouxiophyceae | up to 57 | Li et al. (2011a) |
| *Chlorella zofingiensis* | Trebouxiophyceae | 51.1 | Liu et al. (2011) |
| | | 52 | Liu et al. (2010) |
| *Chlorella emersonii* | Trebouxiophyceae | 63 | Illman et al. (2000) |
| *Botryococcus braunii* | Trebouxiophyceae | 50 | Dayananda et al. (2005) |
| | | up to 75 | Metzger and Largeau (2005) |
| *Scenedesmus dimorphus* | Chlorophyceae | 50 | Chen et al. (2011b) |
| *Scenedesmus obliquus* | Chlorophyceae | 58 | Abou-Shanab et al. (2011) |
| *Pseudochlorococcum* sp. | Chlorophyceae | 50 | Chen et al. (2011b) |
| | | 52.1 | Li et al. (2011b) |
| *Dunaliella tertiolecta* | Chlorophyceae | up to 68 | Takagi et al. (2006) |
| *Neochloris oleoabundans* | Chlorophyceae | 54 | Tornabene et al. (1983) |
| | | 56 | Gouveia et al. (2009) |
| *Nannochloropsis* sp. | Eustigmatophyceae | 55 | Suen et al. (1987) |
| | | 60 | Rodolfi et al. (2009) |
| | | 60 | Jiang et al. (2011) |
| *Monallantus salina* | Eustigmatophyceae | 72.2 | Shifrin and Chisholm (1981) |
| *Phaeodactylum tricornutum* | Bacillariophyceae | up to 57 | Deng et al. (2009) |
| *Nitzschia palea* | Bacillariophyceae | up to 80 | Opute (1974) |
| *Cyclotella meneghiniana* | Bacillariophyceae | 58 | Sicko-Goad and Andresen (1991) |
| *Stephanodiscus binderanus* | Bacillariophyceae | 67 | Sicko-Goad and Andresen (1991) |
| *Isochrysis zhangjiangensis* | Prymnesiophyceae | 53 | Feng et al. (2011a) |
| *Crypthecodinium cohnii* | Dinophyceae | 50 | Ratledge et al. (2001) |
| | | 56 | De Swaaf et al. (2003) |

were screened for oils using old cultures maintained on agar in the culture collection for several months. Many members of this group are important producers of highly unsaturated lipids, often containing specific PUFAs such as EPA or DHA (Khozin-Goldberg et al. 2011).

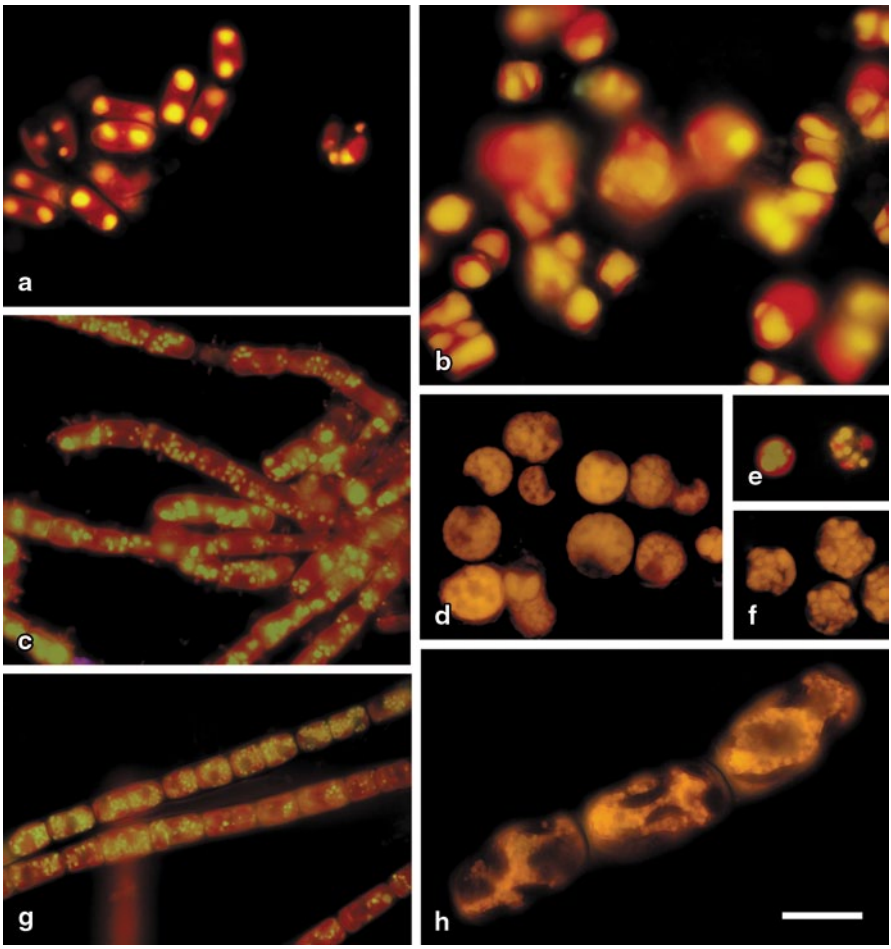


**Fig. 4** Some strains of primary oleaginous microalgal species, *fluorescence images*. Lipids were stained using *Nile Red* (*yellow-crimson*); autofluorescence of chloroplasts is seen as *red* **a** CCALA 384 *Navicula seminulum*, **b** CCALA 765 *Diadesmis gallica*, **c** CCALA 831 *Xanthonema* cf. *debile*, **d** CCALA 278 *Pleurochloris meiringiensis*, **e** CCALA 804 *Nannochloropsis* sp., **f** CCALA 280 *Chlorobotrys regularis*, **g** CCALA 512 *Tribonema aequale*, **h** CCALA 223 *Bumilleriopsis filiformis*. Scale bar = 10 μm. (Original work; previously unpublished)

### 3.1.2 Secondary Oleaginous Species

Secondary oleaginous species (SOS) are defined here as "microalgal species primarily using a storage compound other than lipids (usually starch), but capable of accumulating large amounts of lipids (oils) under specific conditions". SOS are almost exclusively green algae, mostly members of classes Chlorophyceae and Trebouxiophyceae. Some examples of SOS from the collection CCALA are shown in Fig. 5; the screening for oils was carried out similarly to that described above for POS.

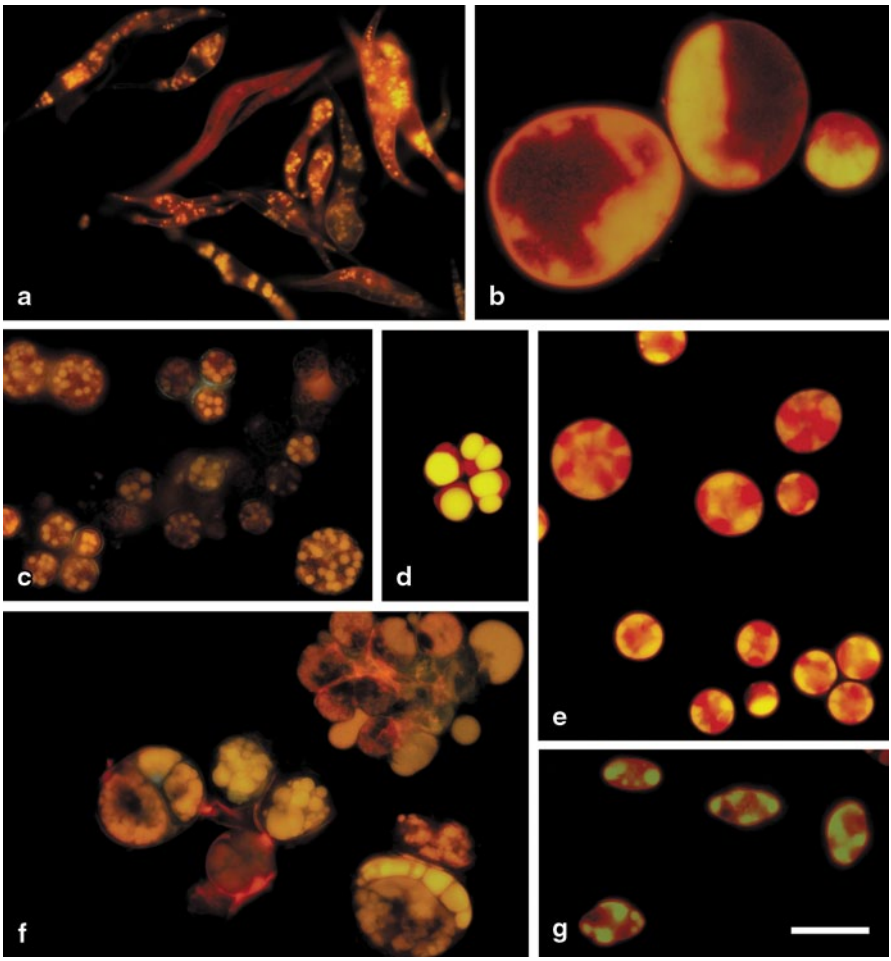


**Fig. 5** Some strains of secondary oleaginous microalgal species, *fluorescence images*. Lipids were stained using *Nile Red* (*yellow-crimson*); autofluorescence of chloroplasts is seen as *red*. **a** CCALA 212 *Ankistrodesmus spiralis*, **b** CCALA 297 *Chlorokybus atmophyticus*, **c** CCALA 780 *Botryosphaerella sudetica*, **d** CCALA 510 *Tetrastrum komarekii*, **e** CCALA 888 *Mychonastes zofingiensis*, **f** CCALA 778 *Botryococcus braunii*, **g** CCALA 397 *Oocystis* cf. *nephrocytioides*. Scale bar = 10 μm. (Original work; previously unpublished)

In SOS, oils are overproduced only when their growth has been retarded, but sufficient energy and carbon remain available. Members of this group usually possess lipids with a higher degree of saturation (Přibyl et al. 2012a) compared to POS. Induction of oil accumulation is rather easy in SOS; some approaches are given in the following chapters.

## 3.2 Methods for Oil Overproduction

As mentioned above, lipid productivity is the most important feature of any microalgal oil production system and increasing it is one of the challenges in making microalgae competitive with oil crop sources. A review showing the lipid productivities of various microalgal production systems (Chen et al. 2011a) is updated here; the most successful experimental achievements in microalgal lipid productivities are summarized (Table 3). As evident from Table 3, the majority of experiments have been based on laboratory cultivation, either photoautotrophic or heterotrophic. Up to date, there are only two large-scale trials achieving substantial lipid productivities under solar irradiation. They used completely different production systems; either POS *Nannochloropsis* sp. cultivated in 110 l closed vertical panels placed outdoors and using a nutrient deficient medium (Rodolfi et al. 2009) or SOS *Chlorella vulgaris* cultivated in a open thin-layer photobioreactor (PBR) placed in a greenhouse under conditions of natural depletion of diluted medium (Přibyl et al. 2012a). However, maximal productivities of both systems were comparable, 0.25 or 0.33 g $l^{-1}d^{-1}$, respectively.

Besides choosing an appropriate oleaginous strain, there are several approaches for enhancement of oil production in microalgae. The traditional way represents manipulation of nutrients, mostly focusing on nitrogen limitation or deficiency. Other possibilities are optimized utilization of energy inputs (light, organic substrates, $CO_2$) or improvements in lipid metabolic pathways using genetic or metabolic engineering tools.

### 3.2.1 Nutrient Deficiency

Observations of intracellular oil droplet-formation under stress (especially nutrient deprivation) have led to efforts to find a 'lipid trigger' where a simple manipulation might greatly increase oil biosynthesis (Singh et al. 2011). To gain nutrient limiting conditions, several approaches are being used. Commonly, a two-phase cultivation process is used. In the first phase, microalgae grow rapidly in nutrient-rich medium up to sufficient biomass density and then are transferred into a nutrient-deficient medium (Rodolfi et al. 2009; Ho et al. 2010; Jiang et al. 2011; Li et al. 2013). For instance, when *Scenedesmus obliquus* was photoautotrophically grown in nutrient-rich medium for 12 days and then transferred to deionized water, the lipid content increased from 12.2 to 39.8 % in the following 12 days (Ho et al. 2010). The most severe limitation of this approach is a substantial light reduction in the relatively dense algal biomass and thus a limited amount of photoautotrophically-fixed energy. In order to enable enough light for autotrophic cultures, optimization of the cultivation process (biomass density, light path and PBR used) is necessary.

The natural depletion of nutrients during batch cultivation overcomes this obstacle; when applied in scale-up processes, it can also reduce costs associated with nutrient manipulations. Nitrogen, whose deficiency is one of the most important

**Table 3** Overview of relevant recent achievements in maximal microalgal lipid productivities; only values exceeding 0.1 g $l^{-1}$ $day^{-1}$ are presented

| Microalgal strain | Cultivation mode | Carbon source | Oil productivity (g $l^{-1}$ $d^{-1}$) | References |
|---|---|---|---|---|
| *Chlorella vulgaris* CCALA 256 (=*Chlorella minutissima* UTEX 2219) | Laboratory, tubes 50 ml<br>Thin-layer PBR, 150 l | $CO_2$ | 1.425±0.135<br>0.326±0.010 | Přibyl et al. (2012a) |
| *Chlorella minutissima* UTEX 2219 | Laboratory, flasks 650 ml | $CO_2$ | <0.155 | Tang et al. (2011) |
| *Chlorella vulgaris* FACHB1068 | Laboratory, 2 l | $CO_2$ | 0.147 | Feng et al. (2011b) |
| *Chlorella zofingiensis* ASU 2 | Laboratory, tubes 300 ml | $CO_2$ | 0.312 | Chen et al. (2011b) |
| *Chlorella* sp. | Laboratory, PBR, 800 ml | $CO_2$ | 0.114±0.016 | Chiu et al. (2008) |
| *Neochloris oleoabundans* UTEX 1185 | Laboratory, bottles 800 ml | $CO_2$ | 0.133 | Li et al. (2008) |
| *Pseudochlorococcum* sp. LARB 1 | Laboratory, PBR 1.2 l | $CO_2$ | 0.35 | Li et al. (2011b) |
| *Nannochloropsis oculata* NCTU-3 | Laboratory, PBR 800 ml | $CO_2$ | 0.142 | Chiu et al. (2009) |
| *Nannochloropsis* sp. F&M-M24 | Outdoor PBR, 110 l | $CO_2$ | >0.250 | Rodolfi et al. (2009) |
| *Nannochloropsis* sp. | Laboratory, columns 1 l | $CO_2$ | 0.41 | Pal et al. (2011) |
| *Chlorella protothecoides* UTEX | Laboratory, flasks 300 ml | Glucose/CPH | 0.932 | Xu et al. (2006) |
| *Chlorella protothecoides* UTEX | Laboratory bioreactors | Glucose | <0.953 | Li et al. (2007) |
| *Chlorella protothecoides* UTEX | Laboratory, flasks 500 ml | JAH | 1.7±0.2 | Cheng et al. (2009) |
| *Chlorella protothecoides* UTEX 255 | Laboratory, flasks 150 ml | Glucose | 0.85 | Shen et al. (2010) |
| *Chlorella protothecoides* UTEX 249 | Laboratory, flasks 100 ml | Glucose/glycerol | 0.25±0.03 | Heredia-Arroyo et al. (2010) |
| *Chlorella zofingiensis* ATCC 30412 | Laboratory, fermenter 3.7 l | Glucose | 1.38 | Liu et al. (2010) |
| *Chlorella zofingiensis* ATCC 30412 | Laboratory, flasks 50 ml | Glucose | 0.263 | Liu et al. (2011) |
| *Chlorella pyrenoidosa* | Laboratory, flasks 250 ml | RSH | 0.62±0.01 | Li et al. (2011a) |

*CPH* corn powder hydrolyzate, *JAH* jerusalem artichoke hydrolyzate, *RSH* rice straw hydrolyzate

oil triggers, can be consumed very effectively by rapidly growing microalgae (Stephenson et al. 2010; Přibyl et al. 2012a). However, the final biomass obtained using this method is usually lower than in the two-phase cultivation processes. Another strategy to enhance lipid productivity is optimization of feeding rates at low nutrient concentrations. When 0.9 mM nitrate (limiting concentration) was intermittently fed ten times during log phase to the green microalga *Nannochloris* sp, in addition to the initial nitrate feed (0.9 mM), the intracellular lipid content (SOS) increased from 31.0 to 50.9 %, compared to those cells cultured in full medium without additional nitrate feeding. Cell concentration, which is usually lower in nitrogen-deficient cultures, did not significantly change (Takagi et al. 2000).

In contrast to dense photoautotrophic cultures, heterotrophic utilization of organic carbon substrates by several microalgae results in a stunning biomass density of up to 160 g $l^{-1}$ (Hyka et al. 2013) without energy limitations. However, such bioconversion of sugars to oils is not economically feasible at the large-scale level. An alternative can be a combination of photoautotrophic growth followed by addition of glucose in log phase. This enhancement of the C/N ratio acts similarly to nitrogen depletion and results in lipid induction (Xiong et al. 2010b).

**Nitrogen** It was proposed that a natural mechanism of altering lipid metabolism in microalgae is the stress response to the lack of bioavailable nitrogen (Tornabene et al. 1983). The absence of available nitrogen ceases microalgal cell division leading to storage of lipid reserves (Packer 2009; Přibyl et al. 2012a). The underlying principle is that insufficient nitrogen for protein synthesis necessary for growth channels excess carbon from photosynthesis into storage molecules and protein content may be reduced (Scott et al. 2010). Indeed, TAGs appear to be synthesized as an early response to growth under conditions where energy input exceeds the cellular capacity for energy utilization (cell growth and division). This situation can result from a variety of adverse environmental conditions such as nitrogen deficiency (Roessler 1990; Mock and Kroon 2002). Oil overproduction in response to nitrogen limitation or deficiency seems to be universal among many microalgal species independent of their classification as either POS or SOS.

The effect of nitrogen deficiency on oil overproduction in microalgae has been known for many decades (Iwamoto and Nagahashi 1955) and this property has been studied in various species and strains. Cultivation of *Neochloris oleoabundans* (SOS) in a nitrogen deficient medium increased lipid content up to 54 % of dry weight (Tornabene et al. 1983). In the green microalgae *Scenedesmus* sp. and *Nannochloris* sp. (both SOS), the intracellular lipid content of cells grown in a nitrogen-limited medium was 0.5 and 1.3 times as high respectively as in those grown in a full medium (Takagi et al. 2000; Ho et al. 2010). When nitrogen was limited (initial N = 4.4 nM) in *Pseudochlorococcum* sp. (SOS) cultures, both lipid content and neutral lipid productivity were higher (52.1 % and 0.35 g $l^{-1}$ $d^{-1}$, respectively) than obtained under N-replete conditions, *i.e.* initial N = 17.6 mM (38.3 % and 0.29 g $l^{-1}$ $d^{-1}$, respectively). Moreover, a conversion of starch to neutral lipids was shown under nitrogen-depleted conditions (Li et al. 2011b). However, in *Chlamydomonas reinhardtii* (SOS), nitrogen-depleted medium substantially induced lipid accumulation

only in low starch and starchless mutants, whereas wild type responded to nitrogen deficiency only slightly (Li et al. 2010b). Nitrogen deficiency in the eustigmatophyte *Nannochloropsis* sp. (POS) promoted lipid (mostly TAG) synthesis *de-novo*; total lipid content was approx. double compared with nitrogen sufficient conditions (Suen et al. 1987). Other work with *Nannochloropsis* sp. however reported only a 14 % increase in lipid content after transferring cultures from nitrogen sufficient to nitrogen-free medium (Jiang et al. 2011). In *Chlorella vulgaris* (SOS), increased lipid content was inversely related to the level of nitrogen in the medium. When media with nitrogen contents of 50 % or 0 % of control were used, lipids comprised 17.5 % or 35.60 % respectively of the biomass, while control cultures contained 12.3 % lipid (Mutlu et al. 2011). In both *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, nitrogen-free medium caused an increase in cellular lipid content, which was significantly higher in high carbon medium (more than 10 times) than cells grown in low carbon medium. The limiting nitrogen concentration for *Chlamydomonas reinhardtii* was determined as 1 mM $NH^{4+}$; lower values led to complete arrest of cell growth and a dramatic increase in cellular lipid content (Deng et al. 2011). In contrast to the above findings, in the haptophyte *Isochrysis zhangjiangensis* a totally opposite effect of nitrogen on lipid production was found. Nitrogen depletion led to the overproduction of carbohydrates instead of lipids, whereas lipids were induced under nitrogen (nitrate) replete conditions. A maximum intracellular lipid content of 53 % of DW was described under conditions of extremely high nitrate concentrations. It was concluded that high nitrate concentrations could be stressful for this species, resulting in unusual oil overproduction (Feng et al. 2011a).

Being one of the most ubiquitous constituents of many important molecules, including proteins and nucleic acids, nitrogen plays a principal role in all living organisms. Therefore, it is not easy to assess its mechanism of influence on lipid metabolism and, more specifically, the apparent correlation between nitrogen deficiency and oil accumulation. Non-specific effects of nitrogen deficiency as a growth-restrictive factor resulting in the accumulation of excess energy in oils was mentioned above. A specific effect of nitrogen-limitation on oil accumulation was shown in oleaginous heterotrophic microorganisms (*Schizochytrium* sp., *Mortierella alpina*). Nitrogen depleted cells up-regulate mitochondrial AMP-deaminase in order to enhance the intracellular nitrogen concentration. Adenosine monophosphate (AMP, substrate for AMP-deaminase) consumption down-regulates another enzyme, isocitrate dehydrogenase, which results in overproduction of citrate, a substrate for ATP:citrate lyase. This cytosolic enzyme uses citrate as a substrate for synthesis of acyl CoA, a limiting precursor for fatty acid biosynthesis, as shown above (Ratledge and Wynn 2002; Ratledge 2004). Although many microalgae are capable of strictly heterotrophic growth and metabolism (Bumbak et al. 2011), this mechanism has not yet been studied in microalgae and so remains speculative.

**Phosphorus** Compared to nitrogen, phosphorus deficiency has been less well investigated, but generally phosphorus limitation has similar effects to nitrogen limitation. A marked increase in cellular lipid content in *Monodopsis subterranea*, represented mainly by TAGs, has been described in response to phosphorus

depletion (Khozin-Goldberg and Cohen 2006). The volumetric amount of TAGs in the phosphorus-stressed diatom *Chaetoceros gracilis* was substantially enhanced compared to controls (2.03 and 0.06 pg cell$^{-1}$, respectively) (Lombardi and Wangerski 1991). Phosphorus limitation resulted in increased lipid content in diatoms *Phaeodactylum tricornutum*, and *Chaetoceros* sp., in haptophytes *Pavlova lutheri* and *Isochrysis galbana* and in the trebouxiophyte *Parachlorella kessleri* (Li et al. 2013). In contrast, lipid content decreased in the green alga *Nannochloris atomus* grown under phosphorus limitation (Reitan et al. 1994). Only a slight increase in lipid content was found in *Chlorella vulgaris* cultured in low-phosphorus medium (50 % of phosphorus), compared to full medium (16.7 and 12.29 % of DW, respectively) (Mutlu et al. 2011). So, it seems that stress caused by lack of phosphorus in green algae is not sufficient to trigger oil accumulation as in heterokontae or haptophyte microalgae. It is evident that induction of oils by phosphorus deficiency is more species dependent that in the case of nitrogen.

**Silicon** Oil induction by silicon deficiency is relevant only in diatoms (Bacillariophyceae) and golden microalgae (Chrysophycae), groups having their cells covered by various silica sheets, scales or frustules. As cultivation of chrysophyte microalgae is difficult, this group is not particularly promising for biotechnological purposes, and only diatoms have been extensively studied. As silicon is a structurally indispensable element for diatom reproduction, its limitation results in growth cessation and consequently in storage compounds accumulating similarly to nitrogen-limited cultures. It was shown that the silicon-stressed diatom *Chaetoceros gracilis* had a substantially higher volumetric amount of TAGs compared to control (2.85 and 0.06 pg cell$^{-1}$, respectively) and even to phosphorus-stressed cells, as mentioned above (Lombardi and Wangerski 1991). Silicon limitation during growth of *Nitzschia frustulum* led to an increased total lipid content as well (Ryu and Rorrer 2010).

Apart from a general stress effect of silicon deficiency on growth processes, there is information on a probable direct effect on enzymatic activity. The activity of acetyl CoA carboxylase (ACC) increased approximately two- and fourfold after 4 and 15 h of silicon-deficient growth, respectively, suggesting that the higher enzymatic activity may partially result from a covalent modification of the enzyme. As the increase in enzymatic activity can be blocked by the addition of protein synthesis inhibitors, it was suggested that the enhanced ACC activity could also be the result of an increase in the rate of enzyme synthesis (Roessler 1988; Roessler et al. 1994).

### 3.2.2 Energy Inputs

As shown in Chap. 2.2, lipid (especially fatty acid) biosynthesis is an energetically demanding process, thus enough energy inputs must be ensured in order that lipids can be synthesized in excess and be stored as oil energy reserves. Microalgae growing heterotrophically are fully dependent on an organic substrate (mostly glucose), a

surplus of which enhances the C/N ratio, and acts similarly to nitrogen depletion as a trigger for lipid accumulation (Xiong et al. 2010b). Photoautotrophically growing microalgae rely on light as the energy source and $CO_2$ as a source of carbon. Both are limiting factors for oil accumulation in microalgae; their levels are strongly suboptimal in most natural environments, with some exceptions such as mineral springs ($CO_2$ or $HCO_3^-$) or neuston (light). This might be one of the reasons that a high cellular oil content is only rarely observed in natural samples of microalgae.

At a low light intensity (40 µmol $m^{-2}$ $s^{-1}$), *Pavlova lutheri* contained FAs esterified mostly in polar lipids whereas at higher light intensities (90–140 µmol $m^{-2}$ $s^{-1}$) FAs were preferentially directed into TAGs (Guedes et al. 2010). The basal level of neutral lipids in the wild type model microalga *Chlamydomonas reinhardtii* was 2–3 % of DW under low light (40 µmol $m^{-2}$ $s^{-1}$) and nitrogen sufficient conditions. When induced under very high light (400 µmol $m^{-2}$ $s^{-1}$) and nitrogen deficient conditions, neutral lipid content gradually increased and reached a maximum of 6.1 % of DW after 4 days. Under the same conditions, the *Chlamydomonas reinhardtii* starchless mutant accumulated 8-fold more neutral lipid than the wild type (Li et al. 2010b). In *Nannochloropsis* sp, apart from other lipid-inducing factors such as nitrogen concentration, a sole high light intensity of 150 µmol $m^{-2}$ $s^{-1}$ resulted in substantially enhanced lipid content compared to low light intensity of 70 µmol $m^{-2}$ $s^{-1}$ (52.4 and 46.1 % of DW, respectively) after 12 days of cultivation (Jiang et al. 2011). It was found in *Pseudochlorococcum* sp. that moderate photoinhibition induced by elevated light intensity was a prerequisite for maximum formation and accumulation of neutral lipid, as the largest amount of neutral lipid was observed in cultures at a very high light intensity (600 µmol photons $m^{-2}$ $s^{-1}$) compared to lower light intensities (Li et al. 2011b).

Various $CO_2$ concentrations (5–70 % v/v) were tested to optimize lipid production in *Scenedesmus obliquus*. It was found that 10 % was the optimal $CO_2$ concentration for cumulative biomass production, and thus for lipid productivity (Ho et al. 2010). Lower $CO_2$ concentrations ranging from 0.5–12 % in air were tested in a marine strain of *Chlorella vulgaris*. Surprisingly, the optimal concentration for maximal lipid content and lipid productivity was found to be only 1 % $CO_2$ (Lv et al. 2010). This low value might be explained by the relatively low light intensity used (60 µmol photons $m^{-2}$ $s^{-1}$), and thus 1 % $CO_2$ was sufficient for photosynthetic C fixation.

### 3.2.3 Genetic and Metabolic Engineering

The most advanced method for overproduction and accumulation of oil is to improve the "wild" microalgal strains. One standard route for improvement is molecular biosciences, involving the introduction of desirable traits by gene and genome engineering. While a few algae can be transformed, the art of introducing foreign DNA into algae is in its infancy compared to the ease with which plant genomes are manipulated. Clean gene replacement approaches, for example by homologous recombination followed by marker elimination, will be very important given the likelihood that algal crops will be cultivated in open raceways or ponds (Merchant et al. 2011).

Genetic engineering of key enzymes in specific fatty acid production pathways of lipid biosynthesis is a promising target for the improvement of both quantity and quality of lipids (Schenk et al. 2008). One of the first attempts to increase the activity of enzymes involved in lipid biosynthesis was accomplished within the Aquatic Species Program funded by the U.S. Department of Energy. The gene for acetyl CoA carboxylase (ACC), a key regulatory enzyme in fatty acid biosynthesis, was cloned and characterized in the diatom *Cyclotella cryptica* (Roessler and Ohlrogge 1993). Genetic transformation of two species of diatoms has been accomplished by introducing chimeric plasmid vectors containing a bacterial antibiotic resistance gene driven by regulatory sequences from the ACC gene from the diatom *Cyclotella cryptica.* The recombinant DNA integrated into one or more random sites within the algal genome and the foreign protein was produced by the algal transformants (Dunahay et al. 1996). This system was used to introduce additional copies of the ACC gene into diatoms in an attempt to manipulate lipid accumulation in transformed strains. These experiments did not, however, demonstrate increased oil production in transformed algal cells (Sheehan et al. 1998).

The possibility of enhanced lipid production by redirecting metabolites from the starch biosynthetic pathway towards lipid synthesis was tested in SOS *Chlamydomonas reinhardtii*. Several low starch or starchless mutants were established using random mutagenesis techniques. One of the mutants defective in the small subunit of ADP-glucose pyrophosphorylase, accumulated neutral and total lipid up to 32.6 and 46.4 % of DW or 8- and 3.5-fold higher, respectively, than the wild type under lipid inducing conditions of high light intensity and nitrogen deficiency (Li et al. 2010b). Transmission electron microscopy revealed numerous starch granules in wild type *Chlamydomonas reinhardtii* and only minute lipid droplets, whereas in the starchless mutant defective in the ADP-glucose pyrophosphorylase gene, no starch granules and numerous large lipid bodies were detected (Li et al. 2010a).

Another starting point for metabolic and genetic engineering of microalgae is the engineering of the photosynthetic light capture machinery in order to improve the solar energy-to-biomass conversion. RNA interference technology was used to down-regulate the expression of light harvesting complex (LHC) proteins in *Chlamydomonas reinhardtii*. The resulting LHC mutant contained less tightly stacked thylakoids. Consequently, the strain exhibited a higher resistance to photodamage and increased light penetration in liquid culture. This newly engineered microalgal strain offers more efficient conversion of solar energy to biomass (Mussgnug et al. 2007) and, potentially, to storage compounds as starch or oil. Moreover, the gene encoding the glucose transporter was successfully introduced into the diatom, *Phaeodactylum tricornutum,* to enhance carbon input into originally obligate photoautotrophic cells (Zaslavskaia et al. 2001).

### 3.2.4 Other

Apart from previously described ways and methods, there are several less used approaches to enhance lipid production in microalgae. Total lipid content in *Chlorella vulgaris* cultures supplemented with a growth-superoptimal concentration of iron

($1.2 \times 10^{-5}$ mol $l^{-1}$ $FeCl_3$) was up to 56.6 % of DW, which was 3–7 times higher than in media supplemented with lower iron concentrations (Liu et al. 2008). The effects of deficiencies of various less studied elements (P, K, S, Ca, Fe) on lipid accumulation were examined in *Chlorella vulgaris*, with sulphur deficiency being the one that most promoted lipid biosynthesis (Deng et al. 2011).

Different salinities (0–100 mM) were tested in *Chlamydomonas reinhardtii*, showing an increase in TAGs proportional to NaCl concentration. At 100 mM NaCl, TAG content reached levels similar to that reached by this strain under nitrogen deprivation (Siaut et al. 2011). A high NaCl concentration (1 M) has been reported to increase intracellular accumulation of TAGs by about 65 % in cells of the marine microalga *Dunaliella salina* (Takagi et al. 2006). Also in the trebouxiophyte *Botryococcus braunii*, TAGs accumulated in response to NaCl in the growth medium (Zhila et al. 2011).

The effects of temperature and pH on lipid accumulation are not such prominent as other factors. Within the range 17–32 °C there were no significant differences in lipid content in the diatom *Nitzschia frustulum*. Slightly significant enhancement was recorded only at a lower temperature of 12 °C (Ryu and Rorrer 2010). The effect of pH on lipid production was investigated in *Chlamydomonas* sp. isolated from an acidic lake. TAG content in cells was greater at pH 1 than at higher pH's, probably due to accumulation of TAGs as storage lipids to prevent osmotic imbalance (Tatsuzawa et al. 1996).

An advanced method for lipid overproduction is the use of fluorescence activated cell sorting based on fluorescent staining of neutral lipids and phenotypic selection of highly fluorescent subpopulations of microalgal cells. Using this method, stable lipid overproducers of *Nannochloropsis* sp. (Doan and Obbard 2011) or *Tetraselmis suecica* (Montero et al. 2011) were isolated from wild type populations.

## *3.3 Production Systems*

The term microalgae is usually used for photoautotrophic, unicellular microorganisms utilizing $CO_2$ as a carbon source for their metabolism. Although certain species are obligate photoautotrophs, others are capable of both heterotrophic and photoautotrophic metabolism, either sequentially or simultaneously (Gladue and Maxey 1994; Lee 2001). However, the trophic level seems to be a relatively flexible physiological feature and can be easily changed by simple genetic engineering methods. Conversion of an obligate photoautotroph into a heterotrophic transformant was successfully accomplished in the diatom *Phaeodactylum tricornutum* by introducing a gene encoding the membrane glucose transporter from human erythrocytes (Zaslavskaia et al. 2001).

With respect to oil production by microalgae, there are two main production systems utilizing different sources of energy and carbon. Photoautotrophic production systems are based on photosynthetic (light dependent) fixation of $CO_2$ into storage lipids (oils) and utilize PBRs of various constructions. The main drawbacks of this approach are associated with a low efficiency of photosynthesis and a limited

delivery of light to individual cells in dense microalgal biomass. Therefore, both maximal biomass density and lipid productivity is relatively low compared to heterotrophic systems. Maximal values of biomass concentration and lipid productivity achieved were over 40 g $l^{-1}$ (Doucha and Lívanský 2009) and 1.425 g $l^{-1}$ $d^{-1}$ (Přibyl et al. 2012a), respectively. The most productive heterotrophic systems utilize glucose as a sole source of both energy and carbon. Maximal biomass concentrations achieved were up to 160 g $l^{-1}$ (Hyka et al. 2013) and lipid productivities of about 1.4–1.7 g $l^{-1}$ $d^{-1}$ (Cheng et al. 2009; Liu et al. 2010). However, heterotrophic production of oils is simply a bioconversion of compounds of lower calorific value (sugars) to higher ones (lipids). So, unless "waste" substrates such as glycerol or molasses are used, this can never become economically feasible on the large-scale level.

Sequential photoautotrophic/heterotrophic cultivation enhanced lipid yield in *Chlorella protothecoides* to values 69 % higher than heterotrophic growth. It was demonstrated that the enzyme Rubisco catalyzed $CO_2$ refixation, enhancing the carbon efficiency from sugar to oil and explaining the higher lipid yield (Xiong et al. 2010b). During mixotrophic cultivation, microalgae undergo photosynthesis and use both organic compounds and $CO_2$ as carbon sources, so the microalgae are able to live under either phototrophic or heterotrophic conditions, or both (Chen et al. 2011a).

### 3.3.1 Photoautotrophic Cultivation

Although coping with lower lipid productivity, photoautotrophic cultivation systems are relatively cheap because they can rely on solar irradiation on a large-scale. Moreover, sequestration of $CO_2$ from industrial sources can bring additional environmental benefits. There are many PBR systems described throughout the literature, both laboratory and outdoor (Doucha et al. 2005; Doucha and Lívanský 2006, 2009; Rodolfi et al. 2009; Chisti 2007; Makri et al. 2011; Chen et al. 2011a). Nevertheless, light quantity is the most limiting factor with respect to energetically demanding lipid biosynthesis.

It was found that natural depletion of elements during photoautotrophic growth of *Chlorella vulgaris* was the most effective way to increase lipid productivity (Stephenson et al. 2010; Přibyl et al. 2012a). When elemental exhaustion to a level enabling induction of lipid production is attained at too high a biomass concentration, the mean light intensity per cell is too low to supply sufficient energy for lipid biosynthesis. So, in order to use this very easy and economically advantageous procedure, it is necessary to grow cell cultures in an appropriately diluted medium. Depletion of nutrients in such a medium is attained at a lower biomass concentration and thus at a higher mean light intensity, which is needed for successful lipid production. This limit can differ in various strains but to keep irradiation of cultures above this limit is one of the most important requirements for lipid production. For instance, in a 7 mm thin-layer algal suspension grown in an outdoor PBR (150 l), the mean light intensity sufficient for lipid production can be attained at very high

biomass concentrations of up to 13 g $l^{-1}$ and relatively low incident light intensity (Li et al. 2013).

To verify laboratory findings, experimentation using large-scale production units is the only way to prove real outcomes of any proposed approach. Unfortunately, to date almost no large scale production studies giving relevant data on outdoor production of lipid-enriched algal biomass have been carried out. The only exceptions are the study with *Nannochloropsis* sp. (Rodolfi et al. 2009), and our works with *Chlorella vulgaris* (Přibyl et al. 2012a) and *Parachlorella kessleri* (Li et al. 2013). The first was accomplished in a closed vertical flat panel PBR; the latter two used a thin-layer PBR. This type of PBR provides further benefits; a high algal density reduces the risk of contamination by other species and decreases the financial input for harvesting the biomass by centrifugation (Doucha et al. 2005; Doucha and Lívanský 2006, 2009; see also the chapter by Doucha and Lívanský in this book).

### 3.3.2 Heterotrophic Cultivation

Heterotrophic cultivation without light and with the controlled addition of an organic source of carbon and energy is similar to procedures established with bacteria or yeasts in multipurpose stirred closed tanks sterilized by heat. To date, only a small number of microalgal species have been cultured heterotrophically in conventional bioreactors (Bumbak et al. 2011) and mostly various *Chlorella* species have been used for oil production experiments. For instance, *Chlorella protothecoides* is able to grow on different substrates and accumulate lipids. The maximal lipid productivities were 0.19, 0.17, and 0.25 g $l^{-1}$ $d^{-1}$ on glycerol, acetate or glucose, respectively (Heredia-Arroyo et al. 2010). In other studies, lipid productivities were calculated for *C. protothecoides* cultures grown on glucose, glycerol and a glucose:glycerol mixture (9:1). It was found that lipid productivity using glycerol was up to 80 % of those using glucose or a glucose:glycerol mixture and specific growth rates were not statistically different among all substrates (O'Grady and Morgan 2011). So, for certain heterotrophic production systems there is a possibility to replace glucose as a substrate by the cheaper glycerol, generated during biodiesel production. Also a remarkably high (900 %) lipid yield of heterotrophically grown *Chlorella zofingiensis* was found compared to photoautotrophically grown cells (Liu et al. 2011). The extraordinary high lipid productivity (1.38 g $l^{-1}$ $d^{-1}$) achieved in the heterotrophic cultivation of *C. zofingiensis* on glucose can be explained by up-regulation of the biotin carboxylase (subunit of ACCase) gene by mono- and disaccharides in the growth medium (Liu et al. 2010). Similar regulation by glucose was found in the stationary growth phase, when lipids accumulated in *Chlorella sorokiniana* (Wan et al. 2011).

*Crypthecodinium cohnii* is a marine dinophyte microalga used for large-scale DHA production. Accumulation of lipids, including DHA, in cultures grown on acetate was higher than on glucose (Ratledge et al. 2001; De Swaaf et al. 2003). This option of FA accumulation raises prospects for oil overproduction in heterotrophic or mixotrophic microalgal systems using acetate as a substrate.

In spite of high biomass and oil productivities using heterotrophic cultivation in conventional stirred bioreactors, there are several drawbacks and constraints. Utilization of organic substrates raises needs for strictly axenic cultures, enhances the risk of their contamination, and often represents an osmotic stress for microalgal cells. Selected cultures must be resistant to shear stress caused by high stirring rates needed to meet the high oxygen demands of heterotrophic cultures. Moreover, there are rheological limitations at the high viscosity of high-density cultures, requirements for expensive ingredients (vitamins, amino acids) and enhanced corrosion of bioreactors using saline media and strains (Bumbak et al. 2011).

### 3.3.3 Mixotrophic Cultivation

As mentioned above, mixotrophic cultures require $CO_2$, an organic substrate, and light. *Chlorella protothecoides* is one of the species able grow in all three cultivation modes. If grown heterotrophically or mixotrophically on glucose, overall lipid productivity was 250 % or 400 % higher, respectively, compared to autotrophic culture. Moreover, mixotrophic productivity of lipids increased with an enhanced initial glucose concentration (Heredia-Arroyo et al. 2010). The optimal glucose concentration for lipid production in a mixotrophic culture of *Chlorella sorokiniana* was about 10 g $l^{-1}$; at higher concentrations, lipid productivity decreased (Wan et al. 2011). Mixotrophically grown *Chlorella vulgaris* was also able to utilize glycerol or acetate, although with significantly lower lipid productivities compared to glucose. No differences in lipid content were found with respect to initial glucose concentration (Heredia-Arroyo et al. 2011).

Although mixotrophic cultivation systems are very productive in the laboratory, this approach is limited for large scale production due to sharing disadvantages of both photoautotrophic and heterotrophic cultivations (demands on both high mean light intensity and organic substrates, contamination risks *etc.*).

## 4 Conclusions and Prospects

In spite of several drawbacks and bottlenecks, microalgae represent a viable alternative to the conventional production of oils from crops. Here, we summarized most effective past and recent efforts in oil production using microalgae, with an emphasis on photoautotrophic cultivation systems. We discussed methods and approaches that affect microalgal oil composition and improve oil productivity using various cultivation systems. This review addresses the issue of oil productivity with respect to the selection of appropriate oleaginous species or strains, optimal ambient conditions, appropriate nutrient status and cultivation modes. We demonstrated that most successful achievements in photoautotrophic oil production have been reached using rapid growing microalgal species capable of accumulating oils in response to nutrient (mostly nitrogen) deficiency under high light and at an optimal temperature.

One of the main challenges for oil production from microalgae is to reduce the high cost of cultivation and downstream processing of the microalgal biomass. Application of the biorefinery concept to microalgal biodiesel production system is a promising approach to cut the overall costs and to provide additional benefits. Biorefining, an analogue of petroleum refining in the petrochemical industry, represents sustainable processing of the biomass. Biorefining employs modern environmentally acceptable methods of so-called green chemistry and biotechnology on a wide range of chemicals, biomolecules, fuels and energy, with no negative impact on the environment. Biorefinery processes can use materials of various origins, mostly waste products, to create highly valued products including those based on oils, as pharmaceuticals, nutraceuticals, animal feed, fertilizers, or higher generation biofuels which do not endanger food supplies. This concept assumes a microalgal PBR located close to sources of waste $CO_2$ (biogas stations, incinerators) and non-toxic wastewater rich in nitrogen and phosphorus (sewage water treatment plants) being used as basal growth medium. Microalgal biomass contains lipids (oil), carbohydrates, proteins, and other minor components such as minerals and vitamins. These components can be processed into value-added products. After oil extraction, the residues, which are rich in carbohydrates, proteins, and minor nutrients can be utilized to produce animal feed. They can also be utilized for biogas production through anaerobic digestion (Cheng and Timilsina 2011). As a possible substrate, a biogas station can use manure of domestic animals, fed in turn by residual microalgal biomass after extraction of oils or high-value products.

All byproducts and additional benefits of biorefining have potential to improve the economics of the microalgae-to-biodiesel process (Cheng and Timilsina 2011). However, to overcome some severe biological constraints, it will be necessary to improve solar energy conversion efficiency, uncouple lipid accumulation from stationary phase and make progress in understanding regulatory factors governing growth and metabolic pathways (Day et al. 2012).

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# Commercial Products from Algae

**Kelly Hudek, Lawrence C. Davis, Jwan Ibbini and Larry Erickson**

**Abstract** The growing interest in algae is associated with current commercial products and future potential biofuel developments. The focus of this chapter is on commercial products from microalgae and macroalgae. This market is estimated to be of the order of billions of dollars per year with more than 20 different commercial products. The largest market is food products, including nutraceuticals and functional foods. There are many refined products; in some cases, there are multiple product streams associated with the separation process. Production occurs in fresh water for some microalgae such as Spirulina. Many macroalgae are grown in salt water environments; there are both managed production systems and harvests from natural areas of seas and oceans. Because of the diversity of growth environments and species, many more potential products are possible, and additional research is to be encouraged. There are many additional locations in the world that can be used to produce beneficial products.

**Keywords** Macroalgae · Microalgae · Nutraceuticals · Products · Seaweeds

**Acronyms**

N Nutraceut
P Pharmaceut

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## 1 Introduction

Algae grow rapidly, produce useful products, and provide environmental benefits. This vast collection of diverse aquatic organisms has received increased attention the past few decades, and for good reason: algae have potential as future biofuels, foods, and other valuable commercial products. Furthermore, algal products can be produced sustainably. This chapter focuses on socioeconomic and environmental considerations of algae while emphasizing the organisms' commercial value.

Algae can create commodity products such as biofuels and foods. While the byproducts of biofuel production do hold potential for future products, this work focuses more on products currently on the commercial market. Items such as nutraceuticals and functional foods created with algae have grown in importance since the year 2000 and there are now significant markets for these products. With the great diversity of algae, there is great promise that algal features may serve to enhance a large variety of current products, and produce new ones. As the currently expanding market for algae-based goods indicates, the future is bright for the research and development of algal products. There are many potential useful products that can be discovered and developed through future research (De Luca et al. 2012).

Concern for the environment and global climate change has increased in recent years, and algae can provide a number of significant environmental benefits. They remove carbon dioxide from the atmosphere, helping to reduce the harmful effects of the gas on climate change and the health of the environment. Algae have also been used to reduce nitrogen and phosphorus concentrations in wastewater before it is fed back to rivers, oceans, or other bodies of water.

In order to produce a commercial product, the algae must be able to be grown at reasonable cost and with appropriate quality control to have a product with sufficient purity to serve customer needs. The processing required to have a consistent product with desired functional properties must be at a cost low enough to compete with other products. Just like any other commercial product, the value of products from algae must exceed the cost of production to have good sales and a growing market.

Algae also contribute to increased sustainable practices. In the last 500 years, agricultural production processes on land have advanced, and there are now new and well-developed approaches to conventional farming operations. Significant progress also stems from the production of commercial products in water. As an aquatic organism, algae can be grown to produce useful products without using very much land. However, commercial operations to grow algae and produce commercial goods must be carried out with appropriate environmental management to avoid degradation of water quality. This work addresses such environmental concerns, demonstrating that algae production is sustainable.

The purpose of this chapter is to bring together information on algal characteristics of commercial value while reviewing progress in the development of algal products.

## 2 Commercial Products from Algae

One of the largest markets for algae is the food industry. Commercial products include algal powders such as Spirulina that are sold as health foods (Venugopal 2009; Yuan 2008; Henrikson 2009); algal products that contain polyunsaturated fatty acids (Venugopal 2009; Shahidi 2008); polysaccharides from microalgae and seaweeds (macroalgae) that provide beneficial dietary fiber and other health benefits (Venugopal 2009; Fitton et al. 2008); carotenoids that serve as sources of antioxidants and provide Vitamin A (Venugopal 2009; Miyashita and Hosokawa 2008; Olaizola 2008; Capelli et al. 2010); algal products that give color to foods and are marketed as food colorants (Delgado-Vargas and Paredes-Lopez 2003); and seaweeds that are used as foods (Venugopal 2009; Yuan 2008).

Algae are used for animal feed, especially in aquaculture where fish, shrimp, oysters, and other seafood are produced in confined environments (Venugopal 2009; Spolaore et al. 2006).

Hydrocolloids, including agars, alginates, and carrageenans, are marketed for their functional use in foods as well as their use in many industrial products (Bixler and Porse 2011; Venugopal 2009). Many processed foods make use of hydrocolloids to provide desired texture, water holding capacity, and as a preservative (Yuan 2008; Bixler and Porse 2011).

Substances from algae also have many applications in scientific research. For example, many cyanobacteria produce phycobiliproteins which are used in analytical chemistry. Telford et al. (2001) describes their use in antigen detection by flow cytometry.

Products from algae are also used in sunscreen, cosmetics, and pharmaceutical products. Table 11.1 provides a list of refined products from algal sources.

There are many different species of algae and there are significantly different natural environments for development and production. Because of this, there have been many research studies on many different products, and some of these have resulted in products that have commercial value as demonstrated by significant markets and sales. Venugopal (2009) and Barrow and Shahidi (2008) have contributed to the effort to identify important studies and significant markets for products from algae. Sales of products from algae are at the billions of dollars per year level (Venugopal 2009; Bixler and Porse 2011).

### *2.1 Health Foods, Functional Foods, and Nutraceuticals*

One of the major commercial markets for algal products is the food industry. Algae have been consumed as foods for centuries in many parts of the world. In the 1950s, the microalgae Chlorella was considered seriously as a food source in light of rapidly growing populations. Better crop efficiency, however, fed the growing population, and microalgae never became a prominent food source.

**Table 11.1** Refined Products from Algae. (Sources: Bixler and Porse 2011; Spolaore et al. 2006; Venugopal 2009; Burja and Radianingtyas 2008; Bhatia et al. 2010; Fitton et al. 2008; Miyashita and Hosokawa 2008)

| |
|---|
| Carotenoids |
| Astaxanthin |
| Beta-carotene |
| Bixin |
| Fucoxanthin |
| Lutein |
| Lycopene |
| Polysaccharides |
| Agar |
| Alginates |
| Ascophyllan |
| Carageenan |
| Fucoidans |
| Furcelleran |
| Polyuronides |
| Polyunsaturated Fatty Acids |
| Archidonic acid (AA) |
| Docosahexaenoic acid (DHA) |
| Eicosapentaenoic acid (EPA) |
| Gamma-Linolenic acid (GLA) |
| Proteins and Amino Acids |
| Allophycocyanin |
| Kainic acid |
| Mycosporine-like amino acids |
| Phycocyanins |
| Phycoerythrins |
| Sterols |
| Desmosterol |
| Fucosterol |
| Isotopically Labeled Compounds |
| Carbon 13 Isotopes |
| Nitrogen 15 Isotopes |
| Hydrogen 2 Isotopes |

In the last 50 years, there has been increased interest and understanding of the benefits of many different chemicals in foods. Food science has advanced during this period, and the importance of diet and exercise has been emphasized in public education.

It's clear that the term nutraceutical comes from combining the terms nutrition and pharmaceutical. It's an appropriate term because in terms of health benefits nutraceuticals usually lie somewhere between food and medication. The U.S. Institute of Medicine defined a nutraceutical compound as "any substance that is a food or part of a food which provides medicinal or health benefits including the prevention and treatment of disease, beyond the traditional nutrients it contains" (Burja and Radianingtyas 2008). Functional foods are closely related, defined as "products derived from natural sources, whose consumption is likely to benefit human health and enhance performance" (Burja and Radianingtyas 2008).

Nutraceuticals may be present in a whole food product such as seaweed; they may also be found in dietary supplements in which the nutraceutical providing the health benefit has been concentrated relative to its concentration in a natural product. Spirulina supplements, in powder or tablet form, are an example of a nutraceutical. Spirulina has been shown to provide "medicinal or health benefits…beyond the traditional nutrients it contains." Some nutraceutical substances that come from algae include antioxidants and carotenoids.

The medical benefits of nutraceuticals include the prevention and treatment of disease. The terms health foods, functional foods, and nutraceuticals are all used to describe many of the food products from algae that have health benefits. Weight reduction, promotion of healthy bones and teeth, cholesterol reduction, disease resistance, improved immune system, anti cancer properties, healthier gut and digestive system, blood pressure control and reduction, heart health, antioxidant properties, and antiviral properties are examples of health benefits associated with different algal food products. The increased popularity of health foods, functional foods, and nutraceuticals associated with algae is due to a better understanding of nutrition and health benefits, commercial availability, increased purchasing power, advances in food processing, and globalization of foods from algae (Venugopal 2009).

## *2.2 Aquaculture and Animal Feed*

Algae are used in commercial aquaculture operations for the production of salmon, shrimp, other fish, and mollusks. The algae supply important nutrients such as polyunsaturated fatty acids (PUFAs), carotenoids, and proteins (Venugopal 2009; Becker 2004). The production of algae for use in aquaculture is often on site, which allows the algae to flow into the aquaculture tanks where they are consumed (Zmora and Richmond 2004). Becker (2004) lists more than 30 species of algae that have been used as feed in aquaculture. Some species are selected because they accumulate large quantities of PUFAs (Cryptomonas sp. and Nannochloropsis sp.) (Becker 2004; Venugopal 2009). Zmora and Richmond (2004) describe several different bioreactors that are used to grow algae that are used for animal feed, including flat plate reactors, polyethylene bag reactors, and open tanks.

Seaweed and fish can be polycultured such that both products are produced in the same growth environment. Significantly higher mean net production rates of seaweed have been observed (Venugopal 2009). The effluents from aquaculture have been fed to seaweed farms where they provide needed nutrients (Venugopal 2009). This provides a good environmental management option for the effluents from aquaculture.

Mollusks, such as oysters, clams, and mussels are fed significant quantities of algae. Many different microalgal species have been used as feed for mollusks (Muller-Feuga 2004). Shrimp farming occurs in the United States, Ecuador, Thailand, China, and other countries of South East Asia. Microalgae may be used in specialized production facilities (Muller-Feuga 2004).

Algae can also be incorporated in feed for animals such as pets and farm animals. The species used the most in animal feed are Spirulina, Chlorella, and Scenesdesmus species. Supplementing the feed of cows, horses, pigs, poultry, and even cats and dogs with algal biomass has had positive results on the health of these animals (Spolaore et al. 2006). In poultry feed, up to 5–10 % can be safely replaced with algal biomass as a partial replacement for conventional proteins. Any higher concentrations, however, can result in undesirable effects such as reduced growth rate and changes in color and flavor of chicken eggs (Becker 2004). In pigs, algae can replace conventional proteins such as soybean or fish meal without serious issues. It has been suggested that at least 33 % of the total protein supplied can be replaced without adverse effects (Becker 2004).

## *2.3 Polyunsaturated Fatty Acids*

Polyunsaturated fatty acids (PUFAs) are important for many aspects of human health. Recently, attention has been focused especially on omega-3 fatty acids because of their association with many health benefits. Fish and other seafood are good sources of these substances, but safety issues arise because fish can also accumulate mercury or other toxins, making them less safe for consumption (Spolaore et al. 2006). Due to a growing world population and overfishing, marine fish are not a sustainable source of PUFAs. Marine microalgae are a source of commercial omega-3 PUFAs, and macroalgae have also been shown to be a significant source of PUFAs (Venugopal 2009). Algae, in fact, are the primary producers of basic fatty acids in marine food webs (Bergé and Barnathan 2005). PUFAs from algae also have the advantage of being vegetarian in nature.

Solvent extraction is used to extract PUFAs from algae. Because of concern for preservation of the product, supercritical carbon dioxide has been used in some cases (Peretti et al. 2003; Venugopal 2009).

Many positive health effects have been linked with the consumption of PUFAs, especially the omega-3s. Venugopal (2009) provides a list of nutraceutical potentials of omega-3 fatty acids. Consumption of these PUFAs can possibly prevent atherosclerosis, arrhythmias, and chronic obstructive pulmonary diseases, reduce blood pressure, reduce symptoms in asthma patients, fight against manic-depressive illness, protect against chronic obstructive pulmonary diseases, alleviate symptoms of cystic fibrosis, prevent relapses in patients with Crohn's disease, prevent various cancers, provide bone health, and improve brain functions in children (Venugopal 2009). The omega-3 Docosahexaenoic acid (DHA) is an important component of tissue in the brain, nervous system, and eye (Venugopal 2009). Because of this, DHA is very important in the development of infants, as well as in growing children. A few studies have shown improvement in behavior of children whose diets have been supplemented with fatty acids, including children with attention deficit hyperactivity disorder (ADHD) (Venugopal 2009; Young and Conquer 2008).

Algae-derived PUFAs, such as DHA and Eicopentaenoic acid (EPA), are already commercially available as supplements that can be purchased at many nutri-

tion stores or pharmacies. The microalga *Crypthecodinium cohnii* is a significant heterotrophic producer of DHA. The lipids of *C. cohnii* contain up to 30% DHA (De Swaaf et al. 2003). The alga *Schizochytrium limacinum* has also been reported as an excellent DHA producer (Chi et al. 2007). A study to test the effectiveness of DHA was conducted in 485 subjects over the age of 55 with age-related cognitive decline (ARCD). The study demonstrated that DHA supplementation, using DHA from *Schizochytrium sp*., improved episodic memory and learning in older adults that were healthy apart from mild memory problems (Yurko-Mauro et al. 2010).

One example of how producing commercial products from algae can accompany the production of renewable fuels is demonstrated by Chi et al. (2007). A major byproduct of biodiesel production is crude glycerol, an impure substance of low economic value. Glycerol was shown to be an inexpensive potential substance with which to produce DHA using Schizochytrium sp.

The production of PUFAs by microalgae can be optimized in many ways. Reducing nitrogen concentration can result in higher fatty acid production. Gamma-linolenic acid content has been increased in Spirulina by adding fatty acids and fatty acid precursors to the production media (Kim et al. 2012). Using light and dark two-stage culture was also beneficial.

### *2.4 Sunscreen Protection of Mycosporine-like Amino Acids*

In the search for sun-protective chemicals, Mycosporine-like Amino Acids (MAAs) have been identified as significant due to their ability to absorb harmful UV radiation. MAAs have been found in bacteria, cyanobacteria, microalgae, macroalgae, and some other multicellular organisms. One of these MAAs is Porphyra-334, which has been isolated from *Porphyra vietnamensis* and investigated for its value as a sunscreen (Bhatia et al. 2010). Cyanobacteria also produce MAAs, and several species that have high yields have been studied (Musher and Fatma 2011). Tao et al. (2008) have shown that MAAs have antioxidative activities, and that they can protect against lipid oxidation. Carreto and Carignan (2011) have reviewed the structure and physicochemical properties of MAAs. A commercial product for use as a sunscreen has been developed (Andes Natural Skin Care 2012).

## 3 Important Species of Microalgae and Cyanobacteria, and their Products

### *3.1 Spirulina*

*Arthrospira platensis* and *Arthrospira maxima*, both of which go by the common name Spirulina, are two species of cyanobacteria that are grown for their commercial value. Spirulina can grow in alkaline waters where competition with other

**Table 11.2** Amino acid composition in protein from Spirulina, cow's milk and whole egg. (Sources include Henrikson 2009, wdatech.free.fr/CSUTECH-t/spirulinafacts.htm, Clement et al. 1967, www.geb.org/food-manufacturers/egg-nutrition-and-trends/nutrient composition#top,www.dairyamerica.com/acid.cfm)

| Amino acid | Spirulina | Cow's milk | Whole egg |
|---|---|---|---|
| Alanine | 7.5 | 3.3 | 5.6 |
| Arginine | 7.7 | 3.4 | 6.0 |
| Aspartic + Asparagine | 11.7 | 7.2 | 10.0 |
| Cystine | 0.9 | 0.9 | 2.3 |
| Glutamic + Glutamine | 13.6 | 19.9 | 13.1 |
| Glycine | 5.1 | 2.0 | 3.4 |
| Histidine | 2.4 | 2.6 | 2.4 |
| Isoleucine | 5.2 | 5.7 | 5.5 |
| Leucine | 7.9 | 9.3 | 8.5 |
| Lysine | 4.2 | 7.6 | 7.2 |
| Methionine | 2.1 | 2.4 | 3.1 |
| Phenylalanine | 4.2 | 4.5 | 5.3 |
| Proline | 4.0 | 9.3 | 4.0 |
| Serine | 4.3 | 5.2 | 7.4 |
| Threonine | 4.5 | 4.3 | 4.8 |
| Tryptophan | 1.4 | 1.3 | 1.2 |
| Tyrosine | 3.8 | 4.6 | 4.1 |
| Valine | 6.0 | 6.4 | 6.1 |

Note that because of rounding, columns do not sum to exactly 100 %. Values reported by different sources may vary by a relative concentration of 10–20 % for any amino acid. There are few primary literature sources for spirulina, most are secondary and of uncertain reliability

organisms is reduced. Productivities of about 12 metric tons/hectare per year can be obtained in open pond systems (Henrikson 2009). Spirulina are high in protein and there are many other nutraceutical and functional food products associated with Spirulina. A representative amino acid distribution in Spirulina is shown in Table 11.2. Values for cow's milk and whole egg are shown for comparison. Spirulina powder is marketed in grocery stores and health food stores, as are Spirulina tablets. It is grown in the United States, Mexico, Japan, Thailand, China, Taiwan, and India (Henrikson 2009). It is also harvested from naturally occurring sources such as Lake Chad (Fitton et al. 2008; Henrikson 2009).

Spirulina is rich in Vitamin A (beta carotene), Vitamin K, and Vitamin $B_{12}$. It also contains an antioxidant rich mixture of at least 10 carotenoids and essential polyunsaturated fatty acids such as linoleic acid (Henrikson 2009). The mineral content of Spirulina depends on the water in which it was grown, but iron, calcium, and magnesium are some of the important minerals that are usually present and give the Spirulina high nutritional value.

Sulfated polysaccharides that are produced by Spirulina have medicinal value as antiviral agents. Viruses to be treated with extracts of sulfate- and phosphate-bound

polysaccharides include the herpes group of viruses and HIV viruses (Burja and Radianingtyas 2008). A dietary supplement for promoting health hormonal balance has been developed using a blend of Spirulina platensis powder with powders of other organisms (Burja and Radianingtyas 2008).

Spirulina and extracts from Spirulina have exhibited therapeutic properties such as cancer prevention, reduction of blood cholesterol levels, and stimulation of the immunological system. Phycocyanins, carotenoids, organic acids, polyunsaturated fatty acids, and sulfated polysaccharides have been identified as substances that may have therapeutic value. Some of these substances act as antioxidants and free radical scavengers that reduce the oxidative stress associated with reactive oxygen species (El-Baky et al. 2008). Supercritical carbon dioxide can be used to extract antioxidants from algae (Mendiola et al. 2008). Spirulina boosts the immune system, which is important in preventing viral infection and cancer (Kralovec and Barrow 2008). A beneficial effect of Spirulina on asthma has been reported (Kralovec and Barrow 2008).

## *3.2 Astaxanthin from Haematococcus pluvialis*

Astaxanthin (3,3'-dihydroxy-ß-carotene-4,4'-dione) is a red-colored pigment carotenoid that is produced commercially for use for aquaculture feeds for salmon and trout as well as for human uses. The green alga, *Haematococcus pluvialis*, produces significant concentrations up to about 3 % dry weight and as much as 390 mg/L of broth. However, one of the challenges of production is contamination. Thus, pure culture photobioreactors have been used for the growth of this organism (Olaizola 2008; Venugopal 2009). Cyanotech Corporation in Hawaii uses a combination of closed photobioreactors and open culture ponds to produce Astaxanthin in Haematococcus. The production process involves growing the algae in the photobioreactors. This is followed by deprivation of nitrate and phosphate, and increased temperature and light intensity. Haematocysts form and Astaxanthin accumulates (Lorenz and Cysewski 2000). The cells appear as shown in Fig. 11.1 when they are growing; their color then changes when the haematocysts form and the Astaxanthin accumulates as shown in Fig. 11.2. The Astaxanthin is separated by crushing the algae and extracting the Astaxanthin; supercritical carbon dioxide has been used for extraction (Venugopal 2009). Ranjbar et al. (2008) have reported good results with a bubble column photobioreactor. Because the commercial market is in millions of dollars per year (Valensa 2009), research to find new strains is continuing (Dragos et al. 2011). While the production using Haematococcus is the most widely used process for human use; for animal feeds, synthetic processes have been developed. A recent economic assessment study reports that the production cost is estimated to be $ 718/kg of Astaxanthin and $ 18/kg of Haematococcus, which is less than the estimated cost of synthesis by the chemical process (Li et al. 2011).

Astaxanthin is used in human health because of its antioxidant activity (Ranjbar et al. 2008; Valensa 2009). Valensa (2009) has a patent in relation to oxidative

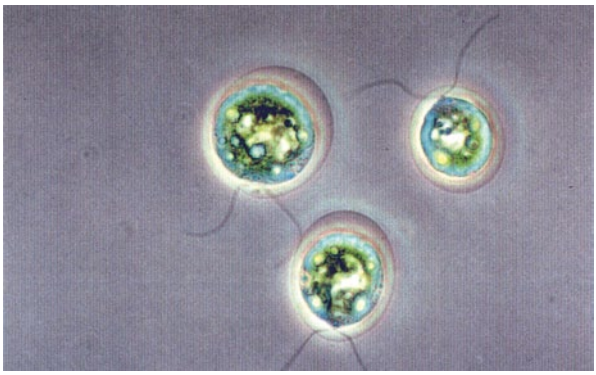

**Fig. 11.1** Green Growing Haematococcus; courtesy of Cyanotech Corporation

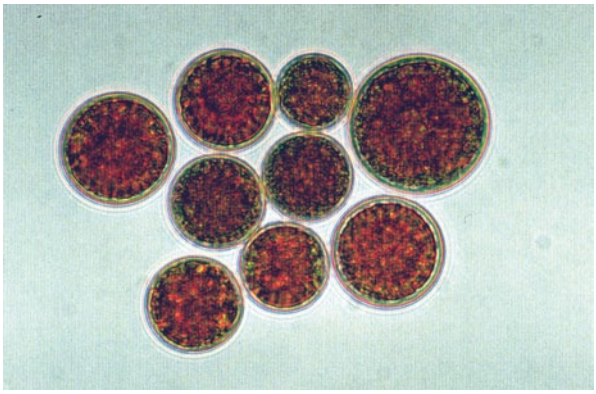

**Fig. 11.2** Red Haematocysts; courtesy of Cyanotech Corporation

stress-related eye health conditions. The antioxidant properties of Astaxanthin protect against oxidative stress, protein degradation, macular degeneration, and reduced visual function in eye health studies (Nakajima et al. 2008; Parisi et al. 2008; Wu et al. 2006; Nagaki et al. 2002). Astaxanthin is also of interest because of its cancer prevention properties (Valensa 2009).

The antioxidant properties of Astaxanthin may provide protective effects which are beneficial with respect to management of neurodegenerative disorders associated with Parkinson's disease (Ikeda et al. 2008). These antioxidant properties of Astaxanthin inhibit the production of inflammatory compounds. Thus, Astaxanthin has anti-inflammatory properties that are of interest in cardiovascular disease and rheumatoid arthritis (Capelli et al. 2010; Choi et al. 2008; Pashkow et al. 2008).

Olaizola (2008) has reviewed the beneficial properties of Astaxanthin as a nutraceutical and functional food. Photoprotective properties associated with skin and eye health, immunomodulating capability, cardiovascular health benefits, and anticancer activity are included. The health benefits are related to the antioxidant properties of Astaxanthin.

## 3.3 Chlorella

Chlorella species are single-celled green alga. Chlorella has been produced commercially since the first pilot project in 1953 (Iwamoto 2004), and is still widely used as a dietary supplement as well as for aquaculture and animal feed (Kralovec and Barrow 2008). It has been grown commercially in photobioreactors and in open ponds, and is produced by more than 70 companies (Spolaore et al. 2006; Iwamoto 2004). The size of the market for Chlorella is of the order of billions of dollars per year (Spolaore et al. 2006).

An important substance in Chlorella is β-1,3-glucan, which is a free-radical scavenger that stimulates immune response. Biological studies have focused on antitumor effects associated with polysaccharides that have immunostimulating activity (Kralovec and Barrow 2008). The conclusion of several studies is that Chlorella is beneficial in suppressing several forms of cancer. Resistance to pathogen invasion has also been observed (Kralovec and Barrow 2008).

Chlorella extracts can lower blood sugar levels, reduce the concentration of blood lipids, increase hemoglobin concentration, and act as a hepatoprotective agent (Burja and Radianingtyas 2008; Iwamoto 2004). The cell wall of Chlorella needs to be disrupted mechanically in order for the beneficial compounds in Chlorella to be available when Chlorella powder is used as a health food.

## 3.4 Dunaliella

Dunaliella species are flagellate green microalgae, and are found in many marine and freshwater habitats. Dunaliella are marketed for their value as a functional food product. The commercial value is due to the high concentration of vitamin A, β-carotene, and the presence of other carotenoids that have value as antioxidants. Products from Dunaliella are also used as colorants in foods (Ben-Amotz 2004; Venugopal 2009; Delgado-Vargas and Paredes-Lopez 2003). Ben-Amotz (2004) has reviewed production methods for Dunaliella. Using intensive cultivation in ponds with constructed raceways with paddle wheels, 200 mg/m$^2$ per day of β-carotene can be produced. Salt concentration and intense solar radiation contribute to successful production of the biomass and β-carotene. Nitrogen deficiency is also used to stimulate carotenoid production.

One of the natural environments where Dunaliella is found is the Dead Sea. The high salt concentration and high solar radiation are beneficial to carotenoid production by Dunaliella in the Dead Sea (AbuSara et al. 2011). Many cosmetic products are available that include minerals and algae from the dead sea.

One of the characteristics of Dunaliella is the thin elastic plasma membrane that encloses the cell. This is easily disrupted for recovery of the carotenoids (Pisal and Lele 2005). The ability to accumulate carotenoids to about 12% of dry weight is another important characteristic. Since Dunaliella has been used as a food for many years, it is recognized as safe for consumption (Venugopal 2009). This is important for industrial exploitation of the products from Dunaliella.

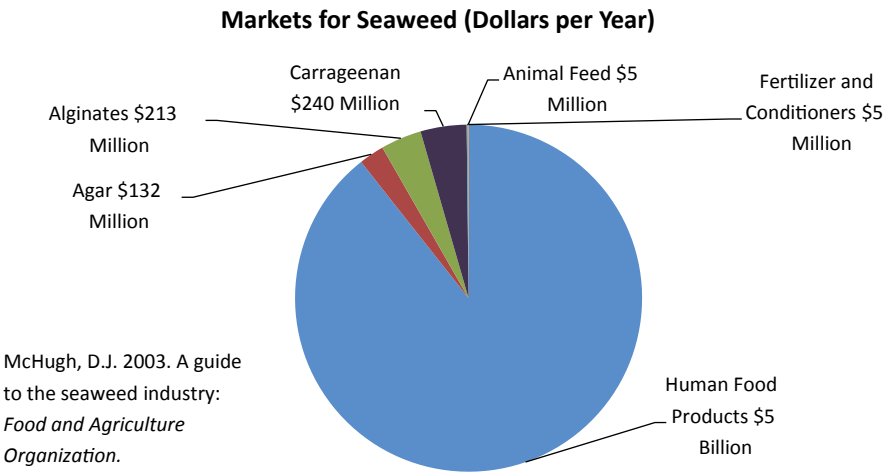


**Fig. 11.3** Global value of seaweed products per year

β-carotene is a precursor of vitamin A which prevents some eye disorders and some cancers. Results of experiments show a positive effect of extracts from Dunaliella with antioxidant properties on health of diabetics, though more study may be needed (Ruperez et al. 2009).

## 4 Important Macroalgae and their Products

Seaweeds are marine macroalgae that produce edible products, industrial hydrocolloids, and nutraceuticals. There are Chlorophyceae (green), Rhodophyceae (red), and Phaeophyceae (brown) seaweeds. The market for seaweed products is worth billions of dollars per year, and the scale of the different markets for seaweed products is shown in Fig. 11.3, using data from McHugh (2003).

The hydrocolloids agar, alginates, and carrageenans have major applications in processed food products such as yogurt, soymilk, and deli meats, to name just a few. Agar has applications as thermally reversible gel in pastry fillings and in bacteriological laboratories. Alginates are used in restructured meats and in pharmaceutical products. Carrageenan has applications as a toothpaste binder, in dairy foods, and water gels (Bixler and Porse 2011). Seaweed hydrocolloid sales exceeded 80,000 metric tons in 2009, with prices in the range from $10–$20 per kg (Bixler and Porse 2011).

Gracilaria and Gelidium are the principal seaweeds for the commercial production of agar. Gracilaria is commercially cultivated in Chile, Indonesia, Thailand, and several other countries. Gelidium is harvested from natural sources in Spain, Portugal, Morocco, Japan, and Mexico (Bixler and Porse 2011). Gracilaria and Gelidium are red macroalgae or Rhodophyceae. Gracilaria has significant amounts of polyunsaturated fatty acids, which have nutraceutical benefits (Yuan 2008).

**Table 11.3** Amino acid composition in protein from Palmaria palmate and Porphyra tenera (Percent of total). (Source: Yuan 2008)

| Amino Acid | Palmaria | Porphyra |
|---|---|---|
| Histidine | 0.5 | 1.52 |
| Isoleucine | 3.7 | 4.34 |
| Leucine | 7.1 | 9.44 |
| Lysine | 3.3 | 4.88 |
| Methionine | 2.7 | 1.19 |
| Phenylalanine | 5.1 | 4.23 |
| Threonine | 3.6 | 4.34 |
| Tryptophan | 3.4 | 2.60 |
| Valine | 6.9 | 6.94 |
| Aspartic acid | 18.5 | 7.59 |
| Glutamic acid | 9.9 | 7.81 |
| Hydroxy Proline | 2.3 | 0.0 |
| Proline | 1.8 | 6.94 |
| Serine | 6.3 | 3.14 |
| Glycine | 13.3 | 7.81 |
| Alanine | 6.7 | 8.03 |
| Arginine | 5.1 | 17.79 |

Alginates are produced by Laminaria japonica and Lessonia nigrescens, brown macroalgae, or Phaeophyceae.

Carrageenan is produced by red macroalgae, or Rhodophyceae, including Gigartina, Sarcothalia, Kappaphycus alvarezii, and Eucheuma denticulatum. Recently farmed Kappaphycus and farmed Eucheuma have been important sources of carageenans (Bixler and Porse 2011).

The interest in seaweeds as sources of nutraceuticals and functional foods was enhanced by epidemiological data that showed that people who consumed seaweeds regularly had greatly reduced mortality rates for coronary heart disease and reduced cholesterol levels (Yuan 2008). Epidemiological studies also showed reduced incidences of breast and prostate cancers among populations that consumed seaweeds regularly (Yuan 2008). Research with animal populations has provided supporting data that show that seaweeds have health benefits. (Yuan 2008).

The seaweeds Palmaria and Porphyra have a long history of being consumed in Japan and other Asian countries, as well as in some European countries and North America. The amino acid composition for these Rhodophyceae is shown in Table 11.3. The amount of protein and the amino acid profile both vary with the growth environment, including the nitrogen concentration, the geographical location, and time of year (Yuan 2008).

Fitzgerald et al. (2011) have reviewed the literature on proteins and peptides from macroalgae and their applications as nutraceuticals and medicinal products. Several bioactive peptides have been isolated from macroalgae, and their functional properties have been reported. Lectins have been isolated from Rhodophyta and used for clinical blood typing. Lectins have been used as analgesics, anti-inflammatory products, antibacterial agents, and in anticancer therapies (Fitzgerald et al.

**Table 11.4** Total carotenoid, fucoxanthin, and fucoxanthinol content (mg/g) in selected Phaeophyceae, brown seaweeds. (Source: Miyashita and Hosokawa (2008). Values are mg/g dry weight.)

| Species | Total Carotenoid | Fucoxanthin | Fucoxanthinol |
|---|---|---|---|
| Ecklonia radiate | 6.85 | 1.65 | 0.24 |
| Carphophyllum maschalocarpum | 6.21 | 1.17 | – |
| C. plumosum | 5.68 | 1.44 | 0.41 |
| Cystophora retroflexa | 4.71 | 0.46 | 0.62 |
| Sargassum sinclairii | 9.79 | 0.54 | – |

2011). Phycobiliproteins have been used as a label on antibodies and other biological molecules. They also have value because of their antioxidant, anti-diabetic, and anticancer properties (Fitzgerald et al. 2011). The investigation of the properties of these proteins provides greater understanding of the benefits associated with a diet in which these proteins are present in algal foods such as seaweed. Fitzgerald et al. (2011) review algal food products with bioactive peptides, including beverages and bakery products.

Seaweeds have significant amounts of dietary fiber, and this is beneficial because of reductions in plasma cholesterol and glucose concentrations (Yuan 2008). The water holding capacity of algal polysaccharides is also beneficial.

The mineral content of seaweeds varies with the growth environment and the season of the year. Seaweeds are considered to be a good source of minerals such as iodine, iron, calcium, and magnesium. There is also the potential for bioaccumulation of lead, cadmium, mercury, and arsenic in seaweed. Thus, the growth environment, especially water quality, is important.

There are important carotenoids in seaweeds. Flucoxanthin is the characteristic pigment of Phaeophyceae. Brown macroalgae produce significant amounts of flucoxanthin and flucoxanthinol (Miyashita and Hosokawa 2008). The concentration varies with the season and the life cycle of these algae. Some representative data is shown in Table 11.4. Flucoxanthin is of interest because of its ability to inhibit the proliferation of cancer cells and its beneficial value as an antiobesity compound (Miyashita and Hosokakawa 2008). Since the brown kelps are grown for their hydrocolloid content, the fucoxanthin has the potential to be recovered as a valuable byproduct.

Pressurized liquid extraction has been used with ethanol to extract fucoxanthin from Eisenia bicyclis, a common edible brown macroalgae (Shang et al. 2011). The optimal conditions of 90% ethanol in water at 110°C, and 2500 psi, were reported for extraction effectiveness. Mise et al. (2011) investigated extraction and drying to obtain a fucoxanthin-rich product of high purity. They reported that the instability of fucoxanthin because of oxidation and heat was a consideration in developing effective commercial products. The effectiveness of the products was evaluated using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay to evaluate the radical scavenging activity of each product. Comparison of drying methods showed that freeze-dried powder was best when evaluated using the DPPH assay (Mise et al. 2011).

The dietary fiber in seaweeds has antiobesity value in the diets of obese individuals (Venugopal 2009). The seaweed polysaccharides have a higher water holding

**Fig. 11.4** Fucus vesiculosus; courtesy of Irish Seaweed Research Group. Copyright Michael D. Guiry, Algaebase

capacity than cellulosic fibers. They can be added to foods that are low in fiber to improve functional properties, such as viscosity, texture, and water binding (Venugopal 2009).

Because of their antibacterial and antiviral activities, the carrageenans are used as effective preservatives in processed foods. Venugopal (2009) has reviewed research on the effectiveness of several algal compounds for control of human pathogens. Alginate dietary fibers stimulate the immune system. Alginates and carrageenans can be used in place of lipids in formulated products to reduce caloric intake in the diet (Venugopal 2009).

## *4.1 Fucus Vesiculosus*

Figure 11.4 is a photograph of the seaweed *Fucus vesiculosus*, which belongs to the group Phaeophyceae, or brown algae, and is more commonly known as bladderwrack. It is high in iodine, and it contains fucoidans and antioxidants. It is used in cosmetics because of anti-aging properties (Fujimura et al. 2002). Dietary fibers from this seaweed, including sulfated polysaccharides, fucans, and alginic acid derivatives, have value for their anticoagulant, anti-inflammatory, antiviral, and antitumoral properties (Ruperez et al. 2002). Polyphenols appear to be important contributors to the antioxidant capacity of powders of Fucus and commercial fucoidans (Diaz-Rubio et al. 2009). Autohydrolysis is a method used to extract fucoidan from the Fucus (Rodriquez-Jasso et al. 2013). The antioxidant activities of brown algae have many applications in the food and cosmetic industries (Tutour et al. 1998).

## *4.2 Chondrus Crispus*

Figure 11.5 is a photo of the red alga *Chondrus crispus*, also known as Irish Moss. This Rhodophycea has a long history as a source of carrageenan. Carageenan from

**Fig. 11.5** Chondrus crispus; courtesy of Irish Seaweed Research Group. Copyright Michael D. Guiry, Algaebase

Irish Moss is a mixture of kappa carrageenan, which forms strong opaque gels with potassium salts, and lambda carrageenan, which creates viscosity suitable for suspensions (Fitton et al. 2008).

*Chondrus crispus* produces compounds for protection from solar radiation. Mycosporine amino acids are UV-absorbing compounds from Irish Moss that can be incorporated into sunscreens (Burja and Radianingtyas 2008). Irish Moss is used on skin and in foods. It is used in ice creams and yogurt as a stabilizer, and in tooth pastes as a binding agent.

## 5 Commercial Production of Algae

### *5.1 Farming of Seaweed*

The commercial production of seaweeds occurs in sea coasts of Korea, Japan, China, the Philippines, Taiwan, Indonesia, Russia, Italy, France, the United States, and Chile (Venugopal 2009). More than 200 species are utilized for useful products. One vegetative propagation method starts with small quantities of the species attached to bamboo poles fixed to the subsurface ground. The macroalgae grow for 60–75 days, with a harvestable yield of 2–8 dry tons per hectare. Specific growth rates are of the order of 0.38/day under early growth conditions (Venugopal 2009). Attached seaweeds generally have higher yields than floating species.

About 60–70 % of the 16 million tons of seaweed produced per year was used for human consumption in 2005 (Venugopal 2009). The hydrocolloids produced from seaweeds account for about 10–15 % of the market. The largest fraction of the production is brown seaweeds. The quantity of seaweed from farmed areas exceeds that harvested from natural production.

**Fig. 11.6** Raceway ponds for growing Spirulina; courtesy of Robert Henrikson; from Earth Food Spirulina. (Henrikson 2009)

## *5.2 Production of Microalgae*

The commercial production of microalgae has been accomplished in several different environments and bioreactor systems. The commercial production of Spirulina has been continuing since 1982 at Earth Rise Farms in California (Henrikson 2009). Ponds with liners, like those shown in Fig. 11.6, are used to grow Spirulina at a high pH, with carbon dioxide bubbled into the water to supply carbon that is needed for growth. In order to avoid contamination, nitrogen, potassium, and other needed nutrients are supplied from sterilized sources. Hu (2004) points out that when Spirulina are cultured at pH 9.5 to 9.8, this enables Spirulina to have a competitive growth advantage that leads to good quality products. Paddle wheels are used to provide mixing and water flow in raceway ponds (Hu 2004; Henrikson 2009).

Spirulina grow optimally at 35–38°C; however, temperature is not controlled in the open ponds. Commercial operations have been set up in the sunny California desert, in Hawaii, and in other locations where sun and temperature are appropriate for good production. At Earth Rise Farms, Colorado River water is used as the source of fresh water.

Filtration is used to separate the Spirulina from the water. Spray drying is one of the more common forms of drying, as it is a quick process and preserves heat sensitive nutrients (Hu 2004; Henrikson 2009).

Dunaliella commercial production with beta-carotene as the product of interest occurs in environments with excellent solar radiation, in brine water with high levels of magnesium (Ben-Amotz 2004). Most production occurs in open ponds or raceways near a good source of sea water. By operating at high brine concentrations, contamination problems are reduced. The use of evaporated concentrated sea water or augmentation by adding salt to achieve the desired salinity has been reported, as has the reuse of the production brine water after harvest (Ben-Amotz 2004). With raceways, productivity can reach 200 mg of beta carotene per square meter per day (Ben-Amotz 2004). Seawater with 1.5 M NaCl, more than 0.4 M $MgSO_4$, and 0.1 M $CaCl_2$ and pH control have been recommended for good growth of Dunaliella. High pH and nitrogen deficiency are both used to enhance beta-carotene production.

Harvesting and separation of the beta-carotene is not well described in the literature for established commercial processes. It is known that extraction has been used to separate the beta-carotene from the other substances which are present (Ben-Amotz 2004). In some cases, the product may contain other carotenoids, including lutein, neoxanthin, zeaxanthin, violaxanthin, and cryptoxanthin (Ben-Amotz 2004).

Chlorella have been produced commercially using mixotrophic production with acetic acid as a source of carbon for growth and product formation. Bacterial contamination is one of the challenges faced by Chlorella producers. With light and carbon sources such as acetic acid, the biomass production is 1.5–2 times higher than for photoautotrophic growth (Iwamoto 2004). The yield of biomass for mixotrophic growth has been analyzed by Lee et al. (1985) and Lee and Erickson (1987). Energetic yields are often higher for mixotrophic growth compared to photoautotrophic growth. Most of the Chlorella production is by mixotrophic mass cultivation (Iwamoto 2004).

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# Recovery of Lipids from Algae

**Dheeban Chakravarthi Kannan and Vikram M. Pattarkine**

**Abstract** One of the crucial steps in generating biofuel from algae is the separation and recovery of lipids from algal biomass. These lipids are eventually converted into liquid biofuel after processing and refining. This chapter presents an overview of extraction techniques and some of the challenges in applying these techniques to industrial-scale algal biofuel production. Lipids are well-encased inside algal cell walls. The aqueous environment of the cells makes it even more difficult to extract the lipids. Hexane extraction is presently the most economical method. Cell-disrupting methods have been attempted as complementary techniques to hexane extraction. Other methods such as super-critical fluid extraction and microwave extraction that may prove better in future are still in developmental stages. The need to dry the algal biomass is a key challenge in hexane extraction. The development of on-site smaller-capacity technologies can be another vital step to enhance industrial-scale biofuel production.

**Keywords** Microalgae · Extraction · Lipid · Extraction efficiency · Hexane extraction · Supercritical carbon dioxide extraction · Extraction cost · On-site technology · Scale-up

**Acronyms**

| | |
|---|---|
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |

## 1 Introduction

Fahy et al. (2009) define lipids as "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and

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polyketides) and/or by carbocation-based condensations of isoprene units (prenol lipids and sterol lipids).” Algal lipids are mainly found as (a) lipid droplets inside the cytoplasm and (b) cell membrane-bound components. Lipid droplets are mostly in the form of triacyl glycerides and fatty acids. Membrane-bound lipids often tend to be phospholipids. They are intracellular components that are well-encased by the cell wall. Some algal species, such as *Botryococcus braunii* also produce straight chain hydrocarbon type of lipids (C8-C30 alkanes and alkenes) that are predominantly extracellular (Wolf et al. 1985).

## 2 Challenges in Algal Lipid Extraction

Typical vegetable oils are interspersed copiously inside the oilseed kernel. The seed kernel is ripe with oil (25–60 % by fresh weight) and even a slight press with fingers is enough to feel the expelled oil (Pramanik 2003; Akintayo 2004). Algal lipids, however, are well-bound by the thick cell walls of individual algal cells. Compared with oilseeds, algal lipid content is comparatively lower—typically 1.5–4 % by fresh weight or 15–40 % by dry weight (Chisti 2007)—but the lipid yield per unit area of cultivation is much higher. Vegetable oil inside oilseeds can often be efficiently extracted by mechanical pressing in a first step; only the remnant needs to be extracted by solvent extraction for complete oil recovery. For algal lipids, mechanical pressing is not effective and more intensive extraction procedures such as solvent extraction are needed from the beginning. The presence of sturdy cell wall and the sparse distribution in larger biomass load presents the major problem for the extraction of algal lipids.

Lipids are inside the aqueous environment of algal cell. This tends to resist the diffusion of non-polar solvents such as hexane, if such a solvent is employed straightaway. If a polar solvent such as methanol is employed before that, it may diffuse inside the cell first and facilitate the diffusion of less polar solvent later. Such polar solvents are not effective by themselves to extract the algal lipids. The algal cells may need to be dried first to facilitate effective extraction employing non-polar solvents directly. Even when the algal cells are dried, the interior cellular components are still hydrophobic; therefore diffusion of a polar solvent first may still be desirable. Also, the quantity of solvent needed to extract lipids from wet cells is normally much more than for dry cells.

The different types of lipids inside the algal cells show varying degrees of extraction effectiveness depending upon the type of solvents used. To effectively extract neutral lipids, non-polar solvents such as hexane are needed. To effectively extract phospholipids, polar solvents such as acetone are required (Halim et al. 2012; Vandana et al. 2001). At times a mixture of solvents with specific composition or a series of solvents may be necessary for effective extraction. Neutral lipids are hydrophobic and have weak van der Waals attraction between the molecules. A non-polar organic solvent such as hexane penetrates into the cell, interacts with

neutral lipid molecules forming the globular droplet through similar van der Waals force, and forms an organic solvent-lipid complex. This complex elutes out of the cell because of the concentration gradient. Some neutral lipids are found as a complex with polar lipids. This complex is in strong hydrogen bonding with proteins in the cell membrane. The van der Waals interaction between non-polar organic solvent and neutral lipids is too weak to disrupt the membrane-associated lipid-protein complexes. But a polar organic solvent such as methanol or iso-propyl alcohol can disrupt such lipid-protein associations through hydrogen bonds with polar lipids. This complex can then similarly elute out from the cell membrane by concentration gradient (Halim et al. 2012; Kates 1986; Medina et al. 1998).

Extraction efficiency can be strain-specific. Some algal species such as *Dunaliella salina* and *Dunaliella tertiolecta* lack the sturdy primary cell wall that makes extraction of intra-cellular lipids difficult. They contain only the secondary cell membrane. Lipid extraction may be easier with such species. Extraction efficiency can also depend on the lipid content; efficiency is normally low for cells with low lipid content.

The kinetics of extraction of lipids out of the cells was reported to follow first order kinetics, attributed to diffusion based on concentration gradient. The solvent: (dry) biomass ratio depends on lipid content and the respective solvent-cellular interaction (strain-specific) (Halim et al. 2012). Increase in temperature has been reported to result in increase in lipid yield in many cases. But in some cases, beyond a certain temperature oxidative degradation may lower the yield (Halim et al. 2012). The extent of lipid extraction by a solvent is also limited by thermodynamic phase equilibrium (Wang and Weller 2006). Multiple washing may be needed to increase the yield beyond a certain limit.

High value metabolites such as astaxanthin, β-carotene, and omega fatty acids are recovered from crude extracts by various chromatographic methods.

## 3 Extraction Methods

### *3.1 Solvent Extraction*

Solvent extraction involves using a solvent (usually non-polar organic, sometimes in combination with polar co-solvents) that permeates inside the algal cells through the cell wall, attempts to fill the cells inside and outside as a continuum, and helps partitioning lipids out of the cells. The algal biomass is then typically separated by gravity. Lipids are recovered by stripping the solvent off. Hexane, cyclohexane (Harun et al. 2010), and heptane (Horst et al. 2012) are commonly used for solvent extraction. Benzene, ether, acetone (Harun et al. 2010), and other polar co-solvents such as methanol (Bligh and Dyer 1959) have also been used.

Folch method (Folch et al. 1957) and Bligh-Dyer method (Bligh and Dyer 1959) employing methanol-chloroform mixtures are generally used as analytical methods.

These methods require large quantities of solvents that are expensive and toxic. Therefore they are not preferred for industrial-scale extraction. Soxhlet hexane extraction method refers to an extraction process where the solvent is heated, percolated, and repeatedly refluxed to extract lipids. This method, however, provides low lipid yields (Mercer and Armenta 2011; Halim et al. 2010).

Hexane extraction efficiencies have been rated around 95 % (Stephenson et al. 2010; Frank et al. 2011). Nagle and Lemke (1989) reported 90 % extraction efficiency for n-butanol and 78 % for hexane/2-propanol (40/60, vol %) (400 g wet biomass (15 % solids), 1200 g solvent, boiling temperature, 90 min). This technology is industrial-scale.

Martek Biosciences Corporation (2003) has employed hexane for extraction of the poly-unsaturated fatty acid docosahexaenoic acid (DHA) from *Schizochytrium* sp. for use as a food ingredient. The report states: "The de-oiled biomass is separated from the oil-rich hexane phase (miscella) by centrifugation and/or filtration. The miscella is chilled and held for a period of time to allow any saturated fats, or other high melting point components (stearins), to crystallize (winterization). The chilled miscella is centrifuged and/or filtered to remove the solid phase. Hexane is then removed from the miscella, leaving behind the winterized oil." Hexane extraction facilities are well-known for vegetable oil extraction (Lundquist et al. 2010).

The need to dry the algal biomass before extraction is the main challenge with solvent extraction. The solvent requirement is much higher (by an order of a magnitude) if algae are not dried. The Aquatic Species Program reported that direct solvent extraction was unlikely to be feasible for wet biomass (Sheehan et al. 1998). Solvents are toxic and pose handling concerns. Compromising the value of high-value byproducts such as nutraceuticals is another concern (Sahena et al. 2009). The options for benign solvents for industrial applications are limited. Also, energy is lost during residual solvent recovery from deoiled algal biomass.

Hexane extraction is presently the most economical option for extracting algal lipids. Other methods may become less expensive in future after further research and development. A study conducted by Lundquist et al. (2010) showed that the cost of hexane extraction, modeled similar to the soybean oil extraction facilities, would depend on the scale of the system. The cost of extraction was calculated to be $60/barrel for a model based on a 100 T/d facility shared by five 100-hectare raceway pond systems. But the cost decreased to ~ $15/barrel for a model based on a 4,000 T/d facility shared by fifty 400-hectare systems. The cost was calculated based on the extraction plant capital cost, operating cost, and the cost to transport algae from harvest site to the extraction plant. The study stressed that development of on-site smaller capacity extraction technologies that could handle algal biomass grown locally were crucial to reduce the cost of algal lipid extraction. This would reduce the considerable cost of transporting harvested biomass to remote large extraction facilities.

### 3.1.1 Complementary Methods

Mechanical Methods

Extraction can be facilitated by physically rupturing or disrupting the algal cells. Such techniques usually complement solvent extraction. Mechanical methods include pressing, grinding with beads that agitate and collide with cell walls, and wet milling. Shen et al. (2009) compared different mechanical disruption methods and concluded that optimum methods would be strain-specific, depending on cellular characteristics. *Scenedesmus dimorphus* (big, bean-shaped and clustered cells) showed the best extraction (19% higher) with wet milling (15 mL concentrated algae, Straub, 100-g (Straub Co., Hatboro, PA), three times). *Chlorella protothecoides* (small and round cells) showed the best extraction (16% higher) with bead-beating (15 mL concentrated algae, bead-beater, Model 3110 BX with 1 mm glass beads (Biospec, Bartlesville, OK), 2 min, twice). Solvent system used was ethanol/hexane (1:1, V/V). Pure hexane resulted in better extraction (16 and 29.7% respectively). Wet milling has large-scale commercial application in starch processing industry, and has been reported to have easy and low-cost operation and maintenance. Bead-beating, even if efficient, has been reported to be limited to small-scale use. Shen et al. (2009) reported that the other methods tested (sonication and French press) were either too expensive or low in lipid recovery. Doucha and Livansky (2008) reported that the extent of disruption in bead milling depended on many variables that determined the nature of collisions between the beads and the algal cells, including shape, size, speed, spatial density, and material properties of the beads, as well as cellular characteristics. The aforementioned Martek process employed wet milling (algal suspension in hexane) as the complementary method (Martek Biosciences Corporation 2003).

Sonication

Extraction can also be ultrasonic-aided, with sonication-induced cavitation acting as an agent of cell disruption (Harun et al. 2010; Shen et al. 2009). Algal cell walls are damaged when cavitation happens near them. This enhances mass transfer and facilitates solvent access to the cell content (Cravotto et al. 2004). The effect has been reported to be much stronger at low frequencies (18–40 kHz) and negligible at 400–800 kHz. Cravotto et al. (2008) reported that ultrasonic assisted extraction proved to be the best when compared with microwave-assisted extraction and Soxhlet extraction for the marine microalga *Crypthecodinium cohnii* rich in DHA. The yield was 25.9% (based on algae dry mass) for ultrasound (8 g sample, 50 mL solvent, 45 °C, 0.5 h) compared with 4.8% for Soxhlet (15 g, 100 mL, reflux, 4 h) and 17.8% for microwave (2 g, 35 mL, 120 °C, pressurized, 0.5 h). Zheng and Hu (2007) reported that ultrasound not only reduced extraction temperature, time, and solvent quantity, but also increased the lipid extraction yield (79.5 and 81.5% extraction efficiencies of DHA and EPA by ultrasound (1 h, 1:4.5 algae mass:solvent

volume); 63.6 and 64.9% by solvent extraction (2 h, 1:5.5)). The method has already found application in the food industry (Cravotto and Cintas 2007). There are some concerns on uncontrolled destruction of biomass that may lyse the lipid molecules randomly, but there has not been much evidence of it. Question marks remain on the scale-up and cost of this method for biofuel production.

### Enzymatic Extraction

Enzymes can be employed to degrade the cell walls (Shah et al. 2004). Cell walls can be limited to partial degradation if needed. Shah et al. (2004) reported a three phase partition system for extraction of oil from *Jatropha curcas* L. seeds using enzyme treatment as a complementary method. It involved simultaneous addition of t-butanol (1:1, v/v) and ammonium sulfate (30% w/v) to the seed slurry (5 g seed/30 mL water). The yield was 82% without enzymatic treatment. With commercial fungal protease enzyme, Protizyme (250 mg, 50 °C, stirring, 1 h, pH 9), the yield was 92%. The yield improved to 97% with sonication (5 min). Soto et al. (2007) reported that enzymatic treatment (0.25% E/S of Olivex–Celluclast (1:1) mixture) improved the oil yield of borage meal from 88 to 95% (45 °C, 20% moisture, 9 hours, double pressing (39.2 MPa), pre-heat (5 min, 70 °C)).

Water itself has been used as a solvent (Institute for Applied Environmental Economics 1995). The enzyme was SP 311, developed by Novo Nordisk, based on *Aspergilus niger*. Lipids suspended in water were easily recovered. Downstream separation of lipids and proteins was much easier than solvent extraction, and their quality was also higher. Enzymatic extraction was much costlier than hexane extraction, and recovery of lipids was lower by 8%.

### Simultaneous Extraction and Transesterification

Simultaneous extraction and transesterification of algal lipids has also been pursued (Belarbi et al. 2000; Lewis et al. 2000) and has been reported to be 15–20% more efficient than the separate extraction and transesterification processes. Molina Grima et al. (2003) showed that the cost of simultaneous extraction-transesterification of a high-value product, omega fatty acids, using a strain with lipid content of 10% (dry basis) was $360/kg of esterified oil for a smaller-scale on-site 8 kg/d oil production facility (80 kg/d biomass). The method has been demonstrated at pilot scale. The cost may be correspondingly lower for larger-scale biofuel system using strains with higher lipid content, eventually approaching numbers similar to those indicated by Lundquist et al. (2010). Similarly, simultaneous extraction and saponification has also been carried out to extract the free fatty acids from wet algal biomass using a mixture of potassium hydroxide and ethanol (Gimenez Gimenez et al. 1998; Robles Medina et al. 1995).

Chromatography

Chromatographic methods are employed to recover high-value metabolites from crude extracts. High pressure liquid chromatography and gas chromatography methods are employed to recover astaxanthin, β-carotene, and omega fatty acids (Molina Grima et al. 2003). Omega fatty acids are recovered by methods such as reverse phase chromatography, silica gel adsorption chromatography, and argentated silica gel chromatography (Belarbi et al. 2000; Gimenez Gimenez et al. 1998; Robles Medina et al. 1995). Supercritical fluid chromatography has been employed to recover astaxanthin, β-carotene, and omega fatty acids (Lim et al. 2002). Membrane-based selective enrichment has been reported to be another alternative (Molina Grima et al. 2003). The chromatographic methods are already in commercial use. For the smaller-scale on-site facility referred to by Molina Grima et al. (2003), argentated silical gel chromatography was used, and the estimated cost of recovering EPA from crude extract was $4,205/kg. This number, however, can be expected to be lower for larger scale as reported by Lundquist et al. (2010). Martek Biosciences Corporation has been marketing DHA as a food ingredient. At present one can obtain omega fatty acid for $75–110 per kg retail (www.vitamins.org).

The scale-up of the cell disrupting complementary methods is yet to be ascertained for viable biofuel production.

## *3.2 Super-Critical Carbon Dioxide Extraction*

This method refers to extracting lipids using carbon dioxide above its critical point (31 °C, 74 atm) (Cooney et al. 2009; Sahena et al. 2009). A supercritical fluid is rated to have excellent extraction ability because it combines solvation contact property of liquids with high diffusion constants of gases. Other advantages of this method are non-toxicity, chemical inertness, and the simplicity of separation of lipids from gaseous carbon dioxide after extraction. Use of a co-solvent such as ethanol has been reported to increase the solvating property of carbon dioxide (Mendiola et al. 2007a; Mendes et al. 2006). The polarity of the solvent is increased and its viscosity is changed. Temperature and pressure are also lowered. One of the advantages of supercritical fluid extraction is the tunable solvent power (Halim et al. 2012). This is a function of density and can be changed by temperature and pressure. Co-solvent and flow rate are the other extraction variables.

Supercritical fluid extraction has been reported to result in higher yields than organic solvents and has higher selectivity for triglycerides (~99 %) (Mendes et al. 2006; Cheng et al. 2011). Supercritical carbon dioxide is non-polar, and addition of a polar co-solvent can aid the yield of polar lipids (Halim et al. 2012). With 20 % ethanol, more than 80 % of phospholipids were reported to be recovered from salmon roe (Tanaka et al. 2004).

Moisture can reduce the effectiveness of supercritical fluid extraction; therefore samples are dried before extraction. Moisture has been reported to impart pasty

consistency to the biomass, and presents a barrier to diffusion of carbon dioxide into biomass and diffusion of lipids out of the cells (Dunford and Temelli 1997; Sahena et al. 2009).

Increase in supercritical carbon dioxide flow results in better contact between the solvent and the lipids, but it can also result in uneven fluid penetration and dead volumes in the vessel (Pourmortazavi and Hajimirsadeghi 2007; Halim et al. 2012). The density of biomass packing as fixed bed is another important factor in extraction. Packing density is related to biomass powder particulate size and volumetric ratio of packing material to biomass powder. Higher packing increases the lipid elution rate, but it can also compromise extraction kinetics by fluid channeling (Pourmortazavi and Hajimirsadeghi 2007).

The cost of supercritical fluid extraction is relatively high because of the high capital costs and the high operating pressures. The cost is likely to come down with time and as more development work is done on this technology. The benign nature of the solvent and the simplicity it offers in downstream processing make this process attractive. The method is still viewed to be only at its initial stages of development given the cost-competitiveness required for biofuel production. Scale-up process design is a key area of development needed for the method. Lowering pressure by using co-solvent may be explored. The method has so far found industrial applications such as

- extraction of
  - tea and coffee (decaffeination)
  - natural products
  - pharmaceuticals
  - herbs
  - essential oils, fats
  - flavors
  - natural colorants (from carrot, alfalfa leaf, sweet potato, tomato, red grape)
  - anti-oxidants
  - oil from nuts (almond, peanut, walnut, hazelnut, pistachios)
  - cholesterol (from egg yolk, meat, milk fat)
- nutrition labelling related fractionated modification
- dealcoholization of alcoholic beverages
- degreasing and dry cleaning

(Mendiola et al. 2007b; Bell 2009; Stewart 2003; Sahena et al. 2009).

Prempiyawat et al. (2011) report that for 93.4% removal of hydrocarbon contaminants (10% wt) from solids (9,000 kg of solids/year, 8 h/d operation), the total capital investment was $ 115,310, the operating cost was $ 5,020/year, and the energy requirement was 3.21 kW. Another study (Gifford et al. 2001) compared supercritical carbon dioxide extraction and hexane extraction for peanut oil as 1.8 GWh/year vs 4.6 GWh/year energy input, $ 6.2M/y vs $ 14M/y operating cost (peanut feed 10 million lb/y; yield 30%; extraction conditions: 550 bar, 55 °C; separation condition: 270 bar).

### 3.3 Microwave Extraction

When algal cells are mixed in a non-polar solvent such as hexane and subjected to microwave treatment, the polar moisture-laden algal cells are preferentially heated and punctured by the increasing temperature and pressure (Balasubramanian et al. 2011; Cravotto et al. 2008). The lipids inside the cells then partition toward the more preferred lipophilic medium, hexane. Thus the entire mixture (predominant portion as solvent) does not need to be heated, and the extraction energy is efficiently and preferentially transferred to the moisture-laden algal cells. An attractive feature of this method is the ability to treat wet biomass. The method has been reported to be rapid, relatively safer (Paré et al. 1997), and needs reduced solvent quantities (Cravotto et al. 2008).

Balasubramanian et al. (2011) reported a 1.2 kW, 2,450 MHz resonant continuous microwave system, designed and optimized for lipid extraction from *Scenedesmus obliquus*. Extraction efficiency of 76–77 % (31 % based on algae dry mass) was reported at 95 °C after 20–30 min (algae:water 1:1 w/w). Cravotto et al. (2008) reported 17.8 % oil yield (based on algae dry mass) for marine microalga, *Crypthecodinium cohnii*, by microwave extraction (2 g sample, 35 mL hexane, 120 °C/pressurized, 0.5 h). Microwave treatment was carried out in a commercial multimode oven operating at 2.45 GHz (Microsynth, Milestone, Italy). The yield was comparable to ultrasound extraction (25.9 %) and Soxhlet extraction (4.8 %) as mentioned earlier. Microwave extraction, along with ultrasound, was mentioned to be promising methods for algal lipid extraction.

The method has already found application in the food industry (Cravotto and Cintas 2007) and in the extraction of essential oils (Lucchesi et al. 2004). Scale-up development is needed for the method to become viable for biofuel industry (Mercer and Armenta 2011). The faster extraction feature of the method has been cited for better economics (Joshi et al. 2010). Nurdin (2007) reported that for the extraction of essential oils from *Mesua ferrea* L. leaves, microwave extraction was about 8 times faster, used 13–18 times less energy, and reduced cost from Malaysian currency RM 11.77 to RM 3.71–5.23 (dry distillation, wet distillation) compared with conventional method (hydro distillation, steam distillation).

### 3.4 Electro-Mechanical Pulsing

In this method, an electric field is applied to aqueous algal suspension in a pulsed fashion. The algal cells are stretched back and forth as the electric field is pulsed. This permeates and punctures the cell wall expelling the intracellular components outside. Lipids can thus be recovered from the intracellular and membrane components (Hebner et al. 2011; Guderjan et al. 2007).

For this method, non-corrosive electrodes need to be used; otherwise metals may leach into solution, potentially contaminating the algal biomass and lowering the by-product value of de-oiled algal biomass. The energy balance of the process is

affected by (a) electrical conductivity of the medium and cellular components, (b) the type of electrode material, (c) spacing and surface area of electrodes, and (d) the type of algae strain.

## 3.5 Extracellular Lipids

Extracellular lipids found in some algae such as the *Botryococcus* species are much easier to extract. Nevertheless, growing these species has proved to be difficult. Their growth rate is slower (about one-fourth the growth rate of *Nannochloropsis oculata*) (Metzger and Largeau 2005). One way to address this is believed to be avoiding colony formation while growing such algae. Also, these types of algae prefer freshwater and brackish water for growth, and may therefore not be able to exploit the abundant cheaper water source, sea water, for mass cultivation.

## 3.6 Live Extraction (Milking)

Researchers have developed a method to extract value components such as β-carotene from the algal strain *Dunaliella salina* while the cell is alive in its growth medium (Hejazi et al. 2004; Mojaat et al. 2008). A separate biocompatible organic phase such as decane and dichloromethane-decane mixture is mixed with the aqueous algal growth phase. The biocompatible organic component extracts products of interest by attaching to the wall of algal cell. The method avoids killing of algal cells and hence maximizes cellular density for continuous production of compounds of interest. It has been reported that the extraction ability of solvent depends on its affinity with the product extracted and on its concentration incorporated in the cellular membrane (Bligh and Dyer 1959). This intriguing extraction technology is in its nascent stages and may take a few years to realize the full potential.

## 3.7 Solventless Extraction Process

Recently Martek Biosciences Corporation (Ruecker et al. 2010) reported a "Solventless Extraction Process." The method refers to the use of an aqueous solvent which contains less than 1% of organic solvent. The method does not require the algae to be dried (50% water). The example reported by Ruecker et al. (2010) refers to 400 gallon batches in which 20 g of 45% KOH were added to hydrolyze proteinaceous compounds in algae. Algal broth was heated to 130 °C for 30 min for cell rupture. It was reported that cell rupture methods could be mechanical or chemical. Thermal method has been reported to be particularly useful for cell walls composed of proteins. The lipids are recovered by successive water washing and centrifuging (light lipid layer and heavy aqueous layer). The crude oil extraction

efficiency ranged from 75–89 % (based on original oil content). The final product was reported to be substantially equivalent to hexane-extracted lipids. The method is believed to be already employed by Martek Biosciences Corporation for the commercial production of DHA (as food ingredient).

## 4 Summary and Concluding Remarks

A variety of techniques exist for extracting lipids from algae, in various stages of development. The most common method currently used is solvent extraction. One of the main challenges with organic solvent extraction is the need to dry the algal biomass before extraction; otherwise the quantity of solvent needed is much higher, which increases processing costs. Development of on-site smaller-capacity processing technologies is crucial for reducing the cost of this technology. To aid solvent extraction process, cell-disrupting complementary methods can be used. Some of the other technologies are in developmental stages and need viable scale-up design. Care must be taken in choosing the extraction method to ensure there is no negative effect on the value of by-products by denaturing, decomposing, or contaminating them. For algae to become a viable biofuel feedstock (a) the energy required to extract and process algal lipids must be much lower than the energy value of the biofuel produced and (b) the capital cost and processing cost must be lower.

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