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Clemens Posten
Steven Feng Chen *Editors*

Microalgae Biotechnology

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Microalgae Biotechnology

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Status, Challenges, Goals

One of the biggest global challenges of the twentieth century is to sustainably supply a growing world population with food, raw materials, and energy in times of climate change. In doing so, biomass plays an important role, as the plants, with the help of sunlight and carbon dioxide from the atmosphere, can produce all these components sustainably and without consuming fossil energy sources. This photosynthesis-based capacity of plants, hence, is the cornerstone of the current bioeconomy concept. However, there are clear limits: The available and even decreasing agricultural area and the yield per unit area that can hardly be increased any further.

Microalgae are a promising—some people say the only—way out of this limitation. Even residual biomass—the so called biomass of the second generation—is available only in limited amounts compared to the huge needs in energy supply. So microalgae have been classified as the biomass of the third generation. The potential for a novel type of biomass production for bioeconomy is enormous. The two major advantages on this strategical level can be identified:

- Per unit area, microalgae can form up to five times more biomass than classical energy plants. Values of 100 t biomass per hectare and year are considered to be realistic.
- No valuable agricultural area is required. It is possible to use practically any areas not claimed for other purposes. This includes dry areas, industrial wasteland, brackish water zones or open seas.

But where are the large-scale production facilities for economically efficient supply of basic foodstuffs, bulk chemicals or chemical energy carriers? Even if the potential of microalgae for biomass supply is quite obvious, there is still a great need for research to develop relevant applications and to find out, how microalgal biomass can be produced in reliable and profitable manner in large scale. This editorial will give a look over current trends, identify existing obstacles, and specify biological and technical research needs necessary for an economically efficient microalgae-based bioeconomy.

Microalgae: Solar Cell Factory for Bio-based Resources of the Third Generation

Microalgae are microscopically small plants naturally occurring in water bodies, such as lakes, rivers or seas. For some time now, microalgae have also been cultivated and used by man. For this purpose, open ponds or closed reactors are applied. There, microalgae grow suspended in an aqueous medium. Apart from the microalgae, this medium contains mineral nutrients only. Some high-quality food or feedstuff supplements and cosmetic products have long been introduced on the market. Examples are the red dye astaxanthin for fish cultivation and polyunsaturated fatty acids (PUFA) for healthy human nutrition. However, the quantity produced worldwide totals some thousand tons only. Several demonstration plants for the production of bioenergy carriers have been built.

Besides the already mentioned advantages the potential of microalgae on the biological level can be summarized:

- Many species grow in saltwater even at high salt concentrations, reducing problems with water supply.
- For many species carbon flux can be partitioned to lipids or carbohydrates without loss of photosynthetic efficiency. Intracellular concentrations are far above those of classical land plants showing high concentrations for example only in seeds.
- Accumulation of pigments namely antioxidants or other strongly reducing compounds is possible thanks the specific cell structure. Similar concentrations cannot be reached in genetically modified heterotrophic microorganisms.
- Microalgae do not have any roots or wooden parts in the sprouts and leaves. Hence, the complete microalga or all its components can be used without any problems.
- The different microalgae species contain multiple different compounds for commercialization, making the biorefinery profitable.

Diversity of Microalgae: The Unexplored Potential

Microalgae do not represent any consistent biological group, but can be divided into several, partly extremely different strains from several phyla. The term microalgae, hence, is understood to comprise forms of microscopically small plants that predominantly live in aquatic habitats. In that sense also the procaryotic cyanobacteria are often included in this term. Of the several hundred thousands of alga species estimated, some ten thousand have been classified so far. Only a fraction of them, i.e. about 20 microalgae, are used for economic purposes.

Comprehensive screening programmes in all parts of the world constantly deliver new strains for strain collections. These strains are then applied in a variety

of high-concentration products. In spite of the application of latest methods, e.g. growth tests in highly parallel microtitre plates, however, the products can be customised to a certain extent and for a few defined environmental conditions only. Often, attention is paid to a certain substance class exclusively. In the second stage, a process-oriented strain selection has to be made. Important criteria are robustness in the bioreactor, temperature stability, or the possibility of specifically excreting valuable substances.

For thousands of years, human beings have been growing higher plants for the production of food, construction materials or fabrics. In the last decades, this process was further analysed and advanced thanks to better insight into life processes. On the one hand, initial diversity converged towards a manageable quantity of useful plants with high yields, such as corn or cotton. On the other hand, a large plant variety developed and was optimised for regional climate conditions or special applications. This step that is referred to as domestication still remains to be accomplished for microalgae screening and strain development within the shortest possible time.

Strain Development: Biological and Technical Optimization

Microalgae-based molecular biological methods are still behind the development in the area of classical microorganisms like bacteria and yeasts. Recently, however, its importance increased strongly. Research concentrates on production of recombinant proteins, a technology that is expected to have major biological and technical advantages when using microalgae. Known products also are in the focus of genetic engineering. Even if photosynthesis as such cannot be improved significantly, it is succeeded in increasing the partly low concentrations of highly valuable substances or in adapting e.g. the fatty acid profiles of the oils produced to the needs of foods, lubricants, or biodiesel.

In addition, process-oriented properties are studied and produced specifically. Algae cells that flocculate “on command” largely facilitate harvest. Another idea is that algae with reduced pigment concentrations utilize light more efficiently during cultivation. Process technology is also influenced by the specific excretion of products from the alga cell. Work in this area does not necessarily lead to the use of the genetically modified strains in practice, but is considered to serve as a model to test the molecular effect and practical use. In view of the high diversity, the findings can then be used in the screening for natural strains.

This development process, known as domestication, is still in an early stage. Strains with interesting products are not yet adapted to the intensive conditions in bioreactors. Comparing the improvement by breeding of terrestrial crops over centuries or the increase of productivity for heterotrophic microorganisms, the high development potential still to be realized becomes obvious.

Products: From High Value Compounds to Bulk Chemicals

Microalgae have been known as food supplements for a long time already. Examples are proteins of high biological quality, vitamins in particular of the B group, polyunsaturated fatty acids, antioxidant pigments, such as carotenoids, minerals, and other substances. The cyanobacterium *spirulina* (biologically correct term *arthrospira*) and the green alga chlorella vulgaris are cultivated for this purpose and marketed in the form of disrupted cells or extracts. *Astaxanthin* from *haematococcus pluvialis* plays an important role as a pigment in the cultivation of salmon. Research additionally covers pharmaceuticals, such as immuno-stimulating polysaccharides or medicine for Alzheimer's disease.

What applies to food supplements also applies to basic foodstuffs. Plant-based biomass cannot be supplied, unless additional agricultural areas are made available. This is where microalgae-based technology comes in. Several international companies in the food production sector deal with microalgae. Work is aimed at offering e.g. colourless, odourless, and tasteless protein components. Polysaccharides, such as alginate or carrageenans, are produced from macroalgae and processed in foodstuff already. They are suitable basic substances for food. They are not to be considered supps only, but can contribute decisively to world food supplies.

Another important application field is animal feed. Facing the overfishing of oceans consensus is given that sustainable aquaculture has to be further expanded. Fish is at the end of food chain, microalgae at the beginning. A healthy fish being healthy for humans as well needs many of the compounds of microalgae. Currently fish a fed with fish meal, what has already run short. Consequently, aquaculture has become a major driver for microalgal biotechnology. Ongoing research is directed to finding and cultivation suitable algae strains especially for this purpose.

Algae-based biotechnology wants to achieve a market share not only in the sector of high-quality products, but also in the area of mass products of medium and low price levels. Discussion focuses on resources for chemical industry, such as monomers for bioplastics and other fine and platform chemicals. Examples are certain fatty acids and isoprene. More than 5 % of the crude oil imported by Germany are used for plastics. This is a market that may be made more energy-neutral by using microalgae.

Renewable energy sources will have the biggest share in the energy mix of the future. An important element will be the use of biomass. Apart from increasingly criticised biofuels of the first generation and the use of residual biomass, microalgae represent another source of sustainable biomass for energy production, which is independent of agricultural production. In analogy to residual biomass, microalgae may be fermented to methane in biogas facilities. Even more interesting is the use of the large oil fraction. It can be processed to biodiesel after extraction and further conversion. Hence, this option yields a liquid fuel urgently required in aviation. Again, biology can directly support process technology. Research focuses on algae that directly produce and excrete hydrocarbons. As a result, extraction and

transesterification may no longer be necessary. Another option is the direct production of hydrogen and bioethanol.

Many algae that have not been studied so far have an enormous potential for use in food, pharmaceutical, cosmetic, and chemical industries. Potential target products, however, have not yet been detected or searched for in the screening process. Genetically modified strains are under investigation for production of recombinant proteins. For *Chlamydomonas* this technology is already quite well developed, for the chloroplast and for the nuclear genome. Problems persist for most of the other strains. In addition genetic modification might be decisive to extend the product portfolio for the energy and chemical substances sectors and help adapt product quality to high standards.

Dialogue between industry with its concrete needs and biology focusing on diversity has to be intensified and broadened to identify the potential in detail. Screening programmes and application of genetic engineering methods developed for model algae to other species will open up further substance classes. Apart from material aspects, strain development also has to focus on technical implementation, robustness, and the capability of excreting valuable substances.

Photobioprocesses: Technologies Along the Value Added Chain

The potential of microalgae has finally to be realized in production plants by process engineering means. Major advantages in terms of production conditions are:

- Microalgae are produced largely automatically by process technologies in open or closed reactors.
- Microalgae do not have any pronounced seasonal growth periods like classical land plants, they can be grown throughout the year depending on the availability of light.
- Growth in closed reactors minimises the use of water. In addition, a reduction of fertilisers and crop protection agents seems to be if possible.
- For recombinant extracellular proteins specific advantages like clear medium for easy separation and strongly reduced virus problems apply.

Of course, the biomass factory of the future will require large areas, as solar energy has to be collected and radiation is quite diluted per se. But this is the only similarity to agriculture. Microalgae production units will appear more like a technical oriented facility. An interesting option is however the coupling between agriculture and algae production.

Photobioreactors: Technical Hub of the Production Process

The photobioreactor is the location, where the microalgae meet with light, CO_2, water, and nutrients and where growth and product formation processes take place.

Open ponds, e.g. raceway ponds, still are the workhorses of commercial microalgae production. They allow for the production of algae biomass at moderate costs and with a moderate energy consumption. Yet, production concentrations and productivities are also low and hardly exceed those of classical agricultural areas. The large surface area exposed to the surroundings is a gateway for contamination, water is lost by evaporation. More ambitious objectives cannot be reached, as the technological development potential is lacking.

These drawbacks might be overcome with the help of closed reactors. The alga suspension is contained in a transparent box through which light may enter. The incident (sun-) light is to be distributed as homogeneously as possible in the suspension. Contrary to a chemical substrate, this cannot be done by mixing. Mutual shadowing effects of the algae produce strong gradients. For this reason, several design criteria have to be observed. For example, the transparent surface area has to be large compared to the medium volume that is to have the form of thin layers. Moreover, microalgae are not capable of processing high light intensities occurring at noontime, for instance. This means that the light has to be "diluted" over a reactor surface that is larger than the footprint area. Two basic reactor designs have been developed, plate reactors and tubular reactors. Several attempts are currently undertaken to make reactor design lower to reduce hydrostatic pressure and to apply light guiding structures to reduce the light gradient inside the medium. Horizontal designs can be regarded as endpoint of this development line.

The capacity of the cells to build complex molecules plays the most important role in the production of moderate amounts of high-quality substances. Energy aspects are less relevant. Artificial illumination may be used. This includes application of colours different from colours. A high "red" fraction e.g. is suitable for photosynthesis and minimizes the energetic effort of the cultivation. Rapid development in the area of LEDs has pushed this technology. Larger foil reactors or smaller glass reactors with external illumination may be purchased on the market. It is also possible to backfit classical reactors with internal illumination systems. In this case, all requirements relating to process control are met. GMP qualification of such a reactor for e.g. the pharmaceutical sector appears to be much easier than for reactors depending on daylight and having large exposed surface areas.

Still, it is a problem to produce algae biomass at low costs and in an energy-neutral manner. One of the reasons are the costs of the reactors per unit area in relation to the gain from collecting sun energy. Research projects relating to inexpensive reactors with a reduced material consumption are to solve this problem in the near future. Like all reactors, photobioreactors for microalgae have to be equipped with mixing and gasification systems. The use of CO_2 with solar energy may be considered a political advantage, but represents a big technical challenge. This particularly applies to low-energy mass transfer into the algae suspension.

Again, this challenge was accepted by industry and academia. The first significantly improved pilot reactors are passing practical tests in demonstration scale at the moment.

Another important aspect is heating up of the reactors by incident sunlight. One way to solve this problem is to select seasonally adapted algae strains. Marine reactors already benefit from cooling by the ocean water. For a more sophisticated technological answer to the problem biologists, bioprocess engineers, and thermodynamics experts cooperate to find ways to store the excessive heat of the day and use it during the night. Patents to filter out the infrared fraction of light are published. That can even be done using transparent photovoltaics and fitting the demand of auxiliary energy by the electricity gained. The complete energy autarkic photobioreactor is a high-level objective but nevertheless it is thinkable. It would be a break through with respect to production costs.

The biomass factory of the future will use large fallow areas for "harvesting sunlight". There, closed reactors will not be protected by greenhouses for reasons of costs. Hence, contamination cannot be prevented completely in the long term. Employing extremophiles is common in open ponds, co-cultivation of different algae are under investigation. Environmentally compatible procedures will have to be established for future production without any herbicides and other polluting measures.

Depending on the specific value added and the location conditions, various designs of photobioreactors will prevail. To ensure economic efficiency, however, few standard models will be envisaged. They will be offered to potential users by special companies. A parameterisation has to be made according to biological/engineering standards. Clear geometrical design criteria are still lacking. Further input from material sciences is needed with respect to UV-resistance, workability or anti-fouling properties. For the usual materials like PMMA or polycarbonate already many different coatings are on the market for different purposes, so progress is likely to happen soon. Measurement, control and the development of process strategies is another fast developing field. Here algorithms from other technical fields like adapted control can be applied to the specific needs of photobioreactors as excellent properties of the medium and the cells for optical measurements on the one hand and fast changing environmental conditions like sun-light, being the "disturbance" in control engineering view.

Downstream Processing: The Difficult Path Towards the End Product

Cell harvesting is the next step in the chain of values added. This technical task of solid-liquid separation may be achieved in principle by flotation, filtration, or centrifugation and yields a pasty product, called slurry, for further processing. Special devices for the separation of microorganisms have been developed and

commercialised for decades. In the case of microalgae, however, a specific problem is encountered. The solid matter content is far below of the values reached by classical fermentation technology. Hence, several times the amounts of water is needed to be processed per harvested microalgal biomass. Moreover, production of chemical energy carriers from microalgae has to be energy-neutral, a requirement that is not encountered elsewhere in biotechnology. Industry has responded to this requirement by developing e.g. novel centrifuges and cross flow devices. To reach the anticipated amounts of biomass, however, further process technology research is required.

Similar to the processing of plant materials, algae processing is followed by necessary or optional steps, such as drying, mechanical dissolution, extraction or chemical conversion. In principle, processes have already been developed for the use of sustainable resources, but they remain to be adapted to algae at least. The pasty structure of the slurry, for instance, cannot be subjected to simple mechanical pressing like rape seed is. In addition, the relatively high water content of the cells makes it difficult to use solvent extraction or hydrolysis processes for biofuel production. Hydrothermal liquefaction or gasification are actively under research and development. On the other hand, algae are much better accessible for many chemical processes, such as energy production in biogas facilities and fractionation, than straw or wood wastes. In this area, research teams are active worldwide.

Biorefinery: Pathways for the Holistic Use of Microalgae-Based Biomass

In an oil refinery complex crude oil is fractionated into different compounds which are fully processed leaving nearly no residual fractions. In analogy, the different compounds of biomass should be isolated and refined in order to gain a complete energetic and material use of biomass. For example, the polyunsaturated fatty acids can be used for human food, while saturated fatty acids can be used for technical purposes. Proteins can in a next step be separated for animal feed, while the residual biomass is finally used energetically in biogas plants.

This concept has to be profiled for different cases. First of all, one of the advantages of microalgae is that they can be used as a whole cell e.g. for human food or animal feed. A small fraction in the daily diet has been shown to deliver minerals and vitamins especially in underdeveloped countries, where only carbohydrate sources are affordable. A basic problem of the biorefinery lies in the different orders of magnitude for the amounts of the produced compounds and the market volume. High value products are usually present only in small fractions of the biomass and are needed only in small amounts. The value of the residual biomass is negligible. The full potential of the biorefinery may be obtained for medium sized plants. In case of bulk chemicals or especially fuel production processes the residual biomass accrues in gigantic amounts which cannot be absorbed

by any market. This holds for the processes currently know delivering a fraction of e.g. 50 % oil in the biomass.

A basic scientific problem is in finding cultivation conditions shifting intracellular stoichiometry to the main product or to by-product ratios best for a given market without losing productivity. High concentrations for carotenoids e.g. are purchased by high light intensities but long cultivation times. Limits for shifting stoichiometry to calculable costs are in most cases not quantitatively known. Innovation lies here in developing strains producing extracellular compounds. This has been shown for polysaccharides and hydrocarbons produced by wild type algae or ethanol formation with genetically engineered strains. Glykolate and isoprene are other candidates. Final target is to partitioning most of the fixed carbon into the product leaving the cells themselves with growth rates close to zero. A side benefit is given by employing biofilms reactors with low mixing rates and saving of cell separation. This concept has been declared as "New Green Chemistry".

Algal biomass can turn out to be an important alternative to fossil fuels. Integrated production and commercialization concepts have to be developed, which are sensible in sustainable and economical aspects. Flexible adaptation to market conditions is one pre-requisite. Doing so, the microalgal based biorefinery has the chance to become the key for an internationally competitive economy. Algae demonstration plants have to be set up by combining the different products and production steps to deliver quantitative numbers for predicting commercial exploitation costs.

Process Integration: Microalgae Production in the Ecological, Economic, and Social Setting

Microalgal production processes have to be carefully be integrated into the environment for sustainability. Some of the advantages of microalgae in terms of process integration are:

- Production processes with microalgae can be integrated comparatively easily into existing energy and material cycles (wastewater, salt water, waste heat, exhaust gases). Cycles can be closed by smart feedbacks.
- Production on land for which no other claims exists will minimize conflicts with other industries or local population.
- Food from microalgae will be more and more accepted starting from taste-, smell-, and color-less preparations. Polysaccharides from macroalgae can be found already in many consumer products.
- Sunny areas with salt water available can be found all over the planet. Microalgae plants will reduce the dependency from oil producing countries and bring working places to structurally weak regions.

It is easy to say that CO_2 for the microalgae comes from industrial plants like gas power stations or lime stone processing. Indeed, it turned out to be a logistic problem as algae cultivation plants will be remote from industrial areas. A promising concepts among others is to couple heterotrophic production like bioethanol processes with microalgal plants to get benefit from CO_2-recycle. Residual biomass from the microalgal biorefinery could be a good carbon source for the heterotrophic stage in the sense of cross-feeding.

Delivery of mineral salts for nitrogen or phosphor supply is a cost factor for production as along as no sensible recycling on the production plants is foreseen. In case of oil production nearly all minerals are basically recyclable from the residual biomass eventually indirectly from the biogas effluent. In the case of protein production nitrogen leaves the plant and has to be replaced. For phosphate a world-wide shortage has been anticipated and needs special attention when whole cells are sold and distributed. This in mind, municipal waste waters are often addressed as feasible sources of N and P. Coupling of microalgal cultivation with aquaculture is often mentioned, because feeding the fish with the algae and recycling the fish waste water including N,P back to the cultivation seems to be sensible. To these scenarios many research project shave been published. However, some technological problems seems to be unsolved like unsuitable stoichiometry of the nutrients or low concentrations making cell retention necessary. Some algae show good abilities for mixotrophic growth to use up organics from wastewater. Another positive issue is the constant temperature of waste water making cooling superfluous. In the same sense cooling water from industry has been proposed for temperature control what means cooling during warm days and warming up in when it is cold to keep growth at its optimum. These aspects have to be studied carefully in life cycle analysis (LCA) to quantitatively assess costs versus benefits taking into account material and energy flows as well as their respective couplings from the intracellular level, over the reactor to the production site and even more to the production environment.

Location Issues: Where to Go Between Light and Shadow, Desert and Sea

The choice of a suitable site is essential for the success of any algal production plant. However, the different way of using microalgae lead to different demands for the production site. Some studies about possible regions show the potential of the microalgae biorefinery but do not reveal a clear preference for certain countries or regions. Solar irradiation is not really a decisive factor. Availability of water, CO_2, nutrients and auxiliary energy are additional points to be considered. In the Sahara about 2.5 times more sun energy per hectare could be used compared to middle latitudes, but this advantage has to be paid by problems in the infrastructure, extremely high irradiation but in only a few hours during the day, and high

temperatures. In mountains close to the equator the picture could be different with moderate temperature all through the year and during day and night.

For high value compounds productivity and production cost play a minor role. The high revenues in relatively small markets allow the application of closed photobioreactors on small areas. Activities in some countries in the middle latitudes are promising in this concern. Further north other points apply like the long days with ideally distributed light intensity during the summer, possibilities for temperature control by low temperature waste heat, and the short distance to CO_2-sources. Studies have shown that here business cases are realistic as production is possible from early spring to late autumn, even if the plant is switched off during winter to save maintenance energy. For further processing a powerful industry is waiting.

Large scale commodities like biofuels are facing a strong cost pressure. Large production capacities are necessary to reach industrial relevance. To fit these constraints large cultivation areas of many square kilometers with high solar irradiation are necessary and access to cheap CO_2 and nutrient sources. Such applications are expected to emerge typically in the southwest of the USA, in the Middle East, in some parts of Asia, or in Australia. One interesting idea are marine photobioreactors. While the availability of salt water inclusive cooling option and free space is obvious, at least in sea areas remote from heavy shipping or sensible fishery areas, wind and waves are challenges.

Regulations: What We Can and What We are Allowed to Do

Just like any other new products, products based on microalgae require approval according to regulations of the respective country. This approval focuses on two levels. First, it is to be demonstrated that the production process does not endanger health of the people working at the production facilities and not adversely affect the environment. Depending on the tonnage produced, comprehensive studies and data sheets have to be submitted according to the pertinent regulations, such as "Registration, evaluation, authorisation, and restriction of chemicals" and "Classification, labelling, and packaging" in the EU. Second, consumer protection has to be guaranteed. Subcategories have been defined depending on the market (food, food supplements, novel food, cosmetics, pharmaceutical products, feedstuff, animal health products). Various requirements have to be met. Allocation to one of these groups may be a problem already. The "EU Novel food regulation" and the "EU Cosmetic regulation" are relevant to algae. In a simplified procedure, for instance, the microalga *odontella aurita* and *astaxanthin* extracts from *haematococcus pluvialis* were approved of as novel food. The (mostly medium-sized) industrial companies consider such approval procedures a high obstacle. Support by politics is needed urgently.

For the transformation into energetic compounds and the envisaged reduction of carbon dioxide emissions, clear regulations and provisions will have to be adopted.

In the transport sector, in which biofuels represent an indispensable element of the sustainable fuel and mobility strategy, many problems have not yet been solved and lead to uncertainties in industry. To meet the political targets and to eliminate uncertainties for the producers of alternative fuels and automotive industry, some points have to be settled by policy, including: Which specifications and minimum requirements have to be met by microbial fuels with respect to adaptation of standards for gasoline and diesel? What are the legal specifications for blending quotas and carbon savings for the envisaged applications and what are the consequences when they are not reached? Such decisions are important for any investigation in algae plants.

Before these points have not been settled, no big change in the transport sector is to be expected.

International Cooperation: Development of Strategic Alliances

Despite all progress in algae-based biotechnology and the current installation of pilot plants to supply biofuels based on ethanol and lipids in the USA, the need for basic and applied research remains in this area. For a holistic use of algae at biorefineries, various biotechnological and process technology disciplines have to cooperate closely. Possibilities of cooperation in research alliances on the national level, however, are limited due to the interdisciplinary character of the topic. While the establishment of research clusters in the European Union is supported under the corresponding research framework programmes, such alliances can hardly be initiated with partners beyond the borders of the European Union and in research-focused nations, such as the USA. Due to the apparently lacking coordination of the funding institutions, hardly any project proposals of this type are invited. Different funding conditions and review procedures exist on the national levels. Applicants would prefer calls to be published not only on a national basis, but by all funding institutions of the target nations at the same time. Proposal, review, and funding could take place in parallel. Establishment of transnational research alliances, including the drivers of the international research community, would result in an innovation boost in the medium and long term. However, efforts to establish such international cooperative ventures by the funding institutions would have to be increased considerably.

Man: Part, Target, and Meaning of the Value Added Chain

Large-scale microalgae production will change the face of the Earth. For this, civil society has to be taken along. Anybody will welcome secure mobility in the coming decades and reduced famine in the world. But where will new jobs be created? In many regions in the world, where hardly any farming is done due to draught and

where no mineral resources are found except for solar energy, microalgae will be a big opportunity. Establishment and operation of the facilities will create jobs and, hence, economic growth. Industrialised countries may contribute their strengths and provide the required process technology. The main concern of industrialised countries is the availability of high-quality biomass for bioeconomy. In addition, it is hoped that production will be distributed over many regions of the world. This will reduce not only material dependence, but also political dependence on fossil resources. For this to come true, the right steps have to be taken today. Technology assessment studies should include this aspect with the balance limits being defined accordingly. Not just since the speech of Obama, microalgae research has moved from science into the focus of the public. Many countries have already taken decisive steps towards creating a future with microalgae on the level of research, also with public funding. Politics can now continue on this way and define framework conditions for research, economy, society, and international relations.

Commercialisation of microalgal biotechnology will go along stepping stones from high value products in small amounts over the medium priced segment to bulk products in plants with increasing size and finally renewable energy. Some of the current trends are outlined in a study of dechema, on which this preface is based. This book will give views to some of the mentioned aspects and will encourage further reading and contributing to bring microalgal biotechnology further forward to make it a benefit for our society.

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

Jin Liu and Feng Chen

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

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

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1 Introduction

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

2 Morphology, Ultrastructure, and Taxonomy

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

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

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

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

3 Growth Physiology

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

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

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

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

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

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

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

4 Mass Cultivation

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

4.1 Photoautotrophy

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

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

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

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

4.2 Mixotrophy

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

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

4.3 Heterotrophy

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

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

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

5 Potential Applications

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

5.1 Chlorella as Human Food and Animal Feed

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

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

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

5.2 *Chlorella as a Source of Carotenoids*

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

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

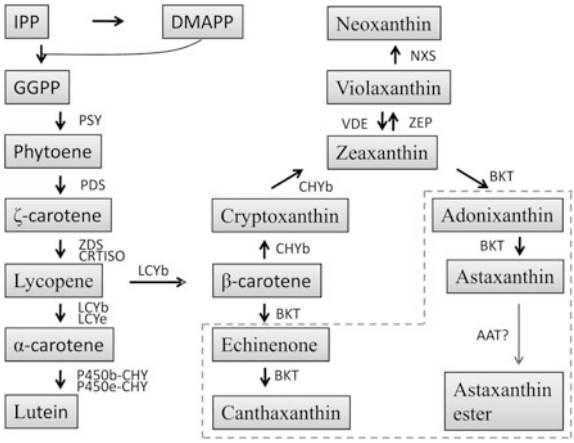

Fig. 1 Schematic diagram of carotenoid biosynthesis in *Chlorella*. In the box is the astaxanthin biosynthetic pathway which is present in *Chlorella zofingiensis*. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *PSY* phytoene synthase, *PDS*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

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

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

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

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

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

5.3 *Chlorella* for CO_2 Biomitigation and Wastewater Bioremediation

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

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

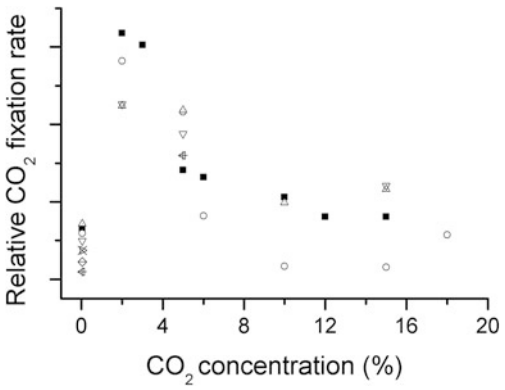

Fig. 2 Relative CO_2 fixation rate of *Chlorella* as affected by CO_2 concentration. Data are based on studies during the past five years

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

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

Table 2 Selected *Chlorella* species reported for wastewater treatment

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

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

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

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

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

5.4 Chlorella as Feedstock for Biofuels

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

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

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

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

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

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

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

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

Table 4 Fatty acid profiles of selected *Chlorella* species

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

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

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

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

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

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

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

5.5 Chlorella as Cell Factories for Recombinant Proteins

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

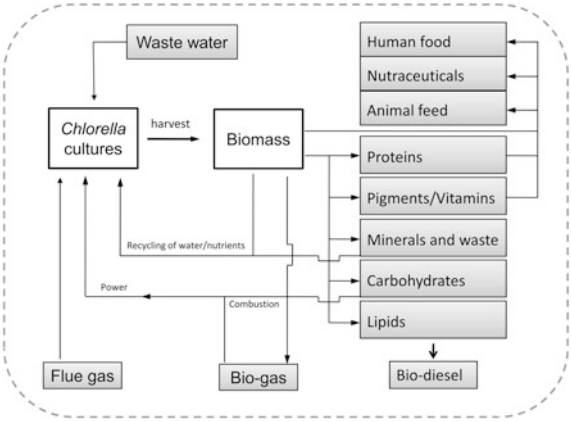

Fig. 3 Schematic illustration of integrated production of biofuels and other products coupled with flue gas and wastewater treatments

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

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

Table 6 Summary of *Chlorella* species for genetic modification

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

[a] Transient expression or unstable transformant

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

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

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

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

6 Conclusions and Future Prospects

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

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Microalgae as a Source of Lutein: Chemistry, Biosynthesis, and Carotenogenesis

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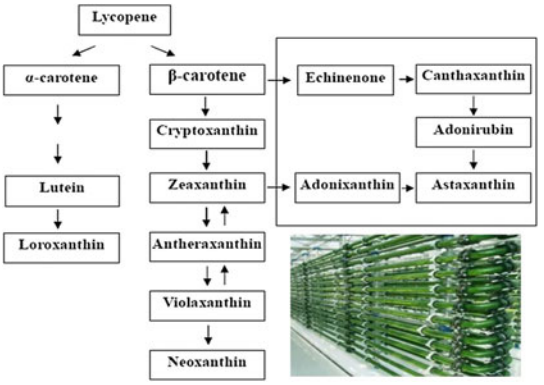
Abstract Microalgae represent a sustainable source of natural products, and over 15,000 novel compounds originated from algal biomass have been identified. This chapter focuses on algae-derived lutein, a group of high-value products. Lutein belongs to carotenoids which have extensive applications in feed, food, nutraceutical, and pharmaceutical industries. The production of carotenoids has been one of the most successful activities in microalgal biotechnology. This chapter gives a mini review of microalgae-based lutein, where emphasis is placed on the biosynthetic pathway and the regulation of carotenogenesis.

Graphical Abstract



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Keywords Biosynthesis · Carotenogenesis · Health effects · Mass cultivation

Contents

1 Introduction

Carotenoids are a group of organic pigments synthesized de novo in higher plants and some other photosynthetic organisms including algae, some types of fungus, and bacteria. They are widely distributed in nature and responsible for a broad variety of colors, such as brilliant yellow, orange, and red of fruits, leaves, and aquatic animals [1]. Thus far, over 700 carotenoids have been identified [2] in which lutein has achieved noticeable attention in both academic and industry research areas. As a natural colorant, lutein has been widely applied in the food industry, especially after the notorious "Sudan red" event [3]. It is also used for coloring fish and poultry, drugs, and cosmetics. In addition, lutein and zeaxanthin are the only carotenoids reported to be present in the eye retina and lens [4]. As these organs are susceptible to oxidant damage, lutein exerts antioxidant action to protect the eyes. These important properties make lutein a highly valuable chemical. According to the report from financial organizations, the global market of lutein is expected to grow to $309 million by 2018 with a compound annual growth rate of 3.6 % (http://www.companiesandmarkets.com/Market/Food-and-Drink/Market-Research/The-Global-Market-for-Carotenoids/RPT988273).

Microalgae are a group of small photosynthetic organisms commonly found in aquatic environments. A number of microalgal species accumulate high contents of carotenoids (including lutein) as a part of their biomass and therefore represent an outstanding natural source of carotenoids. Production of lutein from microalgae is widely studied, for example, *Chlorella protothecoides* [5, 6], *Dunaliella salina* [7], *Scenedesmus almeriensis* [8], and *Galdieria sulphuraria* [9], which has been one of the most successful activities in microalgal biotechnology.

2 Structure

Carotenoids are of isoprenoid origin derived from a 40-carbon polyene chain backbone structure with cyclic groups [10]. There are two types of carotenoids: the hydrocarbon ones are known as carotenes (e.g., α-carotene, β-carotene, and lycopene), and the oxygenated derivatives are named xanthophylls. In the latter, oxygen may be present as OH groups (e.g., lutein), as oxo-groups (e.g., canthaxanthin), or in a combination of both (e.g., astaxanthin). Elements other than carbon, hydrogen, and oxygen are not directly attached to the carbon skeleton in naturally occurring carotenoids [11]. The molecular formula of lutein is $C_{40}H_{52}O_2$, as shown in Fig. 1. The most typical feature of lutein as well as other carotenoids is the long polyene chain. Such a conjugated double-bond system determines the photochemical properties and chemical reactivity that give the basic biological functions of carotenoids, such as antioxidant activities [12].

3 Bioactivities and Impact on Health

The most notable bioactivity of lutein is its antioxidant property. Lutein could be rapidly oxidized by a series of oxidants, which greatly reduces the availability of free radicals to react with other cellular components, such as unsaturated lipids, protein and DNA [13]. Similar to other carotenoid members, the antioxidative power of lutein is attributed to its conjugated carbon double-bond system. This structure allows the quenching of singlet oxygen and scavenging of free radicals. The singlet oxygen-quenching activity includes physical and chemical quenching,

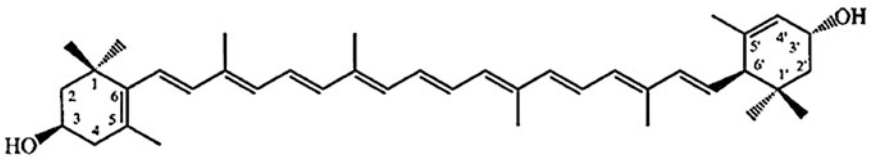

Fig. 1 Structure of lutein

Table 1 Scavenging capacity of carotenoids

Carotenoids	Scavenging capacity[a]			
	$ROO^{\bullet}$	HOCl	$ONOO^{-}$	$HO^{\bullet}$
lutein	**0.6**	**0.97**	**4.81**	**0.78**
zeaxanthin	0.56	1.41	3.87	0.77
β-carotene	0.14	0.71	NA[b]	1.02
lycopene	0.08	0.35	0.4	0.31
fucoxanthin	0.43	1.18	6.26	NA
canthaxanthin	0.04	0.28	0.1	NA
astaxanthin	0.64	1.66	9.4	0.73
α-tocopherol	0.48	1.77	NA	0.37
quercetin	0.84	1.42	5.63	0.97
trolox	1.00	1.00	NA	NA
ascorbic acid	NA	NA	0.41	1.00
cysteine	0.04	NA	1.00	0.02

Peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl), and peroxynitrite anion ($ONOO^{-}$)

Source Adapted from Ref. [14]

[a]The scavenging capacity was calculated considering as reference: trolox for $ROO^{\bullet}$ and $HO^{\bullet}$, cysteine for HOCl, and ascorbic acid for $ONOO^{-}$

[b]NA: no activity was found within the tested concentrations

both of which require very close interaction between lutein and singlet oxygen. The energy transfer reaction absorbs the excited energy of singlet oxygen onto the carotenoid chain, which results in the formation of the carotenoid triplet state. This triplet state can readily return to the stable ground state by releasing the excess energy of singlet oxygen as heat. Rodrigues et al. [14] conducted a membrane-mimicking system to evaluate the scavenging activity of carotenoids against various reactive species based on the fluorescence loss of a fluorescent lipid ($C11\text{-}BODIPY^{581/591}$). The results are shown in Table 1.

The antioxidative properties make lutein a class of important nutrients in health promotion, and there has been ever-increasing evidence supporting their protective effects in preventing or delaying chronic diseases (Table 2). Previous studies indicated that lutein inhibited the oxidation of low-density lipoprotein (LDL), which suppressed the progression of cardiovascular diseases [15, 16]. Dwyer et al. [17] reported that the dietary intake of lutein effectively prevented the occurrence of early atherosclerosis. Seddon et al. [18] reported that there was a direct relationship between carotenoid intake and a decreased risk of age-related macular degeneration (AMD). Among those selected carotenoids, lutein and zeaxanthin were found to exhibit the strongest protective effects. Lutein is also well known to ameliorate the onset or progression of cataracts [19]. Oxidative damage to lens cell membranes is considered to be mainly responsible for the initiation and aggravation of age-related cataracts [20]. Recently, our studies have revealed a novel physiological function of lutein: the antiglycoxidative effect. Glycoxidation, also known as the Maillard

Table 2 Health benefits of lutein

Therapeutic indications	References	Model
AMD[a]	[24, 25]	EIU[b] in Lewis rats CNV[c] in C57BL/6 J mice induced by laser photocoagulation and b-End3, RAW264.7, and ARPE-19 cell lines
Atherosclerosis	[26, 27]	
Retinal neural damage	[28, 29]	
Ultraviolet radiation	[30, 31]	

[a]AMD, age-related macular degeneration
[b]EIU, endotoxin-induced uveitis
[c]CNV, choroidal neovascularization

reaction, is a process in which reducing sugars react spontaneously with amino acids and amino groups of proteins. This reaction is a key factor contributing to the pathogenesis of diabetic complications [21]. We evaluated over 20 microalgal species and found that the extract of several *Chlorella* exhibited a potent antiglycoxidative effect, where lutein was identified as a major effective ingredient [22]. In cultured human-derived retinal pigment epithelial ARPE-19 cells, lutein inhibited the formation of endogenous advanced glycation end products (AGEs) through the suppression of intracellular oxidative stress, for example, nitric oxide, reactive oxygen species (ROS), and lipid peroxidation [23]. These results strongly suggested the significant potential of lutein in the management of diabetic retinopathy.

4 Distribution

Lutein is widely distributed among fruits, vegetables, and flowers, in which marigold flowers are by far the most abundant natural source of commercial lutein. However, a large amount of lutein present in marigold flowers is esterified with half of the weight corresponding to fatty acids [32], and therefore, chemical saponification is necessary in the manufacture of lutein products. In addition, the production of lutein from marigold is also limited by seasons, planting area, and the high manpower cost [33].

Microalgae have long been regarded as an excellent alternative to conventional plant sources as algae-derived lutein is in the free nonesterified form. Green algae *Chlorella* are good examples: *Chlorella pyrenoidosa* can synthesize lutein and other xanthophylls under heterotrophic cultivation using glucose as the carbon source [34]; a high content of lutein was produced by *C. protothecoides* grown in the nitrogen-limited fed-batch culture [5]; and in *Chlorella zofingiensis*, astaxanthin and lutein were biosynthesized in divergent pathways using the common lycopene precursor [35]. Several species of marine microalgae such as *Dunaliella bardawil* and *D. salina*, which have been commonly applied in the production of β-carotene

Table 3 Distribution of lutein in some representative microalgae

Microalgae	Quantity (mg g^{-1})	Reference
Chlorella protothecoides	4.6	[6]
Chlorella protothecoides	5.4	[5]
Dunaliella salina	6.6 ± 0.9	[7]
Scenedesmus almeriensis	5.3	[8]
Galdieria sulphuraria	0.4 ± 0.1	[9]

and phytoene, were also reported to accumulate lutein under specified conditions [36, 37]. Distribution of lutein in some representative algae is summarized in Table 3.

5 Mode of Cultivation

5.1 Photoautotrophic Cultivation

There are two major cultivation modes for microalgae: photoautotrophic and heterotrophic cultures (if a strain is able to grow heterotrophically, a mixotrophic mode may also be developed). Due to the presence of chloroplast, most microalgae grow photoautotrophically through photosynthesis. The idea of photoautotrophic cultivation was first proposed in Germany in the early 1940s, in which CO_2 and light energy are used as the carbon and energy sources, respectively. In the 1990s, Australian researchers proposed the method of "extensive cultivation," which is easy to operate and economical [38]. It consists of large open ponds without CO_2 addition and with minimal control. Facilities are usually located in places where solar irradiance is maximal, cloudiness is minimal, and the climate is warm. The photoautotrophic mode has been commonly employed in the culture of *Dunaliella* for β-carotene production, because this alga can survive under extreme conditions (e.g., high salinity and high temperature) and its cultivation is easily maintained. To reduce the contamination of plankton, hypersaline water is usually used. A two-step approach, namely "intensive cultivation" has also been employed for the large-scale production of *Dunaliella* β-carotene. The aim of stage 1 is to optimize biomass production of cells with a low β-carotene-to-chlorophyll ratio; and in stage 2, the culture is diluted to about one-third to increase the light availability to cells, and carotenogenesis is further enhanced by nitrogen deficiency [39].

Although photoautotrophic cultivation is easily maintained, a high growth rate is barely achieved due to the hostile or unsteady environment. In addition, both light intensity and photosynthesis efficiency are low inside the cultures because of the absorption and scattering of light [40]. As a result, algal growth is largely limited by such inhomogeneous distribution of light. For example, the cell mass production of *Chlamydomonas reinhardtii* only reached 0.57 g cells L^{-1} under photoautotrophic mode with optimized culture conditions [41]. The biomass of *C. zofingiensis*

cultivated photoautotrophically at 68 μmol m^{-2} s^{-1} was confined to 0.72 g L^{-1} [42]. To solve the problems, another culture system, heterotrophic cultivation, has been developed.

5.2 Heterotrophic Cultivation

Heterotrophic cultivation refers to the organism's use of organic carbon substances as carbon and energy sources rather than CO_2 and light to support the cell growth and other physiological events [43, 44]. Due to the elimination of light restriction, cells grow quickly and a high cell mass production can be achieved easily. Thus far, the maximum biomass content achieved in photoautotrophic cultures is 40 g L^{-1} of cell dry weight [45], much lower than that in heterotrophic cultures, where 100 g L^{-1} to greater than 150 g L^{-1} is available [46].

Given the high costs of heterotrophic cultivation, this mode is generally used for the production of value-added compounds from microalgae, such as lutein and astaxanthin (Table 4). When *C. protothecoides* was heterotrophically cultivated, the algal cell dry weight and lutein yield reached 19.6 g L^{-1} and 83.8 mg L^{-1}, respectively, in the fermentor, and even achieved as high as 46.9 g L^{-1} and 225.3 mg L^{-1} using the fed-batch culture strategy [5, 6]. Wu and Shi [51] conducted a heterotrophic cultivation using another *Chlorella* species, *C. pyrenoidosa*; with the artificial neural network (ANN) model employed as the optimization strategy, the highest biomass concentration and the maximum productivity achieved 104.9 g L^{-1} dry cell weight and 0.613 g L^{-1} h^{-1}, respectively. The high cell density can be achieved by the employment of fed-batch, continuous, and perfusion culture strategies that are commonly used in the fermentation of bacteria or yeasts. Fed-batch culture is an effective way to minimize substrate inhibition because a high initial concentration of substrates (e.g., sugars) may lead to growth inhibition. On the other hand, the fed-batch culture cannot overcome the inhibition caused by the toxic metabolites generated during the algal culture. They may accumulate as the cells build up and prevent further enhancement of cell density. Such metabolite-driven inhibition can be eliminated by continuous or semicontinuous cultivation systems, in which the fresh medium is continuously added to a well-mixed culture, while cells or products are simultaneously removed to keep a constant volume. Perfusion culture is a technique combining the advantages of fed-batch and continuous culture systems. Cells are physically retained by a retention device, whereas the spent medium is removed. Meanwhile, fresh medium is fed into the bioreactor to maintain a sufficient nutrient supply. Park et al. [52] established a two-stage mixotrophic culture system for *Haematococcus pluvialis*. By the perfusion process, the biomass density achieved was 3.09 and 1.67 times higher than batch and fed-batch processes, respectively. Zhang et al. [53] established an "attached cultivation" using the immobilized biofilm. Under the optimized condition, the maximum astaxanthin productivity reached 160 mg m^{-2} d^{-1}.

Table 4 Heterotrophic cultivation of microalgae for the production of lutein and astaxanthin

Carotenoids	Strain	Device	Culture mode	Cell concentration ($g\ L^{-1}$)	Productivity ($mg\ L^{-1}\ d^{-1}$)	Yield ($mg\ L^{-1}$)	Reference
Astaxanthin	*Chlorella zofingiensis*	Flask	Batch	10.2	0.7	10.3	[47]
	Chlorella zofingiensis	Flask	Batch	7.5	1.3	11.8	[48]
	Chlorella zofingiensis	Flask	Batch	7.3	1.4	12.6	[49]
	Haematococcus pluvialis	Flask	Batch	NA	0.8	9.0	[50]
Lutein	*Chlorella protothecoides*	Fermentor	Batch	19.6	11.3	83.8	[6]
	Chlorella protothecoides	Fermentor	Fed-batch	46.9	22.7	225.3	[5]

Source Adapted from Ref. [46]

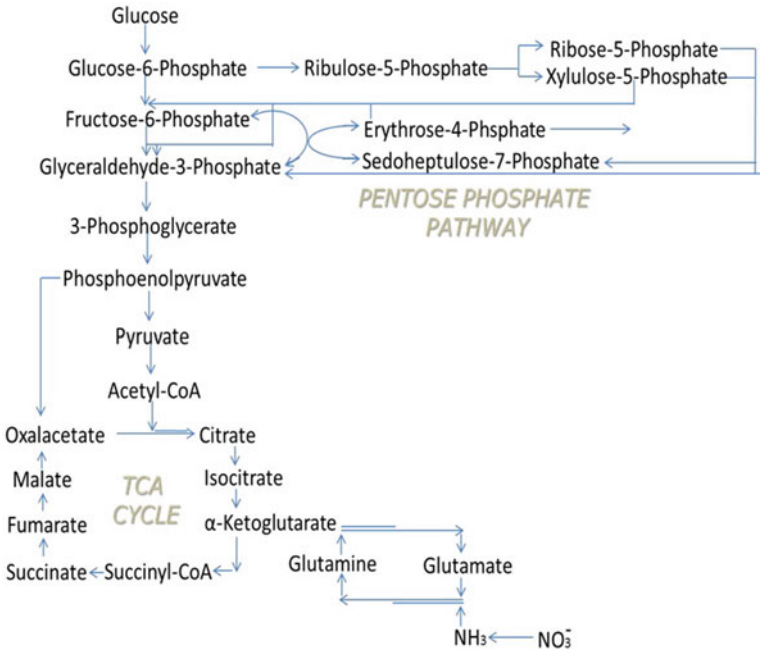

Fig. 2 Heterotrophic metabolism in microalgae (modified from Ref. [55])

The known carbon sources include glucose, glycerol, acetate, fructose, ethanol, and some low-cost media formulations with molasses or carob pulp syrup, as well as wastewater from the sugar or milk processing industries [54]. Among them, glucose is the most superior candidate, stimulating much higher growth rates of microalgae than other carbon sources. Through the comparison between heterotrophic and photoautotrophic modes, it was found that the ATP generated from the former using glucose as the energy supplier exceeded more than 600 % over the latter in which energy was supplied by light [55]. As shown in Fig. 2, in contrast to light conditions where the Embden–Meyerhof–Parnas (EMP) pathway acts as the predominant glycolysis, under darkness, glucose is mainly metabolized via the pentose phosphate (PP) pathway [56]. For example, in alga *Chlorella pyrenoidosa*, the PP pathway accounts for over 90 % of glucose metabolic flux distribution [55].

Heterotrophic cultivation also has some other advantages such as a high degree of process control, low costs for harvesting biomass because of the higher cell densities achieved, and so on [57]. Nevertheless, this is not to say that this cultivation mode is without drawbacks: the number of microalgal species able to grow heterotrophically is limited; the formation of light-induced metabolites will be diminished; contamination and competition with other microorganisms could take place, and so on. Table 5 shows the comparison between photoautotrophic and heterotrophic production of microalgae-derived products.

Table 5 Comparison between photoautotrophic and heterotrophic production of microalgae-derived products

	Photoautotrophic mode	Heterotrophic mode
Available algal species	A lot	Limited
Cell density	Low	High
Cost of production	Low	Highs
Product contents	Low	High
Productivity	Low	High
Scaleup	Easy	Difficult

6 Biosynthesis

Major advances have been made in the elucidation of the carotenoid biosynthetic pathway in bacteria, algae, and higher plants, and it is believed that in these organisms, the biosynthesis of primary carotenoids follows a similar pathway. The biosynthesis process of β-carotene and α-carotene is outlined in Fig. 3, which can be divided into the following steps.

6.1 Formation of Isopentenyl Diphosphate (IPP)

Glyceraldehyde-3-phosphate (GAP) and pyruvate act as the first two precursors. They are utilized to produce 1-deoxy-D-xylulose-5-phosphate (DXP) which is then transformed to IPP through a series of reactions in the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway present in the plastids of plants, cyanobacteria, algae, and certain bacteria [59].

6.2 Formation of Geranylgeranyl Pyrophosphate (GGPP)

GGPP is the first immediate precursor in the lutein biosynthesis pathway, which is synthesized from IPP. At first, IPP is isomerized to its allylic isomer, dimethylallyl diphosphate (DMAPP). Geranyl pyrophosphate (GPP) is then produced by the condensation reaction of IPP and DMAPP. Elongation of GPP by the addition of IPP results in the formation of farnesyl pyrophosphate (FPP) to which is further added another IPP to produce GGPP [60]. These condensation reactions are catalyzed by a single enzyme called GGPP synthase, which was isolated as a soluble and functional homodimer (74 kDa) from the chromoplasts of *Capsicum* [61].

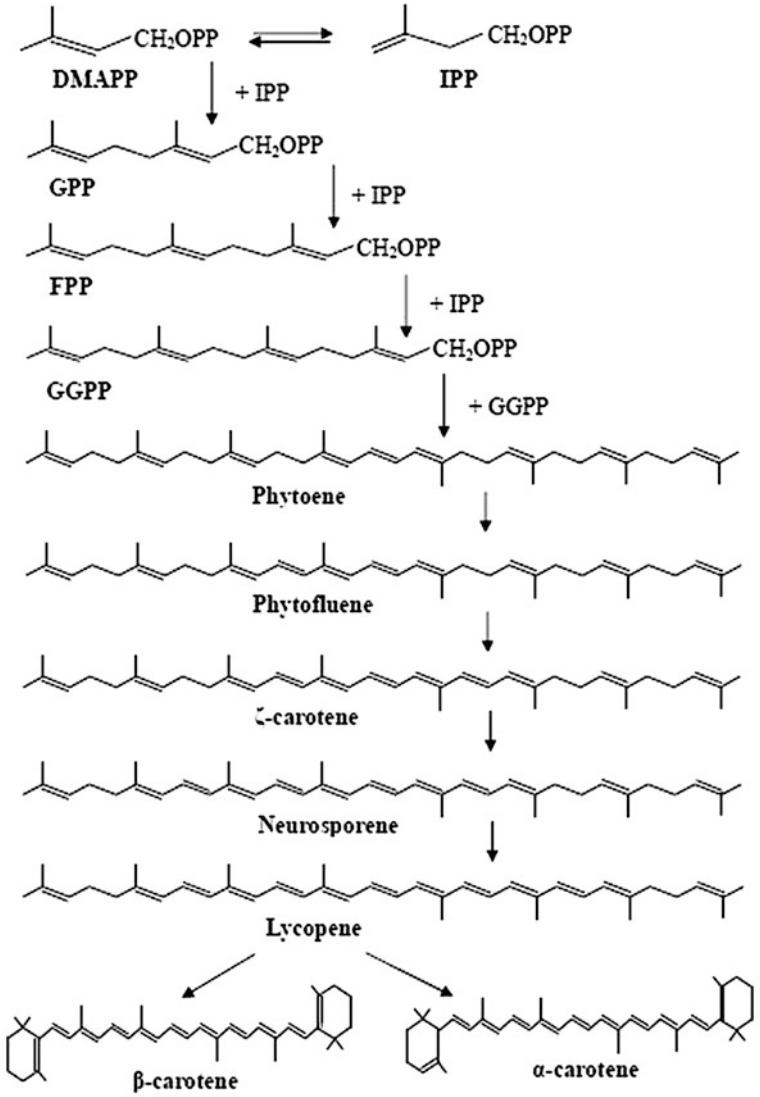

Fig. 3 Carotenoid biosynthetic pathway to form β-carotene and α-carotene (adapted from Ref. [58]). Abbreviations: *DMAPP* dimethylallyl diphosphate; *IPP* isopentenyl diphosphate; *GPP* geranyl diphosphate; *FPP* farnesyl diphosphate; *GGPP* geranylgeranyl diphosphate

6.3 Biosynthesis and Desaturation of Phytoene

Phytoene is synthesized through condensation of two GGPP molecules under the catalysis of phytoene synthase (PSY), a membrane-associated enzyme [62]. PSY has been reported to be a rate-limiting enzyme of carotenoid biosynthesis in

different plant organs, tissues, and locations, such as in canola seeds, ripening tomato fruits, and in marigold flowers [63–65]. In addition, PSY is highly conserved among archaea, bacteria, and eukaryotes [66]. Lycopene is subsequently formed by the conversion of phytoene, which is catalyzed by two desaturases, namely phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) through a series of reactions [67].

6.4 Cyclization of Lycopene

Carotenoid cyclization refers to the formation of a six-membered ring at one or both ends of lycopene and an important branching point of the carotenoid biosynthesis pathway in photosynthetic organisms. One branch leads to the formation of β-carotene and its derivative xanthophylls, such as zeaxanthin and astaxanthin, and the other one leads to α-carotene and lutein in plants and microalgae [68]. The formation of β and ε rings at both ends of lycopene results in the yield of α-carotene under the catalysis of β-cyclase (LCY-b) and ε-cyclase (LCY-e), respectively, whereas the formation of two β-rings at the two ends of lycopene gives rise to β-carotene catalyzed by LCY-b alone [67].

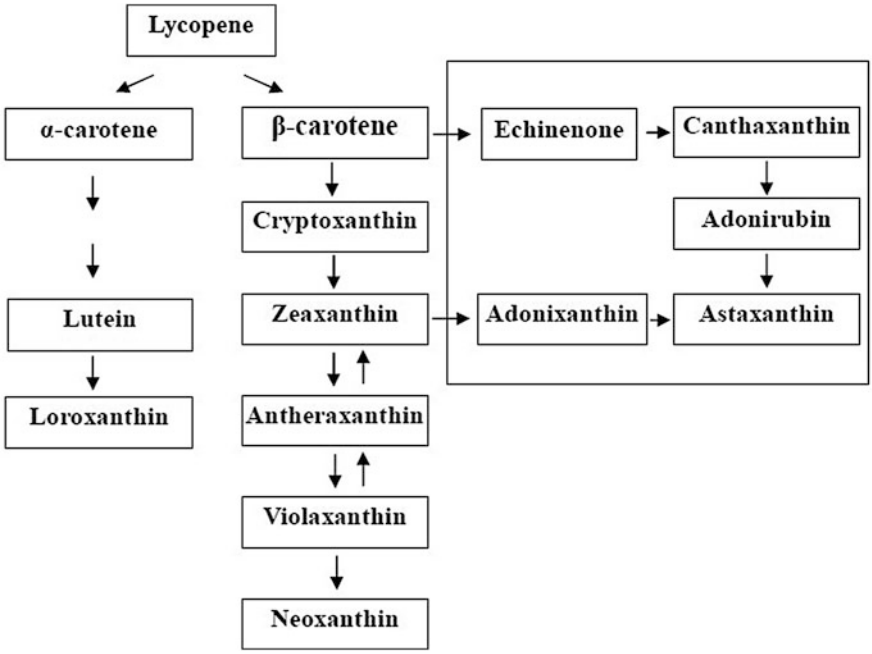

Fig. 4 Schematic diagram of pathway of xanthophyll biosynthesis. In the box is the astaxanthin biosynthetic pathway present in certain algae. Two possible ways are indicated (adapted from Ref. [69])

6.5 Hydroxylation

In higher plants and algae, xanthophylls are enzymically formed through the oxidation of α- and β-carotene. Hydroxylation at the C-3 and C-3′ positions of α-carotene by β- and ε-carotene hydroxylase encoded by genes *CrtR-b* and *CrtR-e* lead to the formation of lutein, whereas hydroxylation of β-carotene by β-carotene hydroxylase alone results in the formation of zeaxanthin [67]. The subsequent epoxidation of zeaxanthin leads to the production of violaxanthin, which is further converted to neoxanthin. Meanwhile, in some algae, additional xanthaphyll biosynthetic pathways could also be present. For example, oxygenation and hydroxylation of β-carotene bring on the synthesis of astaxanthin (Fig. 4).

7 Regulation of Carotenogenesis

7.1 Intercommunication of Cellular Organelles and Retrograde Regulation of Photosynthetic Genes

In higher plants, algae, and other photosynthetic eukaryotic cells, mitochondria, chloroplast, and nucleus are three essential organelles. These cellular components are not isolated or independent, but have interaction and communication with each other. In most cases, chloroplast and mitochondria have their own organelle genome, but these genomes only encode few amounts of proteins. Current proteomics and genomics studies show that these organelles comprise thousands of proteins. Studies in cyanobacterium indicated that most proteins (93–99 %) were encoded by nuclear genes and were transported into the organelles after translation and processing in cytoplasm [70, 71]. Therefore, there must be a mechanism regulating and coordinating the nucleus genome to encode and synthesize the most necessary organelle proteins during cell growth and development.

Anterograde regulation refers to the control of the biogenesis and function of organelles by the nuclear genome and has been widely studied in the past decades. Along with anterograde regulation, retrograde regulation refers to the communication between organelles and nucleus. The functional and developmental organelles send signals to the nucleus in order to activate the necessary genes to meet the particular need of the cell. The detailed retrograde regulation mechanisms in chloroplast and mitochondria remain unclear, and thus far, four organelle retrograde regulation mechanisms have been determined: (1) organellar gene expression (OGE), (2) tetrapyrrole biosynthesis, (3) organellar redox state, and (4) ROS [72].

Johanningmeier and Howell [73] studied the regulation of light-harvesting chlorophyll-binding protein (LHCP) mRNA accumulation in *Chlamydomonas reinhardi*. With the block of late chlorophyll biosynthesis, the intermediate of chlorophyll synthesis, namely Mg-protoporphyrin IX monomethyl ester (Mg-ProtoMe), inhibited by a feedback mechanism the light induction of LHCP

mRNA accumulation. Treatments that presumably resulted in an accumulation of Mg-ProtoMe repressed the expression or transcription of chloroplast genes [74, 75]. Walker and Willows [76] reported the roles of Mg-chelatase in the chlorophyll synthesis pathway. Mg-chelatase is a three-component enzyme comprising subunits ChlD, ChlH, and ChlI, which catalyzes the insertion of Mg^{2+} into the porphyrin ring of Proto IX. Mochizuki et al. [77] observed that the plastid-to-nucleus signal transduction pathway was disrupted in two *Arabidopsis* CHlH mutants but not in ChlI mutants. Also, CHlH was found to be able to bind to deuteroporphyrin even in the absence of the other two cheltase subunits [78]. In this regard, Mochizuki et al. [77] proposed that CHlH may not only serve as the chelatase in tetrapyrrole biosynthesis, but also function as the tetrapyrrole sensor in a chloroplast-to-nucleus signaling pathway. Another possibility is that CHlH could directly participate in the plastid-to-nucleus retrograde signaling pathway by forming a CHlH-tetrapyrrole complex with other cofactors without affecting the tetrapyrrole transport [79, 80].

There is another chloroplast-to-nucleus retrograde regulation pathway derived from the redox and ROS signals in plants and green algae. For redox signal retrograde regulation, two possible sources have been proposed, the redox state of the PQ pool in the photorespiration electron transport chain and light-harvesting complex II (LHCII) kinase. Escoubas et al. [81] proposed that in *Dunaliella tertiolecta*, the redox state of PQ may influence the regulation of nuclear photosynthesis genes, and such retrograde regulation was possibly achieved via a chloroplast protein kinase. Similar regulation mechanisms also exist in higher plants [82]. On the other hand, some research groups also suggested that LHC II kinase, rather than the PQ pool, may serve as the retrograde regulation signal source. Pursiheimo et al. [83] demonstrated that the transcription of genes *Lhcb*, *rbcS*, and *psbA* was positively related to LHCII protein phosphorylation. Bonardi et al. [84] also found that STN7, a dual-function chloroplast thylakoid protein kinase, participated in the chloroplast redox signaling pathway in *Arabidoposis thaliana*. These results indicated that protein kinase may also function as a necessary component in the chloroplast redox signaling transduction pathway.

The retrograde regulation also exists in mitochondria. It is believed that the mitochondrion interacts closely with other cellular organelles, such as chloroplast and nucleus, and a wide range of signaling events in plant cells can be strongly influenced by changes in mitochondrial function. Such changes trigger altered nuclear gene expression by a process of mitochondrial retrograde regulation (MRR) [85]. MRR is manifested at mitochondria dysfunctions induced by mutations, chemical agents, or stresses. Previous studies indicated that plant mitochondrial dysfunction led to the induction of nucleus-located genes encoding proteins for the restoration of mitochondria function. Meanwhile, genes encoding antioxidant enzymes were also activated to detoxify the ROS and protect the cell organelle [85]. The most well-studied MRR thus far is on the expression of gene encoding alternative oxidase (AO) in mitochondria. Two possible signal transduction pathways have been proposed: one involves some intermediates of TCA

cycles such as organic acid 2-oxoglutarate, citrate, and malate, and the other is derived from the ROS produced in mitochondria [86]. MRR is still a poorly understood process and very little is known about the exact factors involved in the signal transduction pathway. Components able to react with ROS in mitochondria or cytosol (e.g., lipids, antioxidant enzymes, or small molecular weight compounds) should be considered as the candidates of primary or secondary signal molecules. Matsuo and Obokata [87] studied the MRR in *C. reinhardtii*. Under heterotrophic and mixotrophic conditions, nucleus-located photosynthetic genes were remote-controlled by a mitochondrial respiratory electron transport chain (RET). When algal cells were cultured under autotrophic and mixotrophic conditions, the controller became a photosynthetic electron transport (PET). These results suggested the cooperation between cellular organelles in algae and indicated the regulatory system of photosynthetic genes changes in response to shifts in the dominant energy source between photosynthesis and respiration.

7.2 Stimulation of Carotenogenesis by Oxidative Stress

7.2.1 Enhancement of Carotenoid Synthesis Induced by ROS

Jahnke [88] reported that the accumulation of carotenoids was one of the responses of microalgae to the oxidative stress induced by UV radiation. When *D. bardawil* was exposed to the UV-A radiation of 70 μmol photons m^{-2} s^{-1} for 84 h, the contents of lutein and zeaxanthin within algal cells increased about threefold and fivefold, respectively [89].

Carotenogenesis in algae could also be stimulated in response to chemically generated oxidative stresses. Kobayashi et al. [90] indicated that Fe^{2+} may serve as an $HO^{\bullet}$ generator via an iron-catalyzed Fenton reaction, and the generated ROS played an essential role in enhancing the carotenoid formation in *H. pluvialis*. This viewpoint was verified by Ip and Chen [49]. When the *C. zofingiensis* culture was supplemented with ROS generators Fe^{2+} (18 μM) and H_2O_2 (0.1 mM), the yield of astaxanthin increased from 9.9 to 12.58 mg L^{-1}. The production of lutein was also enhanced in *C. protothecoides* under heterotrophic cultivation. The lutein content increased from 1.75 to 1.98 mg g^{-1} when 0.01 mmol L^{-1} H_2O_2 and 0.5 mmol L^{-1} NaClO were added to generate $^1O_2$ [91]. Inactivation of antioxidant enzymes is another way to stimulate the formation of carotenoids as they act as nonenzymatic antioxidants. For example, sodium azide is known as an inhibitor of catalase (CAT) and superoxide dismutase (SOD), two important ROS scavengers. Shaish et al. [92] reported that when *D. bardawil* cells were treated with sodium azide, the content of β-carotene increased from 11.7 to 27.5 pg $cell^{-1}$.

7.2.2 Expression Variation of Genes Encoding Enzymes Involved in Carotenoid Biosynthesis After Oxidative Stress Treatment

When the microalgae are under stress treatments such as high light and high salt stress, the expression of genes encoding enzymes involved in carotenoid biosynthesis is upregulated, which subsequently increases the accumulation of carotenoids. According to Steinbrenner and Linden [93], in *H. pluvialis*, all four genes encoding PSY, lycopene cyclase, PDS, and carotenoid hydroxylase showed higher transcript levels in response to increased illumination. Similar research was conducted by Ramos et al. [94] using *D. salina*. The highest steady-state mRNA level of *Lcy-β* (encoding lycopene β-cyclase) was observed in algal cells exposed to high light (500 μmol m^{-2} s^{-1}) or low light (45 μmol m^{-2} s^{-1}) combined with nutrient depletion for 24 and 48 h, respectively. As a result, the cellular β-carotene content increased faster in the case of exposure to high light conditions at 24 h.

7.2.3 ROS Sensing Signaling Cascade Involved in Simulating Carotenogenesis

Due to the high cytotoxic and reactive feature of ROS, their concentration must be tightly controlled. Higher plants and algae can sense the ROS signals and make appropriate cellular responses. The redox-sensitive proteins that reversibly become oxidized or reduced depending on the cellular redox state are essential in the response process [19]. Pfannschmidt et al. [82] indicated that heterotrimeric G-proteins participated in the signaling pathway mediated by ROS. Protein phosphorylation controlled by protein Tyr phosphatases and specific mitogen-activated protein (MAP) kinases are two main well-studied molecular mechanisms of redox-sensitive regulation of protein function in cells [19, 95]. In microalgae, a gene encoding a MAP kinase was isolated from *D. salina* and its expression was found to be affected by changes in temperature and salinity conditions that also influenced the accumulation of β-carotene [96]. Eom et al. [97] detected 16 (11 %) differently expressed genes from *H. pluvialis* that were involved or potentially involved in defense or stress responses. Nedelcu [98] indicated that tumor suppressor protein p53, which plays a major role in cellular response to stress, was also present and mediated cellular responses to stress in two algae, *Volvox carteri* and *C. reinhardtii*.

8 Conclusion and Future Perspectives

Lutein production has been one of the most successful activities in the microalgal industry. As the world's market demand is growing, there are still substantial challenges to be addressed to enhance the production capacity and economics of microalgae-based lutein, which urgently requires the development of microalgal

biotechnology. For example, genetic engineering is a feasible approach to improve the biosynthetic pathway of lutein in microalgae and heighten its cellular accumulation. Another important issue to consider is the cost. As mentioned earlier, although the heterotrophic cultivation allows a higher cell density and lutein productivity, the high costs of glucose hinder its commercial application. It is logical to develop low-cost and effective carbon sources from industrial or agricultural wastes. Finally, the production of lutein may be combined with other algal-based metabolisms (such as oil) and coupled with wastewater treatment and biofixation of carbon dioxide to develop an integrated industrial chain that may increase the process economy and bring environmental benefits as well.

9 Acknowledgments

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Modelling of Microalgae Culture Systems with Applications to Control and Optimization

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Abstract Mathematical modeling is becoming ever more important to assess the potential, guide the design, and enable the efficient operation and control of industrial-scale microalgae culture systems (MCS). The development of overall, inherently multiphysics, models involves coupling separate submodels of (i) the intrinsic biological properties, including growth, decay, and biosynthesis as well as the effect of light and temperature on these processes, and (ii) the physical properties, such as the hydrodynamics, light attenuation, and temperature in the culture medium. When considering high-density microalgae culture, in particular, the coupling between biology and physics becomes critical. This chapter reviews existing models, with a particular focus on the Droop model, which is a precursor model, and it highlights the structure common to many microalgae growth models. It summarizes the main developments and difficulties towards multiphysics models of MCS as well as applications of these models for monitoring, control, and optimization purposes.

Keywords Microalgae · Photobioreactors · Raceways · Modeling · Optimization · Biofuel · CO_2 mitigation

Contents

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1 Introduction

The renewal of phytoplankton-based processes over the last decade has mainly been driven by the great promises of these microscopic plants. Both microalgae and cyanobacteria show a great potential for industrial applications, including food, pharmaceuticals and cosmetics, chemicals, and even biofuel [74]. Contributing the most to this popularity is perhaps the fact that microalgae present high photosynthetic yields compared to terrestrial plants and that certain species can reach a very high lipid content, above 50 % dry weight [73]. With the prospect of achieving lipid productivities several-fold higher than those of terrestrial plants [111], many have started envisioning large-scale microalgae culture systems (MCS) for biodiesel production [22]. Nonetheless, these predictions are often based on crude extrapolations of the productivities obtained in the lab, where conditions differ drastically from those of outdoor production systems, and thus far they could not be confirmed experimentally on pilot- or larger-scale demonstration plants. In this context, mathematical modeling can be a great help to understand, and in turn remedy, the gap between lab-scale observations and the industrial-scale reality. Not only can these models be used for monitoring, control, and optimization of the actual production systems, but they could also drive the choice of a particular microalgae species that is best suited to the local environment or even to a particular season of the year.

Modeling of high-density MCS, together with their control and optimization, proves more challenging than that of bacterial or yeast culture systems. To a large extent, this added complexity stems from the wide range of mechanisms used by microalgae to respond to, or protect themselves from, light and other local environmental factors. This is particularly so in outdoor production systems, where microalgae are permanently subject to unsteady conditions, for example, due to diurnal light and temperature variations. The dynamics induced by this periodic

forcing are difficult to model accurately and also make it hard to devise effective control strategies. Another modeling challenge is tied to the way microalgae access light in order to sustain their growth. Because of light-absorption mechanisms and shadowing effects, a higher microalgae concentration will reduce the amount of light available to the culture. This sets an upper limit on the theoretical (steady) concentration of microalgae, whereby the average growth over the light column is exactly balanced by respiration; that is, the net growth rate is zero. However, predicting this limit is not at all straightforward as microalgae also undergo photoacclimation via the adaptation of their pigments to the available light [2, 65]. In other words, light attenuation, as driven by pigment concentration and cell size, is itself dependent on light. Moreover, under nitrogen-limited growth conditions—often used to stimulate the production of a valuable metabolite [90]—both the pigment composition and concentration vary [42, 96, 108] and the cells increase their size, further affecting light attenuation [102].

Advances in the dynamic modeling of microalgae populations are scattered across various fields, including oceanography, ecology, and biotechnology. An early dynamic model of phytoplankton was proposed by Riley [89] for describing phytoplankton populations on Georges Bank. Quite remarkably, Riley modeled both nutrient- and light-limited growth by considering an exponential decrease of light along depth. A growing number of kinetic models describing the rate of photosynthesis have been proposed ever since, ranging from simple hyperbolic expressions [3] to complex representations accounting for the photoinhibitory effects caused by an excess of light [79, 81, 101, 110]. The processes of nutrient uptake and nutrient-limited growth likewise have been described by a number of semiempirical models [19, 30–32], and these models have later integrated light-limitation effects in order to represent the nonlinear couplings between photosynthesis and nutrient limitation (especially nitrogen) [36, 42, 78].

Regarding MCS, perhaps the first dynamic model of a raceway pond was proposed by Sukenik et al. [105] within the scope of the Aquatic Species Program [99]. This model was later extended to encompass discrete-time photoacclimation dynamics [106]. Other, less detailed, models were also proposed in the meantime [8, 45, 49]. By and large, high-density MCS present many challenges and opportunities for their reliable modeling as well as in applications of these models for control and optimization purposes [10].

This chapter starts by outlining the principles and building blocks of microalgae culture models (Sect. 2). Models that describe the main processes involved in microalgae growth and bioaccumulation are reviewed in Sect. 3, namely carbon and nitrogen internalization (and loss), carbohydrate and lipid storage, and pigment adaptation. The focus in Sect. 4 is on the physical characteristics of MCS, including models of the light distribution, flow pattern, and temperature evolution. Following this review is a discussion about the combination of the biological and physical properties into the overall modeling of outdoor, possibly high-density, MCS together with open issues (Sect. 5). Finally, a number of monitoring, control, and optimization strategies that take advantage of the available mathematical models are considered (Sect. 6).

2 Building Blocks of Microalgae Culture Models

Within the context of bioprocesses, the most natural way of building a model that captures the main process dynamics is to consider conservation principles. Mass, energy, and momentum balances normally come in the form of ordinary differential equations (ODEs) for lumped systems, or partial differential equations (PDEs) in the case of distributed systems, for instance, due to imperfect mixing or advection. An important feature of balance-based models is the presence of conversion terms (e.g., to describe biochemical reactions) as well as transfer terms (e.g., to account for liquid–gas exchange).

In the context of MCS, the solutions to the balance equations characterize concentration fields for key species inside the culture medium (mass balance of nutrient and biomass), along with velocity (momentum balance) and temperature (energy balance) fields in the liquid phase. In addition to transport and accumulation terms, the other critical terms in these equations are those describing:

(i) **Intrinsic biological properties**, including the rates of nutrient internalization, microalgae growth and decay, and intracellular storage. These rates are dependent upon both current and past conditions in terms of culture medium concentration, light, temperature, and so on. Note, in particular, that this dependence is with respect to local conditions in nonhomogeneous culture media.

(ii) **Physical properties**, including the light transmitivity, the viscosity, and the mass and thermal diffusivities in the culture medium. All these properties depend on the (possibly local) compositions of the culture medium and the characteristics of the microalgae cells themselves. They are also dependent on the geometry and mode of operation of the reactor.

The fact that the aforementioned biological and physical properties are strongly coupled and span multiple timescales (ranging from milliseconds to days) makes high-fidelity simulation of an overall microalgae culture system particularly challenging. An important feedback mechanism here is the effect of light on the local growth rate, which modifies the concentration of microalgae, affecting the way light is absorbed and scattered in the culture medium in turn. Even the hydrodynamics in the reactor can have an effect on light distribution as the waves at the surface create a complex air–water interface that can punctually concentrate solar rays. Regarding timescales, light effects on microalgae growth range from milliseconds for photoproduction, to minutes or hours for photoinhibition and photoregulation, and to days or weeks for photoacclimation. On top of this, MCS are subject to various periodic forcings, such as diurnal cycles or mixing inside the reactor leading to fast light variations.

The next two sections give an overview of available models describing the biological and physical properties of MCS. Then, we discuss various ways of coupling these models.

3 Modeling of Intrinsic Biological Properties

The dynamics that underlie microalgae growth—including internalization of the main nutrients (N, P) and carbon (inorganic and/or organic) as well as the effect of light and temperature—can be described in numerous ways.

Detailed metabolic models have been developed by accounting for all available, yet still partial, knowledge about the metabolic pathways of particular microalgae species. These models usually rely upon the principle of *balanced growth*, which precludes internal storage of metabolites. This assumption dramatically reduces the number of kinetic parameters needed to describe the individual reactions in the model. Nonetheless, the fixation of inorganic carbon (CO_2 or bicarbonate) in autotrophic microalgae is dependent on the incoming photon flux, thus this assumption should therefore be limited to constant light culture conditions from a strict point of view. Despite these limitations, some authors have considered using metabolic modeling tools under transient light conditions [23, 57, 98, 113]. Others have expanded the approach to allow for internal storage and reuse of metabolites such as neutral lipids and carbohydrates [24, 58].

A second kind of growth model, often referred to as compartmental models, describes the physiological status of microalgae cells in terms of quotas for key components, such as nutrients, chlorophyll, lipid, carbohydrate, and so on. These macroscopic (semiempirical by nature) models are simpler than their metabolic counterparts, yet they do not call for any assumption about balanced growth. As such, they are well suited for coupling with detailed hydrodynamics models for flow and temperature computations (see Sect. 5), and they are equally well suited for development of model-based monitoring and control strategies (see Sect. 6).

The emphasis in the remainder of this section is more specifically on this latter class of models, focusing successively on nutrient-limited growth, light effects, and temperature effects, and assuming the other factors constant.

3.1 Nutrient-Limited Growth and Decay

In a classical batch experiment, microalgae continue to grow for several days after key nutrients are depleted from the culture medium. This apparent uncoupling between the processes of nutrient uptake (inorganic nitrogen, phosphorus, vitamins, etc.) and growth in microalgae is well documented [95], and it has led to the development of so-called quota models, which account for nutrient storage (pooling) inside the cells. For instance, the internal cell quota q_n of a limiting nutrient (substrate) s can be described by a simple mass-balance equation of the form:

$$\dot{q}_n = \rho(s, \cdot) - \mu(q_n, \cdot) q_n \,, \tag{1}$$

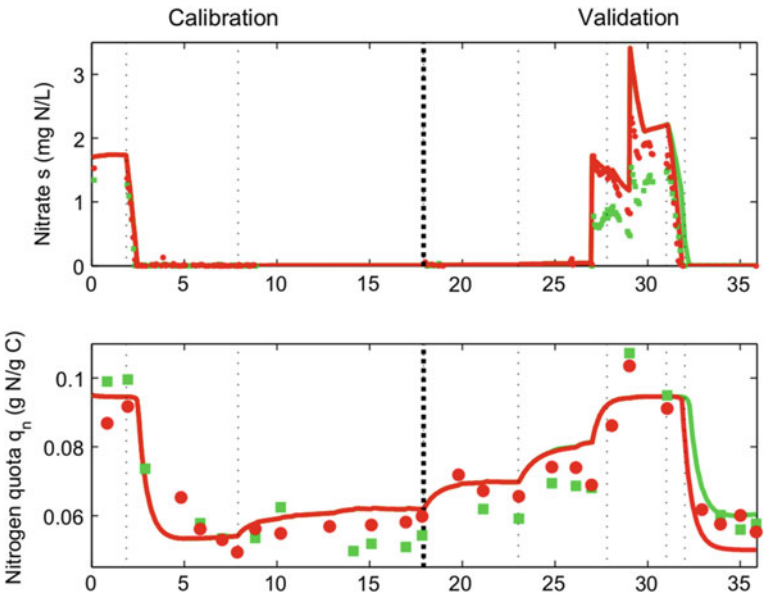

Fig. 1 Comparison between measurements and model predictions of the nitrate concentration (s) in the culture medium and the nitrogen quota (q_n) under nitrogen-limited conditions in a chemostat culture of *Isochrysis* aff. *galbana*. Reproduced from [67]

where ρ and μ denote the uptake rate of nutrient s and the corresponding nutrient-limited growth rate, respectively.[1] The quota q represents the intracellular amount of nutrient per unit cell mass. In the case where nitrogen is the limiting nutrient, for instance, the internal nitrogen quota can be defined in units of g(N) g(C)$^{-1}$.

Initially introduced to describe the limiting effect of Vitamin B_{12} on the growth rate of phytoplankton [30], the Droop model has also been found to predict the effect of macronutrient limitation accurately, including nitrogen or phosphorus limitation, and it has been widely validated [13, 31, 97, 109]; see, for instance, Fig. 1. Although applicable to a single limiting nutrient and constant light conditions only, the simplicity of the Droop model is a big help in practice as it enables detailed mathematical analysis [12, 14, 62]. Moreover, the meaning of its parameters makes it easy to relate to measurable quantities such as minimal and maximal internal quota and maximum growth rate.

The growth rate $\mu(q_n, \cdot)$ in the Droop model is expressed as an increasing function of the internal quota q_n:

$$\mu(q_n) = \mu_\infty \left(1 - \frac{Q_0}{q_n}\right), \tag{2}$$

[1] We use the notation $(\cdot)$ here to recall that these rates can also depend on other key parameters, such as light, temperature, and so on.

where Q_0 stands for the minimal cell quota, below which no growth is possible, and μ_∞ represents the growth rate at an hypothetical infinite quota. Alternative formulations have been proposed on the basis that a minimum (nonzero) internal quota is required in order for microalgae to grow. In a model due to Geider [42], for instance, the growth rate is simply a linear function of the internal quota, $\mu(q_n) = \mu_\infty (q_n - Q_0)$.

The uptake rate $\rho(s)$, on the other hand, is traditionally expressed in terms of Michaelis–Menten kinetics [19]:

$$\rho(s) = \rho_m \frac{s}{s + k_s}, \tag{3}$$

where k_s is the half-saturation constant for substrate uptake associated with the maximum uptake rate ρ_m. In Geider's model [42], an extra multiplicative term of the form $(Q_{max} - q_n)^p$ is appended to the uptake rate expression (3), usually with $p = 1$ [10, 17], so nutrient uptake comes to a stop as soon as the maximum internal quota Q_{max} is reached. Nonetheless, it is not hard to show that the original uptake rate expression (3) defines a maximum quota in a natural way, too. For instance, the internal cell quota is upper bounded by the value $Q_0 + \frac{\rho_m}{\mu_\infty}$ under nonlimiting nutrient conditions ($\rho(s) = \rho_m$) and using the growth expression (2).

In addition to uptake and growth rates, one must account for the loss of carbon via respiration. The corresponding rate of respiration can be expressed as the sum of a basal respiration rate and a term proportional to the cost of biosynthesis. The latter is typically assumed to be proportional to either the growth rate or the uptake rate [42, 92]. Although respiration is often accounted for indirectly as part of the "net" growth rate μ, it becomes necessary to distinguish the respiration rate from the growth rate more specifically when considering the effect of light. One possible approach involves defining the basal respiration as proportional to the cell concentration, while accounting for the rate of biosynthesis in the net growth rate. Note also that nitrogen is assumed to be released at the same rate as carbon in many models [42, 78], meaning that cell mortality and excretion are accounted for in the respiration rate.

3.2 TAG Synthesis

Among the various classes of lipids produced by microalgae, triacylglycerols (TAGs) are considered the preferred class in most applications, including algal-derived biofuel. Many microalgae strains have the ability to accumulate large quantities of lipids in the form of TAGs under environmental stress conditions such as nitrogen starvation. TAGs serve as energy and carbon storage compounds and as an electron sink in situations where the electron supply provided by photosynthesis exceeds the requirements for growth [54].

A number of models describing carbon storage in microalgae cells have become available in recent years [29, 47, 67, 77, 92]. Among them, the model in [67]

presents a simple structure based on the Droop model for representing TAG production in response to nitrogen deprivation and under constant light. It divides intracellular carbon into three pools, namely a functional pool (quota q_f), a sugar pool (quota q_s), and a TAG pool (quota q_l). The carbon fluxes between these pools lead to complex dynamics describing the storage and utilization of both sugars and TAGs. The way the model builds upon the Droop model is via a cascade structure, whereby the pool dynamics depends on the nitrogen quota q_n as well as the uptake rate ρ and growth rate μ:

$$\begin{aligned} \dot{q}_f &= -q_f\,\mu(q_n,\cdot) + (k_1 + k_3)\rho(s,\cdot) \\ \dot{q}_l &= [k_2\,q_n - q_l]\,\mu(q_n,\cdot) - k_3\,\rho(s,\cdot) \\ q_f + q_s + q_l &= 1, \end{aligned} \tag{4}$$

where k_1, k_2, and k_3 are (pseudo-)stoichiometric coefficients. This way, the model inherits the Droop model properties, taking advantage of its track-record validation, and only adding a limited number of extra parameters. Another interesting feature of the model is its ability to reproduce experimentally observed hysteresis in the dynamics of TAG accumulation, as shown in Fig. 2. This behavior contributes to making biolipid optimization complex and rather counterintuitive.

Other recent experimental studies have shown that TAG dynamics can become even more intricate when applying periodic light conditions in combination with

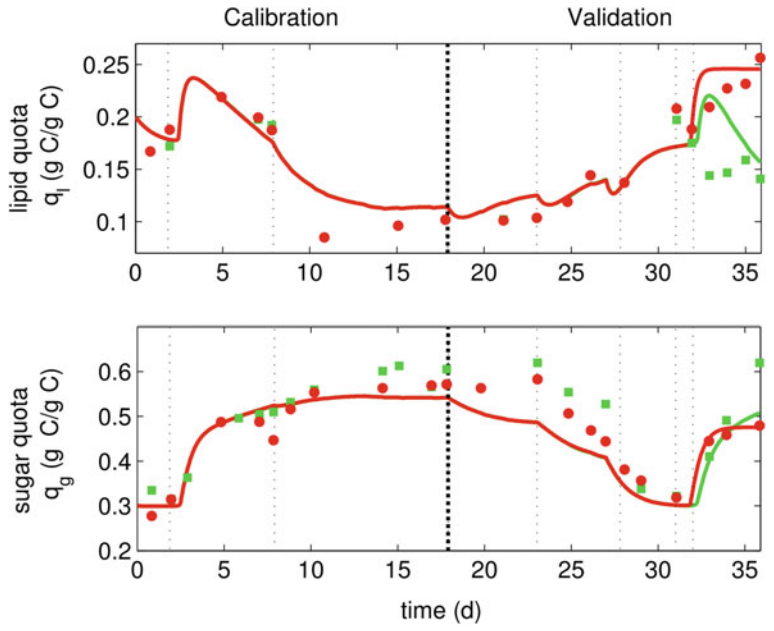

Fig. 2 Comparison between measurements and model predictions of the TAG (q_l) and sugar (q_g) quotas under nitrogen-limited conditions in a chemostat culture of *Isochrysis* aff. *galbana*. Reproduced from [67]

nitrogen deprivation. In particular, TAGs accumulate at a much slower pace than when exposed to continuous light; the lipids that are produced after a nitrogen starvation are consumed during dark phases (probably through a respiration process), thus maintaining the TAG pool at a low level [61]. Understanding how the mechanisms involved in cell synchronization interfere with the dynamics of TAG accumulation, and being able to model these interactions in turn reliably, clearly calls for further research.

3.3 Pigment Synthesis

A key mechanism used by microalgae to adapt their photosynthesis response to the available light involves modification of their pigment content. This photoacclimation strategy takes place at a timescale of days or weeks [25] and it can lead to a dramatic variation in the rate of photosynthesis. For instance, Fig. 3 shows PI-response curves for a microalgae culture preacclimated at different light irradiances, either normalized in terms of carbon or chlorophyll. On top of this, pigment synthesis can be strongly disrupted under nitrogen-limited growth as the pigment content is related to the protein content, which is itself related to the nitrogen status (nutrient quota q_n); this is especially so under complete nitrogen deprivation.

Geider et al. [42] were among the first to introduce chlorophyll as a state variable in their models, in addition to the carbon and nitrogen contents. They expressed the rate of pigment synthesis per carbon unit as proportional to the product between the rates of photosynthesis and nitrogen uptake.

More recently, Bernard [10] presented a model whereby chlorophyll is proportional to the cellular nitrogen content, used as a proxy of the actual protein content:

$$\theta = \gamma(I_0)\, q_n, \tag{5}$$

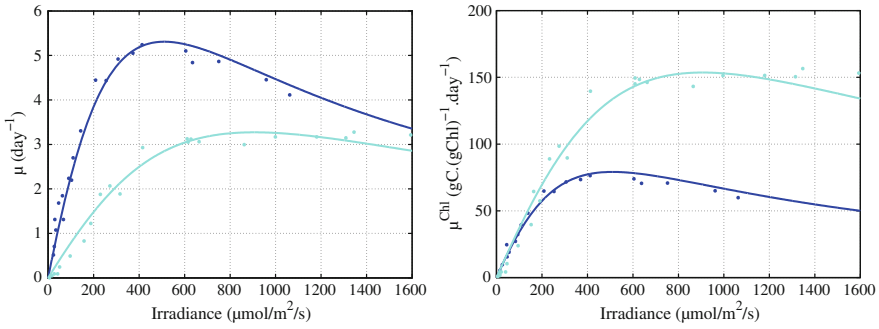

Fig. 3 Comparison between measurements and model predictions of the photosynthetic responses of the diatom *Skeletenonema costatum* under different acclimation states: I_0 = 50 μ mol m^{-2} s^{-1} (*dark points/lines*); I_0 = 1,200 μ mol m^{-2} s^{-1} (*light grey points/lines*). The photosynthesis rate is normalized by carbon (*left plot*) or chlorophyll (*right plot*). Data from [2]

where the chlorophyll quota θ represents the cellular chlorophyll-to-carbon ratio. In this expression, γ is a saturation function of the form

$$\gamma(I_0) := \gamma_{\mathrm{m}} \frac{k_{I_0}}{I_0 + k_{I_0}},$$

parameterized by γ_{m} and k_{I_0}. Moreover, I_0 is a conceptual variable representing the irradiance at which the cells are photoacclimated. In [10], the adaptation mechanism associated with I_0 is assumed to be driven simply by first-order dynamics:

$$\dot{I}_0 = \delta\, \mu(q_n, \cdot)\, [I - I_0], \tag{6}$$

with I the current light irradiance, and δ the photoacclimation rate constant. An alternative way to account for photoacclimation, without introducing the conceptual variable I_0, involves replacing (5) and (6) with:

$$\dot{\theta} = \delta'\, \mu(q_n, \cdot)\, [\gamma(I)\, q_n - \theta]. \tag{7}$$

In yet another model of photoacclimation, Geider [43] proposed the following alternative expression:

$$\theta = \frac{\mathrm{e}^{kT}}{(a - bT)\mathrm{e}^{kT} + cI}, \tag{8}$$

relating the chlorophyll quota θ to the current light irradiance and temperature.

3.4 Light-Limitation Effects

Notwithstanding its importance and significance, the Droop model, as well as other models derived from similar considerations, does not account for the effect of light on the growth rate. As such, it cannot be used directly to describe photolimited microalgae cultures.

Many photosynthesis models describe the effect of light in terms of PI–response curves. They use various proxies, such as the electron transfer rate (ETR), the O_2 production rate, the CO_2 consumption rate, or the growth rate itself. Although all these proxies do not involve the exact same mechanisms, the corresponding PI curves all have the same shapes.

Early photosynthesis rate models [3] considered simple hyperbolic expressions and did not account for photoinhibition by excess light. Such photoinhibition can be represented in a PI curve in either one of two main ways [79, 81, 101, 110]:

- The Platt model [81] is fully empirical and defines an exponential expression of the form $I\mathrm{e}^{-I}$, whereby the growth rate first increases with increasing light intensity up to a certain optimal irradiance, and then decreases from these maximal values as the light irradiance keeps increasing because of photoinhibition.

- The Han model [50], originating in the works of [33, 34, 59], has a stronger physical basis. It describes the chloroplasts in microalgae as arrays of photosynthetic units (PSUs), whereby each PSU is comprised of an antenna complex made up of pigments that is associated with the reaction center of a Photosystem II (RCII). The description of photoproduction and photoinhibition assumes that an RCII can be in either one of three states, namely open, closed, or damaged, and each RCII can transit from one state to another depending on the current light irradiance. An interesting property of the Han model is that the PI–response expression obtained by equilibrating the fast dynamics is of Haldane type.

The PI responses predicted by either of these representations are of course driven by other factors acting on growth, including nutrient limitation, pigment composition, and temperature. Nonetheless, a peculiarity of the chlorophyll-specific growth rate, $\mu^{\mathrm{chl}}(\cdot):=\mu(\cdot)/\theta$, is that the corresponding PI slope at vanishing light irradiance is typically independent of the photoacclimation light, and therefore independent of the chlorophyll quota θ. This property, which was perhaps first highlighted in [82], can be observed on the right plot of Fig. 3.

In particular, the foregoing constant initial slope property is key to understanding the way models that relate photoproduction/photoinhibition to the acclimation state are designed.

- The model by Geider and coworkers [65], which does not account for photoinhibition, expresses the (carbon-specific) growth rate as

$$\mu(I,\theta,q_{\mathrm{n}}) = \mu_{\max}(q_{\mathrm{n}})\left[1-\exp\left(\frac{-\alpha\theta I}{\mu_{\max}(q_{\mathrm{n}})}\right)\right], \tag{9}$$

  where $\mu_{\max}$ stands for the maximal growth rate (dependent on the nitrogen quota q_{n} and realized at high light irradiance); and α corresponds to the (constant) initial slope of

$$\frac{\mu(I,\theta,q_{\mathrm{n}})}{\theta}.$$

- The model by Bernard [10] is based on the Han model and can be expressed in the form

$$\mu(I,\theta,q_{\mathrm{n}}) = \mu_{\max}(q_{\mathrm{n}})\frac{I}{I+\frac{\mu_{\max}(q_{\mathrm{n}})}{\alpha\theta}\left(\frac{I}{I_{\mathrm{opt}}}-1\right)^2}, \tag{10}$$

  from which it is readily checked that the initial slope of $\frac{\mu(I,\theta,q_{\mathrm{n}})}{\theta}$ is again given by α.

At this point, both light- and nutrient-limitation effects can be combined by modulating the growth rate expressions (9) or (10) by a term dealing with substrate limitation, namely, the linear term $(q_n - Q_0)$ for the Geider model and Droop-like kinetics (2) for the Bernard model. Other models coupling chlorophyll production and

photosynthesis have been proposed [36, 40, 78], but they have not been used as much so far. More complex models have also been developed [38, 116], but being more accurate in the detail of the described mechanisms, they comprise more parameters and state variables that render their calibration/validation more difficult, too.

A situation under which the current light-effect models can become inaccurate and should be reconsidered is in the presence of fast-changing light regimes (flashing effect). The way photosynthesis reacts to high-frequency variations in light intensity remains the subject of active research. In order to capture these mechanisms, dynamic models describing the way photons are harvested (at a fast timescale) are required. These models typically work at a lower level by considering the concept of photosynthetic yield [20, 33, 34, 70, 115] and also try to account for nonphotochemical quenching (NPQ) regulation [75].

The effect of light flashes on microalgae growth has been studied experimentally, yet mainly for caricatural light patterns consisting in a succession of on/off periods at varying frequencies. Under such lighting protocols, the Han model—or related models—are found to represent the cell behavior well. We also note that for computational tractability reasons, the fastest timescale can be handled via singular perturbation techniques [21, 88] when considering some typical periodic light forcing [112].

3.5 Temperature-Limitation Effect

Like light, temperature too can play an important role in growth, and it is especially important to take its effect into account when considering outdoor MCS [87]. Two models have mainly been used to describe temperature effects on microalgae growth to date [15, 76].

The model in [15], based on the cardinal temperature model with inflection (CTMI) of [93], is detailed next. The main advantage of this model lies in its calibration simplicity, despite its empirical nature. The growth rate expression $\mu(\cdot)$ developed in the previous sections is simply modulated by a multiplicative switching function $\phi(T)$ taking values in the range $[0, 1]$:

$$\mu(\cdot)\,\phi(T)\,. \tag{11}$$

Moreover, the function $\phi(T)$ is given by

$$\phi(T) = \begin{cases} \dfrac{(T-T_{\max})(T-T_{\min})^2}{(T_{\text{opt}}-T_{\min})\left[(T_{\text{opt}}-T_{\min})(T-T_{\text{opt}})-(T_{\text{opt}}-T_{\max})(T_{\text{opt}}+T_{\min}-2T)\right]}, & \text{if } T \in [T_{\min},\ T_{\max}], \\ 0, & \text{otherwise,} \end{cases} \tag{12}$$

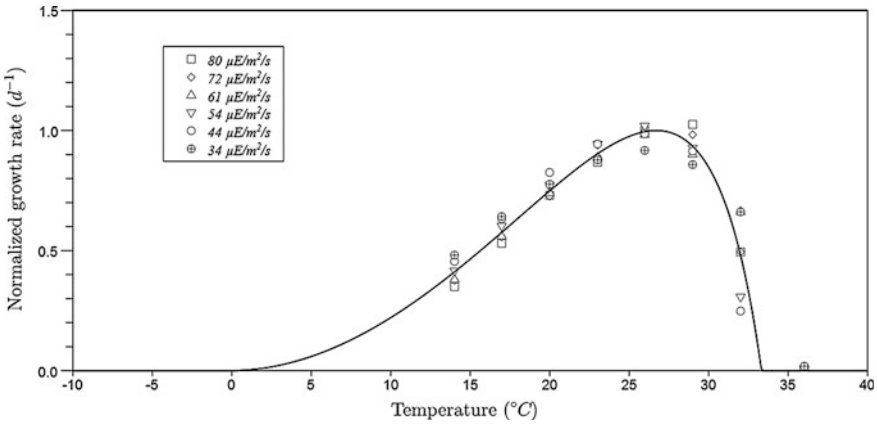

Fig. 4 Effect of temperature on the growth rate of *Nannochloropsis oceanica*: switching function $\phi(T)$ under various light irradiance and temperature conditions. Reproduced from [15]

with $T_{\min}$ and $T_{\max}$, the minimal and maximal temperatures, respectively, at which cells can grow; and T_{opt}, the optimal temperature for growth. Note that the following property must hold in order for the model to present the actual asymmetry that is experimentally observed:

$$T_{\text{opt}} > \frac{T_{\min} + T_{\max}}{2}. \tag{13}$$

Shown in Fig. 4 is a switching function whose parameters $T_{\min}$, $T_{\max}$, and T_{opt} are calibrated under different light and temperature conditions [15].

In addition to having a global effect on growth, temperature is usually assumed to have a similar effect on carbon and nutrient uptake, respiration, TAG synthesis, and the like. In accounting for temperature effects, it is therefore necessary to modulate the corresponding uptake, respiration, or biosynthesis rates, whose expressions have been given in previous subsections, in the same way as in (11).

4 Modeling of Physical Properties

4.1 Light Distribution

The light distribution in a microalgae culture decreases progressively in moving deeper into the culture medium due to photon absorption (mainly by pigments) and diffusion (mainly by the particles in the medium). Because of the complex multi-diffusive nature of the culture medium, classical theories such as the Mie theory do

not readily apply, thereby making the computations particularly challenging. Moreover, the optical properties are dependent on the light wavelength, explaining that green light will be mainly found in the darkest zones of the reactor. A large body of research has been devoted to high-fidelity simulation of the light distribution in such complex media; see, for example, Csogor et al. [28], Fernandez et al. [37], Suh and Lee [104]. In contrast, deriving analytical expressions, which can be used more easily for monitoring and control purposes, proves more difficult.

In the case of simple planar geometry with a 90° illumination angle with respect to the surface, one can use the Beer–Lambert law as a first approximation,

$$I(z,\cdot) = I_0 \exp(-\xi z), \tag{14}$$

where I_0 and $I(z)$ denote the irradiances at the surface level and at depth z, respectively, and ξ is the light attenuation parameter. The latter appears to be mainly correlated with the cell concentration x and the chlorophyll content θx, leading to the following simple approximation:

$$\xi = (a + b\theta)x + c, \tag{15}$$

with a, b, and c the specific light-attenuation coefficients due to biomass, chlorophyll, and background turbidity, respectively.

A key advantage of using the Beer–Lambert approximation is that it allows deriving analytical expressions of the average light and growth rate in simple photobioreactor configurations. Nonetheless, this model does not account for light backscattering, which can become significant in dense microalgae cultures. More accurate radiative transfer models based on the inherent optical properties of microalgae can be used in this context [27, 28, 39, 60, 84, 104].

In the remainder of this subsection, we consider a simple planar geometry of thickness L with a 90° illumination angle with respect to the surface. On application of the Beer–Lambert approximation, one can define the optical depth $\lambda = \xi L$, which reflects the actual amount of light energy absorbed by the culture medium. In particular, we have

$$\lambda = \ln\left(\frac{I_0}{I(L)}\right). \tag{16}$$

Under the additional assumption that all the concentrations are homogeneous (perfect mixing), the average irradiance received by the microalgae across the culture medium is given by

$$\overline{I} = \frac{I_0}{L}\int_0^L \exp(-\xi z)dz = \frac{I_0}{\lambda}[1 - \exp(-\lambda)]. \tag{17}$$

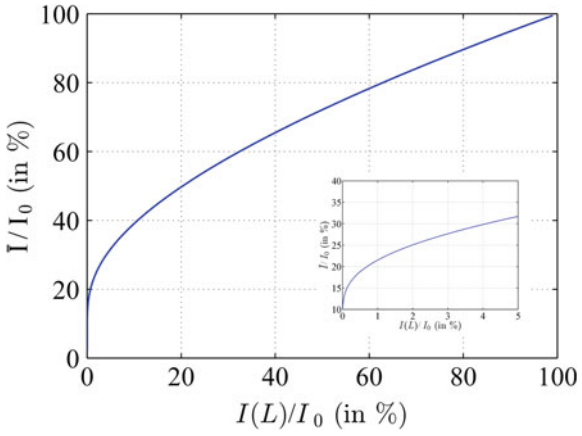

Fig. 5 Average light ratio $\frac{\bar{I}}{I_0}$ as a function of $\frac{I(L)}{I_0}$ in a simple planar geometry with 90° illumination angle

Finally, combining the two previous expressions gives

$$\frac{\bar{I}}{I_0} = \frac{1 - \exp(\lambda)}{\lambda} = \frac{\frac{I(L)}{I_0} - 1}{\ln\left(\frac{I(L)}{I_0}\right)}, \tag{18}$$

which does not depend explicitly on the light attenuation parameter ξ. The evolution of $\frac{\bar{I}}{I_0}$ versus $\frac{I(L)}{I_0}$ is represented in Fig. 5.

4.2 Microalgae Cell Trajectories

Because MCS are both mixed and optically thick at the same time, the microalgae cells are constantly crossing light gradients, which generates fast changes in the light irradiance received by a particular cell. In practice, the cells are exposed to light patterns with characteristic timescales that can be as small as a microsecond [83]. The ability to represent such fluctuations calls for detailed hydrodynamics simulations.

A number of studies have become available in recent years that use computational fluid dynamics (CFD) [71, 83, 85] for characterizing the hydrodynamics in MCS, for instance, based on commercial CFD codes such as ANSYS Fluent. In a second step, Lagrangian trajectories are obtained by integrating the computed velocity field. Although it has been applied mainly to simulate closed photobioreactors (PBRs), this approach has more recently been used to simulate open raceway ponds too [52]. It is because of their larger scale that raceway ponds are

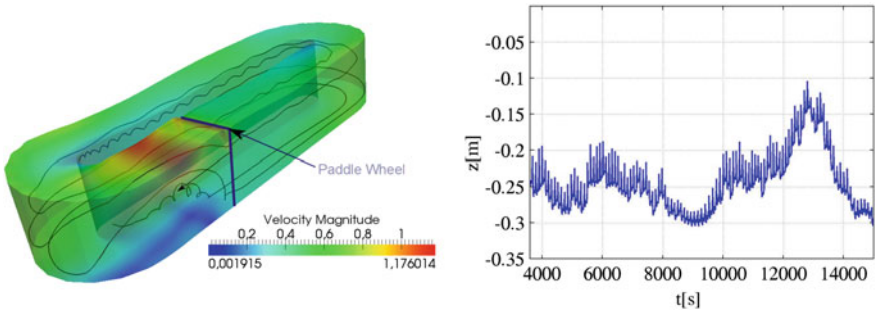

Fig. 6 3D Representation of the velocity field in a raceway pond along with a particular cell trajectory (*left plot*), and variation of the depth of a single cell over time (*right plot*). Reproduced from [52]

more computationally challenging to simulate than PBRs. Using a novel discretization scheme of the Navier–Stokes equations, the model in Bernard [10] has allowed reconstruction of the cell trajectories, and thus of the corresponding received light pattern, over longer time horizons. An illustration of the outcome of such simulations is shown in Fig. 6.

4.3 Temperature Variation

First-principles models describing temperature variations in MCS can be developed based on energy-balance considerations. These models account for the incoming solar energy on the one hand, and the energy dissipated through thermal re-emission, evaporation, and photosynthesis. In particular, this includes the direct and diffuse solar radiation, the radiation from the air and from the ground, the radiation of the culture medium, the evaporation flux, the heat flux in the CO_2-enriched bubbling gas, the conductive flux with the ground surface, and the convective flux at the surface.

Dynamic models that assume an homogeneous temperature of the culture medium have recently been proposed for both photobioreactors [5] and raceway ponds [6]. By taking into account the location, reactor geometry, light irradiance, air temperature, and wind velocity, these models can thus accurately predict the temperature evolution of the culture medium in outdoor facilities throughout a typical day, including the evaporative water losses. When not regulated, it has been shown that the temperature in open raceway ponds (which have a lower thermal inertia and where the benefit of evaporation is quite limited) can peak to values approaching 40 °C, thereby threatening microalgae cell survival. An illustration of these simulation results is presented in Fig. 7.

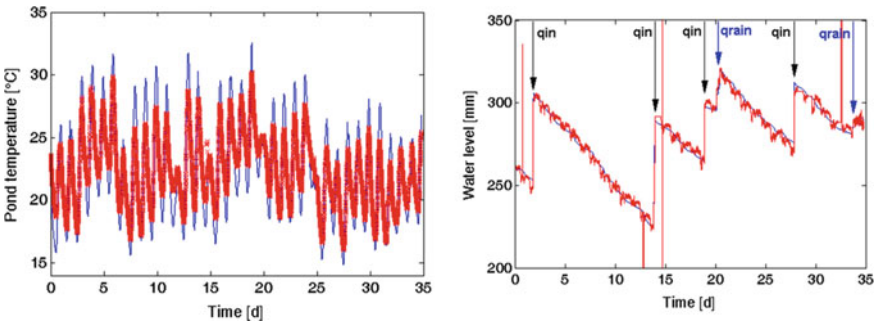

Fig. 7 Simulation of the temperature evolution in a raceway (*blue line*) using a model [6] and comparison with experimental measurements (*red lines*). On the *right-hand side*, the water level is also represented, with the indication of the water supply (*qin*) and rain (*qrain*)

5 Towards Multiphysics Models of Microalgae Culture Systems

5.1 Chemostat Culture

Under the assumption that the culture medium is perfectly mixed, that is, when the cells and limiting substrate concentrations are homogeneous, the main challenge involves characterizing the effect of the light gradient on the growth of the entire cell population. Note that, unlike light effects, the coupling on temperature is rather straightforward in the case where an homogeneous culture medium temperature is assumed.

The following equations describing the evolution of the cell concentration x and substrate concentration s, possibly coupled with the quota Eq. (1) in the classical Droop model, can be derived from mass-conservation principles:

$$\dot{s} = Ds_{\text{in}} - \rho(\cdot)x - Ds \tag{19}$$

$$\dot{x} = (\overline{\mu}(I_0, \cdot) - r)x - Dx, \tag{20}$$

where D stands for the dilution rate; s_{in}, the inlet substrate concentration; r, the basal respiration rate, and $\bar{\mu}(I_0, \cdot)$, the apparent specific growth rate at (surface) irradiance level I_0. It is precisely the determination of $\bar{\mu}(I_0, \cdot)$ that requires special attention here. When separating the timescales of mixing and photosynthesis, two limit situations can be distinguished:

(i) In the situation where mixing is much faster than the photosynthetic processes (infinite mixing assumption), all the cells respond to the mean light intensity $\overline{I}$, as discussed earlier in Sect. 4.1:

$$\overline{\mu}(I_0,\cdot) = \mu(\overline{I}(I_0,\cdot),\cdot)\,. \tag{21}$$

(ii) In the reverse situation where the photosynthetic response is now much faster than the mixing, the apparent specific growth rate corresponds to the average growth rate over the culture depth. For instance,

$$\overline{\mu}(I_0,\cdot) = \frac{1}{L}\int_0^L \mu(I(z,I_0,\cdot),\cdot)\,\mathrm{d}z = \frac{1}{L}\int_{I_0}^{I(L)} \frac{\mu(I,\cdot)}{I}\,\mathrm{d}I, \tag{22}$$

in the case of a simple planar geometry of thickness L with a 90° illumination angle. The rightmost term in (22) provides yet another interpretation of $\overline{\mu}(I_0,\cdot)$ as the average of the yield $\eta(I,\cdot) := \frac{\mu(I,\cdot)}{I}$ over the range of irradiance.

Assumption (ii) has been the basis for the theory of the "light-limited chemostat" [55, 56]. Experimental evidence that the microalgae cells respond to all irradiance levels within the light gradient range can be found in Huisman et al. [55], whereby photosynthetic efficiency is shown to increase with decreasing light intensity, and therefore with culture depth, using fluorescence measurement techniques.

Under this assumption, it can be shown [11] that, if the specific growth rate $\mu(I,\cdot)$ attains its maximal value $\mu_{\max}$ at I_{opt}, then the average growth rate $\overline{\mu}(I_0,\cdot)$ itself will attain its maximal value (which is lower than $\mu_{\max}$) at $I_{\mathrm{opt}}\sqrt{\frac{I_0}{I(L)}}$. Because the latter irradiance level is always greater than I_{opt}, a microalgae culture system with either a higher cell content or a larger water depth shall always be less prone to photoinhibition.

The foregoing considerations are illustrated in Fig. 8 displaying the apparent specific growth rate for various impinging lights I_0 and optical depths ranging from $\lambda = 0$ (limit case that no shading effect occurs) to $\lambda = 10$ (almost complete light attenuation). For example, $\lambda = 3$ corresponds to 95 % of the light being absorbed, either due to a high culture concentration or a thick culture. Note that for such optical depth levels (or higher), the apparent growth rate can be approximated well with Monod-type kinetic rates, keeping in mind that the Monod half-saturation constant should be biomass related. Of course, this does not mean that the microalgae are not subject to photoinhibition effects, but rather that these effects are not directly visible due to the averaging process. Such averaging induces a clear loss of productivity at higher optical depth nonetheless.

5.2 Open Questions

The analysis of a simple chemostat culture in the previous subsection has distinguished two limit cases in order to estimate the apparent growth rate, either as the growth rate at the mean irradiance level, or as the average growth rate across the

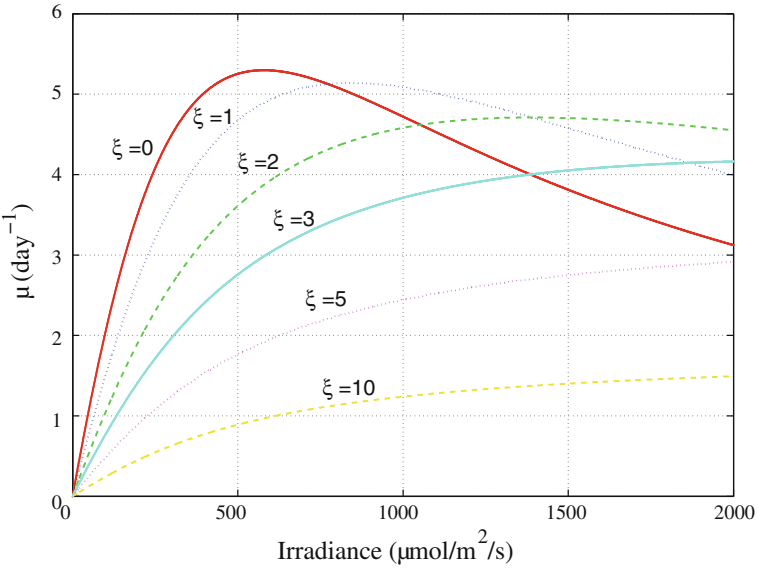

Fig. 8 Apparent specific growth rate (22) in a simple planar chemostat geometry as a function of the impinging light I_0 and for various optical depths λ

culture depth. The reality is probably halfway, yet predicting exactly where it occurs calls for a new paradigm.

The light pattern received by a microalgae cell is strongly influenced by the hydrodynamics in the culture medium [64, 80, 85, 91]. Both the average light and the variation frequency between light and dark phases can indeed differ greatly under different flow regimes, culture concentrations, and so on. This is illustrated in Fig. 9 comparing the simulated light patterns received by a microalgae cell in a tubular photobioreactor at two different cell concentration levels. Although fast-scale photosynthesis models, such as the Han model (see Sect. 3.4), can in principle

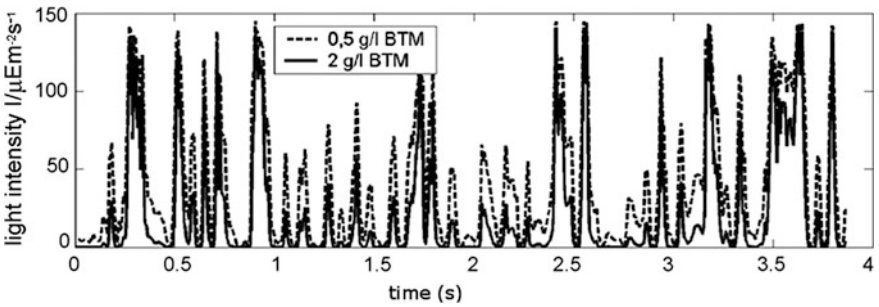

Fig. 9 Simulated light patterns received by a microalgae cell in a tubular photobioreactor at two different cell concentration levels: 0.5 g L^{-1} (*dashed*) and 2 g L^{-1} (*solid*). Reproduced from [80]

be used with this kind of light signal in order to predict the indirect effect of the hydrodynamics on microalgae growth, these models have not been validated with realistic light signals as of yet.

In addition to their effects on fast photosynthetic processes, one should also consider the combined effects of the light gradient and flow mixing on the dynamics of photoacclimation (see Sect. 3.3). The question of the light irradiance at which the cells are photoadapted—that is, which irradiance value I to use in Eq. (6)—is indeed crucial and it still remains open [80, 85, 91, 114].

Finally, in order to predict the daily productivity of a microalgae culture system, one has to combine multiple biological and physical models acting across a wide range of timescales. These models have characteristic timescales ranging from milliseconds for the light reactions, to minutes for the photodamage effect, and to days for the growth and photoacclimation processes. Such an integration has been investigated in Esposito et al. [35], where a coupled Han–Geider model is embedded within a large eddy simulation (LES) framework. Likewise, a coupled Han–Droop model [51] was used for the purpose of raceway light pattern reconstruction and experimental simulation in Hartmann et al. [52].

By and large, the approach that involves coupling detailed hydrodynamics simulators to fast timescale models describing the photosynthetic response holds much promise for a better understanding and prediction of the actual productivity of MCS. However, the added complexity and high computational burden has not yet been compensated by more accurate predictions in the authors' opinion.

6 Towards Model-Based Optimization and Control of Microalgae Culture Systems

6.1 Model-Based Operations Optimization

In parallel with the development of accurate mathematical models describing MCS, an increasing number of studies involving the application of systematic model-based optimization techniques in order to gain process operational insights have started to emerge. A natural optimization criterion in this context appears to be maximizing biomass productivity per unit area, referred to as surface productivity hereafter. Of course, alternative optimization criteria can be considered in the case where TAG synthesis or other biosynthesis is to be optimized.

The following considerations are for a simple planar geometry of thickness L, and our initial focus is for a steady-state microalgae culture under constant impinging light I_0 (and constant temperature). Using the Beer–Lambert law (14) as a first approximation and assuming that the light attenuation parameter ξ varies linearly with the microalgae concentration x, it is not hard to see that the optical depth λ is a function of the cell concentration per unit area $\varsigma := xL$ only (see Sect. 4.1). It follows that the apparent specific growth rate $\bar{\mu}(I_0, \cdot)$ given by (22) is itself a function of ς, and the net surface productivity P can be expressed as

$$P(I_0, \varsigma, \cdot) = (\bar{\mu}(I_0, \varsigma, \cdot) - r)\varsigma. \tag{23}$$

In other words, only the cell concentration per unit area matters according to the metric P: a thin culture (small L) with high cell concentration x is equivalent to a thick culture (large L) with low cell concentration x. Also note that the net surface productivity P in (23) attains its maximum for a finite, nonzero value ς^*, due to the productivity approaching zero for either very small or very large values of ς: in the case of a small cell concentration per unit area, this is because only a tiny fraction of the impinging light is actually absorbed, whereas there is nearly no residual light inside the culture medium when the cell concentration becomes too big.

Using the property that the apparent specific growth rate is approximated closely by Monod-type kinetics in dense cultures having an optical depth $\lambda \geq 3$ (see Sect. 5.1 and Fig. 8), it can be shown [56] that the optimal cell concentration per unit area ς^* maximizing surface productivity is such that the remaining light at depth L corresponds to the compensation light, namely the light at which the growth rate matches the basal respiration rate. Similar conclusions are obtained in [26, 107] by means of different approaches.

Accounting for the light periodicity in determining the optimal productivity introduces yet another layer of complexity. The optimization of cell productivity on a diurnal basis is considered in Akhmetzhanov et al. [1], whereas optimization on account of high-frequency light variations due to mixing and light gradient effects is investigated in Celikovsky et al. [21].

More recently, optimal operation strategies for continuous microalgae cultures have been investigated in Grognard et al. [46]. There the optimal operation is mainly determined by the need to reach a periodic regime, whereby the cell concentration at the end of a 24-h period matches the initial cell concentration, and it is no longer possible for the cell concentration $\varsigma(t)$ to track the optimal cell concentration ς^* for a given impinging light level perfectly. Instead, it is shown that the optimal trajectory $\tilde{\varsigma}(t)$ should be such that it approaches ς^* on average.

6.2 Monitoring and Control

In practice, the goal is often to maintain the culture conditions in such a way that the productivity of microalgae is close to a maximum. A number of simple control actions can be performed that do not interfere with the characteristic timescales of microalgae growth:

- pH Regulation can be achieved via controlling the injection rate of inorganic carbon (typically in the form of gaseous CO_2), for instance, using online pH measurements and a simple PI or PID controller [9, 18]. Alternatively, an MPC controller reducing the CO_2 losses in outdoor photobioreactors was proposed in Garcia Sanchez et al. [41].

- Regulation of the microalgae concentration can be achieved via a feedback control loop measuring the cell density, for example, using an NIR light transmittance sensor, and controlling the injection of fresh growth medium [94]. Turbidostats can also be used for this purpose [72].
- Temperature regulation can be achieved by means of low-level controllers, for instance, by maintaining the culture medium temperature near the optimal growth temperature T_{opt} (see Sect. 3.5). In doing so, one must make sure that the energetic penalty incurred by the temperature control does not overrun the corresponding productivity increase nonetheless. For instance, it has been estimated [5] that the energy needed to downregulate the temperature of a photobioreactor in California to 25 °C (the temperature without control can raise above 40 °C) is about 1.8 GJ $year^{-1}$ m^{-2}, representing the equivalent of 0.3 oil-barrel per meter-square.

A major bottleneck for the implementation of more advanced control and online optimization strategies in MCS appears to be the lack of online sensors that can monitor the biological activity. The idea of using software sensors, also known as observers, that infer key unmeasured bio/chemical variables based on readily available online measurements and a mathematical model of the system, is quite appealing in this context. A high-gain observer was developed in Bernard et al. [16] for estimating both the internal quota and the remaining nutrient concentration. More recently, an interval observer providing confidence intervals on the same inferred quantities and taking into account the discrete nature of the measurements was presented in Goffaux et al. [44]. Regarding TAG synthesis too, a nonlinear interval observer was designed in Mairet et al. [68] for monitoring the internal TAG content of microalgae.

Early work involving the online estimation of microalgae growth, and their subsequent use in closed-loop control strategies in an objective to maximize microalgae productivity, can be traced back to the early 1980s [7, 48]. Since then, other authors have investigated the use of inorganic carbon [4] or oxygen production [103] as a proxy to estimate microalgae growth.

Concerning closed-loop control finally, a simple and near-optimal strategy that enables microalgae productivity levels very close to their theoretical limit was recently presented in [69]. This strategy was derived based on the actual model-based optimal control strategy and involves controlling the optical depth in a raceway pond by applying a dilution rate proportional to the productivity rate. The latter can be inferred based on a software sensor that exploits direct measurements of either the CO_2 injection rate or the O_2 production rate, with the proportionality factor evaluated using online parameter estimation. The proposed strategy could be validated numerically against a model coupling a high-fidelity physical model of an open raceway pond with a biological model accounting for light and temperature limitation effects, photoacclimation, and internal storage of carbon in the form of

TAGs and carbohydrates. Moreover, a mathematical analysis supports the design of this controller by demonstrating its (global) stability with respect to the uncertain initial conditions.

Despite a number of recent advances [53, 66, 69], the availability of robust controllers remains scarce. This can be attributed to the complexity and high-nonlinearity of Droop-like models, that make the derivation and analysis of the controllers particularly arduous. However, the continued development of reliable dynamic models of MCS presents many opportunities for optimization-based control techniques, including nonlinear model predictive control (NMPC) and dynamic real-time optimization.

7 Conclusions

Industrial exploitation of microalgae is just starting, motivated by their huge potential, and by the diversity of innovative applications [86, 100]. However, such photosynthetic organisms whose energy-harvesting strategy is strongly related to light, are more difficult to model than more classical microorganisms (bacteria, yeasts, or fungi). They have a strong aptitude to store nutrients, which motivates the use of quota models (typically the Droop model) that are more complex than the classical Monod model. The microalgae biomass contributes to attenuate light, inducing then a strong coupling between biology (microalgae growth) and physics (radiative transfer properties and hydrodynamics).

Some models exist that can describe separately some of these processes, but there is a clear incentive to develop integrated predictive models, which could realistically predict the behavior of a microalgae culture system, especially in a context of bioenergy production from solar energy. It takes a lot of effort to validate these models over long periods of time, due to the need for extensive measurement datasets. Eventually, these models will support monitoring and optimization of MCS and will guide the development of this promising technology. They will also realistically support quantification and optimization of the reachable productivities, depending on species, type of culture process, period of the year, and location and, therefore, calibrate the corresponding investments. They will also contribute to improve the environmental impact assessment [63] by better quantifying the balance between the requested energy to maintain the algae in suspension and inject CO_2, and the recovered energy through biofuel production.

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Monitoring of Microalgal Processes

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Abstract Process monitoring, which can be defined as the measurement of process variables with the smallest possible delay, is combined with process models to form the basis for successful process control. Minimizing the measurement delay leads inevitably to employing online, in situ sensors where possible, preferably using noninvasive measurement methods with stable, low-cost sensors. Microalgal processes have similarities to traditional bioprocesses but also have unique monitoring requirements. In general, variables to be monitored in microalgal processes can be categorized as physical, chemical, and biological, and they are measured in gaseous, liquid, and solid (biological) phases. Physical and chemical process variables can be usually monitored online using standard industrial sensors. The monitoring of biological process variables, however, relies mostly on sensors developed and validated using laboratory-scale systems or uses offline methods because of difficulties in developing suitable online sensors. Here, we review current technologies for online, in situ monitoring of all types of process parameters of microalgal cultivations, with a focus on monitoring of biological parameters. We discuss newly introduced methods for measuring biological parameters that could be possibly adapted for routine online use, should be preferably noninvasive, and are based on approaches that have been proven in other bioprocesses. New sensor types for measuring physicochemical parameters using optical methods or ion-specific field effect transistor (ISFET) sensors are also discussed. Reviewed methods with online implementation or online potential include measurement of irradiance, biomass concentration by optical density and image analysis, cell count, chlorophyll fluorescence, growth rate, lipid concentration by infrared spectrophotometry, dielectric scattering, and nuclear magnetic resonance. Future perspectives are discussed, especially in the field of image analysis using in situ microscopy, infrared spectrophotometry, and software sensor systems.

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Keywords Image analysis · Microalgal cultivations · Online monitoring · Optical sensors · Software sensors

Contents

1 Introduction: Monitoring Needs for Cultivation of Microalgae

Since the 1980s, increased attention has been given to photosynthetic microorganisms, both eukaryotic microalgae and cyanobacteria, as feedstocks for the production of fuels and chemicals [1–4]. The commercial cultivation of microalgae dates to the 1960s [5]. Both photosynthetic prokaryotes (cyanobacteria or

blue-green algae) and photosynthetic unicellular eukaryotes are often referred to as microalgae. Considerable money and human resources have been invested into research, development, and commercialization of production processes using microalgae. It appears that microalgae have become the most promising prospect for biomass production due to their ability to grow rapidly; produce large quantities of lipids, carbohydrates, and proteins; thrive in poor-quality waters; sequester and recycle carbon dioxide from industrial flue gases; and remove pollutants from industrial, agricultural, and municipal wastewaters [6]. All parts in the microalgal production chain have been subjected to optimization: strain selection and genetic engineering of microorganisms, cultivation conditions and reactors, harvesting procedures and systems, and conversion of biomass components (lipids, carbohydrates, proteins, or whole biomass) into useful and marketable products. In all development and production stages, these subprocesses have to be monitored and controlled. In recent years, monitoring methods that can be used in microalgal processes have been increasingly sought as these processes pose specific challenges that will require both adaptation of existing monitoring methods and development of new approaches. So far, the research efforts observed in the literature have concentrated on high-throughput methods that can process samples drawn from the cultivation rapidly and with high frequency but in the classic offline mode [7–9]. For process automation, however, online, noninvasive monitoring methods minimizing the measurement delay and employing in situ sensors are of high value. In order to be successfully integrated into process control, sensors and the whole measurement chain have to be stable, reliable, and low cost. Therein lies the real challenge.

One of the main objectives of process control in bioengineering is to provide optimal cultivation conditions, thereby increasing the performance (i.e., productivity) of the particular microorganism. The primary condition to achieve this is a fast measurement of key process parameters or variables, preferably in a manner that is noninvasive, in situ, and online. Cultivation monitoring is an integral part of biotechnologies used in pharmaceutical, industrial, or food production, and it is to a certain extent standardized and well established. As cultivation of microalgae has begun to move from relatively small scale (for aquaculture and food production) to large open outdoor units necessary for economical production of raw materials for biofuel production and to large closed outdoor units producing high-end food supplements [10, 11], automated process control on this industrial scale becomes necessary. Efficient process control calls for online monitoring methods that provide the information for control loops with only a short or no delay, can be placed in situ, and require only infrequent maintenance and handling by personnel. In the best of cases, this would be possible not only for standard physicochemical process variables such as temperature, pH, and concentration of dissolved gases and nutrients but also for biological process variables specific to the cultivation of microalgae.

Cultivation of microalgae presents several features different from those of established biotechnological processes, and these lead to specific challenges for the monitoring technology:

- Microalgal cultivation processes that are being commercialized are based on microalgal strains that grow phototropically, heterotrophically, and/or mixotrophically.
- Open bioreactors of outdoor microalgal plants, which are at present the only option for the production of biofuels, require much larger ground surface area (footprint) than comparable facilities for other mass-produced biotechnological products such as beer, enzymes, or antibiotics because of their shallow pond form with depths typically of 20–30 cm. Plants currently in operation or being built reach several tens of hectares of production area [12, 13], translating into total facility volumes up to about 10^5 m^3 with single raceway areas up to 0.9 ha and volumes up to 3000 m^3 (Sapphire Energy; www.columbusnewmexicobiorefinery.com). Raceways with areas up to 5 ha are being tested [14]. The average mixing intensity in such bioreactors is low, possibly with local excesses, leading to substantial spatial gradients in some important process and control parameters, such as pH or concentrations of dissolved O_2 and CO_2 [15], in addition to light gradients caused by light scattering and absorption.
- Closed outdoor photobioreactors (PBRs) producing high-value products, such as food supplements, present another challenge for measurement and control, as their construction is either tubular and the culture is pumped around (a PBR with 500 km of glass tubing in 20 units on 1.2 ha, with total volume of 600 m^3 [11]; smaller modular units are designed by GICON GmbH with 0.3 m^3 volume per unit) or employs plastic bags with only local mixing by CO_2-supplemented aeration (Proviron, Solix [10]). Gradients of pH, CO_2, and O_2 along the PBR axis are more likely to limit growth than in open raceway systems.
- In contrast to the usual biotechnology cultivation processes, which are strictly axenic, large outdoor microalgal cultivations with open bioreactors cannot be run without contamination. Consequently, monitoring of species composition in the culture is of high interest in order to recognize the presence of beneficial (synergistic bacteria), competing (other microalgal strains), or harmful (rotifers, chytrids, amoebas, etc.) biological contaminants at the earliest possible moment in order to take corrective action.
- The product in a microalgal cultivation can be either intracellular (lipids, proteins, sugars) or total biomass. The intracellular products are usually produced in the limitation phase of the cultivation (with exceptions as in the production of ethanol by engineered cyanobacteria [16]). Whole microalgal cells can also be used in food and feed supplements and in aquaculture, obviating the need for biomass processing and intracellular product separation.

When comparing open and closed outdoor PBRs from the business point of view, closed photobioreactors [11] are appropriate for high-value products, including food supplements and essential food components, such as ω-3-unsaturated fatty acids. Production of microalgae for biofuels has still not reached a break-event point, and produced volumes are minuscule compared to total fuel volumes.

At present, the largest demand for sensors in microalgal cultivations is for laboratory-scale PBRs used for basic research, process development, and scale-up. Here, sensors function in a simple environment, the need for repeated calibration is not a concern, advanced methods and sensors can be tested without substantial risks, and comprehensive instrumentation for measurements in the gas, liquid, and biological phase is typical. Many examples of fully instrumented laboratory-scale PBRs can be found, equipped with sensors for light measurement, gas phase analysis (O_2, CO_2), temperature, pH, dissolved O_2 and CO_2, turbidity, optical density, and chlorophyll fluorescence [17–21]. Some of these laboratory-scale PBRs have been developed and instrumented in research institutions (e.g., the short light-path (SLP) panel photobioreactor [22], or the Midiplate flat panel reactor at the Karlsruhe Institute of Technology (KIT) [20, 23]) but many companies marketing standard bioreactors also manufacture photobioreactors (e.g., Infors) while others specialize in the photobioreactor field (PSI [17], bbi-biotech, Subitec, to name only a few European manufacturers).

Ideally, sensors used for microalgal cultivations should be fast, stable, selective, online, and sterilizable (not necessary for open cultivation systems), and the whole measurement chain should need only infrequent calibration. These requirements do not pose problems for monitoring of standard physicochemical variables (temperature, pH, and concentration of dissolved O_2 and CO_2). Here, as in other areas of biotechnology, standard industrial sensors can be employed and improvements concentrate on long-term stability, ability to withstand harsh conditions, and costs. Problems and challenges encountered with these sensors for monitoring of microalgal cultivations do not differ from those in other areas of biotechnology, and the interested reader is referred to reviews on sensors in biotechnology in general, as exemplified by reviews on in situ sensors for pre-sterilized disposable bioreactors [24, 25].

A measurement requirement for phototrophic growth of microalgae that is not encountered in the cultivation of other microorganisms is light intensity. Monitoring light intensity is complicated because of its inherent dependence on spatial coordinates within the photobioreactor and on the biomass concentration. In the case of sunlight, light intensity also changes throughout a diurnal cycle. For this purpose, mathematical models of photobioreactors are often employed that calculate the light intensity based on measured intensity of impinging sunlight and spatial and temporal information about the photobioreactor, its geographic location, and orientation [26, 27]. For a given location, the total yearly light amount can be estimated based on geographic irradiation models [26, 28] or local measurements [29].

As noted above, sensors employed in laboratory-scale photobioreactors operate in a simple environment without having to cope with temperature and humidity extremes, sparse maintenance, and other adverse conditions. Other advantages offered by working at the laboratory scale are the optically clear cultivation medium prepared from laboratory-grade chemicals and clean water, and the transparent reactor walls enabling noninvasive optical measurement with sensors fixed on the outside. Such sensors can be employed not only for reading of optical density and fluorescence [17], of scattered light [30], or for advanced sensors as Raman

spectroscopy [31] but also for physicochemical variables as pH, pO_2, and pCO_2 with fluorescing sensor spots attached inside the reactor or shake flask [30] and read out by fiber optics placed on the outside. Microalgae also have specific advantages for sensor development in contrast to other microorganisms: microalgal cells are larger than bacteria and contain specific pigments absorbing in the visual range but not in the infrared [32], contain organelles small enough for Mie light scattering in the visible (VIS) and near-infrared (NIR) range [33], and the principal pigment, chlorophyll *a*, can be used as a probe of photosynthetic activity thanks to its fluorescence properties [34]. Some of those characteristics remain when transferring the developed sensor to a production-scale PBR: cell properties such as size, pigment content, and organelles do not change much or at all. Other characteristics differ significantly in a scaled-up production reactor, as the mixing intensity that is contributing to keeping clean the reactor walls, or the turbid and discolored cultivation medium containing large dirt particles, especially when wastewater is used as medium [14].

Because almost all specific target products (with the exception of whole biomass used as feed or food supplement) of microalgal cultivations are of intracellular nature, efforts to analyze microalgal cellular contents rapidly and with high sampling rates have increased during the past several years. The real-time monitoring of cellular components is very difficult but high-throughput methods, carried out either serially with high speed or in parallel, on samples drawn from the cultivation in offline mode but with the highest possible degree of automation, aim to close the gap to real-time analysis. Measurement principles employed so far include optical methods as fluorescence spectrometry and flow cytometry (FC), Fourier transform infrared (FTIR), NIR, and Raman spectrometry, colorimetric detection by image analysis, submicroscale gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and dielectrophoresis, as shown in recent reviews [7, 8] and in a publication of the Algae Biomass Organization (ABO) Technical Standard Committee [9]. Attempts to adapt some of these high-throughput methods to online use have been made for Raman spectroscopy [35] and colorimetry [36] but, on the whole, this task is difficult.

In this review, we present cultivation parameters that are of interest for monitoring the photobioreactor, the core component of a microalgal production process, and identify the currently used methods for the online measurement of these parameters as well as methods that could be adapted for online monitoring. Our focus lies with online measurement methods because they can be more easily integrated into automated process control systems. We discuss the application scope, robustness, suitability for online implementation, cost factors, and general advantages and disadvantages of these methods. Control actions resulting from monitoring results are in some cases impossible (e.g., temperature in an outdoor raceway, although temperature can be controlled in outdoor closed pilot reactors by spraying and in laboratory-scale reactors by usual vessel temperature control setups), rather straightforward and well known (e.g., pH by CO_2 injection), or complex and very process specific (e.g., contamination by pests), and we do not include them here.

We use the term "photobioreactor" generally for both open and closed cultivation vessels. Although this term is often reserved for closed reactors systems, we note that a raceway, as well as a closed PBR, fulfills all three criteria included in the term "photobioreactor": it is used for cultivation of living matter, employs light as an energy source, and is a vessel in which growth-related reactions are performed.

2 Process Variables in Microalgal Cultivations

Process variables (or parameters) in bioprocess monitoring can be categorized from two viewpoints: according to their nature as physical, chemical (or physicochemical), or biological, and according to the phase in which they are measured (gas, liquid, or biomass) [37]. From these, the most important parameters in microalgal cultivations influencing growth are the following [38–41]:

- Physical parameters: irradiance (light) in the photosynthetically active range of 400–700 nm (photosynthetically active radiance [PAR]); temperature; mixing intensity.
- Physicochemical parameters: pH; concentrations of dissolved CO_2, O_2, and nutrients (mainly N and P); concentration of extracellular products.
- Biological parameters: biomass concentration; concentration of intracellular products, i.e., biomass composition; physiological state of microalgae as expressed by the current activity of the photosynthetic apparatus, photosynthetic efficiency (PE) and the solar-to-biomass conversion efficiency; cell morphology of the productive species; presence of contamination by other microalgal species, heterotrophic bacteria and fungi, or predator organisms.

Chemical contaminants that do not influence growth but must be kept below prescribed limits because of hygienic or legal considerations (heavy metals, polycyclic aromatic hydrocarbons [PAH], polychlorinated biphenyls [PCB], polychlorinated dioxins and dibenzofurans [PCDD/F], pathogens, etc.) are measured in the final product by classical standardized and legally accredited analytical [42] and microbiological methods, and are not considered here.

The physicochemical and biological process parameters (variables) characterize and influence the process performance and should be controlled (or their control should be facilitated) by design, by initial process conditions, and finally via active process control based on process monitoring by online or offline measurements.

Light, CO_2, and nutrients are limiting process variables that must be kept above a certain concentration or intensity threshold. For phototrophic microalgal cultivations, the basic and most important process consideration is the supply of light. Other process variables must be maintained within an optimal physiological range or below a certain limit to maximize the cultivation productivity: pH, temperature, salinity, dissolved oxygen (DO) concentration, the concentrations of growth inhibitors, and mixing intensity. The intensity of light impinging on the PBR surface and the light intensity profile within the PBR are dependent upon the

environment (intensity of sunlight or of the artificial source) and upon the design of the PBR [15]. As optical properties of the algae suspension change during the process, the light intensity profile within the PBR changes as well [43]. Irradiance at any location within a PBR is given by the photon flux density (PFD) impinging on the photobioreactor surface (be it the wall of a closed PBR or the cultivation medium in a pond/raceway), the light penetration depth, the light intensity profile within the PBR, and the light-dark (LD) cycle frequency [26, 44, 45].

The process variables that can be reliably monitored online using current technologies include primarily the physicochemical variables in the liquid and gas phase: PFD (photon flux density, irradiance), temperature, pH, CO_2, and O_2 concentration in both liquid and gas phase and gas flow rates. Some biological variables in the living phase can be monitored online using measurements of optical density, fluorescence, or imaging; these include biomass concentration and the concentrations of chlorophyll and other pigments, with technology under development to monitor cell count and cell morphology. Cell morphology of microalgae encompasses several characteristics that can be either useful in supporting decisions about the cultivation control (cell size; cell elongation; change in cell form, e.g., straight and helical forms in *Spirulina* or changes during the lifecycle phase; presence of cell clusters in the cultivation medium due to cell division or agglomeration; presence, volume, and position of inclusion bodies in cells), or in detecting contamination by competing or harmful species differing in form from cells of the productive strain. Measurements of biological parameters can be then used to calculate values of other variables characterizing the cultivation state as growth rate, PE, and photosynthetic quantum yield (PQY).

Measurements of physical and physicochemical parameters in smaller and closed PBRs used for research often include the whole spectrum of available sensors and methods with high measuring frequency in the order of minutes or hours; thus, PAR, temperature, pH, concentrations of dissolved CO_2, O_2 and nutrients, and concentration of CO_2 and O_2 in off-gas are monitored. In contrast, measurements in larger, open PBRs (ponds and raceways) used for production are usually limited to incident irradiance, the temperature outside/inside the cultivation, pH, pO_2, and nutrient concentrations; these measurements are not obtained continuously but rather in 1- to 2-day intervals [46, 47]. An exception is a specific example of an outdoor reactor known as the thin-layer inclined PBR, for which measurements of incident irradiance, temperature, pH, pO_2, pCO_2, CO_2/gas flow rate, and CO_2 concentration with an infrared (IR) gas analyzer were reported [48]. These PBRs, first reported in the 1950s [49], are, however, used mostly for research and education purposes and as pilot-scale units. Biological parameters, such as biomass concentration and composition and the presence of contamination, are usually measured offline using culture samples both in small-scale and large-scale PBRs. At present, continuous online measurements of biological parameters include measurement of optical density as a proxy for biomass concentration (laboratory-scale photobioreactors [17, 50, 51]) and the current photosynthetic activity in the culture with a pulse amplitude modulated (PAM) fluorometer in laboratory- and pilot-scale photobioreactors [21, 52, 53].

3 Current Measuring Methods for Online Monitoring of Physicochemical Process Parameters

Physicochemical process parameters that can be monitored online in microalgal cultivations, together with measurement methods and sensor types, are shown in Table 1 (adapted from [54]). The most important physicochemical parameters are the light intensity, temperature, pH, O_2, and CO_2 concentration both in the liquid and gaseous phase, and the concentration of inorganic nutrients. Methods and sensors used for online measurement of these parameters are, with the exception of light measurement, the same as in other biotechnological and chemical industrial processes: Pt-100, pH, pO_2, and pCO_2 electrodes, and O_2 and CO_2 gas analyzers.

3.1 Light Intensity

For most microalgae, the optimal light intensity in the photosynthetically active range (PAR, 400–700 nm) ranges from 10 to 250 $\mu E/m^2/s$, depending on the strain and environmental conditions. This is substantially lower than the intensity of direct sunlight (full sun plus sky), which yields up to 2000 $\mu E/m^2/s$ [55]. PAR constitutes energetically (in W/m^2) about 40 % of the visible sunlight [56]. Note that the light intensity in photosynthesis (the PFD) is always expressed in microEinsteins per square meter per second (1 Einstein is 1 mol of photons) of PAR and not in energy units as W/m^2. The reason lies in the mechanism of photosynthesis where every photon with energy in the correct range leads to the same action and contributes the same energy portion to the energy capture chain in the photosystem II irrespective of its actual energy content. The energy of a single photon exceeding the necessary minimum is dissipated as heat or fluorescence. Radiation sensors used for measuring PAR usually use the $\mu E/m^2/s$ units. The decisive value limiting microalgal growth at every point inside the PBR is the light intensity inside the culture, not the intensity impinging on the surface of the PBR or of the culture medium, because light is attenuated on its path before reaching a microalgal cell by reflection on the PBR surface, by absorption in the PBR wall (if any) or in the cultivation medium, and by scattering on and shading by suspended particles, mostly other cells. In practice, PFD is usually measured on or near the PBR surface (outer wall or liquid surface or its vicinity in the case of a closed and open PBR, respectively) and some type of a mathematical model can then be employed to calculate light penetration into the microalgal suspension to obtain the desired irradiance profile within the reactor [44, 57–59]. Eriksen [39] reviewed some light distribution models used to this purpose. It is also possible to estimate PFD on the reactor surface using mathematical models of solar irradiance that account for geographical location and sun position [26].

Monitoring light intensity inside a PBR is usually only performed for research purposes or to construct light penetration models [43, 58, 60–63]. An example for

Table 1 Physicochemical variables monitored and controlled in a microalgal cultivation online (adapted from [54])

Monitored variable	Sensor type	Acceptable range	What happens when value out of range	Control options	References
Photon flux density (for a single cell)	• Quantum sensor: flat cosine, fiber-optic spherical, PAR dosimeter, integration solarimeter	10–250 μM/m^2/s (optimal); 0–2000 μM/m^2/s (actual)	• Low: slow growth • High: PE sinks at PFD >250 • High: Photoinhibition at >1500	• PBR design • Culture density • mixing	[58, 59, 61, 63, 64, 67, 68, 70]
Temperature	• Thermoelement (Pt-100)	15–35 °C	• Low: slow growth • High: culture death	• Water bath/spraying • Heat exchanger • Shading	[47, 50, 55, 58, 61, 67, 73, 106]
pH	• pH glass electrode • Optical pH sensor • ISFET	7–10	• Growth rate decrease	• CO_2 injection	[46, 47, 55, 56, 61, 68, 79, 81, 94, 135]
pO_2 (liquid phase)	• DO electrode (Clark) • Optical pH sensor • Oximeter	<15–25 mg/L	• High: growth rate decrease	• Aeration	[18, 58, 61, 62, 85, 93, 94]
O_2 (gas phase)	• Paramagnetic analyzer • Polarometric analyzer • Mass spectrometry	Depends on O_2(l) and mixing intensity	High: growth rate decrease	–	[19, 48, 63]
pCO_2 (liquid phase)	• pCO_2 electrode • IR analyzer + flow meter	>0.1 kPa	• Low: growth rate decreases below 0.1 kPa • High: pH decrease, growth inhibition	• CO_2 injection	[18, 59, 74, 85, 89, 156]
CO_2 (gas phase)	• IR analyzer • Mass spectrometer	>0.15 % [85]	• Low: growth rate decreases	• CO_2 feed	[18, 69, 77, 85, 90, 266]
Inorganic nutrients	• UV spectroscopy • Colorimetric assays • Ion-selective electrodes (modified)	Varies with nutrient	• Low: growth limitation; lipid or starch accumulation	• Nutrient addition	[159–161, 163, 164, 267]

DO dissolved oxygen; *IR* infrared; *ISFET* ion-specific field effect transistor; *PAR* photosynthetically active radiance; *PBR* photobioreactor; *PE* photosynthetic efficiency; *UV* ultraviolet

indirect measurement of light intensity profile in an open outdoor thin-layer cascade (TLC) photobioreactor is given in [44] with a light penetration model based on the Lambert-Beer law with an experimentally determined extinction coefficient and PAR measured outside the PBR [48]. It is well known that the Lambert-Beer law is valid only for particle-free systems in which the absorbing component is dispersed on the molecular level (i.e., true solutions). In the aforementioned example [44], the compounded effect of light absorption and scattering on light extinction in a microalgal suspension is accounted for by experimentally determining the extinction coefficient for the algal culture samples taken from the PBR at various biomass concentrations (4–40 g DW L^{-1}), which is then expressed by a function including the thickness of the algal culture layer above the "measured" point and the current cell concentration in the culture. PFD is usually measured for the PAR range by a flat cosine (2π) [58, 61, 62, 64–69] or a fiber-optic spherical (4π) [61, 62, 70] quantum sensor, by a PAR dosimeter [71] or pyranometer [69]. Measurements with a spherical sensor (4π spatial angle; an example: US-SQS/L by Walz GmbH) measure radiation coming from all directions weighted equally and are suitable in situations where the amount of scattered light is very high, as in microalgal suspensions when evaluating exposure of an individual cell to light. When only the intensity of light impinging on a surface is of interest (say, when evaluating the amount of light energy entering a flat panel PBR), a flat plane sensor (2π spatial angle; an example: LI-190SA by LI–COR Inc.) can be used. A flat sensor is practically always "cosine corrected," meaning that radiant flux striking the sensor plane surface at an angle is measured (ideally) with the same intensity as if it were impinging at the surface at zero angle from the normal. Without this correction, the measured intensity of radiant flux with the same energy decreases as its incidence angle moves from perpendicular to parallel to the sensor surface, resulting in severe underestimation of diffuse light. The radiant flux intensity component perpendicular to the sensor surface is proportional to the cosine of incidence angle measured from normal, hence "cosine correction." Properly cosine-corrected sensors provide almost constant response up to the incidence angle of 80°–85°. It is also important to realize that for an individual cell, the light comes in flashes, the intensity and duration of which result from a complex interplay among incident light intensity, cell concentration, light profile within the PBR, and the mixing intensity [15, 27].

3.2 Temperature

Temperature is the second most important process parameter influencing microalgal growth. Temperature optimum differs between microalgal species. Temperatures above the optimal range cause a large drop in PQY [56] and should be avoided [4, 64]. To control temperature in cultivation systems above laboratory scale, several strategies can be employed, such as shading, immersion in a water tank, or evaporative control with water spraying, but in large production systems (e.g., raceways and ponds), no active temperature control is possible. In closed production

photobioreactors with submersed reactor bags (Proviron, Solix [10]), passive control is achieved by immersion in a water tank. Temperature control by shading leads to diminished yields [72, 73]. Temperature is usually measured by a standard industry Pt-100 sensor.

3.3 pH

Without pH control, culture pH will rise in photoautotrophic microalgal cultivations with light intensities above the compensation point in the photosynthesis-irradiance (P/I) curve as dissolved CO_2 is utilized. Each microalgal species has its optimal pH range for growth [64], and changes in pH also affect nutrient availability [38] and thus the growth rate. A typical pH optimum for many species is in the range of 8.2–8.7, with an acceptable range for most cultivated species between pH 7 and 9. Some species are adapted to more acidic or basic environments [74], with the well-known example of *Spirulina* with an optimum of 9.5–10.5 [71] and growing well up to pH 11.5 [75]. pH control is normally carried out by injection of CO_2 into the culture or into the aeration gas stream. In mixotrophic or heterotrophic cultures, acetic acid fed as carbon supply can also be used for pH control [76]. The efficiency of CO_2 utilization used for pH control can be improved by using more sophisticated control algorithms [77, 78]. Reducing pH gradients in the culture by using a pH control algorithm can lead to an increase in the average photosynthesis rate and biomass productivity [78]. As usual in bioprocesses, pH is measured using glass electrodes or optical pH probes [79]. An ion-specific field effect transistor (ISFET) pH probe (for a review of ISFET sensors, see [80]) has been used in a wireless sensor network for monitoring algae cultivations because of its reliability through robustness and stability [81].

3.4 Carbon Dioxide in Liquid and Gaseous Phases

A steady supply of CO_2 as the carbon source is necessary for photoautotrophic growth of microalgae. The CO_2 content of air is 0.03 %, so relying on atmospheric CO_2 in open ponds leads to growth limitations due to low CO_2 diffusion rates from the atmosphere [82]. CO_2 can be supplied by injection into the purge air or by using flue gas from a fossil fuel combustion process [48]. Carbon is the dominant nutrient in terms of amount: the carbon fraction of algal biomass varies between 0.45 in carbohydrate-rich algae and 0.8 in oil-rich algae [15], so, theoretically, at least 1.65–2.93 g CO_2/g biomass must be supplied. The CO_2 partial pressure in the liquid phase should be kept above 0.1–0.2 kPa to avoid limitation [48]. For measuring CO_2 concentration in the liquid phase, classical pCO_2 electrodes can be used [18, 48, 65, 74, 83–85]. However, these require frequent maintenance and calibration and respond slowly to changes in dissolved CO_2 because the rate of diffusion

through the sensor membrane is limiting. Newer pCO_2 electrodes based on dye fluorescence should be more stable than the classical ones [59]. The concentration of CO_2 in the liquid phase can also be estimated from the pH value of the cultivation medium when one realizes that the principle of the Severinghaus pCO_2 electrode is to measure the pH of the bicarbonate solution with CO_2 concentration in equilibrium with the surrounding environment ([86], p. 171 ff.). Bicarbonate is a component of some microalgal cultivation media both in laboratory (BG11, Chu#10) and in production use (Zarrouk's medium for *Spirulina*).

The relationship between CO_2 concentration, pH and water alkalinity is given by the Henderson-Hasselbalch equation [86] for the bicarbonate system, and tables for calculating CO_2 concentration in solutions of known pH, temperature, and alkalinity can be easily found. However, other buffering components (e.g., phosphates) present in the cultivation medium can substantially influence the CO_2–pH dependence, and tests or calibration measurements for the given medium are necessary. An example of using pH measurement as substitute for pCO_2 measurement is given in [87]. For the commonly used Zarrouk's medium for cultivation of *Spirulina* with high bicarbonate content, a mathematical formula for dependence of pCO_2 on pH was theoretically derived and practically validated [74] at two bicarbonate concentrations and two temperatures. An alternative to pCO_2 electrodes is to measure CO_2 in the gas phase after equilibration using an IR analyzer [85] or using a flow cell for the same purpose [88]. Another alternative to pCO_2 electrodes is the OxyGuard dissolved CO_2 analyzer based on IR absorption, which allows direct and continuous measurement, reported for use in fish aquaculture for the concentration range of 2–50 mg/L CO_2 (0.14–3.4 kPa) [89]. CO_2 in the gas phase can be measured by an IR gas analyzer [18, 69, 90]. CO_2 control is usually linked to the pH control by adding CO_2 into the inlet gas, where the CO_2 flow is measured using a mass flow meter and is dosed by a pH controller [18, 56, 61]. Alternatively, measurement of CO_2 in the outlet gas by an IR analyzer is used for CO_2 dosage [18, 69, 85, 90]. Methods and sensors for measurement of dissolved and gaseous CO_2 are reviewed in [91].

3.5 *Oxygen in Liquid and Gaseous Phases*

Oxygen at concentrations above 120–200 % of saturation value (i.e., above ~10–20 mg/L O_2) progressively inhibits microalgal culture growth [15, 92], and this effect is more pronounced at lower temperatures [92]. The DO concentration is a reliable and sensitive indicator of the microalgal culture growth and productivity. Because of its inhibitory effect, oxygen must be continually removed by sparging the culture with air, usually enriched by CO_2; thus, O_2 removal, CO_2 supply, and pH control are combined in one step. Oxygen in the liquid phase can be measured by a Clark electrode, by an optical oxygen sensor [93], or by a luminescence optical DO electrode [94], either directly in the photobioreactor [18, 36, 58, 94–96] or in the degasser or a bypass [61]. Oxygen in the gas phase can be measured in outlet

gas with a paramagnetic [19] or polarometric gas analyzer [48]. Use of a gas analyzer based on mass spectrometry has also been reported [63]. All of these are standard measurement methods used in biotechnology. For determination of the rate of O_2 evolution for the construction of a P/I curve, use of a classical Clark electrode [97] or an Environmental Protection Agency-approved optical DO electrode using the principle of dynamic luminescence quenching [94] is reported.

Insufficient removal of oxygen (lack of mixing and degassing) can lead to high oxygen concentrations in photobioreactors that can easily exceed 200 % of air saturation. Such peaks should be always recorded in order to take a control action. However, many polarographic DO sensors are not able to measure values exceeding 200 % of saturation, as a short survey of sensors offered in the market shows, with reported upper limits mostly in the range of 100–200 %. Only sensors capable of measuring values well over 200 % saturation should be employed for monitoring microalgal cultivations. Optical DO sensors (Presens, YSI) are generally capable of measuring concentrations of up to 450–500 % of air saturation. The accuracy of measurement also decreases above 200 % [98].

3.6 Inorganic Nutrients

The inorganic ions necessary for autotrophic growth of microalgae (other than CO_2) are primarily nitrogen, phosphorous, and iron in the form of nitrate or ammonium salts, phosphate, and iron salts. Limitation by nitrogen or phosphorous is used to induce lipid accumulation in microalgae [67]. On the other hand, high concentrations of ammonia can have inhibitory or toxic effects [99] and high P concentrations can lead to intracellular accumulation [100]. Both elements are thus of interest for monitoring. Commonly, inorganic ions are measured offline by ion chromatography or standard assay methods based on colorimetry.

4 Current Measuring Methods for Online Monitoring of Biological Process Parameters

Biological process parameters that can be monitored online in microalgal cultivations, together with measurement methods and sensor types, are shown in Table 2 (adapted from [54]). The most important biological parameters are the biomass concentration; cell count and cell morphology; concentration of intracellular products (i.e., biomass chemical composition); the physiological state of microalgae as expressed by the current activity of the photosynthetic apparatus, PE, and the light-to biomass conversion efficiency; cell morphology of the productive species; and presence of contamination by other microalgal species or pests. Methods used

Table 2 Monitoring methods of biological variables in microalgal cultivations with online use or online potential (adapted from [54])

Monitoring method	Monitored variable (concentration)	Sensor type	Applicability	Comment	References
Turbidity, multispectral optical density, reflectance	• Biomass concentration	OD sensor Turbidity sensor	Offline and online	Wavelength choice depends upon pigments	[17, 18, 30, 50–52, 95, 103, 105, 106, 112, 240]
Color analysis	• Biomass concentration, cell count • Lipid, pigment concentration	CCD/CMOS camera	Offline and online	Laboratory-scale experiments	[58, 112, 242–245] [36, 58]
In situ microscopy	• Cell number concentration • Biomass concentration • Cell morphology • Population composition (contamination)	Microscope + CCD/CMOS camera	Online	Image-analysis algorithms strain-specific	[93, 118, 120–123, 125]
Absorbance spectrum	• Pigments • Fatty acids	Spectrophotometer	Offline and online	Fatty acid levels may be estimated by correlation to pigment ratio	[101, 102, 140, 141]
Backscattered spectrum	• Biomass concentration • Culture health monitoring • Culture contamination	RGB camera	Offline and online		[136]
Chlorophyll fluorometry	• Photosynthetic efficiency • Quantum yield	Pulse amplitude modulated fluorometer	Offline and online	PAM identifies stress leading to lipid production onset	[15, 17, 21, 49, 53, 55, 70, 130, 134, 268, 269]; YSI, Qubit, bbe Moldaenke

(continued)

Table 2 (continued)

Monitoring method	Monitored variable (concentration)	Sensor type	Applicability	Comment	References
	• RL curve • Lipids • Pigments			Online PAM: [17, 21, 55]	
2D fluorometry	• Biomass concentration • Pigments • Substrates, metabolites	2D spectrophotometer	Offline and online	Multivariate data analysis Currently no applications with microalgae	[24, 171, 175, 176]
Infrared spectroscopy (MIR, NIR, FTIR)	• Lipids • Proteins • Carbohydrates	NIR spectrometer ATR flow system (MIR) Fiber-optic probe	Offline and online	Only offline applications with microalgae	[177, 180, 183–185, 188–190, 192–194] [181, 195, 197]
Raman spectroscopy	• Biomass concentration • Cellular lipids • Glucose • Exopolysaccharides	Raman spectrometer	Offline (single cell and cell ensemble) and online	Online RS: [31, 35]	[8, 217, 225] [226, 227] [31, 35]
NMR spectroscopy	• Lipid content	NMR spectrometer	Offline and online	Online NMR: [166]	[166, 228, 229]
Dielectric spectroscopy	• Lipid content	Vector network analyzer	Offline	Only very high biomass concentrations investigated	[174]
Flow cytometry	• Cell count • Cell size • Lipid content	Flow cytometer Microfluidic cytometer	Offline and online	Online FC (yeast, mammalian cells): [210, 211] Microfluidic FC: [213]	[115, 198] [105, 199, 202, 213] [201, 203–205, 210, 211, 270]

2D two-dimensional; *ATR* attenuated total reflection; *CCD* charge-coupled device; *CMOS* complementary metal-oxide-semiconductor; *FC* flow cytometry; *FTIR* Fourier transform infrared; *MIR* mid-infrared; *NIR* near-infrared; *NMR* nuclear magnetic resonance; *OD* optical density; *PAM* pulse amplitude modulated (fluorometry); *RGB* red, green, blue; *RL curve* rapid light-response curve; *RS* Raman spectroscopy

for online monitoring of biological process parameters are, in contrast to physicochemical parameters, more varied and less standardized, and for many parameters no satisfactory online applicable method exists at this time. Applicable online methods are mostly optics-based, using measurement of optical density (optical absorbance and scattering) at one or more wavelengths, reflectance, in situ microscopy, fluorometry, infrared spectroscopy, and FC. These methods normally require substantial computing power for processing of the primary data to extract the desired information. In the following subsections, only those methods that have been established in online mode, at least in the laboratory, are mentioned. Methods that are not yet established but that have potential for online application are discussed in Sect. 6.

4.1 Biomass Concentration

The standard method for estimating the biomass concentration online is measurement of the optical density (OD) of the microalgal suspension at various wavelengths and calculating the biomass concentration through calibration by offline dry weight determination or microscopic cell counts [48, 55, 101–103]. The selection of measurement wavelength is important because the absorption of light by pigments and organelles can significantly affect the measured extinction [104], and accurate biomass concentration estimation requires that extinction should reflect only light scattering and not absorption [17]. The selected wavelength should therefore lie outside the absorption range of chlorophylls and other cellular pigments (e.g., above 720 nm) or at least in their absorption minimum (e.g., around 550 nm). Chloroplasts and other organelles in microalgal cells, $\sim$2–4 μm in size, also contribute to light scattering as their size lies within an order of magnitude of the light wavelength used in measurements (Mie scattering). It has been shown that cell size influences OD measured at 750 nm, but this effect can be corrected [105].

Commercial turbidity sensors using multichannel light scattering measurement [95, 106], custom flow-through sensors [50–52], photobioreactor-integrated IR sensors [107], photobioreactor-integrated visible light sensors combined with fluorescence measurement [17, 18], or commercial combined sensors (EXO sensor line by YSI, Inc. for water quality monitoring) have been employed. In manual sampling, at least two independent estimates are recommended when monitoring biomass in a mass culture system [108], and this recommendation could be extended to the online measurement in the form of fairly frequent calibration because of sensor fouling. Experimentally, several methods have been tested at the laboratory scale, including automatic sampling and dilution in a flow injection analysis (FIA) system coupled with a spectrophotometer [109, 110]. PAM fluorometry was used for determining the biomass concentration in a shake flask by measuring the in vivo maximum fluorescence yield F_m through the flask bottom after a dark period and assuming proportionality between average F_m and biomass concentration [111]. A study exists in which red, green, and blue (RGB) values in images of algal cultures taken with an

RGB camera demonstrated a correlation with biomass concentration [112], but the color intensities were relatively insensitive to biomass concentration at concentrations typically used in large-scale cultivation systems.

4.2 Cell Count, Cell Morphology, and Contamination

Commercial systems for automatic counting of microalgal cells by image processing were first reported in 1989 [113]. These use various microscopic methods [114] or FC [105, 115] and exist only in offline variants [114]. Devices as Coulter [50] and optical particle [116] counters are also used. A commercial system for online measurement of particle size and shape, including automatic sampling and sample dilution, has been developed by FlowCam [117] but no applications in microalgal cultivations have been reported so far. Another approach to online monitoring of cell count is based on in situ microscope (ISM) technology, which has been developed for monitoring biomass-related parameters (cell count, morphology, and size distribution) in biotechnological processes as well as for monitoring protein crystallization, the stability of enzyme carriers, and enzymatic hydrolysis [93, 118–124]. The ISM method involves processing the online acquired images and calculating particle parameters with only several seconds delay.

The ISM has recently been adapted to monitor microalgal cultivations by adding a flow-through cell [125]. The flow cell is installed in a bypass loop, and the image processing algorithms are adjusted to the required cell type. The information about cell count obtained in this manner can be used for estimating biomass concentration as shown in the case study (Sect. 6.7.3), while data on cell morphology can be employed, at least as supporting information, for identifying culture contamination. Raw images supplied by the device can also be used by process operators for visual evaluation.

For monitoring contamination, only offline methods are available at this time, including classical optical microscopy, dynamic image particle analysis (DIPA) by FC [126], and molecular diagnostics by quantitative polymerase chain reaction (PCR) [127–129].

4.3 Photosynthetic Efficiency and Quantum Yield

Chlorophyll *a* (Chl *a*) fluorescence measurement has become the standard noninvasive method to monitor the photosynthetic performance of microalgal mass cultures [21] and the physiological stress leading to lipid accumulation [130]. Although photosynthetic activity—the driving force of autotrophic growth—can also be monitored by measuring oxygen production and CO_2 uptake, fluorescence methods are faster and more sensitive. However, Chl *a* fluorescence measures only the photosynthetic rate and not respiratory rates, and thus must be supported by

oxygen production measurement [21]. Direct measurement of the Chl *a* fluorescence yield has the advantage of directly assessing the performance of the photosynthetic apparatus as a composite variable that reflects any disturbances in the process and deviations from optimal conditions. The challenge lies in the correct interpretation [131].

There exist two basic Chl *a* fluorescence techniques: the rapid fluorescence induction/relaxation kinetics and the PAM method [132, 133]. The first method, providing the Chl *a* fluorescence induction curve (the Kautsky effect), can be performed only in an offline mode as it requires a dark-adapted culture. The PAM method can measure photosynthetic activity in situ and online using a PAM fluorometer [17, 18, 21, 52, 55, 61, 134], and the result is the photochemical quantum yield of photosynthesis (PQY, Y_{II}, Φ_{PSII}), which characterizes the efficiency of excitation capture by open photosystem II reaction centers [19, 61, 135]. Measurement of photosynthetic activity by Chl *a* fluorescence should be used only in a relative sense, as in following changes in photosynthetic performance in the course of a cultivation, because estimation of absolute rates of photosynthesis in this way is subject to errors [131]. A proxy variable for quantum yield has been suggested in the form of monitoring the red and blue components of light backscattered by the culture [136]. Another measure of the current physiological state is PE, which is defined as the fraction of available incident light that is conserved as chemical energy in produced biomass; it can be calculated from online measurements of the photon flux absorbed in the reactor volume, volumetric biomass productivity, and biomass combustion enthalpy [19, 85, 135]. PAM fluorometry can also be used to measure the so-called rapid light-response curve (RLC), which provides kinetic parameters similar to those of the P/I curve but can be measured within minutes instead of several hours necessary for obtaining the P/I curve [21, 53].

As a practical example, in situ online monitoring of the PQY by a PAM fluorometer together with monitoring differences in O_2 concentration in two locations along an open outdoor TLC photobioreactor could identify failures of CO_2 supply [21]. Online monitoring of the physiological condition of microalgae by means of Chl *a* fluorescence served as a basis for corrective action for the pH control and chemical oxygen demand (COD) load rate in a semi-batch culture of *Chlorella sorokiniana* in phycoremediation of alcohol distillery wastewater [53].

Robust optical fluorescence sensors that measure the concentration of photosynthetic pigments in water are commercially available, with examples from YSI (EXO Total Algae Sensor), Qubit Systems (Algal Online Monitor), and bbe Moldaenke (AlgaeTorch, FluoroProbe). However, these sensors are designed for measuring very low pigment concentrations corresponding to tens of milligrams of biomass per liter as the upper limit.

4.4 Case Study: Decrease in Quantum Yield Monitored by Online PAM Fluorometry

The decrease in quantum yield was monitored by online PAM fluorometry after the onset of glycolate production by *Chlamydomonas reinhardtii* (unpublished data, C. Posten and M. Fresewinkel, KIT). Chl *a* fluorometry using PAM is a well-known, noninvasive, and rapid technique for measuring changes in physiological status and photosynthetic performance in microalgal cultures [55]. The Chl *a* fluorescence yield reflects all changes in environmental conditions that impact the photosynthetic apparatus, such as light availability, nutrient status, temperature, and generally all changes in cellular metabolism forced on cells by stress. In this example, cells of *C. reinhardtii* shift from producing biomass to producing and excreting glycolate, which is intended for use in a subsequent anaerobic methanogenesis stage as a new concept of algae-based biogas production [137, 138].

Measurements were carried out in a Mini-Plate reactor with working volume of 200 mL [139] equipped with temperature control, irradiated from one side with PAR intensity of 200 μE m^{-2} s^{-1}, and with total aeration flow of 150 mL min^{-1}. Gas supply composition was changed at $t = 0$ from 0.8 % CO_2 and 21 % O_2 (growth) to 0.1 % CO_2 and 40 % O_2 (glycolate production), leading to an increase in the culture DO from 20.7 to 42.5 %. The partial pressure of CO_2 was measured by a standard pH-based pCO_2 electrode (Mettler Toledo InPro5000), and DO was measured by an optical sensor (Hamilton Visiferm DO Arc). The glycolate concentration was determined by ion chromatography. Actinic light fluorescence (F_t) was measured continuously on the irradiated side of the reactor, and a saturation pulse (internal probe LED) was applied every 10 min to measure the maximum fluorescence F'_m. The quantum yield of PSII (Y_{II}) was computed using the formula $Y_{II} = \left(F'_m - F_t\right)/F'_m$ [132].

Recorded data on pCO_2, F_t, F'_m, Y_{II} and glycolate are shown in Fig. 1 (pO_2 is not shown as it rises from 20.7 to 42.5 % almost immediately and remains at this value with slight noise). After forcing microalgal cells to produce glycolate by increasing the O_2 concentration in the medium and limiting nutrient supply (CO_2), the quantum yield decreased quickly, along with the concentration of dissolved CO_2. As can be inferred from the pCO_2 data (pCO_2 decreased rapidly but rebounded slightly and remained practically constant afterwards, whereas Y_{II} continued to decrease), at least some part of the quantum yield decrease must be due to the redirection of the algal metabolism. The experiment shows that continuous online measurement using PAM fluorometry is possible. The challenge shifts now to the interpretation of results.

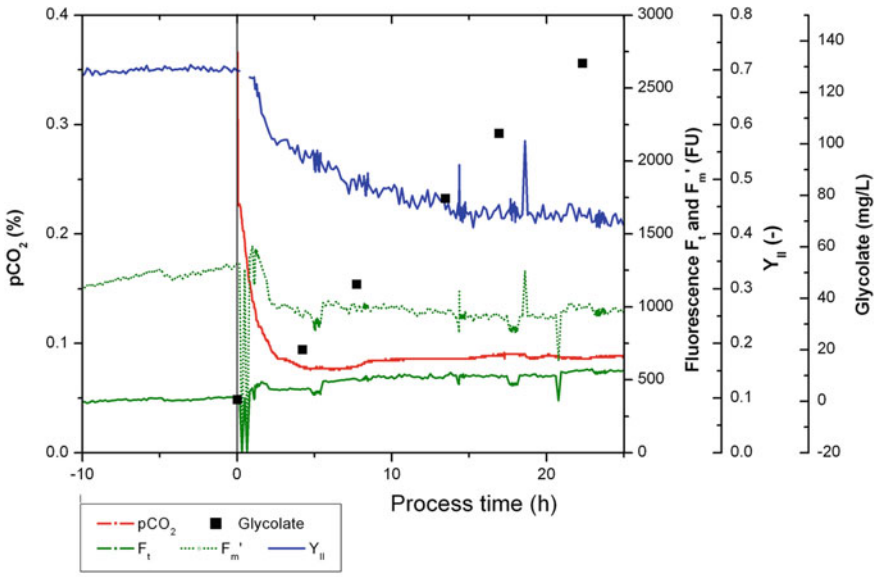

Fig. 1 Decrease in quantum yield measured by online PAM fluorometry after the onset of glycolate production by *C. reinhardtii* (unpublished data, C. Posten and M. Fresewinkel, KIT)

4.5 Biomass Composition

Biomass composition—that is, the concentration of intracellular components including lipids, sugars, pigments, and proteins—is one of the most interesting and important parameters of microalgal cultivations because it relates directly to the reason for cultivating the microalgae. Unfortunately, there are few reliable online measurement methods, and those have only been evaluated on the laboratory scale. Pigments were measured online in a bypass in a spectrophotometer measuring cell [101, 102]. Neutral lipids could be estimated by correlating lipid yield and PE during physiological stress using PAM fluorometry [130], Chl *a* and lipid contents were simultaneously estimated by three-color analysis in culture samples [36]. In that approach, the total fatty acid content was correlated with carotenoid-to-chlorophyll ratio obtained by measuring absorbance spectra of a microalgal suspension [140, 141], and the carotenoid and chlorophyll content were correlated with the color coordinates of a culture sample measured by a colorimeter [58]. The strategy of correlating chlorophyll content with measured multispectral reflectance or fluorescence can be traced back to the work of Lichtenthaler on detection of plant stress by three-color imaging of plant leaves, where changes in reflectance spectra with increasing chlorophyll content can be measured [142–145]. When estimating the chlorophyll content of microalgal biomass using fluorescence, the inner filter effect [146] must be taken into account. This effect distorts measurements by

absorbing both excitation and emitted radiation (either by the fluorophore itself or by other absorbing components) at higher cell concentrations, resulting in a decrease in the detected fluorescence as the sample absorbance increases. The extent of signal distortion depends on the sample-detector geometry and the sample absorbance [147]. The concentrations of biomass, glucose, and oil content could be estimated simultaneously online using support vector regression and several pre-processing algorithms from a Raman spectrum [35].

4.6 Culture Health Monitoring

Continuous monitoring of culture health should give an early warning of adverse environmental conditions in the culture or of impending culture crashes due to biological invaders. Monitoring of culture health can thus be divided into two objectives: monitoring of the physiological state of the cultured producing species, and monitoring of the culture composition and presence of unwanted organisms.

The physiological state of the cultured producers is represented by the culture photochemical efficiency, which can be considered to be a composite variable reflecting the sum of environmental conditions with impact on the photosynthetic apparatus. As previously mentioned, PAM fluorometry measures the current quantum yield of the photosynthetic reactions and can be carried out in situ and online [21, 61, 134], or a proxy for quantum yield can be measured [136].

Today, all standard methods for monitoring biological contamination work only in the offline mode with samples, are labor intensive, and are time-consuming. Assessment of the culture composition focusing on biological invaders can be performed using optical methods, including manual microscopic evaluation [148] and FC [128], metabolic footprint analysis using GC-MS [149] and, more recently, molecular biology methods using organism-specific DNA and a quantitative PCR assay [127–129, 150, 151].

4.7 Concentration of Extracellular Products

Extracellular products of microalgae include hydrogen, ethanol, and exopolysaccharides, and extracellular production of free fatty acids is also being investigated [152]. Measurement of hydrogen on the laboratory scale by mass spectrometry [153] and a polymer electrolyte membrane (PEM) fuel cell [154] has been reported, but there exist standard industrial electrochemical sensors for gaseous hydrogen, such as by Dräger. Analogously, ethanol production on the laboratory scale can be measured by standard ethanol oxidase based analyzer [155] after automated sampling with filtration probe, and standard industrial sensors for measurement of

ethanol in the gas phase can be procured. More within the scope of this review is the online monitoring of the segregation of exopolysaccharides in the course of the *Porphyridium purpureum* cultivation using the shifted-excitation Raman difference spectroscopy with laser excitation at 785 nm [31].

5 Novel Measuring Methods with Potential for Online Monitoring of Physicochemical Process Parameters

Existing sensor systems for temperature, pH, O_2, and CO_2 can measure physicochemical process variables online and are continuously being improved. These systems rely on standard industry-type sensors used generally in biotechnology and can thus take advantage of general progress in this field. For the measurement of pH, O_2, and CO_2, optical sensors based on detection of spectral changes or fluorescence quenching of a suitable organic dye immobilized in a polymeric or sol-gel matrix [24] are manufactured by several companies (PreSens, OceanOptics, Mettler Toledo, YSI, and others). These dyes are also manufactured in sensor spots that can be fixed on the inner surface of transparent vessels or tubing and monitored from outside. Sensor layers can also be located on the tip of an optical fiber [62] or the sensor can be mounted in a classic electrode casing. An example of a novel dissolved CO_2 sensor of this type uses a CO_2-sensitive layer monitored by a fiber-optic charge-coupled device (CCD) spectrometer and has been tested in aquaculture applications [156].

In the case of O_2 and CO_2 measurement, optical sensors can be used for measurements in both liquid and gaseous phases, and they are autoclavable and reusable. An autoclavable fluorescence-based optical pCO_2(l) electrode (YSI 8500) has been used in a predictive control system in a pilot-scale PBR [59]. For measurement of O_2 and CO_2 in the gas phase, standard paramagnetic and infrared gas analyzers are the instruments of choice [62, 69], although mass spectrometric gas analyzers can also be used [63]. In the case of pH measurement, another possibility for sensor improvement is the use of ISFET sensors, which have been commercially available since the 1990s [24, 80]. An ISFET pH probe was used in a wireless sensor network for monitoring algal cultivations because of its robustness and stability [81]. Microfluidic arrays that continuously monitor optical density, DO, and pH, in situ and in real time, have been designed and tested [157, 158].

Technologies for online or at-line nutrient measurement based on membrane filtration exist that have been developed for wastewater treatment processes [159]. These have been used for automated measurements of ammonia, nitrate, and phosphate ions [160, 161], but their use in microalgal cultivation has not been reported. Ion-selective electrodes (ISE) are generally not robust enough for long-term online use and require scrupulous maintenance. The stability of a NH_4^+ electrode in a cultivation of human cells was reported to be 7 days [162]. An array of ISEs with data analysis by artificial neural networks (ANNs) has been developed for the measurement of ammonium, potassium, sodium, chloride, and nitrate for use

in soilless horticulture [163], with a system stability of 6 days. A similar approach has been reported in which the signals of an array of nine off-the-shelf ISEs were processed with ANNs [164], but no stability data were provided.

6 Novel Measuring Methods with Potential for Online Monitoring of Biological Process Parameters

While physicochemical process variables can be monitored online with existing sensor equipment, the same cannot be said about the biomass composition or other biological variables such as cell count, cell morphology, and contamination. This is largely due to the intracellular nature of microalgal products and to the high degree of complexity of cells and their chemical environments, which interfere with many measurement methods. The total biomass concentration (or, properly, particle concentration), which is monitored by measuring turbidity or optical density, is the only biological process variable that can be monitored online with an acceptable degree of accuracy and reliability, although there are always issues of sensor fouling and the resulting drift. As to the photochemical quantum yield characterizing culture photosynthetic health, its change can be measured online without substantial problems using PAM fluorometry [21, 165]. Here, the challenge lies in data interpretation or in their combination with other process data to produce meaningful information about the process state.

Many analytical procedures based on various analytical principles, both established and experimental, are available for measuring the concentrations of cell components. Most of these methods require extensive sample processing and cannot be easily adapted to online monitoring. Even in cases in which sample processing is simple and could be automated, sterile sampling of growth media with suspended cells (necessary for closed photobioreactors) that provides a homogenous and representative sample is difficult and critical. Equipment for sampling cell suspensions can be found on the market (e.g., Trace Analytics, Braunschweig, Germany) but reliable implementation reports are difficult to come by.

The most often used measurement principles include optical methods such as two-dimensional (2D) fluorometry, spectroscopy in the visible and infrared range, Raman spectrometry, and measuring sample color with or without staining. Other measurement principles include NMR spectroscopy, FC, GC-MS, high-performance liquid chromatography (HPLC), pyrolysis, and dielectric spectroscopy. A survey of currently used standard offline methods can be found in the publication of Technical Standards Committee of the Algal Biomass Organization [9] and a review on high-throughput analysis methods [8]. A short overview of the advantages and disadvantages of current advanced methods for lipid (triacylglyceride) determination in microalgae is given in [166]. Apart from classical analytical methods such as GC and HPLC, which currently can be used only for high-throughput offline measurement of lipid content with sampling and sample

processing, pyrolysis in the classical version of thermogravimetry [167, 168] combined with IR spectroscopy [169], MS [167], or GC-MS [170] is also employed for offline determination of algal biomass composition. In recent years, microfabrication has led to the possibility of developing microfluidic array systems for high-throughput analyses. This could be eventually adapted for online implementations as they require only very low volume flows [158] and so could work with a culture side stream.

The methods that seem to be the most promising for adaptation to online monitoring are based on the optical properties of analytes. Measurement of fluorescence in the form of 2D fluorometry, spectroscopy in the visible and infrared range, and Raman spectroscopy can be used for estimation of pigment, lipid, and biomass concentration [8, 24, 35, 171–173]. FC could also be adapted for online monitoring of cell size and shape distributions, culture composition, and identification of invaders and of biomass composition, and some attempts in this direction have already been made [117]. Before FC could be used for online determination of lipid content by cell staining and fluorescence detection, the problem of staining the cells after automated sampling with predictable uniformity would need to be solved. The online or at-line capability of all these methods has already been demonstrated; however, because most of these methods require expensive equipment, bringing them to the level of general applicability requires considerable efforts as to their accuracy, the value of the measured data, and conditions of meaningful and profitable use.

Adapting other measurement methods—NMR spectroscopy, pyrolysis, and dielectric spectroscopy—for online use would probably require more effort than modifying optical methods, but because these principles are already being used in microalgal technology in offline high-throughput analyses, we include them here. In particular, the NMR and dielectric spectroscopy approach described in [166] and [174] is a viable possibility for an at-line installation or online measurement in a bypass because these methods work on unprocessed cells suspended in the cultivation medium.

6.1 2D Fluorometry

In 2D fluorometry, the hardware scans through a range of excitation and emission wavelengths and allows the simultaneous measurement of several analytes. Examples include proteins, vitamins, glucose, biomass, and also metabolites such as ethanol and pyruvate in cultivation media [24]. 2D process fluorometers such as the BioView system (Delta Light and Optics, Denmark) can be modified with a sensor that is attached directly to the cultivation vessel or placed in a bypass/bleed flow and connects to the measuring spectrophotometer via a fiber-optic light guide. A multivariate analysis of the resulting data is necessary because the spectra are complex and overlapping. It has been shown that 2D fluorometry can be employed for online monitoring and control in yeast and bacterial cultivations [171, 175, 176].

In case of specific products to be monitored, simpler fluorometer versions with specific excitation-emission wavelength pairs are possible. Microalgae contain pigments in concentrations that vary with time and cultivation conditions and application of 2D fluorometry has the potential to provide valuable information for process monitoring.

6.2 IR Spectroscopy

Infrared spectroscopy has considerable potential for online monitoring of bioprocesses as it can detect a wide spectrum of organic compounds [172, 177, 178]. The parts of the IR spectrum that are the most useful for monitoring are the mid-infrared region with fundamental vibrations (MIR; 600–4000 cm^{-1}; 17–2.5 μm) and the near-infrared region with harmonics vibrations (NIR; 4000–14,000 cm^{-1}; 2500–715 nm) since this is where organic molecules exhibit their specific signatures. Because of the convoluted nature of spectral signals obtained by IR spectroscopy, extensive data processing with multivariate techniques, such as principal component analysis (PCA), hierarchical cluster analysis (HCA), and partial least squares analysis (PLS), is necessary to extract meaningful information [179–181]. Several compounds can be simultaneously detected by NIR and MIR sensors [182]. However, IR methods for lipid (triacylglyceride) determination in the offline mode require dried biomass samples spiked with lipid standards [183], with a separate calibration for different microalgae species.

A typical application of NIR analysis of microalgae involves obtaining spectra or absorbance data at specific wavelengths from filtered wet [184] and dried [180, 185] microalgal samples. The effectiveness of FTIR to estimate the composition of microalgal biomass in terms of proteins, lipids, carbohydrates, nucleic acids, or silicates has been quantified using isolated components as standards with various levels of success and mostly in dried samples [180, 184–191]. Lipid concentration could be predicted by a regression model using NIR and FTIR spectra of four microalgal species [183]. An online monitoring device for the NIR range, transflexive embedded near-infrared spectroscopy, which is currently marketed by the German company mut AG, has been developed at the University of Kiel using either a flow-through cell or a mountable probe head for at-line measurements of bio-slurry and manure digestion applications. With this device, the free fatty acid content [192, 193] and glycerol [194] in digesting slurry were successfully monitored online.

In the MIR range, an attenuated total reflection (ATR) device was used for simultaneous determination of lipid, protein, and carbohydrate content in powdered freeze-dried microalgal biomass [195]. Effects of sample treatment were studied and conditions necessary to obtain accurate, rapid, and robust estimation of cellular lipid content were ascertained for freeze-dried samples of *N. oculata* [196]. Information on the lipid-to-protein ratio used for estimation of population characteristics could be extracted by a suite of multivariate chemometrics methods from MIR data

(1000–4000 cm^{-1}) measured with whole *Nannochloropsis* cells dried with cultivation medium directly on the ATR crystal [181]. Devices to interface MIR spectrometers to chemical and biological process units have already been developed. One device is an ATR flow cell through which a sample stream flows in the measuring chamber of an FTIR spectrometer [197]; this was used to monitor, online, the methanol concentration in cultivation medium. Another device is an autoclavable optical fiber sensor with an ATR crystal at the tip (e.g., ReactIR by Mettler Toledo and ReactionView by Remspec Corp.).

In all of these online applications of IR spectrometry, soluble extracellular compounds are monitored. In microalgal cultivations, nearly all products are confined within living cells, often within intracellular bodies. Further research is necessary to show whether practicable online monitoring of intracellular products is possible under these circumstances.

6.3 Flow Cytometry

FC in its offline implementation is a multipurpose technique that can extract, from untreated culture samples, information on biomass dry weight, cell count, cell morphology, cell size distribution, cell viability, and presence of biological contamination, and, after fluorescence dye staining, information on cellular components. The use of FC methods in the production of biofuels for monitoring cell count, cell viability, enzyme activity, and lipid concentration has recently been reviewed [115, 198].

The measurement of cell size and cell shape distributions of microalgae by using forward scatter in FC has been shown by Davis [199]. When FC is used for determination of biomass dry weight instead of optical density, the results must be assessed carefully because the correlations among cell count, optical density at 750 nm, and biomass dry weight depend on the growth phase [105]. The cell size and cell shape distributions of microalgae can be measured with FC by forward scatter, and cell viability can be monitored after staining with propidium iodide, which results in fluorescence in cells with damaged cytoplasmic membranes [200]. After fluorescent dye staining (e.g., Nile Red or BODIPY), FC can be used as a fast and reliable method for offline measurement of lipid content [201–204]; the measured fluorescence is calibrated to lipid determination by standard methods as GC. Differentiation between neutral lipids and polar lipids is also possible [204, 205]. This method is, however, limited by uneven dye uptake among cells, leading to inconsistent staining; there is variability between species and growth conditions [206, 207]; and organic solvents or glycerine are required for the dye to penetrate cell walls [208, 209].

Online implementation of FC for monitoring of cell components as intracellular lipids is difficult because of the necessary staining. A flow cytometer coupled with an autosampler and a flow injection system for sample processing has been used for online monitoring in yeast fermentation [210], and an automated cell preparation

system for automatic sample withdrawal, staining, and FC analysis was employed for fed-batch control in mammalian cell cultivation [211]. The latter sample preparation system has been commercialized by MSP Corp. Another commercial system that includes automatic sampling and sample dilution for at-line measurement of particle size and shape has been developed by FlowCam [117]. To date, no applications in microalgal cultivations have been reported with either system. Existing FC online monitoring systems, such as the Cytosense Flow Cytometer [212], are designed for use in marine and lake ecosystems with low to very low cell counts; for use in high-density cultivations, automated dilution would be needed.

A microfluidic cytometer that simultaneously measures forward light scatter, quantum yield through Chl *a* fluorescence, and lipid content by Nile Red staining on single algal cells in real time has been constructed [213]. In that device, cells must be sampled and stained for lipid measurement. Because microfluidics requires only very low volume throughputs, a culture bleed could be considered for connecting such devices.

6.4 Raman Spectroscopy

Raman scattering is used for compositional analysis as a complement to infrared spectroscopy. When applied to biological samples, the key advantage of Raman over IR spectroscopy is its low sensitivity to water content [214]. Raman spectroscopy generally does not require sample preparation and can be used to analyze samples in vivo [215]. Using Raman spectroscopy, the characteristic spectral signatures of proteins, polysaccharides, lipids, pigments, and nucleic acids can be detected. In some cases, biomolecules that are unique to certain algal species can be detected and used for identification [216]. Another advantage of Raman over IR spectroscopy, relevant for bioprocess monitoring, is the possibility to measure through the transparent walls of a PBR and thus to be truly noninvasive [31].

So far, most applications of Raman scattering concerning microalgae are in microspectroscopy, where single living cells are analyzed for pigments, proteins, and lipids using Raman microscopic systems [8, 217]. The high spatial resolution of Raman scattering allows the study of the distribution of lipid bodies in the cell and their beta-carotene content [218–220] and estimation of the lipid body volume in the cell by the amount of beta-carotene [221]. The degree of unsaturation and transition temperatures of lipids, which are important for the biodiesel quality [222, 223] and structural changes of astaxanthin with temperature [224], were investigated using this approach. Individual microalgal cells can be actively sorted in a laser trap after assessing their photosynthetic productivity and biochemical composition in a device combining laser-trapping Raman microspectroscopy and PAM fluorometry [225].

Cellular lipids could be rapidly quantified in lyophilized microalgae powder and microalgae wet paste using near-infrared (1064 nm) Raman spectrometry with accuracy of about 2 W% [226]. Raman spectroscopy has also been employed for

rapid determination of dissolved compounds and nutrients. In a mixotrophic cultivation of *Chlorella*, the glucose concentration was successfully estimated from the Raman spectra of unprocessed microalgal samples using a partial least squares model with pre- and post-processing of spectral data [227]. The latter method could be adapted for online monitoring.

Some attempts in adapting Raman spectroscopy for online monitoring have already been made. The biomass concentration, glucose concentration, and percentage of oil in microalgal biomass were monitored online in a heterotrophic cultivation of *Auxenochlorella* (*Chlorella*) *protothecoides* using an immersion probe inserted into a fermenter port for a near-infrared (785 nm) Raman spectrometer [35]. The collected spectra were preprocessed using combinations of several methods (Savitzky-Golay filter, SNV transformation, Polyfit) and fed into a previously validated multivariate chemometric model built using support vector regression. Glucose and oil concentrations were estimated successfully after the lag phase, and biomass estimates were usable in the entire cultivation range. In another online implementation, the segregation of sulfated exopolysaccharides in the course of the *P. purpureum* cultivation was monitored using shifted-excitation Raman difference spectroscopy with laser excitation at 785 nm [31]. Measurements were performed on supernatant from centrifuged samples, on untreated samples, and in a flow-through cuvette with continuous flow as a final proof-of-concept experiment. Good results in the online monitoring experiment were achieved using the support vector regression method.

6.5 NMR Spectroscopy

NMR spectroscopy has been used to determine the lipid contents of lyophilized biomass [228] and of wet biomass [229]. From the perspective of this review, the most interesting application so far has been the use of liquid state ^{1}H-NMR for noninvasive quantification of the lipid (triacylglyceride, TAG) content within live cultures [166]. Here, living algal cells are sampled in growth medium without any processing and can be returned to the PBR after analysis. Less than 1 mL of culture is needed and the length of one measurement scan is on the order of minutes (several scans are needed for one measurement). A simple flow cell was constructed to allow for continuous data acquisition with frequency of about 50 min per sample. Measurement errors ranged between 3 and 6 % for TAG or FAME equivalent concentrations above 150 mg/L.

6.6 Dielectric Spectroscopy

Lipid accumulation and changes in membrane conductivity alter the dielectric properties of algae cells, and this effect can be used to separate cells with different

lipid contents [230, 231], for separation of viable and nonviable cells [232], and for measurement of the cellular lipid content [174, 233]. It has been shown that different culture conditions (autotrophic vs. heterotrophic) of *A. protothecoides* that lead to biochemical differences also result into significant differences in dielectric properties [234]. An interesting note is that electromagnetic fields (EMF) are known to affect cell biochemistry; the use of EMF in stimulating growth, starch, and lipid production in microorganisms, including algae, has been reviewed [6].

The separation of cells by dielectrophoresis (DEP) is achieved by the effect of the Coulomb force exerted by a nonuniform electric field on a particle having a different permittivity from that of the surrounding medium. The Coulomb force is affected by alterations in cellular composition that lead to changes of cell permittivity. In the case of lipid accumulation, the permittivity of cells decreases. Depending on the imposed electric field frequency, particles of a given composition are pushed toward regions of the highest field gradient or away from it. The field frequency when the force changes direction is called the crossover (or critical) frequency [230].

Experiments have shown that cultures of *Neochloris oleoabundans* with high and low lipid content displayed significantly different DEP crossover frequencies (190 and 125 MHz, respectively) [230]. In that study, a significant step to measuring lipid concentration in living cells using DEP was made by constructing and successfully testing a proof-of-concept microfluidic device for manipulating the movement direction of microalgal cells by imposing a nonuniform electric field. After some design adjustments, this device should be able to sort and eventually count cells according to their lipid content.

The possibility of developing a rapid, noninvasive method for algal lipid measurement by DEP was also investigated in a coaxial transmission line cell built for this purpose [174]. Changes in dielectric properties of a *C. reinhardtii* strain were monitored during lipid accumulation, characterized by the normalized transmission coefficient, which can be measured in unprocessed cell suspension and requires only a commonly available vector network analyzer. The characterization yields a critical (crossover) frequency f_c that decreases with increasing cellular lipid content (estimated by Nile Red fluorescence) but is unaffected by changes in cell concentration. Measurements of lipid accumulation during growth were carried out in the coaxial transmission line cell using centrifuged and resuspended algae cell samples concentrated 37-fold. The influence of cell concentration was investigated in cultures concentrated by factors 13–50. One measurement run required about 30 s to complete. Further investigations would be needed to show whether this method can be used for cell samples in original cultivation concentrations, leading to the possibility to construct a flow-through measurement cell.

One of the cellular characteristics of microalgae is the extensive presence of thylakoid membrane stacks (grana) in the chloroplasts, the site of light-dependent reactions of photosynthesis ([33] p. 132). Theoretical analysis of dielectric spectroscopy assumes a multi-shell cell model in which the effective permittivity and capacitance, two variables with a large impact on the dielectric properties of cells, depend also on the permittivity and capacitance of membranes [174, 230, 235].

Simulations showed that increasing total membrane permittivity (caused, for example, by increasing the grana content in chloroplasts during cultivation, or by different grana content in different microalgal species) leads to a decrease in the critical (crossover) frequency f_c, the measure of lipid content [235]. Changes in membranes due to starvation also influence the dielectric properties of cells [234]. Whether these frequency changes are significant in comparison to changes due to increasing lipid content would have to be elucidated and taken into account when developing the dielectric spectroscopy method further.

6.7 Monitoring of Selected Process Variables with Novel Measuring Methods

Other methods that have been used for measuring selected process variables in bioprocesses, and which could be possibly adapted for online monitoring of microalgal cultivations, are summarized in this section.

6.7.1 Biomass Concentration

The absorption and scattering coefficients and reflectance properties of *Nannochloropsis* in visible (670–680 nm) and NIR (750–950 nm) regions have been investigated with the goal to devise algorithms for biomass dry weight estimation in photobioreactors [236]. Reflectance ratios of NIR/visible were correlated both with the biomass dry weight and chlorophyll cell content with accuracy of about 10 % in the range 1–8 g/L DW. Because optical activity of carotenoids in *Nannochloropsis* species change significantly with respect to Chl *a* in response to nitrogen limitation (i.e., cultures change color) [141, 237–239], this approach was expanded by measuring both the upwelling and downwelling irradiance with a dual-channel spectroradiometer in a culture of *N. salina*, interpreting the acquired spectra with a reflectance model and adding contributions of specular reflections and Chl *a* fluorescence to monitor in real time the chlorophyll concentration and algal growth rate [240]. Variants of OD sensors have also been reported, including NIR absorbance sensors used in computer control of microalgal biomass in a PBR [50, 52] and a hardware sensor using four different monochromatic LED/photodiode pairs with ANN pattern recognition [51].

Digital imaging with image processing aiming at recognition of individual cells has been employed for measuring cell count or biomass concentration. In situ microscopy and its use for online estimation of cell count in a microalgal culture [125] are described in Sect. 6.7.3. In the offline mode, the image processing software HaematoCalMorph was used to extract 25 cell morphology and pigment content characteristics from microscopic color images of *Haematococcus pluvialis.*

These data were used to recognize dead cells, estimate cellular pigment content, and monitor the transition of cells from one type to another [241].

Another possible approach to online cell concentration estimation is the analysis of macroscopic color or light intensity of a microalgal suspension. This strategy does not analyze individual cells and is noninvasive because images of the photobioreactor from the outside are used. Examples include the use of simple correlations of the color intensity in a cultivation flask to estimate biomass concentration [242] and the extraction of the blue component from segmented images of the whole photobioreactor [243]. Another example is the capture of color images of a photobioreactor and using the average gray value or using ANNs to infer concentration values from intensities at selected local points [244, 245].

The idea of measuring through the vessel wall has received new impetus by development of sensor spots for pH and dissolved gases that are interrogated by fiber optics attached externally [79]. Based on this idea, a novel online sensor system for noninvasive monitoring of biomass concentration by turbidity measurement in cultivation vessels has been developed [30]. Scattered light is measured through the vessel wall and correlated to optical density using nonlinear calibration models. For use in strain development in shake flasks, the sensor was integrated into a shaker table and measurement has been synchronized with the movement of the liquid within the flask. The relative measurement error is about 7.5 % for *Escherichia coli* and 12 % for *Saccharomyces cerevisiae*, similar to the error of manual measurements. The risk of contamination and volume decrease by sampling are eliminated with this approach when pH and DO are monitored using sensor spots. Such methods, however, require optically clear cultivation medium and a fully transparent vessel wall without depositions and other distortions, and thus are limited in application to laboratory-scale research reactors.

6.7.2 Cell Count, Cell Morphology, Contamination

For automation of counting cells, recording changes in cell morphology, and early recognition of contamination, the following techniques could be considered to have the capability for online operation: in situ microscopy with a flow cell installed in a bypass to the PBR, which provides real-time microscopic images from the culture [125] for counting cells and recognizing changes in morphology; a capacitance sensor installed in a microfluidic chip suitable for counting and distinguishing both living and dead cells [246]; monitoring the spectral signature of the culture [136] for detecting invasion of a *Chlorella vulgaris* culture by a cyanobacterium at mass ratios as small as 0.08; and an online adaptation of FC using a sampler [210, 211]. Concerning contamination, in situ microscopic methods offer the advantage of providing raw microscopic images of the culture in real time, which can be visually evaluated by operators in parallel with image processing as an additional early warning measure.

The microfluidic chip with a three-dimensional capacitance sensor that detects microalgal cells in a sample suspension flowing through the chip [246] could be

used, in connection with a microflow sensor or adjustable micropump, to monitor cell count in a culture bleed because of small volumes needed. This would diminish the danger of contamination through a bypass.

6.7.3 Case Study: In Situ Microscopy Measuring Cell Count and Cell Size Distribution

An automated alternative to cell counting that can also be implemented as an online device is the ISM. Many versions of ISM have been constructed and applied to monitoring cultivations of yeast, mammalian cells, embryonic stem cells, fibroblasts, and microalgae, and also to characterization of enzyme carriers and crystallization processes, using image analysis and ANNs for extracting information from real-time microscopic images taken inside the cultivation vessel or in a flow cell placed in a bypass [247].

Application of ISM to online cell counting and calculation of cell size distributions has been shown for *C. reinhardtii* and *C. vulgaris* as examples of microalgal cultivations [125]. Cell counts that were computed from online microscopic images in a *C. reinhardtii* (Fig. 2) and *C. vulgaris* (Fig. 3) cultivation are compared with offline optical density data. Image acquisition and processing were carried out in cycles every hour, with 100 images in 1-s intervals in every cycle and averaging the 100 cell counts into the online measured "raw" value for the cycle. The raw data up to the current point were then smoothed with a 12-h asymmetric median filter to

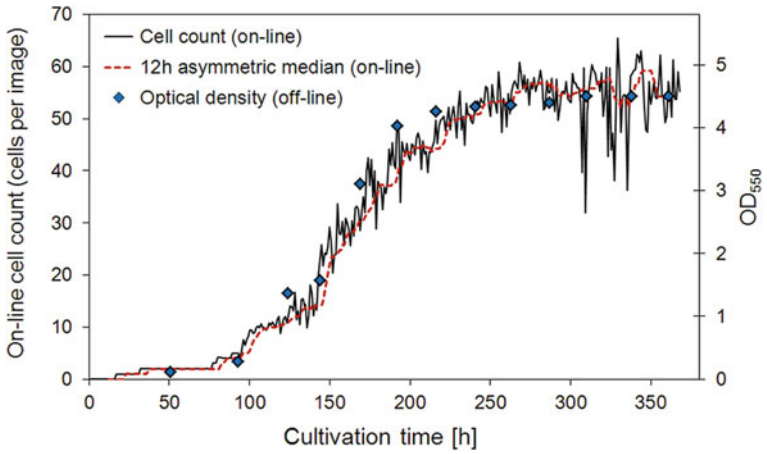

Fig. 2 Cell density calculated from in situ microscope analysis compared with offline cell density measurements (OD_{550}) in a *Chlamydomonas reinhardtii* cultivation. Raw data were smoothed with a 12-h asymmetric median filter. Data as shown in [125]

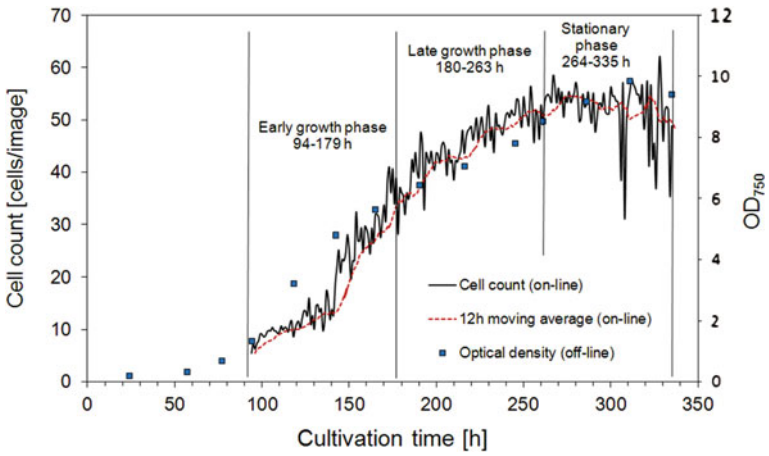

Fig. 3 Cell density calculated from in situ microscope analysis compared with offline cell density measurements (OD_{750}) in a *Chlorella vulgaris* cultivation. Raw data were smoothed with a 12-h asymmetric moving average filter. Data as shown in [125]

produce the output value. The software sensor processing the microscopic images thus has three parts: image processing, yielding cell count in each image and cell size and eccentricity for each recognized cell; averaging of cell counts from 100 images in every cycle; and the asymmetric median filter, producing the unilaterally smoothed cell count value which represents the actual sensor output. Comparison of cell counts obtained in this way with values of optical density measured offline show good agreement of both methods so that offline measurements could be replaced by the online sensor.

The lowest layer of the software sensor, the primary processing of microscopic images, provides information on cell size and morphology—in this case, cell eccentricity—which can be employed for following changes in both parameters during the cultivation. It also allows subsequent calculation of cell size and eccentricity distributions in order to analyze changes in the course of the cultivation, and to characterize cultivation phases or other cultivation parameters. Such an analysis was performed for the same cultivation of *C. vulgaris* that had been used for cell count measurements in Fig. 2. For the cultivation, three growth phases were defined (early growth, late growth, and stationary). Cell size distributions in each of these phases are shown in Fig. 4, and changes in morphology between phases characterized by cell eccentricity are shown in Fig. 5, in both cases expressed as relative frequency of cells within given range of cell size or eccentricity, respectively. After the first 180 h, the cell population became smaller and more elongated, although the eccentricity change was not substantial.

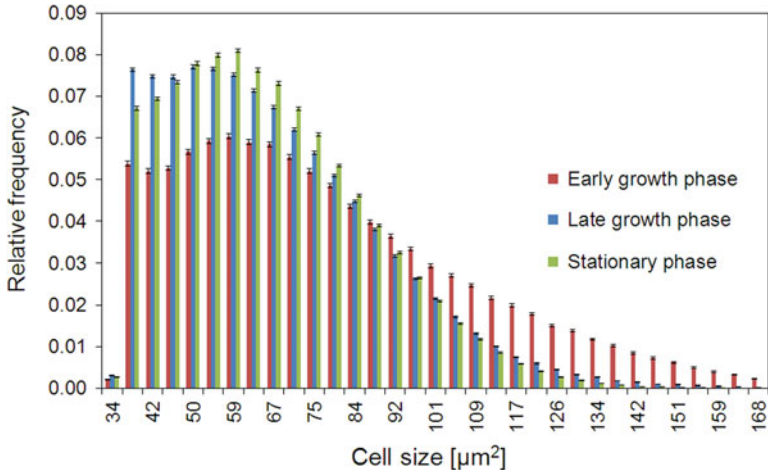

Fig. 4 Cell size distribution in different phases of a *Chlorella vulgaris* cultivation calculated from in situ microscope analysis. Data as shown in the Supplement to [125], doi: 10.1016/j.algal.2013.04.001

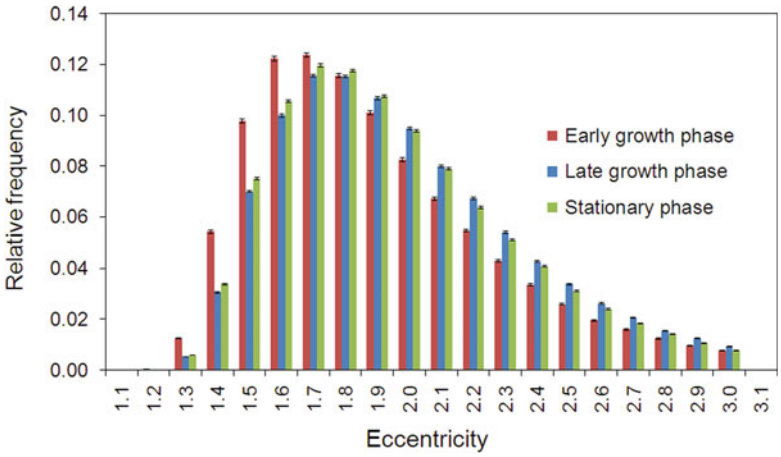

Fig. 5 Distribution of cell eccentricity in different phases of a *Chlorella vulgaris* cultivation calculated from in situ microscope analysis. Data as shown in the supplement to [125], doi: 10.1016/j.algal.2013.04.001

6.7.4 Biomass Composition: Pigment and Lipid Content

Among the optical methods that have the potential to be developed into a noninvasive online version, there is an interesting example of estimating the chlorophyll *a* and lipid contents in microalgae by three-color analysis of culture samples [36]. Total fatty acid content could be correlated with the carotenoid-to-chlorophyll ratio

obtained by measuring the absorbance spectra of microalgal suspensions [140, 141], and biomass, carotenoid, and chlorophyll content have been correlated with color coordinates measured in a culture sample using a colorimeter [58].

As already mentioned, determination of lipids with the NMR spectroscopy [166] was successfully adapted to an online version but the equipment cost would probably be prohibitive, at least at the moment. The dielectric spectroscopy technology noted earlier [174] worked only with highly concentrated biomass samples and its sensitivity would have to be substantially increased for an online adaptation.

7 Software Sensors and Other Computer-Aided Monitoring Methods

In every chemical and biotechnological process, there are process variables that cannot be measured directly because there are no sensors available, or sensors are too expensive, unstable, or inaccurate. In such cases, control theory provides us with a tool (a state observer) for indirect measurement using available hardware sensors capable of working in real time in combination with process mathematical models to estimate values of these process variables. In this way, classical hardware sensors can be replaced by a computer-aided estimator, which, as a secondary benefit, can also reduce signal noise by digital signal filtering. These estimators are usually termed "software sensors," reflecting the fact that they function as real sensors providing "measurement" of the process variable in question.

Essentially, there are two types of estimators: data-driven estimators represented by classical multivariable correlations or ANNs, and process model-driven estimators represented by mass and energy balance models, adaptive observers, and statistical filters based on the Kalman-Bucy filters [248]. In nonalgal bioprocesses, gas analyzers, fluorescence sensors and DO sensors are often used as hardware sensor inputs [249]. In a wider sense, practically all advanced monitoring methods used for bioprocesses could be called software sensors because all measurement methods producing complex signals such as Raman spectroscopy must be accompanied by complex multistep evaluation algorithms to provide meaningful results [227]. Software sensors *sensu stricto* are based either on a mathematical process model with mass and energy balances and process kinetics implemented in an observer or a statistical filter, or employ ANNs (which in itself is a type of correlation machine) trained on process data to map combined signals of several physical sensors to the value of the desired process variable without having to formulate a mathematical model or to recognize and quantify the underlying causal connection. A process estimator providing a single output can be also built as a combination of partial models employing simple correlations, deterministic mathematical models, observers, ANNs and fuzzy logic relations [250, 251].

From the point of view of a biotechnologist managing a production plant, it is desirable to construct sensors measuring process variables or their trends that

characterize the state of the process as it relates to the final product. Simple software sensors serving these needs can be built using algebraic formulas and online measurement of relevant process variables, as the two following ideas demonstrate. Is carbon from CO_2 being channeled into starch or lipids, or wasted in photorespiration, pointing to high O_2 and/or low CO_2 concentration [62]? Calculating the photosynthetic quotient (PQ), an analogue to the respiration coefficient (RQ) in aerobic microbial processes, from online monitoring of CO_2 consumption and O_2 production would provide data to answer that question [62, 252]. What is the concentration of bicarbonate that is used as the carbon source by some microalgae [253]? Bicarbonate could be estimated online by measuring pH and estimating $k_L a$ using the off-gas analytics [90].

In microalgal cultivations, several implementations of the software sensor approach have been attempted to date. As input hardware sensor signals, local irradiance, light distribution represented by reactor images, color intensity, DO concentration, CO_2 balance or concentration, absorbance at one or more wavelengths, and Raman spectrum can be encountered. Biomass concentration is the most often estimated process variable, followed by the lipid and pigment content.

Software sensors estimate the biomass concentration using correlations and measurements of local irradiance [254], color images of light distribution in a photobioreactor [245], and macroscopic color intensity in a cultivation flask [242]. Biomass concentration is estimated together with specific growth rate, PE, and average light intensity using a growth model and DO measurement [66]. Biomass concentration and nitrogen concentration in biomass and in the medium were estimated continuously in cultivations of *Dunaliella tertiolecta* and *Isochrysis galbana* by a continuous-discrete Droop model based an interval observer using intermittent offline biomass measurements [255]. Biomass concentration and specific growth rate estimation in a hydrogen production process with the photosynthetic bacterium *Rhodobacter capsulatus* was carried out with a sliding-mode observer based on Monod and Luedeking-Piret model and measurement of produced hydrogen volume [256, 257]. The Raman spectra signal was employed to estimate the concentrations of biomass and glucose in the medium and the oil content in biomass using support vector regression [35]. The biomass concentration and light intensity in a chemostat with phytoplanktonic cultures were estimated using the concept of quasi-unknown input observers and a Droop model in a rather theoretically oriented work [258]. An interval observer implementing a combination of Monod and Contois kinetics for modeling the influence of light intensity and CO_2-carbonate equilibrium on the growth of *C. vulgaris* was used to estimate the biomass concentration using measurements of dissolved CO_2 [59]. Another type of software sensor estimating biomass concentration consisted of an ANN trained to process concurrent signals from four monochromatic LED/photodiode pairs of different wavelengths installed in a flow cell placed in a bypass [51]. ANNs have also been used to estimate values of biomass concentration and lipid percentage in samples of microalgal cultures grown in wastewater using measurements of spectral fluorescence signatures and absorbance, with some success ($r^2 = 0.7$) [259]. It might be possible to adapt for microalgal cultivations the ANN-based prediction of

concentrations of several cations and anions (ammonium, potassium, sodium, chloride, and nitrate) that were demonstrated in soilless horticulture based on data from an array of ISEs [163, 164].

Estimation of the concentration of lipids and pigments in microalgal biomass uses almost exclusively simple correlations. Examples are the correlation of total fatty acid content with the carotenoid-to-chlorophyll ratio obtained by measuring absorbance spectra in microalgal cultures [140, 141, 260–262], and the use of brightness values of three primary colors for estimation of lipid and pigment content through linear correlations in a laboratory *N. oculata* culture [36]. Estimation of the pigment concentration in microalgal biomass using correlations with spectrophotometric measurements at several wavelengths has often been connected with the lipid concentration [36, 141, 260–262] and can be carried out in a bypass flow cell [101, 102]. Exceptions using more sophisticated approaches are the previously mentioned concurrent online estimation of biomass, glucose, and oil percentage in a heterotrophic laboratory culture of *A. protothecoides* by measuring and processing Raman spectra [35], and adaptive interval observers based on simple process models with Monod and Droop kinetics that are able to estimate the neutral lipid and carbohydrate contents in microalgal cells of *I. galbana* under nitrogen stress in a continuous culture based on measurements of biomass and residual nitrate [263, 264].

8 Perspectives and Outlook for Online Measurements in Microalgal Cultivations

Much of the commentary from our 2013 review of this topic [54] is still pertinent, but there have been several exciting developments since that time. Online measurement of principal physicochemical variables as temperature, pH, pO_2, and pCO_2 in the liquid and gaseous phase is essentially solved, and further efforts concentrate on improving the sensor robustness and reliability and lowering their investment and maintenance costs. This includes developing sensors based on novel measurement principles (ISFET for pH, fluorescence sensors for O_2 and CO_2), and adapting such sensors developed primarily for other bioprocesses to microalgal cultivations. Miniaturization, reliability, robustness, and lowering costs all lead to consideration of optical chemosensors as viable alternatives to classical sensors [93, 265]. Microfluidic chips with built-in microsensors for pH and DO will probably find their place in strain selection and process development [157, 158].

The focus is now shifting to methods for monitoring the most important biological characteristics of microalgal processes—namely, the concentrations of substrates, cells, and cell components that are desired as products. If one considers adaptation of offline methods to online monitoring, there is a challenge that stems from the complexity of the biological phase. In offline analytical methods, this complexity often leads to complicated sample processing steps that cannot easily be

automated for an online application. Therefore, a more fruitful approach may be to develop new measurement methods, including both sensor hardware and effective data processing methods capable of extracting meaningful and valid analysis results from large amounts of noisy and hard-to-interpret raw data. Such data processing methods using multivariate analysis and chemometric models have become an integral part of all modern sensors, which can be thus looked upon as low-level software sensors. Process variables that cannot be measured directly because sensors for such variables do not exist can be measured indirectly by using higher level software sensors, which can be described as computing procedures that can combine values of some easily measured process variables with help of a mathematical process model.

Criteria for an ideal high-throughput method for in vivo monitoring of a cell component, in this case oil content, described in [166], can be also applied for online monitoring methods. Such a method should be nondestructive, applicable to living cells with no sample preparation necessary, have short (seconds to minutes) analysis times, allow for continuous monitoring and correlation with other spectroscopic and physical measurements, and be able to monitor with minimal adjustments a wide range of microalgal strains. When considering these criteria, the most promising methods for online monitoring of biological variables seem to be computer-aided optical sensors measuring multispectral optical density, light scattering, reflectance, fluorescence of pigments and media components, culture color, IR and Raman spectra, and evaluating ISM images. Many of these methods are truly noninvasive as their physical sensors can measure through a transparent vessel wall, while others use flow-through sensor placed in a port or a bypass, although these considerations are important only for closed PBRs. Optical methods can also avoid sampling, which even in nonsterile cultivations in open vessels complicates measurement procedures by containing moving mechanical parts and requiring frequent periodic cleaning and maintenance.

The production plant area is one of the crucial differences between large-scale outdoor autotrophic production of microalgal biomass for pigments or biofuels and large-scale traditional microbial cultivations, such as those producing antibiotics or beer. In a microalgal facility with sensors in situ, it would usually not be possible to carry out physical sample multiplexing in order to use an expensive measurement device to full capacity, so sensors must be relatively low cost and only data are transferred. This again gives preference to optical sensors and methods like spectrometry, fluorometry, IR and Raman spectroscopy, and even in situ microscopy. These methods produce measurements through extensive and sophisticated data processing on relatively cheap hardware. In contrast, some methods require more expensive equipment, such as FC, which in addition would need highly reliable automatic samplers. General experience in the practical aspects of implementing measurement and analytical technology also leads to a preference for optics-based methods because they have a minimum of moving parts. Implementing microfabrication technology with sensors placed in and around microfluidics chips, as reported in the case of a microfluidic cytometer [213], could also lead to lowering hardware costs of more sophisticated measurement methods.

When constructing software sensors, the most extensive signal processing should be carried out at the lowest possible level so that the resulting digital signal emulates a hardware sensor with noise filtered out. Such sensors are easier to validate than a complex sensor concurrently processing several raw process signals. This is a simple lesson taken from the well-known distributed control approach. The resulting signals can be then used directly to evaluate the system state and facilitate forward process control, or can be combined into more complex software sensors on higher level estimating process state variables as biomass concentration or lipid content. An example can be seen in the Sect. 6.7.4 on biomass composition, where signals linked to lipids using different measurement principles—PAM fluorometry, color analysis, and pigment absorbance [36, 58, 130, 140, 141] could be theoretically combined to one software sensor estimating lipid contents using pattern mapping by ANN. Process model-based software sensors also have the beneficial property of providing not only estimates of the current state but also near future predictions of process state variables. Of course, a software sensor using a process model can be only as good as the process model itself and the selection of measured variables.

The question of which biological variables in microalgal cultivations are the most important to be measured or estimated online depends on the respective process and desired products, but a general list of important variables includes the following:

- Biomass concentration (or cell count), which is used to calculate the growth rate: This variable can be estimated online from optical density or turbidity with moderate accuracy; better methods are being investigated.
- Physiological state (as quantum yield): Changes in this variable indicate specific (interruption of CO_2 supply) or nonspecific culture problems. Although this variable can be estimated online using PAM fluorometry, interpretation of measured data is not easy.
- The concentration of products, mostly lipids or pigments, can be estimated online using indirect optical methods (color, absorbance measurement).
- Contamination by competing or harmful microorganisms, which currently can be estimated online only by experimental methods (in situ microscopy, color, spectral signature).

This short list demonstrates the potential for improvement of sensor and measurement technology. The tremendous growth in cheap computing power that can be deployed at-line will facilitate the development of extensive multistep data processing procedures for extracting useful information from noisy and convoluted data supplied by primary sensors, as already seen in NMR, IR and Raman spectroscopy, image processing, and FC. Ample computing power can also be used in training ANNs for mapping process data from reliable standard sensors (temperature, pH, pO_2, pCO_2, OD) to classify cultivation trajectories as “acceptable range” or “danger conditions.” Data from every cultivation run, successful or failed, together with the run evaluation can be added to the database and the ANN can be

retrained. Process operators can use this information during a cultivation run in addition to values and warnings by individual sensors.

Current online monitoring in microalgal cultivations concentrates on physicochemical process variables, for which existing measurement methods and equipment are already standardized, stable, and reliable. Future development will concentrate on miniaturization, lowering of costs including maintenance and improvements in stability. A similar degree of reliability and stability is sought for measurements of biological characteristics and product contents. This is no small task: the biological phase and its interactions with cultivation environment are much more complex, and there are more barriers between the sensor and the measured process variable than with physicochemical variables. On the other hand, reliable online monitoring methods for biological variables such as biomass concentration, physiological state, product concentration, and contamination have a high potential to improve the productivity of microalgal cultivations. At the moment, methods based on the optical properties of the biological phase and its components seem to be the best suited for this challenge, but new technologies may surprise us.

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Photobioreactors in Life Support Systems

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Abstract Life support systems for long-term space missions or extraterrestrial installations have to fulfill major functions such as purification of water and regeneration of atmosphere as well as the generation of food and energy. For almost 60 years ideas for biological life support systems have been collected and various concepts have been developed and tested. Microalgae as photosynthetic organisms have played a major role in most of these concepts. This review deals with the potentials of using eukaryotic microalgae for life support systems and highlights special requirements and frame conditions for designing space photobioreactors especially regarding illumination and aeration. Mono- and dichromatic illumination based on LEDs is a promising alternative for conventional systems and preliminary results yielded higher photoconversion efficiencies (PCE) for dichromatic red/blue illumination than white illumination. Aeration for microgravity conditions should be realized in a bubble-free manner, for example, via membranes. Finally, a novel photobioreactor concept for space application is introduced being parameterized and tested with the microalga *Chlamydomonas reinhardtii*. This system has already been tested during two parabolic flight campaigns.

Keywords Bubble-free membrane aeration · *Chlamydomonas reinhardtii* · Energy efficiency · Microgravity · Mono-/dichromatic illumination · Photobioreactor

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Abbreviations

LED	Light-emitting diode
PCE	Photo conversion efficiency
PBR	Photobioreactor
CELSSs	Controlled ecological life support systems
PAR	Photosynthetically active radiation
BDM	Bio dry mass
ModuLES	Modular life support and energy systems
PFD	Photon flux density
PAM	Pulse amplitude modulation
D	Dilution rate
OD	Optical density
PSII	Photosystem II
CTR	Carbon dioxide transfer rate
OTR	Oxygen transfer rate
CUR	Carbon dioxide uptake rate
OPR	Oxygen production rate
μ	Specific growth rate

Contents

1 Introduction

A major dream for mankind is to spread life beyond Earth into outer space and to other planets. In order to reach distant places, methods sustaining long-term human activities during spaceflight and on space stations are needed.

Photosynthesis as a central process converting light energy to biochemical energy enabled evolution of life: photosynthetic cyanobacteria produced the first oxygen accumulating in the atmosphere leading to the formation of ecosystems on earth. In order to spread life to hostile environments closed systems based on photosynthesis have to be developed.

The first practical approaches based on the idea of using closed controlled life support systems for manned space missions came up early in the 1960s [1] whereas the awareness of the problem emerged after the first successes in the field of cosmonautics by the end of the 1950s [2].

Since then various concepts for biological life support systems were designed and some small subsystems with one or two trophic levels of the food chain were even tested during satellite missions [3, 4] comprised of either only producers such as plants or consumers such as fish. Closed control systems also including men were created and tested during longer periods of several months on the ground. A famous example is the experimental complex of "BIOS-3" [1] in the 1970s.

Future space missions and the establishment of extraterrestrial human colonies (e.g., on Mars) rely on self-sufficient systems able to fulfill the following tasks: a complete purification of water, regeneration of atmosphere (oxygen production, carbon dioxide uptake), and food production and processing, as well as waste recycling [5]. Energy supply may also be taken as a major function of such a system.

Systems capable of fulfilling those major tasks can be classified into physico-chemical and bioregenerative systems whereas current spacecraft life-sustaining systems are based on open-loop physicochemical systems. Analysis proved that hybrid systems incorporating both approaches might be most promising to achieve the above-mentioned goals [5].

Nichoporovich and Semenenko [6] were among the first ones in the 1960s having the idea of using microalgae for designing life support systems. In the following years microalgae and photosynthetic cyanobacteria always played a major role in the development of controlled ecological life support systems (CELSSs), being members of the first trophic level producing oxygen as the essential basis of human and animal life [1, 3, 4, 7]. *Chlorella*, as representative of green microalgae, was also among the species used for the second stage of "BIOS-3" in 1976 [1]. Those approaches differed in numbers of compartments, trophic levels, and complexity. The highest complexity thus far reported relates to the Biosphere-2 experiment in the Arizona desert in 1991 [8].

Design methods for CELSS can be grouped into either holistic or reductionist approaches: the nature of the holistic approach (e.g., Biosphere-2 in Arizona) [8] is a natural evolutionary development of a system where organisms (including human) are assembled in a closed environment and mutual adaption occurs leading to a stable homeostatic ecological system being in equilibrium. In contrast to this, the reductionist approach is based on the division of the entire system into subsystems with the development of external control mechanisms that can come into action if system stability cannot be guaranteed by intrinsic mechanisms. Subsystems are then combined to form the entire system with both internal and external control mechanisms. Disadvantages and advantages of both approaches are discussed elsewhere [5].

This work deals with the potentials of using eukaryotic microalgae for life support systems; their unique features make them a remarkably promising choice for closed (eco-)systems sustaining human life. Moreover, special requirements and

frame conditions in designing photobioreactors for space application are discussed and published concepts are covered in this work. Finally a novel concept of a membrane-aerated, energy-efficient photobioreactor for application in microgravity is introduced. The chosen alga is the green alga *Chlamydomonas reinhardtii*.

2 Potential of Microalgae with Respect to Remote Applications

Extreme habitats such as hot deserts or outer space are in general hostile environments for (human) life and pose stresses on living beings. Environmental factors in space that organisms have to deal with are (i) microgravity, (ii) space radiation, (iii) hypoxia, and (iv) low atmospheric pressure, as well as (v) extreme temperatures [3, 9]. Those extreme habitats basically share the same problems limiting the spreading of life: water, clean air, food, and energy supply [10].

For this reason Polyakov et al. [10] also describe the concept of applying bioregenerative life support systems developed as space technology for establishing sustainable settlements in extreme habitats on Earth, for example, hot deserts which cover one fifth of our land area. This idea was first introduced by Nelson in 2003 [11]. As desertification and soil erosion is a major problem on Earth threatening arable soil, this approach is one possible solution to counter that.

But why do almost all concepts for life support systems rely on microalgae?

In order to establish a sustainable life support system a major requirement is the regeneration of atmosphere, meaning production of oxygen and carbon dioxide removal, while carbon is fixed into complex molecules. This demands an organism capable of photosynthesis, namely higher plants, eukaryotic microalgae, and cyanobacteria. These organisms form the basis of the food chain on Earth and they ensure closing global material cycles of gases and minerals. The driving energy originates from sunlight which is assumed to be the main energy source in space as well. A closed life support system somehow mimics the global material cycles on Earth.

The reasons for using especially eukaryotic microalgae are their unique advantages comparing them with higher plants or cyanobacteria. Several cyanobacterial species appear in filamentous forms hampering bioprocess engineering and proper establishment of suspension cultures. Moreover, some species produce toxins and their production rates might even be elevated under microgravity conditions [12].

In contrast, eukaryotic microalgae show many traits an organism should show on which a first module of a life support system could be based. The most promising characteristics of microalgae are their fast growth rates exceeding those of higher plants and their high photosynthesis rates [13]. Their efficiencies, concerning the conversion of light energy into biomass, also exceed the ones obtained with higher plants [14] up to a factor of 5.

Microalgae cultivations only require light, CO_2, and water with some mineral salts, and in contrast to higher plants they do not need a specific substrate and do not exhibit any long-lasting growth cycles. In addition, for higher plants harvesting could also be a technical problem.

Additionally microalgae have gas exchange characteristics able to meet human requirements [15, 16]. Russian BIOS experiments based on the green alga *Chlorella* proved that a culture volume of 20 L with the reported production rates can sustain the requirements of one man concerning O_2 and water [17].

Moreover, microalgae also have a significant role concerning food: they are used for enhancing the nutritional value of human food and also for animal feed [18] due to their composition, for example, polyunsaturated fatty acids and a high protein content. Undigestable compounds including cellulosic fibers or lignin can be completely avoided. Microalgae could be used as whole cells in a dried form within human food [19].

Concerning their cultivation in photobioreactors microalgae also possess various positive traits. In contrast to filamentous cyanobacteria such as *Arthrospira plantensis* most species of eukaryotic microalgae are unicellular and suspension cultures as well as immobilized cultures can be realized. Microalgae can be grown easily, also in continuous cultures [20–22]. Starch production of *Chlamydomonas sp*. was studied in continuous cultures by Maeda et al. [21], whereas Lamers et al. [22] investigated carotenoid and fatty acid metabolism dependent on a light regime in turbidostat cultures of the halophile green alga *Dunaliella salina*. In general, for continuous microalgae cultures no manual maintenance is required; a complete automated cultivation can be conducted by pure process engineering.

For fresh media supply a media recycling system is needed that automatically refills consumed substrates. Harvesting of microalgae to use algae biomass can be achieved by flocculation (chemical or electroflocculation), or simple centrifugation or sedimentation. The use of hollow-fiber membrane modules for filtration is also reported in space application photobioreactor concepts [23]. The cell-free medium is fed to the media supply unit for dosage of nutrients and filling back into the system to create a closed-loop system.

Even if numerous aspects justify the usage of microalgae as the basis or a first module of a closed life support system, potential obstacles have to be carefully considered and taken into account while designing such a system or just a first part of a whole system. Problems that could endanger a long-term stable continuous culture of microalgae are cosmic radiation and whether genetic stability of algae can be guaranteed for this period.

Cosmic radiation, in species defined, for instance, by Rea et al. [9], could mainly lead to DNA and protein damage either directly by energy deposition or indirectly by generating reactive oxygen species (ROS) through the breakdown of water.

According to Rea et al. [9] the microalga *C. reinhardtii* as an example does not seem to be influenced negatively by increased radiation concerning O_2 evolution and yield of photosystem II (compare to Fig. 1). The ratio of variable fluorescence F_v to maximum fluorescence F_m gives the maximum potential quantum yield of photosystem II (PSII) in dark-adapted cells. The production of oxygen even seems

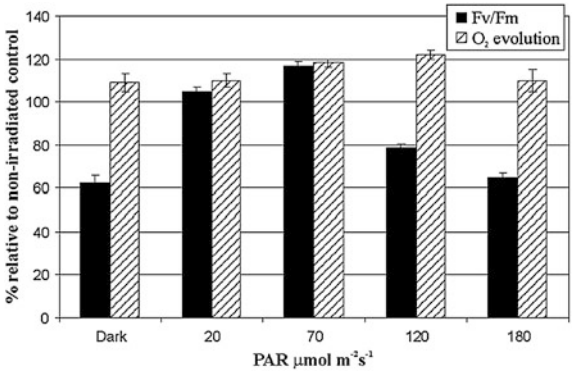

Fig. 1 Maximum potential quantum yield of PSII in dark-adapted cells as F_v/F_m ratio and oxygen evolution of *C. reinhardtii* in relation to control after exposure to fast neutrons at varying light intensities. Figure derived from Rea et al. [9]

to be stimulated. However, they mention that which light the algae were exposed to has an influence, similar to radiation in spaceflight. This could be due to a negative synergistic effect of light and cosmic radiation as high light causes photoinhibtion also producing free radicals.

Gitelson et al. studied UV radiation effects in 2003 on a continuous culture (turbidostat) of *Chlorella vulgaris* [24] to monitor self-restoration/regenerative capacity. They showed that after a decrease of growth rate caused by UV radiation, a growth rate followed which was higher than the initial one. The most damaging outcome was correlated with the highest growth rates, supporting Korogodin's thesis [25] that the most susceptible cell condition to damaging factors is the one with highest growth rates.

Chlamydomonas reinhardtii is among the species showing the highest efficiency of photosynthesis during spaceflight experiments [9]. For this reason, the alga might be a suitable organism for a first microalgae-based module within a life supporting system in spaceflight. *Chlamydomonas* is one of the best-investigated species with respect to physiology and genetics and therefore an ideal model organism, understanding that other species which are better suited for food applications or with higher robustness could be candidates for future practical applications.

Also on the side of technical design of such a system several obstacles have to be taken into account. Space environmental factors of extreme temperatures and temperature changes and low atmospheric pressure are also challenging for process design. Microgravity conditions especially and consequences such as the absence of buyoancy and virtually all convective forces limit design possibilities. Gas exchange has to be accomplished in a complete bubble-free manner. Energy limitation demands a highly energy-efficient system and speaking for the microalgae reactor itself, its yield should be optimized on volume, time, and weight [26]. A major aspect for the design and establishment of a microalgae-based photobioreactor is the question of how and in what quantity light is provided for those

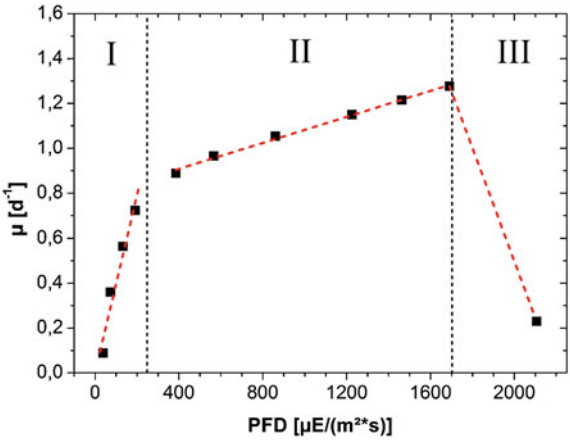

Fig. 2 Typical light kinetics of microalgae. Dependency of specific growth rate μ on light intensity: I_c as compensation point, I_k as optimal irradiance intensity, I_s as saturation of photosynthesis, I_h as inhibition of photosynthesis [27]

photosynthetic organisms to achieve maximum energy efficiency and stability. In order to find a suitable range of light intensity light kinetics of the chosen alga have to be regarded.

Figure 2 shows a typical curve for light kinetics of microalgae [27]. There are three characteristic ranges being:

I. The phase of photolimitation which is characterized by linear increase of photosynthesis rate with increasing irradiance. A first photosynthetic activity can be measured above compensation point I_c, where cell maintenance equals photosynthesis. The optimal point is defined by I_k where only a small further increase of photosynthesis rate can be achieved with increasing light intensity.
II. The photosaturation phase is marked by a constant maximum photosynthesis rate where the efficiency of photons decreases because not every absorbed photon is used for CO_2-fixation as a consequence of limited RuBisCO fixation and capacity of the electron transport chain. Excess energy is dissipated as heat as so-called nonphotochemical quenching NPQ.
III. The phase of photoinhibition is characterized by a decline of photosynthesis rate with increasing irradiance (beyond I_h). Furthermore, production of photoprotective pigments such as carotenoids is induced. The degree of photoinhibition depends on the intensity and duration of light exposure. However, microalgae are capable of adaption to low and high light conditions by so-called photoacclimation.

In photobioreactors light intensity is different in different zones of the reactor and mean growth rates are obtained by integrating along the light path.

A major factor determining efficiency of a first module in a life supporting system is the efficiency with which light energy can be converted into biomass. An important parameter describing this yield is the PCE (Eq. 1). In contrast to the often-used photosynthesis efficiency, PE, photo conversion efficiency also takes into account anabolic reactions following the process of photosynthesis:

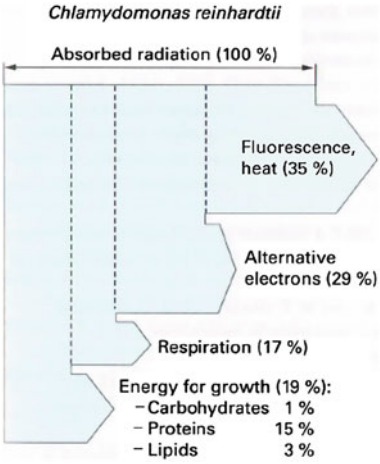

Fig. 3 Energy balance from photon to biomass for *Chlamydomonas reinhardtii* under chemostat conditions with light irradiance at I_k [110]

$$\mathrm{PCE} = \frac{H_X}{I} \tag{1}$$

Equation 1. Definition of the energetic efficiency of microalgae growth

H_X Biomass heat of combustion in *J*; with H_X as specific algae biomass heat of combustion being around 20 MJ/kg [28, 29]

I absorbed light energy in *J*

Maximum theoretical PCE values are reported to be around 9 % for microalgae, whereas higher plants only achieve efficiencies around 4–6 % [14, 30].

Figure 3 shows a balance for energy to biomass conversion for a continuous culture (chemostat enabling optimal cultivation conditions) of *C. reinhardtii* illuminated with an optimal light intensity I_k. Here a value of 19 % is given for the highest possible bound energy in the cells being the upper biological limit.

3 Requirements, Opportunities, and Challenges of Photobioreactors for Space Missions

Reactors for microalgae cultivation in space environments have to fulfill certain additional requirements that they do not necessarily have to fulfill on the ground.

The most important characteristic is a complete bubble-free performance inasmuch as without any buoyancy bubbles would remain in suspension and lead to several problems. Details are given in the following sections.

Another important aspect is environmental pressure acting, depending on the housing, on the reactor and its peripheral devices. For instance, during parabolic flights, overall pressure may drop down to values around 700 mbar or even lower.

Overall pressure drop also influences partial gas pressure and solubilities and may also lead to bubble formation. For this reason pressure has to be monitored carefully during cultivations under microgravity conditions as well as studied for its influence on dissolved gas concentrations and on microalgae physiology during microalgae cultivations on the ground.

The weight of the bioreactor unit is another decisive issue not that important on the ground. This not only concerns the construction as such but the weight of the enclosed water body. Process intensification has to lead to high biomass concentrations and minimization of dark zones.

One requirement for microalgae within life support systems related to space environment is that cell physiology does not have to change during continuous processes (ideally steady-state conditions). A work that is concerned with effects of simulated microgravity conditions on ROS on the cyanobacterium *Anabena sp.* has been done by Li et al. [31]. This cyanobacterium showed higher susceptibility to these ROS under microgravity.

Within this work special attention is paid to illumination of microalgae in life support systems as well as bubble-free aeration via membranes.

3.1 Illumination of Microalgae for Remote Applications

In order to be able to employ algae for remote applications it is necessary to grow them in a very energy-efficient way. Optimum energy transfer has to be approached on the technical and on the biological levels. In order to increase the yield of light to biomass conversion there are several points that can be influenced by process design or genetic engineering. It is self-evident that effects such as light reflection or cell adsorption on technical parts—playing a role in outdoor plants on Earth—have to be avoided completely.

Assuming 100 % radiation absorption, 35 % of energy is lost by fluorescence and heat, which could be decreased using mutants with reduced antenna size (see Fig. 3). Losses of energy can also be caused by quantum energy of photons relating to their energy content U (see Fig. 4) meaning that high-energy photons such as blue photons lead to NPQ to get rid of excess energy which could damage the photosystems. Losses caused by alternative electrons meaning losses within photosynthesis itself during CO_2-fixation, for example, make up another 29 %. By limiting dark zones in the reactor the percentage of absorbed energy used for respiration (around 17 %) can be decreased. The only fixed connection is posed by stoichiometry, meaning that production of biomass and oxygen is coupled with 1 mol CO_2 being converted to 1 mol O_2 resulting in 19 % as the highest possible bound energy in the cells being the upper biological limit.

Concerning efficiency of light into biomass conversion it is also important with which wavelengths of illuminated light these yields have been calculated. Depending on the light color that is used, different photoconversion efficiencies (PCE) will result. One work concerning LED illumination with different colors is

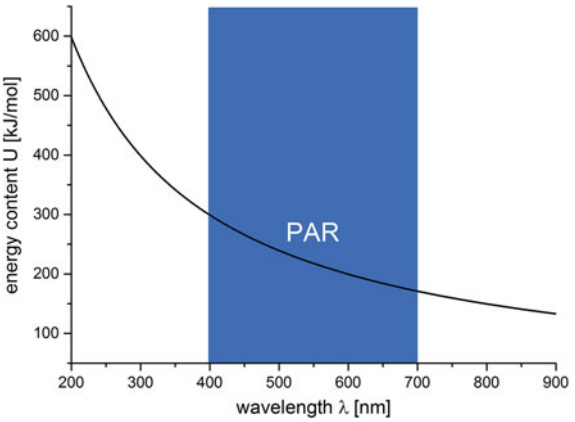

Fig. 4 Energy content U of photons according to their wavelength λ

described by Cerff et al. [32] concerning the growth of the moss *Physcomitrella patens* as a representative of plants. Several studies have also been published on the influence of light colors on the growth of microalgae which is discussed in the following chapter.

For mono- or dichromatic illumination, quantum efficiency based on the energy content of photons according to their wavelength (see Fig. 4 and Eq. 2) has to be taken into account concerning the calculation of efficiencies.

$$U = N \cdot h \cdot \frac{c}{\lambda} \tag{2}$$

Equation 2. Energy content U of photons depending on their wavelength λ

U energy content of photons in J/Einstein or J/mol photon
N Avogadro's number in 1/mol, ($N = 6.022 \times 10^{23}$ 1/mol)
h Planck's constant in J s, ($h = 6.626 \times 10^{-34}$ J × s)
c speed of light in m/s, ($c = 2.998 \times 10^{8}$ m/s)
λ wavelength in nm

A decisive factor is the artificial illumination that is used; if no sunlight is present or if conducting sunlight via fiber-optic structures would be too laborious. In addition to conventional halogen lamps nowadays LED illumination is also used more often for photobioreactors [33–36]. This gives several degrees of freedom with respect to reactor geometry, light color, and possibly light cycles. While having knowledge about the absorption and action spectra of green microalgae, the question arises if illumination with white LED light or halogen lamps is most efficient for biomass yield or if mono- or dichromatic illumination with specific wavelengths should be favored.

In order to determine the wavelengths that should be tested for microalgae growth, knowledge about pigment composition, accessory as well as sensory

pigments, and their physiological role has to be collected and carefully reflected. In our case the microalga of interest is *C. reinhardtii* (wildtype *CC1690*).

3.1.1 Accessory Pigments, Absorption, and Action Spectra of *Chlamydomonas reinhardtii*

The unicellular green alga possesses accessory pigments including chlorophyll a and b as major components of photosystems, as well as carotenoids being essential for cell survival because of their photoprotective and antioxidant properties (acting as quenchers of ROS). Moreover, carotenoids help to broaden the absorption spectrum [37]. Major carotenoids present in *C. reinhardtii* are *β*-carotene, neoxanthin, violaxanthin, and lutein as well as loroxanthin. All of them are associated with photosynthetic reaction centers in the chloroplast of the cell [38]. If cells are subjected to high light stress, a part of violaxanthin is converted to zeaxanthin [39]. In addition to being localized to the chloroplast serving as photosynthetic pigments, carotenoids (*β*-carotene) are also part of the eyespot lens and act as precursor molecules for retinal, the chromophore covalently linked to the rhodopsin photoreceptors of the eyespot being responsible for phototaxis [40, 41].

Evaluation of light spectra is essential where artificial illumination is used and predetermined (e.g., concerning the production of high- value substances, or the use of algae in space application), especially regarding energy optimization. In order to find suitable wavelengths for monochromatic illumination, absorption and action spectra of the alga have to be regarded. Rodríguez et al. [42] detected the following absorption spectrum (Fig. 5) measured on chloroplast thylakoid membrane systems of *C. reinhardtii* wildtype cells with the help of a special apparatus described by Gualtieri et al. [43].

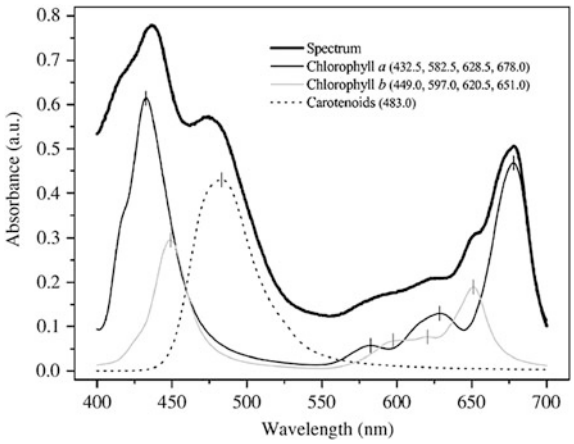

Fig. 5 Absorption spectrum (in vivo) measured by Rodríguez et al. on the chloroplast thylakoid membrane system of *C. reinhardtii* wildtype cells. Spectrum range 400–700 nm, step size 0.5 nm, scan rate 100 nm s^{-1}, 10,000 values per wavelength [42]

It can be clearly seen that in the blue as well as in the red region of the spectrum, absorption is maximal with peaks in the red region around 680 nm. In the blue spectral range the maximal peak is around 435 nm whereas a smaller peak lies around 470 nm. Wavelengths in the blue and red regions of the spectrum seem to be promising light colors for an efficient illumination system of photobioreactors housing microalgae.

Another important question arising from knowledge about accessory pigment composition is whether direct excitation of auxiliary pigments such as chlorophyll b is necessary or if it is sufficient to excite chlorophyll a only.

In general the so-called auxiliary pigments such as chlorophyll b and carotenoids absorb light according to their specific absorption spectra and transfer the resulting energy to chlorophyll a (Förster resonance energy transfer) which drives light reaction of photosynthesis. But does the role of these auxiliary pigments only consist of transferring their absorbed energy to chlorophyll a, or do they have other physiological functions?

One main effect relating to that topic was described in 1960 by Emerson and Rabinowitsch [44], known as the *Emerson effect*.

Emerson described the so-called *red drop* during illumination of a *Chlorella* culture (among others) with wavelengths higher than 685 nm, where chlorophyll a is the only molecule absorbing the light. He determined the quantum efficiency ϕ_{max} in terms of oxygen production, meaning the ratio of produced O_2 mols to mols absorbed light quants. The red drop describes the fact that ϕ_{max} decreases significantly in this long-wave radiation range compared to the efficiency achieved with light at around 600 nm. Also around 650 nm a drop could be seen (Fig. 6).

He concluded that for photosynthesis working with maximum efficiency, it is a prerequisite that light is absorbed by at least two pigments: chlorophyll a and one of the auxiliary pigments. He concluded that there seem to be two in vivo forms of chlorophyll a wherein one form absorbs far-red light and the other form is either

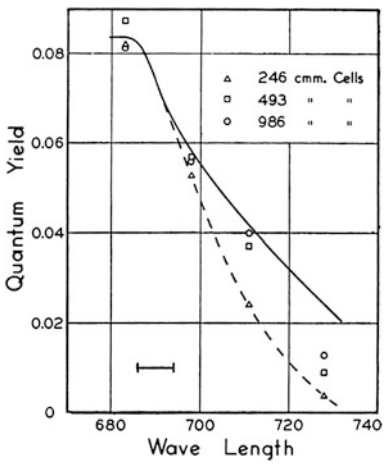

Fig. 6 Red drop for a *Chlorella* culture. The quantum yield declines in the extreme red part of the spectrum where chlorophyll a is the only molecule absorbing light (Emerson and Rabinowitsch 1960 [44])

directly excited by near-red light or indirectly via energy transfer from auxiliary pigments.

Furthermore the so-called *enhancement effect* was also described within the same work. When light at 650 nm and light at 720 nm are used simultaneously for illumination of algae, the quantum yield is as high as with light at 600 nm. This enhancement effect can be explained by the fact that long-wave light around 720 nm can only be used efficiently for oxygen production via PSI if simultaneously light with lower wavelength around 650 (PSII maximum) is there. This effect also appears when these wavelengths are illuminated one after another if the dark time break in between is short; this is called the *Hill effect*.

These experiments led to the conclusion that oxygen production is at PSII which has its absorption maximum in a lower wavelength range of red.

Therefore in general it seems to be necessary that both Chl a and one of the auxiliary pigments are excited to keep photosynthesis running with maximum efficiency.

Chlamydomonas reinhardtii also possesses sensory pigments responsible for phototaxis as well as other physiological functions in addition to those accessory pigments. These also should be taken into account while choosing light wavelengths for high-efficiency microalgae cultures of this green alga.

3.1.2 Sensory Pigments in *Chlamydomonas reinhardtii* and Their Physiological Role

In addition to those accessory pigments, several sensory pigments (photoreceptors) were determined for the green microalga *C. reinhardtii* controlling important physiological functions. In total, seven rhodopsins (whereas the role of two of them is clear, being ChR1 and ChR2, the role of the remaining five is still not known), one phototropin (UV, blue), and two cryptochromes (blue) were identified as sensory pigments in *C. reinhardtii*. Still not proven is the existence of a phytochrome that is sensitive to red light in *C. reinhardtii*. Channelrhodopsins (ChR1, a proton channel and ChR2, a cation channel) acting as light-gated ion channels during photoreception for phototaxis and photophobic response are unusual receptors found in this alga.

3.1.3 Phototaxis as Photomotile Behavior of This Alga

Phototactic behavior was described early in 1867 by Famintzin [45, 46] for *C. reinhardtii*. This movement towards or away (positive and negative phototaxis) from a light source is reported to be strongest in the exponential growth phase and also independent of photosynthesis, meaning that also heterotrophically grown cultures show that behavior [47]. Action spectra for phototaxis shows the highest peak in the blue region (503 nm), whereas only light from near UV up to wavelengths of 550 nm induces a phototactic response [48]. The response, either positive

or negative, is influenced by light intensity (low light mostly positive, high light mostly negative) [49] and other factors such as calcium and magnesium ion concentration [50], cell adaptation to light conditions [51], and rate of photosynthesis [52]. However, in those early days photoreceptor pigments were not yet identified. Later it was found that a rhodopsin has a function for phototropism and interacts with the so-called orange-colored "eye spot" providing information about the direction of the incoming light. Since the 1850s scientists assumed that the orange-colored spot of *C. reinhardtii* cells visible under the light microscope had a function for light perception [46, 53]. With the help of electron microscopic analyses it could be shown that the eye spot consists of multiple layers of carotenoid containing lipid globules localized within the stroma of the chloroplast next to the plasma membrane while being separated from each other by thylakoid membranes [54]. Resulting from a higher refractive index of those vesicles compared to the layers of thylakoid membranes and a distance of 125 nm between them, light beams hitting them at right angles are partly reflected [54]. Only with light beams of wavelengths around 500 nm (four times the distance between the layers) is there a constructive interference [37, 38]. *Chlamydomonas reinhardtii* is also able to determine the angle of incoming light. There are photoreceptors in the plasma membrane next to the eye spot that are shielded from the eye spot from light beams hitting the spot from the back side. Light beams hitting at right angles are amplified by constructive interference and this is maximal at wavelengths of 500 nm. If the angle of the incoming light beam is smaller, constructive interference amplifies light with wavelengths smaller than 500 nm [39].

Phototaxis is also controlled by the cell's circadian clock which provides a rhythm to that photomotive behavior [55].

In 1984 Foster et al. [56] proved that a rhodopsin is the responsible photoreceptor for phototaxis. Also the photophobic stop response is controlled via a rhodopsin with action spectrum peak around 495 nm, which is similar to the phototaxis peak at 503 nm. It could be proven that two proteins act as ion channels, namely channelrhodopsin 1 (ChR1) and channelrhodopsin 2 (ChR2). The action spectra peak of ChR1 is at 500 nm, and that of ChR2 at 460 nm [40, 41]. Therefore it seems to be clear that ChR1 is responsible for phototaxis and the photophobic stop response with an action spectrum peak around 500 nm. The exact function of ChR2 is not known. Others [57] report slightly shifted maxima for rhodopsin A and B (correlating to ChR1 and 2, respectively) being 510 nm and 470 nm whereas A reponds quickly to high light intensities and B slowly to low light (Fig. 7).

Thus for phototaxis light in the UV-blue region with action spectrum peaks around 503 nm seems to be important. This has to be considered for illumination of *C. reinhardtii* cells.

3.1.4 Circadian Clock Provides Rhythm for Phototactic Behavior

Forbes-Stovall et al. [58] describe for strain *CC-124* of *C. reinhardtii* that the cells are highly sensitive to blue light (440 nm) as well as green (540 nm) and red light

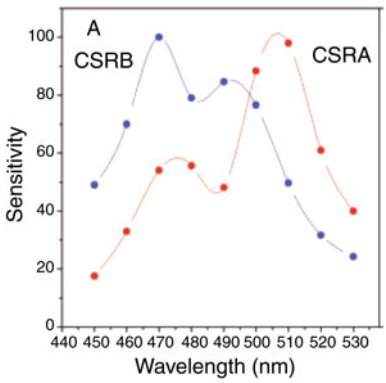

Fig. 7 Action spectra of photoreceptor currents for cells enriched in channel rhodopsin A and B [57]

(640–660 nm) concerning the reset of their circadian rhythm of phototactic behavior. This was also shown before in 1991 for the cell-wall–deficient strain CW15 of *C. reinhardtii* by Kondo et al. [59] for blue and red light but with another peak in the action spectrum for blue at 520 nm. They also assume that the action spectrum peaks are different from that responsible for phototaxis (around 503 nm) and that there are photoreceptors responsible for setting the clock differing from the one leading to phototaxis. They also mentioned that phytochrome could be responsible for the action spectrum peak at 660 nm for resetting the circadian clock of phototaxis, however, this still remains unclear. They conclude that photoreceptors, different from the one responsible for phototaxis, act together in resetting the clock for photomotile behavior, and therefore also for the circadian rhythm of phototaxis wavelengths in the blue region, but also in the green and red range, play a role.

3.1.5 Blue Light not Only Induces Phototaxis…

Blue light not only induces phototaxis and photophobic response in *C. reinhardtii*, but also plays a role for cell division. One mother cell of *C. reinhardtii* can divide into two, four, or eight daughter cells during cell division and there are several critical cell sizes at which cells undergo division [60]. Several hints in the literature suggest that blue light has an impact on cell division. Münzner and Voigt [61] described in 1992 that blue light (no specific wavelength mentioned) induced a delay in cell division in *C. reinhardtii* wildtype cultures (137C mt^+) compared to cultures grown with red and far-red light. Moreover, white light, with which formerly heterotrophically grown cells were illuminated, also caused that effect.

Action spectra for the delay of cell division revealed peaks at 400 and 500 nm, whereas the one at 500 nm is supposed to correspond to rhodopsin which is reported to play a role in phototaxis [56, 62–64].

Oldenhof et al. [65] also report that blue light delays cell division compared to red light ($\lambda > 590$ nm) of *C. reinhardtii* wildtype (cc125 mating type+) cells and

that furthermore cells illuminated with blue light have a more increased size before they undergo cell division than in the control illuminated with red light. They assumed that blue light receptors play a role for controlling the time when cell division starts and inhibit cell division at the minimal critical cell size for cell division. However, their blue light filter also transmitted light with a wavelength higher than 680 nm which makes it difficult to come to a final conclusion.

In 2004 Oldenhof et al. [60] described effects of red and blue light on the time of cell division, the synthesis of DNA, and the presence of cyclin-dependent kinases (CDKs) of *C. reinhardtii* wildtype cultures (cc125 mating type+). Cyclin-dependent kinases (serine/threonine protein kinases) play a role in controlling the progression of the eukaryotic cell cycle. While active, they initiate synthesis of DNA and mitosis. Blue light and white light are reported to delay cell division as well as DNA synthesis compared to red light. Corresponding to that too, CDK activity also occurred later in blue illuminated cultures than in the red control. These effects were proven to be due to blue light. However, the exact mechanisms of how blue light affects these processes still need to be elucidated.

Huang and Beck [66] found that blue light via a phototropin photoreceptor also plays a key role in the sexual life cycle of *C. reinhardtii*. One phototropin gene known in *C. reinhardtii* is Phot wherein the encoded protein possesses two domains sensitive to light, O_2 or voltage (LOV), which are followed by a serine/threonine kinase domain. There are three phases in the sexual life cycle of *C. reinhardtii* that are sensitive to light: gamete formation, the maintenance of mating competence in gametes, and the germination of zygotes. They used wildtype cells (CC124 mt-) and CF14 mt+ as mating partner. The authors conclude that this blue- light–sensitive phototropin primarily controls developmental processes.

Kim et al. [67] report that blue light also has an influence on cell size and cell division of the green alga *Chlorella vulgaris*: blue light illumination (peak wavelength at 450 nm) led to an increased cell size, and red light (peak wavelength at 660 nm) to smaller cells with high cell division activity. Furthermore they applied the wavelengths at different growth stages: when first illuminated with blue light and shifted to red light illumination, higher biomass as well as lipid productivities could be achieved, resulting from that fact that blue light first creates big cells and the shift to red light leads the cells to divide actively.

A blue light receptor also controls several important enzymes, for example, RuBisCO and carbonic anhydrase [68, 69], and furthermore controls reproductive stages [66] and gamete formation [70].

One further blue light receptor that has been identified in *C. reinhardtii* is a cryptochrome (CPH1). The protein encoded is very light-labile and is only present during heterotrophic cultivation in the darkness [66]. Its role still remains unclear.

3.1.6 Red Light

According to Grossmann et al. [71] until now no red light receptor has yet been identified, however, there are certain hints within the genome pointing to its

existence. At least one red light receptor is assumed to exist, but has yet to be found. Red light plays a role in gene regulation and development.

3.1.7 Geometrical Design Aspects of Photobioreactors for Remote Applications Regarding Light

Concerning the design of a photobioreactor for usage in life support systems the question of how the illumination device is implemented in the design is of crucial importance.

Regarding a most simple form of photobioreactor, a plate type of photobioreactor, either illumination from one side or from both sides is possible. Figure 8 shows the typical light distribution for different reactor types that was examined by Jacobi et al. [72]. For the version with single-sided illumination (Fig. 8a), an exponentially decreasing light intensity with the light path can be seen. Details of equations and modeling used for light distribution analysis are given by Jacobi et al. [72]. Using this arrangement for supplying light to the cells, good mixing and a more turbulent flow regime are necessary to minimize the time when a single cell is exposed to dark volume elements with insufficient illumination.

In the case of double-sided illumination (Fig. 8b), no turbulence is necessary as the light is homogeneously distributed within the reactor depth. One cell moving/flowing from one point in the reactor to another is exposed to almost the same incident light intensity in the whole reactor volume. Being able to use laminar flow regimes is also advantageous concerning energy demand especially for space application.

The choice of illumination device and internal energy efficiency (electrical energy to light energy conversion) is also of crucial importance influencing total energy efficiency of the module. For this reason LEDs are chosen for illumination to minimize energetic losses in the form of heat [33, 73]. Further advantages of LEDs are their small size and lightness enabling a good adjustment for various reactor

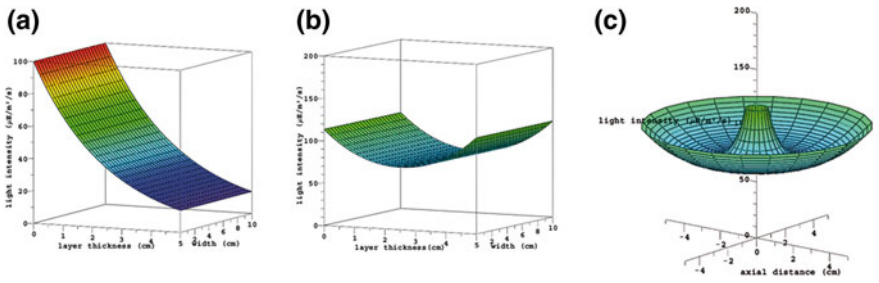

Fig. 8 Typical light intensity distribution in different reactor types for a light intensity of 100 μE/(m^2s) and same absorption coefficients ε and layer thickness *D*. **a** Single-sided illumination of a plate-type reactor; **b** double-sided illumination of a plate-type reactor; **c** cylindrical reactor with outside illumination [72]

structures and sizes. Having a narrow spectral output (half-power bandwidths of about 20–30 nm) and a long durability, LEDs are suitable for absorption-specific illumination in long-term cultivations [33, 73]. Their long lifetime (about one year in continuous PBR operation) [74] especially makes them the best choice among different light sources. Concerning internal efficiency of LEDs the color chosen is also important inasmuch as red LEDs, for example, need 30 % less energy than blue or white LEDs [32].

Being energy efficient also concerns minimizing volume and mass and therefore maximizing the efficiency of the volume inside such a module. This means, concerning a microalgae reactor, maximizing biomass concentration and, on the other hand, volumetric productivity relating to oxygen production [75]. In the case of a one-sided illumination of a plate-type reactor biomass concentration has to be at a value, where there is just no transmission of light on the unlighted side, as transmission of light would mean loss of energy.

In order to reach high biomass concentrations medium composition has to be optimized and feeding strategies have to be developed. As process type, a continuous culture is chosen as optimal conditions can be maintained for a longer time. Biomass concentrations of up to 20 g/L are reported in the literature for photoautotrophic cultivations of a *Chlorella sorokiniana* strain in photobioreactors [76].

Another parameter for optimization is the maximum volumetric oxygen productivity OPR (oxygen production rate) based on reactor volume [75]. Related to batch cultivation, maximal volumetric oxygen productivities in general prevail at the end of the exponential phase when growth changes to a linear phase.

Theoretically a maximum production rate for oxygen of 25–400 fmol O_2 per cell and h can be expected, based on the assumption that one mol chlorophyll can produce 50–400 mol O_2 per h and the chl-content per cell is in the range of 0.5–1 fmol for *Chlorella* [73].

For high-density phototrophic microalgal cultures a high surface-to-volume ratio, efficient light source, efficient light delivery, and an efficient gas transfer are needed. Moreover, a well-balanced medium composition, without accumulation of inhibiting metabolites or other compounds, throughout the process time has to be guaranteed, aiming at high cell density cultivations [73].

In the proposed concept within that work, a reactor geometry based on a plate-type reactor is used, realized as a mini-raceway reactor which can be illuminated either from one side or from both sides. As a bubble-free aeration is a prerequisite under microgravity conditions, in a first approach flat sheet membranes fixed on both sides of the mini-raceway reactor (either horizontally or vertically mounted) are used to supply CO_2 to the cells as well as for removal of O_2 produced. For these reasons the reactor concept for establishing a first module for life support systems based on microalgae has the following characteristics.

- Mini-raceway (ability for single- and double-sided illumination)
- Continuous culture
- Membrane aeration for bubble-free CO_2 supply and O_2 removal

– Illumination by conventional white LEDs in comparison to dichromatic red and blue LED illumination

Details of this concept are depicted in the following sections.

3.1.8 Illumination Concepts and Designs for Biological Life Support Systems in Spaceflight

As reduction of electrical power demand of biological life support systems is of main importance, lighting techniques are of special interest during their development. There are currently studies about hybrid solar and artificial lighting systems conducted by NASA [77]. In addition to LEDs [78] microwave lamps are also focused on because of their excellent power conversion efficiencies [77]. Assuming electrical efficiencies of LEDs amount to 50 % and with a photoconversion efficiency for dichromatic illumination with red and blue of 16 % (for a ratio of 90/10 red/blue photons; see Fig. 10) a total energetic efficiency of 8 % results.

Experiments on cultivation of plants for bioregenerative space applications have shown that a power ratio of red to blue with 90/10 achieves the highest yields in plant biomass [77]. However, it still needs to be elucidated if blue and red photons alone can be used for optimal growth of specific plants that shall be included in closed life support systems [79].

3.1.9 Consequences for Potential Mono- and Dichromatic Illumination of *C. reinhardtii* CC1690 for Remote/Spaceflight Applications

Based on the current knowledge about accessory and sensory pigments and their role in microalgae growth and physiological functions it seems promising to use mono-and dichromatic illumination for the microalga *C. reinhardtii* within the development of a photobioreactor module used for remote applications. This should be tested at several specific wavelengths, according to the action spectrum in vivo; additionally experiments should be conducted testing the influence on growth and PCE while exciting the phototaxis photoreceptor at the corresponding wavelength or even supplemented with blue wavelengths for photoreceptors of the circadian clock of phototactic behavior.

Although mono- and dichromatic illumination have been used for plant cultivation for almost two decades [80] this knowledge has not been widespread within microalgae cultivation aside from physiological studies on photoreceptors and their function and a few studies on cultivation lighting based on colors.

Experiments with red and blue lasers used for cultivation of *C. reinhardtii* in TAP-medium were described by Kuwahara et al. [80]. They tested red laser light with wavelengths of 655 nm and 680 nm, blue light with 474 nm, as well as combinations of each of the red ones with blue. Their control was illuminated with

white fluorescent light. Concerning dichromatic treatments a power ratio of 90 % red to 10 % blue was applied according to common plant lighting techniques [77]. For both dichromatic combinations an increase of cell number was seen compared to monochromatic red illumination and white control. Moreover, it was proven that with red monochromatic illumination with either red laser the same cell count was achieved compared to the white control. Major reasons for the best results for dichromatic illumination with red and fewer blue photons could be the stimulation of high expression or control of important enzymes such as RuBisCO [68] and carbonic anhydrase, the latter playing a major role in CO_2-concentrating mechanisms (CCMs) in that alga. RuBisCO is reported to be under blue light control [68] and that the induction of carbonic anhydrase is regulated by two light-mediated steps [69] whereas one of them requires blue light [69]. Moreover it was shown that the first photosynthesis-dependent step in regulation of induction of that enzyme also required red light [69]. However, Lee et al. [73] used only red LED lights at a peak wavelength of 680 nm in a photoautotrophic cultivation of *Chlorella vulgaris* to achieve high cell densities.

3.1.10 Ground-Based Experiments with Mono- and Dichromatic Illumination

Our first ground-based experiments growing wildtype *CC1690* cells of *C. reinhardtii* under pure phototrophic conditions and illuminated with either mono- or dichromatic red and blue LED lights (see Fig. 9 for emission spectra of LEDs) revealed a higher photoconversion efficiency of pure red as well as red and blue illumination compared to warm-white control. Preliminary results for PCE are shown in Fig. 10.

Detailed and further results of those ground-based lighting experiments are displayed elsewhere.

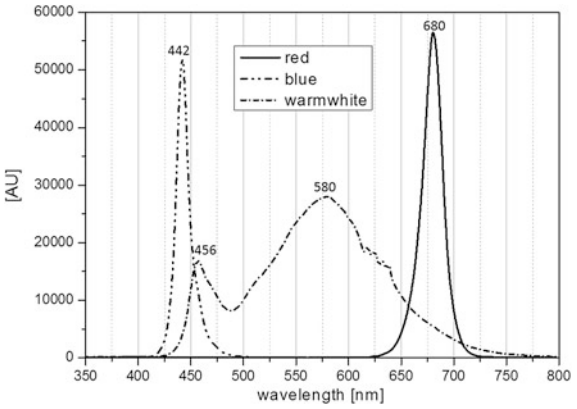

Fig. 9 Emission spectra of blue, red, and warm-white LEDs used during first ground-based tests

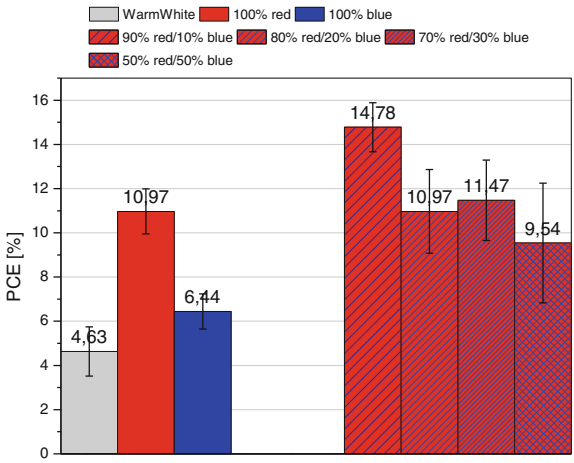

Fig. 10 Preliminary results for photoconversion efficiencies PCE under mono- and dichromatic LED illumination compared to warm-white control. Experiments were run under pure phototrophic conditions in 200 ml working volume plate-reactors with total light intensities of 25 µE/(m^2s) with wildtype CC1690 of *C. reinhardtii* at 25 °C. A warm-white control was compared to pure red and pure blue illuminations as well as red/blue mixtures of 90/10, 80/20, 70/30, and 50/50

3.2 *Aeration of Microalgae for Remote Applications by Membranes*

One of the most important factors influencing the productivity and costs of microalgal cultivations is the provision of carbon dioxide [81–83]. A very simple method to aerate especially large open outdoor systems or lab-scale shaking flasks is the bubble-free surface aeration where CO_2 can enter the liquid cultures via the culture surface as the interface between the gas and liquid phases. A disadvantage is that the mass transport is limited as the liquid surface is size limited [81]. Other conventional methods to supply CO_2 are bubble-based aeration techniques used in bubble columns or airlift reactors [84]. Bubbles of air enriched in CO_2 are also used for mixing. In stirred-tank photobioreactors bubbles are introduced by aeration rings, mixed, and sheared by rotation stirrers of varying type. Moreover, porous membranes can be used for aeration applying a certain pressure leading to bubbles entering the culture broth.

Regarding the application of microalgae-based photobioreactors for space application where no buoyancy is present, an aeration method involving bubbles is not possible. Bubbles would remain in the culture suspension and may interfere with measuring devices and also pumps in the periphery. Furthermore they could also impair cell growth. Moreover, the conventional method of bubble-aeration has in general drawbacks such as the loss of CO_2 in the air caused by a small residence time of gas bubbles inside the liquid and a high resistance for mass transfer from the

gas to the liquid phase [85]. Losses of CO_2 are therefore an important cost factor in cultivations and should be avoided [82, 86]. In photobioreactors with a gas phase cells may also be impaired by the shear stress produced by bursting gas bubbles at the interface [87].

For these reasons it is unavoidable to switch to a completely bubble-free aeration technique. One way to reach that goal may be the aeration via membranes in a diffusion-based manner. Here no pressure difference is applied and mass transport is only driven by a concentration gradient (see Eq. 3) of different partial pressures of CO_2 between the two phases separated by the membrane. CO_2 is transported through the membrane by pure diffusion which can avoid the losses of unused CO_2 to the atmosphere and provide an accurate control of the CO_2 transfer rate obtaining CO_2 directly dissolved in water. This is directly available for the cells without having the resistance of gas–liquid mass transport [82]. Because the liquid and gas phases with bubble-free membrane aeration are not dispersed into each other, the respective flow rate of gas and liquid can be set independently of each other without having limited operating conditions and the total membrane surface area is available even though flow rates might be set to minimum [82].

Sensitive cells also profit highly from bubble-free aeration via membranes due to reduced shear forces. Especially in mammalian cell cultures membrane-aeration has been used for this reason for almost 30 years now [88].

The removal of oxygen that is produced during a phototrophic growth is another very important aspect [82]. High dissolved oxygen concentrations may lead to inhibition of photosynthesis [89, 90] and lead to bubble formation by oversaturation. A critical aspect is the low solubility of oxygen in water, for example, around 8 mg/L at 25 °C with air atmosphere. For this purpose a suitable reactor design, with a certain specific geometry of the membrane and a high selectivity for O_2 related to CO_2, has to be found. Another aspect is the possibility of using different membranes for the CO_2 supply and O_2 removal with different mass transport coefficients for the two gases.

Important characteristics of the membranes closely related to the photobioreactor design are the transparency of the materials, their mechanical stability, and also fouling behavior. The possibility of sterilizing the membrane material in some way also compatible with the rest of the reactor material is of crucial importance. Fouling behavior, for example, is dependent on surface charge, hydrophobicity of the membrane material, cell density, and amount of extracellular polymeric substances (EPS) and other organic matter. Degree of fouling may also depend on the growth stage of algae where exponential growing cells cause less fouling. Membranes possessing negative surface charge may repel equally charged cells whereas the least fouling is found with negatively charged hydrophilic membranes [91]. Fouling of membranes in microalgae reactors is reported to be mainly due to cake formation and to a minor degree by blockage or hydrophobic adhesion of cells [83]. Higher fluid velocity and coating with functional materials such as polyvinyl alcohol PVA can reduce membrane fouling potential during long-term cultivations [83, 92].

Other fields where membranes are applied in microalgae technology are solid/liquid separation (biomass concentration, dewatering, cell retention) and also solute/liquid separation for product recovery and effluent recycling which is problematic or even not possible with other techniques [83]. Common membrane materials used in gas delivery and removal in microalgae technology are polypropylene PP and PVDF as well as silicon-based polymeric membranes, for example, polyvinyltrimethylsilane PVTMS [83].

In summary, membranes have been used for gas delivery and removal in microalgal reactors [83] where one should clearly distinguish between approaches using pressure differences creating small bubbles [90, 93] and concentration-difference–driven processes where the operation is bubble free. For space application relating to energy efficiency and missing buoyancy approaches with bubble-free diffusion-based membrane aeration should be favored.

3.2.1 Mass Transfer Through Membranes in Photobioreactors

In the case of a membrane-aerated photobioreactor a gas phase containing a specific composition is in contact with the membrane, where the liquid phase adjoins on the other side. For both gases, CO_2 and O_2, mass transfer equations with their mass transfer coefficients can be set up.

Based on Fick's law of diffusion and the two-film theory with Henry's law, the equation for the CO_2 transfer rate (CTR) across the membrane is given by:

$$\mathrm{CTR} = k_{\mathrm{L,CO_2}} \cdot a \cdot \left(c^{*}_{\mathrm{CO_2}} - c_{\mathrm{L,CO_2}} \right) \tag{3}$$

Equation 3. Carbon dioxide transfer rate across a membrane for bubble-free aeration

CTR	carbon dioxide transfer rate in mole of $CO_2/(m^3 s)$
$k_{\mathrm{L,CO_2}}$	CO_2 mass transfer coefficient in m/s
a	specific transfer area in 1/m
$c^{*}_{\mathrm{CO_2}}$	CO_2 concentration at interphase in equilibrium in mol/m^3
$c_{\mathrm{L,CO_2}}$	CO_2 concentration in bulk liquid phase in mol/m^3
$\left(c^{*}_{\mathrm{CO_2}} - c_{\mathrm{L,CO_2}} \right)$	concentration gradient acting as driving force in mol/m^3

The constant $k_{\mathrm{L,CO_2}}$ in this case represents the mass transfer coefficient for CO_2 related to the membrane and the boundary layer on the liquid side (resistance by the boundary layer on the gas side can be neglected). The oxygen transfer rate OTR can be calculated analogously.

The selectivity of a membrane towards gases can be expressed in this case via their mass transfer coefficients for gas *A* and gas *B* (determined under exactly the same testing parameters) in the following manner:

$$\alpha_{A/B} = \frac{k_{\mathrm{L,gas}\,A}}{k_{\mathrm{L,gas}\,B}} \tag{4}$$

Equation 4. Selectivity of a membrane for gases *A* and *B*

On the other hand, the carbon dioxide uptake rate (CUR) by the cells must also be described and quantified. Given by stoichiometry of photosynthesis, 1 mol of CO_2 is converted to 1 mol of O_2, resulting in CUR = OPR in molar terms.

The carbon uptake rate of the cells is a physiological characteristic of the culture. It is limited by the metabolic activity of the microalgae, which is in turn limited by photosynthesis and therefore light intensity.

According to the carbon content of the biomass, CUR is directly coupled to biomass productivity. Assuming carbohydrate-rich algae, it is about 1.7 g CO_2 per g biomass produced. This value can rise up to a value of 2.5 g/g. The amount of CO_2 to be transported via the membrane finally depends on the light flux to the culture. Typical values for artificially illuminated reactors are in the range of 2 g/L/d.

The membrane area-to-volume ratio a for quasi-stationary operation can be calculated from CTR − CUR = 0 and depends on the mass transfer coefficient $k_{\mathrm{L,M}}$ of the membrane.

$$a = \frac{A}{V} = \frac{\mathrm{CTR}}{k_{\mathrm{L,M}} \cdot (c^* - c_{\mathrm{L}})} \tag{5}$$

Equation 5. Specific membrane transfer area as area-to-volume ratio

The highest specific membrane transfer areas can be achieved with hollow-fiber modules.

Typical $k_{\mathrm{L,M}}$ values for dense silicone membranes are around 7×10^{-6} m/s for O_2 and around 5×10^{-6} m/s for CO_2. For porous PTFE membranes $k_{\mathrm{L,M,O2}}$ is around 5×10^{-6} m/s, and for CO_2 around 6×10^{-6} m/s.

Factors that influence CTR and OTR are related to design, for example, membrane material, porosity, selectivity of the material for O_2/CO_2, specific transfer area a, and also to culture conditions such as bulk liquid velocity (thickness of boundary layer), salinity, and viscosity of the medium as well as culture temperature.

In the following a short overview of the application of membranes for bubble-free aeration of bioreactors, and especially photobioreactors is given. Furthermore some concepts for membrane-aerated space-photobioreactors are displayed.

3.2.2 Membrane-Aerated Bioreactors

Since the end of the 1980s there have been reports of using bubble-free aeration techniques for shear-sensitive mammalian cells by means of tubular membranes [88, 94–98].

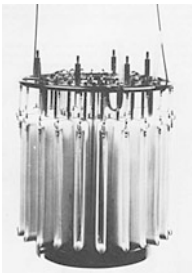

Fig. 11 Diessel reactor with porous Accurel PP hollow-fiber membranes coiled and fixed on carriers [99]. In the production reactors with 20 and 150 l membrane coils were driven eccentrically (for 1 L scale a pendulous motion was applied) and to have homogeneous mixing of microcarriers a spiral agitator was fixed below the membrane carriers

Designs of membrane-aerated bioreactors for animal cell cultures are primarily based on the usage of hollow-fiber membranes. There are several concepts using membrane devices within the reactor.

A hollow-fiber membrane-based bioreactor for bubble-free aeration that has also been patented and known as a *Diessel reactor* (Fig. 11) was described by Vorlop and Lehmann in 1988 [99]. They use porous hollow-fiber Accurel polypropylene membranes for supplying O_2 to mammalian cultures coiled in the form of a basket or fixed to carriers. With the help of a motor the coiled membrane fibers are moved in a tumbling manner. During cultivation they maintain a higher overall pressure on the liquid side than on the gas side in order to prevent bubble formation. Cells were fixed on microcarriers. For scale-up from 1 L lab scale to 20 and 150 L production reactors they used specific membrane area a.

Frahm et al. described a dynamic membrane-aerated bioreactor system based on the usage of hollow-fiber silicone membranes for optimization of oxygen supply for shear-sensitive mammalian cell lines in 2007 [87]. In contrast to conventional rotor–stator systems the membrane fibers themselves are mounted on an oscillating device. This leads to a better circulation of culture broth around the fibers to overcome the limited mass transfer capacity of former designs.

In comparison with conventional rotor–stator systems they report a twofold higher oxygen transport capacity at the same shear forces.

In addition to concepts using membrane devices within the reactor, there are also concepts with membrane hollow fibers or even flat sheets within the periphery of the reactor.

Schneider et al. (1995) [100] compared different types of configurations, all using porous PTFE membranes. The membrane surface was the same for all configurations, but with pure water on both sides of the membrane. In this case mass transfer occurs from liquid to liquid phase. Head space inside the reactor was filled with nitrogen in every case.

Their results for those different configurations indicated that for each design the resistance of the membrane itself to mass transfer could be neglected compared to

the resistance of the boundary layer adjacent to the membrane. For configurations leading to a turbulent flow on the membrane side, this caused the boundary layer to be smaller and therefore the mass transfer coefficient being higher.

Transfer of the elaborated membrane technology for heterotrophic cultures to phototrophic cultures is difficult. Although mass transfer rates are similar between animal cell and microalgal culture, the directions of oxygen and carbon dioxide transfer are reversed. As oxygen is less soluble in water this may lead to problems concerning oversaturation and consequent bubble formation.

3.2.3 Membrane-Aerated Photobioreactors

Bubble-free aeration with the help of membranes has also been used for cultivation of photosynthetic microorganisms although there have been more approaches using pressure difference across the membranes creating small bubbles [90, 93]. The latter are not discussed in this review as they do not seem to be suitable for space application.

Lee and Hing [101] used silicone tubing (various internal diameter and wall thicknesses) in 1989 to supply *Chlorella pyrenoidosa* cultures with CO_2 inside an airtight stirred-tank reactor. The membrane tubing was coiled inside the reactor and different membrane-specific surface areas a and CO_2 partial pressures (mixtures of air and CO_2) on the gas sides were tested. The rate of CO_2 accumulation in cell-free acidic water was maximal when surface area a of the membrane was maximal. They calculated the permeability constant K [m^2/h] of CO_2 diffusion within the membrane material as the comparative parameter (Fig. 12).

With the help of a chosen membrane geometry (length = 80 cm, internal radius = 2 mm) they also used pure CO_2 during cultivation. A peak concentration at small biomass concentrations of 100 mbar (10 % v/v) dissolved CO_2 in the liquid was reached which did not inhibit cell growth. At final cell densities of around 2.6 g/L dissolved CO_2 concentration was almost constant around 1 % pCO_2.

They emphasize that with bubble-free aeration with membrane tubing compared to bubble aeration the CO_2 transfer rate can be actively (at a chosen membrane geometry) controlled by adjusting partial pressure on the gas side. The maximum and minimum transfer rates can be chosen by selecting specific membrane tubing geometries. By choosing appropriate geometries and partial pressures it is even possible to use pure CO_2 avoiding high investment costs for gas mixing in larger scales without being concerned with inhibition at elevated dissolved carbon dioxide concentrations.

Ferreira et al. [82] reviewed the use of hydrophobic microporous membranes for aeration of photobioreactors. Among others, a potential application is the CO_2 supply in microalgal cultures. In the case of hydrophobic porous membranes the pores are filled with gas and the resistance of the membrane to the mass transfer can be neglected in comparison to the resistance of the liquid phase. They mention the disadvantage of pressure drop, laminar flows, and obstruction by solid particles in

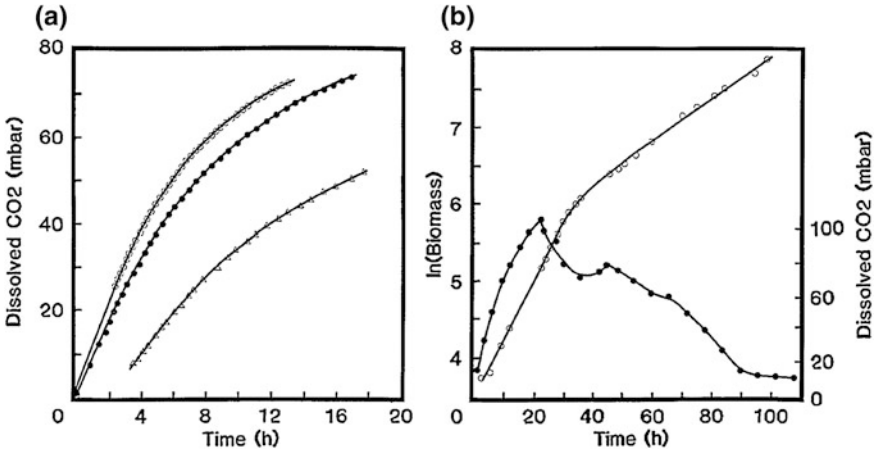

Fig. 12 **a** CO_2 accumulation in acidified deionized water using different membrane areas and different CO_2 partial pressures on the gas side. *Unfilled circle* pCO_2 = 83.8 mbar, A = 418 cm^2; *filled circles* pCO_2 = 83.8 mbar, A = 377 cm^2; *triangles* pCO_2 = 72.5 mbar, A = 377 cm^2. **b** Growth curve of cultivation using pure CO_2: filled circles—biomass concentration; unfilled circles—dissolved CO_2 (mbar) [101]

the liquid by using long fibers with small diameter to meet the needs of mass transfer related to reactor working volume. For gases such as O_2 and CO_2 the resistance of the liquid phase controls the mass transfer [82]. They use polypropylene fibers (pores of 0.2 µmm inner and outer diameters 0.33 and 0.63 mm, surface porosity 69 %; total surface area was 23.8 cm^2) in cultivation with *Chlorella* and compare it to a bubble-aerated culture. One culture vessel was mixed using stirrers and bubble-aerated; the other vessel was pumped (peristaltic pump) through the hollow-fiber module (gas flow rate 4.3 $cm^3\ s^{-1}$) contained in the bypass. For both setups a CO_2 concentration in air of 1 % (v/v) was used; pH and dO_2 were recorded. Both cultures showed similar growth rates, however, they mentioned that dissolved oxygen concentration DO was lower in the vessel with the membrane module even though both systems were open to the atmosphere. So the systems were not closed against the atmosphere which makes it difficult to monitor dissolved gas concentrations in this case and therefore compare the different setups.

In 2001 Carvalho and Malcata also compared [102] the transfer of CO_2 into microalgal cultures by plain bubbling and by hollow-fiber modules (hydrophobic polypropylene fibers, pore size 0.2 µm, 30 cm length, internal diameter 240 µm, total of 7400 fibers, effective area 1.7 m^2, as well as hydrophilic polysulfone fibers, 0.2 µm pore size, 31 cm length, internal diameter 1000 µm, 140 fibers, and effective area of 0.14 m^2). They found higher k_La values for hollow fibers than for plain bubbling. Other advantages are the possibility of circulating the gases and using lower gas pressures thereby reducing costs.

3.2.4 Membrane-Aerated Photobioreactors for Space

Bubble-free membrane-aerated photobioreactors for space application have already been described by various authors [103–105].

A closed membrane-aerated photobioreactor was created and implemented for batch cultivation of the cyanobacterium *Arthrospira platensis* by Cogne et al. [103]. Moreover, a model was designed to predict biomass and pressure during space cultivations within that novel photobioreactor. However, this reactor has not yet been used in parabolic flight tests or satellite missions under long-term microgravity conditions. The reactor itself consists of a cylindrical chamber with a flat sheet membrane separating the gas and liquid compartments (see Fig. 13). The membrane is a porous 0.2 µm PTFE membrane with a thickness of almost 60 µm and a surface

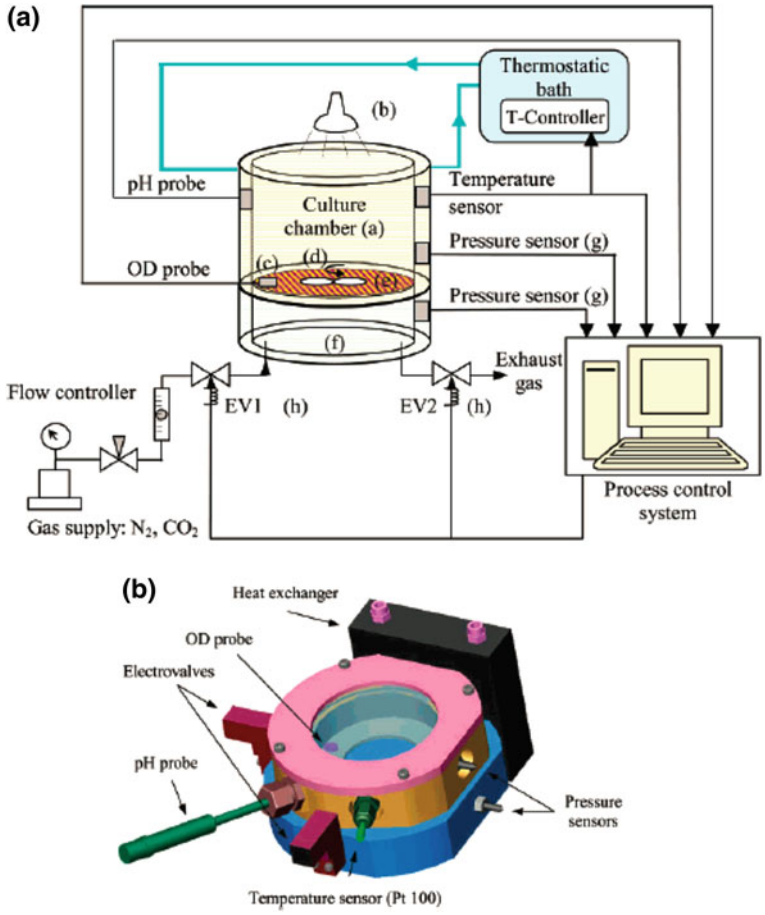

Fig. 13 **a** Scheme of the reactor and its periphery. **b** Online measurements with the help of sensors for pH, OD, and pressure [103]

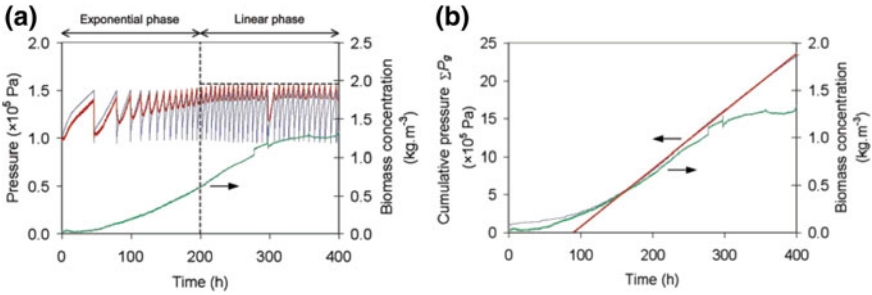

Fig. 14 **a** Biomass concentration and overall pressure in the gas phase during cultivation of *Arthrospira platensis* in this novel membrane-aerated photobioreactor. **b** Cumulative pressure representing O_2 production during cultivation [103]

of 20 cm^2. A disadvantage of porous membranes is the permeability to water vapor which may also lead to volume variations as well as bubble formation inside the liquid phase. Concerning measurement instrumentation the reactor is equipped with an OD (photodiode), pH, and temperature as well as pressure sensor. Mixing was achieved by a magnetic stirrer placed on top of the membrane surface at the bottom of the liquid compartment. Oxygen production was monitored indirectly via the increase of pressure within the gas chamber. When a certain threshold (1.5 bar absolute pressure) was reached the chamber was flushed with nitrogen after the valves at the in- and outlet of the chamber were opened to release the overpressure (see Fig. 14).

During cultivation the pressure drop across the membrane increased until it remained constant at 0.1 bar.

During cultivation, an exponential phase (during the first 200 h) followed by a linear phase (200 h) could be seen (Fig. 14a). Moreover they observed gas bubbles that were formed on the liquid side of the reactor since the first hours of cultivation. They reported a correlation of pressure increase in the gas phase and the increase in biomass concentration in the linear phase of the cultivation; pressure increase was faster after each flushing with nitrogen, due to the higher driving force leading to a temporarily higher mass transfer. This membrane-aerated photobioreactor with online measurements of pH, OD, T, and pressure was first tested on the ground in batch cultivations of the cyanobacterium *Arthrospira platensis* and thereby represented a preliminary system that can be used for developing a final system applicable for space cultivations.

Another ground-based photobioreactor for CELSSs was developed and tested by Ai et al. [104]. The reactor was used for cultivation of the cyanobacterium *Spirulina platensis* (nowadays called *Arthrospira platensis*). Compared to Cogne et al. [103], gas exchange was accomplished with separate membrane hollow-fiber modules for both CO_2 and oxygen. The bioreactor and its periphery consist of nine subsystems: photobioreactor, illumination unit, CO_2 supply unit based on membrane module, O_2 removal unit based on membrane module, a microalgae collection unit, nutrient

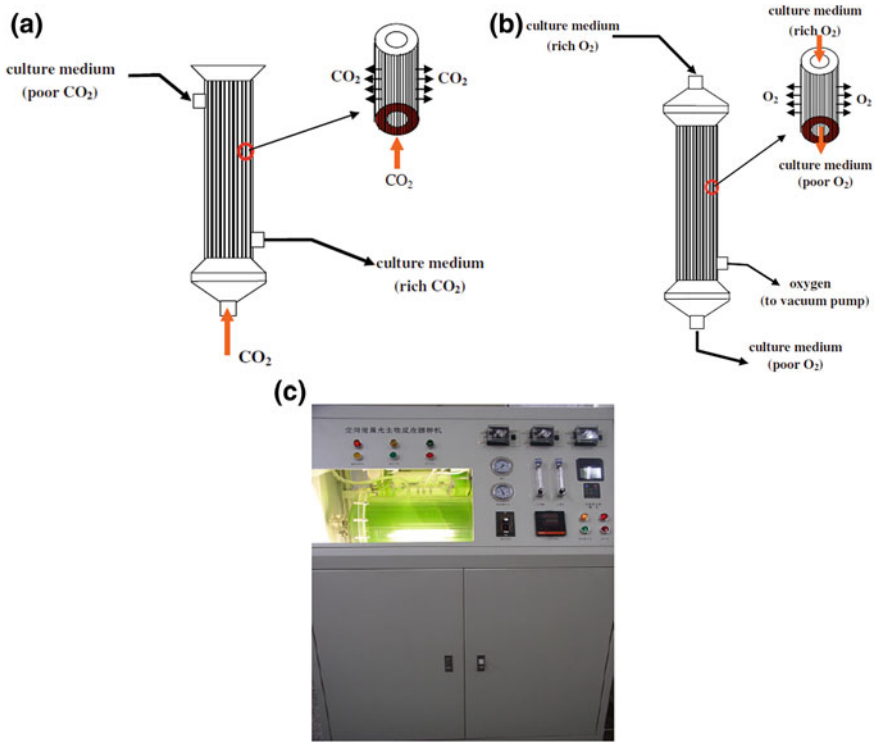

Fig. 15 **a** Membrane unit for supply of CO_2. **b** Unit for removal of O_2. **c** Outlook of the developed reactor [104]

refreshing unit, measurement and control unit, heat exchange unit, and the surrounding cabinet. The bioreactor is illuminated from both sides and the culture broth is circulated through the whole system with the help of a diaphragm pump. Parameters including pH, T, dissolved O_2, salinity, and electric conductivity are measured online and stored by a computer. The microalgae collecting unit is composed of a microfiltration unit. The reactor consists of a transparent PVC tube (d_I = 30 cm) with internal rotating blenders for mixing.

For CO_2 supply they used porous polyethylene fibers (200 fibers, L = 25 cm, d_I = 0.4 mm; Fig. 15a), for O_2 removal PTFE fibers (200 fibers, L = 30 cm, d_I = 1.0 mm; Fig. 15b). Depending on the pH of the medium, the CO_2 membrane module is flushed with pure CO_2 at pH > 9.5; when it falls <8.0 the flushing is turned off again. For O_2 removal an underpressure is created with the help of a pump to force the produced O_2 through the membrane. The vacuum pump is switched on when dissolved O_2 concentration is higher than 10 mg/L; it is turned off again when DO is below 6.0 mg/L. The reactor is sterilized with the help of ozone.

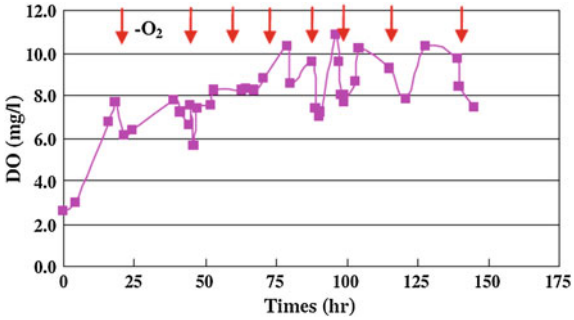

Fig. 16 Dissolved O_2 profile during cultivation. *Arrows* indicate switching on the vacuum pump to start the membrane module for O_2 removal [104]

The temperature of the medium was kept between 28 and 32 °C; at this temperature the saturation concentration for oxygen is (having the atmospheric concentration of gases dissolved in the medium) around 7.5 mg/L for 30 °C and air oxygen concentration (compare to Fig. 16). With pure oxygen as the gas phase around 35.8 mg/L would be soluble at a temperature of around 30 °C. However, the authors do not mention if bubble formation occurred during testing. Furthermore a pressure difference (underpressure) was applied for O_2 removal. So this approach was not purely based on concentration difference acting as the driving force for mass transfer.

In 2012 Farges et al. [105] proposed a concept based on a hollow-fiber PTFE module for microgravity conditions. They relied on hollow-fiber membrane contactor geometries with microporous hydrophobic membranes and used a commercially available one from Gore. However, their module has not been tested yet during algae cultivation but was used in preliminary tests for mass transfer coefficients k_L for CO_2.

There are other concepts with similar approaches concerning membrane aeration. The external gas exchange module of the concept proposed by Brechignac et al. [106] is also composed of hydrophobic membrane fibers (here polypropylene). However, they also proposed a second, very different, option for solving the problem of liquid–gas separation in microgravity (Fig. 17). This concept is based on a cylindrical bioreactor with an axial rotating paddle generating a fluid layer of cell culture medium on the inside of the bioreactor. This would create the possibility of having "normal" ground-based bubble aeration of the culture.

Javanmardian and Palsson [15] describe another photobioreactor primarily designed and implemented for CELSSs (Fig. 18).

The reactor (600 ml volume) was illuminated with xenon lamps at an intensity of 1 mW/cm^2 and fiber optics for light transmission into the reactor resulting in an internal illumination. Cell retention with an integrated ultrafiltration unit also used for exchange of medium enables high cell densities up to 10^9 cells/mL of *Chlorella vulgaris*. Experiments were run in batch and continuous mode. The gas exchange

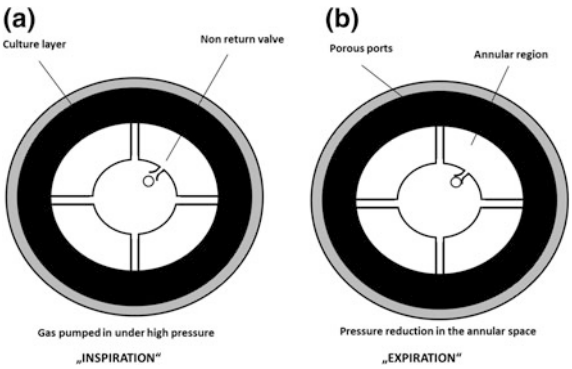

Fig. 17 Scheme of the pseudo-rotating bioreactor aeration concept for bubble-aeration using artificial gravity for gas-liquid separation. Modified after [106]

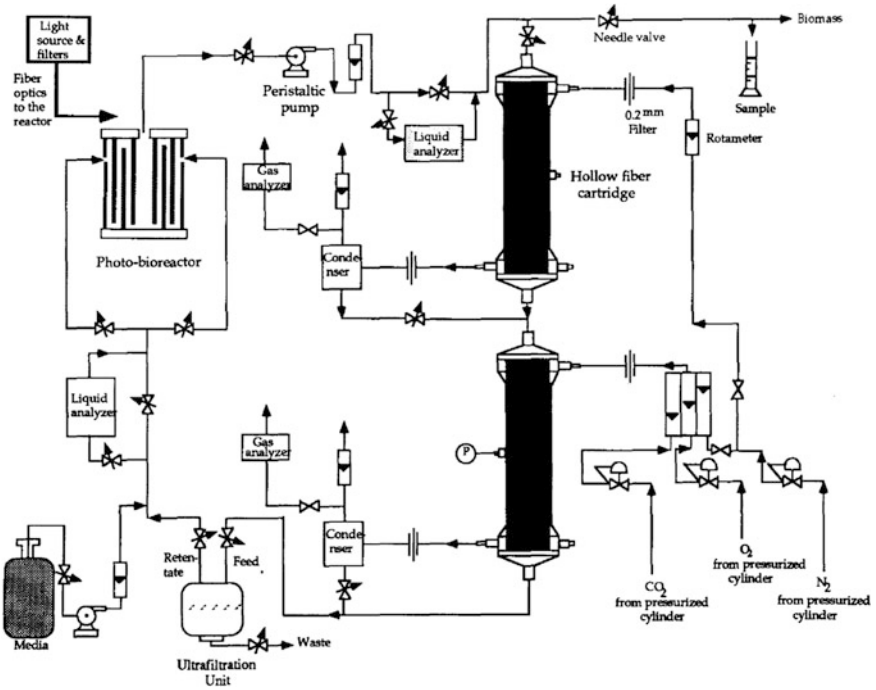

Fig. 18 Photobioreactor concept for application on CELSSs [15]

device was external and realized by hollow-fiber cartridges operating under lower (for O_2 removal) and higher (for CO_2 supply) pressure. So this approach is not purely diffusion-based (Fig. 19).

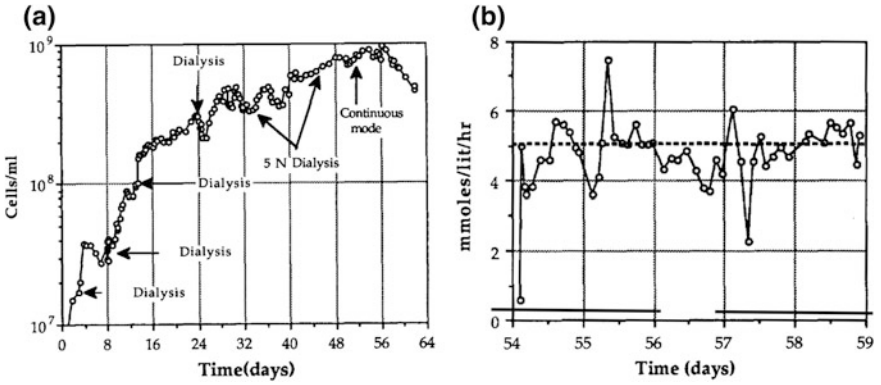

Fig. 19 Growth curve showing batch and continuous phase of cultivation (**a**) and volumetric OPR versus time (**b**) during steady state of continuous mode in photobioreactor designed for CELSS [15]

Volumetric OPRs that were achieved in continuous *Chlorella* culture (cell density at 4×10^8 cells mL^{-1}, dilution rate $D = 0.15\ d^{-1}$) were about 5 mmols $O_2\ L^{-1}\ h^{-1}$ in steady state. At these rates a volume of 200 L would be needed to support the requirement of one human being.

Oguchi et al. [107] describe a closed algae cultivation system based on a membrane module for cultivation of *Spirulina* (cyanobacterium) with an integrated biomass harvesting system operating in semi-continuous mode. Two membrane hollow-fiber modules (hydrophobic and porous, polypropylene, pore size 0.04 μm) acting as O_2 separator as well as CO_2 supplier are integrated in the liquid bypass line. The O_2 module is connected to a vacuum pump that is running continuously with 200 ml/min while the CO_2 valve is open (Fig. 20).

In 1989 Mori et al. also proposed using a hollow-fiber membrane module for gas exchange under microgravity conditions [108] (Fig. 21).

The gas exchange module was composed of porous polypropylene membranes and was run with mixtures of CO_2, O_2, and N_2 on the gas side. Sensors for dissolved CO_2 and O_2 were integrated on the liquid side. They used volumetric concentrations of 10 % CO_2 and 10 % O_2 in the gas stream for optimal growth and gas exchange of the system.

Thus far, no results from parabolic flight or satellite tests with membrane-aerated photobioreactors have been published yet.

3.2.5 Consequences for Potential Bubble-Free Membrane Aeration of Microalgae-Photobioreactors for Remote/Spaceflight Application

In summary, only few reported approaches rely on membrane aeration that is purely diffusion-based; the majority relies on pressure difference.

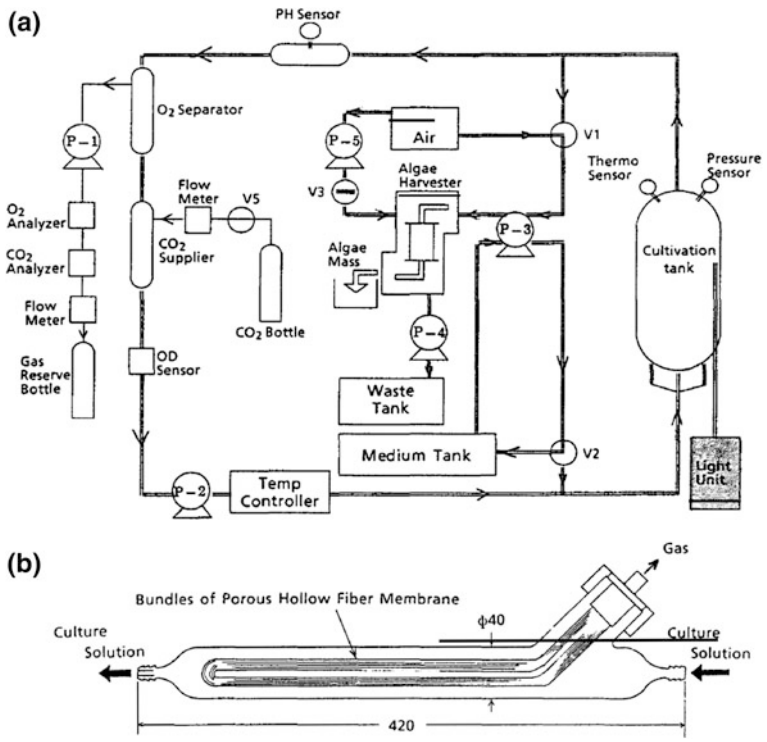

Fig. 20 **a** Scheme of photobioreactor system for CELSSs proposed by Oguchi et al. [107]. **b** Gas separator used in this approach with an effective area of 0.3 m^2

The approach of Cogne et al. [103] was diffusion-based, but bubbles were formed in an early stage of cultivation indicating that some additional factors have to be reflected for realization of complete bubble-free cultivations of membrane-aerated photobioreactor concepts.

The main factor is the selectivity of the membrane which should be higher for O_2 than for CO_2 originating from the stoichiometry of photosynthesis and the different solubilities of these gases in the liquid. Together with a suitable specific transfer area a and gas concentrations these factors may contribute to a bubble-free membrane aeration of microalgae reactors.

4 The ModuLES Reactor

A novel membrane-aerated photobioreactor concept was developed that is aimed for space application. In the proposed concept a reactor geometry based on a plate-type reactor is used, and realized as a mini-raceway reactor that can be

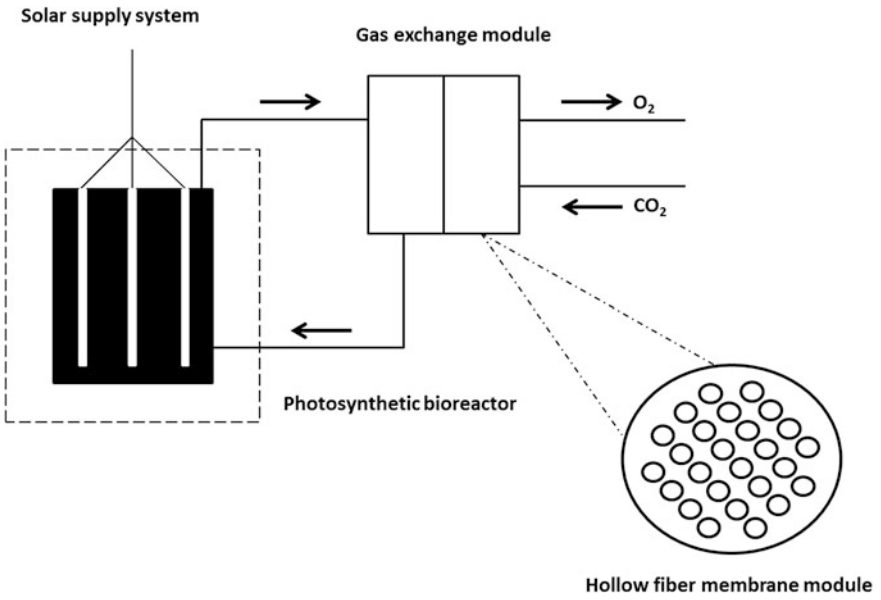

Fig. 21 Approach for a space photobioreactor by Mori et al. Scheme of proposed system with hollow-fiber membrane module for gas exchange. Modified after [108]

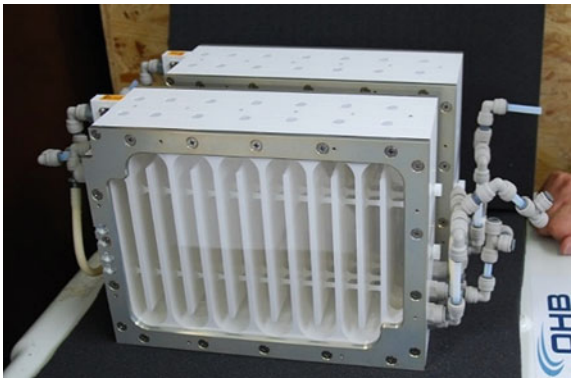

Fig. 22 Reactor geometry of proposed concept for space application

illuminated either from one side or from both sides (Fig. 22). In the first approach single-sided illumination with either warm-white LEDs or dichromatic red/blue LED lighting is used.

A continuous culture technique is chosen in order to maintain constant irradiance at constant cell density and nutrient levels that cannot be accomplished in batch or fed-batch cultures, where cell density changes continuously and increasing self-shading appears, leading to a changed irradiance per single cell.

As bubble-free aeration is a prerequisite under microgravity conditions, in a first approach flat sheet membranes fixed on both sides of the mini-raceway reactor (either horizontally or vertically mounted) are used to supply CO_2 to the cells as well as for removal of O_2 produced.

The system is composed of two gas compartments flushed with different gas mixtures and one liquid compartment (2 L volume) housing the algae. Gas- and liquid-containing compartments possess a meandering shape with the gas flow circulating in countercurrent to the liquid flow.

Mixing is realized by a peristaltic pump installed in the periphery. Fresh medium is fed from storage with the help of another pump into the reactor and algae suspension is pumped at the same rate to a biomass harvesting container that can be coupled to a filtration unit for solid/liquid separation.

Concerning membranes it is possible either to use the same membrane on both sides of the algae compartment or use different membranes adjacent to the two gas compartments. One of the gas compartments contains 5 % CO_2 (v/v) in air for the CO_2 supply to keep dissolved CO_2 in the liquid at a level of 1 %. The other compartment is used for O_2 removal and is flushed with a mixture of 1 % CO_2 in N_2 to create a high driving force for the O_2 transfer across the membrane. During first ground tests and two parabolic flight campaigns a dense flat sheet membrane made of polymethylpentene with 50-µm thickness was used.

For a first hardware test during a parabolic flight campaign illumination was achieved by warm-white LED illumination whereas in a second flight campaign red (emission peak at 680 nm) and blue LEDs (peak at 450 nm) were used for dichromatic illumination at a ratio of 90/10 red/blue photons.

This reactor system is equipped with optical sensors for CO_2 and O_2 partial pressures within the gas loops, as well as optical sensors for dissolved CO_2 and O_2 on the liquid side. Moreover, cell density (as optical density (OD) at 750 nm), pH, temperature, and pressure are measured to monitor the cultivation process. A fluorescence measurement with the help of a PAM sensor is also conducted to detect possible changes in photosynthetic activity upon changed gravity conditions.

A sampling unit is integrated in the periphery of the reactor consisting of 12 syringes pre-filled with RNA fixative. The volume on the liquid side is kept constant, meaning that the sample volume taken (5 mL) is directly balanced with fresh medium from the storage tank. By an automated suction mechanism a representative sample can be taken from the continuously flushed sampling line at a desired time.

Controlled parameters are pH by dosing either CO_2 saturated or unsaturated media and temperature by a feedback-controlled Peltier circuit. Dilution rate D of the continuous process was set constant for parabolic flights and was manually regulated during long-term ground testing.

Technical realization, measurement, and control of this reactor system were accomplished by OHB System AG, Germany.

Details of this concept and results from two parabolic flight campaigns were presented at the 65th International Astronautical Congress, Toronto, Canada [109].

5 Conclusions

Since the beginning of research on life support systems microalgae have played a major role as members of the first trophic level with unique traits suitable for those approaches. In order to maintain microalgae in space, photobioreactors fulfilling special requirements are needed. Illumination and aeration are major fields within photobioreactor design that are important for overall efficiency of such systems.

Mono- and dichromatic illumination is a promising alternative for conventional white illumination which has been used for plant cultivation for longer times.

For microalgae, in addition to physiological studies on photoreceptors and their function, until now only a few studies based on color lighting during cultivation have been published. However, these studies indicate high photosynthesis rates under mono- and dichromatic illumination in the blue and red range and our own first studies have resulted in higher PCE than white illumination.

Bubble-free aeration is a prerequisite for microgravity conditions, which can be realized by membranes either in the reactor itself or in the periphery. However, most reported approaches rely on membrane aeration based on pressure difference, risking bubble formation. In order to avoid that, membrane aeration has to be purely diffusion-based. Also additional factors such as solubility of gases in liquids and selectivity of the membrane material for oxygen and carbon dioxide have to be carefully reflected for realization of a complete bubble-free continuous cultivation inside membrane-aerated photobioreactors.

A novel concept of a membrane-aerated, energy-efficient photobioreactor for application in microgravity was introduced. Lighting was achieved by using dichromatic illumination with red and blue LEDs at a photon ratio of 90/10. This system was preliminarily tested during two parabolic flight campaigns. Sensors and actuators showed a stable performance without interference to cabin pressure or gravity. The microalgal culture was constantly supplied with light and carbon dioxide and showed the precalculated growth behavior. It is proven that the developed reactor concept works as a research tool for microalgae in life support systems in further space missions.

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