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Microalgae Culturing: From Laboratory to Large Scale Units

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Review of DSc. Thesis

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CONCEPTION OF THE THESIS

If we wish to develop biotechnological process based on microalgae cultivation, firstly we have to define a required product and then we can select strain, characterise its physiology, photosynthetic activity and suitable culturing conditions in order to estimate optimum regime not only for growth, but also for high product yield. After the laboratory screening, pilot trials can be carried out in various cultivation units under pre-screened growth conditions in order to confirm suitable/optimal culturing unit and regime. The climatic region also plays a substantial role. Finally, particular culturing unit, regime and region can be decided for large-scale mass production of certain strain (and product). This is a half of the story, the so-called 'up-stream processing', i.e. cultivation of microalgae. The other part of the story is 'down-stream processing', i.e. culture harvesting, cell disintegration, biomass fractionation and product finalisation (Figure 2).

This thesis deals only with the first half of the process – up-stream processing. For that I have selected 22 relevant publications which – in my opinion – contributed to the development of microalgae biotechnology in the Czech Republic as well as worldwide as concerns phototrophic cultivation. The articles concern several key issues of microalgae biotechnology starting in the mid-1990s when I have been entered this field. Focusing my research to microalgae biotechnology, I could employ all the knowledge gained from basic research of photosynthesis in which I was involved since my university studies. Moreover, I could also build on the extensive experience of two generations of microalgae biotechnologists in Třeboň.

In the following chapters I overview some key articles published with my colleagues in which I have mostly played the role of an idea-seeker, fund-riser, researcher, writer and/or corresponding author who communicated with editors. The chapters in the thesis refer to several related topics of microalgae research: (1) design, construction and functioning of cultivation units, (2) biological principles of cultivation, (3) use of photosynthesis monitoring techniques to optimise growth regimes in various culture systems, (4) characterisation of selected strains for production of biomass enriched with some bioactive compounds, and (5) possible future developments.

INTRODUCTION

Microalgae[1] represent a diverse group of microorganisms of tremendous ecological importance, the spread of which is enormous since they inhabit all major ecosystems - from cold, arctic regions, through moderate regions, to extremely alkaline or saline habitats, hot springs and arid soils. They are responsible for almost half of global primary production and form the basis of the food chain in aquatic environments. Furthermore, microalgae represent one of the most efficient converters of solar energy to biomass.

Microalgae have become an ideal platform for the large-scale production of biomass because they are fast-growing in aquaculture, highly-effective, solar-powered 'biofactories'. A dense, well-mixed **mass culture of microalgae[2]** (> 0.5 g biomass per litre) with sufficient nutrition and optimised growth conditions represents an artificial system which behaves quite differently from optically-thin natural phytoplankton populations. Selected species can be cultured as productive strains in various cultivation systems being prone to growth manipulation. In general, these systems are usually optimized to suit certain strains, purposes, or products.

Since its establishment in the 1950s, microalgae biotechnology has shown a range of applications: from the traditional biomass production for human and animal nutrition, technologies for waste-water treatment, products for cosmetics and pharmacy, crop protection in agriculture, and most recently to the possible production of 'third' generation biofuels grown on non-agricultural land using waste nutrients. The variety of high-value bioproducts found in microalgae originates from their wide physiological and functional diversity. Currently, microalgae have been in focus again, mostly as possible sources of nutrition additives, bioactive compounds for pharmaceutical use and next-generation biofuels.

The early cultivation studies of several research groups were summarized in the "bible" of microalgae biotechnology edited by John S. Burlew of the Carnegie Institution in Washington, DC (Burlew 1953). One of the pilot plants for cultivation of *Chlorella* was devised and tested at Arthur D. Little, Inc. in Cambridge, Massachusetts in collaboration with the Carnegie Institution in 1951.

[1] In applied phycology, the term '*microalgae'* is usually used in its broadest sense to mean both prokaryotic cyanobacteria and eukaryotic algae – unicellular or filamentous photosynthetic microorganisms

[2] Well-mixed microalgae culture of high biomass density (>0.5 g per liter) with sufficient nutrition and gas exchange

Pilot cultivation units for microalgae production were constructed in USA, Israel, Germany and Japan. By the mid-1950s it was proven that open outdoor cultivations of fast-growing strains (e.g. *Chlorella* or *Scenedesmus*) were feasible and it substantiated this concept for outdoor microalgae culturing. The overall goal of these early trials was to produce protein for potential human and animal consumption.

Outdoor units for cultivation of microalgae were also built at the Institute of Botany in Třeboň, South Bohemia at the end of 1950s by the research group headed by the plant physiologist Ivan Šetlík. The idea was to grow microalgae on a system of shallow sloped troughs, 2-5 cm deep arranged one below another, to form a cascade, over which microalgae cultures were circulated to secure sufficient mixing for light attenuation and gas exchange to cells (Šetlík et al. 1967, 1970' for a review see Masojídek and Prášil 2010, Masojídek et al. 2015, Grivalský et al. 2019). In 1962-1963, a unique outdoor pilot plant of 900 m^2 was constructed, probably one of the first large-scale units in Europe, at the Laboratory of Algal Production Technology on a new laboratory campus "Opatovický Mlýn" in Třeboň. This original, highly-productive system, known worldwide as the Třeboň's type unit, was constructed as a sloping platforms fitted with transverse baffles where the culture is circulated at a relatively thin-layer (<50 mm) and high flow speed (~0.2-0.3 m s^{-1}).

The philosophy behind the development of this novel system the so called thin-layer cascades (TLCs) was to construct shallow cultivation units in contrast to deep, poorly mixed ponds or raceways. In TLCs the principle of microalgae cultivation was to maintain turbulent flow of a relatively thin culture layer in order to achieve high biomass density and productivity.

CHAPTER 1 CULTURING SYSTEMS

Since the 1950s various cultivation systems and technologies have been developed to grow microalgae mass cultures, using both natural and artificial light. The choice of a suitable cultivation system and the adjustment of the cultivation regime must be worked out for each individual microalgae strain. Crucial variables are the optical depth, turbulence, light acclimation, nutrient availability and metabolite accumulation (Richmond 2004, Grobbelaar 2009).

Two basic approaches to mass production are used: one applies to cultivation in open reservoirs (with direct contact of the microalgae culture with the environment) while the

other involves closed or semi-closed vessels – photobioreactors [3] (PBRs) (for a recent review of cultivation systems, see e.g., Pulz et al. 2013, Zittelli et al. 2013, Acién et al. 2017). Open cultivation systems are natural or artificial ponds, raceways (shallow race-tracks mixed by paddle wheels), circular ponds mixed by rotating arms, or incline-surface cascades). On the other hand, a variety of PBRs (with either natural or artificial illumination) exist consisting of transparent tubes, columns or panels, positioned horizontally or vertically, arranged as serpentine loops, flexible coils, a series of panels or column gardens, in all of which the microalgae suspension is circulated (Malapascua 2018). Microalgae cultivation inevitably starts from strains kept on agar in collections followed by small-scale laboratory cultivation up to inoculation of large-scale plants (Fig. 1) (Barsanti and Gualtieri 2006).

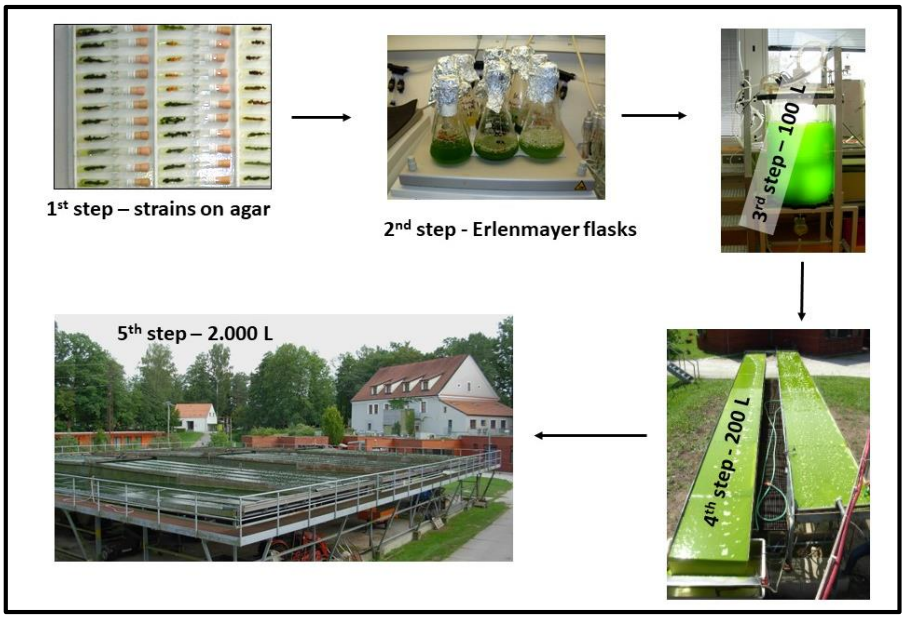

Fig. 1 *The scale-up of microalgae production in stepwise mode: starting with agar and flask culture using a dilution ratio of approximately 1:10 up to pilot and production units*

In every cultivation system, several basic features must be considered: illumination, circulation, and gas exchange (supply of CO_2 and O_2 degassing) as well as a sufficient supply of carbon and nutrients (e.g. Richmond 2013). However, as mass cultures grow in dense suspensions, an efficient mixing is necessary to expose cells to light evenly and to allow for an efficient gas exchange (CO_2 supply/O_2 removal), temperature control and nutrition availability. Generally, biomass production from open units is cheaper than from a culture in closed PBRs, but the use of the open pond is limited to a relatively small

[3] The term photobioreactor is used for closed or semi-closed systems with no direct contact of microalgae culture with the environment illuminated by natural or artificial illumination

number of microalgae species due to a possibility of cross-contamination. From a commercial point of view, the price of the final product is often crucial for consideration.

Open Systems

Numerous variations of open cultivation systems – natural or artificial ponds, tanks, or raceways, or cascades are used for microalgae culturing according to local climate conditions, and materials required (concrete, PVC, fiberglass). In open ponds the culture depth may vary between 10 and 30 cm. The simplest open system is a walled pond, lined with plastic foil and bubbled with air. Microalgae are usually grown at a starting biomass concentration ranging between 0.5 and 1 g L^{-1}. Productivity in these ponds is very low (~1 g dry weight m^{-2} day^{-1}) due to the lack of mixing and low ratio of illuminated surface to total volume (about 10). To improve productivity, tanks, raceways and ponds are mixed by impellers, rotating arms, paddle wheels, or by a stream of CO_2-enriched air supplied into the culture (Grobbelaar 2010).

Due to the limited control of cultivation conditions, open systems are suitable for robust microalgae strains that grow rapidly, or under selective conditions. Open outdoor systems are used for example for *Spirulina* production in Thailand, California, Hawaii, India, South Africa and China. Compared to more sophisticated PBRs, open systems cost relatively less to build and are easy to clean and operate, being more durable with a larger production capacity, but they have intrinsic disadvantages, such as: a susceptibility to microbial contamination, difficulty in managing a suitable culture temperature, massive water loss due to evaporation and a low cell concentration and biomass productivity (Masojídek and Torzillo 2014).

As mentioned in the previous chapter, a novel type of open cultivation unit, thin-layer cascade (TLC) was developed and constructed in Třeboň as a system of sloping smooth planes arranged in a cascade where a shallow culture (<10 mm) was circulated at a high flow speed (~0.5 m s^{-1}) (Doucha and Lívanský 1995, Masojídek et al. 2011a, Masojídek et al. 2015). TLCs were designed as an alternative to deep open ponds or raceways with a culture depth of 10 to 30 cm where diluted cultures of microalgae (0.5-1 g dry wt L^{-1}) are grown under limited mixing and gas exchange. The main advantage of the TLCs was the growth of well-mixed, dense (15-35 g dry wt L^{-1}) culture in a relatively thin-layer.

Since the 1990s, thin-layer systems for microalgae culturing based on the Třeboň's concept have also been constructed in Bulgaria, Greece, Italy, Switzerland, Spain and Portugal (for a review see Masojídek et al. 2015). Concerning the limitations of TLCs as for all open

systems, one has to consider contaminations by other microalgae that limit cultivation trials to robust, fast-growing strains, or those cultured in selective environments both limitations of which are valid for all open cultivation systems. It has become evident that a high growth rate and productivity can be achieved in cultivation systems with low culture thickness due to much higher average cell irradiance compared to deep ponds productivity due to high surface-to-volume ratio: vertical or inclined flat-panel PBRs or sloping platforms, cascades or cascade raceways (for a review see Becker 1994, Masojídek et al. 2015).

Closed Systems

Closed systems, photobioreactors (PBRs) are more flexible (than open systems) as – due to better control of cultivation conditions – can be optimized according to the physiological demands of microalgae species. The most widely adopted outdoor PBRs are tubular, column and flat plate systems. An overview of closed or semi-closed systems and their use for mass cultivation of microalgae outdoors has been widely published (for a review see e.g., Pulz et al. 2013, Zittelli et al. 2013, Acién et al. 2017). They consist of transparent tubes, columns or panels, positioned horizontally or vertically, arranged as serpentine loops, fences, flexible coils, or as a series of panels or columns that act as a photo-stage in which the microalgae culture is continuously circulated or mixed. PBRs can be placed in greenhouses to protect against adverse environmental conditions. Commonly, transparent materials, glass or plastics (Plexiglas, polyethylene, polycarbonate, etc.) are used for the construction (Tredici 2004). In PBRs, the culture suspension is circulated by various pumps, or by air-lifting (injecting a stream of compressed air in an upward-pointing tube). Cooling is maintained by submerging the units in a pool of water, using heat exchangers, or by spraying water onto the PBR surface.

Compared to open systems, PBRs have some advantages: reproducible cultivation conditions, reduced risk of contamination, low CO_2 losses and smaller area requirements. On the downside, closed systems are more difficult to clean, the system must be cooled and degassed effectively since any excessive oxygen produced by the photosynthesizing cultures can reduce growth. Furthermore, the cost of construction is about one order of magnitude higher than that of open ponds (Masojídek and Torzillo 2014).

Cultivation trials in laboratory and pilot units

Since the mid-1990s our team has studied the photosynthetic performance and growth regimes of microalgae mass cultures in various types of laboratory and outdoor cultivation

units, both open and closed (for a review see Masojídek and Prášil 2010). The collaboration with an Italian team at the Centro di Studio dei Microorganismi Autotrofi del CNR in Florence introduced me to closed cultivation systems, tubular PBRs (Torzillo et al. 1996). In the 1990-2010s various experimental and pilot-scale solar PBRs (up to 150 litres) were designed, constructed and tested in our laboratories in Třeboň and Nové Hrady to grow microalgae cultures for various purposes (Masojídek et al. 2003, 2009, Masojídek et al. 2015, Sergejevová et al. 2015)

Several types of PBRs – tubular, panel as well as column – with artificial illumination using various light sources (bulbs, fluorescent tubes, LEDs, solar concentrators, etc.) have been tested to grow various microalgae strains under well-controlled physicochemical conditions (Sergejevová et al. 2015). These PBRs were mostly equipped with precise control of process variables (temperature, irradiance, pH, gas composition, mixing) and accompanied by measurements of photosynthetic performance based on Chl fluorescence. The PBRs with internal illumination make it possible to use light (and energy) efficiently, as the light sources are placed inside the microalgae culture. Recently, 10 and 100-litre PBRs were tested for pilot trials where tubular light sources based on white, high-intensity LEDs diodes were submerged in microalgae cultures (Sergejevová et al. 2015, Malapascua at al. 2019).

In 2000-2010s, close collaboration with the University of South Bohemia resulted in the construction of a unique type of closed tubular PBR which was tested at the biotechnology hall of the Institute of Physical Biology in Nové Hrady. It employed solar concentrators (linear Fresnel lenses) as light sources and the tubular cultivation loop was placed in the focus of Fresnel lenses mounted in a roof of climate-controlled greenhouse (Masojídek et al. 2003, 2009). The dual-purpose system was designed for microalgae biomass production in temperate climate zone under well-controlled cultivation conditions and surplus solar energy was utilised for heating service water. The system was used to study the strategy of microalgal acclimation to supra-high solar irradiance, with values as much as 3-times higher than the ambient value, making the approach unique. In model cultivations, the cyanobacterium *Arthrospira (Spirulina)* was grown, indicating that this organism is tolerant to photoinhibition under sufficient turbulence and biomass density.

At the same period trials were also carried out to tests and compare various types of open systems – ponds, raceways and cascades as well as tubular or flat-panel PBRs for cultivation of microalgae strains: *Nannochloropsis* (Kromkamp et al. 2009, Sukenik et al.

2009), *Phaeodactylum* (Torzillo et al. 2012), *Spirulina* (Silva Benavides et al. 2017), and *Chlorella* (Masojídek et al. 2011a, Jerez et al. 2016a, 2016b, Malapascua et al. 2019).

The advanced TLC unit of 90 m^2 was designed and constructed in 2013 as a modular system in the Institute of Microbiology in Třeboň (Masojídek et al. 2015; patent pending PV 2013-803). Compared to previous units made of fragile glass plates, the cultivation surface was made of stainless steel which is easily cleaned and maintained, avoiding any problems with winter damage and corrosion. The unit was designed to minimise the dark volume of microalgae suspension as the S/V ratio can be operated in the range between 60-180 m^{-1}. The use of this TLC is intended for pilot microalgae production as food and feed additives, especially enriched in certain bioactive compounds (e.g. carotenoids, polyunsaturated fatty acids, etc.), or chemical elements (Se, Cr, Fe, Zn).

Lately, two pilot units, raceway pond (RWPs) and thin-layer cascade (TLCs) have been constructed at Centre Algatech. The cultivation units were made of polypropylene and mounted in greenhouses to protect cultures against cross-contamination and adverse weather conditions. They differ only in circulation devices – paddle wheel or centrifugal pump. A thin-layer raceway pond (TL-RWP) represents a hybrid technology as the culture layer is only 15-20 mm thick with the paddle wheel submerged in a sump (left) while TLC consists of two platforms connected in series where the culture layer of 6-15 mm can be adjusted by declination and pumping speed.

At the University of Almería the R&D cultivation units, three RWPs and two TLCs were constructed to test cultivation of selected microalgae in wastewater (Barceló-Villalobos et al. 2019). The RWPs are 13.5 cm deep with the surface of 80 m^2 and a total volume of suspension is 12 m^3. Two TLCs were also built – these being with an area of 60 m^2 and a volume 2.4 m^3 and a larger one with a surface of 140 m^2 and a total volume 3.4 m^3. Both units are operated at about 20 mm culture layer.

Later, large-scale DEMO1 plants of 1 ha including biomass processing technology was also finished in 2019 and is being tested. At present in 2020, a DEMO 5 plant (area of 5 ha) is being constructed.

CHAPTER 2 CULTIVATION VARIABLES

Microalgae belong among the fastest-growing photosynthetic organisms since their cell doubling time can be as little as a few hours. Biomass production by microalgae is based

on the simple scheme of oxygenic photosynthesis shown below, which determines all the necessary requirements of this biological process:

$$\mathbf{CO_2 + H_2O + nutrients + light\ energy \rightarrow biomass + O_2}$$

In biotechnology, generally, the production of microalgae biomass requires well-defined conditions. The necessary cultivation requirements for the growth of microalgae mass cultures are light, and a suitable temperature, pH, CO_2 and nutrient availability in the growth medium (Fig. 2). Since microalgae mass cultures grow in dense suspensions, sufficient mixing is essential in order to expose cells to light and to allow for an efficient mass transfer (Richmond 2004).

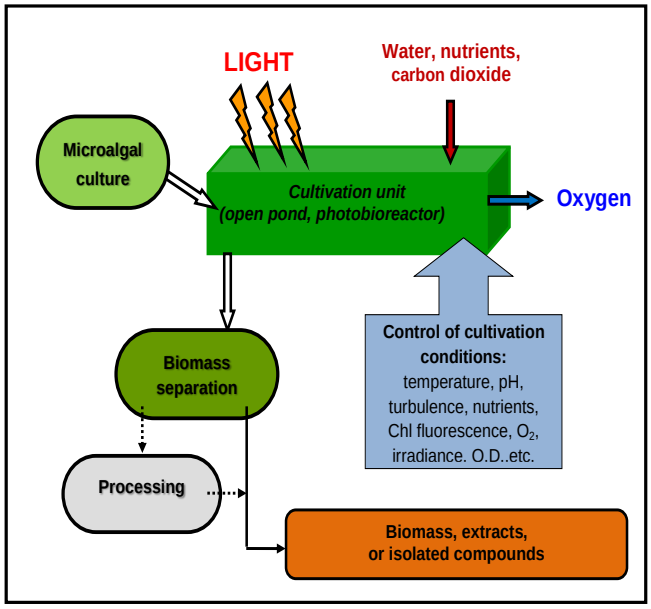

Fig. 2 *A schematic diagram of microalgae cultivation (and biomass processing). The culture is grown in a cultivation unit using an aqueous mineral medium under illumination. However, a minimum biomass concentration corresponding to about 10 g per square meter (~200 mg chlorophyll m^{-2}) is recommended to avoid photo-stress. The cultivation conditions (light, temperature, pH, mixing, nutrient and CO_2 supply, cross-contamination and biological activity) should be controlled. The biomass is separated from the medium and then processed (disintegrated and dried) for use as e.g., a food or feed supplement, or as a source of bioactive substances for pharmacology, cosmetics, biofuels, etc. (from Masojídek and Torzillo 2014).*

Light

In microalgae cultures, light represents major growth-limiting factor in well-maintained mass microalgae cultures (where temperature and nutrients are not so limiting). It is obvious that the averaged amount of photon energy received by each cell is a combination of several variables: photon flux density, cell density, length of optical path (thickness of culture layer) and the rate of mixing (Masojídek et al. 2011a, Grobbelaar 2012, Richmond 2013, Torzillo and Vonshak 2013). Hence, all the factors influencing the microalgae growth should be considered when designing cultivation systems.

Another important aspect is that microalgae cells may utilize strong light only if it is delivered intermittently, in ‘pulses’. A crucial point is the fast turbulence of microalgae culture to induce fast light/dark (L/D) cycling of cells (Grobbelaar 1989, Richmond 2004). In mass microalgae culture it is possible to achieve high photosynthetic yields in full sunlight

when the turbulence and density of cells are adjusted to produce the proper pattern of light intermittence, i.e. the L/D cycles sufficiently short in the order of tens to hundreds of microseconds (10-100 Hz), close to the time scale of the rate-limiting dark reactions of photosynthesis (Janssen et al. 2000, Masojídek et al. 2011a, Zarmi et al. 2013, Masojídek et al. 2015).

Temperature

After light, temperature is the most important variable to control the microalgae culture. Some microalgae strains tolerate a broad range of temperatures between 15 and 40 °C (e.g. *Chlorella*), while freshwater *Eustigmatophyceae* strains (e.g. *Nannochloropsis*) usually require a much narrower range (20°C to 25 °C). However, for the majority of freshwater microalgae the optimum temperature ranges between 25 and 35 °C (Masojídek and Torzillo 2014).

Carbon dioxide

Carbon dioxide serves as the main carbon source and it is usually added on demand; it is the limiting factor in extensive microalgae mass cultures exposed to high irradiances as the ambient CO_2 concentration is very low (0.04%; v/v). Since microalgae are generally found in aquatic habitats, inorganic carbon exists as CO_2, HCO_3^- and CO_3^{2-}. It is common practice that pure CO_2 is added to large-scale cultures to improve growth and yields. However, care must be taken since CO_2 addition can result in the acidification of the medium and must therefore be implemented in a controlled manner (for example, by using a pH-stat).

Dissolved oxygen

The crucial product of photosynthesis is oxygen. Though the oxygen evolution is sometimes overlooked in large-scale cultures, high concentrations of dissolved oxygen in outdoor cultures which can occur especially under high irradiance intensity at midday in outdoor cultures can result in photoinhibition and photorespiration and a reduction in photosynthetic activity and growth. Dissolved oxygen concentrations equivalent to 3-4 times saturation with respect to air are often inhibiting for many microalgae. The maintenance of O_2 levels below this concentration requires degassing, or culture mixing induced by high flow rates. Oxygen over-saturation or anoxia can be alleviated, in part, by good mixing, or aeration of the culture (Malapascua et al. 2014).

Nutrients

Nutrient status can be followed by monitoring the concentration of nitrogen (and other macroelements) and use this as a measure for adding proportional amounts of other nutrients.

In some cases, certain metabolite accumulation might inhibit the growth of microalgae.

CHAPTER 3 CULTURE MONITORING AND MAINTENANCE

Successful cultivation requires continuous monitoring of a culture's physicochemical variables, namely its pH, temperature, dissolved oxygen concentration, and nutrient status. Culture growth can be estimated as changes in optical density, dry weight, cell number, or pigment content.

One of the classical methods for culture control is visual or microscopic examination in order to detect physiological and morphological changes and contamination by other microorganisms. The appearance of 'contaminants' (other microalgae strains as well as protozoa, bacteria, or fungi) might indicate that the cultivated culture has come under unfavourable conditions. Such contaminants often represent one of the major limitations to large-scale production in microalgae cultures, or even culture failure.

Until the late 1980s mass microalgae cultivations were often carried out semi-empirically which has initiated disputes between biotechnologists, physiologists and photosynthetists discussing classical approach vs. photochemical activity monitoring. One, direct approach is to measure photosynthesis *on-line/in-situ* during the diel cycle to monitor the actual situation in a culture. The other possibility is to measure *off-line* using microalgae samples taken from a cultivation unit at selected times.

The Laboratory of Photosynthesis in Třeboň played a substantial role in this development during pilot experiments carried out in cascades and closed PBRs in the Czech Republic, Italy and Israel. Based on previous experiments with crops and microalgae (Masojídek et al. 1991, Corlett et al. 1992, Knoppová et al. 1993), we have worked out procedures to monitor photosynthetic activity of microalgae mass cultures *off-line* and *on-line/in-situ* using chlorophyll (Chl) fluorescence (Torzillo et al. 1996, 1998, Koblížek et al. 1999). Starting in the mid-1990s, Chl fluorescence techniques have become one of the most common and useful approaches used for monitoring of microalgae mass cultures due to its non-invasiveness, ease of use, sensitivity and a wide availability of commercial fluorimeters (Torzillo et al. 1996, 1998, Masojídek et al. 1999, Masojídek et al. 2000,

Vonshak et al. 2001, Masojídek et al. 2001, Baker 2008, Masojídek et al. 2011b, Malapascua et al. 2014). At present, it is relatively easy to gather fluorescence data, but care must always be taken to select and calculate sensible variables. This is particularly true when dealing with microalgae cultures outdoors, where growth limitations, such as light, temperature, and other unfavourable factors can occur side by side. As long as this is kept in mind, Chl fluorescence represents a powerful technique which allows rapid monitoring of physiological status. Though Chl fluorescence represents a rapid technique for microalgae status detection, it must always be accompanied by other physiological measurements.

Chl fluorescence monitoring have often been combined with oxygen production measurements to optimise growth regimes as well as to examine effects of adverse environmental conditions – high irradiance, temperature extremes, high dissolved oxygen concentration and their synergism on microalgae growth (Torzillo et al. 1998, Masojídek et al. 2011a, White et al. 2011, Figueroa et al. 2013, Malapascua et al. 2014). Compared with measurements of O_2 production and/or CO_2 uptake, Chl fluorescence generates analogous information, but it is considerably faster, more sensitive and can provide data on energy distribution between the photochemical and non-photochemical (heat dissipation) processes (for a review see Baker 2008, Masojídek et al. 2011b, Malapascua et al. 2014). Chl fluorescence can be used as a good proxy for photosynthetic performance under certain conditions, oxygen production measurement is a complementary, well-established technique that can distinguish photosynthetic and respiratory rates (Walker 1987, Malapascua et al. 2014).

Presently, two basic Chl fluorescence techniques are used for monitoring photosynthetic efficiency in microalgae mass cultures: rapid fluorescence induction kinetics and the pulse-amplitude saturation (PAM) method (for recent reviews see Maxwell & Johnson 2000, Strasser et al. 2004, Schreiber 2004, Masojídek et al. 2009). While the rapid fluorescence induction kinetics provide us the information on the redox status of the photosynthetic electron transport chain, the PAM-technique gives information on the balance between photosynthetic electron transport and the Calvin-Benson cycle.

Chl fluorescence measurements in our experiments showed that changes of some fluorescence variables can be well correlated with changes of cultivation conditions, physiological status and growth of a given microalgae and the suitability of a selected cultivation system (Torzillo et al. 1996, 1998, Masojídek et al. 2000, 2003, 2009, 2011a,

Malapascua et al. 2014, Ranglová et al. 2019). Several variables can be estimated using the PAM technique (Table 1). The maximal quantum yield (F_v/F_m) is considered as a measure of the maximal photochemical PSII efficiency, an estimate of the physiological state of the culture, the actual quantum yield ($\Delta F'/F_m'$) is an indicator of concrete acclimation of photosystem II (PSII) as it depends on the redox state of the reaction centres and the electron transport rate through PSII, used as a proxy of the photosynthetic capacity and productivity (Figueroa et al. 2013, Kromkamp et al. 2008). The relative electron transport rate rETR (the product of multiplication $\Delta F'/F_M'$ by the photosynthetically active radiation E_{PAR} in the culture) proved to be a simple and reliable variable to estimate the photosynthetic performance of outdoor microalgae cultures and can be well correlated with analogous changes in the daily productivities under different conditions (Torzillo et al. 1998, Masojídek et al. 2000, Masojídek et al. 2010). The so-called non-photochemical quenching NPQ (= $[F_M-F_M']/F_M'$) is, in principle, inversely related to photochemistry ($\Delta F'/F_m'$). It indicates an increased heat dissipation of absorbed energy and is considered a safety valve protecting PSII reaction centres from damage by excess irradiance (Bilger and Björkman 1990).

In practise, we found that a midday-depression of PSII photochemical yields between 20-30% as compared with morning values was essential for well-performing microalgae cultures (Masojídek et al. 2003, Masojídek et al. 2011a, Malapascua et al. 2014). A lower or higher depression of photochemical yields indicated low-light acclimated or photo-stressed cultures, respectively. These results are important from a biotechnological point of view when the growth of outdoor microalgae mass cultures is optimised under varying climatic conditions.

Simple and fast procedures (manuals) for microalgae biotechnologists have been worked out based on Chl fluorescence measurements that can be used to monitor changes of photosynthetic activity and physiological status of microalgae cultures in order to optimise growth (Torzillo et al. 1996, Masojídek et al. 2011b, Malapascua et al. 2014). Recently, we have worked out a rapid, one-day test based on photosynthesis measurements to estimate suitable growth regime for microalgae strains (Ranglová et al. 2019).

In practise, we found that a midday-depression of PSII photochemical yields was between 20-30% – as compared with maximal morning values – is essential for well-performing microalgae cultures (Masojídek et al. 2003, Masojídek et al. 2011a, Malapascua et al. 2014). A lower or higher decrease of photochemical yields indicated low-light acclimated

or photoinhibited cultures, respectively. The night temperatures below 20 °C minimize the respiration losses of biomass to less than 10%. These results are important from a biotechnological point of view when the growth of outdoor microalgae mass cultures is optimised under varying climatic conditions.

***Table 1**. Important variables calculated from Chl fluorescence measurements. F_0, F_V, F_M – minimum, variable and maximum fluorescence in dark-adapted state; F', F_V', F_M' – steady-state, variable and maximum fluorescence in light-adapted state; E – light intensity*

Variable	**Symbol**	**Formula**
Maximum photochemical yield of PSII	F_V/F_M	$F_V/F_M = (F_M - F_0)/ F_M$
Actual PSII photochemical yield	$Y_{II} = \Delta F'/ F_M'$	$Y_{II} = (F_M' - F')/ F_M'$
Relative electron transport rate through PSII (rate of photochemistry)	rETR	$rETR = Y_{II} \times E$
Stern-Volmer coefficient of non-photochemical quenching	NPQ	$NPQ = (F_M - F_M')/ F_M'$

CHAPTER 4 EXPLOITATION OF MICROALGAE – CASE STUDIES

Thousands of strains have been isolated from natural habitats and are kept in culture collections around the world (e.g. UTEX, Austin, USA; University of Tokyo, Japan; Culture Collection of Algae at the Göttingen University; Institute of Botany, Třeboň, Czech Republic; Hungarian Algal Culture Collection, Szechényi István University in Mosonmagyaróvár; Banco Español de Algas, Gran Canaria, etc.). Still, the search in collections and extensive bioprospecting – screening for new production strains with biological activities is carried out by many laboratories and companies.

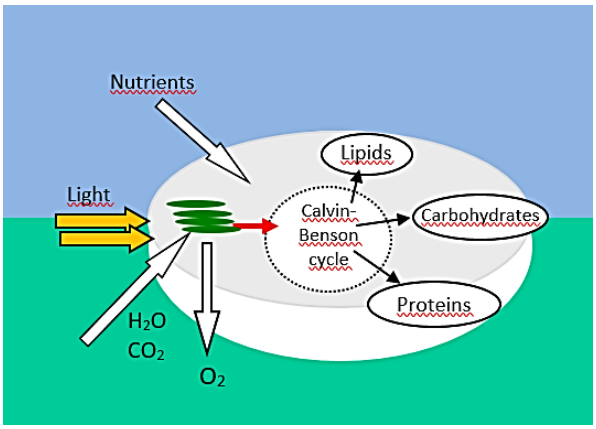

***Fig. 3** Microalgae represents solar-powered "cell factory" for production of three main high-energy compounds of microalgae biomass – proteins, lipids and carbohydrates – as well as other valuable and bioactive compounds – pigments, vitamins, antioxidants, biostimulants, biopesticides, compounds with cytotoxic, antibacterial or antibacterial activity, etc.*

Microalgae cultures are an ideal platform for large-scale production of biomass containing bioactive and valuable substances as they are fast-growing, well-controllable and highly flexible solar-powered 'biofactories' with low nutrient and operating requirements. Due to adaptation to various environments, microalgae produce a spectrum of substances such as proteins, carbohydrates, lipids including polyunsaturated fatty acids (PUFA), pigments, essential aminoacids, antioxidants, raw fibre, vitamins, minerals and others (Fig. 3).

A schematic diagram of microalgae production (up-stream processing) and down-stream processing (biomass separation, disintegration, fractionation, etc.) is shown in Figure 1. After cultivation, the harvesting of single cells or filaments is done by centrifugation, filtration, flotation or flocculation – the selection of the actual harvesting method depending on the dimensions of the organism. Some filamentous and large-cell strains can be separated by filtration using vibrating screens. Although many advances have been made in their cultivation, processing and application since the 1960s to date, only tens of strains have been cultivated in mass cultures (hundreds to thousands of litres on the area of hectares) (Table 2; Masojídek and Torzillo 2014).

In this chapter several case studies and recent projects are shown in which our team has played a significant role. These trials were aimed to potential application of microalgae in important fields of microalgae biotechnology – starting from culture optimisation, the biomass production for human and animal nutrition, microalgae/fish aquaculture, and recently also microalgae use in waste-water treatment, in agricultural applications and for the potential production of biofuels.

Production of Biomass Enriched in Bioactive Compounds

In the past six decades, there have been numerous attempts by researchers and companies to commercialize microalgae biomass production, primarily as a food supplement due to its potential to enhance nutritional value, as probiotics (life-enhancing agents) or so-called functional food (which, in addition to the basic nutritional function, support health). Microalgae biomass is also widely used as a feed supplement for farm and domestic animals (poultry, ruminants, pigs, ornamental birds) and aquaculture (mollusc, crustacean and fish larvae in hatcheries) to improve the quality of products, vitality, health resistance, and colour (e.g. Masojídek and Torzillo 2014, Masojídek et al. 2016).

The recent annual market is 30-40 thousand tons of microalgae biomass (dry weight) for food use is produced mostly in cultures of *Chlorella* and *Arthrospira*, and marketed as health food in the form of powder, tablets, capsules and liquid extracts. It is also added to

bread, snacks, yoghurts, drinks, and beverages as a nutritious supplement or colorant. Numerous trials were carried out in our and cooperating laboratories to contribute to physiological studies and optimisation of growth regimes for biomass production of *Chlorella* (Masojídek et al. 1999, Masojídek and Prášil 2010, Masojídek et al. 2011a, Masojídek et al. 2015, Jerez et al. 2016a, 2016b, Malapascua et al. 2019) and *Spirulina* (Torzillo et al. 1996, 1998, Masojídek et al. 2003, Silva Benavides et al. 2017).

Table 2. The current industrial-scale biotechnology applications of the most exploited microalgae (modified from Masojídek and Torzillo 2014)

Product and Application	**Status**	**Microalga**
Health food, food and feed supplements	Established	*Arthrospira* (*Spirulina*), *Chlorella*
β-Carotene	Established	*Dunaliella*
Astaxanthin	Established	*Haematococcus*
Live food and feed supplements in aquaculture	Established	*Nannochloropsis, Isochrysis, Chaetoceros, Pavlova, Tetraselmis, Phaeodactylum, Skeletonema, etc.*
PUFAs	Established	*Phaeodactylum, Nannochloropsis*
Xanthophylls (lutein, zeaxanthin)	Developing	*Scenedesmus, Chlorella*
Polysaccharides	Developing	*Porphyridium*
Oils, biofuels	Developing	*Botryococcus, Nannochloropsis, Phaeodactylum, mutants of Chlamydomonas & Synechocystis*
Biopharmaceuticals	Developing	*Nostoc, Cylindrospermum, Anabaena*
Biostimulants, biopesticides	Developing	*Chlorella, Scenedesmus, Nostoc*

Due to phototrophic growth, microalgae cells generate reactive oxygen radicals in the photosynthetic apparatus. Therefore, they have developed protective agents and mechanisms, e.g. carotenoids – β-carotene, lutein, astaxanthin, canthaxanthin, violaxanthin, zeaxanthin, neoxanthin and few others that are lipophilic scavengers of reactive radicals. Carotenoids are important antioxidants and also colorants in nutrition. Their physiological function remains to be clarified; however, it is generally believed that they function as photoprotectants *via* the so-called non-photochemical quenching in the adaptation of green microalgae to high irradiance outdoors (Masojídek et al. 2004). In our experiments we examined the interplay between changes in physiological and

photochemical variables and the accumulation of secondary carotenoid astaxanthin in *Haematococcus pluvialis* cultures grown outdoors in photobioreactors under nitrogen deficiency (Torzillo et al. 2003, Masojídek et al. 2009).

As concerns lipidic compounds in microalgae biomass, the most important are fatty acids, especially polyunsaturated fatty acids (PUFAs) which are essential as dietary supplements required for good health to prevent various diseases (e.g. cardiovascular, hypertension, arteriosclerosis, arthritis, etc.) and to boost the immune system. Only microalgae (and some plants) can synthesize PUFAs – linoleic, α-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acid. These have a very promising biotechnological market both for human food and animal feed. Currently, PUFAs, such as carotenoids, have a large market for food and feed applications.

In collaboration with our colleagues, several microalgae strains producing eicosapentaenoic acid (EPA) were studied as concerns their growth regimes in various cultivation units and climate regions: Eustigmatophytes *Monodus* (Vonshak et al. 2001) and *Nannochloropsis* (Kromkamp et al. 2009, Sukenik et al. 2009) in the Negev desert (Israel) and *Trachydiscus* in the Czech Republic (Malapascua et al. 2014). The diatom *Phaeodactylum* was grown in the open pond and tubular PBR in Sesto Fiorentino, Italy (Torzillo et al. 2012, Silva Benavides et al. 2013).

The production of some bioactive compounds in microalgae cultures can be induced by nutrient starvation, for example the increased content of pigments, carbohydrates or lipids (Masojídek et al. 2000, Torzillo et al. 2003, Jerez et al. 2016a). For example, the accumulation of high-energy storage compounds – starch and lipids in *Chlorella* cultures was enhanced by nitrogen and sulphur limitation when exposed to high irradiance (Jerez et al. 2016b). A two-stage strategy (a nutrient replete stage to produce biomass followed by gradual nutrient limitation) might be used to enrich *Chlorella* biomass in lipids, while under either nitrogen or sulphur starvation, both lipid and starch contents were increased. It was accompanied by a decrease of photosynthetic activity.

Aquaculture and Agriculture

Microalgae use is a part of the global aquaculture industry as a number of species are applied in aquaculture, for example: *Nannochloropsis* (Eustigmatophyceae), *Chlorella, Dunaliella* and *Haematococcus* (Chlorophyceae), *Isochrysis* and *Pavlova* (Prymnesiophyceae), *Tetraselmis* (Prasinophyceae) and *Phaeodactylum, Skeletoma* and

Navicula (Bacillariophyceae) (Table 1). The potential of microalgae in aquaculture is mostly to enhance the nutritional value (proteins, carotenoids, polysaccharides, fatty acids, etc.) which positively affects the health and physical condition of the produced organisms. There exist a number of different modes in which microalgae are utilized as feed for rotifers, crustaceans, molluscs and fish in aquaculture – directly as unprocessed cells (*live feed*) to build up the respective food chain, or in the form of plain or processed biomass to be added to the diet.

Apart from the nutritional purposes, the other important impact of microalgae is the improvement of the environment in aquaculture as concerns the production of oxygen, consumption of CO_2 and waste nutrients as well as antibacterial activity. For the larval and juvenile stages of freshwater and marine species, the introduction of phytoplankton to rearing ponds, the so-called '*green-water technique',* has produced much better results in terms of survival and growth than the more traditional clear-water techniques (Neori 2011). Microalgae can also serve as live feed for rotifers as an intermediate stage to feed fish larvae in aquaculture (Yanes-Roca et al. 2018, 2020). Some strains are also used as colorants for breeding of ornamental fish, crustaceans or birds (Gouveia el al. 2003). Among them, the oxygenated carotenoids, xanthophylls astaxanthin and canthaxanthin, are massively used in aquacultures (fish and crustaceans).

In several studies we have applied microalgae biomass (*Chlorella, Scenedesmus, Haematococcus*) enriched in various carotenoids (predominantly lutein, astaxanthin) to improve physical condition and colour of juvenile fish used as ornamentals - albinic form of catfish, *Silurus glanis* (Zaťková et al. 2011), sterlet, *Acipenser ruthenus* (Sergejevová and Masojídek 2012), angelfish, *Pterophyllum scalare* (Kouba et al. 2013) and common carp *Cyprinus carpio* (Stará et al. 2014). The colour of the fish skin is an important parameter in fish breeding, reflecting the momentary physiological status of the fish. Several fish species were compared (e.g. wels catfish *Silurus glanis*, ide *Leuciscus idus*, goldfish *Carassius auratus* and koi carp *Cyprinus carpio*) fed on several diets (varying in carotenoid supplements) as concerns image analysis methods on colour changes (Urban et al. 2013).

Recently, a new project Algae4Fish has started in the framework of INTERREG Programme Austria – Czech Republic which employs a complex approach to fish larvae rearing. The main aim of this project is to develop a three-stage technology – microalgae-rotifers-fish larvae, creating an integrated production system which can be adapted to the

feeding requirements of other fish larvae. In this eco-innovation technology wastes (municipal wastewater, effluent from biogas stations, etc.) are to be used as source of nutrients in microalgae cultivation. The cells will be used as *live feed* to rotifer population and this is applied as *live feed* to fish larvae. The recent trials showed that this procedure can guarantee a significant increase in fish survival rate and as well as improvement physical condition as a key factor for larval husbandry optimization. This pilot project is aimed to pikeperch (*Sander lucioperca*) larvae requirements. It is currently one of fish species targeted by several international initiatives looking for aquaculture diversification within the European Union, due to its market value and fast growth rate in recirculation systems.

Ecological Applications (wastewater treatment, agriculture & biofuels)

Environmental biotechnology has been a rapidly expanding area which is dedicated to the research and application of biological processes for the remediation of contaminated environments (water, soil, air). In the 1990s, a very early attempt was made to use a chimney flue gas produced in a lime kiln as a source of carbon dioxide for microalgae photobioreactors of 10,000 litres in Elbingerode (Germany) (Pulz et al 2013).

Microalgae are suitable for biofiltration as they grow fast and can be easily cultured under favourable climatic conditions. The key substances contributing to water eutrophy, for example, nitrate and phosphate, as well as important industrial and agricultural waste gases (e.g. ammonia and carbon dioxide) are the nutrients required for microalgae culturing. Microalgae cultures, in combination with other microorganisms (co-cultures) have been utilized to treat municipal, agricultural, food and industrial wastes, as well as aquaculture effluents for the reduction of environmental nutrient loads. The symbiotic activity of microalgae and bacteria is a common concept.

Recently our team has become involved in the EU H2020 project SABANA (2016-2021) coordinated by the University of Almería. The aim is to develop and demonstrate an integrated microalgae-based sustainable *biorefinery*[4] to produce a range of *high-value products* (biostimulants, biopesticides used in agriculture, e.g., Ördög et al. 2004, Mógor et al. 2018) and *low-value products* (biofertilizers, aquafeed) for agriculture and aquaculture use by recovering nutrients from wastewaters (municipal wastewater, sewage, pig manure) in continuous mode all year around. The experimental and demo plants

[4] The International Energy Agency defined biorefining as 'the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, materials) and bioenergy'

consisting of outdoor cultivation units are being scaled up to hectares, based on raceway ponds and thin-layer cascades built in southern Spain (Barceló-Villalobos et al. 2019). The task of our team has been optimisation of growth regimes of selected microalgae strains that produce biostimulants and biopesticides using photosynthesis monitoring. For this purpose, two pilot scale cultivation units – TLC and thin-layer RWP were build up and compared at Centre Algatech in Třeboň for culturing of several microalgae strains (single-celled, filamentous, aggregating, etc.).

Biofuels

The Kyoto Protocol implemented the objective of reducing the onset of global warming by lowering greenhouse gas concentrations in the atmosphere to 'a level that would prevent dangerous anthropogenic interference with the climate system'. One of the ways to achieve this goal is to find alternative carbon-neutral sources of energy and fuel that do not cause a further increase of CO_2 emissions into the atmosphere. One of the possibilities is the biotechnological production of the so-called 3rd generation biofuels (do not compete with food or feed crops) derived from microalgae biomass – biodiesel, ethanol, butanol, methane, etc. (Dragone et al. 2010, Saladini et al. 2016). Some species, such as *Nannochloropsis*, or *Chlorella* can accumulate as much as 60% of storage compounds (lipids or polysaccharides) under nutrient limitation.

There are several advantages offered by microalgae over higher plants as a source of biofuels: (1) the microalgae yield of storage compounds per unit of area can greatly exceed that of traditional crops; (2) microalgae can be cultivated in areas unsuitable for conventional agriculture; (3) they can be cultured in controlled aquaculture; (4) microalgae can grow in seawater or in brackish water which is unusable for normal agriculture; and (5) microalgae allow the recovery of phosphorus and nitrogen from wastewater, and the utilization of waste CO_2 (e.g. from flue gas).

Nevertheless, the cultivation of microalgae is still a more expensive process than that of conventional energetic crops (canola, sugar cane, sugar beet, oil palm, etc.). The minimum current cost of microalgae biomass production is about 5-10 US$ per kg, but it still exceeds by one to two orders of magnitude the rate required for economic biofuel production. The cost reduction can be based on suitable or novel strain selection, low-cost photobioreactors and lower energy inputs. However, finding additional value in biomass residues based on the biorefinery principle may greatly enhance the viability of biofuel technology. Nutrient supplies through wastewater treatment, combined with the use of residual biomass for

methane production, could potentially support the likelihood of producing biofuels that could compete with conventional fuels. Efforts to produce biofuels from microalgae are worth to study, but one must be aware that this technology may only become economically viable within 10 to 15 years (Walker 2009).

The other alternative is construction of randomly, or genetically modified strains which overproduce desired compounds. The research objective of the ALGENETICS project in the framework of INTERREG Austria-Czech Republic Research Programme was to employ the cyanobacteria strain *Synechocystis* PCC6803 as a 'cell factory'. The project was specifically focused on the construction of genetically engineered transformants to produce energy-rich compounds – starch and bioethanol – which are not naturally synthesized in this organism. This technology has been completely innovative in the partner countries. Glycogen (natural storage compound in *Synechocystis*), starch and ethanol can be used as a carbon-neutral source to produce transport fuels that do not compete with food crops. Glycogen and starch obtained in biomass can be subjected to acidic hydrolysis at increased temperatures. In this way, polysaccharides in biomass are cleaved to glucose that in turn can be fermented by yeast or bacteria to obtain bioethanol. The obtained bioethanol could be used as a renewable alternative to fossil fuels. This technology should contribute to higher future independence from fossil sources as well as to the reduction of CO_2 emissions to atmosphere.

CHAPTER 5 FUTURE DEVELOPMENTS & OPEN QUESTIONS

What trends should we consider in microalgae biotechnology?

The one, classical approach is the selection of strains with required features (produced compound) that should include: (1) deepening of our biological knowledge of existing microalgae species and their metabolites; (2) discovery of novel producers and potential products; (3) optimization of growth conditions; and (4) improvement of production processes and technology. The technology of advanced screening that involves 'metabolomic' databases, collections as well as field surveys (bioprospecting) in order to identify and even design novel compounds with various biological activities will support the search for new strains and products (Masojídek and Torzillo 2014, Masojídek et al. 2016). The stages needed for optimization rely on the basic biology of microalgae being coupled to bioengineering and will require intensive research.

At present genetic engineering of existing strains represents the other approach to microalgae biotechnology. When we consider the mutation of a microalgae strain, there are two ways to modify an organism in order to enhance its product generation or survival rate under harmful conditions. The first one is the untargeted optimization, where changes in the genome of the organism (non-GMO mutations) are intended to happen randomly, for example due to extensive illumination of UV light, gamma-radiation, or by chemical treatment. The other way is the genetic (targeted) manipulation of a microorganism which leads to a defined modification because the integrated gene and position where it will be integrated, is chosen beforehand.

The last two decades have brought significant advances in microalgae molecular biology. Although most progress has been achieved in a few model systems, we are still not in a position to standardize our molecular and metabolic manipulation of commercially-relevant species. A better understanding is still required of the mechanisms that control the regulation of gene expression in microalgae. Opportunities for novel pathway discoveries and genetic manipulation have to be considered in a biosynthetic perspective.

Modern biology trials frequently involve sampling on a large scale in the form of "omics" data (i.e., genomic, transcriptomic, lipidomic, proteomic, and metabolomic data sets). The arrival of omics technologies has enabled the monitoring of molecular components at both cellular level and genome scale.

High-quality, comprehensive, genome-scale models of photosynthetic microorganisms are powerful tools for studying poorly characterized biological processes. By combining metabolic models with models of gene expression, so called metabolic engineering models enable accurate predictions of growth, macromolecular composition, metabolic fluxes, enzyme efficiencies under nutrient limitation, and more (Thiele et al. 2012, Lerman et al. 2012).

Several original questions remain unanswered and many visions still need to be fulfilled. Some major questions of microalgae biotechnology are still “hot” and open for further research: (i) what is the maximum possible microalgae productivity, (ii) how to increase photochemical efficiency of microalgae culture, (iii) how can it be achieved on a large-scale and in an economic way, (iv) can microalgae cultures accumulate high content of carbon storage products and still grow rapidly? Let us hope that modern approaches will provide positive answers.

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List of author's key publications relevant to DSc. Thesis

(with % of his contribution to individual papers, with journal impact factor listed in 2018 and SCI excluding self-citation)

This thesis is based on the following articles with the reference to individual chapters. Each article is characterised by an abstract, educational and application output, percentage of my contribution and the title page.

I. Torzillo G, Bernardini P, Masojídek J (1998) On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of Spirulina platensis (Cyanobacteria), J Phycol 34: 504-510 **– IF 2.831 SCI 70x**

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V. Masojídek J, Papáček Š, Sergejevová M, Jirka, V, Červený J, Kunc J, Korečko J, Verbovikova O., Kopecký J., Štys D., Torzillo G. (2003) A closed solar photobioreactor for cultivation of microalgae under supra-high irradiance: basic design and performance. J Appl Phycol 15: 239-248 **– IF 2.635 SCI 55x**

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VIII. Sukenik A, Beardall J, Kromkamp JC, Kopecký J, Masojídek J, van Bergeijk S, Gabai S, Shaham E, Yamshon A (2009) Photosynthetic performance of outdoor *Nannochloropsis* mass cultures to extreme environmental conditions – assessment by chlorophyll fluorescence techniques. Aquat Microbial Ecol 56: 297-308 – **IF 2.788 SCI 55x**

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XIII. Silva Benavides AM, Torzillo G, Kopecký J, Masojídek J (2013) Productivity and biochemical composition of *Phaeodactylum tricornutum* (Bacillariophyceae) cultures grown outdoors in tubular photobioreactors and open ponds. Biomass Bioeng 54: 115-122 **– IF 3.537 SCI 50x**

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XVIII. Silva Benavides AM, Ranglová K, Malapascua JR, Masojídek J, Torzillo G (2017) Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in a thin-layer cascade and an open pond. Algal Res 28: 48-56 **– IF 3.723 SCI 3x**

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XXI. Grivalský T, Ranglová K, Câmara Manoel JA, Lakatos GE, Lhotský R, Masojídek J (2019) Development of thin-layer cascades for microalgae cultivation: milestones (review) Folia Microbiol 64: 603–614 **– IF 1.448 SCI 2x**

XXII. Babaei A, Ranglová K, Malapascua JR, Torzillo G, Shayegan J, Silva Benavides AM, Masojídek J (2020) Photobiochemical changes in *Chlorella* g120 culture during trophic conversion (metabolic pathway shift) from heterotrophic to phototrophic growth regime. J Appl Phycol (accepted) **– IF 2.635 SCI**

SUMMARY

Microalgae (both prokaryotic cyanobacteria and eukaryotic algae) represent a diverse group of microorganisms of tremendous ecological importance since they inhabit all major ecosystems - from cold, arctic areas, through moderate regions, to extremely alkaline or saline habitats, hot springs and arid soils**.** Thus, they have developed numerous protective systems against various stressors. Microalgae have been then extensively used as model organisms for basic photosynthetic research as demonstrated by pioneering works by Bessel Kok, Melvin Calvin, Robert Emerson and others. The variety of high-value bioproducts found in microalgae originates from their wide physiological and functional diversity. Therefore, microalgae produce various bioactive substances such as PUFAs, polysaccharides, lipids, antioxidants, or immunologically effective, virostatic, and cytostatic compounds.

In the 1950s microalgae cultures have become an ideal platform for the large-scale production of biomass as they are fast-growing in aquaculture, highly-effective, solar-powered 'biofactories'. Selected species can be cultured commercially as production strains in various cultivation systems being prone to growth manipulation. Since its establishment, microalgae biotechnology has shown a range of applications: from the traditional biomass production for human and animal nutrition, technologies for waste-water treatment, as a source of bioactive compounds for pharmacology and cosmetics, plant treatment in agriculture, and most recently to the possible production of 'third' generation biofuels grown on non-agricultural land using waste nutrients.

In order to develop biotechnological process based on microalgae production, firstly we have to define a required product and then we can select strain, characterise its physiology and suitable cultivation conditions in laboratory experiments to estimate suitable regime not only for growth, but also for high product yield. Here, strain characterisation and screening come to play. Then, pilot trials can be carried out in order to find optimal culturing system, regime and region. Finally, the particular culturing unit, growth regime and region can be decided for large-scale production of certain microalgae strain (and product). This is a half of the biotechnological story, the so-called 'up-stream processing', i.e. cultivation of microalgae. The other part is 'down-stream processing', i.e. culture harvesting, cell disintegration, biomass fractionation and product finalisation.

This thesis deals only with the first half of the process – up-stream processing. To prepare this thesis 22 relevant articles were selected that – in my opinion – contributed to the

research and development of microalgae biotechnology in the Czech Republic (formerly Czechoslovakia) as well as worldwide as concerns phototrophic cultivation.

In five chapters of this thesis I overview some key topics supported by the articles published with my colleagues in which I have mostly played the role of an idea-seeker, team builder, fund-riser, researcher, writer and finally corresponding author who communicated with editors. The chapters refer to several related issues: (i) design, construction and exploitation of culturing systems, (ii) cultivation variables, (iii) culture monitoring to optimise growth regimes, (iv) use of selected microalgae for various applications and (v) future developments and open questions.

In my research activities I have focused on the several issues that significantly influenced microalgae research community:

1. Introduction of the monitoring of microalgae mass cultures using photosynthesis measurements, namely the development of Chlorophyll fluorescence diagnostics
2. Screening, selection and characterisation of microalgae strains using photosynthesis monitoring
3. The correlation of photosynthetic activity and growth (and productivity) as to optimise cultivation regimes of selected microalgae strains in various culturing units
4. Design and construction of various cultivation units, namely thin-layer cascades.

Curriculum Vitae

PERSONAL INFORMATION

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Date of birth - 1952 | Nationality - Czech

Education/Academic degrees

1977 Faculty of Science, Charles University in Prague, MSc in biochemistry
1978-1979 Postgraduate training, Institute of Microbiology, Academy of Sciences, Třeboň, Czech Republic
1980-1984 CSc. degree (= PhD) in microbiology, Institute of Microbiology, Academy of Sciences, Praha, Czech Republic
1984-1985 International Training Course (Topics of Modern Biology), Biological Research Centre, Szeged, Hungary
2005 Habilitation (Associate Professor), cellular and molecular biology and genetics, Faculty of Biology, University of South Bohemia in České Budějovice, Czech Republic
2013 Full-professor, cellular and molecular biology and genetics, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Career/Employment (*positions, dates and employers)*

Institute of Microbiology, Academy of Sciences, Třeboň

2006-onwards Senior researcher, Laboratory of Algal Biotechnology, Institute of Microbiology
1984-1989 Researcher, Laboratory of Photosynthesis, Institute of Microbiology
1989-1990 Post-doctoral fellow (18 months), Biosphere Sciences, King's College London, Great Britain
1991-2005 Senior researcher, Laboratory of Photosynthesis, Institute of Microbiology
2006-2011 Head of the Laboratory of Algal Biotechnology, Institute of Microbiology

University of South Bohemia in České Budějovice

2000-2011 Lecturer and Head of the Division of Biotechnology, Institute of Physical Biology in Nové Hrady, (University of South Bohemia)
2002-2006 Deputy-director of the Institute of Physical Biology
2013-onwards Professor, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic

Expertise

Wide experience in microalgae biochemistry, biophysics, microbiology, biotechnology and plant physiology; with 40 years of experience in the cultivation of microalgae and application of biochemical and biophysical methods to study structural and functional

changes of the photosynthetic apparatus caused by various unfavourable environmental conditions; last 20 years focused to microalgae biotechnology; lectured at international meetings; supervised master and PhD students; managed or co-managed projects from national agencies (Czech Science Foundation, Grant Agency of the Academy of Sciences, Ministry of Education) and was responsible for international projects (NATO Linkage, EU FP5, EU Horizon 2020, INTERREG Czech Republic-Austria) and 5 bilateral cooperation projects (between the Czech Academy of Sciences and CNR Italy); in cooperation with commercial companies participated in the development of a photobioreactor based on solar concentrators (patented), large-scale outdoor equipment for microalgae cultivation and a photobioreactor with internal illumination

Research topics

Cultivation regimes of microalgae; design and construction of various photobioreactors; correlation of photosynthetic activity of microalgae cultures with growth and production of bioactive compounds (biostimulants and biopesticides); diagnostics of photosynthesis by chlorophyll fluorescence, supervising genetic manipulation of cyanobacteria for storage compound production, use of wastewater as nutrient source for microalgae culturing

Membership in Scientific Committees, Boards and Societies

Scientific committee of the International Society for Applied Phycology; Member of the Study Programme Board Biophysics, Faculty of Science, University of South Bohemia in České Budějovice; Member of the Scientific Advisory Board, Faculty of Science, University of J.E.Purkyně, Ústí n. Labem

Publications activity

More than 80 articles in peer-reviewed journals listed in WOS, h-index 30, SCI 2127 (1920 without self-citations), 36 articles published in the field of Biochemistry, Molecular and Cell Biology