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ORIGINAL PAPER

The development of microalgal biotechnology in the Czech Republic

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Abstract Microscopic algae and cyanobacteria are excellent sources of numerous compounds, from raw biomass rich in proteins, oils, and antioxidants to valuable secondary metabolites with potential medical use. In the former Czechoslovakia, microalgal biotechnology developed rapidly in the 1960s with the main aim of providing industrial, high-yield sources of algal biomass. Unique cultivation techniques that are still in use were successfully developed and tested. Gradually, the focus changed from bulk production to more sophisticated use of microalgae, including production of bioactive compounds. Along the way, better understanding of the physiology and cell biology of productive microalgal strains was achieved. Currently, microalgae are in the focus again, mostly as possible sources of bioactive compounds and next-generation biofuels for the 21st century.

Keywords Green algae · *Chlorella* · Biotechnology · Biomass productivity · Mass culture

Abbreviations
DW Dry weight
PSII Photosystem II

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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 extremely alkaline or saline habitats, to hot springs and arid soils. Prokaryotic cyanobacteria, in particular, represent the oldest group of photosynthetic organisms which started the formation of the Earth's oxygenic atmosphere more than 2.5 billion years ago. Microalgae are also responsible for almost half of global primary biomass production and form the basis of the food chain in aquatic environments. Furthermore, they represent one of the most efficient converters of solar energy to biomass.

Algal biotechnology has been closely related to the use of macroalgae (e.g., *Porphyra*), which dates back to the first millennium. In Asia, these species have been cultivated since the Middle Ages, and today this technology represents an industry with an annual turnover of billions of US dollars. For example, the first reports about agar production from *Gracilaria* date back to the 17th century in Japan, and brown algae were already processed for iodine and soda in the 18th century [62].

In nature, water blooms of microalgae can develop in eutrophic reservoirs where phytoplankton populations are occasionally mixed by wind or flux. Even under these optimal situations, biomass concentration is much below 1 g dry matter per liter. Dense, well-mixed mass cultures of microalgae (>0.5 g biomass per liter) represent artificial

[1] In applied phycology, "algae" refers to macroalgae well as microalgae. The term "microalgae" is usually used in its broadest sense to mean both prokaryotic cyanobacteria and eukaryotic algae—unicellular or filamentous photosynthetic microorganisms.

Table 1 Biotechnological applications of the most exploited microalgae

Microalga	Status	Product and application
Arthrospira (*Spirulina*)	Established	Health food, food and feed supplement
Chlorella	Established	Health food, food and feed supplement
Dunaliella	Established	β-Carotene
Haematococcus	Established	Astaxanthin
Nannochloropsis, *Isochrysis*, *Pavlova*, *Tetraselmis*, *Monodus*	Established	Source of lipids, fatty acids, and polyunsaturated fatty acids (PUFAs); aquaculture feed, biofuels
Microalgal biomass in general	Rising	Biofuels—biodiesel, bio-oil, bioethanol, etc

systems with sufficient nutrition and gas exchange, which are completely different from optically thin natural phytoplankton populations with low biomass density, often limited by nutrient and carbon supply.

Natural sources of microalgae readily available for humans are scarce. The cyanobacterium *Arthrospira* (*Spirulina*) was collected by the ancient Aztecs in Mexico as a food additive. In some regions (e.g., Chad in Africa or Myanmar in Asia) smaller amounts are still harvested from natural populations in alkaline subtropical lakes. At present, the bulk of microalgal biomass—about 8,000 metric tons used for biotechnological purposes annually—is produced extensively in cultivation units, where cultures are exposed to light with sufficient mixing and gas exchange (autotrophically), or alternatively grown on organic substrates as a source of carbon and energy (mixotrophic or heterotrophic cultivation).

Numerous cultivation systems have been designed for growth of microalgae since the 1940s. In general, they are optimized to suit a certain strain, purpose or product. Basically, two approaches to mass culturing of microalgae for the purpose of biomass production exist: the first applies to cultivation in large-area open reservoirs (ponds and raceways), while the second represents closed vessels, i.e., photobioreactors[2] or fermentors; for review, see [61, 78]. The first type—open cultivation systems—is represented by natural or artificial ponds, raceways (ponds akin to racetracks), and cascades (i.e., inclined-surface systems). The second type—photobioreactors (closed or semiclosed systems with natural or artificial illumination)—consist of glass or transparent plastic tubes, or panels, positioned horizontally or vertically, arranged as serpentine loops, flexible coils, manifold rows, or "fences," in which the microalgal suspension is continuously circulated [51].

Until recently, most large-scale commercial microalgal production systems employed open systems. However, several large-scale closed systems have been built recently and, for the first time, comparisons of their performance can be made. There are major operational differences between open and closed photobioreactors, and consequently the growth physiology of the microalgae is different between the two systems. Several factors governing growth can, within certain boundaries, be manipulated. Crucial variables are the optical depth, turbulence, light-acclimated state of the organism, nutrient availability, and metabolite accumulation. Each system needs to be optimized for its specific purpose; there is no universal, all-purpose photobioreactor [26, 63]. The choice of a suitable cultivation system and the adjustment of the cultivation regime must be worked out for each individual production strain.

Thousands of microalgal strains have been isolated from natural habitats and are kept in numerous culture collections around the world. However, only a few strains, mostly of aquatic origin, have been cultivated in large-scale production systems of hundreds to thousands of liters. A list of strains and their use is shown in Table 1.

Arthrospira (*Spirulina*) *platensis* is a planktonic filamentous cyanobacterium composed of individual cells (about 8 μm in diameter) that grows in subtropical alkaline lakes with a temperature optimum of about 35°C. In productive cultures, *Arthrospira* is cultivated in shallow mixed ponds or semiclosed tubular photobioreactors in inorganic salts with high concentration of bicarbonate, keeping pH above 9. Its biomass is widely used as a health food and feed supplement containing proteins, fatty acids, phycobiliproteins, carotenoids, polysaccharides, vitamins, and minerals.

The microalga *Chlorella* (green algae *Chlorophyta*) is a cosmopolitan genus with small globular cells (3–8 μm in diameter), including strains with a broad range of temperature tolerance between 15°C and 40°C. *Chlorella* grows autotrophically in an inorganic medium, as well as in mixotrophic and heterotrophic conditions (for example, with addition of acetic acid and glucose). At present, autotrophic production of *Chlorella* is carried out in open ponds, semiclosed tubular photobioreactors, or inclined cascades, since its fast growth prevents contamination by other microalgae. *Chlorella* is the most cultivated eukaryotic alga, since it is widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics

[2] In this article, the term "photobioreactor" is used for closed or semiclosed systems using natural or artificial illumination.

industries. It contains proteins, carotenoids, some immunostimulators, polysaccharides, vitamins, and minerals. The bulk of the microalgal biomass market is represented by *Chlorella* and *Arthrospira*, with annual production of 3,000 and 4,000 t, respectively.

Hypersaline strains of the genus *Dunaliella* have cells about 10 μm in diameter. This microalga produces β-carotene in large amounts, and it is a natural source of carotenoids for some shrimps. The high content of β-carotene makes *Dunaliella* attractive to biotechnologists for large-scale production in shallow, open ponds under high solar radiation.

Haematococcus pluvialis (*Chlorophyta*) is a freshwater, unicellular alga with a rather complex lifecycle. A two-stage process is employed for biomass production. Under stress conditions (nutrient deficiency, salinity, high temperatures in combination with high irradiance), it produces an orange–red pigment, astaxanthin, the important natural colorant for salmonid fish, shrimp, lobster, and crayfish and for the health food market.

Initial period of microalgal mass culture

Mass cultivation of microalgae was pioneered by the Carnegie Institution in the 1940s, and particular attention was paid to the unicellular green microalga *Chlorella pyrenoidosa* (*Chlorophyta*) because of the broad range of environmental conditions under which it can grow, and also because this organism was then extensively used as a model organism for basic photosynthetic research (see pioneering works by Bessel Kok, Melvin Calvin, Robert Emerson, and others). The main aim was to obtain high-protein biomass and to study its possible uses. During World War II, the experience gained in the cultivation of *Chlorella* was applied, namely in the search for an antibacterial substance that might be isolated from the culture of *Chlorella* [1]. In 1947–1948, the possibility of growing *Chlorella* on a large scale for food was seriously considered [10]. The primary cultivation studies of several research groups were summarized in the "bible" of early algal biotechnology, edited by John S. Burlew of the Carnegie Institution in Washington, DC [9]. The first attempts at large-scale microalgal cultivation and design of early pilot plants focused on closed systems in order to isolate cultures from the natural environment. This was a logical consequence of the requirement for controlled growth conditions and to prevent contamination of cultures by other microorganisms. One of the first productive pilot plants for mass cultivation of *Chlorella* was devised and tested at Arthur D. Little, Inc. in Cambridge, MA in collaboration with the Carnegie Institution in 1951 [3]. The cultivation unit (~4,000 l) was constructed from thin-walled plastic tube to form a flat channel of 7–8 cm depth with continuous circulation of the culture by a pump and supply of CO_2 to promote growth. The highest biomass concentration achieved was 1.5 g DW l^{-1}. In Israel, a small-scale pilot plant to produce biomass of *Chlorella* or *Scenedesmus* as green fodder for cattle was set up as a closed, mixed reservoir of 120 l mounted in a greenhouse and taking advantage of climatic conditions with year-round sunlight availability [23]. In Germany, Gummert and coworkers experimented with large-scale cultures of *Chlorella* grown in deep shallow concrete trenches (reservoirs) with plastic lining [27]. These experiments were aimed at evaluating the possibility of utilizing carbon dioxide from waste gases in the industrial district of the Ruhr. The cultures were bubbled with a mixture of air and 1% commercial or "waste" CO_2, which was sufficient to supply carbon and maintain growth as well as to mix the cultures turbulently.

In Japan, at the Tokugawa Institute of Biological Research, an early attempt was made to design a relatively well-controlled outdoor closed system: a tubular photobioreactor to study the growth kinetics of *Chlorella* [75]. The 40-l system consisted of a horizontal loop of glass tubes (diameter 3 cm, length 33 m) and was submerged in a water bath to prevent overheating. The culture was circulated by a pump and aerated by CO_2-enriched air, and the produced oxygen was released in a gas exchange tower. The *Chlorella* culture was grown in batch or semibatch regimes with daily harvest of biomass. In the same laboratory, a "partially enclosed atmosphere system" was constructed using shallow troughs covered with plastic sheets [55]. The advantage of this system was its relatively small depth (2–15 cm) with high linear velocity of suspension flow (6–45 cm s^{-1}), which guaranteed high turbulence of the culture. From the point of view of hydraulics, this system did not differ from the open systems, since the microalgal culture absorbed sunlight and exchanged gases with the atmosphere through its surface. In addition, the cultures were supplemented by overhead artificial illumination. Soon after, the technology of *Chlorella* cultivation for food and feed was worked out in more detail [74].

Microalgal biotechnology in Czechoslovakia

By the mid 1950s it was proven that open outdoor cultures were feasible and that they would probably not suffer from contamination more than any large-scale closed culture system if fast-growing strains (e.g., *Chlorella* or *Scenedesmus*) were cultivated. This is understandable if one realizes that it is practically impossible to keep large installations under sterile conditions. Once the feasibility of open cultures was confirmed, the concept of outdoor microalgal culture was substantiated. This is without doubt due

Fig. 1 Dr. Ivan Šetlík (1928–2009), pioneer and leading figure of algal biotechnology and photosynthesis research in the former Czechoslovakia

to the much simpler design and inexpensive construction of open-type units as compared with closed systems.

With this in mind, the first experimental outdoor units for cultivation of microalgae in the former Czechoslovakia were built at the Botanical Garden of the Slovak Academy of Sciences in the town of Košice at the end of the 1950s. The research group there was headed by plant physiologist Ivan Šetlík (Fig. 1) with interest in factors limiting plant productivity. Initially, the study of microalgae was only a minor research topic, but soon the potentially high productivity of photosynthetic microorganisms was realized and became the main focus of laboratory research [5, 6, 72]. In 1958, the short popular-science movie *Solar Laboratory* was filmed at the Košice Botanical Garden. The film producer, Miro Bernat, liked the idea that microalgal cultivation units would be "fields for the third millennium" and financed construction of the first larger microalgal pilot units (Fig. 2). These "movie" units demonstrated well the potential for outdoor photosynthetic production of microalgal biomass in Central European climate conditions. The first cultivation units were based on the principle of the descending flow surface, constructed as shallow troughs of reinforced polyester resin, arranged stepwise one below another to form a cascade of hydraulic jumps (Fig. 3, originally in [73]). A few months later, these units also caught the attention of Professor Ivan Málek, then director of the Institute of Biology in Prague, who visited Košice. He recognized the potential role of mass microalgal cultivation in the broader aim of managing continuous cultivation of industrial microorganisms and providing alternative sources of protein. With his assistance, the Algological Laboratory of the Institute of Biology was established in January 1960 in Třeboň, a small town in

Fig. 2 Pilot algal outdoor cultivation units constructed in 1958 at the Botanical Garden in Košice (Slovakia)

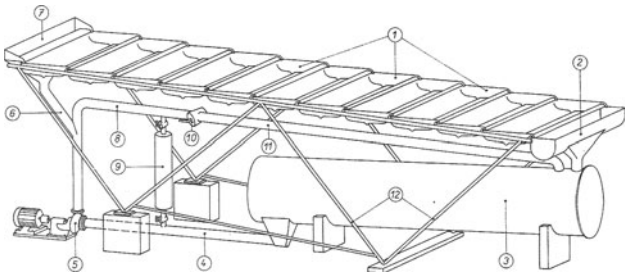

Fig. 3 Schematic diagram of cascade cultivation unit of 12 m^2. The cultivation surface was set up as shallow troughs made of reinforced polyester resin and arranged stepwise (1960)

South Bohemia. Here Ivan Šetlík and his collaborators enjoyed ample support and soon, working with enormous enthusiasm, they developed a number of indoor and outdoor test production units as well as cultivation procedures (Fig. 4). Both Ivan Málek and Ivan Šetlík (Fig. 5) played decisive roles in the formation and establishment of algal biotechnology in the former Czechoslovakia: throughout his whole career, Ivan Šetlík (1928–2009) was a visionary who set research directions not only in algal biotechnology but in photosynthesis in general, while Ivan Málek (1909–1994) in the 1960s provided the necessary backing—infrastructure, support, and international promotion. The new research group in Třeboň was formed on the principle of a complex processes approach to study microalgal productivity; research interests included mathematical modeling of turbulent flow, instrumentation development, biotechnology, physiology, cell biology, algal genetics, and ecophysiology. Soon after, the microalgal biotechnology research was moved to a new laboratory campus "Opatovický mlýn," reconstructed from the former watermill built in 1708 by Augustinian priests.

There, research was aimed at defining the scientific basis for commercial exploitation of microalgae cultivated on a

Fig. 4 Test outdoor cultivation units operating in Třeboň (early 1960s)

Fig. 5 Ivan Šetlík (*left*) and Ivan Málek (*right*) celebrating the first successful cultivation of microalgae in Třeboň (early 1960s)

large scale. In 1962–1963, unique outdoor pilot plants of 50 and 900 m^2 were constructed [73]. The original highly productive units were based on cascades of sloping planes, known worldwide as Třeboň-type cascade units (Fig. 6). The principle of microalgal cultivation was to maintain turbulent flow of a relatively thin layer using corrugated surfaces or a plain surface fitted with baffles. The pilot plant was constructed as dual-purpose units that were used during the winter as a glasshouse for hydroponic vegetable and flower cultivation and in summer for microalgal biomass production.

Compared with reservoirs (open ponds, raceways), with suspension depth of 20–30 cm where dilute cultures of microalgae (0.5-1 g DW l^{-1}) were grown under limited mixing and gas exchange, the main advantage of the cascade system constructed in Třeboň was the growth of a well-mixed thick (10–15 g DW l^{-1}) microalgal suspension

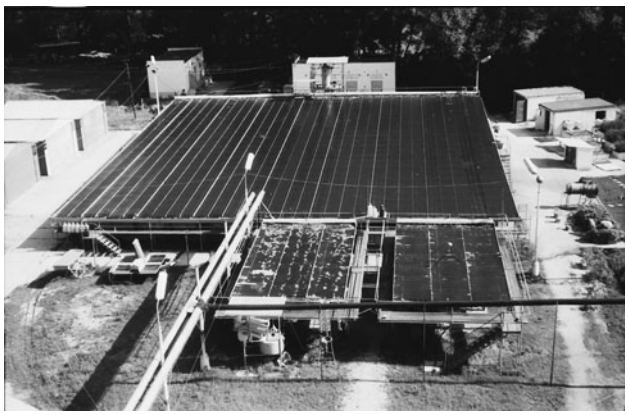

Fig. 6 Semiproduction outdoor algal production units located in the campus of the Opatovický mlýn (mid 1960s)

in a relatively thin layer (1–5 cm). This guarantees high average irradiance per cell and good gas exchange to obtain higher productivity per illuminated surface. Thus, a much lower volume of dense microalgal suspension can be treated at harvest. The units had a plane glass surface, with a slope of 3%, supported by a steel structure. The surface was fitted with transverse baffles 3.5 cm high and 15 cm apart to create intensive turbulence in the microalgal suspension, which moved down the surface at a velocity of 7 cm s^{-1}. The culture was circulated over the surface by an axial-flow pump during the day and was kept in a retention tank at night to reduce heat losses, or during rainfall to avoid dilution by rainwater. The detailed setup of these unique cultivation experiments was described in a newly established journal, *Algological Studies*, which was initiated by Prof. Málek and published in Třeboň [47, 71]. In outdoor cultivation experiments as well as in most laboratory studies, the green microalga *Scenedesmus quadricauda* with large coenobia which settle easily, and later *Chlorella pyrenoidosa* with small, globular cells (2–8 μm in diameter), were used. In the 1960s, the efforts of the laboratory were focused mostly on the technical problems of microalgae mass culture.

Later, in the 1960s and 1970s, cascade thin-layer cultivation units of Třeboň type were also constructed in Poland, Cuba, Bulgaria, and Italy to compare cultivation under various climatic conditions [71, 79, 87]. Collaboration was also established with the biotechnology group of the Istituto di Microbiologia Agraria, Universita di Firenze, which worked with mass microalgae cultures [24, 25].

A project for a large algal production plant and research institute was elaborated in the late 1960s. The plant was to be situated on the opposite side of the Opatovický pond. The proposed layout combined a research and development (R&D) center with biomass production, to create optimal conditions for fast and flexible solution of all problems which are to be expected during the scale-up process [71].

However, the plant and the institute never materialized, and even the existing pilot units were dismantled after only a few years of operation. Following the initial enthusiasm, a certain stagnation in algal research followed, and skepticism about the economical potential of algal mass production took over. In fact, several pilot plants that started operation in the 1960s and 1970s failed to confirm the hopeful prospects derived from the earlier laboratory work of the previous decades. Most of the large-scale tests were abandoned after a few months of operation with the conclusion that, for the time being, large-scale culture could not be economically feasible. The curious thing about this situation is that in no case was a reasonable explanation given for the divergence between the conclusions drawn from basic research and the results obtained with pilot-plant equipment. The only explanation which seemed reasonable was imperfections of the technical equipment and technology; in fact, as was often stated, the conditions obtained for growth of microalgae were rather distant from the optimal ones [73].

However, the main reason for dismantling the pilot production plant in Třeboň was that, after the political turmoil in 1968, Professor Málek was removed from office as a "revisionist," and every activity, even scientific, that he had supported was intentionally suppressed. Partial resurrection of microalgal biotechnology in Třeboň came only at the end of the 1970s, owing to the role of Ivan Šetlík and his collaborators in the space program "Intercosmos," in which they prepared experiments for a Czech astronaut on board the Salyut 6 spacecraft that orbited in 1978. The novel experiments were successful and proved that unicellular *Chlorella* can grow and divide under microgravity conditions of space shuttles [36].

Microalgal photosynthesis and biotechnology after 1989

After the return of democracy in 1989 any direct political influence on Czech science was removed. In the two decades which followed, enormous development in the field of algal biotechnology occurred. In September 1993, the 6th International Conference on Applied Algology was held at Třeboň [2]. It opened new topics and renewed collaborations broken at the end of the 1960s. At this time, a great renaissance of microalgal biotechnology also occurred worldwide. New applications were discussed and new approaches were designed.

Before the turn of the millennium

In the 1980s, it was proven experimentally that cascades (inclined baffled surface with about 3 cm culture layer) circulated by a pump could achieve significantly higher productivity (24.8 g DW m^{-2} day^{-1}) than horizontal raceways circulated by paddlewheels with a culture layer twice as deep (17.2 g DW m^{-2} day^{-1}) when working with the green microalga *Scenedesmus obliquus* [4]. Due to the different culture concentration, areal densities in terms of algal biomass per unit surface were equivalent, but the greater turbulence and better temperature regime of the cascades led to their higher productivity as compared with the raceways.

At the beginning of the 1990s, a third generation of outdoor cascade units for microalgal cultivation was built in Třeboň. Compared with the cascades used in the 1960s, the microalgal suspension in the new cascade units was much thinner—only about 10 mm thick. Instead of densely spaced baffles as described previously [71], plastic rods of 13 mm diameter were placed 1.5 meters apart, and thus the flow velocity could be increased to 0.5 m s^{-1} [17, 18]. Later, it was realized that the inclined-surface system works best if operated as a smooth inclined surface without any baffles where the layer of microalgae is only about 6 mm [44]. This allows achievement of high growth rate up to biomass concentration of 40–50 g DW l^{-1}. Also, cleaning and maintenance were much simpler compared with the baffled system. A 50 m^2 pilot system was tested in the Mediterranean climate where summer productivities were as high as 32 g DW m^{-2} day^{-1}, as compared with Central Europe with productivity maximum of about 23 g DW m^{-2} day^{-1} [16].

Important issues relating to construction design, variation of cultivation regimes, and biomass productivity studies were addressed by measurements of pCO_2 and pO_2 profiles in outdoor cascade units carried out by Karel Lívanský and co-workers [38, 45]. In some experiments, natural gas from an underground source was used for cultivation [44, 45]. These measurements provided detailed results on CO_2/O_2 exchange to optimize the supply of CO_2 for microalgal mass cultures and its utilization in thin-layer open units with long cultivation tracks [37, 39–41, 43]. About 64% of supplied CO_2 was utilized by the microalgal culture, the rest being lost as a result of incomplete absorption in the process of saturation or escape from the suspension into the atmosphere. About 2.73 kg CO_2 was needed for production of 1 kg *Chlorella* biomass. Per 1 g evolved O_2, 1.12 g CO_2 was consumed by the microalgae [42].

Based on fundamental research into the structure and function of photosynthetic membranes carried out at the Laboratory of Photosynthesis in Třeboň in the 1980s and 1990s [56, 57], microalgal biotechnology moved from a semi-empirical to molecular level. New methodology of photobiophysical and biochemical measurements was employed. Laboratory cultures of cyanobacteria and microalgae were examined to define the role of photosystem II (PSII) complex in response to environmental stresses [30, 32, 33].

Since the early attempts in the 1930s, it has been clear that intermittent (pulsed) light is the most important factor for microalgae growth. The amount of photon energy

received by each cell is a combination of several factors: irradiance intensity, cell population density, length of optical path (thickness of culture layer), spectral quality, light absorption, and rate of mixing [63]. Turbulent regime in mass microalgal cultures is essential, since light/dark cycles determine culture productivity. Short pulses of high light intensity can be used with high efficiency if separated by sufficiently long dark periods [22]. Maximal quantum yields were found for light/dark ratios of about 1:10 [31]. In pulsed (intermittent) light regimes, a microalgal culture can utilize a larger fraction of the sunlight reaching a given area. In the 1990s, the introduction of high-intensity light-emitting diodes (LEDs) for scientific use made it possible to measure the effect of intermittent illumination more preciously in the microsecond range. It was proved that photosynthetic rates could be further enhanced if the frequency of intense light pulses was increased from units to thousands of Hz [26, 54, 58]. Lately, a hydrodynamic model of culture in thin-layer cascade units has demonstrated highly turbulent flow allowing rapid light/dark cycles (with frequency of 0.5 s^{-1}) [51].

Following the application of chlorophyll (chl) variable fluorescence measurements in field crops with the aim of correlating changes of photobiochemical activities with productivity (e.g., [13]), chl fluorescence was applied to monitor microalgal mass cultures in situ. This novel approach was qualitatively different from previously used physiological methods, and the photosynthetic and biotechnology groups from Třeboň played a substantial role in its development. The pilot experiments, carried out in cascades and closed photobioreactor systems in the Czech Republic, Italy, and Israel, mostly applied the method of chl fluorescence quenching to examine effects of environmental stresses—high irradiance, temperature extremes, high dissolved oxygen concentration, and their synergism on algal productivity [76, 81]. Online chl fluorescence measurements indicated that changes of daily integrated values of relative PSII electron transport could be correlated well with analogous changes in daily productivity of cultures grown under different conditions [52, 53, 77]. The relative electron transport rate proved to be a simple and reliable parameter for use in estimating the photosynthetic performance of outdoor cultures of microalgae. Thus, in situ chl fluorescence monitoring has proven to be a suitable technique for measuring photochemical performance, being fast, noninvasive, and easy to measure. However, although the theory is well described at present [66, 70, 82, 83], the interpretation of fluorescence signals may not be straightforward, particularly when dealing with microalgae [11, 67, 69].

Experiments in closed photobioreactors as well as open cascade units showed that a midday depression of PSII photochemical yields of 20–30% of maximal morning values is essential for well-performing cultures [49–51, 63]. Lower or higher depression of photochemical yields indicates low-light-acclimated or photoinhibited cultures, respectively. These results are important from a biotechnological point of view in order to optimize the growth of outdoor microalgal mass cultures under varying climatic conditions.

The so-called xanthophyll cycle (light-dependent conversion of violaxanthin to zeaxanthin), was shown to serve as a major, short-term light-acclimation mechanism in higher plants. The role of xanthophylls in thermal dissipation of surplus excitation energy was deduced from the linear relationship between zeaxanthin formation and the magnitude of nonphotochemical fluorescence quenching. Unlike in higher plants, the role of the xanthophyll cycle in green microalgae (*Chlorophyta*) is ambiguous, since its contribution to energy dissipation can vary significantly among species [48, 52]. It was found that the xanthophyll cycle operates in all tested strains (e.g., *Chlorella*, *Scenedesmus*, *Haematococcus*, *Chlorococcum*, *Spongiochloris*); however, its contribution to nonphotochemical quenching was not as significant as in higher plants. It seems that microalgae rely on this dissipative mechanism only at low biomass density.

Another new line of research was focused on microalgal secondary metabolites. The search was for potential producers of secondary carotenoids, extracellular polysaccharides, mycosporine-like amino acids, and polyunsaturated fatty acids. Beside isolation and characterization of these compounds, research also aimed to optimize parameters important for the design and construction of suitable laboratory photobioreactors that would be suitable for overproduction of these bioactive compounds on the scale of hundreds of liters [29, 34, 35, 65, 86].

Because phototrophic microalgae can be cultivated under strictly controlled conditions, they are the ideal choice to incorporate stable isotopes from inorganic C, H, and N sources. Various biochemicals labeled by stable isotopes are used for scientific purposes (molecular structure or physiological investigations), as well as for clinical purposes (gastrointestinal or breath diagnosis tests) [15].

Microalgal biotechnology in the third millennium: future prospects

Since 2000, close collaboration with the Academic and University Center in Nové Hrady resulted in the construction of a brand new type of closed tubular photobioreactor which was based on solar concentrators (linear Fresnel lenses) mounted in a climate-controlled greenhouse on top of the laboratory complex, combining features of indoor and outdoor cultivation units [49, 50]. The dual-purpose system was designed for algal biomass production in temperate climate zone under well-controlled cultivation conditions and with surplus solar energy being used for heating service water. It was used to study the strategy of microalgal acclimation to

supra-high solar irradiance, with values as much as 3.5 times the ambient value. In model cultivations, cultures of the cyanobacterium *Arthrospira* (*Spirulina*) were cultivated at about three times higher solar irradiances (as high as 6 mmol photon m^{-2} s^{-1}) than those usually recorded outdoors in summer, indicating that this organism is tolerant to photoinhibition under sufficient turbulence and biomass density.

A two-stage cultivation process of the green microalga *Haematococcus pluvialis* was investigated with respect to correlations between photochemical activities and astaxanthin production. First, the culture was grown in low-irradiance units, and then exposed to supra-high irradiance when the rate of astaxanthin production was 30–50% higher than in the culture exposed to ambient irradiance. Carotenoid-rich microalgae biomass can be used as a colorant, for example, in ornamental fish aquaculture [88].

The light captured by photosynthetic pigments is roughly ten times higher under full sunlight (2,000 μmol photon m^{-2} s^{-1}) than that required to saturate growth. In other words, as much as 90% of the photons captured by chl antennae are dissipated as heat and fluorescence. Uncritical acceptance of photosynthetic efficiencies of about 10% or even higher [60] inevitably leads to exaggerated estimates of present and future biomass productivity. We can estimate a more realistic figure for maximum photosynthetic efficiency (photon energy converted into biomass energy) of about 4.5% for C3 plants or microalgae by using educated guesswork and detailed consideration of the partial reactions involved (e.g., [7, 8, 84, 91]).

A new type of microalgal bioreactor with precise control of process parameters (temperature, irradiance, gas composition) and online measurement of photosynthetic performance based on chl fluorescence was designed [12] and are now produced commercially (www.psi.cz). In parallel with the design of the new generation of photobioreactors, the study of bioactive compounds in microalgal cultures with potential as pharmacological drug leads has been initiated. The natural product chemistry is extraordinarily diverse, reflecting the exceptional biosynthetic capacities of microalgae. The molecular targets relevant for drug discovery have been generated by nature for millennia, but the technical knowhow to isolate and characterize bioactive compounds has only been available recently [68]. Microalgae represent a large, unexplored source of a variety of chemical structures. Routine methods for evaluation of biological activity in extracts from microalgal biomass as well as culture media have been applied. Lately, new test systems to identify drug candidates were applied for cell-based primary screening of several hundred microalgae strains for antibacterial, antioxidant, fungicidal, allelopathic, antitumoral, wound-healing, and anti-inflammatory compounds as well as enzyme (proteases, acetylcholine esterase) activity inhibitors [21, 28, 46, 59, 80, 85, 89, 90].

Particularly, the search for novel anti-inflammatory substances, able to downregulate increased endothelial chemokine production and adhesion molecule expression as well as tissue damage, holds therapeutic promise [46]. In close collaboration with Austrian partners (IMC Krems), two cell lines were used for detection of anti-inflammatory and wound-healing metabolites from microalgae [85]. The use of new in vitro assays resulted in detection of several compounds with unique structures and potential as novel therapeutics. The search for new acetylcholine esterase inhibitors was successful using primary screening of more than 200 microalgal strains from different habitats during this period of time [89]. Compounds isolated from the cyanobacterium *Nostoc* spp. were structurally characterized [14, 90]. In the field of antioxidant activity a new rapid-resolution separation method was developed [59]. The method was optimized for determination and identification of antioxidants (phenolic compounds and isoflavones) in fmol quantities and submicroliter sample volumes; for example, *p*-hydroxybenzoic, protocatechic, vanillic, syringic, caffeic, and chlorogenic acids, 4-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde were identified in extracts from microalgae strains (i.e., *Spirulina platensis*, *Anabaena doliolum*, *Nostoc* spp., and *Cylindrospermum* spp.) which contain phenolic acids or aldehydes at ppt levels.

Another promising field in current microalgae research is the quest for biofuels. As the rate of fossil-fuel consumption increases to unsustainable levels and accumulation of greenhouse gases in the environment quickly approaches "dangerously high" concentrations, a new bonanza for microalgal biotechnology has started with the goal of economical biofuel production from microalgae. A brief overview of second-generation biodiesel production systems using microalgae has been compiled [64]. To achieve environmental and economic sustainability, fuel production processes are required that are not only renewable but also capable of sequestering atmospheric CO_2. Biodiesel is currently produced from oil synthesized from conventional fuel crops that harvest the Sun's energy and store it as chemical energy. This presents a route for renewable and carbon-neutral fuel production. However, increasing biofuel production on arable land could have severe consequences for global food supply. Second-generation biofuels (biodiesel, bioethanol, and biomethane) produced from microalgae, plant, and forest plantations on vast land areas will have the advantage that they do not compete with food crops. However, current supplies from oil crops and animal fats account for only approximately 0.3% of current demand for transport fuels. In contrast to fuel crops, producing biodiesel from algae is widely regarded as one of the most efficient ways of generating biofuels and also appears to represent the only current renewable source of oil that could meet global demand for

transport fuels. The main advantages of second-generation microalgal systems are that they: (1) have a high photon conversion efficiency (as evidenced by increased biomass yields per hectare), (2) can be harvested batch-wise nearly all year round, providing a reliable and continuous supply of oil, (3) can utilize salt and waste water, thereby greatly reducing freshwater use, (4) can couple CO_2-neutral fuel production with CO_2 sequestration, and (5) can produce nontoxic and highly biodegradable biofuels. Current limitations exist mainly in the harvesting process and in the supply of CO_2 for high-efficiency production. Preliminary studies were carried out on utilization of CO_2 from flue gasses for cultivation of microalgae in outdoor open thin-layer units [19] or in closed cultivation systems [20]. A scheme for a combined process of farm unit size was proposed; this includes anaerobic digestion of organic agricultural waste, production and combustion of biogas, and utilization of flue gas for production of microalgal biomass, which could be used in animal feed.

Throughout the last 50 years, microalgal biotechnology has undergone enormous development. In the former Czechoslovakia, the foundations of microalgal biotechnology were laid in the 1960s by a group of enthusiasts that included Prof. Ivan Málek. Despite the fact that research and practice of microalgal biotechnology had to pass through difficult and critical periods, the foundations proved to be solid. Today, several original questions remain unanswered and many visions still need to be fulfilled. Despite the fact that research and biotechnological priorities have changed, in that microalgae are not considered primarily as the source of proteins or vitamins, but rather of carbon storage products to generate clean energy, the major underlying questions of microalgal biotechnology are still "hot" and open for further research: What is the maximal possible microalgal productivity, and how can it be achieved on a large scale and in an economic way? Can microalgal 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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