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Biological Constraints in Algal Biotechnology

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Abstract In the past decade, considerable progress has been made in developing the appropriate biotechnology for microalgal mass cultivation aimed at establishing a new agro-industry. This review points out the main biological constraints affecting algal biotechnology outdoors and the requirements for making this biotechnology economically viable. One of them is the availability of a wide variety of algal species and improved strains that favorably respond to varying environmental conditions existing outdoors. It is thus just a matter of time and effort before a new methodology like genetic engineering can and will be applied in this field as well. The study of stress physiology and adaptation of microalgae has also an important application in further development of the biotechnology for mass culturing of microalgae. In outdoor cultures, cells are exposed to severe changes in light and temperature much faster than the time scale required for the cells to acclimate. A better understanding of those parameters and the ability to rapidly monitor those conditions will provide the growers with a better knowledge on how to optimize growth and productivity. Induction of accumulation of high value products is associated with stress conditions. Understanding the physiological response may help in providing a better production system for the desired product and, at a later stage, give an insight of the potential for genetic modification of desired strains.The potential use of microalgae as part of a biological system for bioremediation/detoxification and wastewater treatment is also associated with growing the cells under stress conditions. Important developments in monitoring and feedback control of the culture behavior through application of on-line chlorophyll fluorescence technique are in progress. Understanding the process associated with those unique environmental conditions may help in choosing the right culture conditions as well as selecting strains in order to improve the efficiency of the biological process.

Keywords: outdoor cultures, photobioreactors, oxygen stress, photoinhibition, low temperature stress, chlorophyll fluorescence

INTRODUCTION

Outdoor algal cultures are exposed to a variety of changes in environmental conditions. Those changes are taking place in two different time scales. One is the circadian cycle which includes variations in light and temperature in a 24 h cycle. The other one is a seasonal cycle that varies based on the climatic and geographical location of the particular habitat in which the algae are growing. In dense cultures used in algal biotechnology a third cycle is imposed by the intensive mixing system which mainly results in a light-dark cycle which fluctuates in terms of fraction of seconds as compared to the hours or months in the other two cycles.

In order to cope with changing environmental conditions, microalgae have developed diverse mechanisms for sensing and acclimating to changes in their environment [1,2]. Acclimation responses observed include the alteration of light harvesting complex synthesis and degradation in response to changes in light quality and intensity. Such alterations are aimed to help efficiently balance the absorption of excitation energy and the production of reducing power (NADPH) and chemical energy (ATP) with their utilization for growth and cell maintenance. Inability to maintain this balance due to excess excitation of the photosynthetic reaction centers may result in the production of reactive oxygen species which promote photoinhibition and in some case may lead to photo-

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oxidative death. As implied many of the stress responses and adaptive process are associated with the photosynthetic apparatus.

In recent years it has become evident that establishing an economically feasible microalgal biomass production industry requires a significant reduction in the production cost. One way by which this goal can be reached is getting a consistent increase in productivity. For most applications, efficient capture of light energy represents one of the most important factors controlling the culture productivity. Yet, in outdoor cultures the interaction between light and other environmental factors cannot be separated. Hence, detailed studies on the interaction between environmental factors and their possible synergism have to be better understood. This review attempts at outlining the main biological constraints affecting outdoor algal biotechnology.

LIGHT UTILIZATION EFFICIENCY AND PRODUCTIVITY IN MICROALGAL CULTURES

Normally, the growth of many photosynthetic microorganisms gets saturated at about 200 μmol m^{-2} s^{-1}, that is, at about 1/10 of the maximum light intensity recorded outdoors in summer (2,000 μmol m^{-2} s^{-1}). The occurrence of the "saturation effect" has been indicated as one of the most serious limitations to effectively utilize high solar irradiance as already indicated in the early stages of the development of the algal biotechnology [3].

In dense algal cultures the incident light intensity (I_o) on the surface of the culture decreases with the depth, and at a certain depth the light intensity just equals the saturation intensity (I_s). All the light that penetrates to greater depths is utilized with maximum efficiency. At lesser depths only the fraction of the incident light will be utilized.

It has been suggested that the absorptivity of solar radiation by the algal culture can be estimated by employing the Beer-Lambert law, although strictly valid only for monochromatic light:

$$I(_x) = I_{(0)} \exp(-K x) \quad (1)$$

The extinction coefficient K has been assumed to be a linear function of biomass concentration. However, in experimental measurements carried out by the authors on various cultures of microalgae and cyanobacteria, it was found that, while for biomass concentrations lower than 1 g/L of dry weight this assumption does not introduce much inaccuracy, for higher biomass concentrations the absorptivity values are quite different from those obtained on the basis of the assumption. The values of absorptivity of a *Spirulina platensis* culture as function of different biomass concentrations and culture depths are shown in Fig. 1 [4].

The earliest attempt to quantify the effect of light saturation was made by Burlew [3] who presented the equation:

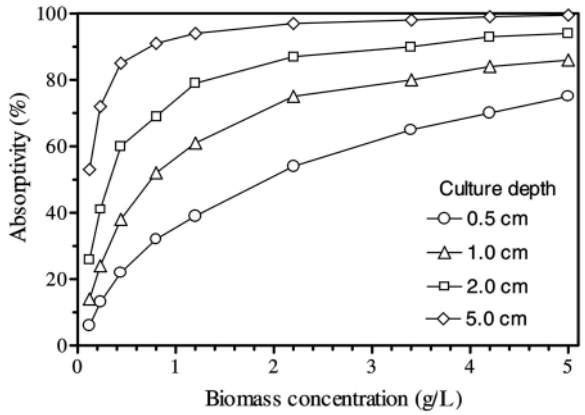

Fig. 1. Absorptivity of a *Spirulina platensis* culture as a function of biomass concentrations for different culture depths. Absorptivity of the culture was measured under sunlight conditions using a Li-Cor quantum sensor (model LI-185 B).

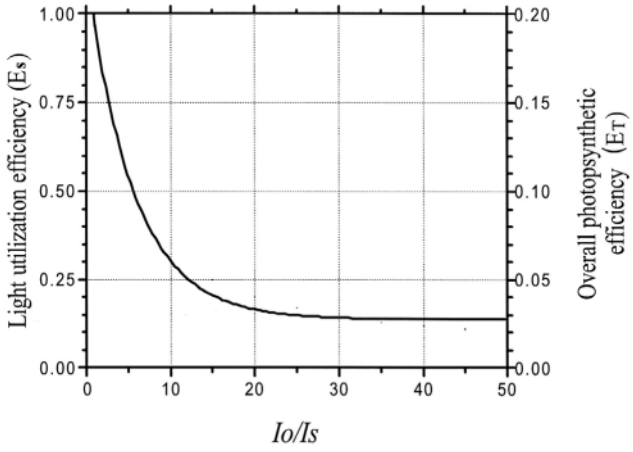

Fig. 2. Light utilization efficiency (*E*s) according to Bush equation, and total photosynthesis efficiency (E_T) for *E*q = *0.2* as a function of I_o/I_s (Ref. [5] modified).

$$E_s = I_s/I_o \; (\ln I_o/I_s + 1) \quad (2)$$

which was attributed to V. Bush. As shown in the graphical representation of the equation (Fig. 2), at a very low light intensity ($I_o/I_s \leq 1$), the light utilization efficiency, *E*s (namely the amount of light absorbed by the pigment antenna) of the photosynthetic apparatus is equal to 1, and falls off first rapidly between $I_o/I_s \leq 20$ and thereafter more gradually when $I_o/I_s > 20$. Assuming the average thermodynamic efficiency of photosynthesis (*E*q), equal to 0.2 in the range of wave length 400~700 nm, the overall photosynthetic efficiency can be also determined from Fig. 2 [5]. It is evident that the light intensity at which the culture growth becomes saturated (I_s) represents an important factor in determining the light utilization efficiency for outdoor algal cultures where I_s represents an independent variable. Hence, the selection of algal strains having a high I_s value to avoid the saturation

effect is desirable. The constraint imposed by the light saturation effect has prompted algal biotechnologists to seek for solutions to attenuate its effect in outdoor cultures. Basically three types of approaches have been proposed:

a) increase of the population density and the mixing rate of the cultures;
b) use of special designs of photobioreactors in which it is possible to improve light utilization efficiency by the culture;
c) search for strains having small antenna size.

The first strategy has been pursued by Richmond and co-workers since the beginning of the outdoor algal biotechnology in Israel [6], and particularly in the recent years with the use of ultra-high cell densities of *Spirulina* and other microalgae in flat photobioreactors. However, even when *Spirulina* cultures were grown at the optimal population density, and with very high mixing rates, it was not possible to prevent the onset of photoinhibition as deducted by a reduction in the F_v/F_m ratio (*i.e.* the maximum photochemical quantum yield of PSII) [7].

The second way to avoid the saturation effect has been proposed in the recent years and consists in the design of sophisticated photobioreactors in which it is possible to promote the so-called "light dilution", an idea that is really not new [3]. The rationale for this proposal is based on the increase of the optical cross section of the photobioreactor, so as a given amount of light impinging on a certain land area occupied by the reactor is distributed on a larger surface (that is, the illuminated area of the culture), thus reducing the saturation effect. If such a condition is realized we can expect an increase in biomass yields per unit of area occupied by the reactor and thus a better efficiency of conversion of light in biomass. Photobioreactors specifically designed to take advantage of the light dilution have been proposed in the recent years [8-10]. Lower PFDs can indeed lead to higher efficiencies as demonstrated by Grima *et al.* [11], but it is worth to point out that an increase in biomass yield (g m^{-2} day^{-1}) does not necessarily involve an increase in volumetric productivity (g L^{-1} day^{-1}) which is strongly affected in a light-limited culture by the light intensity received by the culture. In addition, many of the proposed designs have been tested on very small scale and they are complex and difficult to scale-up at an industrial level in which a low cost production system is desired.

A different concept of photobioreactor design in which light capture system is spatially separated from the reactor was proposed by Mori [12]. Solar beam irradiation in a "clear sky" area may be collected and concentrated by linear Fresnel lenses [12] or parabolic mirrors [13] and channeled via optical fibers into a photobioreactor [14]. Yet, the presence of too many fibers inside the reactor may cause mixing problems. For this reason, distribution plates [15] or rods [16] made of glass or quartz has been proposed. According to Janssen *et al.* [17] air-lift photobioreactors with light redistribution plates equipped with external light collection may be scaled up to 100 m^3 and reach a photosynthetic efficiency of 15% on PAR-basis. However, since the investment costs of this technology is expected to be very high, these photobioreactor designs could be used mainly for the production of high value compounds. Other solutions have been proposed to improve the performance of the microalgal photobioreactor through the realization of a better fluid pattern that is, a better light/dark cycle of the cells inside the tube lumen. This goal has been only partially reached either by using static mixers or by modifying the bioreactor design.

The first approach was first tested by Laws *et al.* [18,19] in open ponds. The static mixers consisted of arrays of foils similar in design to airplane wings placed in the algal flume to create vortices in the culture layer. The authors claim that with this technique they could take advantage of the flashing light effect and thus achieve an increase in the average photosynthetic efficiencies (based on visible light) of a *Cyclotella cryptica* culture in open pond, up to 7.5%. Static mixers were also tested by Ugwu *et al.* [20] in tubular photobioreactors. According to the authors, productivity increased by 40% with a *Chlorella* culture. The second approach was tested by Carlozzi and Torzillo [21] by devising and constructing a strongly curved tubular photobioreactor in which it was possible to generate convective mixing in the bends so as the cells of the core region were carried towards the tube wall and thus received illumination. However, if the higher power required to support such a convective mixing was considered (about 40% higher than conventional reactors) the relatively low increase in biomass yield obtained (17%) became even less impressive. However, the better illumination pattern experienced by cells made it possible to operate with a *Spirulina* culture having a higher biomass concentration. No increase of productivity was observed by Muller-Feuga [22] who tested a swirling flow generated by a tangential inlet of the culture in an annular photobioreactor exposed to artificial light for continuous cultures of *Porphyridium cruentum*. However, it must be pointed out that any obstacle inserted in the tube lumen to generate the desired mixing pattern will inevitably lead to an increase of the power required for culture circulation and to an increase of shear with risk of cell damaging. As a result, the decision of using static mixers or modifying the geometry of tubes must take into account the general economy of the microalgal process.

In conclusion, although some improvement in biomass yield has been obtained in the last 20 years by improving the flow patter, the statement made by Kok in 1953 "*The economic realization of a turbulent flow that would submit individual cells to a favorable flash pattern, rather than to a random distribution of intensity variations, probably is the major engineering problem"* remains actual [3].

Finally the third approach of search for algal strains with small antenna is based on the following rationale. Strains having a small antenna size will minimize light absorption by the first layers of cells, therefore reducing the dissipation of light through non-photochemical quenching and the risk of photoinhibition. This would result in a higher overall photosynthetic productivity in outdoor cultures. Indeed, small antenna size cells are characterized by a higher photosynthesis rate [23-26].

Small antenna size would allow to operate the culture at a higher density and/or culture depth, and consequently an increase in the areal productivity. They may be obtained through acclimation of cells to strong light in the laboratory, however once cells are transferred outdoors they would readily revert to that of normally pigmented cells upon lowering of light intensity during the day and as a result of the increased cell density due to daily growth [27]. Hence developing such mutants may have profound and immediate consequences on the industrial scale biomass production of microalgae where an increase in population density and productivity would allow a reduction of costs. Recently mutants having small antenna size have been obtained from the microalgae *Dunaliella salina* and *Chlamydomonas reinardtii* [28,29]. However, information on their performance in outdoor culture is not available yet.

In certain cases saturating light is a prerequisite to stimulate synthesis of valuable products such as secondary carotenoids. For example *Haematococcus* can accumulate high amounts of astaxathin when cells are exposed to high light and nitrogen deficiency. For this purpose a penthouse-roof photobioreactor equipped with solar concentrators (linear Fresnel lenses) mounted in a climate-controlled greenhouse has been recently constructed in Czech Republic [30]. The photobioreactor can receive irradiance between 2,000 and 7,000 μmol m^{-2} s^{-1} for most of the diel cycle. The authors claim that higher light intensities may induce a faster as well as a higher accumulation of astaxathin in the cells. The photobioreactor has also been tested with very dense *Spirulina* cultures and productivity reached about 33 g m^{-2} s^{-1} in September when light intensity usually declines. This photobioreactor design can yield important information for basic and applied research, especially for investigating the behavior of microalgal cultures under high light irradiance. Furthermore, the light concentrating system makes it possible to extend considerably the cultivation season, and thus is promising for cultivation of microalgae in the northern part of Europe.

OTHER ENVIRONMENTAL FACTORS LIMITING MICROALGAL PRODUCTIVITY OUTDOORS

As discussed above, mass culturing of microalgae outdoors is still facing the problem of over-saturation by light in the upper layers of the culture. As a result photoinhibitory stress may be imposed preventing the cells from achieving the highest productivity required to maintain an economically viable process. Few of the main environmental factors limiting growth are discussed below trying to indicate their relevance to microalgal productivity outdoors. Attempts to assess the relative contribution of the factors limiting productivity of *Spirulina platensis* in open pond raceways throughout the year have been done in the past by Vonshak *et al.* [31] and by Richmond *et al.* [32].

Photoinhibition in Outdoor Mass Culture

In this section we will focus on the physiological and environmental factors associated with the onset and recovery from photoinhibition and its consequences on microalgal productivity outdoors. Even in dense microalgal cultures, the upper layer is exposed to excessive solar radiation. This because cells grown outdoors acclimate to an average PFD, sensed as the average redox state of the plastoquinone pool, which is lower than that at the culture surface. As a result cells develop a relatively large antenna size which increases the risk of photoinhibition when they are shifted from the bottom to the top layer where they are exposed to a PFD which is considerably higher than that photosynthetic apparatus is acclimated to. Even in turbulent flow, a laminar sub-layer develops at the reactor surface. The depth of this sub-layer can be calculated using the Deissler's empirical formula [33]. For example, in a tube with diameter of 6 cm and superficial liquid flow of 0.6 ms^{-1} the thickness of the laminar sub-layer reaches 0.13 mm. Turbulent flow is fully developed only at a depth of 0.67 mm. It is likely that cells close to the illuminated reactor surface are replaced relatively slowly by cells from the core part of the tube. For practical reasons, it is obvious that none of the mixing devices used in such systems is able to induce a light-dark cycle to overlap with the time scale of the flashing light effect attainable in the lab. According to the results gathered by Vonshak and Guy [34], who where the first to demonstrate that dense *Spirulina platensis* cultures grown outdoors undergo a photoinhibitory process, photoinhibition caused a reduction in biomass yield of about 20%. These observations were further confirmed using chlorophyll fluorescence technique in outdoor cultures of *Spirulina* grown in open ponds or in tubular photobioreactors [35-37]. The extent of the photoinhibition can also be influenced by the design of photobioreactors. Cultures in horizontal tubular photobioreactors may experience higher photoinhibitory stress than the ones grown in vertical oriented concentric-tube airlift systems during summer, since they are subjected to higher peaks of irradiance [38]. Photoinhibition in outdoor mass cultures can be detected with high sensitivity from changes in variable chlorophyll fluorescence [36,39]. The F_v/F_m ratio (variable to maximum fluorescence yield) is a convenient measure of the potential maximum quantum yield of PSII, and it has been assumed as a measure of photoinhibition [40]. The F_v/F_m decrease has been found highly correlated to reduction in the quantum yield of O_2 evolution or CO_2 uptake [41]. In the field, F_v/F_m frequently exhibits diurnal depressions that are roughly symmetric to light intensity and are mirrored by corresponding changes in the quantum yield of photosynthesis [42,43]. However, in aquatic systems, as well as in some microalgal cultures, short term photosynthesis measurements may often indicate maximum light-saturated photosynthesis rates (P_{max}) at noon time, that is, in correspondence of the lowest value of F_v/F_m and quantum yield

[44,45]. Beherenfeld *et al.* [44] have demonstrated that in *Thalassiosira weissflogii* adapted to low light that changes in carbon fixation rates are not observed until rate limitation is shifted from the Calvin cycle reactions to electron transport through PSII. In this organism changes in P_{max} were not observed until the reduction of active reaction centers had reached 50% of initial. However, it must be pointed out that algal productivity primarily depends on the light energy conversion efficiency, *i.e.* the absorption and utilisation of light by the photosynthetic apparatus to assimilate CO_2 into dry matter. Therefore, outdoor dense algal cultures are predominantly grown at light limitation and, consequently, their photosynthetic performance would be more dependent on the initial slope rather than on the light-saturated part of the *P/I* curve. Hence, a reduction in the F_v/F_m ratio due to excessive light absorption in the surface layers will inevitably result in a decrease in the biomass yield.

Interaction of Low Temperature and Excess Light

As discussed above, outdoor algal cultures may be usually exposed to a combination of environmental stress. The most common combination is high irradiance and low temperature. It happens that in the morning, while increases in light intensity occur in a range of 1~2 h, the increase of temperature is a much slower process that takes about 4~5 h , particularly in open pond cultures. This kind of de-synchronisation between the two most important environmental factors affecting photosynthesis results in a unique stress condition under which photoinhibition may indeed be induced at a relatively low light intensity, due to the sub-optimal temperature conditions. This problem is particularly relevant in desert areas where culture morning temperature, particularly in open ponds, is far below optimum, even in summer [34,45]. The effect of low temperature on photosynthesis and growth of outdoor cultures of *Spirulina* in tubular reactors has been investigated using saturating pulse fluorescence [35,39]. Diurnal changes in the effective photochemical quantum yield of PSII, $\Delta F/F'_m$, measured in dense *Spirulina* cultures grown at 25°C (that is 10°C below the optimum) showed in the middle of day a reduction of 20% of the morning value which was mirrored by an analogous decrease in the biomass yield. The effect of low temperature on photoinhibition was also investigated in outdoor cultures of *Monodus subterraneus* by measuring the diel changes in photosynthetic oxygen evolution and several photochemical parameters [45]. Cultures were maintained at two temperature regimes. In one, the rise in temperature was initiated in the morning as a result of the solar radiation increase up to the optimal temperature of 28°C, while in the other culture a heating device was used to increase the rate of warming up in early morning. It was found that, although the two cultures were maintained most of the day at the same temperature and light intensity, the culture exposed to sub-optimal temperature, for few hours in the morning, showed a greater decrease in almost all the photosynthetic and chlorophyll fluorescence parameters as compared to the heated one. It was concluded that even a relatively short exposure to sub-optimal morning temperatures induced a photoinhibitory damage. The higher photochemical activity of the heated culture was also reflected in a 60% increase in productivity compared to the non-heated culture.

In open ponds the culture temperature profile can be hardly modified, but it is possible in photobioreactors. In general, a rapid increase in morning culture temperature and a rapid decrease at night can be achieved by either using small-diameter tubes or thin flat-plate and/or by orienting and tilting the photobioreactor at an appropriate angle to the sun [46]. In such photobioreactors, the optimal temperature for growth is reached early in the morning by direct gain of solar heat and thereafter maintained at the optimum by spraying water, or using some other cooling device. Mathematical models to make transient thermal analysis and to estimate the incident solar light energy in different photobioreactor designs have been developed [4,47]. Although in principle tubular photobioreactor should be built with tubes of small diameter, the use of very narrow tubes, in an industrial scale production involving long runs of tubing, may result in hydrodynamic problems, particularly with filamentous microorganisms such as *Spirulina*. Indeed, it can produce a relatively high viscosity suspension, even at a low biomass concentration. In addition, the quick O_2 build-up in the photobioreactor can impose severe limitations to tubing length as it will be discussed later. As a matter of fact, an attempt large-scale production in tubular reactors made with several espaliers of tubes having 1 cm bore size, failed to perform to expectation in the past in Santa Ana, Murcia, Spain (Photobioreactors Ltd.).

Interaction of Low Temperature and High Oxygen Concentration

Oxygen build-up, particularly in closed systems, represents one of the most important factors limiting the productivity of outdoor cultures. Indeed, a number of studies have suggested that reaction involving dioxygen (O_2), resulting in active oxygen species, initiates early destructive processes of photoinhibition [48, for review]. The photosynthetic electron transport system represents the major source of active oxygen species having the potential to generate singlet oxygen, hydrogen peroxide and the superoxide radical [49]. When scavenging of potentially damaging oxygen species is insufficient, photoinhibition can occur.

The combination of high oxygen concentration and high irradiance represents a very common event in outdoor cultures, particularly in closed systems. For example in photobioreactors made with tubes of about 5-cm internal diameter, in well-growing *Spirulina* cultures, the oxygen concentration can increase at a rate of 2~3 mg L^{-1} min^{-1}. This results in an oxygen concentration of up to 70~80 mg/L even with a gas exchange with air every 50 seconds and with a relatively high turbulence rate [35]. The combination of high oxygen concentration and low temperature in outdoor cultures can occur at the beginning of the cooler season, when the culture temperature

Table 1. Effect of oxygen concentration and temperature on biomass productivity and chlorophyll synthesis in *Spirulina platensis* cultures grown outdoors in photobioreactors. SD= standard deviation of triplicate experiments. LO-OT = low oxygen-optimal temperature, HO-OT = high oxygen-optimal temperature, HO-LT = high oxygen-low temperature

Culture conditions	Oxygen concentration (mg/L)	Temperature (°C)	Chlorophyll synthesis (mg L^{-1} d^{-1})	Biomass synthesis (mg L^{-1} d^{-1})
LO-OT	22 ± 2	35	6.02 ± 0.18	570 ± 28
HO-OT	60 ± 19	35	2.57 ± 0.05	380 ± 18
HO-LT	58 ± 16	25	0.22 ± 0.01	230 ± 10

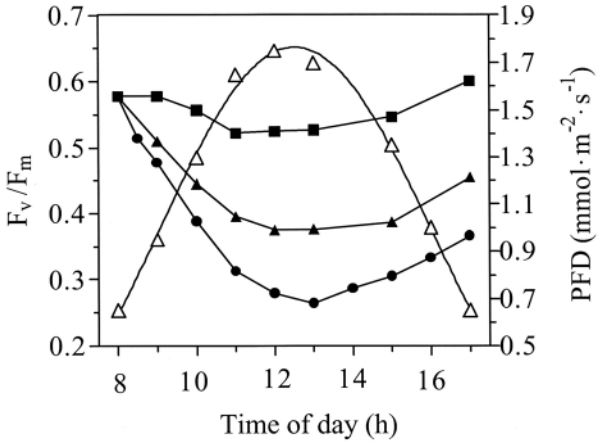

Fig. 3. Effect of oxygen concentration and temperature on the F_v/F_m ratio of *Spirulina platensis* cultures grown outdoors in photobioreactors. (■) Low oxygen-Optimal temperature; (▲) High oxygen-Optimal temperature; (●) High oxygen-Low temperature; (△) PFD.

drops much below the optimum but irradiance is still enough to drive photosynthesis at an appreciable rate. Such conditions can be very common in desert areas where the morning temperature of the culture is far below the optimum while light intensity is high enough to induce photoinhibition. The synergistic effect of high oxygen concentration and low temperature was studied in outdoor cultures of *Spirulina* grown in tubular photobioreactors by using an on-line chlorophyll fluorescence technique [35]. The results showed that the combination of low temperature and high oxygen concentration caused considerable PSII photoinhibition measured as changes in the F_v/F_m ratio, resulting in a strong reduction of the growth of the culture where a mere reduction of 10°C below the optimum was imposed and the oxygen concentration was allowed to rise to 70~80 mg/L (Fig. 3). Photoinhibition reduced the daily productivity of the culture grown under high oxygen stress by about 33%, and that of the culture grown under high oxygen-low temperature stress by 60% (Table 1). The effects of high oxygen and low temperature stress on photosynthesis and productivity were recently studied on both low light and high light acclimated *Spirulina platensis* cultures grown outdoors in tubular reactors in summer [50]. Chlorophyll fluorescence transients and quenching measurements performed during the day showed that PSII photoinhibition was much higher in the low light acclimated cultures than in the high light acclimated ones. In the low acclimated cultures the F_v/F_m ratio declined from 0.6 in the morning to 0.15 in the middle of day. The corresponding values of photosynthetic activity (oxygen evolution) declined to almost zero in the middle of day. However, despite a strong inhibition in both F_v/F_m and photosynthetic activity, particularly in the low acclimated cultures, a significant loss in the net D1 content (about 50%) occurred only in the cells grown under high stress conditions (*i.e.* combination of low temperature and high oxygen). Productivity of low light acclimated cultures was lower than that measured in high light ones, either when they were grown under stress or under optimal conditions. In both low light and high light acclimated cultures, high stress conditions reduced productivity by 37% and 52% respectively, compared to non stressed cultures. The zeaxanthin content was higher in high stress cultures and was reflected by a reduction in the β-carotene content. It was concluded that, since cyanobacteria lack the xanthophyll cycle [51], the increased content in zeaxanthin might provide a defense against photoxidation by scavenging active oxygen species. Moreover, an increased conversion of β-carotene into zeaxanthin, which does not transfer energy to the reaction center, can allow a reduction of the excitation pressure on reaction centers and consequently reduce the extent of photoinhibition.

A midday decline in dissolved oxygen concentration due to photoinhibition was also observed in outdoor cultures of *Pheodactylum tricornutum* grown in tubular photobioreactors, despite the use of high biomass concentrations and a high mixing rate [52]. Furthermore the culture collapsed when the minimum velocity was adopted, apparently due to the onset of photo-oxidation. The high oxygen concentration itself can influence the growth and the photosynthetic activity of the cultures even when light intensity is relatively low or when the biomass concentration is high, as demonstrated in a number of experiments carried out both in the laboratory and outdoors [53-55]. There is a little doubt that oxygen accumulation in the culture represents the main obstacle to photobioreactor scale up. Indeed, in that system the advantage of growing algal cultures at a very high biomass concentration is greatly reduced by oxygen accumulation.

The oxygen inhibition may be counteracted by increasing the speed of the culture, yet very high culture veloci-

ties are not practical because of shear forces that may damage the cells. In addition, the limited strength of materials used for construction of tubes further discourage adoption of high flow rates. Alternatively, diameter of the tubing should be increased to prevent O_2 concentration reaching inhibitory levels. The first to propose a modification in the tubular reactor design to overcome this problem were Richmond *et al.* [56]. They designed and operated a 1,200-L *Spirulina* photobioreactor in which tubes were arranged in parallel and connected by a manifold system. The volume and size of the reactor could be increased without increasing the length of tubes. In such a way scale-up could be achieved without increasing the oxygen concentration in the system. However, even this solution necessarily implies a more powerful pumping system which inevitably increases both investment and running costs. The application of an efficient degassing system to prevent high oxygen accumulation represents a prerequisite for successful design of industrial scale photobioreactors.

In open ponds, when scaled up, inducing turbulence is a major problem affecting both the initial investment as well as the operational cost. An efficient turbulent induction system will also serve well the purpose of removing the oxygen evolved in the photosynthetic process. In relatively small production units, in which high flow rates can be easily obtained, oxygen concentration can be kept at levels of 200% of saturation, while when scaled up a significant reduction in flow rate is observed (10 cm/sec). As a result O_2 concentration in the culture may reach levels up to 500% of saturation. Very little can be done in order to protect algal cultures from oxidative stress. Thus careful attention has to be given to ensure an efficient removal of oxygen from the culture. The possibility to isolate natural strains as well as constructed mutants that can tolerate high oxygen concentrations has not yet been intensively explored.

High Temperature Stress in Outdoor Cultures

Most of the results concerning the effects of high temperature on algal growth have been gathered under laboratory conditions. Short-term responses of photosynthesis to temperature have been frequently used to infer the long-term response of algal growth to temperature, although it is very difficult to relate the short-term response of photosynthesis to that of growth. Indeed, positive net photosynthesis rates, measured in the time scale of minutes, can occur at temperatures well above the upper thermal limit for long-term survival [57 for review]. Since this review deals with outdoor cultures, in this section we will mention only some technical problems related to the temperature control in the culture. As a matter of fact, overheating of the culture represents an important constraint limiting the use of tubular photobioreactors outdoors. Photobioreactors function like solar collectors, therefore in summer days the culture temperature can reach values as high as 45~50°C which are well above the optimum for growth even for thermotolerant organisms such as *Spirulina*. The cost of a cooling device reduces, in fact, the economic advantage of closed systems particularly when typical mesophilic strains are used. The following cooling systems have been tested for mass cultivation of *Spirulina* [58]: (i) Shading of the tubular bioreactor with dark plastic sheets. For an effective control of the culture temperature it was necessary to cover about 80% of the illuminated surface for 5~6 h daily. This caused a strong reduction in the amount of solar radiation received by the culture and consequently in the biomass yield; (ii) Overlapping the tubes in the North-South orientation in order to reduce the amount of light received by the culture in the middle of the day. This system was difficult to install and inadequate for effective control of the culture temperature; (iii) Cooling the culture by spraying water on the surface of the photobioreactor. This cooling device worked very efficiently, however the consumption of water was very high, and this reduced the advantage of closed system in preventing water evaporation. This problem was partially solved by recycling water.

Finally cooling towers have been efficiently tested with large scale tubular photobioreactor in Italy [59]. The system proved to be particularly efficient for *Spirulina.* Yet, a temperature gradient of several degrees along the reactor was inevitably created, namely from the inlet and the outlet of the culture from the tower.

Combining open ponds with closed reactors to avoid cooling systems has been also tested in the authors' laboratory [60]. In principle this solution seems very attractive since one may expect an increase of the morning temperature of the culture in the pond and a reduction of the maximum temperature in the photobioreactor. However, it was found that to maintain midday culture temperature within 35°C in a 25-m^2 tubular reactor in summer, it was necessary to reduce the ratio between tubular and pond parts below 1. In order to reduce the amount of water required for cooling, from 1983 a thermotolerant strain of *Spirulina* (*Spirulina platensis M2*) was utilized. This strain, selected in the author's laboratory, was able to grow up to 42°C and tolerate short temperature exposures up to 46°C.

Cell Fragility

Cell fragility is another key problem of culturing microalgae in photobioreactors [61,62]. As a matter of fact, the choice of the device used for culture recycling represents another important aspect for the successful photobioreactor design. The shearing stress imposed by the circulating device will often dictate whether shear sensitive and fragile cells, such as flagellate or filamentous cianobacteria, can be cultivated in the particular photobioreactor. Among the microalgae and cyanobateria species the sensitivity to shear varies greatly. *Dunaliella* represents an extremely fragile species because it lacks a ridged wall, while other algae species such as *Spirulina* can tolerate relatively high levels of turbulence and sparging with gas. In some case the sensitivity to shear can change during the cell cycle as in the case of *Haematococcus,* a producer of the secondary carotenoid astax-

anthin. This species is particularly sensitive to stress in the green phase during which high shear can cause deflagelation of cells. The resistance to shear greatly increases during the red phase when immobile aplanospores are formed. The physiological parameters of *Chlorella* cultures grown in a tubular loop reactor and recycled either by a centrifugal pump, a rotary positive displacement pump or a peristaltic pump, were compared [63]. The maximum specific growth rate of *Chlorella* cultures recycled either by a centrifugal or a rotary positive displacement pump was lower than that in cultures recycled by a peristaltic pump. The adverse effects on the cells caused by centrifugal or rotary positive displacement pumps were proportional to the rotation speed of the pump. Circulation of *Porphyridium* cultures in a photobioreactor with a screw-pump also strongly increased cell damage [61]. Both peristaltic and membrane pumps have been tested for many years in the authors' laboratories for outdoor culture of *Spirulina* in 500-m tubular reactors. Cultures could be circulated with a peristaltic pump at a speed of 0.3 m/s without any evident damage to the trichomes. However, when the culture speed was increased to 0.8 m/s, the productivity was reduced by 16%. Microscopic observation showed a significant increase in the number of trichome fragments (< 7 μm in length) in cultures circulated at higher speed as a result of the increased frequency of their passage through the pump [64]. According to the authors' experiences, both rotary and screw pumps for circulation of *Spirulina* cultures were found to produce high shear causing trichome breakage (unpublished results). However, it is important to point out that mechanical shear can be evident in small scale photobioreactors in which the cultures pass through the pump very frequently (*e.g.* time scale of seconds) while it can become negligible in long photobioreactors where the time cycle between consecutive passages through the pump is much longer (time scale of minutes). In principle, airlift and bubble column systems seem to be more suitable for the culture recycling of stress sensitive species of microalgae and cyanobacteria. However, it was showed that *Dunaliella* was sensitive to damage by gas bubbles as well as by turbulence in the liquid [65]. Recent experiences carried out by Brindley *et al.* [66] with *Pheodactilum tricornutum* in a small scale bubble column photobioreactor have showed that increasing the air flow rate from 2.0 to 4.0 vvm, the culture productivity strongly declined. The reduction in the culture performance was confirmed by measurement of the F_v/F_m, which decreased from about 0.6 to 0.4. It was concluded that the key factor for pneumatic stress were the bubble-cells interactions and mainly the interactions on the cultures surface. It has been found that for *Pheodactilum tricornutum* values of shear rates greater than 7,000 s^{-1} or microeddies smaller than 45 μm should be avoided since they may affect the productivity of cultures [67]. Actually, high velocities are usually not practicable because of limited strength of material used in constructing photobioreactors. In conclusion the decision of the circulating device requires an interaction between biological and engineering parts.

CONCLUDING REMARKS AND FUTURE PROSPECTS

A better understanding of the interaction between light and other environmental factors governing the growth of dense outdoor microalgal cultures is required in order to be able to reach productivity rates near the theoretical maximum. Hence, a tight cooperation between researchers dealing with photosynthesis stress and biotechnologists is desirable.

Outdoor dense microalgae cultures experience large variations in light intensity due to the changes in daily irradiance and mixing. Although turbulent mixing is aimed to expose the cells to average, uniform irradiance, relatively long exposure of cells to excess light cannot be avoided. On the other hand, a strategy striving to counteract photoinhibition at midday, through an increase in cell concentration, would lead to acclimation of the cells to low irradiance, which may result in an increase in the PSII antenna size and thus to an increased risk of overexcitation. Moreover, the increased fraction of cells deprived of light in the deeper layers would bring about to an increase of energy wasted through respiration.

The effect of photoinhibition on the productivity of microalgal cultures substantially increases if additional stress, *e.g.* sub-optimal temperatures or high oxygen concentration are superimposed. Thus, a larger proportion of the radiation absorbed by the photosynthetic apparatus is dissipated through non-photochemical pathways resulting in a reduced biomass yield. On the other hand, in algal biotechnology warning signals must be recognized as soon as possible in order to prevent a significant reduction in daily productivity or situation which in few days may culminate in the loss of the culture. In this respect, since environmental stress affect the function of the PSII, directly or indirectly, the application of an *on-line* chlorophyll fluorescence technique can represent an useful tool to get rapid evidence of stress conditions affecting the performance of outdoor cultures.

Development of genetically modified strains with small antennae size as well as strains able to withstand to high oxygen concentrations seem to be the most promising ways to circumvent the problem of light penetration in the culture depth and to avoid inhibition of photosynthesis in mass cultures.

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