

The Growth Factors Involved in Microalgae Cultivation for Biofuel Production: A Review

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Abstract

The growing demand for energy and the negative environmental impacts of fossil fuel use are triggering global searches for a renewable and eco-friendly alternative biofuel. Microalgae are considered as one of the most promising feedstocks for biofuel production, due to many advantages including cultivation in non-arable land and being able to grow in wastewater or seawater. That is why; microalgae-based biofuels are regarded as one of the best candidates to replace fossil fuels. There are two main types of microalgae cultivation systems: Open Raceway Ponds and Closed Photobioreactors (PBRs). Due to some limitations in Open Raceways, PBRs have become the most favorable choice for biofuel producers, even though it is costly. To make the process viable, the growth of microalgae for biofuel production should be cost-effective. One way to achieve this goal is to optimize the environmental factors that influence their growth during the cultivation stage to increase the accumulation of bio-compounds of fuel. Algal growth relies mostly on nutrients, CO₂ concentration, pH and salinity, light intensity and quality, temperature and finally mixing, which directly affects all other factors. Thus, before designing PBR, a thorough study on these growth parameters is needed. In the present study, we reviewed and evaluated these growth influencing factors in an extensive way to optimize biofuel production.

Keywords

Microalgae, Biofuel, Photobioreactor, Cultivation, Growth Factors

1. Introduction

The energy crisis is increasing day-by-day due to the heavy industrial development and exponential growth of population. Fossil fuel sources like diesel, petrol, coal natural gas are depleting due to their extensive usage in motor vehicles

and industries. Furthermore, release of huge amount of toxic and harmful gases into the atmosphere pollutes the environment severely because of the continuous use of fossil fuels in different sectors. Level of greenhouse gas (GHG) is also increasing in an alarming rate, releasing CO₂ into the atmosphere and rising temperature which leads to the global warming. Among other gases, CO₂ is considered the major pollutant which damages environment the most. Although the pollutant gases are present in the atmosphere at an endurable rate, due to the emissions from the vehicles and industries, their concentration has increased over the past few decades. Imposing all these negative impacts on the environment, a major climatic change has been observed over the entire globe. At this junction, replacement of fossil fuels with other eco-friendly alternative sources is the best solution to avoid upcoming catastrophe. Microalgae based biofuel could be a very promising alternative in this aspect.

In 1942, Harder and von Witsch [1] first recommended that microalgae could be viable sources of lipids which can be used as food or to produce biofuels. Since then, enormous efforts have been given in research involving microalgae and their bioproducts. Microalgae are considered as one of the most promising sources for bioenergy production now-a-days [2] [3] [4].

The biofuels produced from microalgae have several advantages compared with that from conventional oil seeds. These include the ability to use non-arable land for microalgal cultivation, the higher productivities and possibility to use wastewater and gas flue as source of carbon and nutrients to promote the growth of microalgae [5] [6]. Though microalgae can produce different types of biofuels, such as biodiesel, bioethanol, biohydrogen, syngas, biobutanol, and bioelectricity [7] [8], yet the majority of economic analyses conclude that microalgae biofuels cannot compete with conventional fuels because of its high price [9] [10].

To minimize the production cost, industrialization of microalgae products needs large-scale culture systems. Two major types of microalgae cultivation systems are 1) Open Raceway Ponds (**Figure 1**) and 2) Closed Photobioreactors (PBRs) (**Figure 2**). Though open systems are much cheaper and easier to manage than closed systems, they have many operational problems as well, such as



Figure 1. Open raceway ponds.



Figure 2. Closed Photobioreactors (PBRs).

evaporation, contamination, susceptibility to weather conditions and huge amount of land requirements [11]. On the contrary, these limitations can be overcome by closed systems with high capital costs. In order to reduce these limitations, researchers have been invested a large amount of money in the development of new PBR designs due to the high operational control and the high productivity provided by the PBRs and thus make microalgae-based processes viable [7] [12] [13].

Microalgal growth is influenced by a variety of culture parameters, such as light intensity, pH, salinity, nutrients availability, temperature, CO₂ and dissolved oxygen concentration. Therefore, these culture parameters should be optimized to improve biomass productivity. To design a PBR for optimizing the production of microalgae, a thorough study on growth parameters is important. In the present study, we reviewed and evaluated these parameters for optimizing PBR performance. Since the design of PBR is also responsible for the microalgal growth, a short description of PBR is given at the beginning of the study.

2. Photobioreactor (PBR)

PBRs are artificial cultivation system which favors the growth of selected strain under optimal conditions such as configuration, light, temperature, pH, nutrient, mixing, etc. For commercial microalgal biomass production, closed PBRs, especially tubular PBRs have been used successfully in recent years. Due to good control of culture conditions and high solar radiation availability and thus high biomass productivity, unlike open raceways, tubular PBRs have become a potential system for biofuel production and compounds of high commercial value [14] [15].

A tubular PBR mainly consists of an array of straight transparent tubes, having a diameter of 0.1 m or less and are usually made of plastic or glass [16]. In order to maximize the sunlight capture, these transparent tubes can be arranged in different patterns (e.g., straight, bent, or spiral) and orientations (e.g., horizontal, inclined, vertical, or helical) and thus having different names, such as tubular, helical, horizontal PBR, etc. [17]. However, to scale-up the production,

the tubes are usually arrayed in a horizontal fence-like, which in one hand improves the land utilization, and on the other hand has a better angle for incident light [18].

Since the transparency of the materials and the surface/volume ratio are responsible for the light capture, Glass, plexiglass, polyvinyl chloride (PVC), acrylic-PVC, and polyethylene are the most common materials that are used for PBR construction. All these materials have appropriate transparency for the microalgae cultivations. However, since they all have their merits and demerits, so before using a type, it must need to be evaluated according to the type of process and desired product. Though glass is strong and transparent and very good material for the construction of laboratory-scale PBRs, yet it requires many connection parts for the construction of large-scale PBRs, which could be costly. For this reason, the plastic type, mainly of polyethylene is most suitable for large-scale tubular PBR [19].

PBR can be illuminated by either artificial or natural light. Though the artificial illumination is technically possible, it is expensive compared with outdoor cultivations, which is just viable for commercial production of high added value products [20].

3. Major Factors Affecting the PBR Performance

The salient components for algal growth are a growth medium with a source of light energy for photosynthesis, proper nutrients and CO₂ or air flow. Algal growth is also affected by several environmental parameters such as temperature, pH, salinity, oxygen concentration and processing parameters such as mixing and light intensity. As the culture condition varies from species to species, all of these growth factors must be specified for successful microalgae cultivation for a specific purpose [21].

3.1. Light Intensity and Quality

Availability of the light is the most important factor in the growth and productivity of photosynthetic microorganisms. As light is the main energy input for photosynthetic microorganisms, it must be maximized for better output. However, excess of light particularly coupled with sub-optimal temperature or high oxygen level can damage the photosynthetic apparatus [22]. Therefore, by adequate design of its geometry and orientation, light supply to the cultivation system must be optimized [23] [24]. The amount of light received by the cultured cells is directly related to the carbon influencing the growth rate of the cultures [25]. The growth of microalgae is determined by the photosynthesis rate, which is a direct function of the irradiance to which the cells are exposed inside the culture. **Figure 3** shows the variation of photosynthetic rate with the luminous intensity. As light intensity increases, photosynthesis in microalgae also increases until it reaches a maximum rate at the saturation point (**Figure 4**) [26] [27] [28]. Above the saturation point, the excess light leads to a phenome

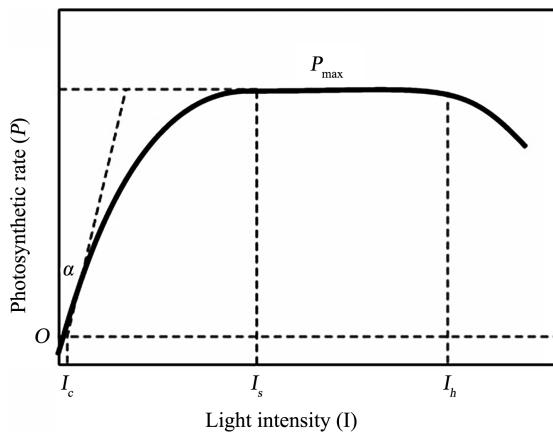


Figure 3. Variation of the photosynthetic rate with the light intensity.

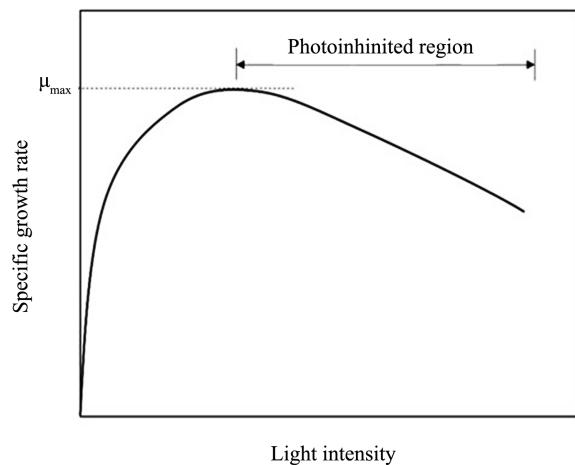


Figure 4. Effect of the light intensity on the specific growth rate of microalgae.

non called photoinhibition. The culture is then said to be photoinhibited. This irradiance is called the inhibition irradiance. For most microalgae, photosynthesis is saturated at 100 to 500 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, although the maximal productivity is obtained at the Average Irradiance (AI) values close to constant irradiance in the range of 50 - 100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [29] [30]. The photoinhibition appears at the irradiances level over 1000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in most strains, although some sensitive strains are photoinhibited at lower irradiances down to 300 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [31].

The irradiance is defined as the amount of radiation reaching a point from all directions in space, at every wavelength. However, only Photosynthetically Active Radiation (PAR), range from 400 to 700 nm, is used by microalgae to perform photosynthesis, whatever the light source is (sun, lamps, LEDs) [32]. Due to mutual shading the irradiance inside microalgae cultures is not homogeneous but a function of light intensity, culture depth, and biomass concentration. Thus cells in the outer part of the culture can be exposed to high irradiances, whereas in the inner part of the culture, cells can be in complete dark. The concept of AI was proposed to solve this problem [33] [34]. According to this concept, the AI at which the cells are exposed to inside a culture is calculated as the volumetric

integral of the corresponding local irradiance in all the points inside the culture. This local irradiance can be calculated by applying Lambert's law for the radiation arriving to the reactor surface. The value of AI is really important because this concept allows normalizing the light conditions in whatever microalgae culture system, allowing calculating an intensive variable representing the "concentration of light" inside the culture. Thus the AI can be used to analyze or model the growth of whatever microalgae as usually performed with bacteria or other heterotrophic microorganisms when considering substrate concentration. In microalgae cultures the growth-irradiance response curves have a hyperbolic shape (**Figure 5**) [35] [36]. In this curve the saturation irradiance (I_s) is the irradiance above which the growth is saturated, whereas constant irradiance (I_k) is the irradiance at which the growth is equal to half of the maximal specific growth rate. Therefore, the influence of light on the growth of any particular strain must be studied in each particular location as it varies from place to place. Gonçalves *et al.* [37] evaluated the effect of light on the growth of microalgae (*C. vulgaris*, *P. subcapitata*, *Microcystis aeruginosa* and *Synechocystis salina*) and nutrients uptake. In the case of *C. vulgaris*, these authors found that the optimum daily irradiance was $208 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

It has been reported that the optimal condition for the growth of microalgae will depend not only on the light intensity but also on the wavelength and the photoperiod to which the cells are exposed [38]. As microalgae need light for their metabolic activity, its growth is strongly influenced by this culture parameter, both in terms of quality (wavelength, the light source used, among others) and quantity (light intensity and light period). The light source can be either artificial or natural (solar). Though the latter being the most economically viable due to its availability, in high value-added cultures, artificial light can also be employed because it allows the precise control of photosynthesis and photoperiod [39].

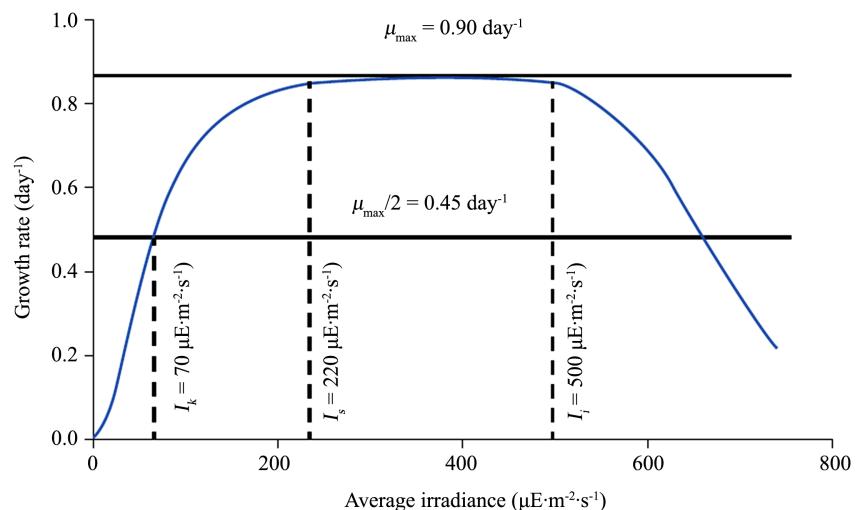


Figure 5. Growth-irradiance response curve of *Scenedesmus almeriensis* indicating the value of characteristics variables for this strain.

Different artificial light sources can be used for microalgal cultivation, such as Light-Emitting Diodes (LEDs), halogen lamps, fluorescent lamps and incandescent bulbs. These light sources vary from each other in energy consumption, spectrum, wavelength distribution and cost. Considering all these characteristics, fluorescent lamps and LEDs are the most commonly used light sources for microalgal production. Comparing with the fluorescent lamps, the use of LEDs has better control of light and the use of different wavelengths, which can be favorable for biomass production [40]. The depth that the light can reach in the culture is independent of the light intensity. Thus, for complementing the natural light, or even for cultures under the artificial light, it is recommended to place LEDs inside the medium to improve the delivery and distribution of photons [41].

Due to the stirring system in microalgae culture tanks, there are the light/dark (L/D) cycles found in the medium. These cycles are responsible for the increase in the photosynthetic conversion and biomass productivity in microalgae, reported by Takache *et al.* [42]. The same authors reported that the efficiency of photosynthesis in *Chlamydomonas reinhardtii* was enhanced when L/D cycles of less than 20 s were applied, with an increase in the growth rate of up to 40%, depending on the conditions of the L/D cycle.

The variation of light wavelength has significant impact in the microalgal growth. The red light can promote higher growth rates with smaller cells and low nutrient uptake. On the other hand, the blue light affects gene expression and some metabolic pathways of microalgae, triggering a high nutrient uptake, but inducing lower growth rates with the larger cells. Due to the lack of phycobilins, green microalgae cannot use yellow and green light effectively [43]. Satthong *et al.* [44] studied the light effect on the growth of *C. vulgaris* TISTR8580. Their obtained results are shown in Figure 6. It illustrates the number of *C. vulgaris* cells at different times and conditions. They have carefully studied the growth of *C. vulgaris* from algae cells cultured under the different light sources, including the white LED light, the red LED light, and the fluorescence light. From the experiments, it was observed that the microalgae are in a state of lag phase during day 0 and day 1. In this period, the algae population remains constant as they are adapting to the new environment. However, the cell density increases dramatically during day 2 to day 3, as compared to day 1. This reflects the typical nature of the exponential growth phase, namely the fast-growing phase. After day 4 to the last day of the experiment, the algae are in a stationary state phase in which the algae population is consistently at maximum. They experimentally found that the white LED light source gives the highest population density on day 14 at 66.63×10^6 cell/ml. Algae under a fluorescent light source with the white light had the highest density on day 14 at 79.33×10^6 cell/ml. The algae under the red LED light source give the highest density on day 3 at 76.83×10^6 cell/ml. Although the cell density of the algae under the red LED light is more than that under white LED light, during the stationary state phase on day 3, the cell density under red LED light is decreased more than that in the case of the white LED light on day 4.

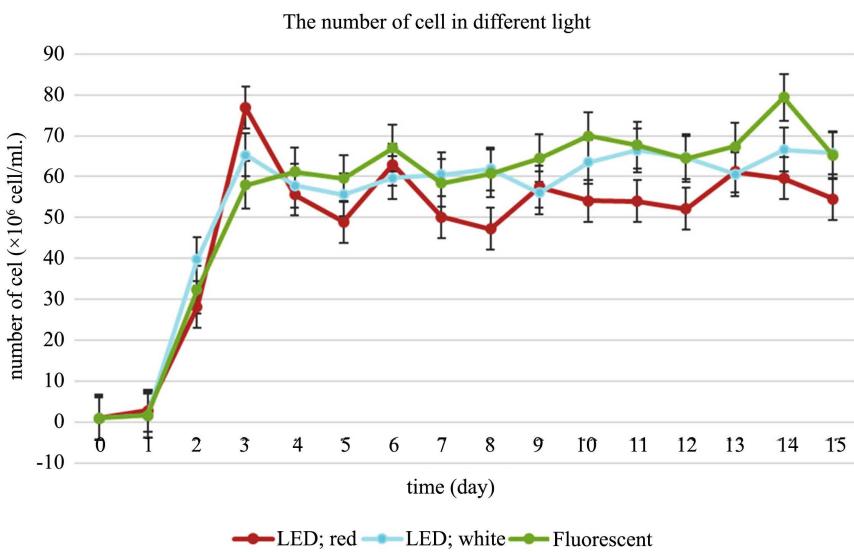


Figure 6. The graph between the density of *C. vulgaris* cell and time using different light sources, 4000 lux, 12 hr: 12 hr L/D cycle, 0.4 vvm air flow rate, with temperature $28^\circ\text{C} \pm 2^\circ\text{C}$.

Figure 7 shows the variation in growth rate of different algae species with their irradiance levels, where the growth rate of only *N. incerta* was studied in presence of the white and blue light. The maximum growth rate of *P. globosa* was obtained at an irradiance level (white light) of $150 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ among the algae species as reported by Sing *et al.* [45] in their review article. The minimum growth rate of *N. incerta* was reported at an irradiance level (blue light) of $150 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Very recently, Esteve *et al.* [46] conducted an experiment with LEDs with different wavelengths: $380 \pm 750 \text{ nm}$ (white), $620 \pm 750 \text{ nm}$ (red) and $450 \pm 495 \text{ nm}$ (blue). They reported that the maximum specific growth rate was obtained by *N. oleoabundans* with white LEDs ($0.264 \pm 0.005 \text{ d}^{-1}$), whereas the maximum biomass productivity ($14 \pm 4 \text{ mg}_{\text{dw}} \text{ L}^{-1}\cdot\text{d}^{-1}$) and CO_2 fixation rate ($11.4 \text{ mg}_{\text{CO}_2} \text{ L}^{-1}\cdot\text{d}^{-1}$) were obtained by *C. vulgaris* (also with white LEDs). Sometimes, combination of different wavelengths yields maximum growth. This is justified by Fu *et al.* [47], who demonstrated that *Dunaliella salina* obtained the highest production of biomass and carotenoids (β -carotene and lutein) with the combined use of 75% of red light (wavelength around 700 nm) and 25% of blue light (wavelength around 400 nm), compared to just the red light.

Metsoviti *et al.* [48] conducted an experiment on the effect of solar irradiance on *C. vulgaris* cultivated in open bioreactors under greenhouse and they found that the increase in solar irradiance led to faster growth rates of *C. vulgaris* under both environmental conditions studied in the greenhouse (in June up to 0.33 d^{-1} and in September up to 0.29 d^{-1}) and higher lipid content in microalgal biomass (in June up to 25.6% and in September up to 24.7%). They have also examined the ratio of light intensity in the 420 - 520 nm range to light in the 580 - 680 nm range ($I_{420-520}/I_{580-680}$) and of artificial irradiation provided by red and

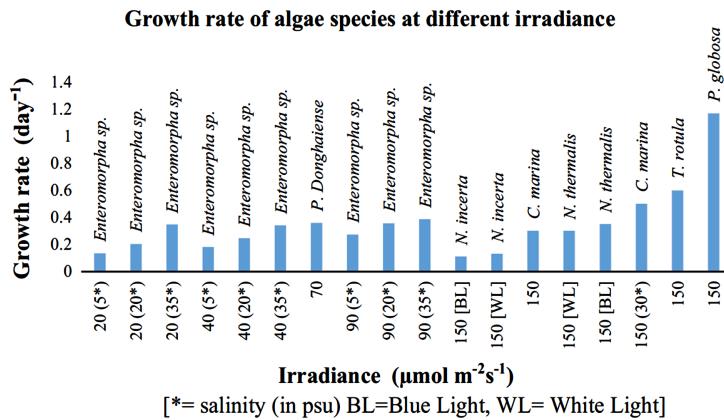


Figure 7. The Growth rate of algae species at different irradiance.

white LED lamps in a closed flat plate laboratory bioreactor on the growth rate and composition. In the experiments conducted in the closed bioreactor, the increase of $I_{420-520}/I_{580-680}$ ratio results an increase in the specific growth rate and the biomass, protein and lipid productivities as well. Additionally, the increase in light intensity with red and white LED lamps resulted in faster growth rates (up to 0.36 d^{-1}) and higher lipid content (up to 22.2%), while the protein, fiber, ash and moisture content remained relatively constant. Overall, the trend in biomass, lipid, and protein productivities as a function of the light intensity was similar in the two systems (greenhouse and bioreactor).

Generally, if the light intensity increases, the microalgal growth also increases up to a photoinhibitory threshold, but it varies among species [49] [50]. Like cell growth, microalgal lipid production is also influenced by the light intensity and it is of particular interest because lipids are the sources of biodiesel. However, increases in light intensity promote or have no effect on lipid production of some species [51] [52], but reduce lipid contents in others [53]. Therefore, studies on the effects of light intensity on lipid production are very important on a species-by-species basis. Lipids, carbohydrates, and proteins are the main ingredients of microalgae [54]. Therefore, if lipid contents increase in a cell, carbohydrates, proteins, or both automatically decrease. An increase in lipids and a decrease in carbohydrate content are often reported due to the nitrogen starvation [55] [56] [57]. Apart from the variable effects on lipid production mentioned above, little is known about how light intensity affects the biochemical composition of microalgae. Therefore, to optimize microalgal lipid production to generate biodiesel, it is important to determine how the production of all three biochemical components changes with light intensity.

To optimize the light intensity for microalgal growth and lipid content various statistical methods have been used by researchers. For instance, the effect of light intensity on *Ettlia* sp. was studied by Kim *et al.* [58] and by conducting tests using response surface methodology with Central Composite Face-centered (CCF) design, they found the optimal light intensity of $730 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ where the maximum biomass productivity was reported as $28 \pm 1.5 \text{ gm}^{-2}\cdot\text{d}^{-1}$. However, maxi-

mum lipid productivity was obtained at $500 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which was reported as $4.2 \pm 0.3 \text{ gm}^{-2}\cdot\text{d}^{-1}$. **Table 1** shows the optimal light intensity for different microalgae species [37].

An increase of lipid production has been reported by *S. abundans* by increasing the light intensity from 55 to $110 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [59]. Similarly, several *Chlorella* species reportedly produce more lipids at a high light intensity ($600 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) than at lower light intensities [60]. For instance, high biomass and lipid content of $20 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was reported by the species *C. vulgaris* [61]. This may be possible at least partly because at high light intensities algae counter photo-oxidation by converting excess photo assimilates into fatty acids [62]. However, some recent studies have found that at high light intensity, lipid contents of various microalgae, including marine strains of *Chlorella*, reduced despite increasing their biomass, which suggest that instead of being stored in the form of lipids, the energy produced was used for cell division [53] [63]. Recently, Nzayisenga *et al.* [64] also found that *C. vulgaris* and *E. pseudoalveolaris* had lower lipid contents when grown at $300 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light than that at lower light intensities, despite increases in biomass (**Figure 8**). Studying with two more species with the highest biomass yields for 15 days, they reported that during the period between 8 and 15 days, fatty acid contents of *S. obliquus* growing at $300 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light doubled, from 5.8% to 11.6%, but changed little at the 50 and $150 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensities (**Figure 8**). In contrast, fatty acid contents of *Desmodesmus* sp. slightly increased during this period under all light intensities. It has been recommended that increases in lipid production under high light intensities may be

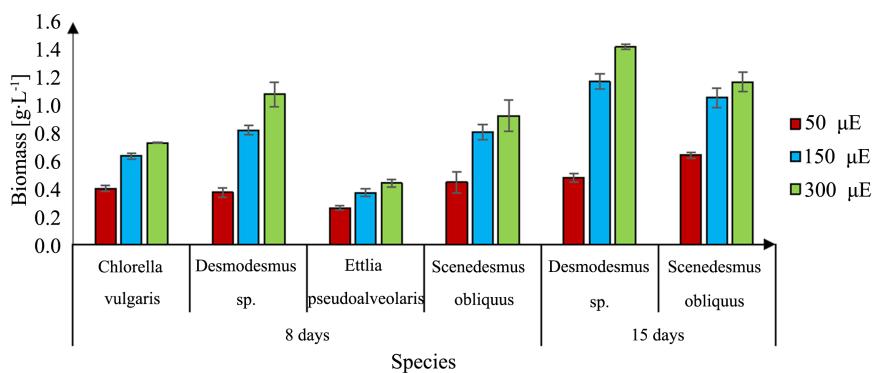


Figure 8. Biomass of the four microalgal strains after growth for indicated times under indicated light intensities: mean \pm standard deviation ($n = 3$ from three separate experiments).

Table 1. Optimal light intensity for different microalgae species.

Microalgae species	Optimal average light irradiance ($\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
<i>C. vulgaris</i>	208
<i>P. subcapitata</i>	258
<i>M. aeruginosa</i>	140
<i>M. aeruginosa</i>	178

partly caused by starvation [65]. However, the fatty acid contents of *Desmodesmus* sp. and *S. obliquus* (grown for either 8 or 15 days) increased with the increases in the light intensity, their protein contents declined, and no significant change observed in their carbohydrate contents [64]. Similarly, reductions in protein contents and increases in lipid contents of *Dunaliella tertiolecta* associated with increases in light intensity have been observed [66]. Their current results also show that higher lipid content is linked to lower protein content, suggesting that lipid synthesis relied mostly on protein degradation or inhibition of protein synthesis [64]. This is also supported by He *et al.* [67], which showed that decrease of protein content under increasing light intensity may be attributed to the consumption of nitrogen. Thus, it is possible that microalgae may have different mechanisms to synthesize fatty acids under high light intensities and/or nutrient starvation, which could affect either protein or carbohydrate content.

In most production systems, as soon as a high cell concentration is reached, the proportion of light zones to dark zones becomes too low and the retention time of cells in the dark area becomes too long, which increases respiration, leading to biomass losses [68]. To avoid photo limitation and maximize the growth, flashing (or pulsed-) light emitting diodes (LEDs) are used recently to generate high-light flashes artificially, which penetrate deep into the culture [69] [70]. The potential benefits of flashing light reported in previous studies differed considerably, and they usually focused on flashing conditions of low frequencies ($f < 100$ Hz) and relatively high duty cycles ($DC > 0.1$) that were used to mimic light regimes in mixed cultures [28] [68]. It was uncertain if flashing light of high frequencies and short duty cycles (e.g., $f > 100$ Hz, $DC < 0.1$) can indeed improve microalgae growth performance. In Schulze *et al.* [70], they reviewed the potential of flashing light to improve microalgal growth and suggested a minimum theoretical frequency threshold of 200 - 333 Hz which is necessary to obtain the biological flashing light effect in microalgae. Very recently, Schulze *et al.* [71] performed another experiment on flashing light applied to *Chlorella stigmatophora* and *Tetraselmis chui* and they have concluded that artificial flashing light does not improve microalgal biomass productivities in photobioreactor, but low frequencies ($f < 50$ Hz) may be still used to improve light harvesting-associated biomolecules production. Similarly, several studies have reported the importance of light intensity on microalgal growth substantiating the increase of biomass concentration under optimal light conditions [72] [73] [74].

3.2. Temperature

Temperature is considered as one of the most significant environmental factors that influence algal growth rate, cell size, biochemical composition and nutrient requirements. Microalgae cultures absorb heat by radiation from the light source used resulting the increase of temperature in the culture. Thus, for a large scale

outdoor culture the irradiance of sunlight and associated temperature is also needed to consider [75]. The optimal temperature for microalgae growth ranges from 20°C to 35°C, although some mesophilic species can endure up to 40°C. Below the optimal temperature the yield of the strain gets reduced, but overheating of the cultures has been identified as critical since it can damage the cells [76]. Therefore, seasonal variations, which lead to the temperature variations during the day/night cycle, have significant effects on microalgal cultivation.

The optimal temperature for *S. almeriensis* is 35°C, but the cultures die at temperatures higher than 45°C (Figure 9) [77]. In case of small-scale reactors, no temperature control is required because the input of heat by radiation is compensated by the output by convection if the air surrounding system is cold enough. However, in outdoor large-scale reactors the solar radiation is high, and additional heat control systems must be used to avoid overheating [78] [79]. Thus, a cost-effective cooling system set-up is necessary for better performance of the PBR.

To prevent overheating of the microalgae cultivation several methods have been tested by researchers. Among them are as follows: 1) using shades with dark-colored sheets [80], 2) cooling by spraying water on the surface of the photobioreactor [81], 3) submerging the entire culture or part of the photobioreactor in a large amount of water [81], and 4) installing a heat exchanger for the photobioreactor [82]. However, shading the PBR greatly reduces the illumination and consequently in the yield of biomass and thus it is inefficient. Though water spraying is one of the most common methods and efficient for cooling, it is only useful in locations with low air humidity and it also increases cultivation costs. On the other hand, controlling temperature by the method of submersion has been demonstrated to promote the average light intensity in the culture.

Several studies have been performed by researchers to show that the temperature has a positive impact on biomass and lipid yield of microalgae [83] [84] [85]. For instance, maximum lipid productivity of $274.15 \text{ mgL}^{-1}\cdot\text{d}^{-1}$ was observed under optimal temperature and pH at 28.63°C and 6.51 respectively for

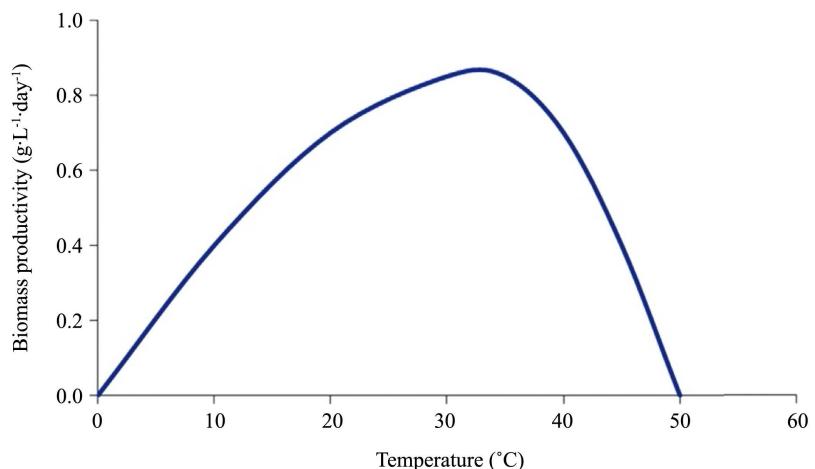


Figure 9. Influence of the temperature in the biomass productivity of *Scenedesmus almeriensis* in continuous cultures at laboratory conditions.

Chlorella protothecoides, which was statistically proven by response surface methodology using Box-Behnken design with smaller p-value ($p < 0.001$) indicating its significance [86]. Gonçalves *et al.* [37] evaluated the effect of temperature on the growth of microalgae (*C. vulgaris*, *P. subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa*) and nutrients uptake. In the case of *C. vulgaris*, these authors found that the optimum temperature for growth was 25°C. According to study conducted by Singh *et al.* [45] some species such as *Chlorella*, *Nannochloropsis*, *Neochloris*, *Scenedesmus*, *Spirogyra*, *Chlamydomonas*, *Botryococcus*, *Haematococcus*, *Ulva* species, few red algae, brown algae and blue-green algae can grow in a temperature range of 20°C - 30°C with the light intensity in the range of 33 - 400 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [87]. It was observed that at the optimal temperature of 25°C and 20°C for *Nannochloropsis oculata* and *Tetraselmis subcordiformis* respectively, the growth rate was higher whereas the neutral lipid concentration was found to be at higher level under 15°C and 20°C for *T. subcordiformis* and *N. oculata* respectively [88]. It is also reported that, at high temperature the cell metabolism disrupts and stop the cell production through enzyme damage [89].

3.3. Nutrients

Conditions of nutrient limitation affect a considerable variation in the biochemical composition of microalgae. An ideal culture medium for microalgae must contain inorganic elements such as Phosphorus (P), Nitrogen (N), and Iron (Fe), among others, which may vary according to the cultivated species. The minimum nutritional requirements needed for the growth of microalgae can be determined by the approximate molecular formula $\text{CO}_{0.48}\text{H}_{1.83}\text{N}_{0.11}\text{P}_{0.01}$ [31]. Thus, the most important nutrients or macronutrients for autotrophic growth are the carbon (C), nitrogen (N) and phosphorus (P) [26]. According to the molecular formula of biomass, it is reasonable to say that about 50% of the biomass is composed of carbon (C) [31]. Carbon is needed in high concentrations, since it is the vital constituent of all organic substances synthesized by the cells, such as carbohydrates, proteins, nucleic acids, vitamins and lipids [26]. Microalgae have inorganic carbon assimilation processes: diffusion ($5.0 < \text{pH} < 7.0$) and active transport ($\text{pH} > 7.0$) [90]. CO_2 and bicarbonates (HCO_3^-) supply are very important in order to achieve high autotrophic production rates [26]. Organic compounds (e.g. sugars, acids and alcohols) can also be used as carbon source for certain species of microalgae that grow in mixotrophic conditions.

Nitrogen is a vital element of structural and operating proteins, the most important element after carbon [81]. It is the second most abundant element in microalgal biomass containing 1% to 14% concentration in dry mass. It is responsible for the formation of proteins, nucleic acids, vitamins and photosynthetic pigments [26]. The assimilation mechanism of nitrate and ammonium (NH_4^+) by microalgae is active transport [90]. Nitrogen is mainly provided as N_2 and in some cases, in the inorganic forms NO_3^- , NO_2^- , NO , NH_4^+ , or in the

organic form, through urea or amino acids [91]. Silva *et al.* [92] evaluated the preferred source of nitrogen (NO_3^- and NH_4^+) for two species of microalgae (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*) and they concluded that the ammonium (NH_4^+) was preferred source of nitrogen for microalgae *C. vulgaris*, since its assimilation by the microalgae involves lower energy consumption [93]. Microalgal growth rate is nearly identical depending on the nitrogen sources used (urea, nitrite, and nitrate). Deficit of nitrogen concentration in the cultivation allows lipids and carbohydrates to be synthesized preferentially [94]. When the microalgae suffer nitrogen shortage, a discoloration of the cells usually occurs (reduction of chlorophylls and carotenoids increase) and a build-up of organic compounds such as polysaccharides and some oils [81]. Goiris *et al.* [95] studied the impact of nutrient limitation in the production of antioxidants in three species of microalgae (*Phaeodactylum tricornutum*, *Tetraselmis suecica* and *C. vulgaris*) and they observed the content of chlorophyll a in biomass was significantly lower when the microalgae were limited by nitrogen.

Phosphorus is another essential nutrient for growth and for many cellular metabolic activities, such as energy transfer, synthesis of nucleic acids, deoxyribonucleic acid (DNA), among others [26]. The concentration of phosphorus can range from 0.05% to 3.3% in dry mass [96]. In wastewater, as well as in natural environments, phosphorus is present in various forms, such as polyphosphate, pyrophosphate, orthophosphate, and metaphosphate [97]. Like nitrogen, phosphorus is also assimilated by the microalgae through active transport [90]. Absorption of this chemical element is energy dependent and it is preferentially added in the form of orthophosphate (PO_4^{3-}) [26]. The composition of biomass production is also influenced by the supply of phosphorus [98]. Internal and external phosphorus supply affects lipids and carbohydrates contents. Moreover, the N: P ratio in the culture medium is also important, as it influences not only the productivity, but also the dominant species in culture [26]. The N: P ratio of 16:1 was first estimated by Alfred C. Redfield in 1934 through the elemental composition of microalgal cells. This ratio is known as Redfield ratio after his name. However, several studies have tested different ratios [99] [100] [101] [102]. Silva *et al.* [92] evaluated the effect of N:P ratio on the growth of microalgae *C. vulgaris* and *P. subcapitata* and the N: P ratios of 8:1, 16:1 and 24:1 were evaluated. For *C. vulgaris*, the N: P ratio of 8:1 was the one that more favored the growth. Reduction of phosphorus may cause pigment accumulation in some microalgae, but the impact is lower than the nitrogen deficiency [81]. Its absent or present at low concentrations can be limiting or affecting the biomass productivity of several microalgae species [103]. Microalgae can amass intracellular reserves of phosphorus, which can be used when phosphate is exhausted in the medium, a behavior known as luxury uptake or accumulation [104]. When the objective is to remove phosphorus from wastewater, it can be used; however, in cultures where synthetic fertilizers are used, luxury uptake should be avoided to maximize the biomass production per mass of nutrients added [97].

Besides the macronutrients already mentioned (nitrogen, phosphorus and potassium), for the adequate growth of microalgae, the medium should contain other nutrients (micronutrients) too. The essential micronutrients are Mg, S, Na, Cl, Ca, Fe, Mo, Mn, Zn, Cu, B and Co, with prominence on magnesium, sulfur and iron (Mg, S and Fe, respectively). Iron is an essential trace element for microalgae growth because of its involvement in the transport of electrons in the process of photosynthesis [105]. Most of these micronutrients can be found in both wastewater and seawater [106]. Fertilizers and salts can also be used as sources of these micronutrients [97].

Table 2 depicts how addition of nutrients increases biomass production and shortens the time of cultivation. The cultivation of *Chlamydomonas* sp. in a Tubular PBR, in Run-1, the time was required for microalgae cultivation (10 days) was longer than Run-4 which only takes 7 days, so that, the growth rate in Run-4 was greater than Run-1. Microalgae obtain foods from the addition of nutrients to support their growth, thus shortening the time of cultivation [107].

3.4. Carbon Dioxide (CO₂)

Carbon dioxide (CO₂) is another important factor that involved in the growth of microalgae production. To produce 1 kg of biomass, microalgae require from 1.8 to 2.0 kg of CO₂ [31]. Considering this ratio, the amount of CO₂ present in the air (0.03%) is not enough to provide the necessary gas pressure in the culture to promote high productivity. Thus, for increasing photosynthetic efficiency in their growth, it is necessary to supply carbon, either in the form of salts, such as bicarbonate, or by injection of CO₂-rich air in the culture [108] [109]. Dúran *et al.* [110] demonstrated that using air injection (600 mL·min⁻¹) in a photobioreactor, microalgae showed optimal growth with up to 20% (volume per volume) of CO₂ present in the injected air, not differing much from the optimal value for microalgae growth. This creates the possibility of using CO₂ from industrial burning; a process that generates on average 5.0% (volume per volume) of CO₂, and depending on the technology and type of fuel used, this concentration may reach up to 20% [111]. This use combines a low-cost source of carbon for microalgae with the reduction of CO₂ emissions to the atmosphere. The supply of CO₂ to microalgae cultures allows increasing biomass productivity, but the reduction of pH, which can inhibit the growth of some species of these microorganisms [112].

Table 2. The effect of adding nutrients against time of cultivation and the microalgae growth rate (μ)/day.

Parameter	Without nutrient (Run-1)	Addition of nutrient (Run-4)
Cultivation time	10 days	7 days
Growth rate (μ)/day (Q = 0.071 L/min)	0.16	0.33
Biomass production (gr/dm ³)	4.892	5.684

For optimizing CO₂ concentration in *Ettlia* sp. to produce maximum biomass and lipid concentration an experiment has been done by Kim *et al.* [58] using response surface methodology with Central Composite Face-centered (CCF) design and they have found 8% CO₂ and 7% CO₂ as optimal concentration for maximum biomass ($28 \pm 1.5 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) and lipid ($4.2 \pm 0.3 \text{ g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) productivity respectively. The optimal concentration of CO₂ was found to be 6.5% for *Chlorella vulgaris* which was statistically determined through response surface methodology with Central Composite Design (CCD) having $R^2 > 0.90$ [113]. However, maximum specific growth rate of 0.310 d^{-1} was reported in a study conducted by Kasiri *et al.* [114] at 22% CO₂ concentration whereas at 35% CO₂ concentration, maximum CO₂ uptake rate of $63.03 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ was observed for *Chlorella kessleri*.

Hadiyanto *et al.* [107] studied the growth rate of *Chlamydomonas* sp. compared with the productivity in terms of carbon dioxide flow rate, as shown in **Figure 10**. It shows that in the variation of 10% and 20% (% v) CO₂ concentration, both growth rate and biomass productivity increase. However, in the variation of 30% and 40% (% v) CO₂ concentration, either growth rate and biomass productivity begin a constant. This shows that bicarbonate ion (HCO₃⁻) at 10% and 20% (% v) concentration, still be converted into biomass by the culture with the help of Carbonic Anhydrate (CA), while the 30% and 40% (% v) CO₂ concentration, CA began to saturate so that the efficiency of CA, in the use of bicarbonate ion, start to decrease.

3.5. Hydrogen Potential (pH) and Salinity

The pH has great significance in microalgal cultures, because, besides affecting the microalgae themselves, it determines the solubility of minerals and CO₂ in the medium [115]. Several factors such as composition and buffering capacity,

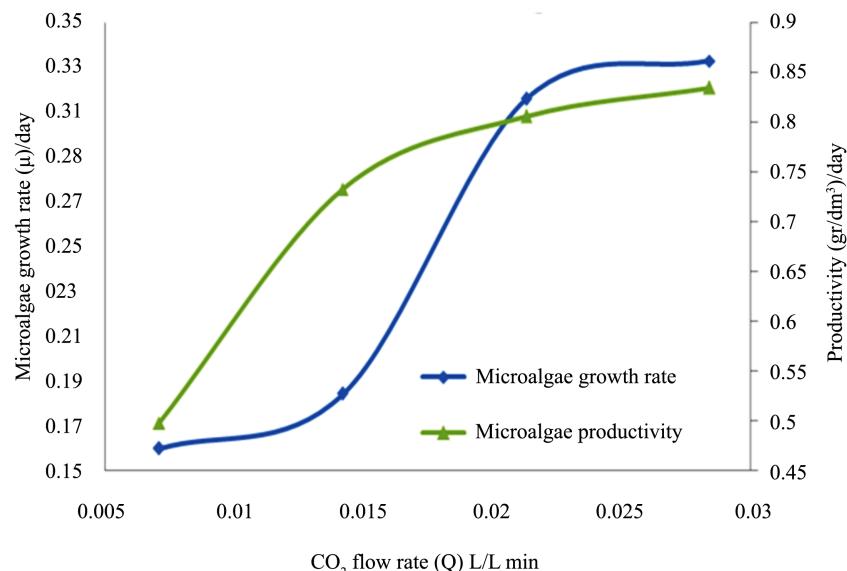


Figure 10. Growth rate and productivity of microalgae.

amount of dissolved CO₂, temperature and metabolic activity of the cells may influence the pH of the culture medium [116]. Levels of tolerance to the pH of the culture medium vary species to species, which may affect the growth rate, but the most common pH values for microalgae culture vary from 6 to 8 [27] [117]. Most microalgae usually tolerate wide pH intervals, but beyond this interval the yield is greatly reduced. Optimal pH values for microalgae range from neutral to slightly alkaline (7.0 - 10.0), although some species have optimal pH at acidic values below 3.0 [118] [119]. The optimal pH ranges from 7.5 to 8.5 and the biomass productivity is strongly decreasing at pH above 9.0 in the case of *Scenedesmus almeriensis* (**Figure 11**) [77].

The hydroxide ion (OH⁻) accumulates in the growing medium, leading to a gradual increase of pH during the photosynthetic CO₂ fixation [26]. This shifts the chemical equilibrium of the inorganic carbon present in the medium towards the formation of carbonates (CO₃²⁻). However, they are not the preferred carbon source for microalgae [120]. On the other hand, a decrease of solution pH moves the chemical equilibrium towards the formation of CO₂, which is considered one of the preferred carbon sources for microalgae. Nevertheless, this process can lead to the release of CO₂ into the atmosphere, decreasing the concentration of this nutrient extremely important for the cultivation of microalgae.

Addition of nitrogen to the culture also influences the pH. When nitrogen is provided in the form of ammonium, the solution pH increases by decreasing the concentration of nitrogen available for microalgae [121] [122]. High pH values shift the chemical equilibrium of ammonium for the production of ammonia which can be released into the atmosphere due to the aeration of the culture, reducing nitrogen availability for microalgae.

The concentration of phosphorus in culture medium can also be influenced by elevated pH, as it can lead to precipitation of phosphate (in the forms of calcium phosphate, iron phosphate and aluminium phosphate) and therefore limit the amount of phosphorus available for microalgae [122] [123].

The pH can directly affect the microalgae, as the pH of microalgal cytoplasm is neutral or slightly alkaline, and enzymes are pH-sensitive and may be inactive

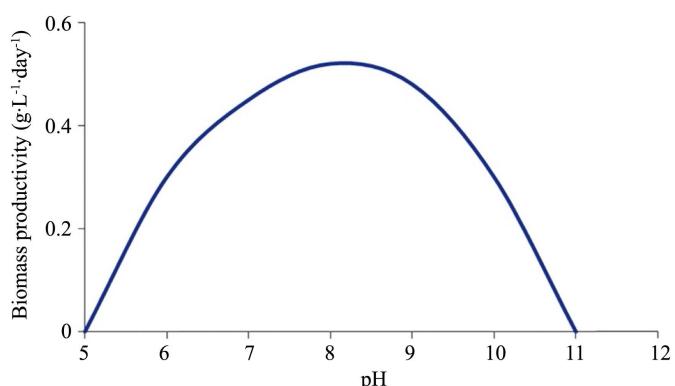


Figure 11. Influence of pH in the biomass productivity of *Scenedesmus almeriensis* in continuous cultures at laboratory conditions.

in acidic conditions [124]. Therefore, extreme pH conditions can cause the disruption of many cellular processes, which may lead to the collapse of culture [93].

Tripathi *et al.* [125] studied the effect of pH on the growth of *Scenedesmus* sp. in a pH range from 7 to 10 and concluded that the optimal pH for this species was 8. Munir *et al.* [126] examined the pH effect on the growth of two microalgae species (*Spirogyra* sp. and *Oedogonium* sp.) in a range of 6.5 - 9.0, achieving the highest growth at pH 7.5 for both species. Wu *et al.* [127] studied the effect of pH on the growth of *Scenedesmus* sp. LX1. by varying the pH from 5 to 11 and observed that, for pH values of 7, 9 and 11, there was no significant difference of growth in the cultures. However, there was a significant limitation in the growth of the microalgae at pH 5.

The variation of pH can be reduced by using buffers in the cultures, but for large-scale systems, it is costly. As CO₂, when dissolved, reduces the pH of the medium, pumping atmospheric air (0.03% of CO₂) or CO₂-enriched air through the aeration of the cultures can regulate pH variation in the culture [128].

Figure 12 shows the growth curves of *Chlorella sorokiniana* DOE1412 and CO₂ addition during PBR cultivation at different pH values. Data are the average

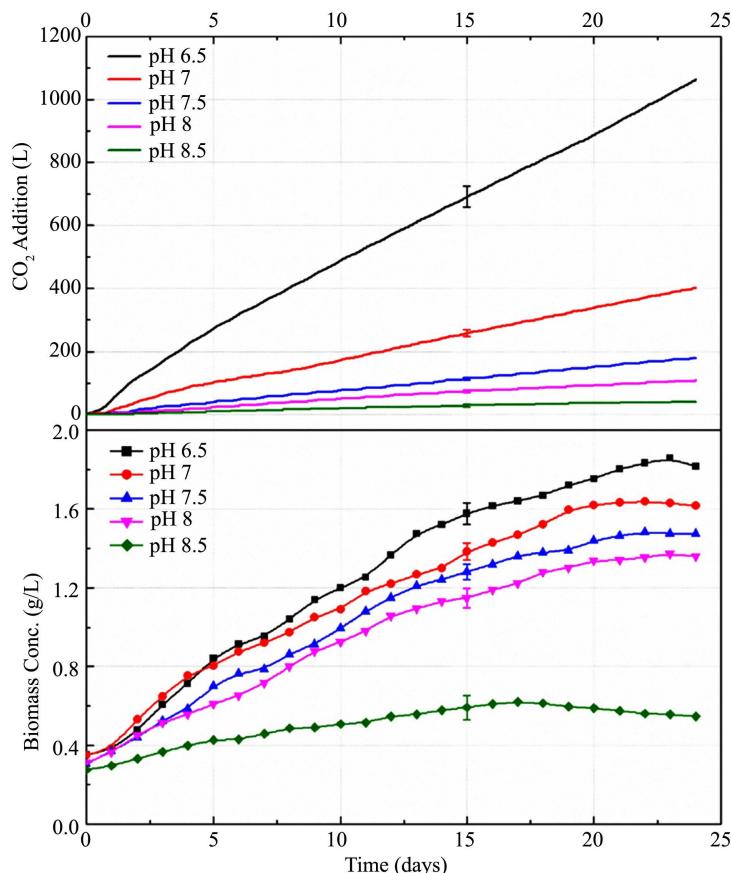


Figure 12. Growth curves for *Chlorella sorokiniana* DOE1412 and CO₂ addition for cultures at different pH in a 90 L PBR. Data are the average of duplicates. \pm S.D. lines at $t = 15$ day are shown.

of duplicates. DOE1412 exhibited a longer linear growth phase with lower growth rates in these larger reactors, which were light limited. The linear growth rates calculated using the first ten days of data were 0.091 g/L-day for pH 6.5, 0.074 g/L-day for pH 7, 0.068 g/L-day for pH 7.5, 0.061 g/L-day for pH 8, and 0.023 g/L-day for pH 8.5. Biomass growth rates decreased with increasing pH from 6.5 to 8.5, with a significant drop in rate at a pH of 8.5 (about a quarter of the rate at pH 6.5) [115]. However, the lipid content was not a function of pH in the PBRs as no significant differences were found (See **Figure 13** oneway ANOVA: $p > 0.05$). Overall, the lipid content was slightly lower at the larger scale (25.7% by mass), which is often observed. These values were similar to the observations of Moheimani [129].

Salinity is another factor that requires attention, during microalgae cultivation because in open culture it tends to increase due to the intense evaporation, increasing their concentration in the medium. Some species of microalgae, especially those found in freshwater environments, are very restricted in terms of salinity. In general, based on their tolerance to salinity microalgae can be divided into three categories: oligohaline, when they can develop only in water with low salinity (maximum salinity between 0.5 and 5 g·kg⁻¹); mesohaline, when they develop in environments of moderately saline water, with salinity between 5 and 18 g·kg⁻¹, and polyhaline, when they can develop in highly saline water, with salinity between 18 and 30 g·kg⁻¹ [130].

3.6. Mixing

The mixing plays a key role in the balance of gases and pH of the system. Sufficient turbulence of microalgae cultures minimizes the existence of gradients that can limit the performance of the cells. Thus mixing reduces the gradient of nutrients in the culture broth, avoids cell sedimentation in the system, and forces the cells to move between dark to light zones, enhancing photosynthesis [131]. It helps to facilitate heat transfer and avoid thermal stratification by ensuring all cells of the population to have uniform average exposure to light and nutrient.

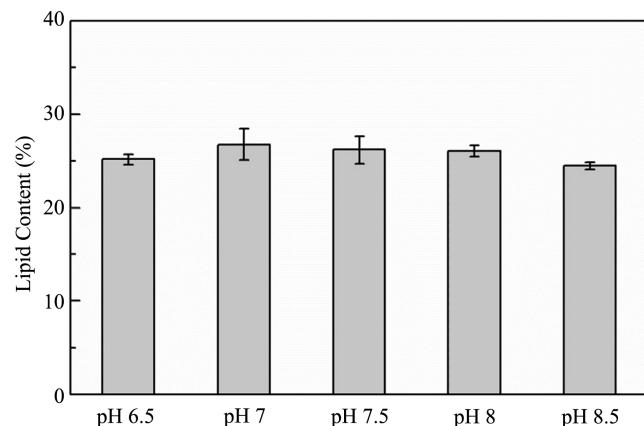


Figure 13. Lipid content for *Chlorella sorokiniana* DOE1412 grown at different pH in a 90 L PBR. Data are shown as means \pm S.D., $n = 4$.

The mixing is usually provided by aeration with CO₂-enriched gas bubbles or pumping, mechanical agitation, or a combination of these means in the tubular PBR. Since, some of the species do not tolerate vigorous agitations, so care must be taken in choosing the appropriate system [19] [132]. The energy supplied imposes a cost, whatever the mixing method is, which must be optimized. Furthermore, excessive mixing can damage cells and consequently reduce the growth of the culture. Thus aeration and/or agitation by pneumatic and mechanical devices may produce cell damage if microalgae are susceptible to hydrodynamic and mechanical shear forces, thus impacting culture performance [133] [134]. Main factors determining shear sensitivity are the type of microalgae (the presence of fragile flagellate), composition and thickness of the cell wall, intensity and nature of the shear stress, and adequacy of culture conditions to which the cells are exposed (pH, temperature, irradiance, etc.) [131]. The shear rates for a single phase flow and multi-phase flow have been thoroughly studied by Deb *et al.* [135] [136].

In a study conducted by Sánchez *et al.* [137], it was noticed that in raceway culture systems stirred by paddles, the daily growth of *Isochrysis galbana* microalgae culture was double when compared to the system without stirring (8.8×10^5 and 4.0×10^5 cells mL⁻¹·d⁻¹). This shows the importance of stirring the culture medium in the industrial processes of microalgae production.

Sobczuk *et al.* [138] investigated the mixing effect on biomass concentration for *P. tricornutum*. Their results are shown in Figure 14. Initially, the biomass concentration remained constant and steady at an agitation speed of 150 rpm (*i.e.* impeller tip speed of $0.68 \text{ m}\cdot\text{s}^{-1}$). After that the agitation rate was increased stepwise from 150 to 550 rpm. The biomass concentration changed and attained new steady states for each step change in the agitation rate. The increase in the agitation rate increased the biomass concentration up to a maximum impeller speed of 350 rpm (impeller tip speed of $1.56 \text{ m}\cdot\text{s}^{-1}$) even though the dilution rate was held constant at 0.0139 h^{-1} throughout the experiment. Further increase in the agitation speed declined the biomass concentration, but stable steady states were achieved up to the highest impeller speed investigated.

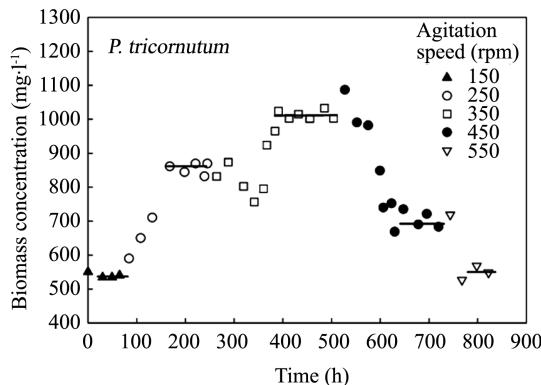


Figure 14. *P. tricornutum* biomass concentration versus time in the continuous culture at various impeller agitation speeds. Horizontal lines indicate steady states.

4. Conclusions

There is no doubt that microalgae have tremendous potential as a source of bio-fuel, food, feed and high value bio-compounds. Since they are rich in lipids, proteins, and carbohydrates, which are the sources of many beneficial products for mankind, in the near future, microalgae will generate clean energy and third generation biofuels, thus contributing to sustainable development both environmentally and economically. However, there are still limitations in the productivity of microalgae. Therefore, more researches needed to further improve the existing technology. For instance, more advanced culturing techniques incorporating with novel biotechnology should be developed to increase the productivity of microalgae. As the algal biofuels will play a vital role in future, attention should be given on the following:

- Optimization of the culture growth parameters is necessary for high yields of biomass and lipid content.
- Development of modern technology for large-scale industrial production of biofuels along with wastewater treatment.
- Improvement of the CFD simulations and mathematical modeling-based process, before industrial trial and scale-up.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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