Process Biochemistry 76 (2019) 171–177

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Engineering strategies for the enhancement of *Nannochloropsis gaditana* outdoor production: Influence of the CO_2 flow rate on the culture performance in tubular photobioreactors

L. Moraes[a], G.M. Rosa[a], A. Morillas España[d], L.O. Santos[b], M.G. Morais[c], E. Molina Grima[d], J.A.V. Costa[a,*], F.G. Acién Fernández[d]

[a] Laboratory of Biochemical Engineering, College of Chemistry and Food Engineering, Federal University of Rio Grande, 96203-900, Rio Grande, RS, Brazil
[b] Laboratory of Biotechnology, College of Chemistry and Food Engineering, Federal University of Rio Grande, 96203-900, Rio Grande, RS, Brazil
[c] Laboratory of Microbiology and Biochemistry, College of Chemistry and Food Engineering, Federal University of Rio Grande, 96203-900, Rio Grande-RS, Brazil
[d] Department of Chemical Engineering, University of Almería, 04120, Almería, Spain

ARTICLE INFO

Keywords:
Carbon dioxide
Flow rate
Microalgae
pH control
Photobioreactor

ABSTRACT

The aim of this study was to evaluate the influence of the CO_2-specific flow rate on pH control, CO_2 use efficiency and biomass and lipid productivity of *Nannochloropsis gaditana* cultivated in outdoor tubular photobioreactors of 3.0 m^3. In that study was evaluated the injection of CO_2-specific flow rates ranging from 0.4 to 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}. The use of low CO_2-specific flow rates, from 0.4 to 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, has enabled pH control. The highest biomass productivities and CO_2 use efficiency were obtained using the flow rates of 1.2 and 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}. The highest lipid productivities were verified in experiments with flow rates of 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, followed by 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}. A high CO_2-specific flow rate increases CO_2 loss and reduces the performance of the cells due to inadequate pH control. By contrast, a low CO_2-specific flow rate prevents adequate pH control and the performance of the cultures. Considering the results provided, an optimal CO_2-specific flow rate (1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}) was obtained for outdoor photobioreactor operations to produce *N. gaditana*, thus contributing to cost reduction.

1. Introduction

Microalgae are microorganisms that, through photosynthesis, produce biomass that can be used in food, feed and cosmetics [1] for the extraction of high added-value biocompounds [2] and in biofuel production [3,4]. Additionally, these microorganisms can be applied to wastewater treatment [5] and flue gas cleaning because they use nutrients from the effluents as a source of nitrogen, phosphorus and carbon for their growth [5–7].

Microalgae can be produced in both open and closed reactors. Open reactors (ordinarily the raceway type) are commonly used in the production of robust and not easily contaminated microalgae, mainly strains such as *Spirulina* and *Dunaliella* [8]. The most widespread use of this type of system occurs mainly due to its simplicity, relative low cost of construction and operation, with the possibility of scale up [9,10]. Generally, open reactors have minimal control of the operating conditions because they are more susceptible to contamination [11]. Closed reactors (photobioreactors) are the most recommended system to produce biomass of non-robust strains usually rich in high added-value compounds [12]. Among them, tubular photobioreactors are the most extended ones. Although photobioreactors have a higher cost of construction than open systems, the bioreactor design is considered the most promising, because it has greater control of cultivation parameters, resulting in higher biomass productivities and lower contamination risks [13].

The increase in microalgae biomass productivity can be achieved by the supply of mainly carbon, in addition to nitrogen and phosphorus, among others, regardless of the photobioreactor configuration. Carbon dioxide (CO_2), which can be injected pure or from flue gases, is used as a carbon source and to control pH in photobioreactors. When pure CO_2 is supplied, the carbon source can represent up to 30% of biomass production costs [14]. Owing to the high cost of pure CO_2, its utilization efficiency is an important aspect that must be considered when producing microalgae at an industrial scale. Studies have shown that losses can reach up to 50%, but improved design parameters and operating conditions can minimize these losses, reducing it below 20%, especially

* Corresponding author.
E-mail address: jorgealbertovc@terra.com.br (J.A.V. Costa).

https://doi.org/10.1016/j.procbio.2018.10.010
Received 2 October 2018; Accepted 14 October 2018
Available online 15 October 2018
1359-5113/

when using advanced control strategies [15,16].

Nannochloropsis gaditana is a marine strain characterized by its fatty acid profile and high lipid content. Thus, this microalga strain is proposed to produce biodiesel and have applications in aquafeed and as a source of polyunsaturated fatty acids [17]. Previous studies have reported that *N. gaditana* can be cultivated under outdoor conditions in raceway and tubular photobioreactors [18,19]. However, the productivity and quality of the produced biomass are better when using tubular photobioreactors [19]. In addition to the photobioreactor design, certain factors, such as the CO_2 supply for pH control and carbon source, need to be evaluated for microalgae growth. Thus, it is important to evaluate the effects of the operational conditions (such as the CO_2 flow rate and concomitant pH variation) in outdoor microalgae production.

As described above, the aim of the study was to evaluate the influence of different CO_2-specific flow rates on pH control, CO_2 use efficiency and biomass and lipid productivity by *N. gaditana* cultivated in outdoor tubular photobioreactors. Additionally, we sought to identify the optimal conditions to maximize the performance of the system to enlarge the potential for large-scale production.

2. Material and methods

2.1. Microorganisms and culture medium

The marine microalga used was *Nannochloropsis gaditana*, which belonged to the collection of cultures of the research group "Marine Microalgae Biotechnology Group of University of Almería (Spain)". The culture medium used was prepared using artificial seawater (30 g L^{-1} of NaCl), agricultural fertilizers (g L^{-1}) [$Ca(NO_3)_2$ (0.8), KH_2PO_4 (0.25), $MgSO_4$ (0.3)] and micronutrients (mg L^{-1}) [B (0.23), Cu (0.09), Fe (2.10), Mn (1.17), Mo (0.07) and Zn (0.35)] [20]. The components of the culture medium were dissolved in water and were filtered through an ultrafiltration system comprising a 0.02 µm pore membrane (SFP 2860; DOW™ Ultrafiltration Modules, DOW®, China), followed by sterilization with ozone. The inoculum of *N. gaditana* was propagated in the laboratory and then on a pilot scale (outdoor conditions) in bubble column photobioreactors (total volume: 0.1 m^3) located inside a greenhouse, with constant aeration and CO_2 supply on-demand for pH control (7.8–8.0).

2.2. Tubular photobioreactors

The experiments were carried out in pilot-scale tubular photobioreactors of 3.0 m^3 (working volume of 2.6 m^3), located inside a greenhouse at the "*Estación Experimental Las Palmerillas*" property of Fundación CAJAMAR (Almería, Spain). The pilot plant comprises ten tubular photobioreactors, these ones consisting of solar light receivers and a bubble column, as previously described by Fernández et al. [21]. The light receivers comprising tubes of 19 m in length and 0.09 m in diameter, united in a loop configuration, with a total horizontal length of 400 m. The bubble column was 3.5 m high and 0.5 m in diameter, with a work volume of 0.45 m^3. This column was used to eliminate gases accumulated in the system (mainly oxygen) to allow the entrance of the culture medium and temperature control. The temperature of the culture was maintained below 30 °C by passing water at room temperature, with a flow rate of 1.5 $m^3 h^{-1}$ in stainless steel heat exchangers located inside the bubble column. The continuous recirculation of the culture (with a velocity of 0.9 m s^{-1}), between the column and light receivers, was performed using a centrifugal pump installed in the bottom of the column. Each photobioreactor is equipped with a dissolved oxygen probe (OD5120; Crison, Barcelona, Spain), pH and temperature probe (pH5083 T; Crison, Barcelona, Spain), which are located at the end of the loop and are connected to a multimeter (MM44; Crison, Barcelona, Spain). The solar radiation received was measured using a thermoelectric pyranometer connected to an AC-420 adapter (LP-02; Geónica S.A., Spain). The probes were connected to a transmitter (Labjack U12) and data acquisition software (Daqfactory, Azeotech, Arizona, USA) [22,23].

2.3. Operation conditions

The experiments performed consisted of varying the CO_2-specific flow rates at 0.4, 1.2, 1.9, 3.1 and 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} to determine the effect on the pH control and carbon supply during the production of *N. gaditana* in tubular photobioreactors. Independent of the CO_2-specific flow rate, the pH of the culture was controlled at 8.0 by the on-demand CO_2 supply. That control was realized by on-off algorithm, in which opens the CO_2 valve at a pH higher than 8.1 and closes it at a pH below 7.9. The pH sampling time was 10 s, which was lower than the cycling of the reactor that was 4 min. The CO_2-specific flow rates were monitored by flow meters (FR4500; Key Instruments, USA), and the gas was injected into the cultures at a working pressure of 0.2 kgf cm^{-2} using on/off solenoid valves. The CO_2 was injected on-demand by the control system using an on/off controller, only during the light period (when solar radiation > 30 W m^{-2}), to control the pH at the setpoint (8.0). Air was supplied to the bubble column at a flow rate of 200 L min^{-1} for the desorption of gases, mainly oxygen, accumulated in the culture. The cultures were carried out in June (spring-summer) in tubular photobioreactors as described above. The cultures were assessed in the semicontinuous mode at a dilution rate of 0.16 d^{-1}. The supernatant was recovered after centrifugation, followed by separation of the biomass and recirculation after nutrient supplementation and cleaning (ultrafiltration + ozone).

2.4. Analytical determinations

The biomass concentration (C_b) was determined by vacuum filtration of 50 mL of culture in a 0.7 µm pore-size glass microfiber filter (Whatman™). The biomass was washed with 50 mL of ammonium formate solution 1.25% w v^{-1} and was dried in an oven at 80 °C for 24 h.

The fluorescence of chlorophyll or the maximum quantum yield (Fv/Fm) was measured to determine the physiological status of the cells. For this analysis, a 3.5 mL aliquot of culture was kept in the dark for 15 min; later, the value of the Fv/Fm ratio was measured in a fluorimeter (AquaPen AP 100; Photon Systems Instruments, Czech Republic) [23].

The cultures were evaluated daily by microscopic observation (Leica, ICC50 HD, Germany) to verify that the strain used was the dominant microorganism throughout the experimental period.

The total inorganic carbon concentration (TIC; g L^{-1}) in the inlet and outlet culture medium was determined using an analytical kit (Hach-Lange LKC 381, Germany) and photometer (Dr Lange LASA 50, Germany) [7].

2.5. Harvesting and biomass composition

The biomass of each experiment was recovered from the liquid medium by centrifugation (GEA, Germany) and then was dried by freeze drying (FD80; Cuddon Freeze Dry, New Zealand) and was stored at −20 °C. The lipids were extracted with a chloroform: methanol mixture (1: 2) and were quantified according to the colorimetric method proposed by Marsh and Weinstein [24], which uses the standard curve of tripalmitin [25]. The moisture content in the biomass was determined by official methodology [26], and the lipid concentration was expressed on the dry basis.

2.6. Independent and response variables

The independent variable was the imposed CO_2-specific flow rate, whereas environmental variables that could not be controlled such as solar radiation and temperature were measured and registered online.

Direct response variables included the dissolved oxygen and pH of the culture, in addition to the number and duration of CO_2 injections and the overall mass flow of CO_2 supplied. These variables were also measured and registered online using data acquisition software (Daqfactory; Azeotech, Arizona, USA).

The biomass productivity (P_b, g L^{-1} d^{-1}) was calculated according to the following equation: $P_b = C_b D$, where Cb is the biomass concentration (g L^{-1}) and D is the dilution rate (d^{-1}) [12]. The CO_2 demand by the cultures (D_{CO_2}, g d^{-1}) was calculated according to Eq. (1), where P_b (g L^{-1} d^{-1}) is the biomass productivity, V_{work} (L) is the working volume of the photobioreactor, x_{cbm} is the mass fraction of carbon in the biomass (considered 0.5 g_{carbon} $g_{biomass}^{-1}$), and MM_{CO_2} (g mol^{-1}) and MM_C (g mol^{-1}) are the molecular weights of carbon dioxide and carbon, respectively.

$$D_{CO_2} = P_b V_{work} x_{cbm} \frac{MM_{CO_2}}{MM_C} \quad (1)$$

The CO_2 use efficiency (E_{CO_2}, % w w^{-1}) was calculated according to Eq. (2) from the ratio of the CO_2 demand (D_{CO_2}, g d^{-1}) and CO_2 mass rate (m_{CO_2}, g d^{-1}) injected into the cultures.

$$E_{CO_2} = \frac{D_{CO_2}}{m_{CO_2}} \quad (2)$$

The ratio between the CO_2 supply and biomass production ($R_{CO_2/biomass}$, g_{CO_2} $g_{biomass}^{-1}$) was calculated according to Eq. (3), where m_{CO_2} (g d^{-1}) is the CO_2 mass rate injected into the cultures, P_b (g L^{-1} d^{-1}) is the biomass productivity and V_{work} (L) is the photobioreactor working volume.

$$R_{CO_2/biomass} = \frac{m_{CO_2}}{P_b V_{work}} \quad (3)$$

The lipid concentration was evaluated in the lyophilized biomass of each experiment, while the lipid productivity (P_{lipid}, mg L^{-1} d^{-1}) was determined according to Eq. (4), where P_b is the biomass productivity and f_{lipid} is the lipid fraction present in the biomass.

$$P_{Lipid} = P_b f_{Lipid} \quad (4)$$

2.7. Statistical analysis

The results of the experiments were assessed by analysis of variance (ANOVA) followed by Tukey's test for the comparison of means at a 90% confidence level.

3. Results and discussion

This study evaluated the effect of CO_2-specific flow rates on pH control, biomass productivity, and CO_2 use efficiency of *N. gaditana* cultures carried out in tubular photobioreactors under outdoor conditions. All experiments were carried out in June (spring-summer) to ensure that the cultures were exposed to similar environmental conditions such as solar radiation and temperature. Fig. 1 shows the daily variation of dissolved oxygen, solar radiation, temperature and pH in a typical solar cycle. The maximum value of solar radiation in the daylight period was 564.8 W m^{-2}, the temperature ranging from 24 °C to 34 °C as extreme values during the experimentation time. The average temperature in the daylight period was 31 °C, while that during the night was 27 °C. The available cooling system prevents the temperature from increasing above 35 °C, which could strongly reduce microalgae growth. The increase in the culture temperature during the solar cycle occurs by the absorption of solar radiation as heat. Therefore, this increase may cause overheating of the culture, resulting in the inhibition of growth and even cell death [23]. Zitteli et al. [27] verified that the highest biomass productivity (0.8 g L^{-1} d^{-1}) of *Nannochloropsis* sp. in tubular photobioreactors under outdoor conditions was achieved in the spring when the temperatures ranged from 28 °C to 29 °C. Similarly, San

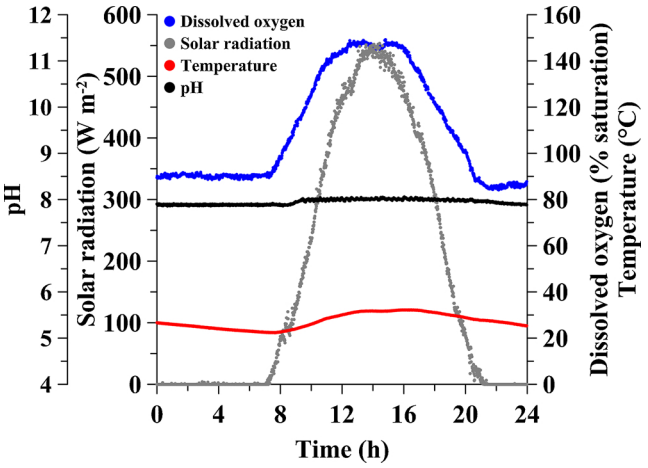

Fig. 1. Variation in dissolved oxygen (D.O.), solar radiation, temperature and pH of the culture during the production of *Nannochloropsis gaditana* in tubular photobioreactors at a 0.16 d^{-1} dilution rate, when operating at a CO_2-specific flow rate of 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}.

Pedro et al. [18] observed the highest biomass productivity (0.19 g L^{-1} d^{-1}) in *N. gaditana* cultures using raceway reactors under outdoor conditions, at temperatures of 29 °C.

The data in Fig. 1 show that, due to photosynthesis, the concentration of oxygen dissolved in the culture follows the sunlight intensity outline. During the night, the dissolved oxygen concentration was 89% Sat., lower than complete saturation with air due to cell respiration. The dissolved oxygen increases concomitantly with solar radiation, reaching a maximum value of 149% Sat. between 12 and 16 h. After 18 h, with the decrease in solar radiation, the dissolved oxygen concentration also declines gradually. According to these results, there was no high accumulation of dissolved oxygen in the culture that can damage the performance of the cells. Thus, the mass transfer capacity on the bubble column was higher than the oxygen production rate by the microalgae under the conditions analyzed. According to Ippoliti et al. [23], dissolved oxygen concentrations above 300% Sat. can be achieved when producing *Isochrysis* T-ISO in tubular photobioreactors at noon (12 h-highest solar radiation), indicating that the photobioreactor has insufficient mass transfer capacity to remove the oxygen produced. Under these conditions of a high dissolved oxygen concentration, photorespiration is enhanced, reducing the biomass productivity. The value of dissolved oxygen achieved in tubular reactors is highly dependent on the strain and operational conditions. According to Molina et al. [28], the dissolved oxygen concentration is increased up to 200% Sat., when producing *Phaeodactylum tricornutum* in tubular photobioreactors but is reduced at noon when the solar radiation exceeds 1500 μE m^{-2} s^{-1}, due to the occurrence of photoinhibition. Experiments performed in the springtime do not show a decline in the dissolved oxygen at noon, and the cause was attributed to lower photoinhibition during the springtime when solar radiation is generally less than that in the summer.

In addition to temperature and dissolved oxygen, the carbon supply and pH control are major factors determining the performance of microalgae cultures [12]. According to Acién et al. [29], the on-demand injection of CO_2 was demonstrated to be an adequate method for pH control and the carbon supply in microalgae production processes. The required carbon can be supplied by gaseous streams containing high CO_2 concentrations at a low flow rate or, alternatively, using low CO_2 concentration streams at high flow rates. Additionally, the reliability of the system may also vary depending on the CO_2 utilization capacity related to biomass production and minimizing carbon losses to the atmosphere [7].

The results observed (Fig. 2) for all the CO_2-specific flow rates (0.4 to 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}) evaluated that the pH was controlled

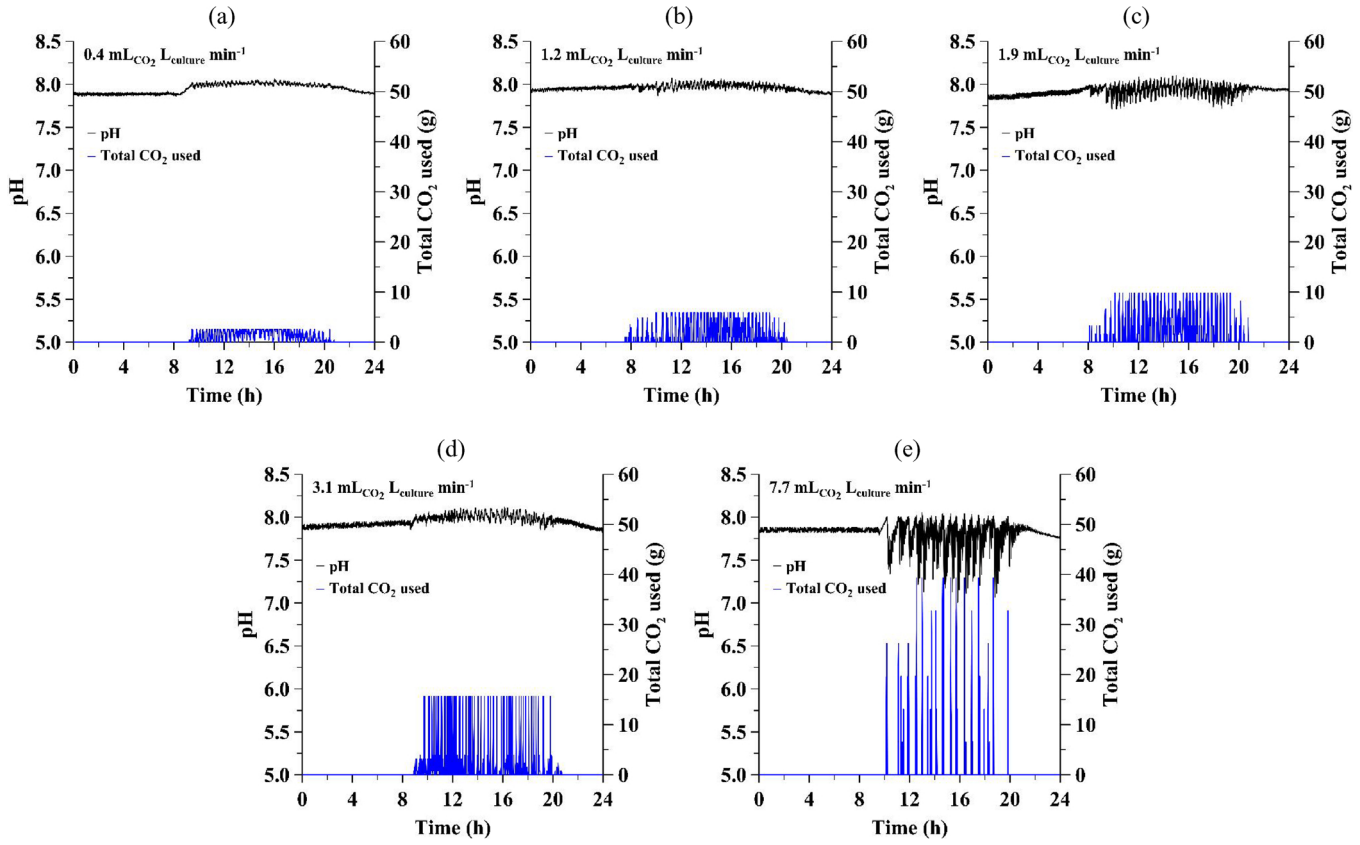

Fig. 2. Daily variation in the pH and total CO_2 used for pH control in the *Nannochloropsis gaditana* cultures, operated in the semicontinuous mode and carried out with CO_2-specific flow rates of 0.4 (a), 1.2 (b), 1.9 (c), 3.1 (d) and 7.7 mL_{CO_2} $L_{culture}$ min^{-1} (e).

Table 1
Experimental values of pH, chlorophyll fluorescence (Fv/Fm), biomass productivity (P_b), CO_2 mass rate (m_{CO_2},) CO_2 demand (D_{CO_2},) CO_2 use efficiency (E_{CO_2}), ratio between the CO_2 supply and biomass production ($R_{CO_2/biomass}$), CO_2 valve activation, and lipid concentration in biomass and lipid productivity (P_{Lipids}) as a function of the CO_2-specific flow rate in *Nannochloropsis gaditana* cultures.

Parameter	CO_2-specific flow rate (mL_{CO_2} $L_{culture}^{-1}$ min^{-1})				
	0.4	1.2	1.9	3.1	7.7
pH Min	$7.85^{a} \pm 0.01$	$7.85^{a} \pm 0.03$	$7.69^{b} \pm 0.03$	$7.69^{b} \pm 0.04$	$7.03^{c} \pm 0.03$
pH Max	$8.07^{b} \pm 0.01$	$8.08^{a,\ b} \pm 0.01$	$8.11^{a} \pm 0.01$	$8.12^{a} \pm < 0.01$	$8.04^{b} \pm 0.01$
pH Mean	$7.99^{a} \pm 0.03$	$7.98^{a,\ b} \pm < 0.01$	$7.93^{b} \pm 0.01$	$7.98^{a,\ b} \pm < 0.01$	$7.78^{c} \pm 0.03$
Fv/Fm	$0.70^{a} \pm 0.01$	$0.70^{a} \pm < 0.01$	$0.70^{a} \pm 0.01$	$0.68^{a} \pm < 0.01$	$0.69^{a} \pm 0.03$
P_b (g L^{-1} d^{-1})	$0.21^{b} \pm 0.01$	$0.22^{a,\ b} \pm 0.01$	$0.24^{a} \pm < 0.01$	$0.16^{c} \pm < 0.01$	$0.12^{d} \pm < 0.01$
m_{CO_2} (g d^{-1})	$966.3^{c} \pm 14.1$	$1389.8^{a,\ b} \pm 180.7$	$1665.6^{a} \pm 10.6$	$1496.3^{a,\ b} \pm 1.56$	$1356.7^{b} \pm 42.6$
D_{CO_2} (g d^{-1})	$996.9^{b} \pm 44.0$	$1032.5^{a,\ b} \pm 37.7$	$1100.3^{a} \pm 1.57$	$754.4^{c} \pm < 0.01$	$549.8^{d} \pm 15.7$
E_{CO_2} (% w w^{-1})	$103.0^{a} \pm 6.1$	$74.0^{b} \pm 5.7$	$67.0^{b} \pm 0.5$	$50.4^{c} \pm 0.1$	$41.0^{c} \pm 2.4$
$R_{CO_2/biomass}$ (g g^{-1})	$1.75^{a} \pm 0.10$	$2.42^{b} \pm 0.23$	$2.71^{b} \pm 0.02$	$3.57^{c} \pm < 0.01$	$4.45^{d} \pm 0.27$
CO_2 valve activation	$526^{a} \pm 7.07$	$380^{b} \pm 14.9$	$285^{c} \pm 18.4$	$274.5^{c} \pm 6.36$	$71^{d} \pm 11.3$
Lipids (% w w^{-1})	$18.8^{c} \pm 0.3$	$14.6^{d} \pm 0.4$	$16.3^{d} \pm 1.4$	$21.1^{b} \pm 0.6$	$23.1^{a} \pm 0.6$
P_{Lipids} (mg L^{-1} d^{-1})	$39.5^{a} \pm 0.9$	$32.1^{b} \pm 0.1$	$39.1^{a} \pm 2.0$	$33.8^{b} \pm 0.3$	$27.7^{c} \pm 0.8$

Identical superscript letters in the same line indicate that the means were not significantly different at a 90% confidence level ($p > 0.10$).

below the setpoint value of 8.0, including at noon with the highest photosynthetic activity. When using low CO_2-specific flow rates, from 0.4 to 1.2 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, the lowest variations of pH are observed because the supplied CO_2 is approximately consumed at the same rate that it is supplied (Fig. 2a-b). When increasing the CO_2-specific flow rate to 1.9 and 3.1 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, higher variations of pH are observed, with decreases up to 7.7 in the morning because the excess of CO_2 supplied reduces the pH of the culture after injections (Fig. 2c-d). The variations of pH are highly relevant when providing 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} of CO_2; in this case, the minimum pH reached was 7.03 (Fig. 2e). The number of activations of the on/off valve is reduced proportionally from more than 500 when providing 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} of CO_2 to less than 100 when providing 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} of CO_2, presenting a significant difference between the experiments ($p < 0.10$) (Table 1). This finding is important because the lifetime of the valves is limited; thus, many actuations imply a lower useful life of the equipment. The reduction of valve activations is interesting because it contributes to energy savings, increased equipment life and reduction of the CO_2 injection time [30,31]. However, increasing the CO_2-specific flow rate can result in pH variations (pH gradients), which influence the CO_2 use efficiency and biomass productivity. This phenomenon was observed by Santos et al. [7]: when a

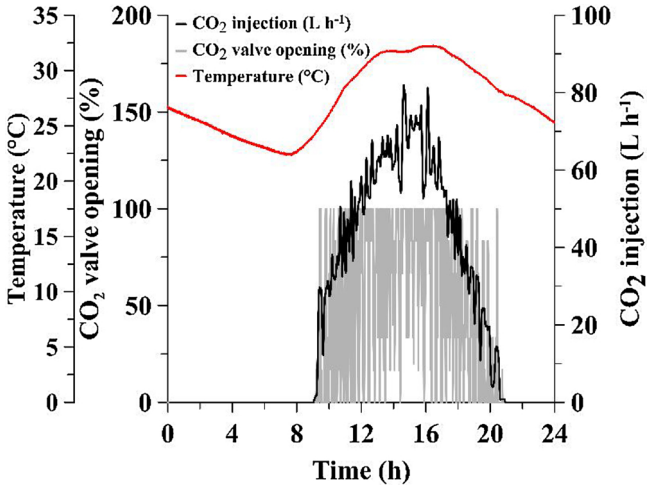

Fig. 3. Daily percentage variation in the position of the valve for CO_2 addition, CO_2 injection, and temperature of the *Nannochloropsis gaditana* cultures carried out in the semicontinuous mode, using a CO_2-specific flow rate of 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}.

higher CO_2 mass is injected, either by a high molar fraction or injection flow rate, there may be a delay between the pH measurement in the reactor and valve opening for the CO_2 supply. In that sense, more carbon would be supplied than required by the system, possibly affecting cultivation performance.

The frequency of which the CO_2 valve is open can be used to determine the total CO_2 injected that, in an ideal setting, must to be the same as the net CO_2 demand by the cultures. Fig. 3 shows that, when a CO_2-specific flow rate of 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} was used, the valve to CO_2 injection was open almost all the time. At any flow rate, the frequency of opening was higher at noon than in the morning and afternoon. Thus, the CO_2 injection rate shows the typical variation of sunlight, achieving its maximum value at noon with up to 80 L h^{-1} of CO_2 injected. Arbib et al. [32] reported that it is possible to correlate the intervals of the flue gas injections in the culture with the photosynthetic activity of the microalgae cells. Thus, solar radiation was demonstrated to be the environmental parameter that directly influences the pH of the cultures. The results reported by Godos et al. [6] indicated that the highest frequency of flue gas additions (10% CO_2) in *Scenedesmus* sp. cultures was verified with the increase in solar radiation.

The experimental maximum, minimum and average values of the pH for each of the analyzed conditions are summarized in Table 1. The experimental data showed that the minimum pH remains higher than 7.69 at CO_2-specific flow rates lower than 3.1 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} but is reduced to 7.03 when the CO_2-specific flow rate is higher than this value, presenting a significant difference from that in the other experiments ($p < 0.10$). The maximum pH values ranged from 8.04 to 8.12. Although the data presented a significant difference between the treatments ($p < 0.10$), this variation was small. The mean pH values were similar, ranging from 7.99 to 7.78 ($p > 0.10$), for CO_2-specific flow rates of 0.4 to 3.1 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}. In the experiment using a CO_2-specific flow rate of 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, the average pH of the cultures was 7.78, significantly different from that in the other treatments ($p < 0.10$). These pH values are recommended for microalgae production, especially for *N. gaditana*. However, the pH gradients of the cultures negatively affect the performance of the cells. As reported by Rubio et al. [15], Berenguel et al. [33]and Santos et al. [7], when CO_2 is injected at high concentrations, pH gradients may occur within the reactor that may affect the culture. The increase in pH during the light period is due to photosynthesis; thus, according to Grobbelaar [34], the CO_2 consumption by microalgae results in the accumulation of OH^- ions in the medium and, consequently, in the increase in pH. The on-demand injection of CO_2 allows the replacement of CO_2 consumed and maintenance of the pH; however, if excessive CO_2 is injected, the pH is reduced in excess, thus damaging the cells. According to Pawlowski et al. [30], the pH of cultures is influenced mainly by two phenomena, CO_2 supply and CO_2 uptake, which are functions of light availability. The CO_2 supplied contributes to the formation of carbonic acid (H_2CO_3), causing a reduction in the pH of the culture. On the other hand, microalgae in the presence of light perform photosynthesis, consuming CO_2 and producing O_2, increasing the pH of the cultures.

The experimental values of Fv/Fm do not show damage of the photosynthetic apparatus because these values were higher than 0.65, even with the pH variations in culture (Table 1), with no significant difference ($p > 0.10$) for this response among the experimental conditions. However, the biomass productivity shows a clear reduction when operating at CO_2-specific flow rates higher than 3.1 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, when the higher pH gradients were verified. Thus, the biomass productivity slightly increases from 0.21 to 0.24 g $L^{-1}d^{-1}$ when the CO_2-specific flow rate is increased from 0.4 to 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} and is reduced to 0.16 and 0.12 g $L^{-1}d^{-1}$ when the CO_2-specific flow rate is increased to 3.1 and 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} (Table 1). Among the three lowest utilized CO_2-specific flow rates, the lowest ($p < 0.10$) biomass productivity was observed when using a CO_2-specific flow rate of 0.4 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, likely due to a carbon limitation effect. The highest values of biomass productivity were verified in the experiments with CO_2-specific flow rates of 1.2 to 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}, with a significant difference ($p < 0.10$) from that of the other treatments.

Independent of the CO_2-specific flow rate used, the total inorganic carbon concentration (TIC) in the inlet (0.119 g L^{-1}) was lower than that in the outlet because the CO_2 supplied oversaturated the culture medium with respect to air saturation. However, when using low CO_2-specific flow rates (0.4–1.2 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}), the TIC concentration in the outlet fraction was lower (0.147-0.151 g L^{-1}) than when using the optimal CO_2-specific flow rate of 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} (0.157 g L^{-1}). At higher CO_2-specific flow rates (3.1–7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}), the TIC concentration in the outlet fraction was higher (0.167-0.169 g L^{-1}) because less carbon was fixed by the microalga; thus, it persisted in the culture medium. Acién et al. [14] reported that, in microalgae cultures, the TIC concentration in the culture medium outlet is higher than that in the inlet, likely due to the added CO_2 for the control of pH remaining in the liquid phase but being lost with the culture medium when dilution is performed.

To determine the total amount of CO_2 effectively fixed into the biomass, an overall mass balance must be performed. The CO_2 mass rate (m_{CO_2}) increased from 966.3 to 1665.6 g d^{-1} when the CO_2-specific flow rate was increased from 0.4 to 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} ($p < 0.10$). However, the increase in the CO_2-specific flow rate (3.1–7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1}) caused an m_{CO_2} decrease from 1496.3 to 1356.7 g d^{-1} (Table 1). Thus, the flow rate of the CO_2 mass injected is a direct function of biomass productivity. The estimated CO_2 demand shows a similar tendency, increasing from 996.9 to 1100.3 g d^{-1} when the CO_2-specific flow rate increases from 0.4 to 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} ($p < 0.10$), but it decreases to 754.4 and 549.8 g d^{-1} when the CO_2-specific flow rate increases up to 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} ($p < 0.10$) (Table 1). The CO_2 use efficiency decreases from 100% to 42% when the CO_2-specific flow rate increases from 0.4 to 7.7 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} ($p < 0.10$) (Table 1). The experiments using the CO_2-specific flow rates of 1.2 and 1.9 mL_{CO_2} $L_{culture}^{-1}$ min^{-1} showed statistically similar CO_2 use efficiencies ($p > 0.10$). The efficiency does not only consider the CO_2 lost to the air by stripping but also considers the carbon accumulated in the culture medium; thus, it is a global efficiency value. The only approximation performed to calculate this efficiency is to consider a constant carbon content into biomass; because it is produced in the continuous mode, this approximation is quite reasonable. When considering the injection of CO_2 into microalgae cultures, it is assumed that microalgae comprise 50% of

carbon on average as biomass [34]. Therefore, it is necessary to provide a minimum of 1.8 kg of CO_2 to produce 1 kg of biomass [14]. The data obtained from experimental measurements show that the real values ranged from 1.75 to 4.45 $g_{CO_2}\ g_{biomass}^{-1}$ (Table 1), due to different phenomena occurring such as CO_2 absorption in the culture medium, CO_2 losses and reduction of biomass production by inadequate pH value/gradients. Under the optimal conditions found in the CO_2-specific flow rates of 1.2 and 1.9 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$, the optimal values were 2.42 and 2.71 $g_{CO_2}\ g_{biomass}^{-1}$, respectively. These results agree with those previously reported by Acién et al. [14] using *Scenedesmus almeriensis*. These authors verified the results with a CO_2 use efficiency of 74.5% and 2.34 $g_{CO_2}\ g_{biomass}^{-1}$. Chai and Zhao [35] verified a reduction of the CO_2 removal efficiency with the flow rate increase, from 2.5 to 15 $mL_{mixture}\ L_{culture}\ min^{-1}$ (10% CO_2), in cultivations of *Chlorococcum* sp. The authors attributed this result to the reduction of the interfacial area between gas and liquid, suggesting that it can be improved by minimizing the bubble size at a lower CO_2-specific flow rate. In that sense, Ryu et al. [36] evaluated the effect of the injection of one CO_2 concentration (5%) on the different aeration flow rates and verified that, when the aeration rate was increased, there was a decrease in the CO_2 use efficiency by microalgae. The authors also evaluated the bubble size and concluded that, when the bubble size is larger, the mass transfer efficiency of the gas was decreased due to the reduction of the gas-liquid interface. Santos et al. [7] and Godos et al. [6] evaluated CO_2 transfer and carbon balance in raceway reactors and observed that an increased CO_2-specific flow rate resulted in the supply of more carbon than required by the microalgae cultures. According to the authors, this may contribute to the greater accumulation of this gas in the liquid medium and, consequently, higher losses to the atmosphere. Likewise, Moraes et al. [37] evaluated the effect of the gas mixture flow rate containing air and CO_2 (CO_2-specific flow rate fixed at 6 $mL_{CO_2}\ L_{medium}\ min^{-1}$) in *Spirulina* sp. LEB 18 cultivation and observed a reduction in the biomass productivity and E_{CO_2} with an increase in the flow rate from 50 to 300 $mL_{mixture}\ L_{culture}\ min^{-1}$.

In the present work, the lipid concentration in *N. gaditana* biomass varied between 14.6% and 23.1% (Table 1). The highest lipid concentration ($p < 0.10$) was verified in the experiment with injection of 7.7 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$. Thus, it was possible to observe that the use of a high CO_2 flow rate and occurrence of pH gradients may have caused a stress condition for the cultures, contributing to the increased lipid content of *N. gaditana*. However, when analyzing lipid productivity, it was possible to verify that the highest results, with significant difference among other results ($p < 0.10$), were found in the experiments with 0.4 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$ ($39.5\ mg\ L^{-1}\ d^{-1}$) and 1.9 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$ ($39.1\ mg\ L^{-1}\ d^{-1}$). The increase in the CO_2 flow rate from 0.4 to 7.7 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$ resulted in a reduction of almost one-third in lipid productivity in *N. gaditana* cultures. According to Acién et al. [29], when microalgae are submitted to stress conditions, they can trigger the accumulation of lipids in the biomass. However, these conditions do not contribute to cell growth. In that sense, in addition to assessing the lipid content produced, one should consider lipid productivity. According to Chen et al. [38], lipid productivity is an important factor that needs to be evaluated because it allows verification of the potential of microalgal biomass application as raw material for biodiesel production.

In summary, the experimental values obtained demonstrate that CO_2 must be provided to *N. gaditana* cultures to avoid carbon limitation and inadequate pH. However, the CO_2 gas flow rate employed, independent of the photobioreactor, is not an arbitrary parameter. To enhance the CO_2 use efficiency, the CO_2-specific flow rate utilized in cultures must be minimized, but excessive reduction may have a negative effect on the biomass productivity. Thus, it is necessary to perform global analysis of the culture parameters (biomass productivity, photosynthetic efficiency, CO_2 use efficiency, CO_2 demand and biomass composition) and process (pH control, CO_2 valve activations, carbon mass supplied) to identify the most suitable experimental condition for the development of the production system.

4. Conclusion

The CO_2-specific flow rate used for carbon supply and pH control in *Nannochloropsis gaditana* cultures in tubular photobioreactors under outdoor conditions influenced the performance of the entire process. In that sense, in addition to defining the CO_2-specific flow rate to control the pH, it is necessary to analyze the existence of pH gradients, carbon use efficiency and biomass productivity in depth as a function of the cultivation operational conditions. Therefore, the use of a CO_2-specific flow rate of 1.9 $mL_{CO_2}\ L_{culture}^{-1}\ min^{-1}$ was considered optimal to produce *N. gaditana* in tubular photobioreactors under outdoor conditions.

Acknowledgements

The authors would like to thank CAPES (Coordination for the Improvement of Higher Education Personnel) for support from the Sandwich Doctorate Scholarship Abroad (PDSE) under the process Number: 88881.131625/2016-01, the European Union's Horizon 2020 Research and Innovation Program under Grant Agreement No. 727874SABANA and the Program of Support to Publication of the Academic Production/PROPESP/FURG/2018.

References

[1] J. Trivedi, M. Aila, D.P. Bangwal, S. Kaul, M.O. Garg, Algae based biorefinery—How to make sense? Renew. Sustain. Energy Rev. 47 (2015) 295–307.
[2] M.G. Morais, B.S. Vaz, E.G. Morais, J.A.V. Costa, Biologically active metabolites synthesized by microalgae, Biomed. Res. Int. (2014) 1–16.
[3] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306.
[4] J.A.V. Costa, M.G. Morais, The role of biochemical engineering in the production of biofuels from microalgae, Bioresour. Technol. 102 (2011) 2–9.
[5] E. Posadas, M.D.M. Morales, C. Gomez, F.G. Acién, R. Muñoz, Influence of pH and CO_2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways, Chem. Eng. J. 265 (2015) 239–248.
[6] I. de Godos, J.L. Mendoza, F.G. Acién, E. Molina, C.J. Banks, S. Heaven, F. Rogalla, Evaluation of carbon dioxide mass transfer in raceway reactors for microalgae culture using flue gases, Bioresour. Technol. 153 (2014) 307–314.
[7] T.D. Santos, J.L. Mendoza-Martín, F.G. Acién Fernández, E.M. Molina, J.A.V. Costa, S. Heaven, Optimization of carbon dioxide supply in raceway reactors: influence of carbon dioxide molar fraction and gas flow rate, Bioresour. Technol. 212 (2016) 72–81.
[8] G.C. Zittelli, N. Biondi, L. Rodolfi, M.R. Tredici, Photobioreactors for mass production of microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult. Appl. Phycol. Biotechnol. second ed., Wiley Blackwell, Oxford, 2013, pp. 225–266.
[9] J.L. Mendoza, M.R. Granados, I. de Godos, F.G. Acién, E. Molina, C. Banks, S. Heaven, Fluid-dynamic characterization of real-scale raceway reactors for microalgae production, Biomass Bioenergy 54 (2013) 267–275.
[10] B. Wang, C.Q. Lan, M. Horsman, Closed photobioreactors for production of microalgal biomasses, Biotechnol. Adv. 30 (2012) 904–912.
[11] I. Fernández, F.G. Acién, J.M. Fernández, J.L. Guzmán, J.J. Magán, M. Berenguel, Dynamic model of microalgal production in tubular photobioreactors, Bioresour. Technol. 126 (2012) 172–181.
[12] E. Molina-Grima, F.G. Acién, F. García-Camacho, Y. Chisti, Photobioreactors: light regime, mass transfer, and scaleup, J. Biotechnol. 70 (1999) 231–247.
[13] F.G. Acién, E. Molina, A. Reis, G. Torzillo, G.C. Zittelli, C. Sepúlveda, J. Masojídek, Photobioreactors for the production of microalgae, in: C. Gonzalez-Fernandez, R. Muñoz (Eds.), Microalgae-Based Biofuels and Bioproducts - From Feedstock Cultivation to End-Products, Woodhead Publishing Series in Energy, Cambridge, 2017, pp. 1–44.
[14] F.G. Acién, J.M. Fernández, J.J. Magán, E. Molina, Production cost of a real microalgae production plant and strategies to reduce it, Biotechnol. Adv. 30 (2012) 1344–1353.
[15] F. Camacho Rubio, F.G. Acién Fernández, J.A. Sánchez Pérez, F. García Camacho, E. Molina Grima, Prediction of dissolved oxygen and carbon dioxide concentration profiles in tubular photobioreactors for microalgal culture, Biotechnol. Bioeng. 62 (1999) 71–86.
[16] F.G. Acién, F. Garcia Camacho, J.A. Sanchez Perez, J.M. Fernandez Sevilla, E. Molina Grima, Modeling of biomass productivity in tubular photobioreactors for microalgal cultures: effects of dilution rate, tube diameter, and solar irradiance, Biotechnol. Bioeng. 58 (1998) 605–616.
[17] A. San Pedro, C.V. González-López, F.G. Acién, E. Molina-Grima, Marine microalgae selection and culture conditions optimization for biodiesel production, Bioresour. Technol. 134 (2013) 353–361.
[18] A. San Pedro, C.V. González-López, F.G. Acién, E. Molina-Grima, Outdoor pilot production of *Nannochloropsis gaditana*: Influence of culture parameters and lipid production rates in raceway ponds, Algal Res. 18 (2015) 205–213.
[19] A. San Pedro, C.V. González-López, F.G. Acién, E. Molina-Grima, Outdoor pilot-scale production of *Nannochloropsis gaditana*: Influence of culture parameters and

lipid production rates in tubular photobioreactors, Bioresour. Technol. 169 (2014) 667–676.
[20] J. Camacho-Rodríguez, M.C. Cerón-García, C.V. González-López, J.M. Fernández-Sevilla, A. Contreras-Gómez, E. Molina-Grima, A low-cost culture medium for the production of *Nannochloropsis gaditana* biomass optimized for aquaculture, Bioresour. Technol. 144 (2013) 57–66.
[21] I. Fernández, F.G. Acién, M. Berenguel, J.L. Guzmán, G.A. Andrade, D.J. Pagano, A lumped parameter chemical-physical model for tubular photobioreactors, Chem. Eng. Sci. 112 (2014) 116–129.
[22] G.A. De Andrade, D.J. Pagano, J.L. Guzmán, M. Berenguel, I. Fernández, F.G. Acién, Distributed sliding mode control of pH in tubular photobioreactors, IEEE Trans. Control Syst. Technol. 24 (2016) 1160–1173.
[23] D. Ippoliti, A. González, I. Martín, J.M.F. Sevilla, R. Pistocchi, F.G. Acién, Outdoor production of *Tisochrysis lutea* in pilot-scale tubular photobioreactors, J. Appl. Phycol. (2016) 3159–3166.
[24] J.B. Marsh, D.B. Weinstein, Simple charring method for determination of lipids, J. Lipid Res. 7 (1966) 574–576.
[25] D.L. Holland, P.A. Gabbott, A micro-analytical scheme for the determination of protein, carbohydrate, lipid and RNA levels in marine invertebrate larvae, J. Mar. Biolog. Assoc. U.K. 51 (1971) 659–668.
[26] AOAC, Official Methods of Analysis of the Association of Official Analytical Chemists, 17th ed, AOAC, Maryland, 2000.
[27] G.C. Zitteli, F. Lavista, A. Bastianini, L. Rodolfi, M. Vincenzini, M.R. Tredici, Production of eicosapentaenoic acid by *Nannochloropsis* sp. Cultures in outdoor tubular photobioreactors, J. Biotechnol. 70 (1999) 299–312.
[28] E. Molina, J. Fernández, F.G. Acién, Y. Chisti, Tubular photobioreactor design for algal cultures, J. Biotechnol. 92 (2001) 113–131.
[29] F.G. Acién Fernández, C.V. González-López, J.M. Fernández Sevilla, E. Molina Grima, Conversion of CO_2 into biomass by microalgae: how realistic a contribution may it be to significant CO_2 removal? Appl. Microbiol. Biotechnol. 96 (2012) 577–586.
[30] A. Pawlowski, J.L. Mendoza, J.L. Guzmán, M. Berenguel, F.G. Acién, S. Dormido, Effective utilization of flue gases in raceway reactor with event-based pH control for microalgae culture, Bioresour. Technol. 170 (2014) 1–9.
[31] A. Pawlowski, I. Fernández, J.L. Guzmán, M. Berenguel, F.G. Acién, J.E. Normey-Rico, Event-based predictive control of pH in tubular photobioreactors, Comput. Chem. Eng. 65 (2014) 28–39.
[32] Z. Arbib, J. Ruiz, P. Álvarez-Díaz, C. Garrido-Pérez, J. Barragan, J.A. Perales, Effect of pH control by means of flue gas addition on three different photo-bioreactors treating urban wastewater in long-term operation, Ecol. Eng. 57 (2013) 226–235.
[33] M. Berenguel, F. Rodríguez, F.G. Acién, J.L. García, Model predictive control of pH in tubular photobioreactors, J. Process Control 14 (2004) 377–387.
[34] J.U. Grobbelaar, Inorganic algal nutrition, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult. Appl. Phycol. Biotechnol. John Wiley & Sons Ltd, Oxford, 2013, pp. 123–133.
[35] X. Chai, X. Zhao, Enhanced removal of carbon dioxide and alleviation of dissolved oxygen accumulation in photobioreactor with bubble tank, Bioresour. Technol. 116 (2012) 360–365.
[36] H.J. Ryu, K.K. Oh, Y.S. Kim, Optimization of the influential factors for the improvement of CO_2 utilization efficiency and CO_2 mass transfer rate, J. Ind. Eng. Chem. 15 (2009) 471–475.
[37] L. Moraes, G.M. Rosa, B.B. Cardias, L.O. Santos, J.A.V. Costa, Microalgal biotechnology for greenhouse gas control: carbon dioxide fixation by *Spirulina* sp. at different diffusers, Ecol. Eng. 91 (2016) 426–431.
[38] B. Chen, C. Wan, M.A. Mehmood, J.S. Chang, F. Bai, X. Zhao, Manipulating environmental stresses and stress tolerance of microalgae for enhanced production of lipids and value-added products–A review, Bioresour. Technol. 244 (2017) 1198–1206.