Outdoor Large-Scale Cultivation of the Acidophilic Microalga *Coccomyxa onubensis* in a Vertical Close Photobioreactor for Lutein Production

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Abstract: The large-scale biomass production is an essential step in the biotechnological applications of microalgae. *Coccomyxa onubensis* is an acidophilic microalga isolated from the highly acidic waters of Río Tinto (province of Huelva, Spain) and has been shown to accumulate a high concentration of lutein (9.7 mg g^{-1}dw), a valuable antioxidant, when grown at laboratory-scale. A productivity of 0.14 g L^{-1} d^{-1} was obtained by growing the microalga under outdoor conditions in an 800 L tubular photobioreactor. The results show a stable biomass production for at least one month and with a lutein content of 10 mg g^{-1}dw, at pH values in the range 2.5–3.0 and temperature in the range 10–25 °C. Culture density, temperature, and CO_2 availability in highly acidic medium are rate-limiting conditions for the microalgal growth. These aspects are discussed in this paper in order to improve the outdoor culture conditions for competitive applications of *C. onubensis*.

Keywords: carotenoids; extremophiles; microalgal biotechnology

1. Introduction

The production of microalgal biomass efficiently is a key prerequisite in the production process of valuable compounds from microalgae. Large-scale production of biomass requires the processing of high volume of cultures and the use of both indoor and outdoor systems, which may differ in geometry, culture engineering principles, and variable ambient conditions. An outdoor system offers the advantage well defined cultivation infrastructures and suitable latitude-dependent environmental conditions, particularly light and temperature, which may add significant economic advantages for biomass production [1,2].

Tubular photobioreactors, open raceway systems, and flat panel systems, have so far been widely recognized as suitable for large-scale microalgal production under outdoor conditions [3,4]. The choice of the culture system is highly influenced by the characteristics of the specific microalgal species, such as its robustness, growth rate, and the value and purity requirements of the target product to be obtained. Tubular and panel photobioreactors systems should be suitable to carry out the massive production of non-robust microalgal species, where the risk of contamination by undesired microorganisms and fluctuation of specific culture parameters, such as oxygen concentration and temperature can be

minimized to a certain level [5]. Corrosion of equipment due to acidity might be a problem which, however, is overcome by using reactor components made of resistant-to-acid materials. In addition, slow-growth rate microalgal species are adequate for cultivation in photobioreactors which allow for a high control of growth conditions close to optimal values [1,6,7]. On the contrary, highly robust species, such as *Chlorella vulgaris* and *Dunaliella salina*, are commercially grown in raceway open ponds with a very low level of control of temperature and microbial contamination, thus reducing operational costs without dramatic reduction in the theoretical maximum algal biomass productivity [8,9].

Lutein is a compound consumed worldwide, mainly as a food colorant. Lutein sales amount to 150 million dollars in the USA only. Currently, lutein is obtained from marigold petals, with a low lutein content of 0.03% (*w/w*) [10,11]. Actually, the only possible other sources with sufficient content to be considered for lutein production are certain strains of microalgae, several of them considered as potential lutein sources based on their lutein content which ranges from 0.5% to 1.2% dry weight [10].

Coccomyxa onubensis is a relatively slow-growth rate acidophilic microalga, which has been grown outdoor in a 6.0 L photobioreactor for production of lutein [12]. Among the potential applications of this microalga, an antibacterial effect against human pathogens has been reported by Navarro et al. [13], and microalgal fatty acids were suggested to be involved in the antibacterial activity. Although the mechanisms through which fatty acids may exert bactericidal activity are not fully understood, it has been reported they may promote membrane damage resulting in nutrient uptake alteration and cellular respiration inhibition [14]. In addition, this microalga could be also attractive as a nutritional supplement for food and feed industries [14]. These data indicate the potential of *C. onubensis* as source of valuable compounds and highlight the interest of producing its biomass at large-scale. In this work we investigated the growth of the microalga in an 800 L vertically stacked tubular photobioreactor, and this creates a step forward in the way for the efficient and economically feasible production of enriched *C. onubensis* biomass. To the best of our knowledge, this is the first example of biomass production of an acid-tolerant microalga in an outdoor tubular photobioreactor and offers experimental details of what should be taken into account for the successful pilot-scale production.

2. Materials and Methods

2.1. Microalga and Culture Medium

Coccomyxa onubensis ACCV1 was isolated from the acidic waters of the Tinto river, Huelva (Spain), phylogenetically characterized and deposited in the Experimental Phycology and Culture Collection of Algae at the University of Goettingen in Germany (SAG) with the stock number SAG 2510 [15]. According to the chemical composition of the acidic water in its natural environment, the medium used for maintaining axenic cultures of the microalga in the culture room was prepared at pH 2.5 by modifying the K9 medium described by Silverman and Lundgren [16]. The culture medium was prepared in our laboratory with the following chemical composition: 22.67 mM K_2SO_4, 1.34 mM KCl, 2.87 mM K_2HPO_4, 4.31 mM $MgCl_2$, 22.65 mM KNO_3, 0.09 mM $CaCl_2$, and 5 mL of Hutner's trace elements solution prepared in our laboratory as indicated in [15]. The microalgal cultures were grown in Erlenmeyer flasks (Fisher Scientific S.L., Madrid, Spain) in an algal room at 25 °C bubbled with air containing 2.5% (*v/v*) CO_2 and continuously illuminated by white fluorescent lamps.

For large-scale cultivation in indoor plastic bags and in an outdoor tubular photobioreactor, a culture medium based on an NPK-fertilizer solution (Agralia Fertilizantes S.L., Huesca, Spain) was used in order to reduce nutrient costs. The 1 L of culture medium (NPK-medium) contained 0.25 mM NO_3^-, 0.45 mM NH_4^+, 0.4 mM P_2O_4, 0.64 mM K_2O, and 0.4 mL of a commercial micronutrient solution (Microfer Complex, Fercampo, Málaga, Spain).

Previous experiments at laboratory scale unveiled a more efficient use of urea by the microalga, as compared to other common nitrogen sources used to grow microalgae, including nitrate, nitrite, or ammonium [15,17]. Thus, the final medium was supplemented with 8 mM urea (Panreac Química

SLU, Barcelona, Spain) which also provides carbon to the microalga, 100 μM $FeCl_3$, and pH was adjusted to 2.5.

2.2. Cultivation of C. onubensis in Indoor Air Fluidized-Bed Plastic Bags

The microalga was grown in two plastic bags (Plásticos Gamaza, Santander, Spain) of 400 L each, having 60 cm diameter, 2.1 m height, and 200 μm thickness, fully transparent to photosynthetically active radiation (PAR) (Figure 1A), and using the NPK-medium as described in the previous section. Two conditions of initial biomass concentration were assessed: 0.31 g L^{-1} (C1, Figure 2A) and 0.57 g L^{-1} (C2, Figure 2A). The bags were maintained indoors at 25 °C and continuously bubbled with CO_2-enriched air (2.5% *v/v*) through air diffusers placed at the bottom of the bags. The cultures were continuously illuminated with white fluorescent lamps that yielded 150 μE m^{-2} s^{-1} of incident light on the surface of the bag. The incident light intensity on the surface of the culture bags was measured using a photoradiometer (HD9021, Delta OHM, Padova, Italy).
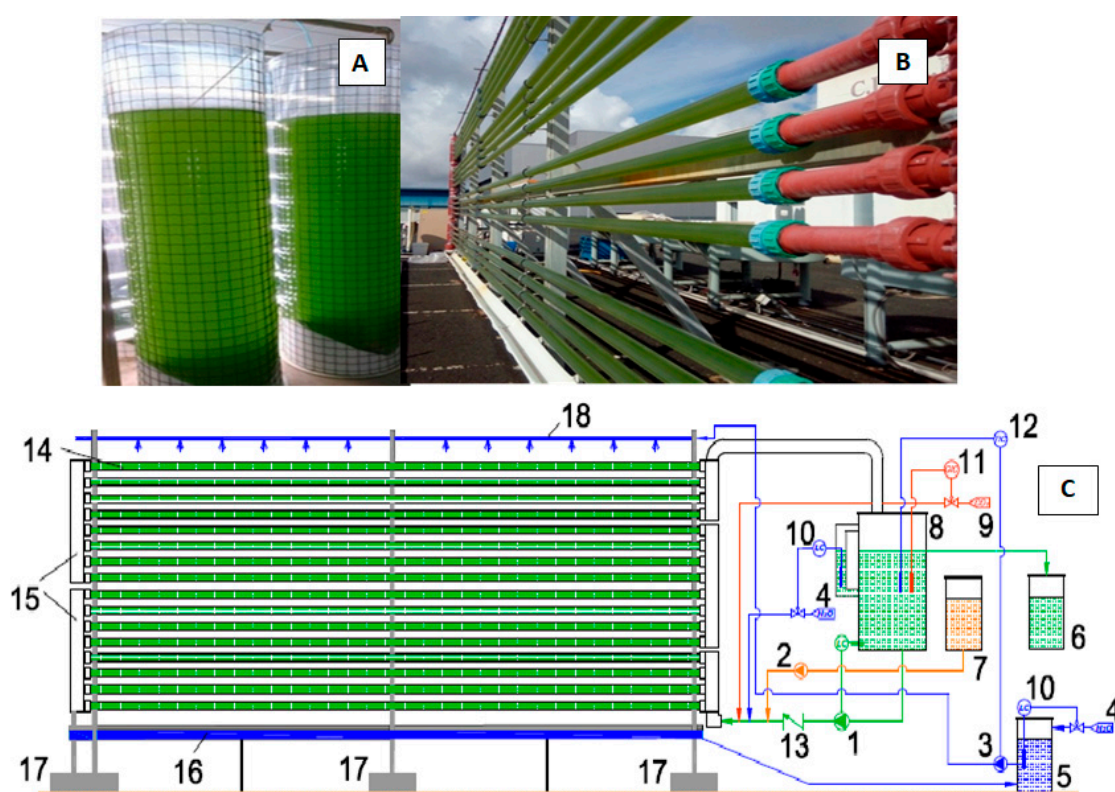

Figure 1. Cultivation systems for large-scale production of *Coccomyxa onubensis* biomass. Indoor 400 L cultivation bags (**A**); outdoor 800 L vertical tubular photobioreactor (**B**); configuration of the photobioreactor is detailed in (**C**): (1) culture pump; (2) nutrient pump; (3) cooling water pump; (4) water inlet; (5) cooling water tank; (6) harvested biomass tank; (7) nutrient tank; (8) degasser tank; (9) CO_2 inlet; (10) level controller; (11) pH controller indicator; (12) temperature controller indicator; (13) non-return valve; (14) glass pipe; (15) manifolds; (16) cooling water collector; (17) pipe support; (18) cooling pipe with sprinklers. The systems are located at CIDERTA (University of Huelva, Spain).
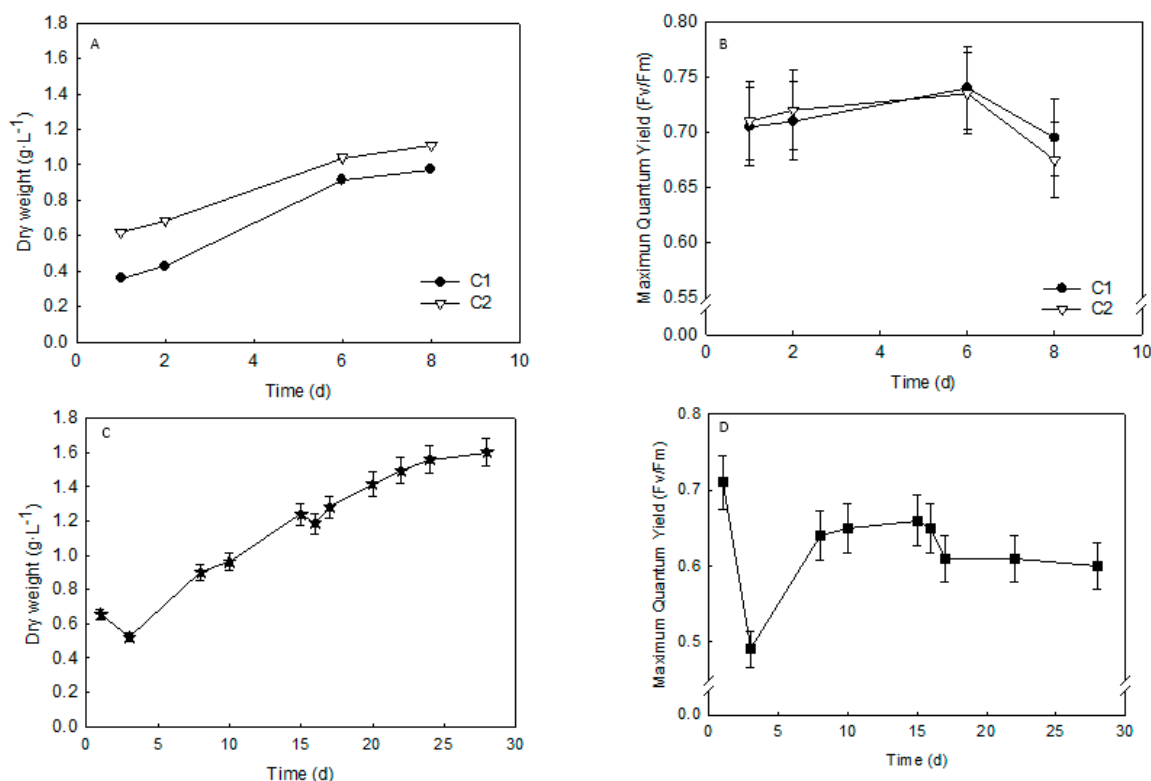
Figure 2. Growth of *C. onubensis* cultures in batch indoors and in an outdoor photobioreactor (PBR) system. Time-course evolution of growth in batch cultures (**A**) and photosynthetic capacity (maximum quantum yield, (**B**)). Time course of growth in an outdoor PBR (**C**). Photosynthetic capacity in photobioreactor culture (**D**). Two conditions of initial biomass concentration were assessed: 0.31 g L^{-1} (C1) and 0.57 g L^{-1} (C2). Details of the experimental set-up and parameter determination are described in Section 2.

2.3. Cultivation of C. onubensis in an Outdoor Vertical Tubular Photobioreactor

The microalga was cultured at a pilot scale in an 800 L outdoor vertical tubular photobioreactor, using the same NPK-medium previously described. The system consists of 16 horizontal glass tubes with 6 cm internal diameter vertically stacked and connected mutually by means of manifolds on both sides of the tubes (Figure 1B). The glass tubes were kindly provided by Schott (Schott Glass Iberica SL, Barcelona, Spain). The photobioreactor is equipped with temperature and pH control systems, which allowed keeping the temperature below 25 °C and pH between a range of 2.5–3 (Figure 1C). Reactor components were made of resistant-to-acid materials. This design was particularly based on the conclusions made from the work of Vaquero et al. [12] in a 6 L outdoor tubular photobioreactor (PBR).

For replication of outdoor cultivation trials, the limitation exists that only one large PBR system was available. Thus, it was not possible to replicate experiments under the same outdoor conditions. However, the trial of growth outdoors allowed to understand that the acidophilic microalga grow stably in an PBR system outdoors and also to approach the first data of productivity.

The culture broth was placed inside a 1500 L tank and pumped to the bottom manifold of the photobioreactor. The broth circulates through the four bottom tubes and reaches the large common manifold at the side extreme. Subsequently, the broth enters the four tubes above and flows in opposite direction, reaching the manifold at the extreme of the tube. The flow pattern was repeated until the broth reached the manifold placed at the highest position, finally returning to the tank where it was degassed before being again pumped back to the photobioreactor (Figure 1C).

pH was measured continuously using the pH/ATC transmitter DMM-4000/pH (Design Instrument, Barcelona, Spain) equipped with a pH sensor (SG900CD, SENSOREX, Inc., Los Angeles, CA, USA). Temperature was measured continuously using a temperature sensor (PT100). Both controllers were

equipped with a data acquisition system ICP-COM type. CO_2 was automatically injected when the culture pH exceeded 3.5. The cooling system (see below) was set to turn on automatically when the culture temperature exceeded 24 °C.

The temperature control was carried out by dripping water over the tubular system. The water used for cooling was collected by a channel in the low part of the photobioreactor and returned to the cooling water tank. This tank was placed underground, thus reducing water losses.

2.4. Harvesting of Microalgal Biomass

After the cultivation period, the microalgal biomass was harvested by continuous flow centrifugation at 250 L h^{-1} and 8400 rpm using an industrial centrifuge (KA–6, GEA Westfalia Separator, Oelde, Germany). The biomass was frozen at −20 °C for 24 h and subsequently lyophilized in a freeze drier (FD8512, IlShin BioBase, Ede, The Netherlands). The powder was then vacuum packed and stored at −80 °C until further use.

2.5. Growth Measurements and Productivity

In all cultures of this study, the algal growth evolution with time course was daily assessed by following dry weight measurements which were carried out by taking 10 mL samples of each culture. The samples were filtered through glass microfiber filters of 47 mm diameter and 0.7 μm pore size (MFV–5, Filter-Lab, Barcelona, Spain). The filters containing wet algal biomass were dried in an oven at 100 °C for 24 h [18].

2.6. Maximum Photosystem II Quantum Yield (Fv/Fm)

The photosynthetic efficiency was evaluated by measuring the chlorophyll fluorescence in dark-acclimated cells, considered as the maximum photosynthetic efficiency of photosystem II (F_v/F_m). This parameter was determined using a portable pulse amplitude modulated fluorometer (AquaPen-C AP-C 110, Photo Systems Instruments, Drasov, Czech Republic), according to the method previously described [19].

2.7. Pigment Analysis

Pigments were extracted with methanol and measured spectrophotometrically as described in Ruiz-Domínguez et al. [20]. Specific carotenoids—lutein and β-carotene—were separated by HPLC equipped with a diode-array detector (L-7420, TermoQuest, CA, USA) and a RP18 column (LichroCart RP18, Merck KGaA, Darmstadt, Germany), 5 μm, size 250 × 4 mm. In the mobile phase, solvent A was ethyl acetate and solvent B was acetonitrile and water (9:1, *v/v*) (mobile phase flow rate was 1 mL per minute). Carotenoids detection was at 450 nm and the carotenoids were quantified using lutein and β-carotene standards supplied by DHI-Water and Environment (Hørsholm, Denmark) [20].

2.8. Statistics

In this study, one outdoor experiment was carried out from 8 March 2017, to 4 April 2017. Unless otherwise indicated, the data presented in this manuscript are the means of three independent samples.

3. Results and Discussion

3.1. Batch Cultivation of C. onubensis in Plastic Bags Indoors

Cultivation of *C. onubensis* in two plastic bags of 400 L each in the indoor condition was first investigated to analyze the growth stability and the average of biomass productivity. These cultures served as a source of active biomass for inoculation to the outdoor tubular photobioreactor. Figure 2A shows the growth of *C. onubensis* cultivated indoor in batch cultures in plastic bags. Two different initial biomass concentrations were assessed by differential dilution (*v/v*) of the mother culture: 1/2 (control) and 1/1, resulting an initial biomass concentration in the cultures of 0.31 g L^{-1} (C1, Figure 2A),

and 0.57 g L^{-1} (C2, Figure 2A), respectively. The culture with the lowest initial biomass concentration showed the fastest growth and it resulted in a higher biomass productivity. On the contrary, the culture with the highest initial biomass concentration rapidly attained maximum dry weight (Figure 2A).

A maximal lutein concentration of 9.7 mg g^{-1}dw was obtained, which leads to a maximal lutein productivity of 0.9 mg L^{-1} d^{-1} in plastic bags indoors.

The maximum biomass productivity in culture bags indoors achieved values of 0.09 g L^{-1} d^{-1} (Table 1) for experimental condition C1. In this condition, a maximum lutein concentration of 9.7 mg g^{-1}dry weight was obtained, leading to a maximum lutein productivity of 0.87 mg^{-1} d^{-1}. The experimental condition C2 resulted in a maximum biomass productivity of 0.07 g L^{-1} d^{-1}. Thus, condition C1 was slightly more efficient.

Table 1. Comparative productivity and lutein content of biomass in different acidophile and non-acidophile microalgae. "PBR" means photobioreactor.

Microalga	Growth System	pH	Productivity (g L^{-1} d^{-1})	Lutein (mg g^{-1})	Reference
C. onubensis	Indoor plastic bag 400 L	2.5	0.09	9.7	Present work
C. onubensis	Outdoor PBL 800 L	2.5	0.14	10.0	Present work
C. onubensis	Laboratory conditions 1 L	2.5	1.80	6.0	[18]
C. onubensis	Outdoor PBR 6 L	2.5	0.40	4.8	[12]
Chlamydomonas acidophila	Laboratory conditions. High light	2.5	0.75	-	[21]
Chlamydomonas acidophila	Laboratory conditions. Low light	2.5	0.08	-	[21]
Galdieria sulphuraria	Laboratory conditions	2.0	0.27	-	[22]
Muriellopsis sp.	Laboratory conditions 1 L	7.5	1.60	6.0	[23]
Muriellopsis sp.	Outdoor tubular	7.5	13.0 [†]	5.0	[24]
Nannocloropsis sp. CCAP 211/78	Outdoor PBR 6 L	7.5	0.71	-	[25]
Scenedesmus almeriensis	Laboratory conditions. High light	7.0	0.95	5.3	[21]
Scenedesmus almeriensis	Outdoor tubular 4000 L	7.0	0.29	4.5	[10]

[†] Units: g m^{-2} d^{-1}.

The cultures in the plastic bags are light-limited due to the large bag size, which reduces dramatically the intensity of light in the culture core. In this respect, from our own experience in bags a linear correlation exists between *C. onubensis* growth rate and light intensities below 400–500 µE m^{-2} s^{-1}. Therefore, maintaining large amounts of *C. onubensis* biomass growing actively in the linear growth phase (dry weight between 0.6 and 1 g L^{-1}) even at a lower growth rate might reduce nutrient consumption and operational costs (Figure 2A).

A major constraint in cultivating microalgae at acidic pH values is the low CO_2 solubility at low pH. It means that the cultivation system design and operation mode must be such that the supplied CO_2 is homogeneously distributed throughout the cultivation system and CO_2 losses are minimized. These conditions will influence the biomass production yield of an acidotolerant or acidophilic microalgal species [6]. However, the results of previous cultivation assays of *C. onubensis* in small-scale tubular photobioreactors showed the long CO_2 retention time in the reactor, thus, indicating the convenience of using closed tubular photobioreactors for their production [12,26].

3.2. Cultivation of C. onubensis in a Tubular Photobioreactor Outdoors

The growth of acidophilic or acidophilic microalgae at acidic pH largely depends on the inorganic carbon availability in the form of CO_2 [27,28]. At low pH, CO_2 has a very low solubility and, consequently, CO_2 supply in open systems like raceway open ponds may easily result in massive losses of CO_2 to the atmosphere. The cultivation in closed photobioreactors (i.e., tubular systems) can avoid large losses of CO_2 and additionally allow for a more efficient use of CO_2 by the cultivated algal species [29]. In addition, the vertical configuration of a tubular photobioreactor results in lowered photoinhibition compared to the horizontal disposition [12]. Accordingly, a vertical tubular photobioreactor which allows for better control of main cultivation parameters was selected for this study.

The *C. onubensis* biomass was initially produced in batch cultures, such as in plastic bags in indoor conditions, and used as an inoculum for culturing *C. onubensis* in an 800 L tubular photobioreactor outdoors. The initial biomass concentration in the tubular photobioreactor was set at 0.65 g L^{-1} and the pattern of growth is observed in Figure 2C. A biomass productivity of 0.14 g L^{-1} d^{-1} was achieved (Table 1). A maximal lutein concentration of 10 mg g^{-1}dw was obtained, which leads to a maximal lutein productivity of 1.42 mg L^{-1} d^{-1}. The outdoor trials for *C. onubensis* production described in this manuscript were carried out in March, a month of moderate maximum and minimum temperatures of approximately 26 and 5 °C on average, respectively. The culture medium pH was adjusted continuously to 2.5, which is optimal for *C. onubensis* growth [15]. In outdoor cultures, Vaquero et al. [18] observed a noticeable effect of temperature on *C. onubensis* growth. Thus, in order to maintain the culture temperature at 25 °C in the photobioreactor, water was trickled over the glass tubes from thin perforated pipes placed at the top of the vertical tubular photobioreactor. Water was collected at the bottom of the system and recirculated (Figure 1B,C).

As shown in Figure 2C, culture adaptation to outdoor conditions resulted in a slight decrease in biomass density after the first two days, which correlates with the slight decrease of maximum quantum yield from 0.7 to 0.5 within that time period. The outdoor conditions greatly differ from indoor conditions, particularly the light intensity which is much higher in the outdoor systems. Consequently, an adaptation period is required for the cultures to perform efficiently. During the adaptation period to the outdoor high light intensity, the maximum quantum yield decreases temporarily to 0.5, which did not affect further growth performance in the reactor. Zijffers et al. [30] reported that maximum photosynthetic efficiency values below 0.6 indicate a significant loss of culture viability. Nevertheless, an average value of approximately 0.65, typically found in healthy algal cultures, was recorded after the first few days. Afterwards, the maximum quantum yield of *C. onubensis* roughly remained constant within the range 0.60–0.65 (Figure 2D).

Lutein content in the microalgal cells may vary along the growth cycle in the PBR, depending on a number of factors and the algal species [31], thus the mode of cultivation in the PBR system, the dilution rate, and/or the right moment for harvesting should be eventually optimized to get enhanced productivities. The cultivation system might influence the lutein accumulation in the cells, as light path varies largely depending on the production system, for instance the tubular photobioreactors light path is usually shorter than that in raceway open ponds [32]. In microalgae lutein may accumulate under relatively low irradiance levels as, for instance, proven in *Chlorella* [33], which suggests that the low volumetric productivity shown in this paper might still be enhanced in dense cultures.

Overall, the results suggest that *C. onubensis* can be produced massively in outdoor tubular photobioreactors, at acidic pH values, during the springtime in southern Europe, seemingly without photoinhibition if the production is carried out at suitable biomass concentrations. Non-optimized biomass and lutein productivities of 0.14 and 1.42 g L^{-1} d^{-1}, respectively, were achieved, which can still be further improved. For instance, heating was not implemented during the night and the outdoor cultures were produced under natural light–dark cycles. The productivity should be expected to increase in cultures subjected to 24 h illumination and temperature control, thus implementing artificial light and heating overnight. Nevertheless, these experiments should be carried out only once the impact on process economy has been assessed. In addition, we hereby suggest that while performing the outdoor cultures at higher biomass concentrations in summer, major constraints, i.e., very high temperature and light irradiance, could be evaded by shading the tubular photobioreactor during midday hours and by cooling the system continuously as described in the previous section.

Since, so far, no reports are available on outdoor cultivation of acidophilic microalgal species, the comparison of the data obtained in this study with outgroup data is not possible. Nevertheless, in general, the productivity of *C. onubensis* is higher than that reported for other microalgae, including some acidophilic species, at a laboratory scale (Table 1). According to the *C. onubensis* productivity results obtained in the laboratory conditions and in low volume outdoor tubular reactors [12] (Table 1),

there is still room for improvement of the productivity in high volume outdoor reactors if appropriate cultures conditions are used and prior adaptation of the microalgal species is carried out.

A further economic analysis should be done to assess the potential of outdoor production of *C. onubensis* in PBR for eventual production of lutein-enriched biomass. In this sense, a complete set of productivity data should be obtained throughout the year from the PBR operating in semicontinuous mode and with the optimal biomass concentration range.

4. Conclusions

The capacity of an acidic extreme environment microalga, *C. onubensis*, for massive production in outdoor photobioreactors was evaluated. The production in acidic medium minimizes the risk of biological contamination and reduces the loss of algal productivity associated with microbial proliferation. *C. onubensis* can be produced massively in outdoor tubular photobioreactors, at acidic pH values, during the springtime in southern Europe, seemingly with limited photoinhibition if the production is carried out at suitable biomass concentrations. Non-optimized, maximal biomass and lutein productivities of 0.14 g L^{-1} d^{-1} and 1.4 mg L^{-1} d^{-1}, respectively, were achieved, which can still be further improved. The optimization of process engineering at extreme acidic pH, including pH-control and the use of acid-resistant materials, is a key challenge to maximize the productivity of *C. onubensis* and their derived products in photobioreactors.

References

1. Forján, E.; Navarro, F.; Cuaresma, M.; Vaquero, I.; Ruíz-Domínguez, M.C.; Gojkovic, Z.; Vázquez, M.; Márquez, M.; Mogedas, B.; Bermejo, E.; et al. Microalgae: Fast-growth sustainable green factories. *Crit. Rev. Environ. Sci. Technol.* **2015**, *45*, 1705–1755. [CrossRef]
2. Holdmann, C.; Schmid-Staiger, U.; Hirth, T. Outdoor microalgae cultivation at different biomass concentrations—Assessment of different daily and seasonal light scenarios by modeling. *Algal Res.* **2019**, *38*, 101405. [CrossRef]
3. Mata, T.M.; Martins, A.; Caetano, N.S. Microalgae for biodiesel production and other applications: A review. *Renew. Sustain. Energy Rev.* **2010**, *14*, 217–232. [CrossRef]
4. Norsker, N.H.; Barbosa, M.J.; Vermuë, M.H.; Wijffels, R.H. Microalgal production: A close look at the economics. *Biotechnol. Adv.* **2011**, *29*, 24–27. [CrossRef] [PubMed]
5. Acién-Fernández, F.G.; Fernández-Sevilla, J.M.; Molina-Grima, E. Photobioreactors for the production of microalgae. *Rev. Environ. Sci. Biotechnol.* **2013**, *12*, 131–151. [CrossRef]
6. Molina, E.; Fernández, J.; Acién, F.G.; Chisti, Y. Tubular photobioreactor design for algal cultures. *J. Biotechnol.* **2001**, *92*, 113–131. [CrossRef]
7. Madhubalaji, C.K.; Ajam, S.; Sijil, P.V.; Mudliar, S.; Chauhan, V.S.; Sarada, R.; Rao, A.R.; Ravishankar, G.A. Open Cultivation Systems and Closed Photobioreactors for Microalgal Cultivation and Biomass Production. In *Handbook of Algal Technologies and Phytochemicals*; CRC Press: Boca Raton, FL, USA, 2019; pp. 178–202.
8. Ben-Amotz, A. Industrial Production of Microalgal Cell-Mass and Secondary Products-Major Industrial Species: *Dunaliella*. In *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*; Richmond, A., Ed.; Blackwell Publishing: Hoboken, NJ, USA, 2007; pp. 273–280. [CrossRef]
9. Liu, J.; Hu, Q. Chlorella: Industrial Production of Cell Mass and Chemicals. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*; John Wiley & Sons: Oxford, UK, 2013; pp. 329–338. [CrossRef]

10. Fernández-Sevilla, J.M.; Acién-Fernández, F.G.; Molina-Grima, E. Biotechnological production of lutein and its applications. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 27–40. [CrossRef]

11. McClure, D.D.; Nightingale, J.K.; Sachin Black, A.L.; Zhu, J.; Kavanagh, J.M. Pilot-scale production of lutein using *Chlorella vulgaris*. *Algal Res.* **2019**, *44*. [CrossRef]

12. Vaquero, I.; Ruiz-Domínguez, M.C.; Mogedas, B.; Vilchez, C.; Vega, J.M. Production of lutein-enriched biomass by growing *Coccomyxa* sp. (strain *onubensis*; Chlorophyta) under spring outdoor conditions of southwest Spain. *J. Adv. Biotechnol.* **2016**, *6*, 828–834. [CrossRef]

13. Navarro, F.; Forján, E.; Vázquez, M.; Toimil, A.; Montero, Z.; Ruiz-Domínguez, M.C.; Garbayo, I.; Castaño, M.A.; Vílchez, C.; Vega, J.M. Antimicrobial activity of the acidophilic eukaryotic microalga *Coccomyxa* sp. (strain *onubensis*). *Phycol. Res.* **2017**, *65*, 38–43. [CrossRef]

14. Navarro, F.J.; Forján, E.; Vázquez, M.; Montero, Z.; Bermejo, E.; Castaño, M.A.; Toimil, A.; Chagüaceda, E.; García-Sevillano, M.A.; Sánchez, M.; et al. Microalgae as a safe food source for animals: Nutritional characteristics of the acidophilic microalga *Coccomyxa onubensis*. *Food Nutr. Res.* **2016**, *60*. [CrossRef] [PubMed]

15. Fuentes, J.L.; Huss, V.; Montero, Z.; Torronteras, R.; Cuaresma, M.; Garbayo, I.; Vílchez, C. Phylogenetic characterization and morphological and physiological aspects of a novel acidophilic and halotolerant microalga *Coccomyxa onubensis* sp. nov. (Chlorophyta, Trebouxiophyceae). *J. Appl. Phycol.* **2016**, *28*, 3269–3279. [CrossRef]

16. Silverman, M.P.; Lundgren, D.G. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. *J. Bacteriol.* **1959**, *77*, 642–647. [CrossRef]

17. Casal, C.; Cuaresma, M.; Vega, J.M.; Vílchez, C. Enhanced productivity of a lutein-enriched novel acidophile microalga grown on urea. *Mar. Drugs* **2011**, *9*, 29–42. [CrossRef] [PubMed]

18. Vaquero, I.; Vázquez, M.; Ruiz-Domínguez, M.C.; Vílchez, C. Enhanced production of a lutein-rich acidic environment microalga. *J. Appl. Microbiol.* **2014**, *116*, 839–850. [CrossRef] [PubMed]

19. Cuaresma, M.; Janssen, M.; Vílchez, C.; Wijffels, R. Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency. *Bioresour. Technol.* **2011**, *102*, 5129–5137. [CrossRef]

20. Ruiz-Domínguez, M.C.; Vaquero, I.; Obregón, V.; de la Morena, B.; Vílchez, C.; Vega, J.M. Lipid accumulation and antioxidant activity in the eukaryotic acidophilic microalga *Coccomyxa* sp. (strain *onubensis*) under nutrient starvation. *J. Appl. Phycol.* **2015**, *27*, 1099–1108. [CrossRef]

21. Spinola, M.V.; Díaz-Santos, E. Microalgae Nutraceuticals: The Role of Lutein in Human Health. In *Microalgae Biotechnology for Food, Health and High Value Products*; Springer: Singapore, 2020; pp. 243–263.

22. Hirooka, S.; Miyagishima, S.Y. Cultivation of acidophilic algae *Galdieria sulfuraria* and *Pseudochlorella* sp. YKT1 in media derived from acidic hot springs. *Front. Microbiol.* **2016**, *7*, 2022. [CrossRef]

23. Del Campo, J.A.; Rodríguez, H.; Moreno, J.; Vargas, M.A.; Rivas, J.; Garcia-Guerrero, M. Lutein production by *Muriellopsis* sp. in an outdoor tubular photobioreactor. *J. Biotechnol.* **2001**, *85*, 289–295. [CrossRef]

24. Blanco, A.M.; Moreno, J.; Del Campo, J.A.; Rivas, J.; Guerrero, M.G. Outdoor cultivation of lutein-rich cells of *Muriellopsis* sp. in open ponds. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1259–1266. [CrossRef]

25. De Vree, J.H.; Bosma, R.; Janssen, M.; Barbosa, M.J.; Wijffels, R.H. Comparison of four outdoor pilot-scale photobioreactors. *Biotechnol. Biofuels* **2015**, *8*, 215. [CrossRef] [PubMed]

26. Vaquero, I.; Ruiz-Domínguez, M.C.; Márquez, M.; Vílchez, C. Cu-mediated biomass productivity enhancement and lutein enrichment of the novel microalga *Coccomyxa onubensis*. *Process. Biochem.* **2012**, *47*, 694–700. [CrossRef]

27. Gross, W. Ecophysiology of algae living in highly acidic environments. *Hydrobiologia* **2000**, *433*, 31–37. [CrossRef]

28. De Farias-Neves, F.; Hoinaski, L.; Rubi-Rörig, L.; Bianchini-Derner, R.; de Melo-Lisboa, H. Carbon biofixation and lipid composition of an acidophilic microalga cultivated on treated wastewater supplied with different CO_2 levels. *Environ. Technol.* **2019**, 3308–3317. [CrossRef]

29. Molina-Grima, E.; Acién-Fernández, F.G.; García-Camacho, F.; Chisti, Y. Photobioreactors: Light regime, mass transfer, and scale-up. *J. Biotechnol.* **2009**, *70*, 231–248. [CrossRef]

30. Zijffers, J.W.F.; Schippers, K.J.; Zheng, K.; Janssen, M.; Tramper, J.; Wijffels, R.H. Maximum Photosynthetic Yield of Green Microalgae in Photobioreactors. *Mar. Biotechnol.* **2010**, *12*, 708–718. [CrossRef]

31. Gerloff-Elias, A.; Spijkerman, E.; Pröschold, T. Effect of external pH on the growth, photosynthesis and photosynthetic electron transport of *Chlamydomonas acidophila* Negoro, isolated from an extremely acidic lake (pH 2.6). *Plant Cell Environ.* **2005**, *28*, 1218–1229. [CrossRef]

32. Huang, Q.; Jiang, F.; Wang, L.; Yang, C. Design of photobioreactors for mass cultivation of photosynthetic organisms. *Engineering* **2017**, *3*, 318–329. [CrossRef]

33. Gong, M.; Bassi, A. Investigation of *Chlorella vulgaris* UTEX 265 cultivation under light and low temperature stressed conditions for lutein production in flasks and the coiled tree photo-bioreactor (CTPBR). *Appl. Biochem. Biotechnol.* **2017**, *183*, 652–671. [CrossRef]