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Production of *Scenedesmus almeriensis* Using Pilot-Scale Raceway Reactors Located inside a Greenhouse

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Abstract: The microalga *S. almeriensis* was produced in spring and summer using 80 m^2 raceway reactors located inside a greenhouse in Almería, Spain. This microalga was selected because it is a fast-growing and robust strain with potential applications in the production of functional foods and feeds or as a high-value agricultural biostimulant. Overall, the biomass productivity obtained in summer was 24.9 ± 0.9 g·m^{-2}·day^{-1}. This value was higher than that obtained in spring, with an average value of 21.4 ± 1.3 g·m^{-2}·day^{-1} ($p < 0.05$). The F_v/F_m value of the cultures at the stationary phase was comparable and around 0.6, which is the optimum of this strain. No major differences in the macromolecular composition of the biomass were observed between seasons, with an average protein, lipid, ash, and carbohydrate content of 37.9, 4.6, 10.8, and 46.7%, respectively. The data reported herein were used to validate a previously described model. The differences between the experimental and the predicted biomass productivities were below 5% in spring and 8% in summer, although a larger dataset is needed to validate the model. Overall, results supported the robustness of the selected strain and its utilisation in different industrial sectors.

1. Introduction

Microalgae are gaining increased importance in the context of global bioeconomy. These microorganisms are mainly being used as foods or nutraceuticals [1]. However, despite the large number of microalgal strains currently available in culture collections, only a limited number of strains are being mass cultured for industrial applications. These include, but are not limited to, *Arthrospira platensis* and *Chlorella vulgaris* used as food [2] or *Dunaliella salina* and *Haematococcus pluvialis* which are commercially produced as sources of β-carotene and astaxanthin, respectively [3–5]. Microalgal strains used as food in the EU must comply with Regulation (EU) 2015/2283 on novel foods. Due to their long history of use, *A. platensis* (commercially known as spirulina) and *C. vulgaris* do not need to comply with this regulation. Other strains such as *Tetraselmis chuii* can also be used as food in the EU [6]. In the United States, the consumption of microalgae is regulated by the Food and Drug Administration (FDA), which recognise food additives and ingredients as GRAS, the acronym for the phrase "generally recognised as safe". *Arthrospira*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Schizochytrium*, *Porphyridium cruentum*, and *Crypthecodinium cohnii* have GRAS status [7]. Most countries accept the consumption of microalgae following the FDA recommendations. Microalgae are used to produce food not only directly as food ingredients but also indirectly, as feed ingredients [8] or as high-value agricultural products, namely biostimulants and biopesticides [9]. Due to their high content in proteins and other valuable bioactive molecules, microalgae are excellent sources of feed ingredients. In addition, biostimulants are one of the top trends in agriculture, especially in organic

cropping systems. The biostimulant activity of microalgae is due to their content of phytohormones (e.g., auxins, cytokinines, ethylene, gibberellins) [10].

Due to their small size, microalgae are not harvested from the environment but are produced in controlled facilities using photobioreactors. The most widely used photobioreactor designs are raceway reactors. Today, approximately 90% of the microalgal biomass being commercialised is produced using raceway reactors [11]. Their main advantages are their simplicity, ease of scale-up, and low construction costs. Indeed, raceway reactors are the most cost-effective cultivation system and their low productivity is compensated by their low capital and operating costs (and high economic value of microalgae) [12]. One of the main challenges of microalgae production is the difficulty of up-scaling biological processes. Most of the studies conducted to date assessed biomass productivities using laboratory-scale or pilot-scale photobioreactors that are not representative of industrial practices. There is an urgent need for up-scaling microalgae production processes to predict industrial productivities and estimate production costs.

The aim of this study was to validate the production of the strain *Scenedesmus almeriensis* using 80 m^2 raceway photobioreactors during two seasons, spring and summer. *S. almeriensis* was selected because of its huge potential to achieve commercial success. Due to its high content in lutein, this strain has been suggested as a potential functional food ingredient [13]. This microalga also showed potential for use as a feed ingredient promoting the gut functionality of fish [8] and as a biostimulant in agriculture [9]. In addition, *S. almeriensis* is a robust strain that can be produced using wastewater and different reactor designs [14,15] which has been suggested as key to reduce production costs [16].

2. Materials and Methods

2.1. Microalga Used and Culture Media Composition

The selected strain was *S. almeriensis* (CCAP 276/24). The strain *S. almeriensis* was initially isolated from a photobioreactor exposed to high temperature (45 °C) and irradiance (2000 µmol·m^{-2}·s^{-1}) conditions [17]. Previous reports demonstrated that *S. almeriensis* can adapt to pH, temperature, and salinity values ranging from 7–10, 26–40 °C, and 0–5 g NaCl·L^{-1}, respectively. Moreover, the used strain shows no signs of photoinhibition up to 1625 µmol·m^{-2}·s^{-1} [18].

The inocula of *S. almeriensis* were produced at 23 ± 2 °C, pH 8.0 ± 0.1, and 150 µmol·m^{-2}·s^{-1} in batch mode until a concentration of 1 g·L^{-1}. Then, the inocula were scaled up to a final volume of 80 L using pH-controlled outdoor bubble column photobioreactors placed inside a greenhouse. The culture medium was formulated using commercial fertilisers instead of analytical-grade chemicals. The composition of the medium was 0.90 g·L^{-1} NaNO$_3$, 0.18 g·L^{-1} MgSO$_4$, 0.14 g·L^{-1} K$_2$PO$_4$, and 0.03 g·L^{-1} Karentol® (Konegard, Barcelona, Spain). Karentol® is a commercial solid mixture of micronutrients that include boron, copper, iron, manganese, molybdenum, and zinc.

2.2. Photobioreactors Used

The biomass production was conducted using three 80 m^2 raceway reactors located inside a greenhouse (Figure 1) at the Institute for Agricultural and Fisheries Research and Training (IFAPA) in Almería, Spain. The three reactors were identical and were operated simultaneously under the same environmental conditions (Figure 2). The operating volume of the reactors was 11.8 m^3 and the depth of the culture was 0.13 m. Biomass production was initially conducted in batch mode until the stationary phase was achieved and then in semi-continuous mode with a dilution rate of 0.3 day^{-1}, which is the optimum for these reactors in spring and summer [19]. Semi-continuous production was conducted for 20 days. Temperature, pH, and dissolved oxygen concentration were monitored using 5083 T and 5120 probes (Crison Instruments, Barcelona, Spain) connected to an MM44 control-transmitter unit (Crison Instruments, Barcelona, Spain) and Labview data acquisition software (National Instruments, Austin, TX, USA).
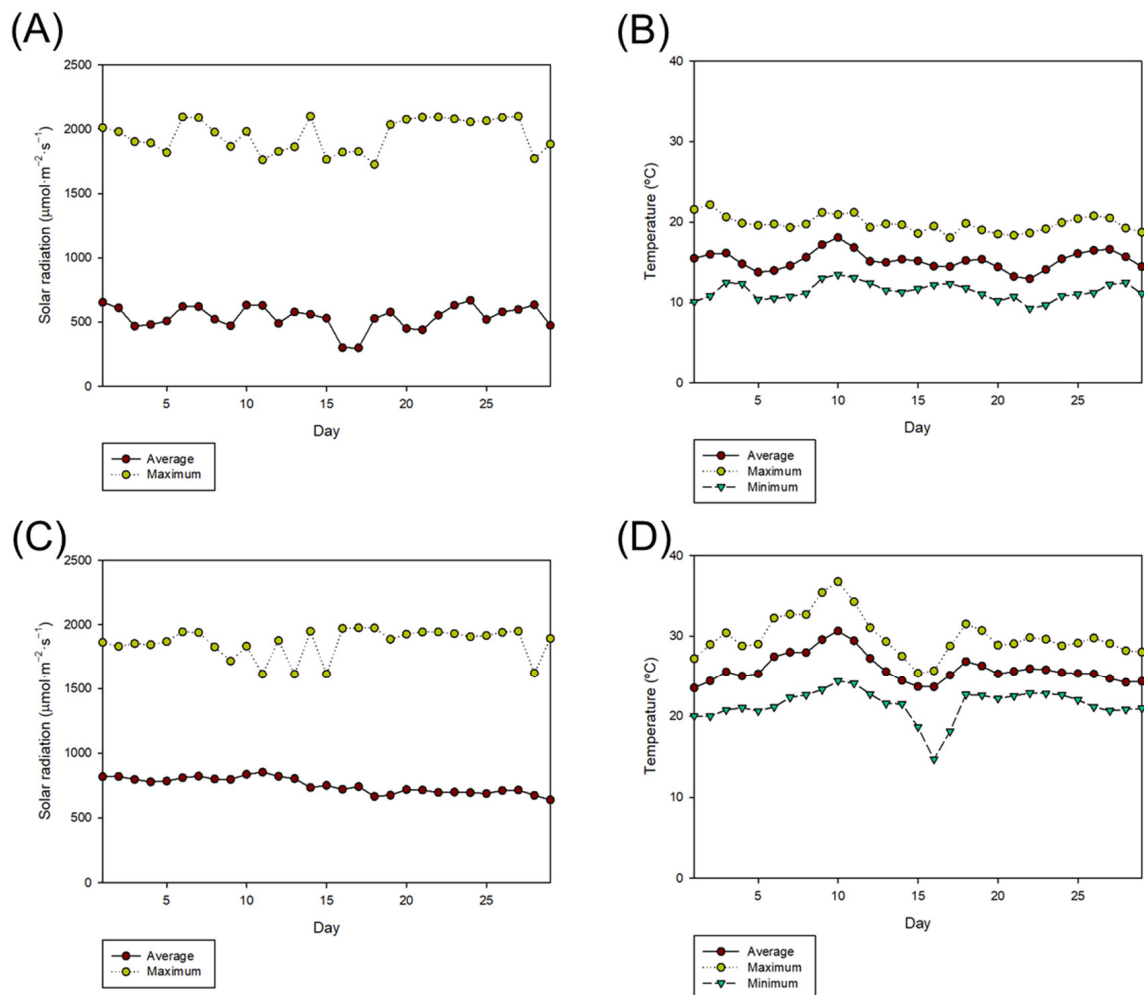
Figure 1. Photobioreactors used.

Figure 2. (**A**) Solar radiation and (**B**) temperature during biomass production in spring and (**C**) solar radiation and (**D**) temperature during biomass production in summer.

The pH was controlled and kept constant at 8.0 ± 0.1 by on-demand injection of carbon dioxide. Water evaporation was compensated daily by addition of freshwater. The culture was harvested using an industrial SSD 6-06-007 GEA separator (GEA Westfalia Group,

Oelde, Germany). The harvested biomass was immediately frozen and freeze-dried. The dried powder was vacuum-sealed and stored at −20 °C until further analysis. The nutrient content of the water effluent was analysed to ensure that the culture was not limited in terms of nutrients.

2.3. Analytical Determinations

The biomass concentration was measured by dry weight filtering 50 mL of culture through 1 μm filters and drying it in an oven at 80 °C for 24 h. The biomass productivity was calculated as the product of the biomass concentration and the dilution rate. The chlorophyll fluorescence ratio (F_v/F_m) was determined using an AquaPen AP 100 fluorometer (Photon System Instruments, Drásov, Czech Republic) after a dark adaptation period of 5 min.

The biomass extinction coefficient (K_a) and the average irradiance inside the culture (I_{av}) were calculated as described previously [19]. Spectrophotometric determinations were conducted using a Genesys™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Barcelona, Spain).

The lipids were extracted following the Folch method using chloroform:methanol (2:1 *v/v*) as the solvent and the total lipid content was calculated gravimetrically [20]. The crude protein content was determined by the Kjeldahl method following acid digestion using a nitrogen-to-protein conversion factor of 5.95 [21]. The ash content was determined by calcination of the biomass to a constant weight in a muffle oven at 600 °C for 12 h [22]. The carbohydrate content was determined by difference.

The pigment contents, namely chlorophyll-a (Ch_a), chlorophyll-b (Ch_b), and total carotenoids (TC), were estimated spectrophotometrically using the equations proposed by Wellburn [23]. Briefly, 100 mg of freeze-dried biomass were suspended in 40 mL of acetone and sonicated for 10 s using a UP400S ultrasonic processor (Hielscher Ultrasonics GmbH, Teltow, Germany) operating at 400 W and 24 kHz. The extract was stirred at 150 rpm for 5 min and centrifuged. The supernatant was evaporated under a nitrogen stream, resuspended in methanol, and the pigment concentration was calculated using:

$$Ch_a\left(\mathrm{mg \cdot L^{-1}}\right) = 16.72 \cdot A_{665} - 9.16 \cdot A_{652} \tag{1}$$

$$Ch_b\left(\mathrm{mg \cdot L^{-1}}\right) = 34.09 \cdot A_{652} - 15.28 \cdot A_{665} \tag{2}$$

$$TC\left(\mathrm{mg \cdot L^{-1}}\right) = \frac{1000 \cdot A_{470} - 1.63 \cdot Ch_a - 104.96 \cdot Ch_b}{221} \tag{3}$$

where A_{665}, A_{652}, and A_{470} are the optical density of the extract at 665, 652, and 470 nm, respectively. Two technical replicates were conducted per natural replicate and results are expressed on a dry weight basis.

The concentration of N-NO$_3^-$ and P-PO$_4^{3-}$ at the inlets and outlets of the reactors was measured using official methods approved by the Spanish Ministry of Agriculture [24]. Briefly, N-NO$_3^-$ and P-PO$_4^{3-}$ were measured spectrophotometrically by measuring the absorbance at 220–275 nm and the phospho-vanado-molybdate complex, respectively. All these determinations were conducted daily and in triplicate per natural replicate.

2.4. Model Validation

Experimental results were compared to those predicted by a recently published model developed following a surface response methodology [25]. The model allows predicting biomass productivity in raceway reactors as a function of the dilution rate and the depth of the culture. The model enables the estimation of the biomass productivity in the south of Spain in spring using the following equation:

$$P_b\left(\mathrm{g \cdot m^{-2} \cdot day^{-1}}\right) = 5.90 + 113.41 \cdot D - 47.82 \cdot h - 145.43 \cdot D^2 \tag{4}$$

In summer, biomass productivity can be estimated using the following equation:

$$P_b \left(\text{g·m}^{-2} \cdot \text{day}^{-1} \right) = -0.23 + 149.57 \cdot D - 6.73 \cdot h - 192.00 \cdot D \cdot h - 142.74 \cdot D^2 \qquad (5)$$

where D is the dilution rate (day^{-1}) and h is the depth of the culture (m).

2.5. Statistical Analysis

Normality and homoscedasticity of the variables within each group were checked. The data were analysed using one-way analysis of variance with JMP 13 (SAS Institute Inc., Cary, NC, USA). A Tukey pairwise comparison of the means was carried out to identify where sample differences occurred ($p < 0.05$).

3. Results

The environmental conditions during biomass production are shown in Figure 2. Briefly, the average environmental temperature during spring and summer was 15.2 ± 1.1 and 25.9 ± 1.7 °C, respectively. Maximum temperature values were on average 19.3 ± 1.6 and 29.3 ± 2.6 °C, respectively. Similarly, the average solar radiation that reached the cultures in spring and summer was 559 ± 82 and 741 ± 64.4 µmol photons·m^{-2}·s^{-1}, respectively.

These environmental conditions allowed achieving biomass productivities of 21.4 ± 1.3 and 24.9 ± 0.9 g·m^{-2}·day^{-1} in spring and summer, respectively (Figure 3). F_v/F_m values were comparable in both seasons and around 0.6, which is the optimum for *S. almeriensis* produced in raceway reactors.
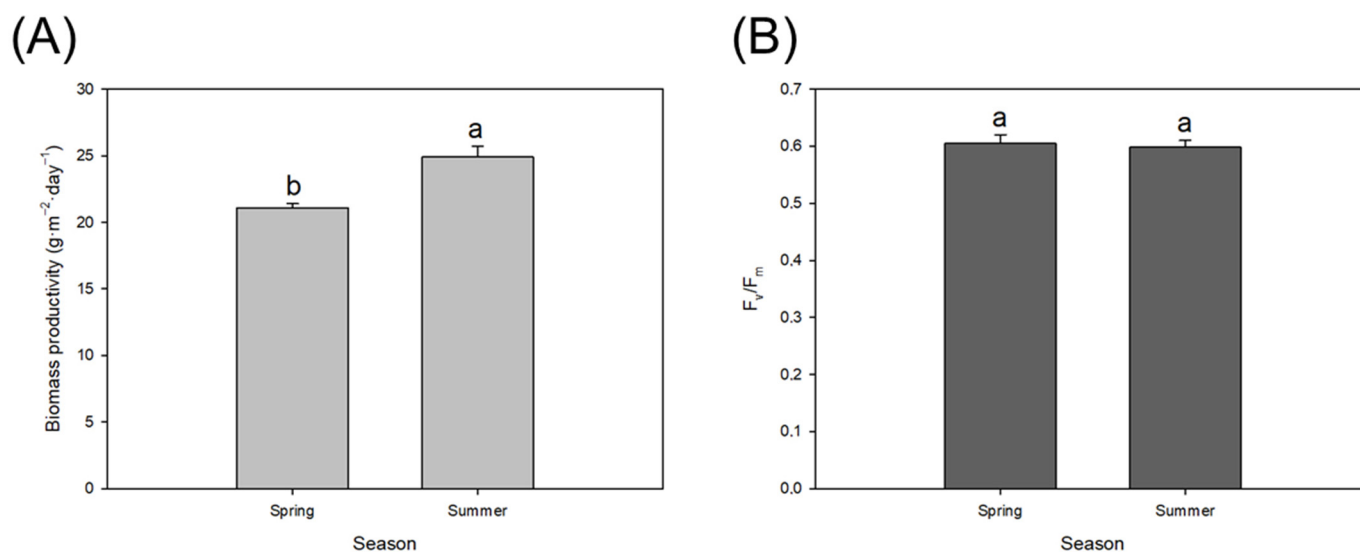
Figure 3. (**A**) Biomass productivity and (**B**) F_v/F_m values during the steady state. Values represent the means of three independent determinations $\pm$ SD. Different letters indicate significant differences between seasons. The criterion for statistical significance was $p < 0.05$.

The biomass productivity values were compared to those obtained using a model that predicts biomass productivities as a function of culture depth and the dilution rate [25]. The comparison between the experimental and predicted biomass productivity values is shown in Figure 4. The model used was developed using wastewater instead of freshwater and commercial fertilisers as the nutrient sources. Overall, the differences between the experimental and predicted biomass productivity values were around 5% in spring and 8% in summer, suggesting the validity of the model to predict biomass productivity in the south of Spain.

The macromolecular composition of the produced biomass is summarised in Table 1. Overall, no major differences were observed between the composition of the biomass produced in spring or in summer. The protein content of the biomass was on average 37.9%.

The lipid and ash contents were calculated as 4.6 and 10.8%, respectively. Moreover, no differences were observed between the total chlorophyll content of the biomass produced in spring and summer (9.9 ± 0.3 mg·g^{-1}). However, the total carotenoid content in summer was estimated as 3.9 ± 0.5 mg·g^{-1}, higher than that obtained in spring with an average value of 2.5 ± 0.2 mg·g^{-1} ($p < 0.05$).

Table 1. Macromolecular composition of *S. almeriensis*. Results are the average of three independent determinations $\pm$ SD. Different letters indicate significant differences. The criterion for statistical significance was $p < 0.05$.

Season	Protein (g·100 g^{-1})	Lipid (g·100 g^{-1})	Ash (g·100 g^{-1})	Carbohydrate (g·100 g^{-1})
Spring	38.0 ± 0.2	4.6 ± 0.3	9.9 ± 0.3	47.5 ± 0.7
Summer	37.8 ± 0.4	4.6 ± 0.5	11.6 ± 2.4	45.9 ± 2.5
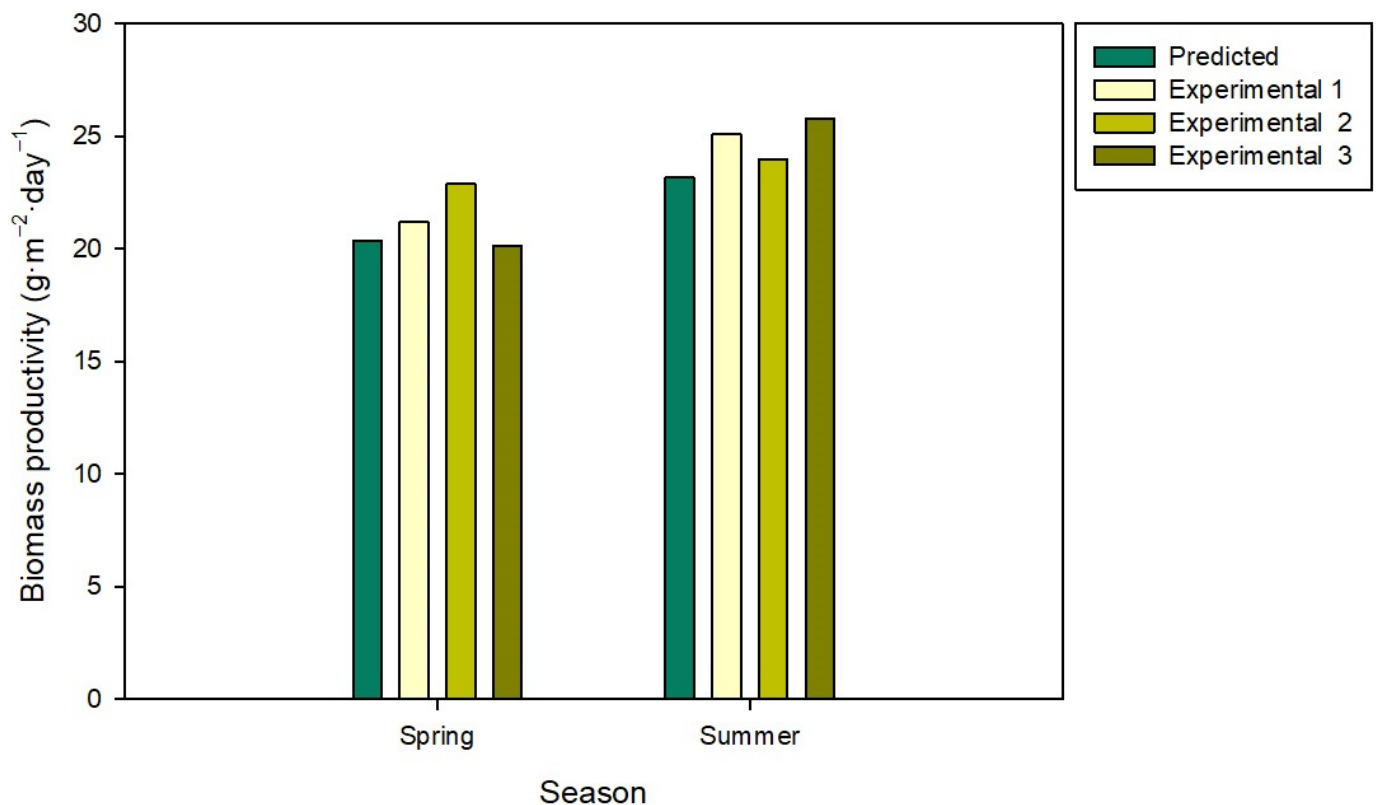
Figure 4. Differences between predicted and experimental biomass productivities. Predicted values were obtained using an SRM model for spring and summer, respectively [25].

4. Discussion

The environmental conditions of the region during biomass production and the design of the photobioreactor allowed achieving biomass productivity values of 21.4 ± 1.3 and 24.9 ± 0.9 g·m^{-2}·day^{-1} in spring and summer, respectively. The higher productivity obtained in summer can be attributed to more favourable environmental conditions. Indeed, the optimal temperature for *S. almeriensis* is 25 °C [18] and the average temperature in summer was 25.9 °C. The biomass productivity of the process has a striking effect on the production cost of the biomass and it is highly sensitive to geospatial factors [12]. The productivity values reported here were in line with those obtained in previous reports using raceway reactors. For example, the biomass productivity of a 100 m^2 raceway reactor operated at a culture depth of 0.1 m and a dilution rate of 0.2–0.3 day^{-1} in spring led to a

biomass productivity of 20–25 $g \cdot m^{-2} \cdot day^{-1}$ [26]. Similarly, biomass productivity values from 20 to 25 $g \cdot m^{-2} \cdot day^{-1}$ were reported for a 80 m^2 raceway reactor during spring and summer [19]. Similarly, the biomass productivity values reported herein were also comparable to those obtained when producing microalgae using wastewater and other waste streams instead of freshwater. For example, the productivity of a 32 m^2 (0.12 m culture depth) raceway reactor producing *Scenedesmus* sp. in summer was 24 $g \cdot m^{-2} \cdot day^{-1}$ when operated using centrate from anaerobic digestion as the sole nutrient source and a dilution rate of 0.3 day^{-1} [27]. Similarly, a previous study utilising the same photobioreactor used herein but operated with wastewater instead of freshwater reported maximum biomass productivities of 25.1 $g \cdot m^{-2} \cdot day^{-1}$ in summer [14]. The results demonstrate the robustness of the produced strain that can grow well independently of the type of water used. Economic viability is of key importance to achieve commercial success [28]. Annual studies are needed to assess the techno-economic performance of the reactors. However, production of microalgal biomass using raceway reactors is feasible as most of the biomass produced today is produced using this system [16].

The quantum yield or the F_v/F_m value of the cultures was monitored daily. The F_v/F_m value, shown in Figure 2, represents a non-invasive measurement of photosystem II (PSII) activity [29]. A decrease in the quantum yield indicates that the culture is subjected to a stress condition such as excess light [30], lack of nutrients [31], or presence of toxins or heavy metals [32]. The F_v/F_m values are just an estimation of the status of the culture. The optimal value for most microalgal strains ranges from 0.6 to 0.7. Previous studies reported decreased F_v/F_m values in summer because of reversible damage to PSII components [33]. Values close to 0.6 have been reported for this same strain in spring and summer, demonstrating that the microalgae were growing without stress conditions. *S. almeriensis* is highly resistant to harsh environments, especially in terms of solar radiation [18].

In the present study, the biomass productivity obtained when operating the reactors in spring and summer was compared with that predicted using a recently published model based on a response surface methodology [25]. A response surface methodology explores the relationship between several explanatory variables and one or more response variables. In the used model, the explanatory variables used were the dilution rate and the depth of the culture, which are the main operational parameters in raceway reactors [34]. Moreover, because of the strong impact of environmental conditions on the performance of microalgae, the above-mentioned study developed one model for each season. This is a simple model to predict biomass productivity based only on operational conditions, other aspects related to the biology of microalgae and environmental conditions would better predict biomass productivity [35,36]. In the present study, the difference between the predicted and experimental values was lower than 5% in spring and 8% in summer, suggesting the validity of the model. It is important to highlight that a larger dataset is needed to confirm these findings.

The effect of environmental conditions on the macromolecular composition of the produced biomass is shown in Table 1. The composition of the biomass can be strongly affected by environmental and operational conditions [34,37]. The median macromolecular composition of microalgae was reported to be 32.2% protein, 17.3% lipid, 15.0% carbohydrate, 17.3% ash, and 5.7% RNA [38]. These values were calculated using a hierarchical Bayesian analysis of data compiled from the literature. In the present study, as highlighted before, no differences were observed in the protein, lipid, ash, and carbohydrate content of the produced biomass. The composition of the produced biomass was comparable to that of *Scenedesmus* sp. produced in controlled laboratory-scale photobioreactors [39]. The protein content of the produced biomass was around 38%, which compares well with other food products rich in protein such as legumes (approximately 26%). Although the protein content of the biomass was high, other microalgae have higher protein content. For example, the cyanobacterium *A. platensis* or spirulina can accumulate over 60% of protein on a dry weight basis [40]. *A. platensis* is a prokaryotic cyanobacterium and not a eukaryotic microalga. Cyanobacteria have a higher protein content than microalgae [38].

Moreover, despite not affecting the total chlorophyll content, the different environmental conditions during the two production periods promoted the synthesis and accumulation of carotenoids. In the present study, only the total carotenoid content was determined. Further studies will identify the carotenoid profile of the biomass, focusing on lutein which has anti-inflammatory properties and other beneficial effects, especially on eye health [41]. Carotenoids are ubiquitous and essential pigments in photosynthesis. These compounds absorb in the blue–green region of the solar spectrum and transfer the absorbed energy to chlorophylls. By doing this, they expand the wavelength range of light that is able to drive photosynthesis and are therefore of key importance for photosynthetic organisms. The higher solar radiation values in summer probably triggered the synthesis and accumulation of carotenoids. Other microalgae such as *H. pluvialis* respond similarly to high light availability and promote the synthesis and accumulation of carotenoids and, in the case of *H. pluvialis,* astaxanthin [5]. As highlighted above, *S. almeriensis* shows potential for being used as an ingredient in the development of functional foods because of its high content in lutein. Although some food products have been developed [13,42], *S. almeriensis* is not yet permitted as food. Further studies will assess the safety of the produced biomass and will facilitate the incorporation of this valuable microalga into foods.

5. Conclusions

The microalga *S. almeriensis* can be scaled up using raceway reactors and commercial fertilisers as the sole nutrient source. Biomass productivities were comparable to those of previous studies using similar photobioreactor designs. An increase in biomass productivity in summer was observed when compared to spring because of more favourable environmental conditions. The composition of the produced biomass suggests the potential of *S. almeriensis* as a novel food or feed ingredient because of its high protein content, which compares favourably with current protein sources such as soybean.

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