



Production of Lutein-enriched Biomass by Growing *Coccomyxa* sp. (strain *onubensis*; Chlorophyta) under Spring Outdoor Conditions of Southwest Spain

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ABSTRACT

Coccomyxa sp. (strain *onubensis*) is an acidic environment microalga, which was grown under spring outdoor conditions of Huelva province (southwest, Spain). A 6 L pilot tubular photobioreactor was used to determine the influence of the environmental light and temperature conditions on the microalga growth and its intracellular content of carotenoids. Cloudy days supplied the adequate light intensity (maximum 1070 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and temperature (maximum 32°C) regimes for a biomass productivity of 0.28 $\text{g L}^{-1} \text{d}^{-1}$ with an intracellular content of lutein (4.8 mg g^{-1} dry weight) and eventually β -carotene (0.8 mg g^{-1}) obtained at the end of the day evening. This work supplies guidelines for a large-scale outdoor culture of *Coccomyxa* sp. to supply lutein-enriched biomass, with the advantage of the growth at pH 2.5, which preserves cultures from contamination.

Keywords

Coccomyxa sp.; Lutein; β -Carotene; Eukaryotic microalgae; Tubular photobioreactor.

Academic Discipline And Sub-Disciplines

Biology; Biotechnology

SUBJECT CLASSIFICATION

Biotechnology; Bioprocesses

TYPE (METHOD/APPROACH)

Outdoor bioproduction; Bioprocess engineering; High-added value compounds production

INTRODUCTION

Commercial interest of microalgae increased during the past decades due to its biomass, which is considered as a rich source of a vast group of chemicals with applications in food, nutritional, cosmetic, pharmaceutical and fuel industries [1].

The utilization of microalgae for humans is centuries old (*Nostoc* in Asia and *Spirulina* in Africa and Mexico), however, large-scale commercial production of biomass from microalgae date only from the 1950s when *Chlorella* and *Arthrospira* began to be considered as new alternatives for foods [2,3,4]. In outdoor cultures, the availability of nutrients, light intensity and temperature are the main parameters that influence microalgae biomass production [5,6]. Light received by cells is a function of the incident light intensity on the surface of the reactor, the path length of the light from the surface to any point inside the culture, and the light attenuation produced by the cells themselves (mutual-shading effect) inside the reactor [7].

When irradiance intensity is low, the microalga photosynthetic machinery receives fewer photons than it can manage; and consequently, the growth of alga is light-limited. However, photosynthetic efficiency is usually high because the largest part of the absorbed light energy by the microalga had converted into biomass chemical energy [8]. Conversely, when irradiance intensity was high, the cultures were saturated for its photosynthetic activity; and then photooxydative damage may occur in the cells producing important degradation of photosystem II, thus, amazing cells viability. Microalgae have mechanisms to prevent photooxydation and some of them have been related with carotenoids production due to its antioxidant nature. Depending on microalga sensitivity to light, the irradiance intensity may induce the accumulation of carotenoids in the biomass [9].

Some studies have reported the influence of environmental parameters on biomass productivity from acidophilic microalgae; however, very few of them were done under outdoor conditions [10,11]. One of the main reasons that can explain this fact is because this kind of microalgae apparently cannot compete for biomass production with mesophilic ones due to their low growth rate [12]. However, *Coccomyxa* sp. exhibits a relatively high growth rate under acidic conditions, as compared with other acidophilic microalgae; thus, it is a good candidate for outdoor cultivation with biotechnological purposes.

The effect of outdoor light intensity and temperature regimes of spring weather at Huelva province was studied on the biomass productivity and carotenoids accumulation of *Coccomyxa* sp. using an experimental tubular 6 L photobioreactor. The reported results provide hopes regarding the use of this microalga for large-scale production of lutein-enriched biomass.

MATERIALS AND METHODS

Microalga and growth medium

The unicellular eukaryotic microalga *Coccomyxa* sp. (strain *onubensis*) was isolated from the acidic waters of the Tinto river, which runs through a mining area in Huelva (Spain), and has very special features that include a low pH and high concentrations of heavy metals, such as iron, copper, magnesium and aluminium. The microalga has deposited in the Göttingen (Germany) stocks culture, with the number SAG 2510.

An axenic culture of this microalga has obtained by streaking it on a basal agar 1.75% (w/v) medium at pH 2.5, followed by transfer to liquid medium. According to the chemical composition of water in its natural environment, the microalga has grown at a pH of 2.5 in a modified K9 medium prepared with the following composition: 3.95 g K_2SO_4 , 0.1 g KCl, 0.5 g K_2HPO_4 , 0.41 g $MgCl_2$, 2.29 g KNO_3 , 0.01 g $CaCl_2$ and 5 ml of Hutner trace elements solution. The microalga cultures were bubbled with air containing 5% (v/v) CO_2 . Incident light intensity on the cultures has measured at the surface of the photobioreactor using a photoradiometer Delta OHM (mod. HD9021).

Photobioreactor

The microalga has grown autotrophically in a pilot tubular photobioreactor (PBR) with a total length of 13 m made up with transparent acrylic pipes of 21 cm (inside diameter) and 25 cm (outside diameter) that included a degasser vertical column that acted to avoid dissolved oxygen accumulation in the culture and a spray cooling system to regulate temperature. In addition, the PBR has an inlet to bubble air enriched in CO_2 . The total volume capacity of the PBR was 6 L, of which 4.5 L were within the pipes and 1.5 L within the degasser. The flow rate through the tubes was 6.0 L min^{-1} . A schematic diagram of the photobioreactor used in this study is shown in Figure 1.

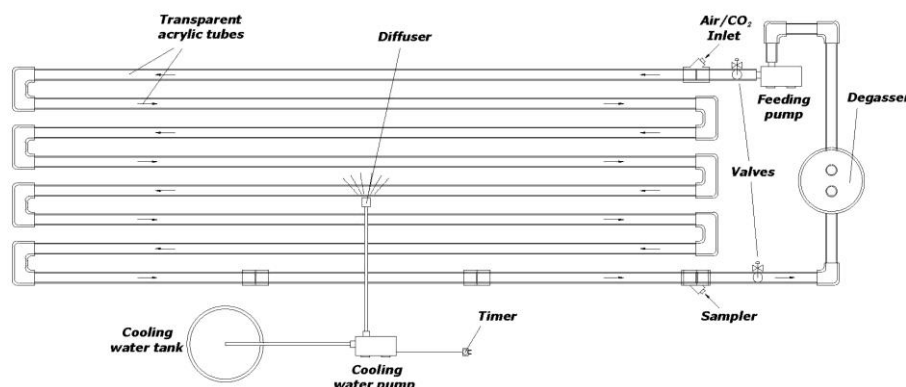
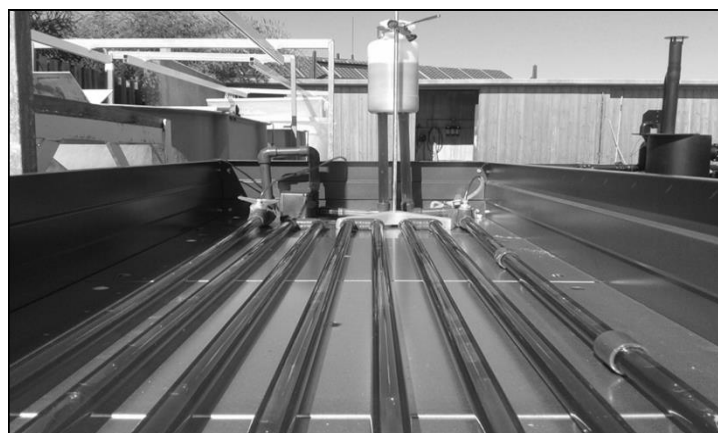


Fig 1: Picture and diagram of the 6 L pilot tubular photobioreactor used for the growth of *Coccomyxa* sp. cultures.

Outdoor culture conditions

The regimens of incident light intensity and temperature on the microalga cultures are presented in Figures 2 and 3, respectively. Four experimental working conditions were used in these studies and they are the following:

VHLT Culture. The microalga growth proceeds under light and temperature conditions of a typical spring sunny day (late April) at the study site in Huelva province (Southwest, Spain). The cell culture endured moments of high-level of light intensity (approximately $1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and maximum temperature of approximately 40°C at the early afternoon. This culture underwent at “very high light and temperature” conditions, thus the name VHLT.

VHL Culture. The incident light intensity profile on the PBS was similar to that used for VHLT culture, but temperature has moderated with a spray cooling system, and maximum did not exceed 32°C . This culture underwent at “very high light” conditions, thus, it has been referred to as VHL.

HL Culture. The microalga culture had submitted to the irradiance intensity and temperature change profiles typically produced during a spring cloudy day at the study site. The cell culture had exposed to a maximal light irradiance of $1070 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the surface of the reactor, but the maximum temperature reached in the culture was 32°C . This culture supports “high light” conditions (HL).

ML Culture. To limit the incident light intensity on the microalga cultures during the spring sunny days, the reactor was shaded with a mesh, and the algal culture was exposed to a maximal light intensity of $650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and temperature of 36°C . This culture supports “moderate light” conditions (ML).

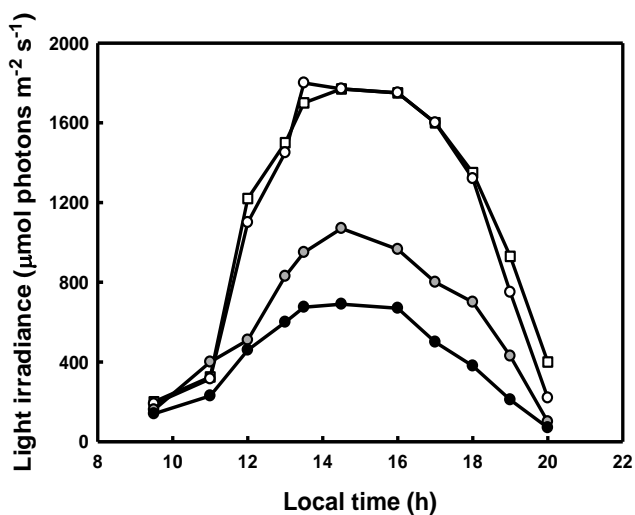


Fig 2: Incident light intensity profile on *Coccomyxa* sp. outdoor cultures. It shows the incident light intensity profile on VHLT (white circles), VHL (white squares), HL (grey circles) and ML (dark circles) cultures.

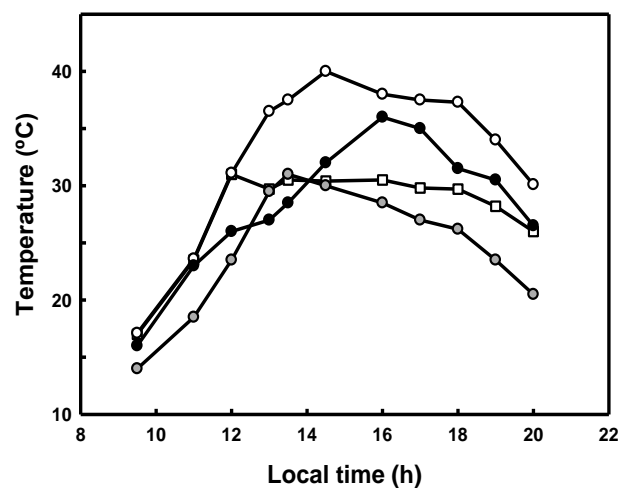


Fig 3: Temperature profile in *Coccomyxa* sp. outdoor cultures. It shows the temperature profile corresponding to VHLT (white circles), VHL (white squares), HL (grey circles) and ML (dark circles) cultures.

Dry weight measurements and growth rate calculations

Dry weight measurements and growth rate calculations were determined as previously described [13].

Quantum yield (Q_Y)

To evaluate the photosynthetic performance, chlorophyll fluorescence measurements have been considered as the maximum quantum yield, $Q_Y (F_v/F_m)$, of the PSII photochemistry. This parameter was determined using pulse amplitude modulation (PAM) 210, Walz, Germany), according to the method previously described [14].

Chlorophyll and carotenoids determination

Pigments were extracted using 1 mL aliquots of the cultures. The cells were spun down for 8 min at 13000 rpm, suspended in 1 mL of methanol, shaken vigorously for 1 min, and heated at 60°C in a water bath for 5 min. The suspension has then centrifuged for 8 min at 4400 rpm and pigments were in the supernatant. Carotenoids were separated from chlorophylls and were identified by HPLC following the method described by Young *et al.* [15]. An RP-18 column (TeruoQuest, Thermo products, UK) was used with a mobile phase including ethyl-acetate (as solvent A) and acetonitrile and water (9:1, v/v) (solvent B). Standards (DHI, <http://c14.dhigroup.com>, Denmark) and their corresponding calibration curves have been used as reference to identify and quantify both lutein and β -carotene.

Measures of dissolved oxygen

Dissolved oxygen (DO) concentration in the culture is the result of an equilibrium between the oxygen-producing processes (e.g., photosynthetic activity) and oxygen-consuming processes (e.g., aerobic respiration and chemical oxidation) and the rates at which oxygen is added to, or removed from, the system by aeration or degassing, respectively. DO concentration was measured at the surface of the degasser using a portable oxymeter (Crison, OXI 45+).

Statistics

Each experiment was performed three times and the observed tendency was clear and coherent, but since outdoor conditions may slightly change from one day to another, the presented results correspond to one outdoor experiment done in a typical day conditions. Experimental results were obtained as mean \pm S.D. of three parallel measures. A multifactorial analysis of the variance (ANOVA) was executed using the data obtained from each extract. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Effect of outdoor light intensity and temperature profiles on maximum quantum yield and biomass productivity of *Coccomyxa* sp. cultures

Figure 4 shows the maximal Q_Y of PSII in the *Coccomyxa* sp. cultures under different experimental conditions used in this work. The VHTL and VHL cultures exhibited Q_Y under 0.60, with values as low as 0.50, during daytime with highest illumination, indicating that cells are under light-induced stress. On the other hand, the Q_Y values reached by HL and ML cultures are always over 0.70 and 0.60, respectively, which is compatible with non-stressed cells in the photobioreactor. The cells in these cultures exhibited healthy aspect (data not shown).

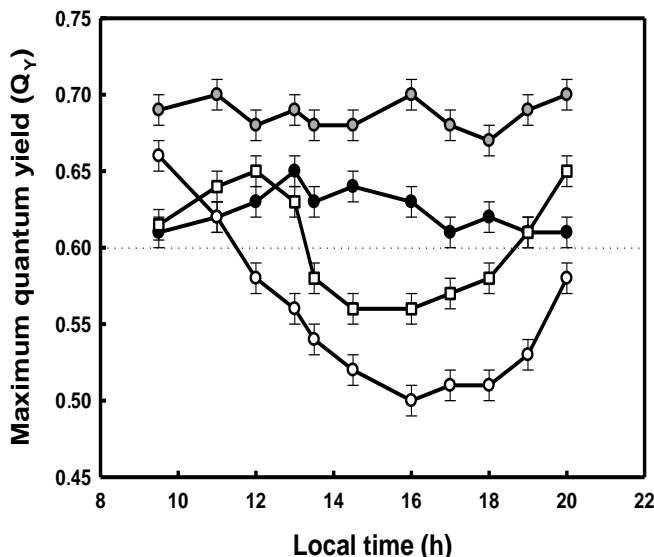


Fig 4: Maximum quantum yield, Q_Y (Fv/Fm), of PSII in *Coccomyxa* sp. outdoor cultures. The initial biomass concentrations in the cultures were 0.80-0.85 g L⁻¹. When indicated measurements were done through triplication using aliquots of the corresponding culture, data were given as mean values \pm SD. Other experimental conditions are displayed in Figure 2.

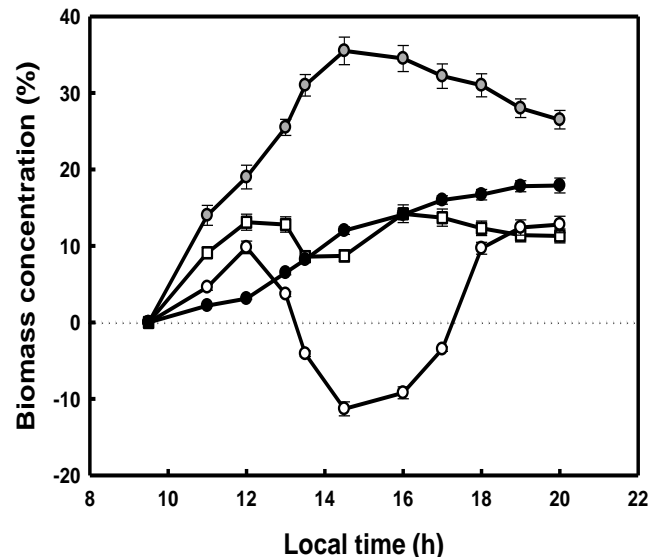


Fig 5: Biomass productivity of *Coccomyxa* sp. outdoor cultures. Data shown correspond to VHTL (white circles), VHL (white squares), HL (grey circles) and ML (dark circles) cultures. Other conditions as in Figure 4.

Figure 5 shows the diurnal relative biomass change in outdoor *Coccomyxa* sp. cultures. After 10 h of growth, the biomass increased by 12% (w/w) in both VHTL and VHL cultures, however, VHL culture exhibited higher biomass than VHTL culture during most part of the day, indicating that moderate temperature profile was better than high temperature regime for outdoor growth of *Coccomyxa* sp. The biomass productivity in HL and ML cultures differed significantly from each other during the daylight time, and a net increase of 35.0 and 15.5% (w/w), respectively had been observed at 14:30 h local time.

Biomass productivity of *Coccomyxa* sp. was studied by growing the microalga for several days in HL and ML cultures (data not shown). After four days of growing, the obtained biomass productivity was 0.28 g L⁻¹ d⁻¹ and 0.14 g L⁻¹ d⁻¹, which supposes growth rate of 0.34 and 0.12 d⁻¹, respectively. In HL culture, the DO (observed at the end of day solar time) increased gradually (data not shown) until 37% (v/v) with respect to the initial value.

Evolution of intracellular content of lutein in *Coccomyxa* sp. cultures

The effect of light and temperature on the intracellular lutein content had been studied in outdoor cultures of *Coccomyxa* sp. (Figure 6). At high light intensity, VHTL and VHL cultures, lutein content was below 4.0 mg g^{-1} , while in ML culture the cells showed the maximum lutein content of 5.4 mg g^{-1} at 14:00 h local time, and HL culture showed 4.8 mg g^{-1} . However, during evening, a content of $4.8 \text{ mg lutein.g}^{-1}$ was obtained in both ML and HL cultures. The highest lutein/chlorophyll ratio of 0.37 was observed at the largest incident light intensity on the PBR in the VHLT culture, while a ratio of 0.25 was observed in the other cultures, all along the experiment. In addition, the intracellular β -carotene content was 1.1 mg g^{-1} (ML culture) and 0.8 mg g^{-1} (HL culture).

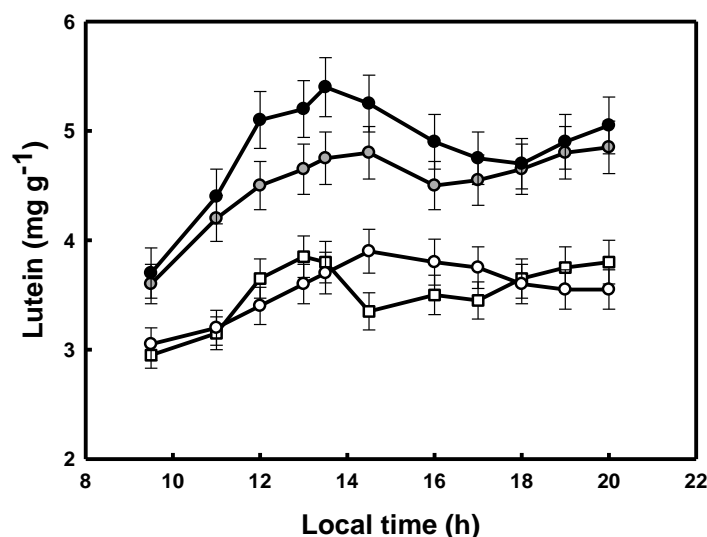


Fig 6: Intracellular content of lutein in *Coccomyxa* sp. outdoor cultures. It shows the lutein content in cells of the corresponding cultures. Conditions as in Figure 4 and 5.

DISCUSSION

Microalga photosynthetic performance, Q_y , competes with the process of fluorescence and heat loss for excitation energy in the pigment antenna of PSII. According to the Q_y values obtained for *Coccomyxa* sp. cultures, the VHLT and VHL regimes induce an important stress in microalgal cells during daytime corresponding to highest incident light intensity. However, from 18:00 h ahead, a gradual recovery of Q_y value was produced in both cultures until 0.6, which may indicate that *Coccomyxa* sp. cells overcome the light-induced stress. It is widely accepted that Q_y value of a healthy microalga culture range between 0.70 and 0.80 [16]. Many photosynthetic organisms exhibit typical midday depression in the photosynthetic yield and they recovered well from this stress when the intensity of incident light was attenuated (Figure 4; [17]). *Coccomyxa* sp. grows well at a maximum temperature of 30°C , and seems to have difficulties when temperature increases at 40°C , particularly if the incident light intensity on the culture is high. Conversely, some microalgae can grow well at temperature as high as 40°C [18].

In short time (10 h) experiments, maximal biomass productivity was obtained at the early evening in HL culture (cloudy days), indicating that a maximum light irradiance of $1070 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on the PBR surface is well tolerated by the microalga ($Q_y = 0.68$, Figure 4). These data, and those observed in VHTL and VHL cultures, suggest that incident light intensity on the surface of the culture is a critical factor for outdoor *Coccomyxa* sp. biomass productivity, and the cultures should be shadowed with a mesh in sunny days to preserve them from light-induced stress. In experiments extended during four days, an average of $0.28 \text{ g L}^{-1} \text{ d}^{-1}$ of biomass productivity of *Coccomyxa* sp. had been obtained in HL culture conditions strategy as compared with a productivity of $0.14 \text{ g L}^{-1} \text{ d}^{-1}$ corresponding to ML cultures. These data could have been improved by supplying to the culture with an organic carbon source, such as urea, in order to avoid the loss of biomass produced during the night [19]. Outdoor microalgae productivity data change significantly depending on photobioreactor type used for the culture, and data ranging between 2.70 and $0.05 \text{ g L}^{-1} \text{ d}^{-1}$ have been reported for *Spirulina platensis* and *Haematococcus pluvialis*, respectively, using tubular photobioreactor [20].

One of the major problems to consider for the design and operation of a closed photobioreactor is the accumulation of dissolved oxygen in the culture of microalga [21]. In the case of *Coccomyxa* sp. HL culture, the photosynthetically generated oxygen during the PBR performance increased until a maximum of 37% (v/v) with respect to the initial value, but the microalga biomass production had not been negatively affected.

Lutein and β -carotene behave in *Coccomyxa* sp. as light harvesting pigments, in cultures with low-intensity incident light, and further, they may act as cells protectors against photooxidative damage eventually produced when cultures were submitted to high light intensity. Lutein has garnered increasing attention because it helps to prevent or diminish human age-related macular degeneration (AMD). Currently, lutein is obtained from marigold flowers, which content ranges between 0.06-1.2 % (w/w) [22]; however, reports of lutein-producing microalgae raise the question if they become an



alternative to plant flowers as natural sources of this valuable pigment [23]. *Coccomyxa* sp. in HL culture regime may reach the reasonable good lutein content of 4.8 mg.g^{-1} at the day evening (Figure 6) and it has the additional advantage of a biomass productivity higher than any other microalga culture. With some exceptions, the lutein content found in other microalgae used to be under 5.0 mg.g^{-1} [23].

Temperature is a more important parameter than light intensity influencing the intracellular content of β -carotene in microalga cells [24], which is consistent with the data observed with *Coccomyxa* sp. Outdoor ML culture conditions (maximum temperature reached by the culture was 36°C) were the most adequate to reach optimum intracellular β -carotene content (between $0.9\text{-}1.0 \text{ mg.g}^{-1}$). However, in HL cultures (maximum temperature reached 32°C) the β -carotene content reached, at the daylight evening, was 0.8 mg.g^{-1} , while the biomass production was two-fold that obtained with ML cultures, which favour the selection of cloudy days, as the best culture conditions for β -carotene production with *Coccomyxa* sp. The best microalgae known to produce β -carotene is *Dunaliella salina*, with $8.3 \text{ mg.L}^{-1} \text{ d}^{-1}$, but in this case lutein content was very low [25].

CONCLUSIONS

The findings in this work open possibilities for the production of lutein and β -carotene-rich biomass with the microalga *Coccomyxa* sp. in continuous outdoor cultures using a tubular photobioreactor. Spring cloudy days in Huelva province (Southwest Spain) supplied the best environmental regimes of incident light intensity on the culture and temperature profile for the growth of microalga. However, in this area, sunny days are predominant at that time, and the outdoor PBR cultures should be shadowed with a mesh to preserve cells from photooxidative damage. Additionally, the biomass would have been harvested at the daylight evening when the intracellular lutein and β -carotene concentrations are high.

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