



RESEARCH ARTICLE

SCREENING INDIGENOUS MICROALGAE STRAINS FOR OUTDOOR PRODUCTION OF
SECONDARY CAROTENOIDS AND FATTY ACIDS

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ABSTRACT

The exploration of indigenous microalgae species with biotechnological potential is growing due to advantages in their use. In this context five locally isolated species of microalgae were screened for secondary carotenoid and fatty acids production. The species were cultivated outdoors in cylindrical vertical photobioreactors to evaluate their growth potential and analyze the composition of the biomass produced in terms of fatty acids and secondary carotenoids. As expected, summer was the most productive season for all the evaluated parameters. *Scenedesmus spinosus* presented the maximum specific growth rate, but *Chlorococccum* sp. was the most interesting strain due to its high production of both unsaturated fatty acids and secondary carotenoids. The values obtained compared favorably with those of commercially used strains suggesting that prospection and screening of indigenous strains for outdoor production has good potential to detect microalgae with industrial applications.

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INTRODUCTION

In the last few years, there has been renewed interest in microalgae as commercial sources of high-value compounds, driven in part by the attempts to develop commercially viable biofuels (see Borowitzka, 2013; Nascimento *et al.*, 2013; Talebi *et al.*, 2013). This has resulted in the recognition of the microalgae strategic importance for biomaterials production by the European SET-PLAN (strategic energy technological plan, see Gouveia *et al.*, 2015). As a result of the key value chain of these organisms for the bioeconomy, the biotechnology of microalgae has gained considerable progress and relevance, with several high-value products already well established in the market, and clear opportunities for additional new products (Borowitzka, 2013).

Carotenoid production represents nowadays one of the most successful markets of microalgae, mainly on the claim of their proven antioxidant properties (Campo *et al.*, 2007). Carotenoids are associated with various health benefits, such as prevention of age-related muscular degeneration, cataract, certain cancers, rheumatoid arthritis, muscular dystrophy and cardiovascular problems. All this emphasized the interest in finding microalgae species with high carotenoid content

(Ahmed *et al.*, 2014). Under unfavourable culture conditions, it has been reported that some microalgae have the ability to synthesize very high amounts of a complex mixture of secondary carotenoids, especially astaxanthin, canthaxanthin and echinenone (see revision in Ahmed *et al.*, 2014).

Microalgae have also long been of interest as sources of fatty acids, especially the long-chain polyunsaturated fatty acids (PUFAs) such as γ -linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Borowitzka, 1988; Ratledge, 2010). According to Moheimani (2013) the ideal microalga must grow well under high cell density and varying outdoor environmental conditions, and have a high biomass productivity and a high oil content (25–30 %).

Indigenous algae are adapted to the prevailing regional abiotic and biotic factors and are, therefore, good candidates for bioresource production and waste mitigation (Wilkie *et al.*, 2011). This work aims promoting the biotechnological use of indigenous microalgae through the evaluation of the outdoor culture potential of five indigenous species, and the analyses of the composition of the biomass produced in terms of secondary carotenoids and fatty acids.

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MATERIALS AND METHODS

Microalgae strains

The Azores archipelago (36° 55' to 39° 43' N; 24° 45' to 31° 17' W), has many freshwater bodies, where many microalgae species with a cosmopolitan distribution are known to occur (Gonçalves, 2008). In this study, five different indigenous microalgae strains (*Chlorococcum* sp. Meneghini, *Desmodesmus communis* (E.Hegewald) E.Hegewald, *Scenedesmus obliquus* (Turpin) Kützing, *Scenedesmus spinosus* Chodat, and *Monoraphidium pusillum* (Printz) Komárková-Legnorová) were tested for outdoor cultivation in vertical photobioreactors.

The isolates are deposited at the Ruy Telles Palhinha (AZB) Herbarium microalgae culture collection of the Department of Biology of University of the Azores. Stock cultures were made and maintained in batch cultures (600 mL) in the laboratory at a temperature of 23 ±2 °C and illumination was provided by 4 cool-light fluorescence lamps (18 W) on 12:12 h light: dark period for 15 days.

Culture media

The strains were all cultured in a commercial fertilizer medium composed of two stocks, as follows: Macronutrients - N 20 %, P₂O₅ 5 %, K₂O 4 %, MgO 4.5 %; Micronutrients - S 6 %, B 0.08 %, Cu 0.016 %, Fe 0.16 %, Mn 0.08 %, Mo 0.0016 %, Zn 0.05 %. The final concentration of the culture media was made of 0.2 mL/L of macronutrient stock and 0.2 mL/L of micronutrient stock. Media were autoclaved at 1.21 atm for 20 min before use. Analytical grade sodium bicarbonate (NaHCO₃) was used as an inexpensive carbon source in all experiments at a concentration of 0.84 g L⁻¹ (White *et al.*, 2013).

Photobioreactors for outdoor production

Outdoor batch cultivation was carried out in tubular bubble column photobioreactors constructed from transparent acrylic plastic. The cylinders, with a working volume of 6.5 L, were 10 cm in diameter, 100 cm in length and had a conical base to avoid sedimentation. A 6 mm air tube connected to an air distributor was installed at the base of each photobioreactor to allow effective air circulation and function as a sampling port. Another tube of the same diameter was located on the cover of each photobioreactor to allow the output of the air injected. The air for culture aeration was produced by a vortex blower, previously filtered through a HEPA filter.

Cultivation

The 20 photobioreactors were distributed to prevent shading between them. Their inoculation was carried out at the end of the day to facilitate the photoadaptation of the cultures. Three 15-days production cycles were conducted in each of three different periods of the year: summer, autumn and winter. The autumn represents an intermediate season, similar to spring in terms of local conditions of light and air temperature. All assays were performed outdoors with no artificial control of temperature and illumination, and followed in a daily basis.

Biomass production

The biomass concentration was determined daily by dry weight measurements. For this purpose, 25 ml of microalgal suspension was filtered through previously dried and pre-weighted 0.2 µm fibreglass filters and oven-dried at 100 °C for 24 h. After drying, the filters were cooled to room temperature in a desiccator and weighed.

Microalgae growth was assessed through the specific growth rate and productivity rate, following Mohsenpour and Willoughby (2013):

$$\mu = [\ln(X_t/X_0)]/(t-t_0)$$
$$P = (X_t - X_0)/(t-t_0)$$

where μ is the specific growth rate (day⁻¹), X is the biomass concentration (g L⁻¹), t is the number of days and P is the productivity rate (g L⁻¹ d⁻¹).

Biomass composition

At the end of each experiment, the photobioreactors were placed in the dark 24 h for sedimentation. Following this period 90% of the culture was discarded and the rest was centrifuged at 3000 rpm for 2 min. The wet biomass was frozen at -80 °C and then lyophilized to perform subsequent analysis.

To estimate the carotenoid content, about 1 g of dried microalgae biomass was crushed in a ball mill for 3 min 50 s, using eight 10 mm balls, at a frequency of 25 s⁻¹. Approximately 30 mg were placed into round bottom centrifuge tubes with cap. The extraction was performed by adding 2 ml of acetone, glass beads (425-600 µm) and kept in an ice bath for 10 min. After this, the tubes were stirred 2 min using a vortex unit, the samples were centrifuged at 3.000 rpm for 5 min, and the supernatant was collected. The previous extraction steps were repeated on the cell residue until the solvent and biomass were colourless. Total carotenoids were quantified by spectrophotometry by measuring the 470, 645 and 662 spectra's. The calculations of chlorophyll a, b and carotenes were done according to the application of the formulas of Costache *et al.* (2012). This methodology was done in duplicate and each reading was taken twice.

Fatty acid composition of the different samples was analyzed by Gas Chromatography. The transesterification of the fatty acids was done by adding 2 ml from a mixture of methanol/acetyl chloride (95:5 v/v) and 0.2 ml of internal standard solution from heptadecanoic acid in petroleum benzine 60°-80 °C (5 mg mL⁻¹) to approximately 100 mg of freeze dried microalgae biomass. The combination was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80 °C for 1 h. After cooling to room temperature, the vials were diluted with 1 ml of water and 2 ml of n-heptane and let settle for 15 min. The upper layer was recuperated, dried over anhydrous Na₂SO₄ and filtered into a vial. The filtrate was analyzed by gas chromatography, equipped with a flame ionization detector (FID).

RESULTS

Biomass production

Growth curves of the five microalgae strains (Fig. 1) show an adaptation period ranging from three to five days followed by an exponential growth phase and ultimately a stationary phase.

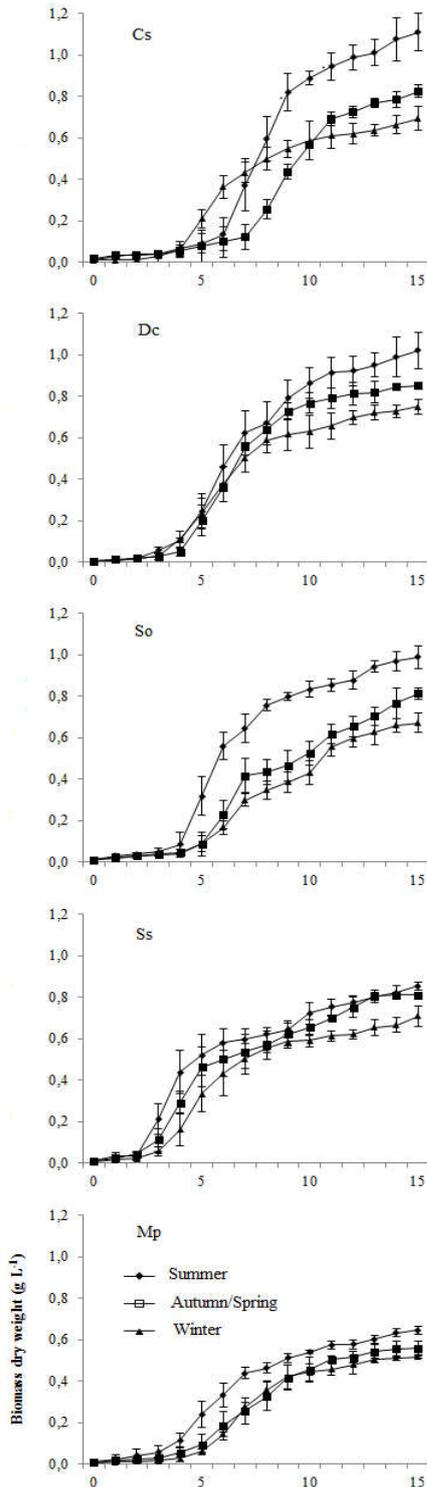


Figure 1 Growth curves (mean ± standard deviation) of the five microalgae strains during summer, autumn/spring and winter. *Chlorococcum* sp (Cs), *D. communis* (Dc), *S. obliquus* (So), *S. spinosus* (Ss) and *M. pusillum* (Mp).

Total biomass accumulation (Table 1) was significantly higher in summer. Biomass accumulation varied among species (Fig. 2), with *M. pusillum* having a significantly lower biomass than any of the other species.

Table 1 Global biomass production. Significant differences from an analysis of variance followed by the post-hoc Newman-Keuls test are marked with different letters

| | | Summer | Autumn/Spring | Winter |
|--|------|--------|---------------|--------|
| Total Biomass (g L ⁻¹) | Mean | 0.924 | 0.771 | 0.668 |
| | SD | 0.176 | 0.115 | 0.088 |
| | Sig | A | B | B |
| Specific growth rate (d ⁻¹) | Mean | 1.043 | 0.846 | 0.670 |
| | SD | 0.124 | 0.077 | 0.123 |
| | Sig | A | B | C |
| Productivity rate (g L d ⁻¹) | Mean | 0.092 | 0.070 | 0.059 |
| | SD | 0.019 | 0.015 | 0.012 |
| | Sig | A | B | B |

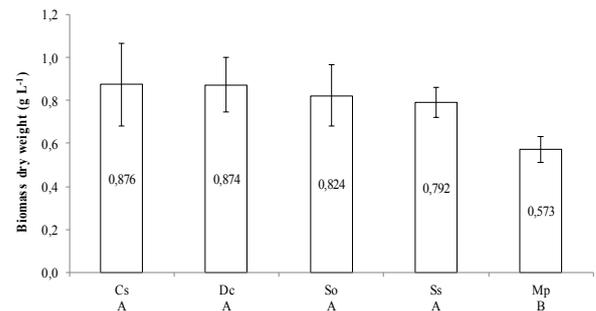


Figure 2 Maximum biomass accumulation. Species as in Figure 1; significance as in Table 1.

Global maximum specific growth rate was significantly different between all seasons (Table 1) being highest in summer and lowest in winter. No significant differences were, however, recorded between species (Fig. 3).

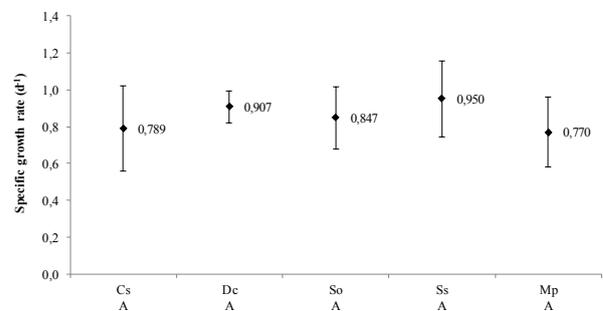


Figure 3 Maximum specific growth rates. Species as in Figure 1; significance as in Table 1.

Global biomass productivity rates were significantly higher in summer (Table 1). Differences between autumn/spring and winter were not significant. Species productivity rates (Fig. 4) were significantly higher in *S. spinosus* and *D. communis*, compared with *M. pusillum*.

Secondary carotenoids

The production of secondary carotenoids was significantly higher in the summer (Table 2). No significant differences were found between autumn/spring and winter. Species responsible for the highest production of carotenoids (Fig. 5) were *Chlorococcum* sp., *S. obliquus* and *M. pusillum*.

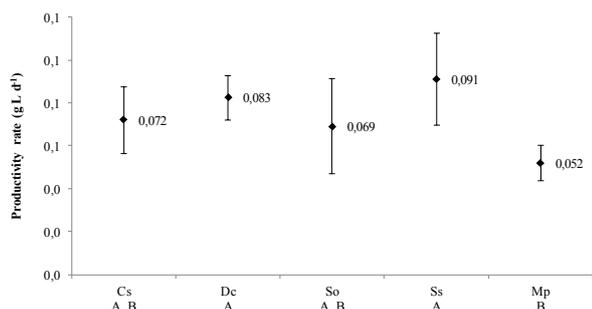


Figure 4 Highest biomass productivity rates. Species as in Figure 1; significance as in Table 1.

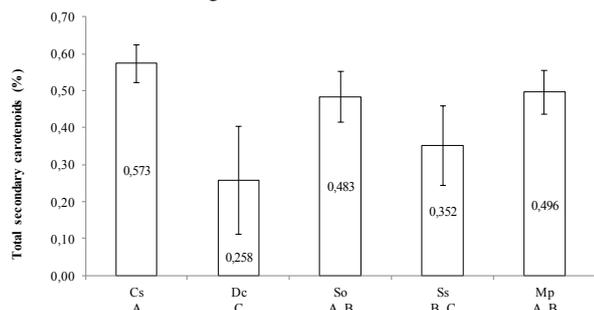


Figure 5 Total secondary carotenoids as a percentage of dry weight. Species as in Figure 1; significance as in Table 1.

Table 2 Secondary carotenoids and fatty acid contents as a percentage of dry weight. Significance as in Table 1

| | | Summer | Autumn/Spring | Winter |
|---------------------------------|------|--------|---------------|--------|
| Total secondary carotenoids (%) | Mean | 0.536 | 0.406 | 0.355 |
| | SD | 0.076 | 0.149 | 0.138 |
| | Sig | A | B | B |
| Total fatty acids (%) | Mean | 15.5 | 11.7 | 9.12 |
| | SD | 7.052 | 3.207 | 2.566 |
| | Sig | A | AB | B |

Fatty acids

The total fatty acid contents of the biomass harvested was significantly higher in the summer than in winter (Table 2). No significant differences were found between these two seasons and autumn/spring. *Chlorococcum* sp. produced significantly higher amounts of fatty acids than all the other species (Fig. 6). No significant differences were observed among these other species.

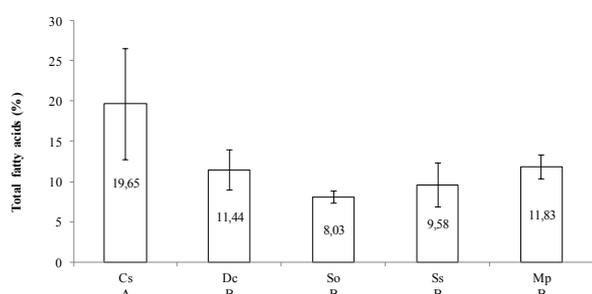


Figure 6 Total fatty acids as a percentage of dry weight. Species as in Figure 1; significance as in Table 1.

The fatty acid profile of the biomass harvested from the different species (Table 3) reveals different patterns for each acid. The most abundant acids in all studied species were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and α -linolenic acid (C18:3). *D.*

communis produced significantly more saturated fatty acids whereas *Chlorococcum* sp. produced significantly less than the other species. *Chlorococcum* sp. produced an order of magnitude of monounsaturated acids higher than all the other species. Its polyunsaturated acid production was average, with the highest percentages recorded on *S. obliquus* and *M. pusillum*.

DISCUSSION

This work demonstrated that outdoor cultivation of microalgae in the Azores is possible throughout the year. As expected growth was highest in summer, the season in which the production of secondary carotenoids and fatty acids also increased.

The maximum productivity registered in the present study for *S. spinosus* (0.134 d^{-1}) was similar to the one reported by Chen *et al.* (2014) for *C. vulgaris* in 50 L outdoor photobioreactors (0.07 to 0.11 d^{-1}), but lower than the one reported by Moheimani (2013) also for *C. vulgaris* in outdoor bag photobioreactors (0.27 - 0.58 d^{-1}). This may be due to the fact that this later cultivation was semi-continuous with a dilution rate of 20 to 60% and harvesting every 1 to 3 days. Continuous cultivation, indeed, enables culture density optimization with a consequent increase in productivity (Coelho *et al.*, 2014).

The annual average specific growth rate achieved for the studied *Chlorococcum* sp. of 0.798 d^{-1} , with a maximum value of 1.13 d^{-1} , is a promising result since values obtained are near those reported by Zhang *et al.* (1997) culturing the same genus in optimized indoor conditions (maximum value of 1.58 d^{-1} at $30 \text{ }^\circ\text{C}$ and a pH of 8).

The production of secondary carotenoids obtained in the present study ranged from 1.7 mg/L in *D. communis* to 5.0 mg/L in *Chlorococcum* sp, clearly within industrially interesting concentrations. Orosa *et al.* (2000) suggested that *Neochloris wimmeri* and *Chlorella zofingiensis*, with production values of 2.8 mg/L, were promising species for the industrial production of secondary carotenoids, based on a study of five Chlorophyceae under inductive laboratory conditions.

In terms of dry weight concentration, the present study demonstrated the capacity of producing up to 0.65% of total secondary carotenoids in summer, using and indigenous strain of *Chlorococcum* sp. Unpublished data shows that this concentration can be as high as 1%. These concentrations compare or exceed those reported for other species. Mir3n *et al.* (2002) obtained 0.5% total carotenoids for *Phaeodactylum tricorutum* cultivated in photobioreactors, and Blanco *et al.* (2007) reached 1% for *Muriellopsis* sp. growing in outdoors open tanks (1%), in a production only feasible during 9 months a year. Yuan *et al.* (2002) had already demonstrated that *Chlorococcum* sp. could produce large amounts of secondary carotenoids, including astaxanthin, adonixanthin, canthaxanthin, lutein and β -carotene. In Malaysia Zhang *et al.* (1997) reported that a locally isolated *Chlorococcum* sp. could accumulate astaxanthin and its esters as secondary carotenoids achieving concentrations of 0.52%, similar to those obtained in the present study.

Table 3 Fatty acid profiles (% composition) of the studied species. Significance as in Table 1.

| | Chlorococcum sp. | | D. communis | | S. obliquus | | S. spinosus | | M. pusillum | |
|-----------------|------------------|------|-------------|------|-------------|------|-------------|------|-------------|------|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Saturated | 35.67 | 2.12 | 82.46 | 7.28 | 64.25 | 6.32 | 67.75 | 5.98 | 59.74 | 4.17 |
| Sig | A | | B | | C | | D | | C | |
| 10:0 | 0.88 | 0.1 | 1.23 | 0.24 | 0.98 | 0.09 | 0.91 | 0.08 | 1.1 | 0.1 |
| 12:0 | 1.25 | 0.25 | 2.13 | 0.37 | 2.15 | 0.42 | 3.09 | 0.4 | 1.32 | 0.13 |
| 14:0 | 1.17 | 0.21 | 4.34 | 0.48 | 2.93 | 0.62 | 3.81 | 0.28 | 1.76 | 0.51 |
| 16:0 | 17.66 | 0.72 | 35.65 | 2.71 | 25.86 | 1.27 | 27.46 | 2.06 | 18.46 | 0.76 |
| 18:0 | 13.36 | 0.72 | 37.25 | 3.23 | 31.39 | 3.87 | 30.45 | 3.02 | 33.94 | 2.27 |
| 20:0 | 0.35 | 0.03 | 0.69 | 0.12 | 0 | 0 | 0.4 | 0.05 | 0 | 0 |
| 22:0 | 0.7 | 0.06 | 0 | 0 | 0.59 | 0.04 | 0.7 | 0.04 | 1.65 | 0.28 |
| 24:0 | 0.29 | 0.01 | 1.17 | 0.13 | 0.36 | 0.02 | 0.92 | 0.05 | 1.5 | 0.13 |
| Monounsaturated | 34.46 | 2.91 | 2.99 | 0.48 | 1.86 | 0.1 | 2.92 | 0.16 | 2.86 | 0.61 |
| Sig | A | | B | | B | | B | | B | |
| 16:1 | 3.8 | 0.34 | 2.53 | 0.45 | 1.65 | 0.09 | 2.49 | 0.13 | 2.48 | 0.54 |
| 18:1 | 30.46 | 2.55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20:1 | 0.2 | 0.01 | 0.46 | 0.03 | 0.21 | 0.01 | 0.43 | 0.03 | 0.38 | 0.07 |
| Polyunsaturated | 29.87 | 1.52 | 14.55 | 1.16 | 33.89 | 2.3 | 29.33 | 2.25 | 37.4 | 1.52 |
| Sig | A | | B | | C | | A | | C | |
| 18:2 | 10.49 | 0.49 | 4.44 | 0.56 | 6.42 | 0.96 | 5.57 | 0.55 | 5.94 | 0.72 |
| 18:3 | 19.37 | 1.03 | 10.11 | 0.59 | 27.47 | 1.34 | 23.76 | 1.7 | 31.4 | 0.79 |

The studied *Chlorococcum* has, therefore, potential for local commercial production and it could be considered an alternative to *Haematococcus pluvialis*, the freshwater species most widely used for industrial carotenoid production, generally with a content of 1.5-3% (Campo *et al.*, 2007).

Chlorococcum sp. was also seen to be the most promising of the studied taxa for fatty acid production (29 % in summer). The obtained values are lower than the ones reported by Harwati *et al.* (2012) but it is worth considering that these authors were working under optimized conditions (56%). Similar values to the ones obtained in the present study were reported by Moheimani (2013) for *C. vulgaris* (19-30%) and by Blanco *et al.* (2007) for *Muriellopsis* sp. (23.5%). Higher values were, however, reported by Chen *et al.* (2014) for *C. vulgaris* (44%).

The fatty acid profile obtained for *Chlorococcum* sp. in the present study revealed a proportion of mono and polyunsaturated fatty acids (PUFAs), namely oleic (C18:1), linoleic (C18:2) and linolenic acids (C18:3), similar or slightly higher than the ones reported by Chaichalerm *et al.* (2012) for *Chlorococcum humicola* and by Harwati *et al.* (2012) for *Chlorococcum* sp. These MUFAs and PUFAs are well known to be a source of healthy food fats (Harwati *et al.*, 2012).

From all the studied species, *Chlorococcum* sp. is the most interesting one given its higher growth rates and final concentrations of both carotenoids and high quality fatty acids. Its high proportion of monounsaturated and polyunsaturated relative to the saturated fatty acids, some reported as having high market values (e.g. γ -Linolenic acid, Koller *et al.*, 2014), indicates this taxon as a source of healthy food fats (Harwati *et al.*, 2012). This species was seen to grow well in the vertical tubular-type photobioreactors used in this study. This is in agreement to Zhang *et al.* (1997) that have also shown that *Chlorococcum* sp. grows well in tubular photobioreactors, and that it is a good candidate for mass production in outdoor culture systems due to its tolerance in terms of temperature and pH. These authors demonstrated that 30 °C was the optimal growth temperature and that growth was negatively affected with higher values.

The Azorean climate seems therefore to have the appropriate conditions to allow an outdoor production of *Chlorococcum* sp. throughout the year in vertical photobioreactors.

This paper has shown the potential of an indigenous strain of *Chlorococcum* for the production of both secondary carotenoids and fatty acids confirming the importance of local prospection and screening to detect microalgae with commercial applications in outdoor production. Further studies encompassing culture optimization, including stress induction, are recommended for a scale up process for industrial production.

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Compliance With Ethical Standards

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Conflict of Interest: Emanuel Xavier declares that he has no conflict of interest; José Azevedo declares that he has no conflict of interest; Alberto Reis declares that he has no conflict of interest; Ana Neto declares that she has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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