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Evaluation of two Portuguese strains of *Botryococcus braunii* as biofuel feedstock

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ACOI 58 and ACOI 1257, two different Portuguese strains of colonial green microalga *Botryococcus bruanii* Kutz obtained from Coimbra Collection of Algae (ACOI), were evaluated on their potential for biofuel production, which was assessed from batch culture in photobioreactors under indoors and outdoors conditions using pretreated waste water and semisynthetic culture media. In the experiments, the maximum specific growth rate achieved was 0.4 day^{-1} with doubling times ranging from 2 to 7 days in the fastest growing phase. However, both strains showed ability to absorb nutrients in waste water cultures, to grow and accumulate oil. The maximum lipid extraction on a dry matter basis was 36% in CHU media and 29% in waste water cultures, which revealed the promising potential of these *B. braunii* isolates to be used in biofuel production applications. This is also supported by the predominance of oleic (C18:1, 42 to 63%) and palmitic acid (C16:0, 8 to 18%) in the lipid extract, since the methyl esters of these fatty acids (FAME) are ideal for biodiesel production. In summary, although these strains of *B. braunii* have presented a typical growth for this species, they produced considerable lipid content and were able to grow in waste water and under outdoors conditions that warrants further investment in their study.

Key words: Microalga, *Botryococcus braunii*, wastewater, photobioreactors, biofuels.

INTRODUCTION

Environmental adversities and depleting of fossil fuels have become one of the major human concerns worldwide and the use of these fuels as main energy source is now widely accepted as unsustainable for the future. Therefore, numerous research has been conducted into alternative fuels production and several biofuel candidates have been proposed as a potential to eliminate the vulnerability of energy sector at the same time to contribute to the mitigation of CO_2 emissions and its effects on the environment (Serra and Zilberman,

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2013; Ndimba et al., 2013). In this context, biofuels production from natural renewable resources is widely considered the most sustainable options among which microalgae are an outstanding candidate for biomass and biofuel production. Additionally, they could be a solution to the food vs. fuel competition controversy, as its high photosynthetic capacity is an effective process for carbon sequestration, lipid accumulation and their ability to grow using poor quality waters do not compete with food crops for arable land (Sawayama et al., 1994; Chisti, 2007; Mata et al., 2010; Singh et al., 2010; Kumar et al., 2011). Several microalgae species received great attention in the last decades thus, aiming the production of a wide range of biofuels and others value-added products as revised and listed by Mata et al. (2010). Among these species, the most prominent are those with high lipid content and high biomass productivity namely, from genus *Botryococcus*, *Chlorella*, *Euglena*, *Dunaliella*, *Crypthecodinium*, *Nannochloris*, *Nannochloropsis*, among others.

Regarding the biodiesel production from microalgae much attention has been focused on *Botryococcus braunii* Kützing, a cosmopolitan colonial green alga occurring in both temperate and tropical fresh water environments because of its ability to accumulate unusually high levels of hydrocarbon, in the range of 15 to 35% of dry weight (Metzger and Largeau, 2005), with up to 86% dry weight of cell material being combustible oil, which accumulate mostly in its colony matrix (Yamaguchi et al., 1987; Chisti, 2007). This species is usually classified into three major groups (races A, B, and L) based on the chemical structures of the specific hydrocarbon they synthesize: race A is characterized by n-alkadiene and/or n-trienes and their derivatives with odd carbon number (C23–C33) (Metzger et al., 1985); race B synthesizes specific C_nH_{2n-10} triterpenes, known as botryococcenes (C30–C37) and methylated squalenes (C31–C34) (Huang and Poulter, 1989); and race L that synthesizes a single tetraterpenoid hydrocarbon called lycopadiene (Metzger et al., 1990). Recently, some strains producing C_{20} saturated n-alkane was discovered and classified into race S (Kawachi et al., 2012). The slow-growing rate of *B. braunii* has seriously hampered its application for biofuel production (Metzger *and* Largeau, 2005; Cheng et al., 2013). In particular, cultivation by most cost-effective non-axenic outdoor system for large-scale production is hampered, mainly due to the proliferation of other fast-growing green algal and cyanobacterial contaminants (Ioki et al., 2012). However, this species of microalgae has tremendous potential to be used as a renewable biomass feedstock and it is recognized as one of the potent renewable feedstock for production of liquid hydrocarbons (Ashokkumar *and* Rengasamy 2012). Thus, the production of this liquid hydrocarbon requires further optimization studies on *B. braunii* biomass cultivation, as well as the isolation and proper characterization of new native strains with significant growth rates, ability to produce high lipids content and capable of being grown in poor quality waters.

The objective of the present study was to investigate the potential of two strains of *B. braunii* microalgae indigenous to Portugal as renewable feedstock for biofuel production. To do so, the biomass cultivation was investigated using both waste water and synthetic culture media under indoor controlled conditions and outdoor weather conditions. Lipid accumulation profile and nutrients removal efficiency from wastewater was accessed for indoors culture.

METHODS

Microalgae strain and preparation of inoculums

Two strains of the microalga *B. braunii* Kützing (Chlorophyta; Chlorophyceae; Botryococcaceae) were used in this study, both provided by ACOI - Coimbra Collection of Algae (http://acoi.ci.uc.pt) - with the designations ACOI 58 and ACOI 1257. The first strain was originally isolated in 1975 from a freshwater reservoir in Porto da Castanheira, Poço dos Basílios and the second was collected in 1997 from Erva da Fome dam, Serra de Estrela, both locations in central Portugal. Henceforth, in this study, each strain will be named, strain 1 and strain 2, respectively. Stock cultures of these strains were made in 125 ml Erlenmeyers containing 50 ml artificial BBM medium (Stein, 1973) and maintained inside a Fitoclima 750 E culture chamber (Aralab) at 22°C approximately 55 µmol photons $m^{-2}.s^{-1}$ provided by cool-white fluorescent illumination with a 12:12 h light:dark cycle. The cultures were subcultured monthly and kept free of contaminants. To prepare the inoculums (seed culture), some subcultures were transferred to a growth room where they were acclimatized to the experimental culture conditions (23 ± 1°C; 100 to 110 µmol photons $m^{-2}.s^{-1}$; 14:10 h light:dark cycle cool-white fluorescent illumination). The subcultures volume was doubled fortnightly with artificial BBM medium addition.

Culture medium, mass cultivation conditions and experimental design

For all experimental cultures carried out in this work, transparent polyethylene photobioreactors were used (diameter 12.5 cm, vol. 2 L). These photobioreactors were cylindrical with a conical bottom and were kept hanging vertically. On the side a tap was placed for sampling and the aeration/homogenization was provided by an aquarium pump (1.5 l/min filtered air) placed at the conical bottom of the photobioreactors.

Two artificial culture media BBM and CHU-10 (Stein, 1973) and a pre-treated waste water media (PWW) (Table 1) were used; the latter was a courtesy of Lisbon waste water treatment station (SIMTEJO, S.A.). For all cultures, sterile materials and solutions were used, culture media were autoclaved (121°C, 1 bar, 20 min), glass materials were sterilized by dry heat (180°C, 1 H) and the photobioreactors were subjected to UV radiation for 20 min just before inoculation in a laminar flow hood (ESI, FluFrance). Indoors and outdoors experiments were carried out using the two *B. braunii* strains and the three culture media mentioned above; all in duplicate. The indoor experiments were carried out in the culture chamber at the Department of Plant Biology, Faculty of Science,

Table 1. Chemical characteristics of the pre-treated wastewater.

Variable	Value
$NO_3 + NO_2$ (N $\mu g.L^{-1}$)	3001
NH_4 (N $\mu mol.L^{-1}$)	64
PO_4 (P $\mu g.L^{-1}$)	2420
pH	7.9

University of Lisbon (FCUL) and maintained for 50 days at constant temperature (23±1°C) and illumination (110 to 120 $\mu mol.s^{-1}.m^{-2}$) provided by cool-white fluorescent lamps with a photoperiod of 14/10 light/dark. Outdoors experiments used the same conditions and design, except for temperature and lighting that were subject to summer climatic variations and were carried out at the FCUL campus.

Sampling, **biomass analysis and harvesting**

Samples (10 ml) for biomass and lipid content analysis were taken every 4 days. The biomass ($g.L^{-1}$) was determined by filtering 5 ml of algal culture using pre-weighed 4.7 cm (1.2 µm pore) Fisher glass fiber filters. Each filter with algal biomass was dried at 65ºC for 8 h, cooled to room temperature in a vacuum desiccator and weighed gravimetrically. At the end of the 50 days of culture all biomass was harvested by centrifugation at 8000 rpm, 16°C 20 min (Routine 35R, Hettich) and frozen at -80°C for later proceedings.

Determination of lipid content increase

The variation in lipid content over the experimental period was determined by Nile red method (Lee et al., 1998; Elsey et al., 2007). Per sampling, 1.5 ml of each algal suspension was stained with 25 µl solution of Nile Red dissolved in acetone (0.25 $\mu g.ml^{-1}$) and vigorously vortexed and incubated in the dark for 10 min. Finally, a fluorimetric reading was performed using a fluorescence microplate reader (SpectraMax Gemini EM) with a 490 nm excitation filter and a 585 nm emission filter. The relative fluorescence intensity of Nile Red was obtained after subtraction of both the autofluorescence of algal cells and the fluorescence intensity of Nile Red alone in the medium.

Extraction and estimation of total lipid content

The biomass harvested and frozen after each culture (section 2.3) was lyophilized for 48 h (Cryodos Telstar freeze dryer) and the lipid fraction was extracted by organic solvents using an adaptation of the Bligh and Dyer (1959) method. Lyophilized biomass of each sample was ground in a mortar, weighed and transferred to a 50 ml Falcon tube. There, 5 ml of chloroform and 10 ml of methanol were added per gram of biomass and the mixture was sonicated for 10 min. Afterwards 5 ml of chloroform and 9 ml of distilled water were added and the mixture was sonicated again for another 5 min. After two hours extraction, samples were vortexed for 30 s and centrifuged (10 min, 5000 rpm, 16°C) for phase separation. The lower organic phase was carefully collected into a round bottom glass flask, previously dried, weighed, and then the extraction process was repeated with the addition of 10 ml of chloroform. The combined organic phases were washed with 20 ml of 5% (w/v) NaCl solution, evaporated on a rotary evaporator (Yamato Hi Tec RE-51, Water Bath BM-51) to dryness and the total lipids were measured gravimetrically. The entire extraction process was done in triplicate.

Fatty acid analysis

The dry lipid extracts were dissolved in 5 ml of hexane, transferred to a GC vial and stored in a freezer until further analysis, which was performed as described by Nyberg (1986) and Indarti et al. (2005) with minor adjustments. Briefly, 3 ml of each extract were introduced into glass vials and 5 ml of a methanol/benzene/sulfuric acid solution (20/1/1) were added.

The mixture was heated at 80°C for 2 h in a water bath for the methanolysis process. After cooling, 5 ml of distilled water was added to each vial and the mixture was vigorously vortexed. The resulting methyl esters of the methanolysis reaction were extracted three times with 1 ml hexane, concentrated under nitrogen flow and then subjected to chromatography on a silica column; the esterified fatty acids were eluted from the column with 5 ml of pentane. Then, these samples were again concentrated under nitrogen flow and analyzed by Gas-Liquid Chromatography (GLC) and by Gas-Liquid Chromatography associated with Mass Spectrometry (GLC/MS) for the identification of the fatty acids in the extract.

GLC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with two FID's, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m×0.25 mm i.d., film thickness 0.25 µm; J and W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m×0.25 mm i.d., film thickness 0.15 µm; J and W Scientific Inc.). Oven temperature was programmed for a range of temperatures between 50 to 300°C with a rate of 5°C min^{-1} and then held isothermal for 10 min; injector and detector temperatures were 290 and 300°C, respectively; the carrier gas, hydrogen, was adjusted to a linear velocity of 30 $cm.s^{-1}$. Samples were injected using the split sampling technique at a ratio 1:50; percentage composition of the lipids was computed by the normalization method from the GC peak areas, which were calculated as mean values of two injections of each sample, without using response factors.

The GLC-MS unit consisted of a Perkin Elmer Autosystem XL gas chromatograph equipped with DB-1 fused-silica column (30 m×0.25 mm i.d., film thickness 0.25 µm; J and W Scientific Inc.), and interfaced with Perkin Elmer Turbomass mass spectrometer (software version 4.1). Oven temperature was programmed exactly as the CGL analysis described above; transfer line temperature was 280ºC; ion trap temperature was 220ºC; the carrier gas, helium was adjusted to a linear velocity of 30 $cm.s^{-1}$; split ratio was 1:40; ionization energy was 70 eV; ionization current was 60 µA; scan range was 40 to 600 µm; scan time was 1 s. The identity of the compounds was determined by comparison of their retention times and their mass spectra with those of commercially available standards and by comparison with a library of mass spectra was developed in the laboratory.

Nutrient analysis in the PWW

For nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) quantification in the pretreated waste water (PWW), the samples were filtered through glass fiber filters (Fisher) and then analyzed using a Tecator 5020 FIAstar Analyser equipped with a Hitachi U-3200 spectrophotometer. The nitrate and nitrite concentrations were determined according to the method of Grasshoff (1976) and the method of Bendschneider and Robinson (1952), respectively. The phosphate was quantified by the method of Murphy and Riley

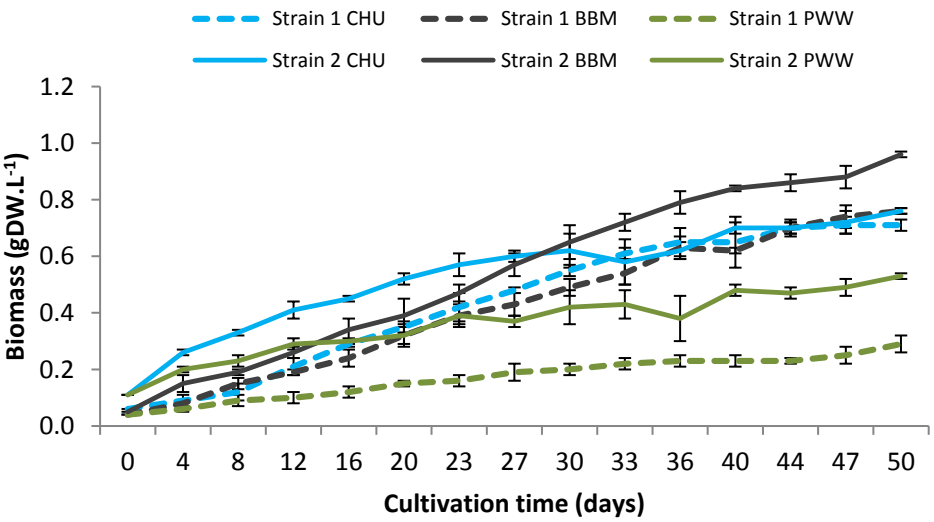

Figure 1. Growth curves of *B. braunii* under indoors culture conditions.

(1962). Ammonia concentration was determined using filtered PWW samples according to the method of Koroleff (1969) using a spectrophotometer UV/visible (UV-1603, Shimadzu). These tests were performed with samples collected at the beginning and end of the cultures in PWW, in order to analyze the capacity of both strains of algae to remove these nutrients.

Statistical analysis

Data were expressed as average values ± standard deviation in which cultivation experiments were carried out in duplicate and all samples were analyzed at least in triplicate. The statistically significant differences between values (if applicable) were evaluated by analysis of variance and t-test using the excel data package.

RESULTS

Indoors cultures

Biomass growth and lipid content

Both strains showed continuous growth throughout the culture time (Figure 1). However, there were substantial differences in the three different media in particular, growth rate and biomass concentration. The specific growth rate during the fastest growing period (day^{-1}) were 0.4 and 0.2 in the CHU media, 0.3 and 0.2 in the BBM media and 0.2 and 0.1 in PWW media, for the strain 1 and strain 2, respectively. Although the first strain had higher specific growth rate in the fastest growing phase, if we consider the entire growth period, the second strain registered higher rates in all culture media tested. The final biomass concentrations achieved (g DW.L^{-1}) were 0.71±0.02 and 0.76±0.01 in the CHU media, 0.76±0.01 and 0.96±0.01 in the BBM media and 0.29±0.03 and 0.53±0.01 in the PWW media for strain 1 and strain 2, respectively. The growth of both strains in the waste water used as culture medium (PWW) was clearly slower than in synthetic culture media thus reaching biomass concentrations at the end of culture period at roughly half of the obtained in synthetic media. Higher biomass concentrations and higher productivity for strain 2 on both synthetic culture media, CHU and BBM were observed. This indicates a different behavior for the two strains under similar conditions. Both strains accumulated lipids (Figure 2). In general, lipid content of the cultures in BBM medium did not reach half of the corresponding lipid content obtained in the cultures in CHU and PWW media. Surprisingly, the cultures in wastewater had higher lipid content (Figure 2) despite having much lower biomass growth than the cultures in synthetic media (Figure 1). In general, although there were marked fluctuations in lipid contents, the strain 2 seems to have accumulated higher lipid content than the strain 1, at least in the late stage of culture.

Nutrients removal in wastewater medium

The characteristics of treated wastewater collected from SIMTEJO plant are listed in Table 1. Note that only the pH and the compounds nitrate, nitrite, ammonia and phosphates have been analyzed since this effluent was already pretreated for dumping in the river, thus the levels of heavy metals and other toxic compounds are controlled by the company. The removal of nutrients in the cultures of *B. braunii* using pretreated wastewater

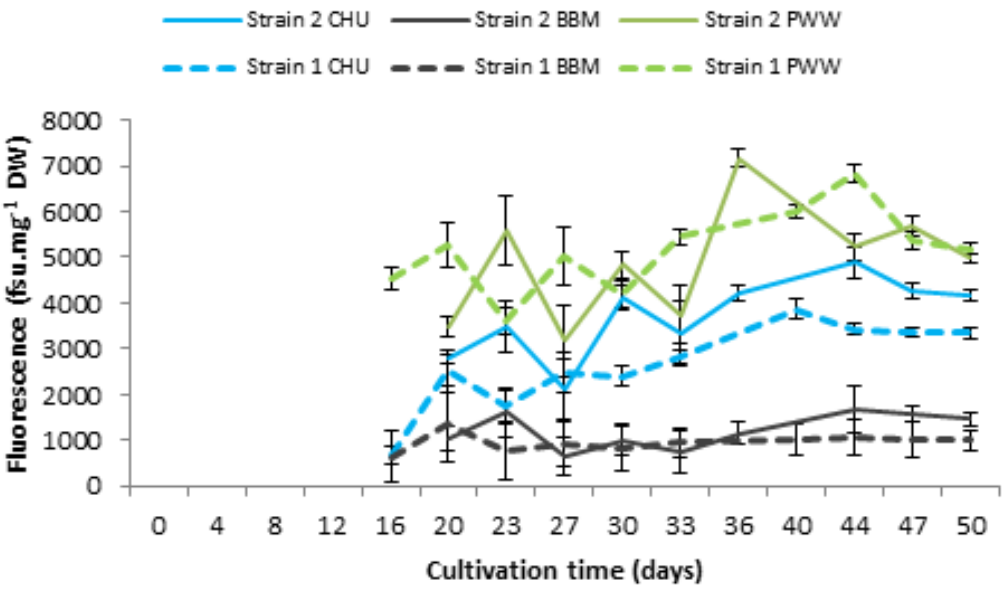

Figure 2. Kinetics of lipid accumulation in indoors cultures.

Table 2. Initial and final concentrations of nutrients and their corresponding percentages of removal in Indoors cultures of *B. braunii* in PWW.

Variable	Strain 1			Strain 2		
	Initial	Final	Removal (%)	Initial	Final	Removal (%)
Total phosphates (µg.L^{-1})	2419	488	80	2420	263	89
Nitrate + nitrite (µg.L^{-1})	3001	9	100	3001	1.83	100
Ammonia (µmol.L^{-1})	64	0.17	100	64	0.09	100

*The terms initial and final refer to nutrients concentrations at the beginning and at the end of the trials, respectively. Values represent mean of a duplicate.

(PWW) was determined by comparing the concentrations of the compounds mentioned above in the culture medium at the beginning and at the end of the cultures. The results presented in Table 2 shows that an almost complete absorption of these nutrients took place during the culture period. The absorption of phosphate was 79.8 and 89.1% for strain 1 and strain 2, respectively, while the absorption of nitrogen compounds was over 99% for both strains.

Outdoors cultures

The cultures grown outdoors followed the same experimental design used in indoors cultures but the difference in this case was that the cultivation was done outside with the photobioreactors exposed to natural sun light and subjected to summer weather variations. As shown in Figure 3, the start of all the cultures had a long adaptation period, after which all cultures of strain 1 were developed, while for the strain 2 the cultures in the PWW medium was lost. The final biomass concentrations obtained by the strain 1 cultures in CHU medium were almost twice than those found in the cultures of this strain in BBM and PWW media; the concentrations reached 1.0±0.03, 0.58±0.03, and 0.57±0.04 g/l (dry weight), respectively. For strain 2, growth was delayed in all cultures. In the CHU medium a biomass concentration of 0.8±0.01 g/L (dry weight) was obtained at the end of the cultivation period, while the culture in BBM medium grew little reaching a density not exceeding 0.2 ± 0.03 g/L (dry weight).

Total lipid content and fatty acid analysis

As depicted in Figure 2, the lipid content was found to vary according to the culture age but stagnant at the end of exponential growth. Results for the lipid extracts of dry biomass grown indoors are shown in Table 3, where we

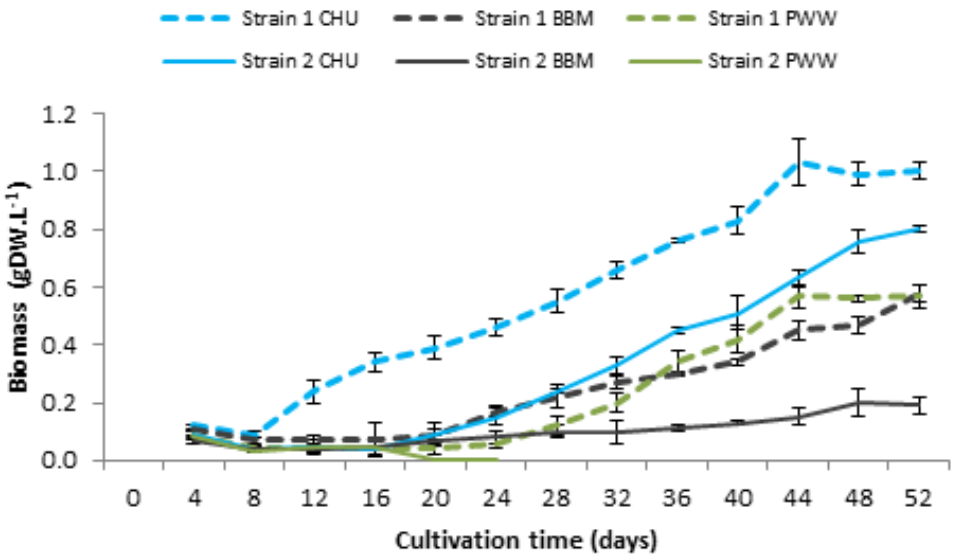

Figure 3. Growth curves of *B. braunii* under outdoors culture conditions.

Table 3. Lipid contents* of the indoors cultures at the end of the growing period.

Variable	CHU		BBM		PWW	
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
Lipid extract (%)	18.38±3	36.05±0.2	13.19±2	18.71±2	17.12±1	28.91±0.1

*Values represent mean ± SD of three replicates.

Table 4. Fatty acids profile of the strains 1 and 2 of *B. braunii* in the indoors cultures (nd not detected).

Variable	Fatty acids proportion (%)					
	CHU		BBM		PWW	
Fatty acids	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
16:1	0.5	1.3	4.1	2.8	1.1	2.7
16:0)	11.8	9.8	11.8	18.1	12.9	8.2
18:2	19.8	2.6	7.0	17.8	5.0	1.8
18:1	49.8	62.9	43.3	42.3	65.8	43.3
18:0	1.9	0.4	2.8	4.0	1.9	nd
20:1	3.9	6.7	0.6	2.0	3.4	8.0
22:1	2.6	nd	4.2	2.4	2.8	10.9
24:1	0.6	0.6	5.7	nd	0.5	nd
26:1	nd	2.6	nd	0.6	nd	6.7
28:1	2.9	10.2	7.0	1.7	2.8	8.7

can see that the maximum lipid content of 36% was obtained from strain 2 in CHU medium. We can also see that higher extracted lipid content was obtained for this strain in the three culture media tested.

In Table 4 the fatty acid profiles determined for the lipid extracts of both *B. braunii* strains in all conditions tested indoors are shown. As seen for both strains and in all culture media tested, the oleic acid (C18: 1) was the major constituent of the fatty acids with relative proportions between 42 and 66%. The second most dominant fatty acid was palmitic (C16: 0) with a relative percentage between 8 and 18%. There were some

variations in the fatty acid profile of both strains under the influence of the different culture media used, especially in C18:2, C18:1 and C28:1. However, the most obvious observation is that oleic acid and palmitic acid were the dominant constituents of the fatty acids obtained in all extracts of both *B. braunii* strains.

DISCUSSION

For the past four decades, microalga have been listed as one of the most credible options for fuel oil and hydrocarbon exploration in view of the challenges to be faced in post-peak fossil fuels production, as well as, in mitigating adverse environmental effects derived from the fossil fuel use. A special interest has been dedicated to the microalgae *B. braunii* due to its ability to accumulate large amounts of hydrocarbons and ether lipids (Nascimento et al., 2013). However, the viability of algae-based biofuel industry depends on the selection of adequate strains in regards to profitable yields and oil quality, therefore, searching for new strains is still an essential issue. Thus, in the study herein, we conducted a pioneering investigation with two *B. braunii* strains indigenous to Portugal and we evaluated their ability of being grown in poor quality waters in indoors and outdoors conditions.

From a macroscopic point of view, the main visible change observed in the cultures was their color. Initially, the cultures carried out indoors gradually developed an intense green color passing to green tint brown at the end of the cultivation time. In the outdoors cultures, the color change was much more evident and faster, indicating the presence of intense orange pigments throughout the growing period.

By microscopic observations, it was noted that both algal strains formed colonial clusters where cells were attached to each other by stretches through several distinct clumps of cells, as described for typical cultures in aqueous-suspended condition (Metzger and Largeau, 2005). For both strains, the cell membranes were thickened and showed some colorless droplets in the outer walls during cultivation. According to Largeau et al. (1980), this might indicate an accumulation of hydrocarbons. However, strain 1 formed small colonies which readily disaggregated with stirring while strain 2 formed relatively bigger and more consistent colonies and were stirring resistant, resulting in a viscous and thick appearance throughout the culture time. The lyophilized biomass of strain 1 appeared as an orange-green or green powder, while strain 2 formed a greenish-brown sticky mass.

For biomass growth, results presented show that both strains did not have vigorous growth in all media tested resulting in moderate biomass productivity. This weak growth has been explained as a consequence of the colony morphology formed by microalgae cells embedded in the extracellular matrix which limits CO_2 and mineral salts diffusion to the cells. Another factor considered to be the cause of poor growth is the synthesis and accumulation of several lipid and hydrocarbons that do not seem to be available as reserve compounds and as such consume much of the photosynthetic energy assimilated to the detriment of growth (Belcher, 1968; Wolf, 1983).

Although the growth dynamic of these cultures under controlled conditions (indoors) was very similar to that reported by many studies for the same species under similar conditions (Dayananda et al., 2006; Rao et al., 2007; Órpez et al., 2009), we have obtained slightly different growth and productivity for the same strain in two different media of what may be related to a more rapid depletion of mineral nutrients in CHU medium than in BBM medium. The comparison of the biomass concentrations obtained for the two strains showed that for both culture media, CHU and BBM, the strain 2 had a higher productivity, which indicates a different behavior of the two strains towards similar conditions. This had also been observed by Li and Qin (2005), that *B. braunii* strains from different locations behave in different ways towards the same culture conditions, in other words, the growth and lipid productivity was specific to each strain. So, it is essential to conduct research into new strains to exploit the potential of this species which is considered very promising as feedstock for the production of biodiesel and other compounds of commercial importance (Sawayama et al., 1994; Chisti, 2007; Dayananda et al., 2010).

Comparing the biomass growth dynamics in all indoors cultures with their lipid accumulation, it was noticed that the lipid content showed slightly increase by the end of the biomass exponential growth after which there was an attenuation pattern also obtained by Kojima and Zhang (1999) and Talukdar et al. (2013). Thus, we can even say that lipid productivity showed a positive correlation with biomass growth, which indicates that more efficient hydrocarbon production, can be achieved under the culture conditions enabling higher biomass growth.

As shown in the results, the *B. braunii* strains used in this work revealed ability to accumulate high lipid content when grown on wastewater without adding mineral nutrient and showed high efficiency in removing nutrients. Considering this finding associated with the fact that *B. braunii* has a wide tolerance to pH alterations [6 to 9 according to Dayananda et al. (2006)] and the cost of medium preparation still accounts for approximately 20% of the total cost in the whole oil production process according to Shiho et al. (2012); we can hypothesize the possibility of using waste water as culture medium in large scale, coupled to the primary treatment systems of urban waste water in order to obtain cheap feedstock for biofuels production simultaneously with removing

nutrients that contribute to the eutrophication of the water bodies receiving the effluent. This idea is also supported by those of Sawayama et al. (1992), An et al. (2003) and Órpez et al. (2009) who noticed notable amounts of hydrocarbon and biomass yields using only wastewater as the culture medium.

In addition to the finding mentioned these *B. braunii* strains also showed ability to grow on outdoors conditions, without any control of light and temperature. However, the initial green color of the cultures have become more red or orange along the cultivation time, and is probably associated with synthesis and accumulation of carotenoids, especially β-carotene (Belcher, 1968), thus being a strategy of resistance/survival in adverse conditions. Certainly, the climatic conditions that were felt during the culture period in midsummer, especially the high temperature and light intensity negatively influenced the outdoors cultivation of these two Portuguese strains of microalgae *B. braunii*, since these are two of the most important factors that affect the biomass and hydrocarbons production in this species (Kojima and Zhang, 1999; Qin, 2005). Even so, the yield obtained in these cultures in CHU medium suggests that these organisms can be exploited for biomass cultivation under outdoor culture conditions, although optimization of these conditions seems to be necessary for each strain. Furthermore, the algal carotenoids are well known for their various pharmaceutical, nutraceutical and cosmetic applications (Dayananda et al., 2010), which increases the range of opportunities for these two *B. braunii* strains once the potential co-valorization could add to the economic effectiveness of biofuel production.

Since the main application exploited for this microalgal species has been lipid and hydrocarbon production for biofuel, we determined the lipid content in the biomass obtained under indoors controlled conditions. As can be seen in Table 3, lipid content varied between 13.2 and 18.4% for strain 1 and between 18.7 and 36% for the strain 2. The lipid contents obtained herein with the strain 2 are competitive with those of the other isolates obtained under similar conditions, namely, 32% by Wolf et al. (1985), 38.6% by Singh and Kumar (1992), 32.2% by Lee et al. (1998) and 11 to 17.9% by Órpez et al. (2009). Kojima and Zhang (1999) reported 50% lipid content obtained in photobioreactors supplemented with 1% CO_2. A wide range of lipid content results has been obtained for various *B. braunii* strains and some with importance for biodiesel production were summarized in Tasić et al. (2016). Although values of lipid content up to 80% has been reported (Li and Qin, 2005; Chisti, 2007), most of the values reported for *B. braunii* was in the range of 15 to 40% dry weight, which indicates the capability of the strains used herein.

The fatty acid profile revealed that the oleic (C18:1) and the palmitic (C16:0) acids together represented 50 to 78% of the total fatty acids contained in the organic-solvent extracts. The predominance of these two fatty acids has also been reported by Dayananda et al. (2006, 2007) for different *B. braunii* strains. Since, palmitic, stearic, oleic and linolenic acid are the most common fatty acids contained in biodiesel (Knothe, 2008), several authors (Canakci and Van Gerpen, 2001; Miao and Wu, 2006; Xu et al., 2006) consider these medium-chain fatty acids (C16 and C18) ideal for high quality biodiesel, making these bio compounds alongside hydrocarbons the parameters that mostly direct and accurately assess the potential of *B. braunii* as a feedstock for biodiesel production.

Given the results presented, we can argue in support of coupling the production of these *B. braunii* strains and wastewater treatment. Since the cost and availability of water and nutrients represent the main challenge of microalgae mass cultivation, municipal wastewater as an efficient source of both nutrients and water can represent a double add-value. This approach for nutrient removal and recovery could be essential to achieving increasingly stringent discharge standards and simultaneously contribute to the cost-effectiveness of algal biofuels/bioenergy development. We have shown that these two *B. braunii* strains were extremely efficient, removing up to 89% of total phosphorus and nearly 100% nitrogen from wastewater, leading to nutrient concentrations that would meet European discharge standards. Coupling microalgae cultivation to secondary wastewater effluent treatment could considerably reduce the associated environmental impacts and energy input for mixing and aeration since the oxygen is produced by photosynthesis. However, this approach would consider functional polycultures, even because wastewater cannot be completely sterilized in a practical and cost-effective way. Therefore, exploratory experiments will be required in order to understand if these *B. braunii* strains will naturally dominate in wastewater and optimize its potential for the production of algal biomass.

Conclusion

Herein, we revealed that although the Portuguese strains of *Botryococcus braunii* have presented a typical growth for this species, they produced considerable lipid content up to 36% of the dry biomass. Furthermore, both strains showed ability to grow in wastewater, produce oil and remove nutrients that contribute to the eutrophication. The fatty acids profile showed the predominance of oleic (C18:1, 42 to 63%) and palmitic acid (C16:0, 8 to 18%), which are considered ideal for the production of high-quality biodiesel. In short, the results obtained in this work for both strains pointed them as excellent candidates for wastewater profitable use in high-quality biodiesel production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

An JY, Sim SJ, Lee JS, Kim BW (2003). Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. J. Appl. Phycol. 15:185-191.

Ashokkumar V, Rengasamy R (2012). Mass culture of *Botryococcus braunii* Kütz under open raceway pond for biofuel production. Bioresour. Technol. 104:394-399.

Belcher JH (1968). Notes on the Physiology of *Botryococcus braunii* Kiitzing. Archiv für Mikrobiologie 61:335-346.

Bendschneider K, Robison NJ (1952). A new spectrophotometric determination of nitrite in seawater. J. Mar. Res. 11:87-96.

Bligh EG, Dyer WJ (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Canakci M, Van Gerpen J (2001). Biodiesel production from oils and fats with high free fatty acids. Trans. ASAE 44:1429-1436.

Cheng P, Ji B, Gao L, Zhang W, Wang J, Liu T (2013). The growth, lipid and hydrocarbon production of *Botryococcus braunii* with attached cultivation. Bioresour. Technol. 138:95-100.

Chisti Y (2007). Biodiesel from microalgae. Biotechnol. Adv. 25(3):294-306.

Dayananda C, Kumudha A, Sarada R, Ravishankar GA (2010). Isolation, characterization and outdoor cultivation of green microalgae *Botryococcus* sp. Sci. Res. Essays 5:2497-2505.

Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2006). Influence of nitrogen sources on growth, hydrocarbon and fatty acid production by *Botryococcus braunii*. Asian J. Plant Sci. 5(5):799-804.

Dayananda C, Sarada R, Usha Rani M, Shamala TR, Ravishankar GA (2007). Autotrophic cultivation of *Botryococcus braunii* for the production of hydrocarbons and exopolysaccharides in various media. Biomass Bioenerg. 31:87-93.

Elsey D, Jameson D, Raleigh B, Cooney MJ (2007). Fluorescent measurement of microalgal neutral lipids. J. Microbiol. Methods. 68:639-642.

Grasshoff K (1976). Methods of Seawater Analysis. Verlag Chimie, New York.

Huang Z, Poulter CD (1989). Tetramethylsqualene, a triterpene from Botryococcus braunii var. Showa. Phytochemistry 28:146-1470.

Indarti E, Majid M, Hashim R, Chong A (2005). Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. J. Food Compost Anal. 18:161-170.

Ioki M, Ohkoshi M, Nakajima N, Nakahira-Yanaka Y, Watanabe MM (2012). Isolation of herbicide-resistant mutants of *Botryococcus braunii*. Bioresour. Technol. 109:300-303.

Kawachi M, Tanoi T, Demura M, Kaya K, Watanabe MM (2012). Relationship between hydrocarbons and molecular phylogeny of *Botryococcus braunii*, Algal Res. 1:114-119.

Kojima E, Zhang K (1999). Growth and hydrocarbon of microalga *B. braunii* in bubble column photobioreactors. J. Biosci. Bioeng. 87:811-815.

Koroleff F (1969). Direct determination of ammonia in natural waters as indophenol blue. International Council for the Exploration of the Sea (ICES) Comm. Meet. Pap. 1969/C: 9; revised 1970:19-22.

Kumar K, Dasgupta CN, Nayak B, Lindblad P, Das D (2011). Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria. Bioresour. Technol. 102(8):4945-4953.

Largeau C, Casadevall E, Berkaloff C, Dharnliencourt P (1980). Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii.* Phytochemistry 19:1043-1051.

Lee SJ, Yoon BD, Oh HM (1998). Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. Biotechnol. Technol. 12:553-556.

Li Y, Qin JG (2005). Comparison of growth and lipid content in three *Botryococcus braunii* strains. J. Appl. Phycol. 17:551-556.

Knothe G (2008). Designer biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels 22(2):1358-1364.

Mata TM, Martins AA, Caetano NS (2010). Microalgae for biodiesel production and other applications: A review. Renew. Sust. Energ. Rev. 14(1):217-232.

Metzger P, Largeau C (2005). *Botryococcus braunii*: A rich source for hydrocarbons and related ether lipids. Appl. Microbiol. Biotechnol. 66(5):486-496.

Metzger P, Allard B, Casadevall E, Berkaloff C, Couté A (1990). Structure and chemistry of a new chemical race of *Botryococcus braunii* that produces lycopadiene, a tetraterpenoid hydrocarbon. J. Phycol. 26:258-266.

Metzger P, Berkaloff C, Couté A, Casadevall E (1985). Alkadiene and botryococcene producing races of wild strains of *Botryococcus braunii.* Phytochemistry 24:2305-2312.

Miao XL, Wu QY (2006). Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. 97:841-846.

Murphy J, Riley JP (1962). A modified single solution method for the determination of phosphate in natural waters, Anal. Chim. Acta 27:31-36.

Nascimento IA, Marques SSI,Cabanelas ITD, Pereira SA, Druzian JI, de Souza CO, Vich DV, de Carvalho GC, Nascimento MA (2013). Screening microalgae strains for biodiesel production: Lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. BioEnergy Res. 6:1-13.

Ndimba BK, Ndimba RJ, Johnson TS, Waditee-Sirisattha R, Baba M, Sirisattha S, Shiraiwa Y, Agrawal GK, Rakwal R (2013). Biofuels as a sustainable energy source: An update of the applications of proteomics in bioenergy crops and algae. J. Proteom. 93:234-44.

Nyberg H (1986). GC-MS methods for lower plant glycolipid fatty acids. In Gas Chromatographyl/Mass Spectromandry, Linskens HF, Jackson JF (ed). Springer-Verlag: Berlin 3:67-99.

Órpez R, Martínez ME, Hodaifa G, Yousfi F, Jbari N, Sánchez S (2009). Growth of the microalga *Botryococcus braunii* in secondarily treated sewage. Desalination 246:625-630.

Qin J (2005). Bio-hydrocarbons from algae: Impacts of temperature, light and salinity on algae growth. A Report for the Rural Industries Research and Development Corporation, Australian Government. RIRDC Publication No 05/025 and RIRDC Project No SQC-1A. p. 18.

Rao AR, Sarada R, Ravishankar GA (2007). Influence of CO2 on Growth and Hydrocarbon Production in Botryococcus braunii. J. Microbiol. Biotechnol. 17(3):414-419.

Sawayama S, Inoue S, Yokoyama S (1994). Continuous culture of hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. Appl. Microbiol. Biotechnol. 41:729-731.

Sawayama S, Minowa T, Dote Y, Yokoyama S (1992). Growth of the hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. Appl. Microbiol. Biotechnol. 38:135-138.

Serra T, Zilberman D (2013). Biofuel-related price transmission literature: A review. Energ. Econ. 37:141-151.

Shiho M, Kawachi M, Horioka K, Nishita Y, Ohashi K, Kaya K, Watanabe MM (2012). Business evaluation of a green microalga *Botryococcus braunii* oil production system. Procedia Environ. Sci. 15:90-109.

Singh A, Smyth BM, Murphy JD (2010). A biofuel strategy for Ireland with an emphasis on production of biomethane and

minimization of land-take. Renew. Sustain. Energ. Rev. 14:277-288.
Singh Y, Kumar HD (1992). Lipid and hydrocarbon production by *Botryococcus* spp. under nitrogen limitation and anaerobiosis. World J. Microbiol. Biotechnol. 8:121-124.
Stein J (1973). Handbook of Phycological methods. Culture methods and growth measurements. Cambridge University Press. P 448.
Talukdar I, Kalita MC, Goswami BC (2013). Characterization of the biofuel potential of a newly isolated strain of the microalga *Botryococcus braunii* Kützing from Assam. Bioresour. Technol. 149:268-275.
Tasić MB, Pinto LFR, Klein BC, Veljković VB, Filho RM (2016). *Botryococcus braunii* for biodiesel production. Renew. Sust. Energ. Rev. 64:260-270.
Wolf FR (1983). *Botryococcus braunii*. An Unusual Hydrocarbon-Producing Alga. Appl. Biochem. Biotechnol. 8:249-260.
Wolf FR, Nonomura AM, Bassham JA (1985). Growth and Branched Hydrocarbon Production in a strain of *Botryococcus braunii* (chlorophyta). J. Phycol. 21(3):388-396.
Xu H, Miao XL, Wu QY (2006). High quality biodiesel production from a microalga chlorella protothecoides by heterotrophic growth in fermenters. J. Biotechnol. 126:499-507.
Yamaguchi K, Nakano H, Murakami M, Konosu S, Nakayama O, Kanda M, Nakamura A, Iwamoto H (1987). Lipid composition of a green alga, *Botryococcus braunii*. Agric. Biol. Chem. Tokyo 51:493-498.