Title: Improvements in the cultivation of *Botryococcus braunii* using commercial fertilisers.

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Abstract

Agricultural fertilisers (NPKs) have been recognised as an alternative to make microalgae cultivation cheaper as well as simpler in terms of the preparation of the medium. *Botryococcus braunii*, a green microalga, has the almost unique capacity to accumulate and excrete large amounts of long chain hydrocarbons and/or interesting groups of polysaccharides which can be further converted into bio-products. However, limitations in growth are currently hindering its industrial production. In this work, the use of different agricultural fertilisers (NPKs) was evaluated for the cultivation of two *B. braunii* races (A and B) in terms of productivity and final media labour and cost. Results corroborated that fertilisers-based media are easier to prepare and their prices are considerably lower compared to common culture media. At the same time, a good growth performance and photosynthetic efficiency can be maintained, and carbohydrate and hydrocarbon productivities can be further enhanced. However, special attention should be given to each particular strain since different behaviour in growth and metabolite production can be observed depending on the media composition.

The significantly higher productivities obtained, together with the important reduction in media price when using commercial fertilisers, and the advantages related to the easiness to prepare the culture media based on NPKs represent an important achievement for the development of an industry based on these renewable products.

Keywords: *Botryococcus braunii,* fertilisers/NPK, cost-reduction, hydrocarbons, polysaccharides, productivity improvement.

1. Introduction

Culture media optimisation is an important factor to be considered in microalgal cultivation due to the current high cost associated with the biomass and metabolites production. Previous research studies showed that in the development of microalgal products, one of the major tasks is to select a suitable culture medium, so that the quality and quantity of biomass can be enhanced (Gong and Chen 1997; Ilavarasi et al. 2011; Wong et al. 2017; Bermejo et al. 2020). The choice of such medium mainly depends on factors as its chemical composition or the desired major metabolite accumulation (Borowitzka 2005). However, to obtain biomolecules with high added value at a commercial level, it is necessary to massively produce biomass, which implies a high demand of optimum analytical grade reagents and a considerable consumption of time for the preparation of the culture media (Liu and Hu 2013). All this also has the disadvantage of raising the production costs of microalgae cultures (Molina-Grima et al. 2003; Borowitzka 2005). In this sense, strategies such as using alternative media should be undertaken to decrease costs without compromising the productivity and nutritional values of the culture. With this purpose, some studies have been carried out over the last years with agricultural fertilisers, as well as with wastewater, as culture media for different species of microalgae (Nayak et al. 2016; Silva-Benavides 2016; Faé-Neto et al. 2018; Schneider et al. 2018; Jbari et al. 2020; Ali et al. 2021). Wastewater has been used as an efficient source of nutrients, with the added benefit of reducing the environmental impact by treating the water. Although for certain applications, such as bioenergy production, the use of wastewater might be preferable, the applications of the final biomass or by-products aimed at human consumption are not recognised as safe (Molinuevo-Salces et al. 2019). In this regard, it is important to consider other affordable alternatives to the use of conventional chemicals. In addition, microalgae cultivated in

agricultural fertilisers have been reported to produce, in general, more biomass and a higher fixation of CO_2 compared to those grown in wastewater, which is of great importance from the point of view of the global warming (Schneider et al. 2018). It has been also recognized that the replacement of chemical compounds by fertilisers is a way to make microalgal cultivation even easier and cost-effective (Scardoelli-Truzzi and Sipaúba-Tavares 2017). However, few results can be found in literature, although it is being implemented elsewhere where cultivation at pilot or large scale takes place.

Botryococcus braunii is a green colony-forming microalga which has the almost unique capacity to synthesise, accumulate and excrete large amounts of long-chain hydrocarbons and/or interesting groups of polysaccharides, such as exopolysaccharides (EPS) (Gouveia et al. 2017). Such metabolites can be further converted into bio-products and, accordingly, the attention of researchers has increased in attempts to exploit *B. braunii* as a renewable source of products (Banerjee et al. 2002; Li and Qin 2005). However, despite its potential, the slow growth rate of this microalga hinders its industrial application.

In the present work, with the purpose of increasing *B. braunii* biomass and metabolites productivities and make the process cost-effective and more efficient, it was proposed to cultivate two different races of such microalga (A and B) in culture media based on agricultural fertilisers (NPKs). Although the use of different commercial low-cost nutrients has been previously reported for *B. braunii* (Kurinjimalar et al. 2017), to the best of our knowledge, it is the first attempt to cultivate *Botryococcus* with commercial agricultural NPKs. The algae selection was made based on a previous study that demonstrated their potential as polysaccharide (race A) and hydrocarbon (race B) producers (Gouveia et al. 2017). As a first approach, these microalgae were cultivated with different nitrogen sources in order to identify the preferred one and, therefore, to know which fertilisers could be more suitable to be used. Subsequently, the suitability of

several fertilisers containing the preferred nitrogen source was assessed. Finally, the fertilisers resulting in the highest productivities for both *B. braunii* races were decided to be further tested at different nitrogen concentrations to check if productivities could be enhanced.

2. Material and Methods

2.1. Microalgal strains and culture conditions

Botryococcus braunii race A (CCALA778) and race B (AC761) were provided by the Culture Collection of Autotrophic Organisms, Trebon, Czech Republic and the Laboratoire de Chimie Bioorganique et Organique Physique, Ecole Nationale Supérieure de Chimie de Paris, France, respectively. Both strains were cultivated in 1L Roux flasks inside a culture room at a temperature of 25 ºC. Continuous fluorescent illumination was provided at an incident light intensity of 100 µmol photons m^{-2} s^{-1} (Photosynthetically Active Radiation; PAR), and the cultures were bubbled with air containing 2.5% (v/v) CO_2 as carbon source. Each *Botryococcus* strain had a different reference media based on the conclusions of a previous study where productivity and sustainability were enhanced by the modification of Chu 13 media (Bermejo et al. 2020). *B. braunii* race A had M44 as reference medium ($CaCl_2 \cdot 2H_2O$ 734 µM; $MgSO_4 \cdot 7H_2O$ 811 µM; K_2HPO_4 602 µM; KNO_3 22 mM; FeNaEDTA 40 µM; $Na_2Mo_4 \cdot 2H_2O$ 1 µM; final pH 7.2), and *B. braunii* race B had M35 as reference medium ($CaCl_2 \cdot 2H_2O$ 734 µM; $MgSO_4 \cdot 7H_2O$ 811 µM; K_2HPO_4 3.3 mM; KNO_3 22 mM; FeNaEDTA 10 µM; Na2Mo4·$2H_2O$ 1 µM; final pH 7.2).

The composition of the different agricultural fertilisers used in this work are presented in Table 1. In order to avoid nutritional deficiencies related to the presence of calcium and magnesium, elements that naturally can be found in the tap water which would be used

123 to prepare culture media at large scale, the media prepared with fertilisers were
124 supplemented with these elements at a concentration equal to that of the reference
125 medium of each *B. braunii* race. Likewise, the cultures were supplemented with a solution
126 of commercial micronutrients (Agralia AG Complex, Agralia Fertilisers, S.L, Spain)
127 according to the concentration of iron present in each reference medium
128 (Online Resource 1).
129 In order to assess both *B. braunii* races growth and metabolite production in the different
130 culture media, an adaptation period, through repeated-batch mode, was needed before the
131 experiment. This has been reported to be essential to get biomass growth comparable to
132 that of the controls (Voltolina et al. 1998). Cultures were maintained in an optical density
133 range between 0.5 and 1 approximately, measured at 750 nm, via punctual dilutions.
134 Several cycles were carried out in order to ensure the steady state was reached.
135

136 **2.2. Maximum photosynthetic efficiency**

137 Maximum photosynthetic efficiency of photosystem II (PSII), also known as maximum
138 quantum yield of photosystem II ($Qy = F_v/F_m$), was assessed measuring the chlorophyll
139 fluorescence in dark-acclimated cells for 20 minutes according to the method described
140 by Cuaresma et al. (2011). This parameter was determined using the pulse amplitude
141 modulation (PAM) technique by an AquaPEN AP-100 device (Photon System
142 Instruments, Czech Republic).
143

144 **2.3. Biomass dry weight determination**

145 Biomass concentration was determined by filtering aliquots of cell cultures through pre-
146 weighted 2.7 μm glass microfiber filters (Filter-Lab MFV4, Filtros Anoia, Barcelona,
147 Spain) and washing with deionised water. The filters containing the algae were dried at

100 °C for 24 h and placed 1 h into a desiccator to cool down prior to weighing them. Dry weight was the result of the difference between the filter weight before and after the filtration step.

2.4. Productivity calculation

Volumetric productivities of biomass and each pertinent compound of interest (carbohydrates and hydrocarbons) were calculated applying the following equation:

$$P_v = \frac{(C_t - C_{t-x})}{\Delta_{t(t,t-x)}}$$

where $C_t - C_{t-x}$ is the increase in biomass or metabolites concentration (g L^{-1}), during a determined period of time in the linear phase of the growth ($\Delta_{t(t,t-x)}$).

2.5. Carbohydrate and hydrocarbon determination

Metabolites content in *B. braunii* race A and race B was determine as described by Bermejo et al. (2020). Carbohydrates content in *B. braunii* race A was measured according to a colorimetric method based on Dubois principle (Dubois et al. 1956). First, 3-10 mg of lyophilised biomass was hydrolysed through the addition of HCl 2.5M and its incubation at 100 ºC. Subsequently, a neutralisation with NaOH 2.5M was carried out. Finally, the absorbance measured spectrophotometrically at 483nm after the addition of phenol and H_2SO_4 allowed the quantification of carbohydrates.

The extraction of hydrocarbons in *B. braunii* race B was accomplished in accordance with the method reported by Folch et al. (1957) and Bligh and Dyer (1959). Hydrocarbons were identified and quantified by gas chromatography (Agilent 7890A, FID detector, United States) after the incubation of at least 0.5 g L^{-1} of biomass in organic solvents (methanol:dichloromethane).

Hydrocarbon's quantification was carried out through gas chromatography (Agilent 7890A, FID detector, USA) following the methodology developed by Bermejo et al. (2020). A calibration curve with a squalene pattern (SUPELCO 442785, C30 analytical standard, USA) at concentrations between 50 and 850 ppm was previously made in order to quantify the amount of hydrocarbons present in the samples. The concentration of hydrocarbons was calculated using the calibration data and the area obtained in the chromatograms for each sample.

2.6. Statistical Analysis

The presented data are the average value of two experimental replicates and three analytical replicates in each experiment and they were expressed as means with ± standard deviation (SD). Mean values are presented in the corresponding figures with error bars as standard deviation. The variations in productivities of biomass and metabolites among the different culture media were evaluated using one-way analysis of variance (ANOVA) after having checked that the data met the requirements for it. If ANOVA results were significant, comparisons among means were followed by a post-hoc Tukey's multiple comparison tests, with a confidence level of 0.05. The statistical analyses were performed using Minitab version 18 (Minitab® Statistical Software).

3. Results

3.1. Culture of *B. braunii* races A and B in different commercial fertilisers

In order to optimise the algal cultivation process, several agricultural NPKs were evaluated. However, the majority of commercial fertilisers contain ammonium, and to a greater extend urea, instead of nitrate as source of nitrogen. Thus, a preliminary test was carried out to evaluate the ability of *B. braunii* to grow in culture media in which nitrate

197 (nitrogen source of the reference media) was substituted by ammonium or urea. The
198 experiment demonstrated that *Botryococcus* was able to grow in all the nitrogen sources
199 tested. Nevertheless, whereas in cultures with ammonium the biomass growth was
200 reduced by 38.6%, cultures with urea presented significantly higher growth compared to
201 those with nitrate as nitrogen source (Fig. 1).

202 Once it was shown that urea was a suitable nitrogen source to grow both *B. braunii* strains,
203 the selection of adequate commercial fertilisers for their cultivation was carried out
204 accordingly. At the same time, due to the necessity of a further reduction in the media
205 cost, and considering that a certain degree of nutrients limitation has been proven to
206 enhance metabolite accumulation in *Botryococcus* (Bermejo et al. 2020), the different
207 fertiliser-based media were prepared with reduced concentrations of nitrogen compared
208 to the reference media (see Table 2). Repeated-batch cultivation was carried out to adapt
209 the algae. During this adaptation period it was observed that whilst *B. braunii* race B was
210 able to grow in all the fertilisers, the race A grew with only two of the five selected
211 fertilisers (2.4-4.8-6 and 8-6-6). The rest of the fertiliser-based media resulted in a
212 progressive decrease in the maximum photosynthetic efficiency of the cells that
213 eventually led to the culture collapse (Fig. 2a).

214 Regarding biomass and metabolites productivities (carbohydrates and EPS in race A, and
215 hydrocarbons in race B), calculated at the end of the repeated-batch process, both strains
216 cultivated in 8-6-6 fertiliser-based medium presented the highest metabolites results
217 (Table 3) with no significant differences in biomass respect to the reference. *B. braunii*
218 race B registered 45% higher hydrocarbon productivity respect to its reference medium.
219 And in the case of the race A, although the carbohydrate value did not present significant
220 difference, it showed 42% higher EPS productivity compared to its reference medium.
221 Several studies have demonstrated that EPS produced by microalgae present important

antioxidant, antitumor, and anti-inflammatory activity (Yingying et al. 2014; Talero et al. 2015; Zhang et al. 2019), and some of them also perform as biomaterial and biolubricant (Arad et al. 2006). Moreover, it has been proposed that a solution to facilitate the production of high-value products from microalgae is to reuse the cell mass for continuous production (Hejazi and Wijffels 2004), which is easier if the metabolites have been excreted to the culture medium as it is the case of the EPS. Considering the above, having higher amount of excreted carbohydrates downplays the slightly lower total carbohydrate productivity obtained for *B. braunii* race A.

3.2. Media cost calculation and selection of the best fertiliser-based medium for both *B. braunii* races

Considering the obtained results (Table 3), it is evident that culture media composition substantially influences the productivity of *B. braunii* in terms of metabolites accumulation. But another point to bear in mind in order to select the best medium it is the final cost. Thus, the price of all fertiliser-based media was calculated and compared to the price of the reference medium for each microalga (estimated from technical grade reagents costs). As it can be seen in Table 4, fertiliser-based media presented much lower prices than their respective reference culture media. Therefore, considering the easiness of operation when preparing medium based in agricultural fertilisers, their lower price, and that both *B. braunii* races resulted in better productivities of interesting metabolites, 8-6-6 fertiliser-based medium arose as a promising medium for large scale *Botryococcus* cultivation.

3.3 Assessment of *B. braunii* races A and B culture in different 8-6-6 fertiliser-based medium concentrations

The previous experiment revealed the potential and suitability of the commercial fertiliser NPK 8-6-6 to cultivate *B. braunii* races A and B. In this regard, it was decided to further

evaluate the amount of such fertiliser to be used in order to see the effect in the productivity and/or reduce the media cost. Fertiliser concentration was adjusted to one-half (8-6-6(1/2)) and 2-fold (8-6-6(2x)) the concentration used previously with each *B. braunii* race (see Table 2), and which was used as reference in this experiment. Repeated-batch cultivation was carried out to adapt the algae to the new concentrations of 8-6-6 fertiliser-based medium. During more than 800 hours of cultivation, pH was maintained at 7.2 ± 0.5 and maximum photosynthetic activity of PSII was daily monitored. Biomass dry weight and metabolites content (total carbohydrates, EPS and hydrocarbons) were determined during the last growing cycle once the cultures were considered to be adapted to the growing conditions.

3.3.1. *B. braunii* race A

Minor differences were observed in photosynthetic efficiency between the fertiliser-based media 8-6-6 and 8-6-6(1/2), which showed values above 0.6. However, photosynthetic efficiency of the culture with the fertiliser 8-6-6(2x) was continuously decreasing since the beginning of the experiment until the culture death (Fig. 3a).

Biomass volumetric productivities showed 38.8% lower value in 8-6-6(1/2) when compared to the reference fertiliser 8-6-6 (Table 5). However, regarding the effect of the fertiliser concentrations in the biochemical composition of *B. braunii* race A, the results showed a significant improvement. The final total carbohydrate and EPS volumetric productivities for the fertiliser 8-6-6(1/2) were 13% and 25% higher, respectively, than those obtained with the reference 8-6-6.

3.3.2. *B. braunii* race B

Regarding the maximum photosynthetic efficiency of PSII (F_v/F_m) of *B. braunii* race B (Fig. 3b), it can be noticed that culture with the fertiliser 8-6-6(2x) followed the same pattern during the entire experiment, with efficiency values above 0.6. Nonetheless,

maximum photosynthetic efficiency values of 8-6-6 and 8-6-6(1/2) showed a steep decrease in the middle of each growing cycle, being more prominent for the second one, although it could be recovered after each dilution step. In terms of volumetric hydrocarbon productivity, fertiliser-based medium at the highest concentration also resulted in the major value at the end of the experiment (Table 5).

4. Discussion

In the present work, *B. braunii* races A and B were able to grow in different nitrogen sources (nitrate, ammonium and urea). However, the culture with urea showed the best biomass production results from 200 hours, being the value at the end of the process significantly higher compared to the culture that contained nitrate (Fig. 1). Ammonium might have been expected to be the preferred compound as it is the reduced form of nitrogen, and it requires less energy for assimilating into amino acids. Nevertheless, the results obtained in our study with *B. braunii* were in accordance with other works which already described a high rate of urea uptake and urea assimilation for many species of algae as compared to other nitrogen sources (Brennan and Owende 2010; Campos et al. 2014; Kim et al. 2016; Minyuk et al. 2020). Urea is a cheap and highly enriched organic compound containing 46% of nitrogen and 20% of carbon that can be easily used by most microalgae after being degraded to ammonium and bicarbonate via urease (Solomon and Glibert 2008).

The selection of commercial NPKs was made taking into account the observed preference of *B. braunii* by the urea, and the maximum photosynthetic measurement allowed to determine their suitability in the culture of both races. A loss of cell viability was observed when the race A was cultivated in the NPKs 18-6-6, 4-10-10 and 12-6-4(3) which might be due to the higher concentration of ammonium in those three fertilisers-based media in comparison with the other two in which the alga grew normally (NPKs 2.4-4.8-6 and 8-

297 6-6). In aqueous solution, ammonium ion (NH_4^+) exists in equilibrium with the un-ionised
298 or free ammonia (NH_3), which has been reported to be toxic to several photosynthetic
299 organisms, according to the dissociation equation: $NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$
300 (Abeliovich and Azov 1976). Because of the equilibrium, increasing one of them
301 automatically increases the other. Furthermore, this equilibrium mainly depends on pH.
302 Thus, as the algal density increased, the pH in the medium also increased due to carbon
303 dioxide assimilation. This condition might have forced the reaction to move to the left,
304 increasing the concentration of ammonia which has a toxic effect on the microalga growth
305 and, consequently, might affect the microalgal photosynthetic efficiency. In the case of
306 *B. braunii* race B, it was able to grow in all the fertilisers (Fig. 2b) since the amount of
307 them used was considerably lower than for the race A. Therefore, the consequently lower
308 ammonium concentration did not affect the metabolism of the alga.

309 Among all the fertilisers tested, culture media based on NPK 8-6-6 resulted in the best
310 metabolite (EPS and hydrocarbon) productivities. It is known that nutrient availability
311 plays an important role in the growth and biochemical composition of microalgae (Rasdi
312 and Qin 2015; Bermejo et al. 2020). Nitrogen and phosphorus are the major elements that
313 can affect their metabolism, and the ratio N:P has been considered an effective strategy
314 to shift their chemical composition. According to Redfield ratio (Redfield 1934), the
315 better metabolite results obtained with the fertiliser 8-6-6 indicated that these races of
316 *B. braunii* rather need nitrogen (N:P < 16) than phosphorus (N:P >16) limitation to
317 accumulate a higher amount of their interest compounds. This is in agreement with other
318 works in which *Chlorella* and *Scenedesmus* were grown with sufficient phosphorus under
319 N-starvation for lipid accumulation (Chu et al. 2013, 2014). In this sense, comparing the
320 composition of the selected fertilisers to both reference media (Table 2), the fact that the
321 medium based on fertiliser 8-6-6 presented a much lower nitrogen concentration could

have strengthened the increase in the production of the metabolites of interest. A study carried out by Singh and Kumar (1992) demonstrated that nitrogen limitation, as well as other stress conditions, may promote the biosynthesis of hydrocarbons and carbohydrates in *B. braunii*. However, nitrogen starvation can present many negative effects in microalgae cultivation such as damaging the synthesis of proteins participating in photosystems PSI and PSII, and decreasing the synthesis of photosynthetic pigments (Markou et al. 2012b). The fact that no negative effect was found in the biomass productivity neither in the photosynthetic activity of *Botryococcus* when using the fertiliser 8-6-6 (Fig. 2) can be considered a positive outcome compared to other N-limiting media (Benvenuti et al. 2015).

During the second approach, carried out to assess the potential of the fertiliser 8-6-6 in the cultivation of *B. braunii*, the cellular collapse observed for the race A when the amount of the fertiliser was the double (8-6-6(2x)) might be attributed, once again, to the higher concentration of ammonium in that medium since, as it was previously mentioned, this compound is correlated to the presence of ammonia which is known to be toxic for algae (Abeliovich and Azov 1976). By contrast, it was able to grow under a lower concentration of the fertiliser 8-6-6 (8-6-6(1/2)) and, although the biomass production was reduced respect to the reference concentration, a significant improvement in the metabolite production was observed. This is in accordance with other studies that have demonstrated that a high degree of nitrogen limitation can have a stronger impact on biomass productivity and growth than on carbohydrate production (Hopkins and Hüner 2009; Markou et al. 2012; Bermejo et al. 2020). It is important to address that the reduction in biomass in the case of 8-6-6(1/2) culture is accompanied by a considerable easiness of operation, besides a major reduction in price due to the lower amount of medium used, which is very beneficial at large-scale production. On the other hand, the

media containing unconsumed fertilisers must be discarded in a proper way in order to reduce the environmental footprints (Pugazhendhi et al. 2020). Thus, a lower initial fertiliser concentration would facilitate its subsequent removal at the end of the cultivation process. All this, together with the significantly higher carbohydrate content, makes 8-6-6 fertiliser-based medium at half concentration an interesting candidate to be evaluated at large-scale for this race A of *B. braunii*.

In the case of the race B, the decrease in the maximum photosynthetic efficiency values showed in the middle of each growing cycle with the fertiliser 8-6-6(1/2) and 8-6-6, might be indicative of cellular stress due to the nutrient limitation at which the biomass could be exposed, especially in the case of the fertiliser 8-6-6(1/2). It has been described that photosynthetic efficiency is highly dependent on the supply of nutrients in algae (Zhao et al. 2017; Li et al. 2020). The existence of nutrient limitation is also supported by the fact that the biomass productivity results for 8-6-6 and 8-6-6(1/2) cultures were 17.4% and 31.7% lower respectively than the productivity obtained with double concentration of fertiliser (Table 5). It is known that nitrogen limitation or starvation can enhance lipid and/or carbohydrate production but, depending on the species of microalgae and the degree of limitation, it can also result in chloroplast degradation, structural damage of photosystems, and a decrease in photosynthetic efficiency, that can eventually negatively affect the accumulation of these metabolites (Li et al. 2016, 2020). It is therefore essential to find the most suitable initial nitrogen supply to avoid that the desired stress produced by a low content of nitrogen becomes too harmful for the photosynthetic capacity of the algae. In this study, such nitrogen concentration was that in the fertiliser 8-6-6(2x), in which the microalga showed the best balance between biomass and hydrocarbons production. Hence, the fertiliser 8-6-6(2x) should be considered an outstanding candidate to cultivate *B. braunii* race B, especially at large-scale.

Overall, differences were observed in biomass and metabolite production among the two *B. braunii* races depending on the culture medium (reference/fertiliser-based media) and/or the concentration of its components. This behaviour is in agreement with several studies carried out with other microalgae, supporting that requirements for nutrients may vary as the algae belonging to different species and genus (Lagus et al. 2004; Praba et al. 2016; Colusse et al. 2020). Therefore, the selection of the most suitable culture medium for a particular microalga is crucial to maximise its cultivation yield. In this context, our study found the use of commercial agricultural NPKs a feasible and cost-effective approach for the production of *Botryococcus* rich in products of interest while preserving biomass growth.

Conclusions

In this study, the advantages of using commercial fertilisers to cultivate *Botryococcus braunii* were demonstrated. Two races of *B. braunii* (A and B) were successfully grown in several fertiliser-based media showing a considerable improvement in their productivities. Carbohydrate production was increased for the race A when it was cultivated in fertiliser-based media that contained lower amount of nutrients compare to its reference medium. However, in the case of the race B, as the nitrogen concentration in its reference medium was already low, the highest hydrocarbon productivity was reached with a fertiliser-based medium containing similar nutrients concentration as the reference has. This different behaviour depending on the microalgae and media composition emphasises the suitability of optimisation studies.

It can be concluded, from the enhanced productivity results of this work, and considering the major benefits related to media preparation and their lower cost, that the use of fertilisers should be taken into account as a promising tool for the feasible production of *Botryococcus*.

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552 **Caption to Figures**

553 **Fig. 1.** Evolution of biomass growth for *B. braunii* race A cultures with different nitrogen
554 sources; (●) nitrate (NO_3^-); (○) ammonium (NH_4^+); (▼) urea ($CO(NH_2)_2$). Error bars
555 show standard deviation of replicates. (*) indicates differences at the significance level
556 $p<0.05$ (Tukey's pairwise test) respect to the final biomass value obtained with NO_3^- as
557 source of nitrogen.

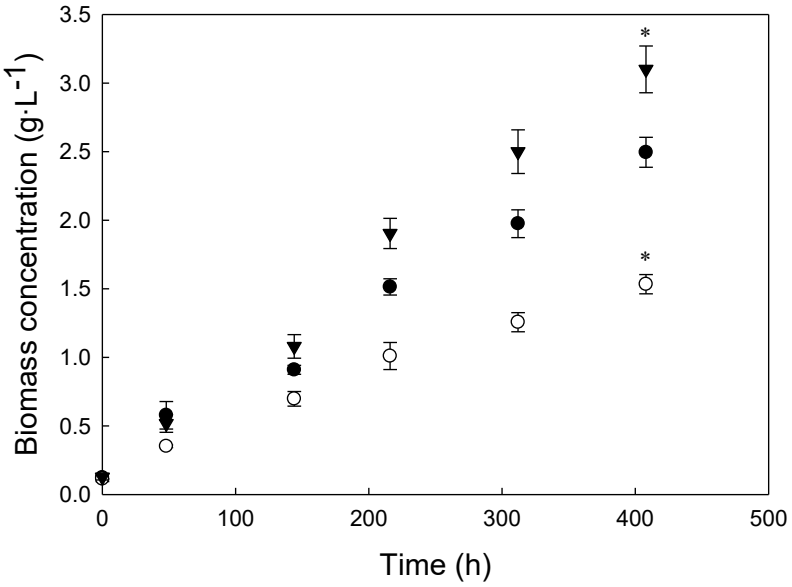

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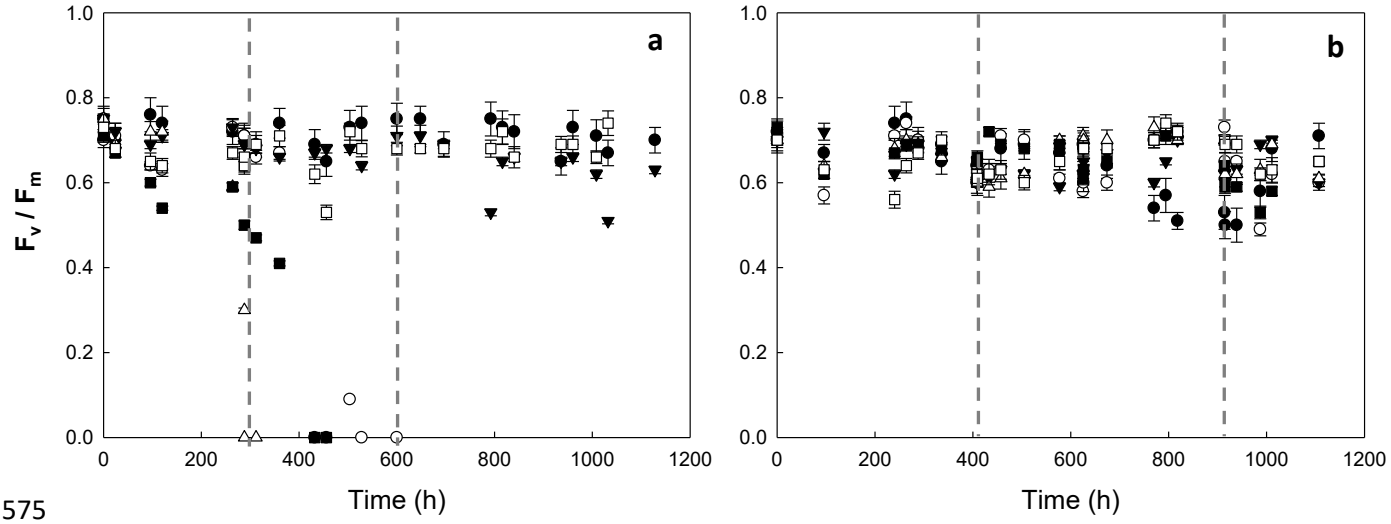
Fig. 2. Evolution of maximum photosynthetic efficiency of PSII (F_v/F_m) for *B. braunii* race A (a) and race B (b) grown in their respective reference culture media and in the selected fertiliser-based media, during the repeated-batch cultivation; (●) reference medium; (○) NPK 18-6-6; (▼) NPK 2.4.-4.8.-6; (▲) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6. Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.



583 **Fig. 3.** Evolution of maximum photosynthetic efficiency of PSII (F_v/F_m) for *B. braunii*
584 race A (a) and race B (b) during the repeated-batch cultivation with different
585 concentrations of 8-6-6 fertiliser-based medium. (●) NPK 8-6-6; (○) NPK 8-6-6(1/2);
586 (▼) NPK 8-6-6(2x). Dash lines represent the punctual dilutions carried out and error bars
587 show standard deviation of replicates.

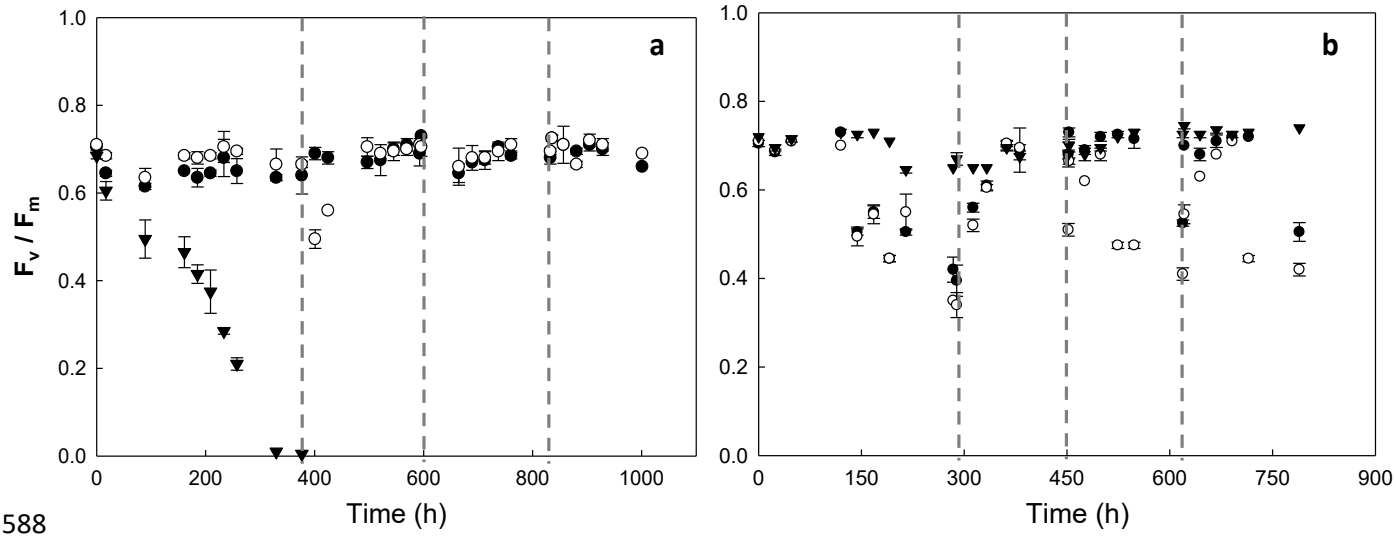


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Table 1. Code and composition of the different commercial fertilisers used for the optimization of *B. braunii* cultivation. All the fertilisers used in this study were kindly provided by Fertiberia S.A. (Spain).

NPK	N $mol \cdot L^{-1}$	P $mol \cdot L^{-1}$	K $mol \cdot L^{-1}$	N / P	Nitrogen source	Other nutrients
12-6-4(3)	5.54	0.50	1.01	11.02	9% urea, 3% ammonium	S (0.45 M)
4-10-10	2.66	0.84	2.55	3.15	4% ammonium	
18-6-6	8.01	0.52	1.58	15.28	15.9% urea, 2.1% ammonium	
2.4-4.8-6	1.08	0.38	1.44	2.84	1.6% nitrate, 0.8% ammonium	
8-6-6	3.63	0.49	1.49	7.35	6% urea, 2% ammonium	

Table 2. Composition of the reference culture media and the fertiliser-based media (NPKs) for *B. braunii* race A and race B. The volume of the commercial NPKs and micronutrients solution (AG complex) used in the preparation of the media is shown in $mL \cdot L^{-1}$. The ratio nitrogen/phosphorus (N/P) is shown, as well as the final media composition in millimole per litre (mM).

	***B. braunii* race A**					***B. braunii* race B**				
	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]
Reference	---	22.25	0.60	37	0.04	---	3.96	0.60	6.70	0.05
12-6-4(3)	2	11.09	1.01	10.98	---	0.50	2.77	0.25	11.08	---
4-10-10	5	13.30	4.23	3.14	---	1	2.66	0.84	3.17	---
18-6-6	1	8.01	0.52	15.40	---	0.25	2	0.13	15.38	---
2.4.-4.8.-6	1	10.84	3.82	2.84	---	2	2.17	0.76	2.85	---
8-6-6	2	7.27	0.99	7.34	---	0.50	2.18	0.30	7.27	---
AG Complex	0.044	---	---	---	0.04	0.060	---	---	---	0.05

Table 3. Biomass and metabolites volumetric productivities (P_V) of *B. braunii* race A and race B, expressed as grams per litre of culture broth per day, at the end of the cultivation in different fertiliser-based media.

Culture media	*B. braunii* race A			*B. braunii* race B	
	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	**Carbohydrates P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**EPS P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**Biomass P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**Hydrocarbons P_V** ($g \cdot L^{-1} \cdot d^{-1}$)
Reference	$0.283^{ab} \pm 0.023$	$0.169^{a} \pm 0.002$	$0.033^{a} \pm 0.005$	$0.163^{a} \pm 0.002$	$0.049^{a} \pm 0.002$
18-6-6	---	---	---	$0.158^{a} \pm 0.001$	$0.060^{b} \pm 0.001$
2.4-4.8-6	$0.250^{b} \pm 0.008$	$0.138^{b} \pm 0.004$	$0.018^{b} \pm 0.002$	$0.160^{a} \pm 0.002$	$0.058^{b} \pm 0.004$
4-10-10	---	---	---	$0.163^{a} \pm 0.003$	$0.046^{ac} \pm 0.002$
12-6-4(3)	---	---	---	$0.145^{b} \pm 0.002$	$0.040^{c} \pm 0.001$
8-6-6	$0.312^{a} \pm 0.027$	$0.165^{a} \pm 0.003$	$0.047^{c} \pm 0.003$	$0.161^{a} \pm 0.004$	$0.071^{d} \pm 0.003$

$^{a, b, c, d}$ Different superscript letters indicate differences at the significance level $p<0.05$ (Tukey's pairwise test) within the different culture media.

Table 4. Comparison between fertiliser-based media (NPKs) and reference media costs for *B. braunii* race A and race B. Media costs per kg of biomass of *B. braunii* race A are only indicated for the fertilisers 2.4-4.8-6 and 8-6-6 since such race was not able to grow in the rest of the NPKs. The prices were calculated according to the real price of the commercial fertilisers and micronutrients solution (AG Complex), and the prices of the reference media were calculated based on those of the different technical grade chemicals from Sigma-Aldrich website (2021).

	Price of commercial solutions used		***B. braunii* race A**				***B. braunii* race B**			
	NPK	AG Complex	NPK	AG Complex	Final Price		NPK	AG Complex	Final Price	
	(€/ton)	(€·L^{-1})	(mL·L^{-1})		(€·L^{-1})	(€·kg^{-1} biomass)	(mL·L^{-1})		(€·L^{-1})	(€·kg^{-1} biomass)
Reference	---	---	---	---	0.255	41.13	---	---	0.073	45.60
18-6-6	274	4.8	1	0.044	0.000485	---	0.250	0.058	0.000347	0.22
2.4-4.8-6	240		10		0.002611	0.47	2		0.000758	0.47
4-10-10	226		5		0.001341	---	1		0.000504	0.31
12-6-4(3)	230		2		0.000671	---	0.500		0.000393	0.28
8-6-6	210		2		0.000631	0.09	0.500		0.000383	0.24

673 **Table 5.** Volumetric productivities (P_V) of carbohydrates and hydrocarbons for *B. braunii*
674 race A and B, respectively, expressed as grams of biomass produced per litre of culture
675 broth and per day. P_V were calculated at the end of the adapted phase of cultivation in
676 different concentrations of the fertiliser 8-6-6.

Culture media	*B. braunii* race A			*B. braunii* race B	
	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	**Carbohydrates P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**EPS P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**Biomass P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**Hydrocarbons P_V** ($g \cdot L^{-1} \cdot d^{-1}$)
8-6-6	$0.394^{a} \pm 0.015$	$0.229^{a} \pm 0.009$	$0.064^{a} \pm 0.003$	$0.224^{a} \pm 0.015$	$0.087^{a} \pm 0.002$
8-6-6 (1/2)	$0.241^{b} \pm 0.003$	$0.259^{b} \pm 0.010$	$0.081^{b} \pm 0.006$	$0.185^{a} \pm 0.005$	$0.055^{b} \pm 0.003$
8-6-6 (2x)	---	---	---	$0.271^{b} \pm 0.025$	$0.100^{c} \pm 0.005$

677 [a, b, c] Different superscript letters indicate differences at the significance level $p<0.05$ (Tukey's pairwise test)
678 within the different concentrations of the fertiliser 8-6-6.
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