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Predicting biomass and hydrocarbon productivities and colony size in continuous cultures of *Botryococcus braunii* showa

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HIGHLIGHTS

- Models predict biomass, hydrocarbon and colony size of *B. braunii* showa cultures.
- Tool to optimize and maximize *B. braunii* showa biomass and hydrocarbon yields.
- Maximum theoretical values were validated experimentally.

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ABSTRACT

Mathematical models were developed to predict biomass and hydrocarbon productivities and colony size (ouputs) of *Botryococcus braunii* showa cultures based on light intensity, temperature and dilution rate (inputs). These models predicted the following maximum values: biomass productivity, 1.3 g L^{-1} d^{-1}; hydrocarbon productivity, 1.5 mg L^{-1} d^{-1}; colony size, 320 μm under different culture conditions respectively. These values were confirmed experimentally. Additionally, the combination of inputs that simultaneously maximize all the possible outputs combinations were determined. The prediction for biomass-hydrocarbon-colony size were 1 g L^{-1} d^{-1}, 12.05 mg L^{-1} d^{-1} and 156.8 μm respectively; biomass productivity-hydrocarbon productivities: 1 g L^{-1} d^{-1} and 13.94 mg L^{-1} d^{-1} respectively; biomass productivity-colony size: 1 g L^{-1} d^{-1} and 172.8 μm respectively; hydrocarbon productivity-colony size: 9 mg L^{-1} d^{-1} and 240 μm respectively. All these predictions were validated experimentally. These models might be very useful to implement a *Botryococcus braunii* showa large scale production.

1. Introduction

The colonial microalgae *Botryococcus braunii* (Chlorophyta, Trebouxiophyceae) that dwells from fresh to brackish water displays a unique characteristic, cells are embedded in a matrix of sugars and hydrocarbons naturally excreted (Weiss et al., 2012). This distinctive characteristic has attracted the attention to *B. braunii*. The hydrocarbons accumulated in the extracellular space have been suggested for exploitation as renewable source for biofuels and other chemicals as olefins (Banerjee et al., 2002; Uno et al., 2015) in milking-like extractions (García-Cubero et al., 2018a–d). *B. braunii* strains have been classified in 4 races according to the hydrocarbons profile: race A (chain length of C_{25}-C_{31}), B (C_nH_{2n-10}, n = 30–37), L ($C_{40}H_{78}$ or tetraterpene) and S (C_{18}-C_{20}) (Banerjee et al., 2002; Kawachi et al., 2012). Among them, *Botryococcus braunii* showa (race B) is considered one of the most promising strains owing to its high hydrocarbon contents mainly produced on batch cultivations (Gouveia et al., 2017; Yoshimura et al., 2013).

To date, a small number of pilot scale experiments have been performed with *B. braunii* rendering low biomass and hydrocarbon productivities mainly because of its typical low growth rate (Al-Hothaly et al., 2015; Bazaes et al., 2012; Wang et al., 2013). One way to overcome these low yields is to optimize culture conditions. Response Surface Methodology (RSM) is a collection of statistical tools widely spread in the improvement of processes and applied in recent years for optimization of culture conditions for different microalgae for which limited information on growth kinetics under different conditions is available (Ghosh et al., 2015; Marudhupandi et al., 2016). RSM offers several

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advantages over single-parameter optimization approach, which is time consuming and generally leads to incomplete information and results (Marudhupandi et al., 2016). Therefore, RSM might be used to predict and maximize biomass and hydrocarbon productivities under different culture conditions. In addition, in the particular case of *B. braunii*, RSM can be used to lead a specific size of colony, trait that has been proved to have a role in downstream processes as large colonies increase the floatability of the algae (Furuhashi et al., 2016a,b). Improvement of medium composition and polyhydroxybutyrate production of different *B. braunii* strains has been done using RSM previously (Dayananda et al., 2005; Kavitha et al., 2016; Tran et al., 2010). However, this tool has not yet been applied to hydrocarbon and biomass production of *B. braunii* showa.

In this work, we studied the effect of the major culture parameters (light intensity, temperature and dilution rate (continuous operation) (De Vree et al., 2015; Ruiz et al., 2016)) in biomass and hydrocarbon productivities, and colony size of *B. braunii* showa. This allowed to formulate different predictive models that might be used to establish a cost efficient and sustainable hydrocarbon production based on *B. braunii* showa.

2. Material and methods

2.1. Growth medium and culture conditions

Botryococcus braunii showa (University of Bielefeld, Germany) was grown photo-autotrophically on modified Chu 13 medium (Dayananda et al., 2007) supplemented with KNO_3, Vitamin B_{12}, Thiamine and Biotin (12 mM, 0.1 μM, 3.26 μM, 0.11 μM respectively; final adjusted pH 7.2) under sterile conditions in lab-scale flat panel air-lift photobioreactors with an optical path of 14 mm and working volume of 0.4L and 0.03 m^2 illuminated surface area (AlgaeMist®) (Breuer et al., 2013). An airflow of 0.5vvm (0.2L min^{-1}) was set to mix the cultures. The pH (set point 7.2) was controlled by injection of CO_2 on demand. A continuous mode (dilution) during the light hours of the photoperiod (12D:12 N) was selected (Photo-Chemostat). Illumination was done in blocks, light intensity is immediately set to the setpoint after the night period. The dilution rates, temperature and light intensity at each experiment were determined by the experimental design (Table 1).

2.2. Analytical methods

Samples were always taken at the same time of the day (after the first 6 h of light). Biomass concentration was determined by dry weight (dw) as described by Vejrazka et al. (2011) in triplicate (technical replicates). The harvested volume was measured every day to check the dilution pump, which was calibrated at the beginning of each experiment. It was assumed that cultures achieved a steady state (μ = D) after harvesting 10 times the reactor's volume (4 L), followed by 10 days of stable dry weight determinations. Samples for determination of hydrocarbon content and colony size were taken in triplicate on 3 different days during the steady state phase (technical replicates). The samples were mixed with hexane (5:1v/v) was heated (80 °C) for 5 mins. After the solution was cooled to room temperature, the solution was mixed in a programmable rotator Multi Bio RS-24 (Vertical rotation 41/16; Reciprocal rotation 60/10; Vibro 5/5) for 10 min. Afterwards, the solution was centrifuged (rcf 2500*g*, 10 mins) to separate hydrocarbons in the top layer from the rest of the broth. The hydrocarbons were determined in a semi-quantitative way by GC-FID using a ZB-5Ms column (Phenomenex®) with He as carrier gas (flow 1 ml min^{-1}). The injection temperature was 350 °C, thus the temperature of the oven was 80 °C for 5 mins, followed by an increase of 5 °C min^{-1} until it reached 350 °C (for 10 mins). Squalene ($C_{30}H_{50}$) (Sigma-Aldrich S3626) was used in the calibration standards (2000-1000-500–200 mg L^{-1}) and solutions of alkane standard in toluene (C_{21}-C_{40}) as references (Alkane solution Sigma-Aldrich 04071). For this reason, the hydrocarbon content is referred to squalene-equivalent (sq_{eq}). Colony size was measured as volume weighted mean using a multi-sizer (Mastersizer 2000, Malvern Instruments) and assimilated to the diameter of an equivalent-sphere (μm) (García-Cubero et al., 2018a–d; Gouveia et al., 2019). All the experiments were done in biologic replicates (n = 3).

Table 1
Levels analysed for each factor (culture parameter).

Factor	Level		
	−1	0	+1
Temperature (°C)	15	22.5	30
Light intensity ($\mu mol_{ph}\ m^{-2}\ s^{-1}$)	600	1200	1800
Dilution rate (d^{-1})	0.1	0.2	0.3

2.3. Calculations

Once cultures achieved a steady state, biomass and hydrocarbon productivity were calculated according to Eqs. (1) and (2) respectively:

a) Biomass productivity

$$P(t) = C(t)*D \quad (1)$$

where P(t) is biomass productivity (g L^{-1} d^{-1}) at time t, C(t) is biomass concentration (g L^{-1}) and D is the dilution rate over 24 h (d^{-1}).

b) Hydrocarbon productivity

$$Phc(t) = Chc(t)*D \quad (2)$$

where Phc(t) is the hydrocarbon productivity (mg L^{-1} d^{-1}) at time t, C(t) is hydrocarbon concentration (mg L^{-1}) and D is the dilution rate over 24 h (d^{-1}), so corrected for 12 h of dark period with no dilution.

2.4. Experimental design, statistical analysis and response surface methodology (RSM)

A Box-Benhken experimental design was used (3 factors; 3 levels each, Table 1) to generate RSM models for each variable (biomass and hydrocarbon productivities and colony size). The levels tested for each factor were selected based on information inferred from literature (Bazaes et al., 2012; Cabanelas et al., 2015; Sakamoto et al., 2012; Yoshimura et al., 2013). A set of 15 experiments with different combinations of each factor were performed (45 in total because of biologic replicates) (Table 2). The statistical analysis of the results was done using the software Statgraphics® Centurion XVI. The data were fit to the following polynomic regression Eq. (3) (second order):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (3)$$

where Y is the response of each variable (biomass and hydrocarbon concentrations and colony size), β_0, β_i, β_{ii}, β_{ij} are constant coefficients and X_i, X_j are the independent variables (light intensity, temperature and dilution rate). The polynomic equations were used for plotting the response surface of biomass and hydrocarbon productivities and colony size and to determine the combination of factors for optimizing the variable response. Overall desirability index *($0 \leq D_i \leq 1$; 0 = low desirability; 1 = high desirability)* was used for the multiple response optimization. Differences between and among groups were highlighted using One-way ANOVA. Pearson correlation coefficient (r) was used to test the linear correlation between variables. Durbin-Watson (*DW*) test was used to detect autocorrelation in the residuals. The Pareto chart was used to determine the interactions and importance of the factors. The significance level of all the tests was 5%. The culture conditions predicted by the models to maximize each specific outcome (biomass and hydrocarbon productivities and colony size) or all their possible

Table 2
Set of the experiments and results (biomass concentration and productivity, hydrocarbon concentration and productivity, colony size) of the three-levels Box-Benhken experimental design once a steady state was achieved in continuous cultures.

Experiment	Temperature (°C)	Light intensity ($\mu mol_{ph}\ m^{-2}\ s^{-1}$)	Dilution rate (d^{-1})	Biomass concentration (g L^{-1})	Biomass productivity (g L^{-1} d^{-1})	Hydrocarbon concentration (mg L^{-1})	Hydrocarbon productivity (mg L^{-1} d^{-1})	Colony size (μm)
1	22.5	1200	0.2	2.10 ± 0.03	0.42 ± 0.00	63.66 ± 0.07	12.73 ± 0.01	138.013 ± 2.1
2	30	1200	0.3	1.67 ± 0.08	0.50 ± 0.02	17.62 ± 0.03	5.29 ± 0.00	232.5 ± 1.3
3	30	1800	0.2	2.45 ± 0.09	0.49 ± 0.02	29.82 ± 0.05	5.96 ± 0.01	232.64 ± 0.9
4	22.5	600	0.3	0.33 ± 0.00	0.10 ± 0.00	34.20 ± 0.1	10.26 ± 0.03	157.47 ± 3.2
5	22.5	1800	0.1	11.8 ± 0.10	1.18 ± 0.01	177.6 ± 0.08	17.76 ± 0.00	103.19 ± 1.7
6	22.5	1200	0.2	2.15 ± 0.02	0.43 ± 0.00	55.22 ± 0.03	11.04 ± 0.00	152.22 ± 2.5
7	22.5	1800	0.3	0.43 ± 0.09	0.13 ± 0.03	8.56 ± 0.09	2.57 ± 0.03	283.21 ± 3.6
8	15	1200	0.3	0.07 ± 0.01	0.02 ± 0.00	49.87 ± 0.1	14.96 ± 0.03	108 ± 2.1
9	30	1200	0.1	10.3 ± 0.15	1.03 ± 0.01	40 ± 0.02	4 ± 0.00	119.36 ± 1.9
10	15	1200	0.1	0.5 ± 0.01	0.05 ± 0.00	18 ± 0.03	1.8 ± 0.00	109 ± 1.4
11	30	600	0.2	0.2 ± 0.00	0.04 ± 0.00	11.6 ± 0.00	2.32 ± 0.00	126.17 ± 2.1
12	15	600	0.2	0	0	0	0	0
13	15	1800	0.2	0.15 ± 0.01	0.03 ± 0.00	51.53 ± 0.01	10.30 ± 0.00	50 ± 0.8
14	22.5	600	0.1	0	0	0	0	0
15	22.5	1200	0.2	1.9 ± 0.10	0.38 ± 0.02	48.69 ± 0.05	9.74 ± 0.01	180 ± 4.1

simultaneous combinations (biomass productivity-hydrocarbon productivity-colony size; biomass productivity-hydrocarbon productivity; biomass productivity-colony size; hydrocarbon productivity-colony size) were later validated empirically.

3. Results and discussion

Establishing an industrial production of hydrocarbons by *B. braunii* cultures, requires knowledge on optimal cultivation conditions that enhance productivity. Although some efforts have been done recently, most of these works only addresses culture medium optimization and extraction procedures, with limited information on operational culture procedures (Dayananda et al., 2007, 2005; Sim et al., 2001). RSM can provide a better strategy to optimize a process or the yield of one specific product (Marudhupandi et al., 2016). For instance, RSM approaches have been applied with success previously in *B. braunii* Kütz for polyhydroxybutyrate production (Kavitha et al., 2016). In our work, the major goal was to determine the culture conditions to optimize biomass and hydrocarbon productivities respectively in continuous cultures at steady state.

Although our previous publications showed the feasibility of continuous cultivation of *B. braunii* (García-Cubero et al., 2018a–d), this has seldom been done and a few examples have been reported to date (García-Cubero et al., 2018b; Jin et al., 2016). We selected photo-chemostat operations as it offers several advantages for modelling the microalgal behaviour (García-Cubero et al., 2018b). Once a steady state is achieved, the variation of the microalgal output (biomass and/or hydrocarbon productivities and colony size) is motivated directly by the effect of changing a single culture parameter (light, temperature or dilution rate in our study). Hence, this operational mode renders easier the modelling process and provides a better quality in the results (García-Cubero et al., 2017). The selection of culture parameters was done on basis of those reported as being the more critical at large scale (De Vree et al., 2015) with a direct effect in the productivity and therefore production costs (Ruiz et al., 2016). In indoor lab scale photo-bioreactors, we simulated a broad range of outdoors and large-scale conditions reported for *B. braunii* cultures in literature (Bazaes et al., 2012; Cabanelas et al., 2015; Sakamoto et al., 2012; Yoshimura et al., 2013), in order to have the maximum applicability of our results for industrial production.

3.1. Modelling biomass productivity at steady state in continuous cultures of B. braunii showa

From the experimental data obtained for biomass concentration, the biomass productivity was determined in each culture condition assayed (Table 2). Thus, it was possible to model biomass productivity at steady state. Temperature and dilution rate showed a statistical influence in biomass productivity (*p-value* < *0.05*). No correlation in the residuals were observed (*DW* > *0.5*). Temperature exhibited a positive effect on biomass productivity (when temperature increased, biomass productivity did too); contrary, dilution rate displayed a negative effect on biomass concentration (when dilution rate increased, biomass concentration decreased) (Fig. 1A). The data were fitted to the equation (3) with the following regression coefficients to obtain the quadratic eq. (4) ($R^2 = 0.88$):

$$Y_1 = -2.293 + 0.169X_1 + 0.001X_2 - 1.162X_3 - 0.0029X_1^2 + 0.00002X_1X_2 - 0.166X_1X_3 - 0.0000003X_2^2 - 0.003X_2X_3 + 15.375X_3^2 \quad (4)$$

where Y_1 is biomass productivity (g L^{-1} d^{-1}) once a steady state is achieved, X_1 is temperature (°C), X_2 is light intensity ($\mu mol_{ph}\ m^{-2}\ s^{-1}$) and X_3 is dilution rate (d^{-1}). The model predicts a maximum biomass productivity of 1.35 g L^{-1} d^{-1} under the following culture conditions: temperature 30 °C, light intensity 1800$\mu mol_{ph}\ m^{-2}\ s^{-1}$ and dilution rate 0.1 d^{-1} (Fig. 2A). The data obtained empirically under the same conditions for model validation was 1.33 g L^{-1} d^{-1}. (Table 3).

B. braunii is characterized by presenting a low growth (Gouveia et al., 2017). Our results indicate that it is feasible to cultivate *B. braunii* showa in photo-chemostat in the range of dilution rates from 0.1 to 0.3 d^{-1}. Higher dilution rates were not tested as according to literature, this microalga is not characterized by high growth rates (Cabanelas et al., 2015) and therefore they can lead to a wash out of the cultures. Cultivation in a continuous way might be the cornerstone for establishing a continuous hydrocarbon production based on *B. braunii*. Our results also reveal the existence of a link between biomass and hydrocarbon content

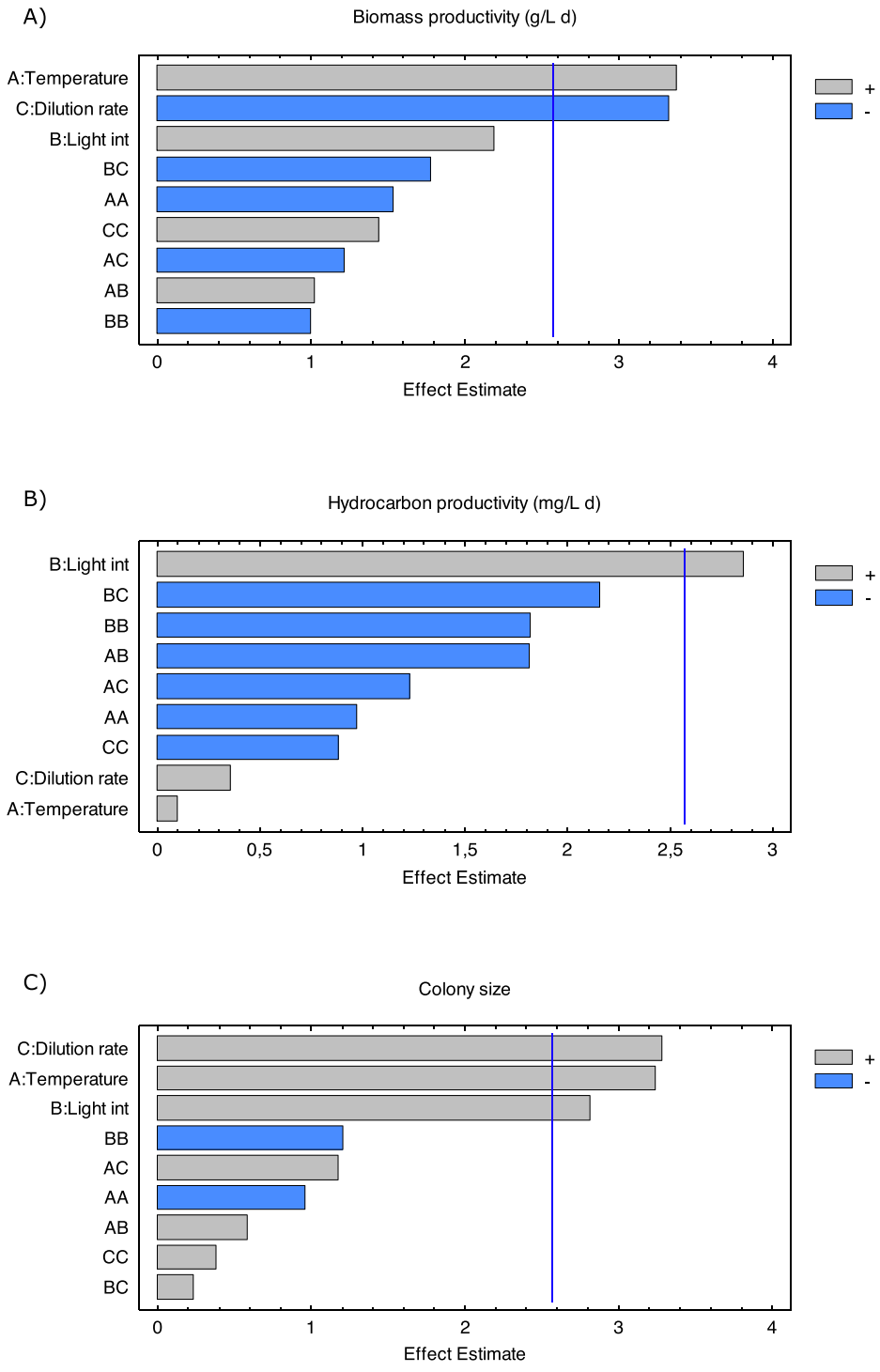

Fig. 1. Pareto charts for the standardized effects of the different factors individually and combined on biomass productivity at steady state in continuous cultures (A), hydrocarbon productivity at steady state in continuous cultures (B) and colony size at steady state in continuous cultures (C) (Legend: A = Temperature; B = Light intensity; C = Dilution rate; AB combined effect of Temperature-Light intensity; AC = Combined effect of Temperature-Dilution rate; BC = combined effect of Light intensity-Dilution rate).

at steady state, complementing the linear relationship already established in *B. braunii* between intracellular lipids and extracellular hydrocarbons (Jin et al., 2016). Likewise, our data indicates that the biomass productivity is negatively affected by high dilution rates and positively by temperature. In photo-chemostat mode, the increase of dilution rate leads to higher biomass productivities (García-Cubero et al., 2017). Nevertheless, this correlation might changes at really high dilution rates leading to a reduction of productivity. High dilution rates lead to higher I_{av} per cell (García-Cubero et al., 2017) and photoinhibition and oxidative stress can occur (Khorobrykh et al., 2020; Ugya et al., 2020), leading to a decrease in biomass productivity, as observed with *B. braunii* showa in this work. In addition to light, our results show that the increase of temperature exhibits a notable and positive effect over *B. braunii* showa. The optimal temperature determined in this study for achieving the highest biomass productivity is 30 °C. This feature makes *B. braunii* showa a strain suitable to be cultivated in a large range of location worldwide as it has been already proposed for other microalgal species (Béchet et al., 2017; Han et al., 2016; Ras et al., 2013). Also, this trait might allow to be more tolerant regarding temperature control for an industrial exploitation of *B. braunii*. The maximum values for biomass productivity predicted by our models and, later validated experimentally, range from 1.1 g L^{-1} d^{-1} for the multiple parameter

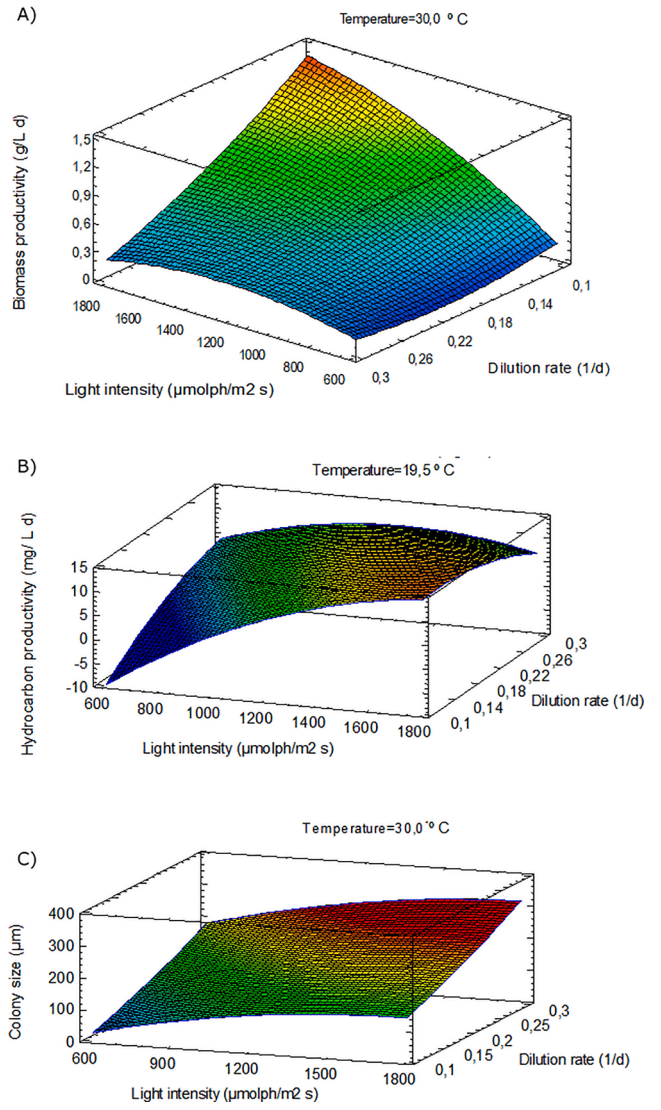

Fig. 2. Response surface plot for A) biomass productivity at steady state in continuous cultures when temperature is controlled at 30 °C; B) hydrocarbon productivity at steady state when temperature is controlled at 19.5 °C; and C) colony size at steady state when temperature is controlled at 30 °C. (Footnote: decimals are noted by commas in the figure).

optimization to 1.3 g L^{-1} d^{-1} as the highest for the independent optimization. These values are in line with the results under heterotrophic conditions published by Wan et al (Wan et al., 2019) and higher than others reported in literature for different *B. braunii* strains (Bazaes et al., 2012; Gouveia et al., 2019; Gouveia et al., 2017).

3.2. Modelling the hydrocarbon productivity at steady state in continuous cultures of B. braunii showa

In similarity to biomass productivity, the effect of different combinations of independent factors (light intensity, temperature and dilution rate) on hydrocarbon productivity in continuous cultures at steady state was studied (Table 2). The analysis of the data showed that only the parameter light intensity showed a statistical influence in hydrocarbon productivity (*p-value* < *0.05*). No correlation in the residuals were observed (*DW* > *0.5*). Light intensity exhibited a positive effect on hydrocarbon concentration (when light intensity increased, hydrocarbon concentration did too) (Fig. 1B). The data were fitted to equation (3) with the following regression coefficients to obtain the quadratic Eq. (5) ($R^2 = 0.82$):

$$Y_2 = -114.337 + 3.936X_1 + 0.078X_2 + 287.924X_3 - 0.043X_1^2 - 0.001X_1X_2 - 3.96X_1X_3 - 0.00001X_2^2 - 0.0865X_2X_3 - 221.692X_3^2 \quad (5)$$

where Y_2 is hydrocarbon productivity (mg L^{-1} d^{-1}) once a steady state is achieved, X_1 is temperature (°C), X_2 is light intensity (μmol_{ph} m^{-2} s^{-1}) and X_3 is dilution rate (d^{-1}). The model predicts a maximum hydrocarbon productivity of 14.1 mg L^{-1} d^{-1} under the following culture conditions: temperature 19.5 °C, light intensity 1800μmol_{ph} m^{-2} s^{-1} and dilution rate 0.12 d^{-1} (Fig. 2B). The data obtained empirically under the same conditions for model validation was 15.8 mg L^{-1} d^{-1} (Table 3). In addition, the empirical data showed a relationship between biomass and hydrocarbon concentration was determined (*r = 0.73; p-value* < *0.05*)

These results show that high light intensity positively affects hydrocarbon productivity. However, Wang et al (Wang et al., 2019) published that *B. braunii* IPE 001 decreased its content (reduction of up to 15%) in long-chain hydrocarbons at high light intensity conditions. This divergence in results might be based on the fact that their experimental setting was in batch conditions. In batch cultivations, all culture conditions vary with time including the average light per cell (I_{av}) which decreased with increasing biomass concentration. Therefore, we highlight the importance of working under steady state conditions when modelling. Other culture strategies might be proposed for increasing the hydrocarbon productivity in *B. braunii* like N-starvation in oleaginous microalgae species (Del Río et al., 2017; Del Río et al., 2015), nutrient stress such as P or K (Manchanda et al., 2019) or high CO_2 environments (Cheng et al., 2020; García-Cubero et al., 2018c). In any case, a deep overview of the cellular biochemical pathways of *B. braunii* is recommended to improve hydrocarbon productivity (Blifernez-Klassen et al., 2018; Wichmann et al., 2020). Our model suggests a maximal hydrocarbon productivity of 14 mg L^{-1} d^{-1}. The experimental validation of the model resulted in 12% higher productivity (up to 15.8 mg L^{-1} d^{-1}). These values might be considered as low hydrocarbon productivities when compared to other publications, where it was achieved a hydrocarbon content of up to 35% dwc (Wan et al., 2019; Wang et al., 2013). Nevertheless in those publications, the hydrocarbon determinations were performed under severe hydrolytic/extraction conditions, determining therefore both intra and extracellular hydrocarbons, but avoiding the survival of the cell. Oppositely, we performed mild hydrocarbon extractions by using hexane and low temperatures to ensure a higher survival rate of cells. In this way, our models are susceptible of being applied in an industrial scenario for non-destructive applications (also known as milking) (R. García-Cubero et al., 2018d; Jackson et al., 2020, 2019).

Table 3
Maximum values predicted and experimental data once a steady state was achieved in continuous cultures for each variable under the optimal culture conditions.

Variable	Factor			Maximum value predicted	Experimental value
	Temperature	Light	Dilution rate		
Biomass productivity (g L^{-1} d^{-1})	30	1800	0.1	1.35	1.33 ± 0.2
Hydrocarbon productivity (mg L^{-1} d^{-1})	19.5	1800	0.12	14.1	15.8 ± 0.08
Colony size (µm)	30	1800	0.3	320	317.5 ± 1.2

3.3. Modelling the colony size at steady state in continuous cultures of *B. braunii showa*

Last, the effect of the different combinations of the independent factors (light intensity, temperature and dilution rate) on the size of the colonies was studied (Table 2). The statistical analysis of the results showed that all factors have an influence on the size of the colonies (*p-value* < 0.05). No correlation in the residuals were observed ($DW > 0.5$). Dilution rate, temperature and light intensity all exhibited a positive effect on colony size (when dilution rate, temperature and/or light intensity increased, colony size did as well) (Fig. 1C). The data were fitted to equation (3) with the following regression coefficients to obtain the quadratic Eq. (6) ($R^2 = 0.87$):

$$Y_3 = -240.356 + 15.338X_1 + 0.193X_2 - 791.476X_3 - 0.429X_1^2 + 0.003X_1X_2 + 38.046X_1X_3 - 0.00008X_2^2 + 0.0939X_2X_3 + 961.783X_3^2 \quad (6)$$

where Y_3 is colony size (μm) once a steady state is achieved, X_1 is temperature (°C), X_2 is light intensity ($\mu mol_{ph}\ m^{-2}\ s^{-1}$) and X_3 is dilution rate (d^{-1}). The model predicts the largest size of colonies (320 μm) under the following culture conditions: temperature 30 °C, light intensity 1800$\mu mol_{ph}\ m^{-2}\ s^{-1}$ and dilution rate 0.3 d^{-1} (Fig. 2C). The data obtained empirically under the same conditions was 317.5 μm (Table 3).

The causes for variation in colony size of *B. braunii* are still unknown because most of the research done to date has been focused on improving disruptive methods of the colonies in order to enhance the hydrocarbon extraction (Furuhashi et al., 2016a,b; Tsutsumi et al., 2018). Research is needed to understand the biology behind variations in the size of the colonies. Nevertheless, some observations have been done along the years. Among them, salinity, light intensity or temperature have been proved that influence the colonial size (García-Cubero et al., 2018a; Rao et al., 2007; Van Den Berg et al., 2019; Zhang and Kojima, 1998). In addition, it has been published a correlation between size of the colonies and hydrocarbons extractability in *B. braunii*, showing that the smaller the colony size is, the better hydrocarbon extraction occurs (Tsutsumi et al., 2018). Therefore, the colony size might play a role in the downstream process of *B. braunii*. Our results show that the combination of high light intensity, high dilution rate and high temperature leads to the largest colonies of *B. braunii* showa, with an average diameter of 320 μm. This result is in line with Berg et al (Van Den Berg et al., 2019), where similar sizes of colonies (300–400 μm) were found during light acclimation experiments. Although the colony size is influenced by culture conditions, it is also strain specific. In recent works with *B. braunii* CCALA778, we showed that colony size varied between 33 and 108 μm (R. García-Cubero et al., 2018a). Gouveia et al reported an average colony size values of 80 μm with the same strain (Gouveia et al., 2019). The colony size of *B. braunii* showa is considered in this work as it may affect hydrocarbon milking-like processes. It has been demonstrated that the size of the colony might affect the availability of hydrocarbon extraction in non-disruptive approaches (Jackson et al., 2020; Mehta et al., 2019).

3.4. Optimization of multiple responses at steady state in continuous cultures of *B. braunii showa*

Once RSM models were made for each single variable (biomass productivity, hydrocarbon productivity and colony size), the combination of factors (culture parameters) that lead to a simultaneous maximum yield of the different variables was determined. Using the desirability index plot (Fig. 3), it was possible to establish the culture conditions that lead for a multiple response optimization at steady state in continuous cultures. This tool is used when is sought to combine simultaneously the maximum possible yields of the different outputs. In this work, we focused in to find the culture conditions that lead to a simultaneous maximization of all possible combinations of the culture outputs (biomass and hydrocarbon productivities and colony size). The

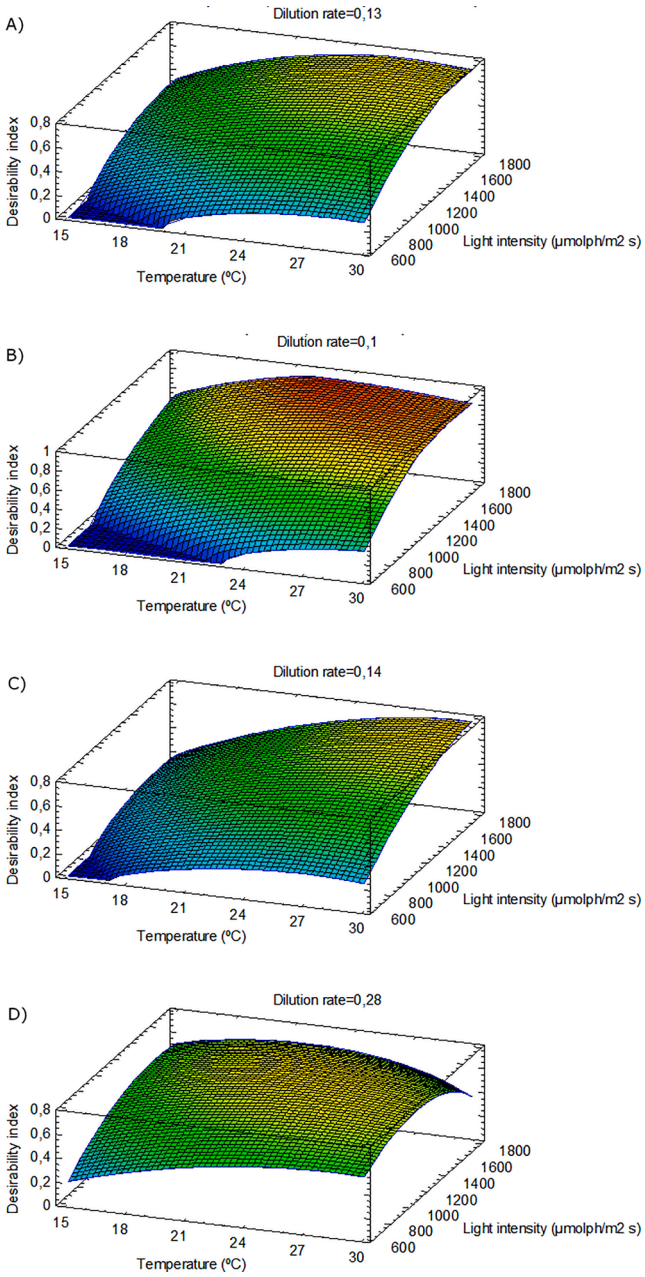

Fig. 3. Desirability index surface plot for the multiple response optimization at steady state: A) biomass productivity-hydrocarbon productivity-colony size (Dilution rate = 0.13 d^{-1}), B) biomass productivity-hydrocarbon productivity (Dilution rate = 0.1 d^{-1}), C) biomass productivity-colony size (Dilution rate = 0.14 d^{-1}), and D) hydrocarbon productivity-colony size (Dilution rate = 0.28 d^{-1}). (Footnote: decimals are noted by commas in the figure).

maximum partial values for each variable combination at the high desirability index (D_i) are described in Table 4. Henceforth, it was possible to optimize biomass productivity, hydrocarbon productivity and colony size responses simultaneously resulting in the following predicted maximum values: 1.0 g $L^{-1}\ d^{-1}$; 12.05 mg $L^{-1}\ d^{-1}$; 154.3.3 μm respectively, under the following culture conditions: temperature 26.7 °C, light intensity 1695$\mu mol_{ph}\ m^{-2}\ s^{-1}$ and dilution rate 0.13d^{-1} (D_i = 0.75). The empirical data obtained for this combination of factors were 1.1 g biomass $L^{-1}\ d^{-1}$; 12.9 mg hydrocarbon $L^{-1}\ d^{-1}$ and a colony size of 154 μm. The maximum values predicted for biomass and hydrocarbon productivities at steady state after a simultaneous optimization are 1.0 g $L^{-1}\ d^{-1}$ and 13.9 mg $L^{-1}\ d^{-1}$ respectively under the following culture conditions: temperature 21.9 °C, light intensity

Table 4
Maximum values predicted and experimental data once a steady state was achieved in continuous cultures for each variable in the different combinations of factors for multiple response optimization at the highest desirability index (D_i).

Variable Combinations	D_i	Factor			Maximum value predicted			Experimental value		
		Temperature	Light	Dilution rate	Biomass productivity (g L^{-1} d^{-1})	Hydrocarbon productivity (mg L^{-1} d^{-1})	Colony size (μm)	Biomass productivity (g L^{-1} d^{-1})	Hydrocarbon productivity (mg L^{-1} d^{-1})	Colony size (μm)
Biomass productivity-Hydrocarbon productivity-Colony size	0.75	26.7	1695	0.13	1.0	12.05	156.8	1.1 ± 0.6	12.9 ± 0.1	154 ± 3.3
Biomass productivity-Hydrocarbon productivity	0.93	21.9	1800	0.1	1.0	13.9	–	1.1 ± 0.3	17.1 ± 0.1	–
Biomass productivity-Colony size	0.78	30	1800	0.14	1.0	–	172.8	0.9 ± 0.5		177.1 ± 0.9
Hydrocarbon productivity-Colony size	0.74	24.2	1336	0.28	–	9.0	240		9.3 ± 0.5	234.4 ± 4.1

1800μmol$_{ph}$ m^{-2} s^{-1} and dilution rate 0.1d^{-1} ($D_i = 0.93$). The empirical data obtained for this combination of factors were 1.1 g biomass L^{-1} d^{-1} and 17.1 mg hydrocarbon L^{-} d^{-1}. The maximum values predicted for biomass productivity and colony size are 1.0 g L^{-1} d^{-1} and 172.8 μm respectively under the following culture conditions: temperature 30 °C, light intensity 1800μmol$_{ph}$ m^{-2} s^{-1} and dilution rate 0.14 d^{-1} (D_i = 0.78). The empirical data obtained for this combination of factors were 0.9 ± 0.5 g biomass L^{-1} d^{-1} and 177.1 ± 0.9 μm for colony size. Last, the simultaneous maximum values predicted for hydrocarbon productivity and colony size are 9.0 mg L^{-1} d^{-1} and 240 μm under the following culture conditions: temperature 24.2 °C, light intensity 1336μmol$_{ph}$ m^{-2} s^{-1} and dilution rate 0.28 d^{-1} ($D_i = 0.74$). The empirical data obtained for this combination of factors were 9.3 mg hydrocarbon L^{-1} d^{-1} and 234.4 μm for colony size.

RSM has shown to be an useful tool to improve microalgal biotechnology processes such as polyhydroxybutyrate production, harvesting or medium optimization of *B. braunii* (Dayananda et al., 2005; Kavitha et al., 2016; Zheng et al., 2012). The models presented in the current study permit yield optimization of either one single parameter and their possible combinations. By performing a simultaneous optimization of biomass productivity, hydrocarbon productivity and colony size, the economic gap that impedes an hydrocarbon industry based on *B. braunii* cultures might be overcome.

4. Conclusions

In this work, Response Surface Methodology (RSM) was used via Box-Behnkem experimental design to formulate three mathematical models based on different cultivation parameters (inputs: light intensity, temperature and dilution rate) to describe biomass and hydrocarbon productivities and colony size (outputs) in continuous cultures of *B. braunii* showa. Hence, it was possible to maximize the response for each single output as well as to optimize the combined response of every possible combination of those outputs. These models are a useful tool to optimize the yield of *B. braunii* showa under different culture conditions, being the cornerstone for process optimization towards future large-scale operations.

Author's contribution

Rafael García-Cubero: Conception, experimental design and set-up, experimental work, analysis and interpretation of the data, statistical analysis of the data and drafting of the manuscript. **Dorinde M.M. Kleinegris:** Analysis and interpretation of the data, critical revision of the manuscript and final approval of the manuscript. **María J. Barbosa:** Obtaining of funding, analysis and interpretation of the data, critical revision and final approval of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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