Extracellular hydrocarbon and intracellular lipid accumulation are related to nutrient-sufficient conditions in pH-controlled chemostat cultures of the microalga *Botryococcus braunii* SAG 30.81

Jian Jin, Catherine Dupré, Jack Legrand, Dominique Grizeau*

LUNAM Université, CNRS, GEPEA, Université de Nantes, UMR 6144, CRTT, 37 bd de l'Université, BP 406, 44602 Saint-Nazaire Cedex, France

*Corresponding author

Highlights:

1. Effect of pH in continuous cultures studied in successive steady-states.
2. Hydrocarbon productivities in *B. braunii* cultures were pH-dependent.
3. Increase of biomass and hydrocarbon productivities induced by 3N6P-AF-6 medium.
4. Extracellular hydrocarbon content appears related to total intracellular lipid content.
5. Hydrocarbon productivities were related to steady-state DIC speciation.

Abstract

Here we investigate the effect of environmental pH on biomass and hydrocarbon productivity in photoautotrophic cultures of *Botryococcus braunii* SAG 30.81 (race A). Successive steady-states of continuous cultures were used to study the effects of pH controlled by carbon dioxide feeds in a closed photobioreactor. At a fixed dilution rate of 0.2 d^{-1} , hydrocarbon productivity was found to be pH-dependent in the range 5.5 to 8.0, with an optimum at pH 6.5. pH variations from pH 6.0 to pH 8.0 had little effect on biomass productivity, nutrient requirement for biomass production, photosynthetic yield and pigment ratios, whereas acidic pH 5.5 conditions induced a slight decrease in biomass and hydrocarbon productivities as well as stressing the cells. The pH effect occurred even after a threefold increase in nitrate and

phosphate concentrations in the feed culture medium, resulting in biomass productivity up to 8.9 g m⁻² d⁻¹ and hydrocarbon productivity up to 0.5 g m⁻² d⁻¹. Dissolved inorganic carbon data suggest that a maximum hydrocarbon content of 5.7% dry weight was reached at and above 1 mmol L⁻¹ dissolved CO₂ and that hydrocarbon production was linearly related to CO₂ up to 2.5 mmol L⁻¹, whatever the N and P enrichment of AF-6 culture medium.

Keywords: *Botryococcus*; pH effect; continuous culture; dissolved CO₂; hydrocarbon; lipids

1. Introduction

Microalgal biofuels are gaining increasing attention as an alternative energy source. Among microalgae, *Botryococcus braunii* strains have emerged as the most promising potential renewable resource due to the huge amount of extracellular hydrocarbon (HC) they produce, which can reach 75% of dry cell weight [1]. Strains of *B. braunii* are classified into four races, A, B, L and S, according to nature of the hydrocarbons they produce[2,3]. Hydrocarbon oils of *B. braunii* make a suitable feedstock material for hydrocracking to transport fuels [1]. CO₂ supplementation improves growth and hydrocarbon oil production in *B. brauni* cultures [4–6], which means that *B. braunii*, like other photosynthetic microalgae, can bio-mitigate CO₂ from industrial exhaust gases to help reduce greenhouse gas emissions [7].

However, successful mitigation of CO₂ and improved production of hydrocarbon oils depend on CO₂ tolerance and the associated decrease in culture pH. Environmental pH is one of the key operational parameters driving algal growth, as it is known to affect photosynthetic activity, availability of inorganic nutrients through carbon and metal speciation, potential enzyme activities within cell walls, and oil production rates [8]. Low pH could influence intracellular pH and thereby modify enzyme activities [9], and acidification of the chloroplast is known to cause inhibition of photosynthesis [10]. This makes it vital to achieve appropriate

pH control to improve the productivity of microalgal cultures, as demonstrated for the triacylglycerol-accumulating microalgae *Nannochloropsis salina* [8]. Nevertheless, few studies have investigated the influence of pH on *B. braunii* cultivation [4,6,11–13]. It has been reported that maximal production of both biomass and hydrocarbon could be achieved at a pH around 6.3 [4] or at pH 7.5 [12], whereas another study showed higher biomass could be obtained at pH 6.0 but that hydrocarbon content showed little response to pH in the range 6.0 to 8.5 [11]. Other studies have demonstrated pH effects on pigments [13] and growth rate [6] but did not address pH effect on hydrocarbon production. It appears that *B. braunii* race A are generally cultivated at around pH 7.5 [14] whereas *B. braunii* race B strains are cultivated at around pH 6.5 [6]. However, in these studies, culture pH was only adjusted before cultivation [11,12,15] or monitored [4,6], never held constant by either automatic pH regulation or stoichiometrically-balanced growth media as was done for triacylglycerol-accumulating microalgae [16]. Moreover, these experiments were often carried out in batch cultures for relatively short durations. Batch cultures are non-ideal model systems as they carry a number of inherent drawbacks for quantitative studies on the effects of environmental parameters on microalgal culture performances. Chemostat cultivation makes a far better tool for studies on quantitative aspects under controlled cultivation conditions.

We therefore investigated the effect of pH on biomass and hydrocarbon productivities in continuous controlled cultures of a *B. braunii* race A strain. Chemostat cultures were used at a fixed dilution rate to determine the relation between culture performances at steady-state, and environmental pH was regulated by automatic CO₂ injection into the cell suspensions. This is the first time this approach has been applied to investigate the behavior of *B. braunii* cultures. A change in nitrate and phosphate nutrient composition of the feeding medium was used to compare the effects of increasing residual nitrate and phosphate concentrations in the cultures on biomass and hydrocarbon productivities according to pH setpoints.

2. Materials and Methods

2.1. Strain and medium

Experiments were conducted with the strain *B. braunii* Kützing SAG 30.81 obtained from the Culture Collection of Algae at Gottingen University (Sammlung von Algenkulturen, SAG). This strain is classified as a race A strain as identified by their characteristic hydrocarbons using GC-MS [11,15,17] and further confirmed by comparison with a typical race A strain LB 572 using random amplified polymorphic DNA analysis [18]. The strain was kept on buffered AF-6 medium with 2-(N-morpholino) ethanesulfonic acid (MES, 2 mmol L⁻¹) agar plates and sub-cultured in a 300 mL column. The subcultures were regularly checked for absence of bacteria and fungi by controls on Plate Count Agar (Fluka).

The modified AF-6 medium was used for preliminary cultivations [19]. Final concentration contains NaNO₃ 140 mg L⁻¹, NH₄NO₃ 22 mg L⁻¹, MgSO₄·7H₂O 30 mg L⁻¹, KH₂PO₄ 10 mg L⁻¹, K₂HPO₄ 5 mg L⁻¹, CaCl₂·2H₂O 10 mg L⁻¹, FeCl₃·6H₂O 1 mg L⁻¹, Fe-citrate 2 mg L⁻¹, citric acid 2 mg L⁻¹, Na₂EDTA·2H₂O 8 mg L⁻¹, micronutrients (MnCl₂·4H₂O 200 µg L⁻¹, ZnSO₄·7H₂O 40 µg L⁻¹, CoCl₂·6H₂O 8 µg L⁻¹, Na₂MoO₄·2H₂O 20 µg L⁻¹, NH₄VO₃ 1 µg L⁻¹, H₂SeO₃ 5 µg L⁻¹) and vitamins (biotin 2 µg L⁻¹, thiamine HCl 10 µg L⁻¹, vitamin B₆ 0.1 µg L⁻¹, vitamin B₁₂ 1 µg L⁻¹). Before autoclaving, pH was adjusted to 6.6 using KOH. For a first set of experiments, feed medium for continuous cultures was AF-6 medium without MES buffer but enriched with two-fold phosphate concentration (2P-AF-6) in order to avoid the phosphate limitation observed during preliminary batch cultures. A second set of experiments was done with a 3-fold sodium nitrate 6-fold phosphate concentration-enriched medium (3N6P-AF-6, without MES) to improve biomass production.

2.2. Culture system and experimental set-up

2.2.1 Batch culture

Experiments were conducted in 300-mL bubble columns. Aeration was provided by injecting 0.2 μm -filtered air at the bottom of the columns at a rate of 0.1 vvm (volume per volume per minute). Continuous photon irradiance was provided by cool-white fluorescent lamps (Philipps, Master TLD 18W) and its intensity-adjusted to 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ as photosynthetic photon flux density (PPFD) corresponding to photosynthetically active radiation (PAR). Measurements were performed at the surface of the columns using a Li-Cor light meter (LI-250A). Batch cultures were maintained at a temperature of $23 \pm 2^\circ\text{C}$.

2.2.2 Continuous culture

The torus photobioreactor (PBR) system (Fig. 1), as described by Pruvost et al. [20], was sterilized by vapor stream. Working volume (V_w) was 1.4 L with a light incident area A_l of 0.035 m^2 and a A_l/V_w ratio of 25 m^{-1} . Speed of the axial flow impellers was 200 rpm. The chemostat cultures were fed with a medium flow rate (F) of 0.28 L d^{-1} , corresponding to a dilution rate $D = F/V_w$ of 0.2 d^{-1} .

Temperature was regulated at $23 \pm 1^\circ\text{C}$. The photobioreactor was continuously illuminated at a photon flux density of 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in the 400–700 nm waveband (PAR). Air flow rate was 450 mL min^{-1} corresponding to an aeration rate of 0.32 vvm. pH was controlled by automatic CO_2 injection (Mettler Toledo M200 pH-controller) for values set sequentially at 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, after reaching a steady-state at the operating pH (mean duration of 10 days).

Fig. 1. Sketch map of the torus PBR system. At sampling, stopper 1 is open and stopper 2 is closed. At other times, stopper 1 is closed and stopper 2 is open. Sampling tube and harvest bottle were autoclaved (121°C, 25 min) before being connected. Controller 1 (CO₂ flow controller) will be open when the pH value detected online is higher than the setpoint pH value, or closed when the pH value is lower than setpoint pH. Controller 2 (air flow controller) is open all the time at a constant air flow rate (450 mL min⁻¹), with culture pH controlled at the setpoint pH ± 0.1 pH unit.

2.3. Analytical methods

2.3.1 Biomass concentration and productivity

Biomass concentration (X , g L⁻¹ or g m⁻²) was determined by dry weight (dw) measurement as described in [21]. Amounts of samples (10 mL) were filtered over pre-weighed Whatman GF/F-grade glass fiber filters and washed with distilled water. The filters were dried at 105°C for 24 h in aluminum trays, cooled down in a desiccator, and dry weight was measured. Biomass productivity P_x (g L⁻¹ d⁻¹ or g m⁻² d⁻¹) was calculated using the formula:

$$P_x = D \cdot X, (\text{g L}^{-1} \text{ d}^{-1} \text{ or g m}^{-2} \text{ d}^{-1})$$

2.3.2 Pigment contents

The samples (2 mL) were first centrifuged (12,100 g, 10 min, room temperature) then the cells were suspended in 2 mL methanol solution (90 % v/v) by sonication and vortexing (18,000 rpm) followed by incubation in the dark for 2 h. The supernatant was clarified by centrifugation (12,100 g, 10 min, room temperature) and the optical densities at 470 nm, 652.4 nm, 665.2 nm and 750 nm (, Perkin Elmer Lambda 2S spectrophotometer) were used to determine the concentrations of chlorophylls (Chl, including chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) and carotenoids (Car) using the spectrophotometric equations of Lichtenthaler & Buschmann [22] and those below:

$$\text{Chl } a = (OD_{665.2nm} - OD_{750nm}) \cdot 16.82 - (OD_{652.4nm} - OD_{750nm}) \cdot 9.28, (\text{mg L}^{-1})$$

$$\text{Chl } b = (OD_{652.4nm} - OD_{750nm}) \cdot 36.92 - (OD_{665.2nm} - OD_{750nm}) \cdot 16.54, (\text{mg L}^{-1})$$

$$\text{Car} = \frac{1000 \cdot (OD_{470nm} - OD_{750nm}) - 1.91 \cdot \text{Chl } a - 95.15 \cdot \text{Chl } b}{225}, (\text{mg L}^{-1})$$

2.3.3 Photosynthetic activities

In vivo chlorophyll fluorescence of photosystem II (PSII) was measured with a Water-PAM (Walz GmbH, Germany). Samples from steady-state cultures were dark-adapted for 20 min to determine F_o then exposed to a saturating pulse (800 ms; irradiance of $\sim 2400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) to determine F_m . Photochemical efficiency (F_v/F_m) was calculated with WinControl-3 Software (Walz GmbH). For determinations of photosynthesis-irradiance (P/I) curves, samples of microalgal cells were exposed to 8 incremental levels of irradiance (75, 109, 165, 245, 346, 479, 773 and $1127 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), each with a 3-min light-adaptation duration [23]. Electron transport rate (ETR) vs photosynthetic active radiation (PAR) values were fitted as P/I curves according to Platt's equation [24], i.e.:

$$\text{ETR} = \text{ETR}_{\text{mPot}} \cdot \left(1 - e^{-\frac{\alpha \cdot \text{PAR}}{\text{ETR}_{\text{mPot}}}}\right) \cdot e^{-\frac{\beta \cdot \text{PAR}}{\text{ETR}_{\text{mPot}}}}$$

where α is initial slope of P/I curve, which is related to quantum efficiency of photosynthesis (electrons/photons), and β is photoinhibition parameter. The values of α , β and ETR_{mPot} are estimated by the fitting procedure.

Maximum ETR (ETR_{max} , $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$) is determined by the equation:

$$ETR_{max} = ETR_{mPot} \cdot \left(\frac{\alpha}{\alpha + \beta}\right) \cdot \left(\frac{\beta}{\alpha + \beta}\right)^{\frac{\beta}{\alpha}}$$

2.3.4 Nutrient concentrations

2.3.4.1 Dissolved inorganic carbon concentrations and calculation for $CO_{2(aq)}$

Dissolved inorganic carbon (DIC) was determined using a TOC Analyzer (Shimadzu, TOC-5000A). The supernatants of the samples were injected after centrifugation (12,100 g, 10 min, 10°C). The relative concentrations of all DIC species, dissolved carbon dioxide ($CO_{2(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) in the culture were calculated from the DIC and the pH values at each steady-state with equations derived from the following reactions:

$$[DIC] = [CO_{2(aq)}] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}];$$

At constant pressure and temperature, the following equilibria between $CO_{2(aq)}$, H_2CO_3 , HCO_3^- and CO_3^{2-} species apply:

$$\text{where } K_0 = \frac{[H_2CO_3]}{[CO_{2(aq)}]}, K_1 = \frac{[HCO_3^-] \cdot [H^+]}{[H_2CO_3]}, K_2 = \frac{[CO_3^{2-}] \cdot [H^+]}{[HCO_3^-]},$$

The calculations used the constants from Millero & Roy [25,26]. At equilibrium, $K_0 \approx 1.7 \times 10^{-3}$; then only a small fraction, less than 1% of the dissolved CO_2 , is converted to H_2CO_3 , with the main fraction as aqueous molecular $CO_{2(aq)}$. K_1 and K_2 were calculated from the following equations:

$$pK_1 = \frac{6320.813}{T} + 19.568224 \cdot \ln T - 126.34048$$

$$pK_2 = \frac{5143.692}{T} + 14.613358 \cdot \ln T - 90.18333$$

$$\text{Then, } [CO_{2(aq)}] = [DIC] \cdot \frac{[H^+]^2}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2}$$

2.3.4.2 Nitrate and phosphate concentrations

Nitrate as N-NO₃ and phosphate as P-PO₄ concentrations were determined in the culture medium using an ionic chromatography system including a cationic chromatograph (Dionex-1100-IonPac) equipped with a CG16 guard column and CS16 separation column, an anionic chromatograph (Dionex-ICS 900-IonPac) equipped with a AG9-HC guard column and AS9HC separation column, and an external AMMS (Anion-ICE MicroMembrane Suppressor 300, Dionex) supplied with sulfuric acid (H₂SO₄, 25 mmol L⁻¹, 1.8 mL min⁻¹). Eluents were a solution of 6 mmol L⁻¹ Na₂CO₃ and 1 mmol L⁻¹ NaHCO₃ at a flow of 0.9 mL min⁻¹ for anionic chromatography and a solution of 20 mmol L⁻¹ H₂SO₄ at a flow of 0.9 mL min⁻¹ for cationic chromatography. Detection was by conductivity, and data acquisition and processing were accomplished on Chromeleon software. Nutrient requirement for biomass production (R_{SX}, mg gX⁻¹) in terms of biomass production was calculated as:

$$R_{SX} = \frac{S_{in} - S_{out}}{X}, \text{ (mg gX}^{-1}\text{)}$$

where: S_{in} is ion concentration of the fresh medium flow into the photobioreactor and S_{out} is ion concentration of culture sampled from the PBR outlet.

2.3.5 Hexane extract contents

Hexane extracts, regarded as representative of crude hydrocarbon, were measured using a gravimetric method [4]. The lyophilized biomass was soaked in n-hexane at room temperature

then sonicated and vortexed (18,000 rpm) for 5 minutes. The upper hexane layer containing hydrocarbon was collected by centrifugation (12,100 g, 10 min, 10°C), and the supernatants were evaporated under a gentle flow of nitrogen gas. Dried hexane extracts were weighed to determine percent of biomass dry weight.

2.3.6 Total lipid and hydrocarbon contents

Total lipids and hydrocarbons were determined according the method described by Ishimatsu et al. [27]. Quantity of total lipids was determined gravimetrically and expressed as percent of biomass dry weight. After drying and weighing for the calculation of total lipids, the remaining residue (total lipids) was dissolved in a small amount of hexane and then purified by chromatography on a silica-gel column. Four bed volumes of n-hexane were applied to the column, and the purification of hydrocarbon was confirmed by thin-layer chromatography (TLC) using squalene as standard. The eluate was evaporated and dried under a gentle flow of nitrogen gas, and quantity of purified hydrocarbon content [HC] (% dw) was determined. Hydrocarbon productivity was calculated as:

$$P_{HC} = P_X \cdot [HC], (g L^{-1} d^{-1} \text{ or } g m^{-2} d^{-1})$$

and specific rate of hydrocarbon production was determined as:

$$q_{HC} = \frac{P_{HC}}{X}, (gHC gX^{-1} d^{-1})$$

2.4. Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). Data was processed by R software to test homogeneity of variance using leveneTest (Car package). Differences between groups were analyzed statistically using one-way analysis of variance (ANOVA) and Tukey's 'Honest Significant Difference' method (TukeyHSD) at 95% family-wise confidence level.

3. Results and Discussion

The effect of pH on efficiency of *B. braunii* cultures was essentially studied using continuous cultures after preliminary assays in batch cultures to determine the value of the dilution rate and the enrichment level of the basic AF-6 culture medium. The results thus correspond to N and P-enrichment levels of the culture medium at a fixed dilution rate. All data obtained under continuous cultures correspond to steady-state equilibrium, i.e. < 10% variation on at least 10-day periods.

3.1. Preliminary characterization of *B. braunii* batch cultures in AF-6 medium

Preliminary cultures were maintained in 300 mL columns with AF-6 medium, as this medium has been demonstrated as suitable for *B. braunii* [27]. During 12-day batch cultures, pH increased from 6.6 to 9.5, although the AF-6 medium contained 2 mmol L⁻¹ MES, a pH buffer. This corresponds to a specific pH variation from 1.8 UpH to 4.2 UpH per g biomass produced. This alkalization of the culture medium, routinely observed in any microalgal cultures with nitrate as N-source, has been reported in other *B. braunii* cultures [11,13].

Ionic analysis of the culture supernatants after 12-day incubation periods indicated that almost all the initial phosphate concentration (103 µmol L⁻¹), was consumed (Fig. 2a). Another study has already shown that phosphate was a potent limiting factor in batch cultures of *B. braunii* 765 with an initial concentration of 175 µmol L⁻¹ [4]. The residual concentrations of the other macronutrients after the 12-day cultivations were still relatively high, such as nitrate at about 300 µmol L⁻¹, except for DIC which in some cultures was under 2 mg L⁻¹. These measured DIC values indicated that inorganic carbon could be limited. Then, during the first set of experiments, the continuous culture was fed with a 2-fold phosphate-enriched AF-6 medium, named 2P-AF-6. Moreover, the end of the log phase seemed to coincide with pH values over 7.5 (see Fig. 2b). Therefore, the pH effects on *B. braunii* biomass and hydrocarbon production

Fig. 2. Variations of (a) biomass, X, and phosphate concentrations and (b) Ln X and pH as a function of time in a *B. braunii* batch culture. Culture medium was AF-6. The residual concentrations of the other nutrients were non-negligible after 13 days of incubation.

were studied with microalgae grown in a photobioreactor where pH was regulated using controlled injection of CO₂ gas flow. Through this approach, the inorganic carbon supply of the cultures was provided, as after injection a fraction of the CO₂ gas dissolves as CO₂ (aq) according to Henry's law and is then converted to HCO₃⁻ and CO₃²⁻. The dilution rate of the continuous cultures was fixed at 0.2 d⁻¹, as the specific growth rates of *B. braunii* cultures are usually in the range 0.1 d⁻¹ to 0.3 d⁻¹, with a maximal growth rate of 0.5 d⁻¹ [6].

3.2. Characterization of *B. braunii* continuous cultures in 2P-AF-6 medium

3.2.1 Biomass and hydrocarbon productivities in 2P-AF-6 medium as a function of pH

To highlight the effect of environmental pH on production rates, a continuous culture of *B. braunii* was started in a 1.4 L photobioreactor. All the parameters were fixed, i.e. irradiance ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), temperature (23°C), stirring (200 rpm), aeration rate (0.32 vvm) and 2P-AF-6 feed medium at a dilution rate of 0.2 d^{-1} , except the pH value which was changed stepwise in 0.5 unit increments from pH 5.5 to pH 8.0. The biomass and lipid concentrations were determined after reaching a steady-state at each pH value. The effects on biomass and total lipid productivities were deduced from these steady-state values.

Fig. 3. Biomass and total lipid productivities as a function of pH in steady-state continuous cultures fed with 2P-AF-6.

Environmental pH had a significant effect on total lipid productivity ($P < 0.05$) while having little effect on biomass productivity (Fig. 3). According to the geometric features of the PBR, maximal biomass productivity reached $5.2 \text{ g m}^{-2} \text{ d}^{-1}$ and total lipids areal productivity reached

2.2 g m⁻² d⁻¹ at pH 6.5. Environmental pH not only had an effect on total lipids productivity but also on the total lipids and hexane extract contents of dry biomass (Fig. 4). Total lipid contents were significantly different according to whether microalgal biomass was produced at a pH over or under the threshold of 7.0.

Fig. 4. Total lipids and hexane extracts contents as a function of pH in steady-state continuous cultures fed with 2P-AF-6.

The hexane extracts are generally regarded as crude hydrocarbon fractions. As shown in Fig. 4, in contrast with total lipids, the highest values for hexane extract contents were obtained at pH 6.5. A change of just one pH unit, from pH 7.5 to 6.5, was enough to induce a significant ($P < 0.05$) increase in total lipid and hexane extract contents. Hexane extract content was between 19.9% dw and 36.8% dw, which is consistent with Dayananda et al. [15] who found that hexane extract as hydrocarbon ranges from 15% to 50.6% dw of the same SAG 30.81 strain. Note that the hexane extracts could contain other lipids, such as triacylglycerol and aldehydes, and that hydrocarbon accounted for only 20.4% of hexane extracts from an Austin

Fig. 5. Comparison of biomass productivity, purified hydrocarbon productivity, nutrient consumption at pH 6.5 and nutrient consumption at pH 7.5 in steady-state continuous cultures fed with 2P-AF-6 or 3N6P-AF-6. a) biomass and hydrocarbon productivities; b) N and P residue concentrations. N = nitrogen; P = phosphate.

strain of *B. Braunii* [28]. In order to quantify the exact hydrocarbon content of this strain, we purified the hydrocarbon using silica gel chromatography.

In addition, pH also had a significant effect on purified-hydrocarbon productivity, which increased fourfold with the change from pH 7.5 to pH 6.5 (Fig. 5a).

At pH 6.5, hydrocarbon productivity reached up to $0.291 \text{ g m}^{-2} \text{ d}^{-1}$ with a hydrocarbon content of 5.7% dwh whereas at pH 7.5, hydrocarbon productivity was only $0.065 \text{ g m}^{-2} \text{ d}^{-1}$ and with a much lower hydrocarbon content of 1.3% dw. Moreover, hydrocarbon-specific production rate was significantly higher at pH 6.5 ($11.1 \text{ mg HC g X}^{-1} \text{ d}^{-1}$) than at pH 7.5 ($2.7 \text{ mg HC g X}^{-1} \text{ d}^{-1}$; $P < 0.05$). The hydrocarbon contents measured in this study were not so high, peaking at 5.7% dw, they are still within the range of values reported in the literature. Hydrocarbon contents in *B. braunii* race A biomass are generally from 1% dw to 10% dw, although reports claim some race-A strains accumulate up to 61% hydrocarbons while others accumulate less than 0.1% dw [2]. As hydrocarbons are mainly accumulated in the extracellular matrix of *B. braunii*, it was possible to estimate intracellular lipid contents by subtracting purified hydrocarbons content from total lipids content [2]. The calculation of the ratio of extracellular hydrocarbon to intracellular lipids as a function of pH then confirmed that optimal pH was around pH 6.0–6.5 (Fig. 6).

According to the literature, one of the main precursors for hydrocarbon biosynthesis in *B. braunii* race A is oleic acid, probably via an elongation mechanism involving conversion to very-long-chain fatty acids, as revealed by comparative gene expression analysis in a race A strain of *B. braunii* [29,30].

Fig. 6. Ratio of purified hydrocarbons to intracellular lipids as a function of pH in steady-state continuous cultures fed with 2P-AF-6. Intracellular lipids = total lipids – purified hydrocarbon.

There could therefore be a relation between intracellular lipid accumulation and extracellular hydrocarbon accumulation in *B. braunii* cultures. Indeed, when taking in account only the pH range from 6.0 to 8.0, there is a linear relation between extracellular hydrocarbon and the intracellular lipid contents:

$$[HC] = 0.534 \cdot [\text{Intracellular lipid}] - 13.398, R^2 = 0.939$$

The assumption of a linear relationship between hydrocarbon and lipid contents is only valid for pH values between 6.0 and 8.0. This relationship is not valid for the values corresponding to pH 5.5. The hypothesis for this is that the physiological state of *B. braunii* cells could be modified when the algae is grown in such acidic environmental conditions.

3.2.2. Steady-state photosynthetic activity at pH from 5.5 to 8.0

As *B. braunii* SAG 30.81 was cultivated under photoautotrophic conditions, photosynthetic activity is essential to provide energy and reductive carbon for biomass and hydrocarbon production [31]. The photosynthetic characteristics of the cell populations were determined at

each steady-state. The photochemical efficiency of photosystem II (PSII), expressed as F_v/F_m and ETR_{max} , was relatively unaffected by pH of the culture from pH 6.0 to 8.0, even if a slight increase was observed at pH 6.5 for the continuous culture fed with 2P-AF-6 (Fig. 7)

Fig. 7. Steady-state F_v/F_m and ETR_{max} of the P/I curve as a function of pH in continuous cultures of *B. braunii* at a fixed dilution rate of 0.2 day^{-1} . Means $\pm$ SD. P/I curve = photosynthesis-irradiance curve.

The parameters F_v/F_m and ETR_{max} are commonly used to evaluate the impact of stresses such as nutrient or heat stress on microalgae [32,33]. In our experimental conditions, the absence of any sharp decline in F_v/F_m and ETR_{max} values suggests *B. braunii* were not

stressed at any pH in the range 6.0 to 8.0 (Fig. 7). However, the slight decrease of Fv/Fm at pH 5.5 indicates an inhibition of microalgal photosynthesis by this more acidic environment—an effect that could be related to the decrease in biomass concentration at pH 5.5. The decrease of extracellular pH induced by a high CO₂ supply could also affect intracellular pH, although green microalgae are known to maintain their intracellular pH at around 7.2 even when grown in relatively acidic media of pH down to 3.0 [34]. For example, when cells of *Chlorococcum littorale* were transferred from low CO₂, i.e. 0.04% in air, to high CO₂, i.e. up to 40%, intracellular pH value decreased from 7.0 to 6.4 with an environmental pH value of 5.5 [9]. This acidification induced an inhibition of photosynthetic carbon fixation in *C. littorale* cells exposed to excess concentrations of CO₂ [9].

3.2.3. Steady-state pigment composition at pH from 5.5 to 8.0

Microalgae like *B. braunii* have two major classes of pigments, chlorophylls (Chl) and carotenoids (Car), that are generally associated to photosynthetic activities, except in some race B strains that accumulate Car within the extracellular matrix [35]. The race A strain SAG 30.81, like other green algae, contains Chl *a*, Chl *b* and Car. Chl *a*, Chl *b* and Car contents are known to depend on culture conditions, particularly nutrients and light. Eroglu et al. [36] observed values from 0.38% to 0.86% (w/w) for chlorophylls and from 0.10% to 0.54% (w/w) for carotenoids according to species of *B. braunii* grown under air. Sydney et al. [7] found 13.05% (w/w) pigments in *B. braunii* SAG 30.81 cultivated under CO₂-enriched (5%) air. As shown in Table 1, the pigment contents obtained here at each steady-state were within the range of values obtained by Ranga Rao et al. [5], at 1.16% w/w total Chl and 0.27% w/w Car. Chl *a*/Chl *b* ratio and Car/Chl ratios are widely used indicators of photosynthetic cell response to changes in irradiance, nutrients, temperature or salinity [37]. Plant cells present Chl *a*/Chl *b*

ratios of 3.2–4.0 when exposed to high irradiance and 2.5–2.9 when grown under low irradiance [38].

Table 1

Steady-state pigment composition of biomass as a function of pH in continuous cultures of *B. braunii* at a fixed dilution rate of 0.2 d⁻¹.

	Feeding medium	Total Chl (%dw)	Chl <i>a</i> / Chl <i>b</i> (mol / mol)	Total Car (% dw)	Car / Chl (w / w)
pH 5.5	2P-AF-6	1.3±0.1*	2.1±0.2	0.24±0.02*	0.19±0.02
pH 6.0	2P-AF-6	2.0±0.2	2.3±0.2	0.37±0.03	0.19±0.02
pH 6.5	2P-AF-6	2.1±0.1	2.4±0.3	0.37±0.03	0.18±0.01
pH 7.0	2P-AF-6	1.8±0.1	2.2±0.1	0.33±0.02	0.18±0.01
pH 7.5	2P-AF-6	1.8±0.2	2.3±0.3	0.33±0.02	0.18±0.01
pH 8.0	2P-AF-6	1.7±0.1	2.2±0.2	0.32±0.02	0.19±0.02
pH 6.5	3N6P-AF-6	2.2±0.2	2.1±0.3	0.38±0.03	0.18±0.01
pH 7.5	3N6P-AF-6	1.8±0.2	2.1±0.2	0.34±0.02	0.19±0.02

Means ± SD. Chl = chlorophylls; Car = carotenoids; * = significantly different from other conditions (P < 0.05).

As reported for other Chl *b*-containing plant cells, the Chl *a*/Chl *b* values of microalgae are generally between 2 and 3, with little if any effect of pH [39].

The Car/Chl ratio (0.24) of *B. braunii* obtained by Ranga Rao et al. [5] in cultures fed at a 2% CO₂ concentration is relatively close to our data. Note that a lower Car/Chl ratio (0.15) was also observed in other *B. braunii* species in absence of CO₂ supplementation [36]. The evidence thus suggests there was apparently no stress from pH 5.5 to 8.0, as environmental

pH had no significant influence on Chl *a*/Chl *b* and Car/Chl ratios. However, under steady-state, total chlorophyll and carotenoid contents were significantly decreased at pH 5.5 ($P < 0.05$), thus confirming that the cells were stressed by the more acidic conditions (Table 1).

3.2.4. Nutrient requirement for biomass production at steady-state from pH 5.5 to 8.0

As residual concentrations of macronutrients—mainly phosphate and nitrate—in the outflow from the continuous culture were relatively low (Fig. 5b), we cannot exclude that the pH effect could be related to some nutrient limitations. Nutrient limitations are known to induce physiological responses, particularly in certain photosynthetic characteristics related to PSII [40]. Indeed, nitrogen (N-NO_3^-) and phosphorus (P-PO_4^{3-}) count among the main components

Table 2

Nutrient requirement for biomass production (Rxs mg g X^{-1}) during growth in steady-state continuous cultures at a fixed dilution rate of 0.2 d^{-1} but at different pH levels.

	Feed medium	N-NO_3^-	P-PO_4^{3-}
pH 5.5	2P-AF-6	57.5 ± 3.2	13.5 ± 1.2
pH 6.0	2P-AF-6	59.0 ± 2.2	13.0 ± 1.0
pH 6.5	2P-AF-6	56.5 ± 4.0	12.0 ± 0.9
pH 7.0	2P-AF-6	58.5 ± 2.8	12.5 ± 1.0
pH 7.5	2P-AF-6	61.5 ± 2.6	12.5 ± 1.1
pH 8.0	2P-AF-6	63.5 ± 4.4	13.5 ± 1.0
pH 6.5	3N6P-AF-6	42.2 ± 3.8	15.9 ± 1.2
pH 7.5	3N6P-AF-6	48.1 ± 5.1	16.6 ± 1.5

Means $\pm$ SD.

of many microalgal culture media as well as the most effective factors for regulating secondary metabolism in microalgal cells [41].

N and P requirement for biomass production were calculated for all the successive steady-states of the continuous culture fed with 2P-AF-6 medium (Table 2). pH changes had no significant effect on nutrient consumption yields determined from data on each steady-state continuous culture.

3.3. Biomass and hydrocarbon productivities at pH 6.5 and 7.5 with the 3N6P-AF-6 medium

We led a second set of experiments characterized by threefold-higher N and P concentrations in the feed medium (3N6P-AF-6) and focusing on two successive steady-states at pH 6.5 then 7.5. In these conditions, N and P concentrations increased in the PBR outlet regardless of pH setpoint (Fig. 5b). As shown in Fig. 5a, both biomass and hydrocarbon productivities were increased by feeding the culture with the 3N6P-AF-6 nutrient-enriched medium compared to 2P-AF-6 medium. Biomass and hydrocarbon productivities increased up to $8.9 \text{ g m}^{-2} \text{ d}^{-1}$ and $0.50 \text{ g m}^{-2} \text{ d}^{-1}$, respectively, at pH 6.5, and up to $8.5 \text{ g m}^{-2} \text{ d}^{-1}$ and $0.15 \text{ g m}^{-2} \text{ d}^{-1}$, respectively, at pH 7.5. Note that the increase in purified hydrocarbon productivity was related to the increase in biomass productivity by increasing the N and P supply. Indeed, purified hydrocarbon content showed no significant change, at 5.6% dw at pH 6.5 and 1.7% dw at pH 7.5. These data confirm the results of Xu et al. [42] showing that higher biomass and hydrocarbon productivities in *B. braunii* cultures were obtained by increasing the N and P supplies. In contrast, according to these data, the effect of pH on biomass and hydrocarbon contents was similar whatever the N and P concentrations.

However, nitrogen requirement for biomass production significantly decreased when the culture medium contained threefold more N (Table 2). It seems that enriching N and P in the feed medium could decrease the N needs for biomass. Sydney et al. [7] worked on the same *B.*

braunii SAG 30.81 strain as here and reported a relatively similar nitrogen requirement for biomass production of 40.72 mg g X⁻¹. However, phosphorus requirement for biomass increased slightly but significantly in the cultures grown on 3N6P-AF-6 medium (Table 2). This observation could be interpreted by a relatively classical luxury phosphate uptake process, with intracellular accumulation of polyphosphate as an intracellular nutrient pool in different microalgae [43], particularly in *B. braunii* [44]. Further studies are needed to improve the formulation of a culture medium for an optimal production of biomass for the different *B. braunii* strains.

There was no significant difference in Fv/Fm and ETR_{max} between feeding with 2P-AF-6 and 3N6P-AF-6 media in steady-state continuous cultures at pH ≥ 6.0 (Fig. 7). Total lipid productivity was significantly higher at pH 6.5 (3.4 g m⁻² d⁻¹) than pH 7.5 (2.8 g m⁻² d⁻¹). Hydrocarbon productivities were also significantly higher at pH 6.5 (P < 0.05) regardless of media used (2P-AF-6 or 3N6P-AF-6)(Fig. 5a).

In addition, extracellular hydrocarbons-to-intracellular lipids ratio was also higher at pH 6.5 than at pH 7.5, in line with the results on feeding with 2P-AF-6 medium. Extracellular hydrocarbons and intracellular lipids were fitted together with data previously obtained and appeared to follow a linear relationship:

$$[\text{HC}] = 0.557 \cdot [\text{Intracellular lipids}] - 141,$$

although with a low of R² value (= 0.769). Either way, this trend needs to be confirmed.

There are relatively few published studies on *B. braunii* cultures within photobioreactors, and only two in which pH was controlled by automated CO₂ injection, at setpoint values of pH 7.2 and 7.5, respectively [7,14] ; see Table 3). Our data at pH 7.5 were similar to results from another study at pH 7.2 on the same strain but much higher on biomass productivity and lipids

Table 3

Comparison of *B. braunii* biomass productivity, total lipid productivity and hydrocarbon productivity during studies within pH-controlled photobioreactors.

Strain	Race	pH control	Biomass productivity (mg L ⁻¹ d ⁻¹)	Hydrocarbon productivity (mg L ⁻¹ d ⁻¹)	Total lipid productivity (mg L ⁻¹ d ⁻¹)	Reference
GUBIOTJTBB1	--	7.5	13	6.8 ^a	7.4	[14]
SAG 30.81	A	7.2	207 ^b	--	68	[7]
SAG 30.81	A	6.5	223	12.5 ^c	84	This study
SAG 30.81	A	7.5	212	3.8 ^c	70	This study

^a, hexane extract as crude hydrocarbon; ^b, average productivity calculated from figure; ^c, purified hydrocarbon; --, no data available.

productivity and different on hydrocarbon productivity compared to strain GUBIOTJTBB1 at pH 7.5. This difference could be due to other cultivation conditions or could be strain-dependent. Further, our study found that the highest hydrocarbon and total lipid productivities were reached at pH 6.5, but pH is also known to influence the speciation and bioavailability of different nutrients in the culture media, particularly DIC forms.

3.4 Relation between dissolved carbon dioxide and hydrocarbon production

Hydrocarbon is considered as a growth-associated product in *B. braunii* cultures [6]. However, our preliminary results suggest that environmental pH can stimulate hydrocarbon production with little effect on biomass productivity. The large increase (>70%) in biomass concentration found here when continuous cultures were fed with 3N6P-AF-6 medium compared to 2P-AF-6 medium indicates that certain N and P limitations control biomass accumulations without change of hydrocarbon content within the PBR. The controlling factor here was not light

availability, as PFD at the PBR was constant. Indeed higher productivities could be reached by increasing PFD and decreasing the light path within the PBR, as already shown in a trickle-film configuration [2].

B. braunii hydrocarbon content is mainly related to its exocellular hydrocarbons [1,45]. Indeed, the biomass of this colonial species is essentially composed of cells surrounded by a complex extracellular matrix of polymerized hydrocarbons [46] that accumulates liquid hydrocarbons, mainly alkadienes and alkatrienes in race A strain SAG 30.81. The extracellular fraction has been evaluated to about 95% of total hydrocarbon [36]. We can use these characteristics to assume that the liquid hydrocarbons as exocellular products could be dissociated from the biomass and the matrix. Then, as biomass concentration at steady-state seems relatively unaffected by pH compared to the change in hydrocarbon biosynthesis, the stimulation of hydrocarbon productivity at pH 6.5 could be triggered by either increased hydrocarbon biosynthesis activities, the excretion process, or the adsorption capacity of the matrix. In the first case, pH 6.5 would be optimal for hydrocarbon biosynthesis activities that resulting in overproduction. Another hypothesis is that this stimulation could be caused by an effect of external pH on internal pH, as some intracellular enzymes, such as the decarbonylase, associated with hydrocarbon biosynthesis, are highly pH-dependent [47]. In the second case, pH 6.5 could impact the rate of globule content exocytosis through the plasma membrane and cell wall of *B. braunii*. In the third case, the polymer's capacity to trap extracellular hydrocarbon would presumably be pH-dependent, like the effect of NaCl on efficiency of hydrocarbons extraction from *B. braunii* biomass observed by Furuhashi et al. [48]. Moreover, we cannot rule out an effect of pH on chemical composition of the liquid hydrocarbon fraction.

Fig 8. Inorganic carbon dioxide composition of the supernatant of *B. braunii* cultures in 2P-AF-6 and 3N6P-AF-6 media at pH 6.5 and 7.5 at the constant dilution rate of 0.2 d⁻¹; CO_{2(aq)} values were calculated from DIC data.

However, we observed that with pH controlled by CO₂ injection, DIC concentrations in the supernatants of steady-state cultures seemed to be relatively constant (i) at pH 6.5 and 7.5 regardless of feed medium (2P-AF-6 or 3N6P-AF-6) (Fig. 8), and (ii) between pH 6.0 and 8.0, with a net RSD of 13% and very little fluctuations of DIC concentration during each steady state (RSD ≤ 6.8%). Thus, at first glance, there was visibly no DIC limitation of growth and hydrocarbon production occurred in our experimental conditions. On the basis of biomass elemental composition, stoichiometry equations on *B. braunii* have shown that hydrocarbon-enriched biomass production needs about 30% more CO₂ for strain BOT 22 than for *Chlorella* sp. or *Spirulina* sp. [2]. Not only the necessary amounts but also species of DIC have to be provided to drive increased growth of hydrocarbon-enriched biomass. The significant difference in hydrocarbon production at pH 6.5 vs pH 7.5 could thus be related to the effect of pH on inorganic carbon speciation, i.e. CO_{2(aq)} or H₂CO₃, HCO₃⁻, and CO₃²⁻ [49–52]. Fig. 9 shows that hydrocarbon accumulation in *B. braunii* colony biomass of seems to be limited at dissolved CO₂ concentrations < 1 mmol L⁻¹. A maximal hydrocarbon content of 5.7% dw was

reached at and above this concentration. This DIC range is comparable with other DIC values related to biomass productivities in cultured microalgae such as *Isochrysis galbana* and *Phaeodactylum tricornutum*, where DIC limitations but not CO₂ limitations were observed for biomass productivity but not metabolite productivity at DIC values < 0.5 mmol L⁻¹ [53]. After factoring in all the data obtained here but excluding data with physiological inhibition on cells at pH 5.5, hydrocarbon productivity appears linearly related to CO_{2(aq)} up to 2.5 mmol L⁻¹, whatever the N and P content of the culture medium (Fig. 10), according to the relation:

$$P_{\text{HC}} = 0.146 \cdot \text{CO}_{2(\text{aq})} + 0.077, R^2=0.869$$

Fig. 9. Hydrocarbon content of *B. braunii* biomass as a function of residual CO_{2(aq)} in the culture under steady-states conditions from pH 6.0 to 8.0 in continuous cultures fed with 2P-AF-6 or 3N6P-AF-6; CO_{2(aq)} values calculated from DIC data.

Fig. 10. Relation between hydrocarbon productivity and residual dissolved carbon dioxide in the culture medium at each steady state; CO_{2(aq)} values calculated from DIC data.

As a result, pH effects on hydrocarbon productivity are probably related to how pH influences distribution of DIC speciation, and CO_{2(aq)} could be the key driver of hydrocarbon production.

4. Conclusion

Successive steady-state continuous cultures of *B. braunii* SAG 30.81 at a constant dilution rate have shown that hydrocarbon productivity but not biomass productivity is pH-dependent in the range 5.5 to 8.0. At pH 5.5, the inhibition of PSII activities could be related to a reduction in hydrocarbon production. Maximal hydrocarbon productivities were obtained at pH 6.5 in two modified AF-6 media with nitrate and phosphate enrichments. The pH effect on hydrocarbon production was shown to be mainly related to dissolved CO_{2(aq)} concentration. This study also indicated that in unstressed, i.e. stable PSII activity, microalgae, extracellular hydrocarbon accumulation appeared to be linked to intracellular lipid accumulation. Further comparative studies between race-A and race-B *B. braunii* strains cultivated at different dilution rates and PFD could valuably help determine how dissolved CO₂ availability, hydrocarbon productivity and intracellular lipid productivity are co-related.

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