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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

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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 CO_2 and that hydrocarbon production was linearly related to CO_2 up to 2.5 mmol L^{-1}, whatever the N and P enrichment of AF-6 culture medium.

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]. CO_2 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 CO_2 from industrial exhaust gases to help reduce greenhouse gas emissions [7].

However, successful mitigation of CO_2 and improved production of hydrocarbon oils depend on CO_2 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

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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 CO_2 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 $NaNO_3$ 140 mg L^{-1}, NH_4NO_3 22 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 30 mg L^{-1}, KH_2PO_4 10 mg L^{-1}, K_2HPO_4 5 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 10 mg L^{-1}, $FeCl_3{\cdot}6H_2O$ 1 mg L^{-1}, Fe-citrate 2 mg L^{-1}, citric acid 2 mg L^{-1}, $Na_2EDTA{\cdot}2H_2O$ 8 mg L^{-1}, micronutrients ($MnCl_2{\cdot}4H_2O$ 200 μg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 40 μg L^{-1}, $CoCl_2{\cdot}6H_2O$ 8 μg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 20 μg L^{-1}, NH_4VO_3 1 μg L^{-1}, H_2SeO_3 5 μg L^{-1}) and vitamins (biotin 2 μg L^{-1}, thiamine HCl 10 μg L^{-1}, vitamin B_6 0.1 μg L^{-1}, vitamin B_{12} 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 18 W) and its intensity-adjusted to 150 μmol photons m^{-2} 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 − 250 A). Batch cultures were maintained at a temperature of 23 ± 2 °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 ± 1 °C. The photobioreactor was continuously illuminated at a photon flux density of 150 μmol photons m^{-2} 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).

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:

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

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

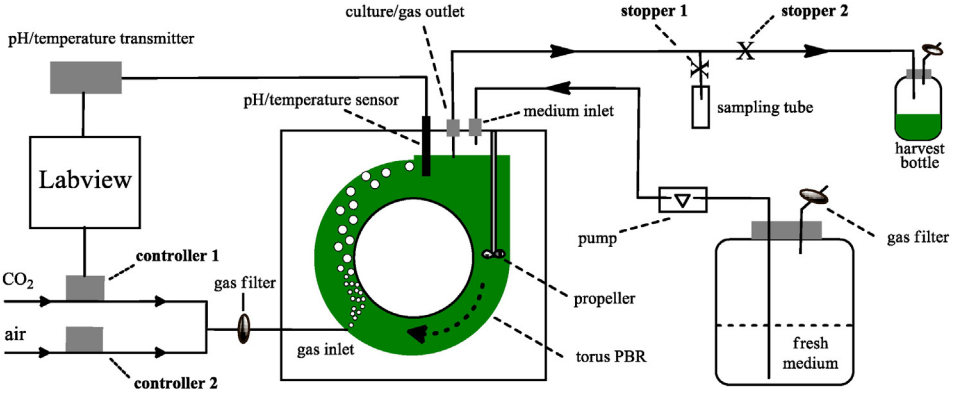

Fig. 1. Sketch map of the torus PBR system. At sampling, stopper 1 is open and stopper 2 is closed. At other times, stopper 1 is closed and stopper 2 is open. Sampling tube and harvest bottle were autoclaved (121 °C, 25 min) before being connected. Controller 1 (CO_2 flow controller) will be open when 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.