

# Effect of bicarbonate on growth of the oleaginous microalga *Botryococcus braunii*

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## Abstract

The effect of bicarbonate, produced by the enzymatic hydration of CO<sub>2</sub> from post-combustion fumes, was investigated on *Botryococcus braunii* growth. The NaHCO<sub>3</sub>, supplied to cultures in the role of inorganic carbon source is proposed as a more eco-sustainable alternative to gaseous CO<sub>2</sub>. The salt was provided to the cultures at the final concentration of 0.5-1.5-2.5 g L<sup>-1</sup>. The growth rate was considered for specific time intervals (T0-T5, T5-T10 and T0-T10) showing values significantly higher in the culture supplemented with 2.5 g L<sup>-1</sup> bicarbonate. The doubling times were also considered in all experimental cultures showing a faster doubling for the period T0-T5. The increase in pH drives the increase in growth in the experimental conditions in which the salt was added. The results suggest that bicarbonate is able to promote the algal growth, therefore it can be considered a valid alternative to CO<sub>2</sub> gas.

## Introduction

In the scientific society, a growing attention on microalgae as an alternative and renewable energy source is increasing. The green colonial microalga *Botryococcus brauni* (Trebouxiophyceae, Chlorophyta) is considered one of the promising feedstocks for biofuel production. It is able to make hydrocarbons and to form vast floating blooms suggesting the prospective for a large-scale cultivation and a simplified harvesting.<sup>1-3</sup> *Botryococcus* is ubiquitous species spread on all continents, except Antarctica, in freshwater, brackish and saline lakes in temperate, tropical and alpine climates.<sup>3,4</sup>

According to the literature, microalgae contain less than 5% of dry weight as hydrocarbons, while in *Botryococcus braunii* it can reach to 60-75% of dry weight,<sup>5-7</sup> however, the growth of *B. braunii* is slower than other most common utilized microalgae such as *Chlorella* sp., *Dunaliella*,

*Nannochloropsis* sp., or cyanobacteria *Spirulina*.<sup>8,9</sup> Therefore, to develop strategies to obtain *Botryococcus* cultures with a faster growth rate could represent a goal for commercial purposes.

The main factors that affect microalgae growth rate are temperature, salinity, light intensity, nitrogen and CO<sub>2</sub> availability.<sup>1</sup> About CO<sub>2</sub>, a correct supply of inorganic carbon is essential for photosynthetic activity and then for biomass production.<sup>4</sup> Although microalgae are able to grow even just by using atmospheric CO<sub>2</sub> (~400 ppm), the growth rate and cell productivity can be improved by supplementing inorganic carbon.<sup>10-12</sup> Nowadays, the principal carbon source for microalgae cultivation is the CO<sub>2</sub> gas; however, insufflation of dioxide carbon could be expensive and inefficient since much of CO<sub>2</sub> was lost into the atmosphere.<sup>12</sup>

In the present study, we propose the use of bicarbonate, produced by the enzymatic hydration of carbon dioxide of the post-combustion fumes, as chipper and eco-friendly alternative of inorganic C source for microalgae cultivation. The bicarbonate spent in the experiments, derived from a mimetic-selective trap based on carbonic anhydrase from *Mytilus galloprovincialis*,<sup>13,14</sup> capable of converting exhaust fumes, rich in CO<sub>2</sub>, in a bicarbonate solution.<sup>15-17</sup>

Here, we evaluated, for the first time, the effect of different concentrations of bicarbonate supply on cell growth in *Botryococcus braunii*.

## Materials and Methods

*Botryococcus braunii* (strain 807-1) was acquired from the algae collection at Goettingen University (SAG).

The experimental cultures were grown, in triplicate, in 2 L glass flasks, under controlled conditions of temperature (26±1°C), light (120-135 μmol s<sup>-1</sup> m<sup>-2</sup>) and continuously bubbled of filtered air at a flow rate of 80-100 L h<sup>-1</sup>. The Bristol medium (NaNO<sub>3</sub> 2.94 mM, CaCl<sub>2</sub> 0.17 mM, MgSO<sub>4</sub> 0.3 mM, K<sub>2</sub>HPO<sub>4</sub> 0.43 mM, KH<sub>2</sub>PO<sub>4</sub> 1.29 mM, NaCl 0.43 mM) was used to cultivate the microalgae; further nitrogen was not added to the cultures daily, while different supplementation of bicarbonate (as NaHCO<sub>3</sub> 0, 0.5, 1.5, 2.5 g L<sup>-1</sup>, dose effect) was added, in a single administration at T0.

*B. braunii* cultures were daily monitored, and the growth was spectrophotometrically evaluated by measuring the optical density (OD) at 650 nm.

The specific growth rate was calculated using the following formula:  $\mu = 1/T (\ln N_2 -$

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Contributions: GS, laboratory setting and manuscript writing; CMAB, statistical analysis and manuscript writing; SC, experimental plan.

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$\ln N_1)$ , where T is the time (days) of observation, N<sub>2</sub> is the OD<sub>650</sub> reading at time t<sub>2</sub>, and N<sub>1</sub> is the OD<sub>650</sub> reading at time t<sub>1</sub>.

The doubling time or time required to achieve a doubling of the number of viable cells was calculated, according to Yoshimura *et al.*,<sup>18</sup> by dividing  $\ln(2)$  (=0.693) by the value of specific growth rate.

The data were analysed by two-way analysis of variance (ANOVA) (dose effect, time effect and interaction) followed by the Tukey's multiple post-hoc test using SAS software (2009). The level of significance was P<0.05.

## Results

The growth of *B. braunii* experimental cultures was monitored for 10 days (T0-T10) as changes in optical density (OD) at 650 nm (Figure 1). At T10 values of growth were significantly (P<0.01) higher in the culture supplemented with 2.5 g L<sup>-1</sup> NaHCO<sub>3</sub>, (OD 3.14±0.10) compared to the culture control (NaHCO<sub>3</sub> 0 g L<sup>-1</sup> OD 2.45±0.28) whereas no statistical differences were observed among the other experimental conditions (NaHCO<sub>3</sub> 0.5 g L<sup>-1</sup>

OD  $2.58 \pm 0.33$ ;  $1.5 \text{ g L}^{-1}$  OD  $2.63 \pm 0.18$ ) (Figure 1). Indeed, starting from the 5<sup>th</sup> day, the cultures added with  $2.5 \text{ g L}^{-1}$  of  $\text{NaHCO}_3$  significantly increase the OD value both with respect to the control ( $P < 0.01$ ) and also to the  $\text{NaHCO}_3$  level equal to  $0.5 \text{ g L}^{-1}$  ( $P < 0.01$ ) (Figure 1).

Specific growth rate of *B. braunii* varied with  $\text{NaHCO}_3$  concentration (Table 1). The growth rate under bicarbonate supplementation was calculated for the entire experimental period (T0-T10) and for specific time intervals (T0-T5 and T5-T10). Considering the first period (T0-T5), the highest growth rate was determined by  $2.5 \text{ g L}^{-1}$  of bicarbonate ( $P < 0.05$ ) respect cultures without or with lower level, whereas for the second period (T5-T10) the best growth rate resulted in the  $0 \text{ g L}^{-1}$   $\text{NaHCO}_3$  cultures ( $0.12 \text{ day}^{-1}$ ), with value significantly higher than  $1.5 \text{ g L}^{-1}$  ( $P < 0.05$ ) (Table 1). The doubling times were also calculated in all experimental cultures, demonstrating that during the period T5-T10 the cultures showed an improved doubling (Table 1). Therefore, we can assume that after T5, the  $\text{NaHCO}_3$  administered at time 0 runs out, and therefore the cultures growth continues without the bicarbonate drive.

Figure 2 shows the variations of pH in the culture medium after the addition of bicarbonate. The pH at the start of the experiment ranging about from 7.72 ( $0 \text{ g L}^{-1}$   $\text{NaHCO}_3$ ) to 8.00 ( $2.5 \text{ g L}^{-1}$   $\text{NaHCO}_3$ ). At the end of experimental period (T10), the pH values were significantly higher ( $P < 0.05$ ) in the cultures treated with bicarbonate, respect to the control ( $10.09 \pm 0.25$  vs  $10.30 \pm 0.05$ ,  $10.31 \pm 0.03$  and  $9.50 \pm 0.32$  respectively for  $0.5 \text{ g L}^{-1}$   $\text{NaHCO}_3$ ,  $1.5 \text{ g L}^{-1}$   $\text{NaHCO}_3$ ,  $2.5 \text{ g L}^{-1}$   $\text{NaHCO}_3$  and  $0 \text{ g L}^{-1}$   $\text{NaHCO}_3$ ).

## Discussion and Conclusions

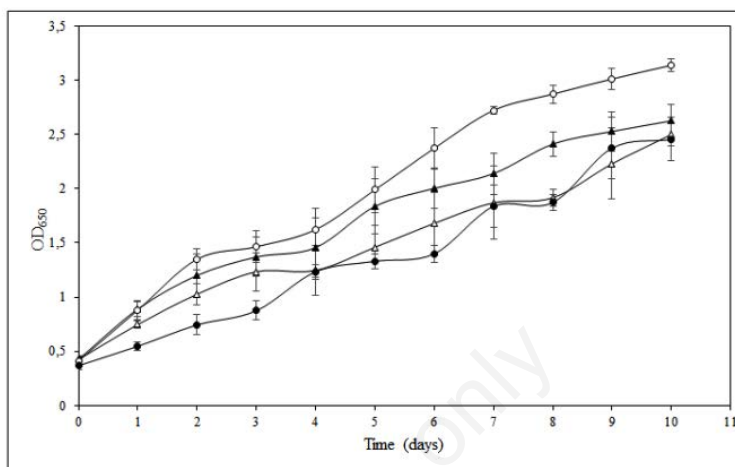
In recent years, microalgae belonging to *Botryococcus* species have caught attention for their ability to produce and accumulate hydrocarbons at a high rate per unit of dry cell weight.<sup>2</sup> However, the cultivation of *B.*

*braunii* is limited by its slow growth rate and doubling time.<sup>2</sup> Therefore, to quicken the microalga growth rate would be a great goal for large-scale cultivation.

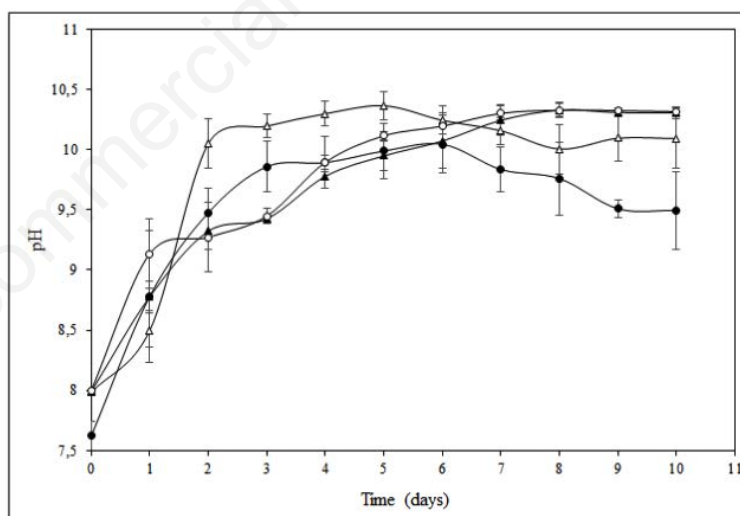
According to previous studies, the addition of  $\text{NaHCO}_3$  to algal culture can show beneficial effects on cell growth, improving

the production of algal-biomass and enhance the biofuel production potential.<sup>2,19</sup>

In our experiment, we found that *B. braunii* well-tolerate sodium bicarbonate concentration at least  $2.5 \text{ g L}^{-1}$ , as well demonstrated also by the rise of growth rate. As verified by White *et al.* (2012),<sup>11</sup>



**Figure 1.** Growth curves for *Botryococcus braunii* batch cultures supplemented with  $\text{NaHCO}_3$   $0 \text{ g L}^{-1}$  (filled circle);  $\text{NaHCO}_3$   $0.5 \text{ g L}^{-1}$  (empty triangle);  $\text{NaHCO}_3$   $1.5 \text{ g L}^{-1}$  (filled triangle);  $\text{NaHCO}_3$   $2.5 \text{ g L}^{-1}$  (empty circle). Error bars represent SD ( $n=3$ ).



**Figure 2.** pH curves for *Botryococcus braunii* batch cultures supplemented with  $\text{NaHCO}_3$   $0 \text{ g L}^{-1}$  (filled circle);  $\text{NaHCO}_3$   $0.5 \text{ g L}^{-1}$  (empty triangle);  $\text{NaHCO}_3$   $1.5 \text{ g L}^{-1}$  (filled triangle);  $\text{NaHCO}_3$   $2.5 \text{ g L}^{-1}$  (empty circle). Error bars represent SD ( $n=3$ ).

**Table 1.** Means of specific growth rates (SGR) and doubling times (DT) for different periods and in different conditions of sodium bicarbonate supplementation.

$\text{NaHCO}_3$	Specific Growth Rate ( $\text{day}^{-1}$ )			Doubling Time (days)		
	Days 0-5	Days 5-10	Days 0-10	Days 0-5	Days 5-10	Days 0-10
$0 \text{ g/L}$	0.242 <sup>a</sup>	0.122 <sup>b</sup>	0.182 <sup>a</sup>	2.529 <sup>a</sup>	5.797 <sup>ab</sup>	3.499 <sup>ab</sup>
$0.5 \text{ g/L}$	0.246 <sup>ab</sup>	0.115 <sup>ab</sup>	0.180 <sup>a</sup>	2.847 <sup>a</sup>	6.078 <sup>b</sup>	3.851 <sup>ab</sup>
$1.5 \text{ g/L}$	0.291 <sup>ab</sup>	0.072 <sup>a</sup>	0.182 <sup>a</sup>	2.390 <sup>a</sup>	9.868 <sup>b</sup>	3.814 <sup>a</sup>
$2.5 \text{ g/L}$	0.315 <sup>b</sup>	0.092 <sup>ab</sup>	0.203 <sup>a</sup>	2.208 <sup>a</sup>	7.871 <sup>b</sup>	3.412 <sup>a</sup>

Within SGR columns and within DT rows, values followed by the same superscript letters do not differ significantly by the Tukey test ( $P < 0.05$ ).

the threshold level of tolerability of  $\text{NaHCO}_3$  supplemented, is species specific.

Many green microalgae have shown to tolerate high sodium ion concentration, in fact, *Chlorella vulgaris* exhibited an optimum tolerance of bicarbonate at  $1.0 \text{ g L}^{-1}$ ,<sup>10</sup> while the marine microalgae *Nannochloropsis salina* and *Tetraselmis suecica* have revealed increase of cell density and growth rates in cultures supplemented with  $\text{NaHCO}_3$  5.0 and  $1.0 \text{ g L}^{-1}$  respectively.<sup>11,12</sup>

The bicarbonate ion ( $\text{HCO}_3^-$ ), present in culture media, has low cell membrane diffusion capacity. However, many microalgae are able to actively transport bicarbonate ions via plasma membrane to the cytosol where the enzyme carbonic anhydrase (CA) maintains a stable flux of  $\text{CO}_2$  to RuBisCO (Ribulose Biphosphate Carboxylase/Oxygenase) for photosynthetic activity.<sup>4</sup> The fact that *Botryococcus* drives higher growth in  $\text{NaHCO}_3$  enriched media let us to suppose that the microalga has an efficient system of inorganic carbon concentrating mechanism which allows it to take advantage of bicarbonate. However, as the increase in growth rate and the improvement of doubling time is limited above all to the first 5 days of the experiment, this suggests that the amount of  $\text{NaHCO}_3$   $2.5 \text{ g L}^{-1}$  is not sufficient to maintain a good growth rate for the entire duration of the experiment. Interestingly, in our study, the microalgae culture growth results correlated with the pH rise at the end of the experiments (T10). The increase in pH was mostly due to the effect of bicarbonate. However, starting from T4-T5, in accordance with the increase in the doubling time values, the significant differences on the pH are smoothed, confirming that the bicarbonate added to the T0 is no longer sufficient or has been consumed.

It has been demonstrated in *Chlamydomonas reinhardtii*, *Dunaliella parva* and *Anabaena variabilis* that the removal of inorganic C from culture medium by photosynthetic uptake and the consequent  $\text{O}_2$  evolution, leads to an increase in extracellular pH.<sup>20-23</sup> Furthermore, for *Botryococcus braunii*, a  $\text{pH} \geq 10.00$  does not seem to have negative effect on cells growth.

In conclusion, the addition of dissolved sodium bicarbonate to cultures of *Botryococcus braunii* can promote a boost to growth. In the development of strategies to cultivate microalgae in eco-sustainable way, the use of sodium carbonate from eco-sustainable technologies is to be considered a suitable alternative to the use of gaseous  $\text{CO}_2$  that at the same time could contribute to  $\text{CO}_2$  mitigation processes.

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