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Effects of Sodium Bicarbonate on Biomass and Carbohydrate Production in *Synechococcus* PCC 7002

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Microalgae and cyanobacteria have attracted much attention for developing new biofuels production technologies. In particular, cyanobacteria seem to be promising in view of third generation bioethanol production. The most used source of inorganic carbon for algal cultivation is concentrated CO_2 in air. However the use of this source can account of up to 50% of the biomass production costs. Bicarbonate salts, supplied to the culture medium, may be a valid alternative as source of inorganic carbon for photosynthetic microorganisms able to capture, use and recovery carbon from this substrate. In this study *Synechococcus* PCC 7002 was used in order to test the capability of this strain to exploit bicarbonate and to elucidate the effect of such a carbon source on carbohydrates production. Batch experiments were carried out, using automatic control of pH and temperature (set to 8.5 and 28 °C, respectively). The concentrations of sodium bicarbonate used were 5.5, 11, 22, 44 and 88 g L^{-1}. The cyanobacteria was able to grow in all concentrations of bicarbonate presenting high potential for production of biomass, with DCW 6 g L^{-1} in 44 and 88 g L^{-1} and productivity as high as 1.12 g L^{-1} day^{-1}. An accumulation of carbohydrates was observed up to a maximum of 25% using 88 g L^{-1} of sodium bicarbonate, however this value was reached under nitrogen starvation in the stationary growth phase, when the carbon source was still limiting, because all the bicarbonate ion was completely consumed in all experiments.

1. Introduction

The use of algae, microalgae and cyanobacteria for third-generation biofuels production (bioethanol, biodiesel and biogas) has many advantages over higher plants utilized to produce first- and second-generation biofuels. This is thanks to their faster growth, their capability of growing under different growth conditions including the use of wastewater, their reduced need for water and other resource inputs, and the possibility of not occupying arable lands for cultivation.

Photosynthetic microorganisms with potential for bioethanol production are selected primarily in accordance with their ability to accumulate carbohydrates. The biochemical composition of microalgae under normal conditions (without nutrients limitation) is characterized by a wide percentage range of proteins (30–50 %), carbohydrates (20–40%) and lipids (8–15 %) (Cardoso et al., 2011; Ho et al., 2013). This biochemical flexibility (capacity of lipids and carbohydrates accumulation) is depending on the species and on the environmental and nutritional conditions. The main environmental factors influencing carbohydrate accumulation are light intensity, pH, salinity and temperature, while the nutritional factors include the availability and source of nitrogen, carbon, phosphorus, sulfur, and iron (Chen et al., 2013; Markou et al., 2014).

Currently, cyanobacteria have attracted much attention and investments, since they are started to be considered a new source of energy (Silva et al., 2014). The phylum cyanobacteria or division Cyanophyta is a group of oxygenic bacteria that obtains energy by photosynthesis. They are commonly referred to as blue-green algae, even though the term algae is usually associated to eukaryotic organisms. Most of cyanobacteria species are terrestrial but there are some marine species as well. *Spirulina*, *Chlorococcus*, *Gloeocapsa*, *Synechocystis* and *Synechococcus* are some examples of genera grouped into the cyanobacteria phylum.

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The autotrophic growth of photosynthetic microorganisms requires a source of inorganic carbon, usually provided as CO_2. CO_2 can be supplied to the cultivation system (1) by pumping air, (2) by pumping air with concentrated CO_2 (generally 5%), (3) in the form of bicarbonate salts. In atmospheric air, CO_2 concentration is very low, about 0.04%, so that the amount of air need to aerate the culture is a lot, resulting in a high energetic consumption associated with the large amount of energy for pumping (Markou et al., 2014). These costs are related to technological aspects of capture, compression, transportation, temporary storage problems and loss of gas (Chi et al., 2011) and can reach up to 50% of biomass production costs (Chisti, 2013).
An alternative would be to use bicarbonate salts as a carbon source, according to a process called Bicarbonate-based Integrated Carbon Capture and Algae Production System (BICCAPS) (Chi et al., 2013). Bicarbonate salts have high solubility in water compared to CO_2 (for example, $NaHCO_3$ solubility > 90 g L^{-1} at 25 °C) and it is expected that their use efficiency is higher than CO_2 (Markou et al, 2014) .The use of aqueous solutions of bicarbonate for algal cultivation should result in lower costs than CO_2, which requires intensive energy for compression (Chi et al., 2011).
In this work we studied the tolerance of *Synechococcocus* PCC 7002 to sodium bicarbonate concentration evaluating its biomass production and carbohydrates accumulation in batch conditions, as well as their relationship with nutrients such as N and P, in view to evaluate this alternative carbon source for cyanobacterial cultivation.

2. Materials and Methods

2.1 Cyanobacteria Cultivation

Synechococcus PCC 7002 was grown in a Basal A medium (Bernstein et al., 2014) at a temperature of 28°C. The pre-inoculum was cultivated in flasks at approximately 100 ± 5 µmol photons m^{-2} s^{-1} and held in the exponential phase with pH ranging between 8–9. For the experiments, sodium bicarbonate was used as the only carbon source in concentrations of 5.5, 11, 22, 44 and 88 g L^{-1}, respectively. Batch experiments were performed in 250 mL-working-volume glass vertical tubes, with a 5 cm diameter, continuously mixed by a stirring magnet placed at the bottom of the bottle. The effective light intensity was measured with a radiometer DeltaOhm HD2102.1 positioned at mid distance between the reactor and lamp. Each batch experiment started with an initial microalgae inoculation of OD_{750} = 0.2–0.3.

2.2 Cultivation System

The cultivation system was composed of a water thermostatic bath, a stirring apparatus, illumination source (light bulb MAX8, 70 W, 1180 lumen and color temperature 2900 K), providing light only on one side, and a pH sensor in the reactor. The temperature was set to 28 °C and pH to 8.5. The pH sensor (D.O. Apparecchiature Elettroniche) was connected to a pH regulation system, which controls a chromatographic pump (LC-20AT Prominence), activated when the pH in the reactor exceeded the set point, by using a concentrated HCl solution (2M) to adjust the pH value. The HCl concentration was chosen to avoid substantial volume changes in the reactor.

2.3 Analysis

Synechococcus PCC 7002 growth was monitored daily by measuring the optical density at λ = 750 nm (UV-visible Spectro, Spectronic Unicam®). The dry cell weight (DCW) or dry weight was measured by using cellulose acetate filters of 0.45 µm (Whatman®) at the end of the growth curve (stationary phase). Filters were pre-dried for 10 min at 105 °C in order to remove any moisture. Biomass was filtered and dried for 2 h at 105 °C and then weighed to measure the dry weight, then expressed as grams per liter. All experiments were performed in at least two independent biological replicates.
The carbonate species concentrations were analyzed daily using a common titration method, consisting of the use of two different pH-sensitive dyes, phenolphthalein, and bromocresol green (Sigma-Aldrich®), to determine CO_3^{2-} and HCO_3^- concentrations in the solution (Gris et al., 2014). The carbohydrate content was measured by anthrone method (Morris, 1948; Trevelyan and Harrison, 1952).
To analyze nutrients consumption, samples of culture were filtered (cellulose filter of 0.2 µm) and ammonium ($N–NH_4$) and phosphate ($P–PO_4$) concentrations were measured daily using analytical kits based on standard methods in water and wastewater (APHA, 1992). Ammonium was measured by the absorbance (at 420 nm) of an indophenolic complex produced by the reaction of ammonia with phenolic derivatives (St. Carlo Erba Reagenti ® (Italy) code 0800.05405). Orthophosphates were measured by a modified analytical method using absorbance (at 706 nm) (Sforza et al., 2014).

2.3 Growth and Bicarbonate Assimilation Model

To evaluate the kinetics of absorption of bicarbonate (bicarbonate ion, carbonate, total) and also the cell growth, a first order kinetics represented by Eq. (1) and (2):

$\frac{dS}{dt} = -kt$ (Bicarbonate Consumption) (1)

$\frac{dX}{dt} = \mu t$ (Biomass production) (2)

where dS/dt is the substrate consumption rate (g L^{-1} day^{-1}), k is the absorption rate (day^{-1}), dX/dt is the cell production rate (g L^{-1} day^{-1}) and μ is the growth rate (day^{-1}).

It was carried out a non-linear regression by Eq. (3) to evaluate substrate inhibition:

$$k\ or\ \mu = (k\ or\ \mu)_{max} \cdot \frac{S}{\left(K_S + S + \frac{S^2}{K_I}\right)} \quad (3)$$

where Ks is the semi-saturation constant (g L^{-1}) and K_I is the substrate-inhibition constant (g L^{-1}). Experimental data were fitted to calculate the parameters values.

3. Results and Discussion

For aquatic photoautotrophs, such as cyanobacteria and algae, the availabitliy of CO_2 in liquid phase is low, leading the HCO_3^- bicarbonate ion to be the predominant dissolved inorganic carbon (DIC) specie at neutral or slightly alkaline pH. Cyanobacteria cells are able to import HCO_3^- using specific cellular transporters. In the cells HCO_3^- is concentrated into the carboxysome where it is converted to CO_2 by the activity of different carbonic anhydrases (CA) (Price, 2011; Kamennaya et al., 2015). In order to test the possibility to cultivate *Synechoccocus* PCC 7002 by using bicarbonate ion as a source of carbon instead of CO_2, batch experiments were carried out with different concentrations of sodium bicarbonate, ranging between 5.5 and 88 g L^{-1}. The effect of bicarbonate concentration on potential of biomass production and carbohydrates accumulation was also verified.

When carbon is used by cyanobacteria in the form of bicarbonate ion, its dissociation leads to the formation of H^+ ions with a consequent pH increase (Eq. 4).

$$CO_{2(aq)} + H_2O \overset{pK1}{\longleftrightarrow} H_2CO_3^* \overset{pK2}{\longleftrightarrow} HCO_3^- + H^+ \overset{pK3}{\longleftrightarrow} CO_3^{2-} + 2H^+ \quad (4)$$

(pK_1 = 3.6, pK_2 = 6.3 pK_3 = 10.3)

Thus, in order to avoid cell stress due to these pH changes, the pH needs to be maintained between 7–9 using automatic pH control or buffers. An automatic pH control was necessary since no buffer was used in the culture medium, as it was previously found that *Synechoccocus* PCC 7002 did not grow in the presence of HEPES and Tris-HCl buffers, probably, because buffers exhibit toxicity to some cyanobacterial species (Morales et al, 2002).

Under constant pH, the cyanobacteria grow, suggesting a direct absorption of bicarbonate ion (dominant carbon specie at range 7.5–9.0). Maximum values of OD_{750} 10–12 were reached (Figure 1). The use of very high concentrations of sodium bicarbonate caused a slowdown of cell growth (especially at 88 g L^{-1} of sodium bicarbonate), probably due to substrate inhibition or unbalanced osmotic pressure.

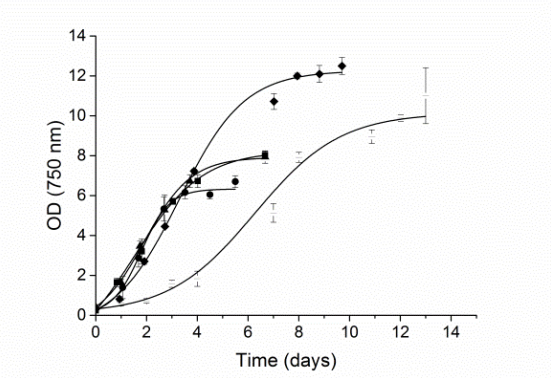

Figure 1: Growth curves of Synechococccocus PCC 7002 under different concentration of sodium bicarbonate: (●) 5.5 g L^{-1}, (■) 11 g L^{-1}, (▲) 22 g L^{-1}, (♦) 44 g L^{-1} and (–) 88 g L^{-1}.

Few authors (i.e., Chi et al., 2013) applied high concentrations of bicarbonate salts (*Euhalotece* ZM 001 14 – 160 g L^{-1}), while lower concentrations (< 15 g L^{-1}) were studied on *Chlorella prothotecoides* (Gris et al., 2014), *Scenedesmus sp.* (Pancha et al., 2015) *Scenedesmus obliquus* (Guangmin et al., 2014),

Thermosynechococcus sp. (Su et al., 2012). Not all the species of microalgae and cyanobacteria are able to exploit large amounts of bicarbonate: despite they have the necessary carriers, they are often inhibited by the substrate concentration (Qiao et al., 2015). Selecting and optimizing the growth conditions of these tolerant bicarbonate species is a recent approach and can substantially reduce production costs.

In our experiment, higher values of DCW were obtained with 44 and 88 g L^{-1} but the highest specific growth rate was found between 5.5–22 g L^{-1} sodium bicarbonate, indicating that between 22–44 g L^{-1} substrate inhibition started (Figure 2A and 2B).

As regards, carbohydrates accumulation, it was clear that while the concentration of nitrogen was not limiting (between 5.5 and 22 g L^{-1}) few carbohydrates were formed (Figure 2C and 2D). But when the amount of N in the stationary phase was low or limiting (22, 44 and 88 g L^{-1} of sodium bicarbonate), a linear accumulation of carbohydrates started, reaching values around 25%. However it is known that *Synechococcus sp* can accumulates up to 50% (% DCW) of carbohydrates with CO_2 as a carbon source (Mollers et al., 2014), and using 88 g L^{-1} was still not a satisfactory condition for carbohydrates production. This is an operational limitation due to the solubility of this salt in water.

The use of N starvation is one of the most common methods for accumulation of high energetic value substances in microalgae (lipids and carbohydrates) (Gonzales-Fernandez and Ballesteros, 2012) mainly for the carbohydrate production (Ho et al., 2013). On the other hand, phosphorus was completely absorbed during the cultivation in all experiments (data not shown).

In all of our experiments, final concentrations of bicarbonate in the medium were approximately 0 (Figure 2D), but in the experiment at 88 g L^{-1} loss of gas bubbles was observed, probably due to the conversion of bicarbonate into CO_2 at high salt concentrations.

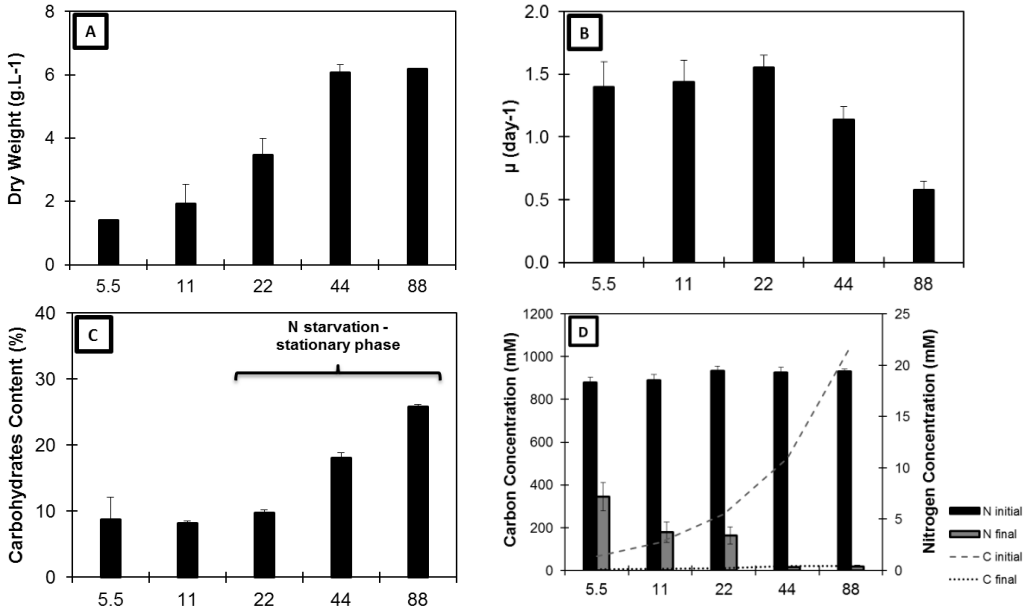

Figure 2: Parameters of Synechococcus Cultivation. A) Dry cell weight, B) Growth rate, C) Carbohydrates content and D) Nutrient consumption.

Synechococcus sp. PCC 7002 showed high bicarbonate absorption, growing well between 5.5 and 88 g L^{-1}, although at 88 g L^{-1}, longer cultivation times were necessary. Productivity values ranged between 0.43–1.12 g L^{-1} day^{-1} (Table 1), with maximum productivity achieved at 22 g L^{-1}, 1.12 g L^{-1} day^{-1}, which is quite high and makes it very attractive for biofuel applications.

The kinetic results are shown in Table 2. In summary, It was observed that with increasing concentration of sodium bicarbonate the assimilation rate of bicarbonate and the cell growth rate decreased above 22 gL^{-1}, showing that there was growth inhibition.

By the constants of inhibition it is noticed that in 21.69 g L^{-1} the absorption of sodium bicarbonate started difficulty, and above the 28 g L^{-1} cell growth was compromised. Stoichiometric relationships found were: 6.9 g sodium bicarbonate → 1 g biomass and 5.1 g bicarbonate ion → 1 g biomass, showing high potential of microalgae for fixation of ion bicarbonate and production of biomass, however, using sodium bicarbonate in batch conditions for carbohydrates production we did not find the maximum value mentioned in literature

(Mollers et al., 2015) requiring optimizations and adaptations of cultivation (fed-batch or continuous cultivation).

Table 1: Productivities of Synechococcocus PCC 7002 cultivation in batch using sodium bicarbonate.

Bicarbonate Sodium Concentration (g L^{-1})	Productivity (g L^{-1} day^{-1})	Days to reach stationary phase
5.5	0.4375	3.6
11	0.4825	3.7
22	1.1229	3.3
44	0.9059	6.6
88	0.6155	9.8

*Productivities were calculated in a time space between the starting of cultivation and the first day of the stationary phase.

Table 2: Kinetics constants for substrate consumption and cellular growth.

Bicarbonate Sodium Concentration (g L^{-1})	$k_{carbonate}$ (day^{-1})	R^2	$k_{bicarbonate}$* (day^{-1})	R^2	k** (day^{-1})	R^2	μ (day^{-1})	R^2
5.5	0.6406	0.9883	0.5357	0.9424	0.6196	0.9671	1.510	0.9783
11	0.6632	0.8510	0.5766	0.9871	0.6817	0.9851	1.611	0.9787
22	0.7889	0.9181	0.748	0.9632	0.6855	0.8873	1.552	0.9938
44	0.3191	0.7464	0.3188	0.8419	0.4516	0.9659	1.210	0.9977
88	0.3134	0.8948	0.2911	0.9738	0.2951	0.977	0.733	0.949

*ion bicarbonate, **total = carbonate + ion bicarbonate

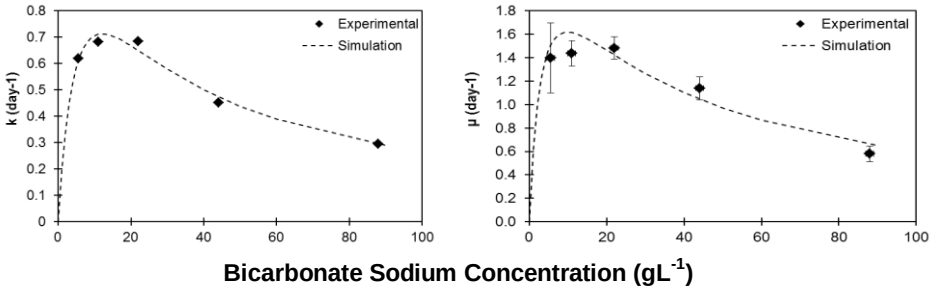

Figure 3: Fitting of Substrate Inibition for Sodium Bicarbonate by Synechococcocus PCC 7002.

Experimental data plotted in Figure 4 were correlated by equations (5) and (6) obtaining:

$$k = k_{max} \cdot \frac{S}{\left(K_S + S + \frac{S^2}{K_I}\right)} = 1.5071 . \frac{S}{\left(6.7346 + S + \frac{S^2}{21.6887}\right)} \qquad R^2 = 0.9525 \qquad (5)$$

$$\mu = \mu_{max} \cdot \frac{S}{\left(K_S + S + \frac{S^2}{K_I}\right)} = 2.7401 . \frac{S}{\left(3.4336 + S + \frac{S^2}{28.5306}\right)} \qquad R^2 = 0.9766 \qquad (6)$$

4. Conclusion

The cyanobacterium *Synechococcus* PCC 7002 was able to accumulate notable amounts of bicarbonate and to produce high amount of biomass (reaching 6 g L^{-1} of dry cell weight and a maximum productivity value of 1.12 g L^{-1} day^{-1} at 22 g L^{-1} of sodium bicarbonate), even though substrate inhibition was noticed when 44 and 88 g L^{-1} were used. Clearly it is a species with high potential for biomass production. However, the range of bicarbonate concentration (5.5–88 g L^{-1}) was still limiting the carbohydrate content achieved which was around 25%, instead of 50% found in literature. It was also observed that accumulation of carbohydrates occurred when there was a limitation of nitrogen in stationary phase.

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