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Effect of Carbon–Nitrogen Ratio for the Biomass Production, Hydrocarbons and Lipids on *Botryoccus braunii* UIS 003.

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The genus *Botryococcus* compiles a variety of green microalgae which accumulate large quantities of hydrocarbons, this genus is classified in four types or races (A, B, L and R) based on chemical structure of hydrocarbons. Race B has been acknowledge due to its hability to accumulate triterpene hydrocarbons C_{30}-C_{37} best known as botriococene and methylated squalene C_{31}-C_{34} which are considerate as candidates for biofuels production; however, one of the main problems that face biofuels production using this alga as feedstock is the continuous production, both lipids and hydrocarbons; that is why it's mandatory to find the best carbon and nitrogen source that maximizes biomass and total lipid production.
It was found that by adjusting the carbon/nitrogen ratio using sodium carbonate (Na_2CO_3) it was possible to substantially improve the production of biomass from 1 to 2 g/L in 15 days thus doubling the production of biomass in the same time; however, both the lipids and hydrocarbons production will not be affected positively showing a significant reduction from baseline.

1. Introduction

The high levels of Botryococenes on *Botryococcus braunii* Race B Kützing, F.T. (1849) and it is ability to self agegate and create natural blooms has raised expectations on the possibility to use this alga as a sustainable feedstock for biofuels production and other high valuable chemicals (Metzger & Largeau, 2005). However, in order to improve lipids concentration (especially hydrocarbons) and biomass production, the alga require high concentrations of carbon source (CO_2, glucose sodium acetate, etc).
According with literature the addition of carbon to the mass cultivation of microalgae represents the main limitation (Tapieh & Bernard, 1988; Olaizola et al, 1991) because high concentrations may inhibit growth or accumulation of certain metabolites; on the other hand low concentrations can limit it (Rados et al., 1975). These ranges (inhibitory and limiting) vary from species to species, so concentration must not only be lower than a certain value which satisfies the algae need for carbon, but also not to exceed in order to avoid a massive loss which ultimately can lead to unnecessary waste (Cheng et al., 2006) and significantly raise production costs.
Many studies has been published since the Bloom at Darwin River in 1979, there are several barriers to the mass cultivation of these particular microalgae. First this alga has a slow growth rate therefore bacteria, cyanobacteria and even other microalgae may colonize an open system (Metzger & Casadevall, 1992).
Studies such as those published by Dayananda *et al.,* (2005, 2006 and 2007) and Ranga Rao (2007, 2010), shed light on how to operate a low-contamination crop in open ponds. There are several studies on the improvement of the culture medium, ranging from carbon source (Tran et al, 2010; Tanoi et al, 2011; Bazaes et al, 2012; Sarada Rao, 2012), nitrogen (Dayananda et al., 2007), phosphorus (An et al., 2003), even the effect of the concentration of CO_2 in the botriococene deposition (Ge et al., 2011).
Others authors such as Ruangsomboon (2011) evaluate several nutrients (nitrogen, phosphorus and iron), light intensities and light/dark cycles, finding that as in other algae by decreasing the initial concentration of

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nitrogen the final concentration of lipids can be improved. However, each of these variables was assessed independently; therefore, the effect of their interaction on the deposition on lipids is still unknown.
When it comes to process optimization on culture media, one of the most troublesome the production of microalgae nutrient is nitrogen, due to its consumption rate is important in metabolic regulation for biomass production (Solovchenko et al, 2008; Takagi et al., 2000). In addition, its consumption rate is linked to the assimilation rate of carbon, according to Huppe and Turpn (1994) up to 55% of the whole carbon assimilated by the algae is committed to nitrogen metabolism, due to this, it is necessary to evaluate how these two elements affect the overall biomass production and lipid deposition on this algae.

2. Methodology

2.1 Microalgae culturing

A Novel strain of *B. braunii* Race B was solated from an artificial pond in Colombia, the alga was maintained on on 500 mL Bold Basal Culture Media (BBM) (Bischoff and Bold 1963) on 1000 mL flask and mixed using filtered air (0.2 μm membrane filter) with 1% (v/v) of CO_2.

2.2 Carbon/Nitrogen ratio

In order to prove the effect of Carbon and Nitrogen ratio a 3^3 Central composite Design was applied using STATISTICA® 7.0, After 15 days of culture, 100 mL of algae were mixed with 100 mL of BBM on 500 mL flasks containing different concentrations of $NaNO_3$ and KNO_3 (Table 1).

Table 1: Variables obtained for the Design of Experiments 3^3

Exp	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
KNO_3 (g(L)	0	0.75	0.38	1.54	0.75	1.5	0.38	1.5	0.75
Na_2CO_3 (g/L)	0.94	0.49	0.63	0.94	.094	1.26	12.6	0.63	1.39

2.3 Biomass, lipids and hydrocarbons quantification

For biomass quantification every 5 days over 15 days 20 mL of culture medium were filtered using Whatman GF/C (pre-combusted for 1 hour at 100°C), filters were dried for 1 hour at 100°C followed by 12 hours in desiccator until constant weight.
For the extraction of lipids a modification of Eroglu & Melis (2010): 10 mL of culture were mixed with 5.7 mL of Bligh & Dyer solution and 50 mg 0.5 mm glass beads on 50 mL Falcon tube and homogenized with vortex for 15 minutes at top speed, 7 mL of Bligh & Dyer solution were added for a second round of extraction. The mixture was centrifuged at 3400 rpm for 15 minutes in order to separate the biomass from the lipidic phase, 3 mL of analytical chloroform were added and the mixture was left in a refrigerator for 24 hours. After 24 hours the water layer was removed, the lipid phase was transferred to pre-weighed glass tubes and dried at 40°C under low-pressure nitrogen atmosphere and weighed to obtain the total weight of extracted lipids.
For the quantification of hydrocarbons a modification of Eroglu & Melis (2010) was applied as follows. 20 mL of culture were mixed with 10 mL of Analytical Heptane and 50 mg 0.5 mm glass beads on 50 mL Falcon tube and homogenized with vortex for 15 minutes at top speed, another 5 mL of Heptane were added for a second round of extraction. In order to separate the biomass from the hydrocarbons phase 10 mL of distilled water were added to the mixture and centrifuged at 3400 rpm for 15 minutes. The hydrocarbon phase (upper layer) was removed using 5 mL micropipette, transferred to a 2 mL quartz cuvette, and measured on a spectrophotometer at 190 nm.

3. Results and discussion

Biomass concentration using the carbon/nitrogen ratio is presented in Figure 1. All experiments show a significant increase from day 10. At day 15 the best results for the production of biomass were the F, D, and I reaching about 1 g/L, this results are consistent with those described by Rao and Sarada (2012) who found that a concentration of 0.1% (w/v) of Na_2CO_3 can positively affect biomass production.

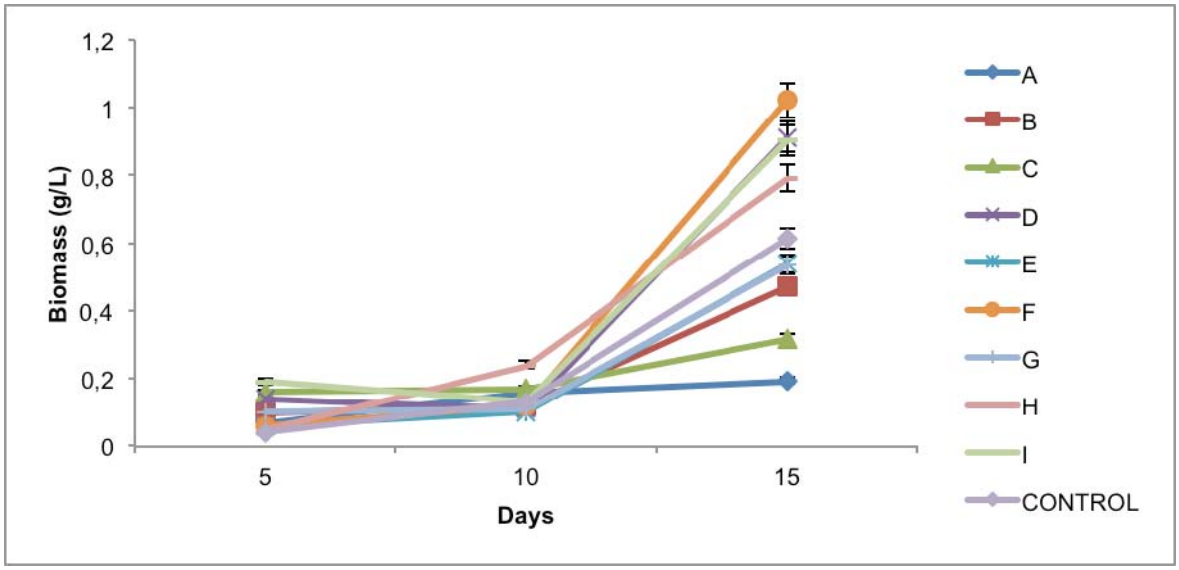

Figure 1. Biomass production

According with the surface response for lipids and hydrocarbons (Figure 2) In order to increase lipid accumulation high concentrations of Na_2CO_3 (> 1.3 g/L) and low concentrations of KNO_3 are required (<0.2 g/L), on the other hand to promote hydrocarbon, slightly lower concentrations of Na_2CO_3 (0.8 to 1.2 g/L) and a higher concentration of KNO_3 (0.6 to 1 g/L) are required. Given the above results it is possible to assume that hydrocarbon accumulation occurs early, requiring high concentrations of nitrogen, while the accumulation of other lipid occurs later when the nitrogen source has been consumed. However the need for high concentrations of carbon for each of the above metabolites shows that the carbon source must be added several times to maintain optimal levels favoring the synthesis thereof.

According to Zhila *et al* (2005) under nitrogen deprivation (specifically KNO_3) the concentration of certain polar lipids in *B. braunii* changes drastically especially on oleic acid; in addition according to Cheng *et al* (2013) under nitrogen limitations the final content of lipids and hydrocarbons increase substantially, while the chemical profile of hydrocarbons remains almost the same, the ratio of lipid changes especially on the oleic and linoleic acid.

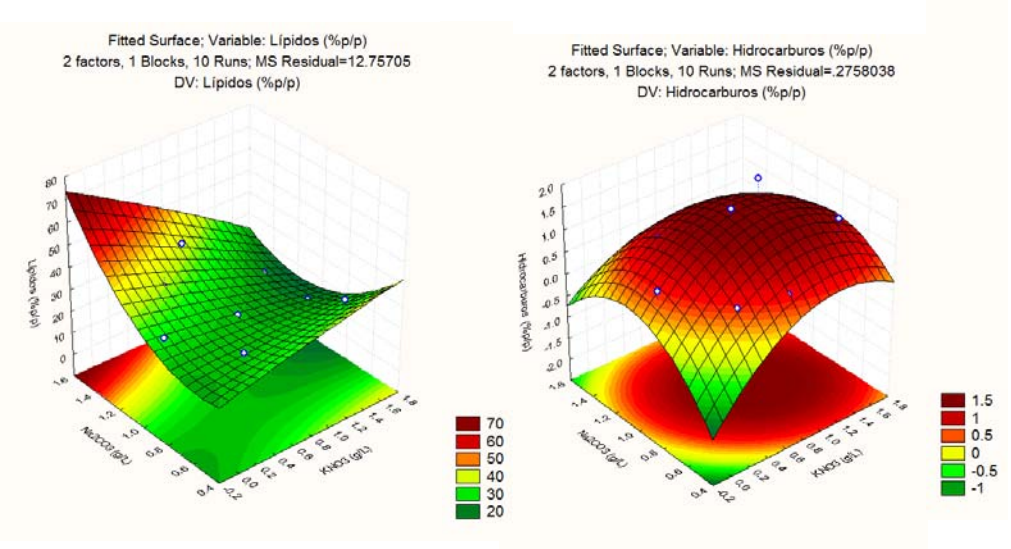

Figure 2. Surface response for lipids (left) and hydrocarbons (right)

Considering the results obtained using Na_2CO_3 three new experiments for the improvement of biomass production, and lipid and hydrocarbon accumulation were proposed (Table 2).

Table 2. Best concentrations using carbon/nitrogen ratio.

	KNO_3 (g/L)	Na_2CO_3 (g/L)
A	0	0.94
B	0.75	0.49
C	0.38	0.63
CONTROL	1.50	0

In Figure 3 the results for biomass concentration are exposed. After 15 days of culture is possible to obtain up to 2 g/L, considering that according to the initial data can be obtained in 15 days to 1 g/L, the improvement in the C/N allowed a doubling on the biomass produced.

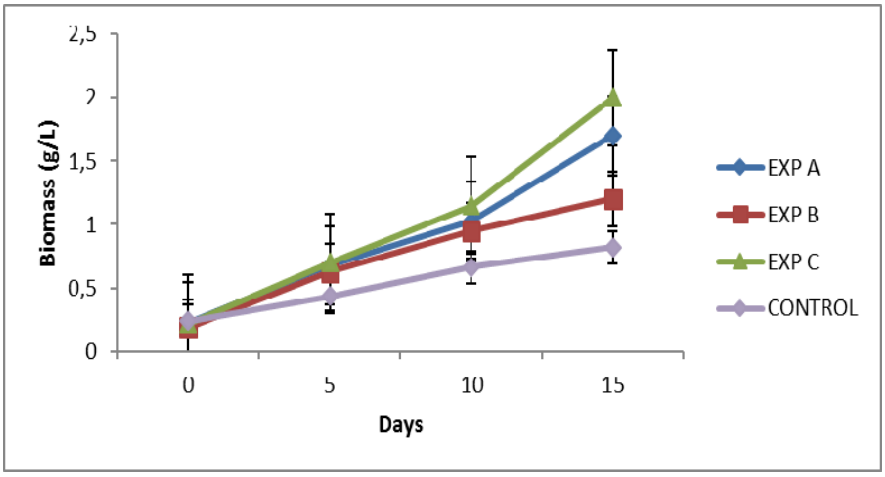

Figure 3. Production of biomass using improved C/N ratios

Even with significant results in biomass production both production of lipids and hydrocarbons (Figure 4 and 5) were not affected positively presenting a significant reduction from baseline. Therefore it is possible to conclude that a single addition of Na_2CO_3 is sufficient to positively influence the production of biomass, but not in the production of lipids and hydrocarbons. According to these results, it would be necessary to add a certain amount of Na_2CO_3 daily basis, for ensuring a significant increase in the lipid and hydrocarbons deposition.

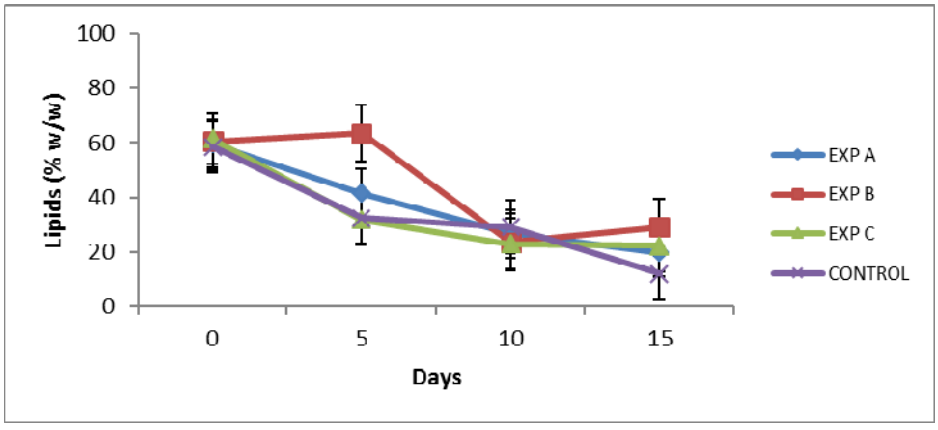

Figure 4. Production of lipids using improved C/N ratios

However, it is necessary to monitor the effect of a considerable addition of this carbon source, since an excess of sodium would occur, which could result in growth inhibition. According with Furuhashi *et al* (2013), the use of high concentrations of seawater adversely affect the production of biomass; therefore, a culture with continuous or semi-continuous addition of Na_2CO_3 can reduce significantly biomass production.

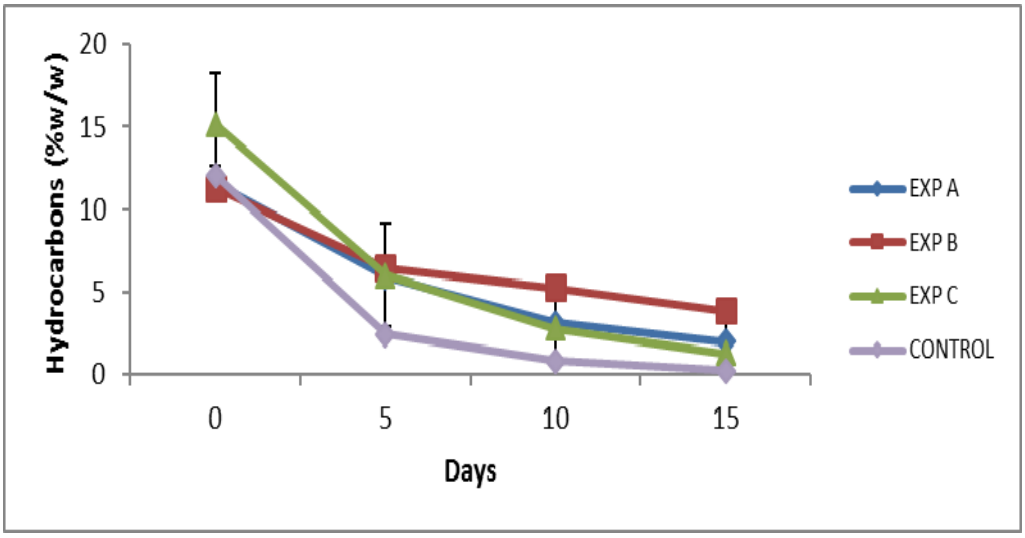

Figure 5. Production of hydrocarbons using improved C/N ratios.

4. Conclusions

By adjusting the carbon/nitrogen ratio using sodium carbonate (Na_2CO_3) as carbon source it was possible to substantially improve the production of biomass from 1 to 2 g/L in 15 days, thus doubling the production of biomass in the same time. However, both the lipids and hydrocarbons production were not affected positively showing a significant reduction from baseline making it possible to conclude that a single addition of Na_2CO_3 is sufficient to positively influence biomass production but not in the production of lipids and hydrocarbons. Further studies should be foused on how this carbon source can be used effectively in order to stabilize the accumulation of lipids and hydrocarbons.

Acknowledgments

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