IMPROVEMENT OF LAB–SCALE PRODUCTION OF MICROALGAL CARBOHYDRATES FOR BIOFUEL PRODUCTION

MEJORAMIENTO EN LA PRODUCTIVIDAD DE CARBOHIDRATOS MICROALGALES A ESCALA–LABORATORIO PARA LA OBTENCIÓN DE BIOCOMBUSTIBLES

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ABSTRACT

This work studied the improvement of biomass and carbohydrate (glucose and xylose) lab–scale productivity in *Chlorella vulgaris* UTEX 1803 through the use of the carbon/nitrogen ratio. In order to do so, mixotrophic cultures were made by the modification of initial concentration of CH_3COONa (5, 10 and 20 mM) and $NaNO_3$ (0.97, 1.94 and 2.94 mM). All treatments were maintained at 23 ± 1°C, with light/dark cycles of 12h : 12h for 5 days.

It was found that in addition to the carbon/nitrogen ratio, time also influences the concentration of biomass and carbohydrates. The treatment containing 10 mM acetate: 1.94 mM nitrate, reached a concentration of 0.79 g/L of biomass, 76.9 μg/mL of xylose and 73.7 μg/mL of glucose in the fifth day. However, the treatment containing 20 mM acetate: 0.97 mM nitrate produced 1.04 g/L of biomass, 78.9 μg/mL of xylose and 77.2 μg/mL of glucose in the third day, while in the same day the treatment containing 0 mM acetate: 2.94 mM nitrate, produced 0.55 g/L of biomass, 40.2 μg/mL of xylose and 31.3 μg/mL of glucose.

The use of carbon/nitrogen ratios improved biomass productivity (from 0.55 to 1.04 g/L) as well as xylose (from 40.2 to 78.9 μg/mL) and glucose (from 31.3 to 77.2 μg/mL) concentration, representing an improvement of up to two times the production of both biomass and carbohydrates in only 3 days of culture.

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RESUMEN

En este trabajo se estudió el mejoramiento en la concentración de biomasa y carbohidratos (glucosa y xilosa) en *Chlorella vulgaris* a escala laboratorio utilizando la relación carbono/nitrógeno; para lograr esto, se realizaron cultivos mixotróficos utilizando modificaciones en la concentración inicial de *CH₃COONa* (5, 10 y 20 mM), y *NaNO₃* (0.97, 1.94 y 2.94 mM). Los tratamientos fueron realizados a 23±1°C, con ciclos luz–oscuridad 12h : 12h, durante 5 días.

Se encontró que además de la relación carbono/nitrógeno, el tiempo influye en la concentración de biomasa y carbohidratos. Al quinto día el tratamiento 10 mM acetato: 1.94 mM nitrato alcanzó una producción de 0.79 g/L de biomasa, 76.9 μg/mL de xilosa y 73.7 μg/mL de glucosa, sin embargo, al tercer día el tratamiento 20 mM acetato: 0.97 mM nitrato produjo 1.04 g/L de biomasa, 78.9 μg/mL de xilosa y 77.2 μg/mL de glucosa, mientras que el tratamiento 0 mM acetato: 2.94 mM nitrato produjo 0.55 g/L de biomasa, 40.2 μg/mL de xilosa y 31.3 μg/mL de glucosa.

Al implementar la relación carbono/nitrógeno, es posible mejorar las productividades de biomasa (de 0.55 hasta 1.04 g/L), xilosa (de 40.2 hasta 78.9 μg/mL) y glucosa (de 31.3 hasta 77.2 μg/mL), representando un mejoramiento de hasta 2 veces en biomasa y carbohidratos en 3 días de cultivo.

Palabras clave: *Chlorella vulgaris, Cultivo mixotrófico, Glucosa, Xilosa, Microalgas, Biomasa, Tratamiento con acetato de sodio, Tratamiento con nitrato de sodio.*

RESUMO

Neste trabalho estudou–se o melhoramento na concentração de biomassa e carboidratos (glucose e xilose) em Chlorella vulgaris a escala laboratório utilizando a relação carbono/nitrogênio; para conseguir isto, realizaram–se culturas mixotróficas utilizando modificações na concentração inicial de CH₃COONa (5, 10 e 20 mM), e NaNO₃ (0.97, 1.94 e 2.94 mM). Os tratamentos foram realizados a 23±1°C, com ciclos luz–escuridão 12h : 12h durante 5 dias. Encontrou–se que além da relação carbono/nitrogênio, o tempo influi na concentração de biomassa e carboidratos; no quinto dia o tratamento 10 mM acetato: 1.94 mM nitrato alcançou uma produção de 0.79 g/L de biomassa, 76.9 μg/mL de xilose e 73.7 μg/mL de glucose. Entretanto, no terceiro dia o tratamento 20mM acetato: 0.97 mM nitrato produziu 1.04 g/L de biomassa, 78.9 μg/mL de xilose e 77.2 μg/mL de glucose, enquanto que o tratamento 0 mM acetato: 2.94 mM nitrato produziu 0.55 g/L de biomassa, 40.2 μg/mL de xilose e 31.3 μg/mL de glucose. Ao implantar a relação carbono/nitrogênio, é possível melhorar as produtividades de biomassa (de 0.55 hasta 1.04 g/L), xilose (de 40.2 até 78.9 μg/mL) e glucose (de 31.3 até 77.2 μg/mL), representando um melhoramento de até 2 vezes em biomassa e carboidratos em 3 dias de cultura.

Palavras chave: *Chlorella vulgaris, Cultura mixotrófica, Glucose, Xilose, Microalgas, Biomassa, Tratamento com acetato de sódio, Tratamento com nitrato de sódio.*

1. INTRODUCTION

Microalgae are considered one of the most versatile micro–organisms in terms of their size, shape and ecological function (Vonshak, 1990). In addition, they are able to convert solar energy into chemical energy through photosynthesis (Hosikian, Lim, Halim & Danquah (2010). As a result, they have been recognized as a source of different products, and recently, as a raw material for biofuel extraction (Chisti, 2007) including oil–derived Biodiesel (Sheehan, Dunahay, Benemann & Roessler, 1998; Banerjee, Sharma, Chisti & Banerjee, 2002; Gavrilescu & Chisti, 2005), photobiologically produced biohydrogen (Melis, 2002; Fedorov, Kosourov, Ghirardi & Seibert, 2005; Kapdan & Kargi, 2006). Biomethane produced by the anaerobic digestion of microalgal biomass (Spolaore, Joannis–Cassan, Duran & Isambert, 2006) and bioethanol derived from algae (micro and macro–algae) (Soojin *et al*., 2011; Zhou *et al.*, 2011) However, the idea of using microalgae as a fuel source is not new (Chisti, 1981; Nagle & Lemke, 1990; Sawayama, Inoue, Dote & Yokoyama, 1995), but it has gained greater relevance due to oil prices, political instability in producing countries, environmental challenges and, more significantly, the emerging concern about global warming which is associated with the burning of fossil fuels (Gavrilescu & Chisti, 2005). Although they could potentially provide a solution to this global problem. works such as the study by Gonzalez and Kafarov (2011) demonstrated that the production of microalgae focused only on biodiesel production has a high cost, making it necessary to fully use their various metabolites (carbohydrates, proteins and triglycerides, among others) that can be later transformed into high added value products and biofuels through the application of other processes. An important aspect for the use of microalgae under the biorefinery concept is the increase in biomass productivity. This has been achieved by variations in the microalgae culture conditions where the type and concentration of nutrients, temperature, light intensity, photoperiod, genetic manipulation, etc., are assessed (Thompson, Guo & Harrison, 1992; Molina *et al.*, 1995; Fábregas *et al.*, 1996).

Most of the published studies regarding carbohydrate extraction have been focusing on macroalgae such as *Ulva* sp. and *Enteromorpha* sp. which have carrageenan, alginate, fucoidan and agar–agar (Lahaye,1991; Lahaye & Robic, 2007; Lahaye, Gómez, Jimenez & Garcia, 1995; Ray, 2006), as well as several of these polysaccharides in *Ulva clathrata* (Hernández–Garibay, Zertuche–González & Pacheco–Ruíz, 2011). In the case of *Chlorella* sp. Zhou *et al*. (2011) found that cellulose, hemicellulose and starch accounted for 35.28, 10.19 and 20.25% of dry biomass respectively; a more detailed study found that up to 22% of its total biomass are carbohydrates, mainly rhamnose, arabinose, xylose, mannose, galactose, glucose and uronic acid (Zhongquan, Gizaw & BeMiller. 2012). Even knowing the detailed composition in sugars (mono and polysacharides), effect of the concentration of some nutrients in the amount of carbohydrates present in the biomass of *C. vulgaris* is unknown.

It has been considered that the ratio between carbon and nitrogen influences biomass production (Flynn, 1991; Flynn, Davidson & Cunningham, 1993; Huppe & Turpin, 1994) and lipid content (Illman, Scragg & Shales, 2000; Scragg, Illman, Carden & Shales, 2002; Lin & Lay, 2004). Nitrogen assimilation (both as NO_3^- and as NH_4^+) in microalgae is an expensive process that is closely related to carbon metabolism in autotrophic and heterotrophic cultures (Turpin *et al.*, 1995; Turpin 1991) since up to 55% of the assimilated carbon is committed for the assimilation and metabolism of nitrogen (Huppe & Turpin, 1994).

The initial concentration and the rate of assimilation of certain nutrients such as nitrogen are important in cellular metabolic regulation. Nitrogen is a stress factor in algae growth, and its limited supply could significantly change the composition of the organism (Solovchenko *et al.*, 2008; Takagi, Watanabe, Yamaberi & Yoshida, 2000).

In the case of *Chlorella vulgaris* studies have mainly focused on the increase in lipid content (Illman *et al.*, 2000; Scragg *et al.*, 2002; Heredia–Arroyo, Wei & Hu, 2010). However, there are no studies that evaluate the effect of nitrogen or carbon (or both) on the rate and concentration of carbohydrates for this or any other species. Even knowing about its existence, it is not possible to generalize the carbon/nitrogen relation in microalgae, given that there is a potential to disrupt

these organisms simply by altering the lighting level during sampling, sample manipulation and experimental incubations (Hitchcock, Goldman & Dennett, 1986). There are also differences between microalgae species and groups both in the rate and magnitude of the responses and the threshold that makes these responses significant (Flynn, 1991). However, in order to obtain high concentrations of specific metabolites such as lipids protein and carbohydrates it is necessary to find the specific carbon: nitrogen ratio the purpose of this study is to improve on lab–scale the production of carbohydrates present in *Chlorella vulgaris* through the implementation of the carbon: nitrogen ratio.

2. METHODS

Culture Conditions

Chlorella vulgaris UTEX 1803, from the collection of algal cultures at the University of Texas (Austin, TX, USA) was used. Initially, the strain was maintained in laboratory–scale photobioreactors using Bold Basal culture media (BBM) whose composition is (M): *NaNO$_3$* (2.94 X 10^{-3}), *MgSO$_4$.7H$_2$O* (3.04 X10^{-4}) *NaCl* (4.28 X 10^{-4}), *K$_2$HPO$_4$* (4.31 X 10^{-4}), *KH$_2$PO$_4$* (1.29 X 10^{-3}), *CaCl$_2$.2H$_2$O* (1.70 X 10^{-4}) and micronutrients (M) *ZnSO$_4$.7H$_2$O* (3.07 X 10^{-5}), *MnCl$_2$.4H$_2$O* (7.28 X 10^{-6}), *MoO$_3$* (4.93 X 10^{-6}), *CuSO$_4$.5H$_2$O* (6.29 X 10^{-6}), *Co(NO$_3$)$_2$.6H$_2$O* (1.68 X 10^{-6}), *H$_3$BO$_3$* (1.85 X 10^{-4}), EDTA (1.71 X 10^{-4}), *KOH* (5.53 X 10^{-4}), *FeSO$_4$.7H$_2$O* (1.79 X 10^{-5}). 2.5 L Cylindrical airlift reactors (14 cm of internal diameter and 35 cm height) with 2 L of culture were used. The culture was mixed using filtered air (0.2 μm membrane filter) flow of 0.6 L/min without any additional *CO$_2$* supply.

Sodium acetate and sodium nitrate concentrations in mixotrophic cultures were varied (Table 1) based on the results obtained by Hernández–Benitez and Rosas–Oviedo (2011). Each of the experiments (including the control) was inoculated with approximately 0.4 g/L (in dry weight) of fresh biomass and performed in triplicate for 5 days under light:dark cylces of 12:12 hours The cultures were mixed using filtered air (0.2 μm membrane filter) flow of 0.6 L/min without any additional *CO$_2$* supply. Initial pH, temperature and light intensity were 7 ± 1, 23 ± 2°C y 480 ± 1 μmol photons/m^2/s.

Sample Analysis

Quantification of biomass, xilose, glucose concentration and phosphate consumption rate were measured by triplicate in the morning and evening for five consecutive days.

Table 1. Mixotrophic cultures for C. *vulgaris*.

Sodium nitrate \ Sodium acetate	5mM	10mM	20mM
0.97mM	T1	T2	T3
1.94mM	T4	T5	T6
2.94mM	T7	T8	T9

Biomass Quantification

For measurements of biomass concentration, a sample of 6 mL of each culture was taken. Subsequently sample was diluted to a ratio: 1:8 sample:distilled water. The diluted sample was measured at 500 nM using a *Spectroquant Pharo* 300 spectrophotometer (Merck). The optical density (OD) was correlated to the amount of biomass (g/L) in accordance with the *Equation 1*:

$$C_B = 1.4602 * DO500 \qquad (1)$$

Determination of Carbohydrates

The Phenol–Sulphuric acid colorimetric method (Dubois *et al.*, 1956) was used. This method allows the differentiation of the various types of sugars present in the sample and is more sensitive than methods such as the DNS. A 1 mL sample of each culture was centrifuged for 20 min at 3400 rpm to separate the biomass from the liquid phase. To avoid a possible interference generated by *chlorophylls*, pigments were extracted from the separated biomass through the addition of 10 mL of ethanol. The mixture was subsequently heated for 10 min at 25°C in a water bath and centrifuged again for 20 min at 3400 rpm. Two phases were obtained after centrifugation, the liquid phase corresponding to the extracted pigments and the pigment–free biomass pellet. To avoid the color of the pigments altering the measurements, 0.3 g of activated carbon were added to the liquid phase, then shaken for a few seconds and filtered. Subsequently, 1 mL of 5% phenol solution
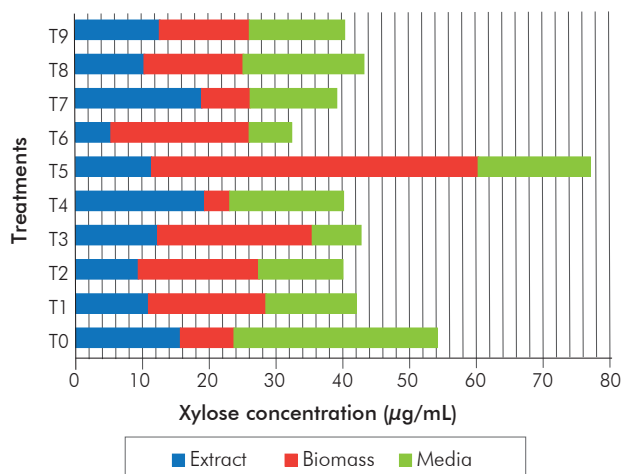
Figure 1. Xylose concentration on the fifth day of culture for the nine treatments.
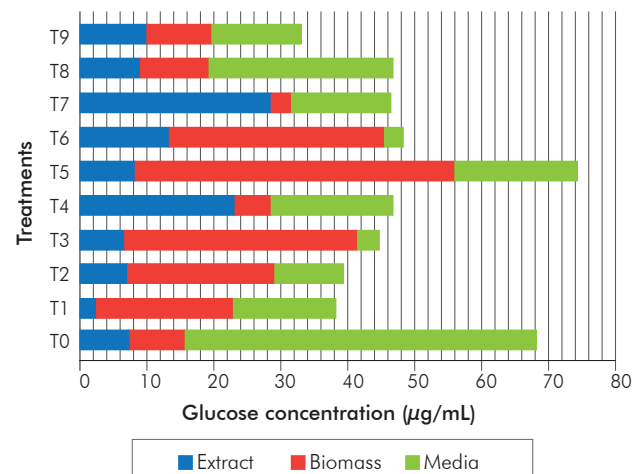

Figure 2. Glucose concentration on the fifth day of culture for the nine treatments.

and 5 mL of 95.5% sulphuric acid were added to each phase. Finally, each of these samples was transferred to the colorimetric cells. Absorbance at 480 nm was measured for identification of xylose and 490 nm for identification of glucose, in a *Spectroquant Pharo* 300 spectrophotometer (Merck®). The spectrophotometric absorbance was converted to carbohydrate concentration using a calibration curve established for xylose and glucose respectively (Annexes A and B).

Determination of Phosphate Consumption Rate

The standard wastewater determination methods book (Clesceri, Greenberg & Eaton, 1999) was used for this measurement. Following this procedure, a 30 mL sample was taken from each treatment, centrifuged for 20 min at 3400 rpm and then vacuum filtered using a 0.45 μm Wattman nylon filter. 10 mL of metavanadate–molybdate solution (which consist of 8.33 g/L of ammonium molibdate and 4.16 g/L of ammonium metavanadate) was added to each sample, and then diluted to a 50 mL volume with distilled water. Finally the sample was measured in the spectrophotometer at a wavelength of 470 nm.

Statistical Analyses

The data obtained were analyzed using the SPSS 15.0, STATISTICA 7.0 statistical package and STATGRAPHICS centurion XVI.I. Normality (Shapiro–Wilk) and analyses of variance (ANOVA) tests were performed in order to establish differences and similarities among the different analyzed variables.

All analyses were performed with a significance level of 95%.

3. RESULTS

Concentrations of xylose and glucose after 5 days are shown in Figures 1 and 2. Treatment T5 (10 mM sodium acetate, 1.94 mM sodium nitrate) presented the highest concentrations of xylose (76.9 μg/mL) and glucose (73.7 μg/mL), the total xylose produced was distributed as follows: 48.9 μg/mL (63.6%) in the biomass, 11.21 μg/mL in the pigments extract (14.6%) and 16.8 μg/mL in the culture media (21.8%). Similarly, the total glucose produced was distributed as follows: 47.7 μg/mL in the biomass (64.7%), 8.04 μg/mL in the pigments extract (10.9%) and 17.9 μg/mL in the media (24.4%). On the other hand, the highest concentration of carbohydrates present in the culture media was obtained in the control treatment (T0: 0 mM sodium acetate, 2. 94 mM sodium nitrate), with 56% xylose and more than 70% glucose, showing that the changes made in the treatments allow sugars to remain in the microalgae.

During the experimentation time there were no significant differences (F: 0.264, p: 0.8510) in glucose and xylose concentrations, both in the morning and in the evening, for each of the different treatments. However, there were significant differences among the treatments (F: 3.03, p: 0.0004).

Although in the different treatments the reached xylose and glucose concentrations during five days of culture are significant, it is also possible to obtain similar (or higher) values of carbohydrates on the pigments–free biomass after three days of culture (Figures 3, 4 and 5). This phenomenon occurred only in treatments T3, T6 and T9, which have the highest concentration of sodium acetate (20 mM) in comparison with the other treatments. Form this treatments the highest concentrations were found in T3 (20 mM sodium acetate, 0. 97 mM sodium nitrate), with values for xylose of 58.3 μg/mL in the biomass after pigments extraction, 6.49 μg/mL on the pigments extract and 14.1 μg/mL in the culture media for a total of 78.95 μg/mL of xylose; in the case of glucose it was found values of 63.32, 6.75 and 7.13 μg/mL obtained from the biomass after pigment extraction, on the pigments extract and culture media, respectively, for a total of 77.21 μg/mL of glucose (Figures 5 and 6).
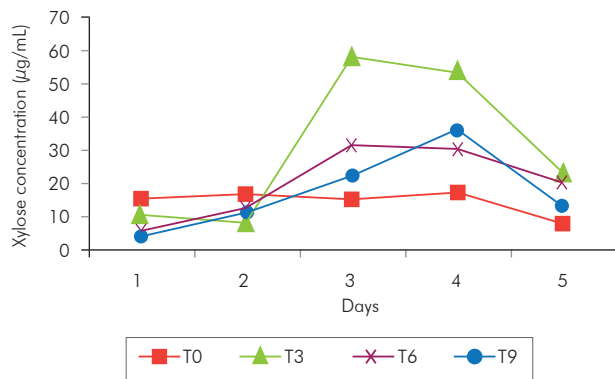

Figure 3. Xylose concentrations for treatments T3, T6, T9 and T0 (control) during the five days of culture.
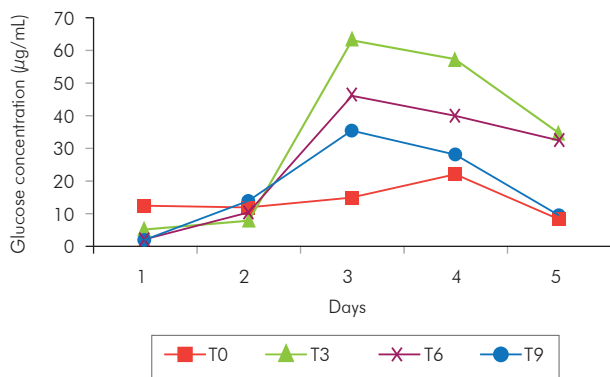

Figure 4. Glucose concentrations for treatments T3, T6, T9 and T0 (control) during the five days of culture.
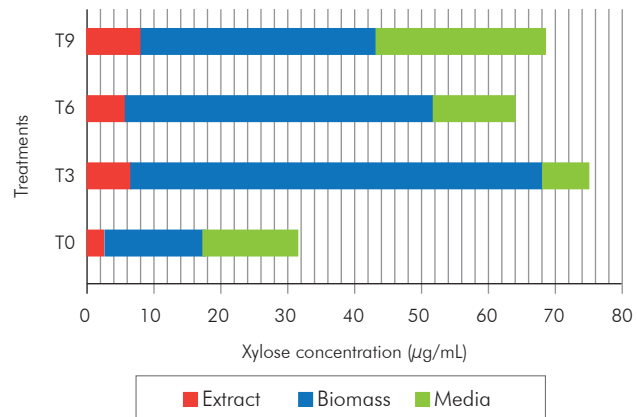

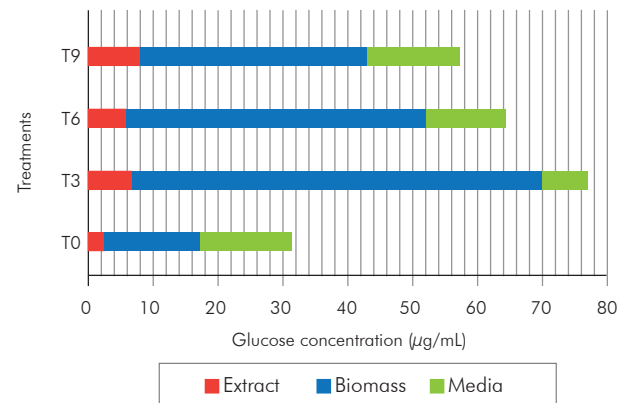**Figure 5.** Xylose concentration on the third day of culture for treatments T3, T6, T9 and T0.

Figure 6. Glucose concentration on the third day of culture for treatments T3, T6, T9 and T0.
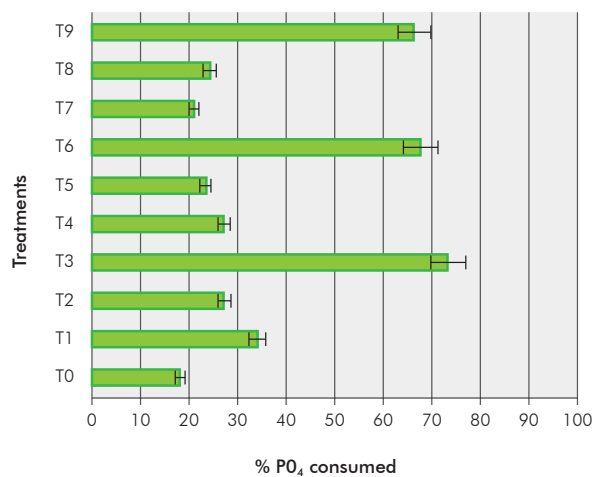

Figure 7. Phosphate consumption of cultured *Chlorella vulgaris* in all treatments.
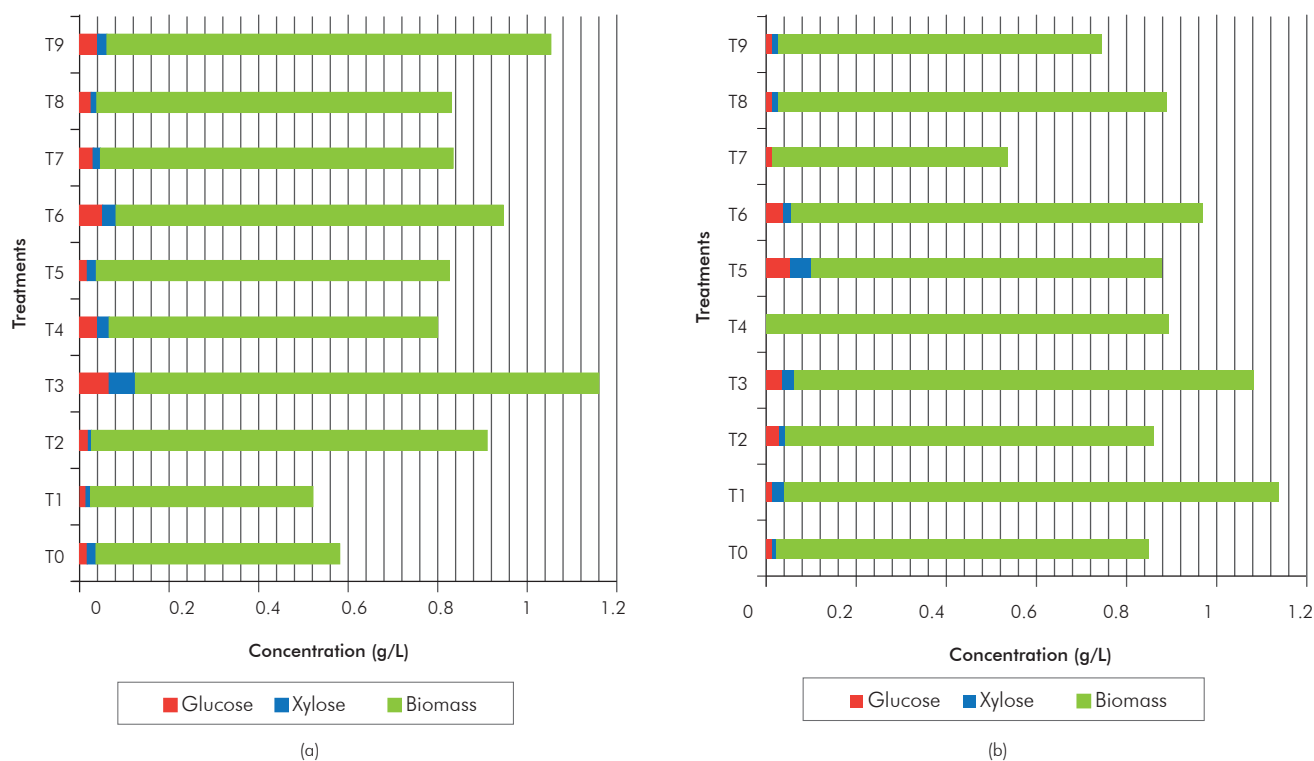
Figure 8. Concentration of sugars in the biomass and biomass concentration of *Chlorella vulgaris* for all treatments on the third (a) and fifth (b) days of culture.

Results shown that the amount of sodium acetate is directly proportional with phosphate consumption to each of the treatments (Figure 7). In addition, statistical analysis shows significant differences in phosphate consumption and among the different treatments (F: 213.6, p: 0.00), however there is no relation between the phosphate rate consumption and biomass concentration. The treatment with the highest phosphate consumption in the culture media (76%), highest biomass concentration (1.04 g/L) and highest carbohydrate concentration (xylose 0.6 g/L and glucose 0.06 g/L) was T3, followed by T6, with a phosphate consumption of 67% and biomass, xylose and glucose concentration was 0.86 g/L, 0.03 g/L and 0.05 g/L, respectively, and finally, T9, consumed 66% phosphate with a concentration of 1 g/L of biomass, 0.02 g/L of xylose and 0.04 g/L of glucose.

Table 2. Total xylose and glucose concentrations on the third and fifth days of culture for the treatments with highest sugar concentrations (T3, T5, T6 y T9) and control (T0).

	Xylose		Glucose	
	Day 3	Day 5	Day 3	Day 5
	% in Biomass (w/w)	% in Biomass (w/w)	% in Biomass (w/w)	% in Biomass (w/w)
T3	7.60	4.10	7.50	4.30
T5	6.60	9.70	7.90	9.30
T6	5.90	3.40	7.40	5.20
T9	4.70	5.40	6.90	4.50
T0	5.60	6.40	4.30	8.10

Table 2 shows a comparison between the treatments with the highest concentration of sugars (T3, T5, T6 and T9) and the control (T0). It can be observed that on the third day of culture, the amounts of xylose and glucose range between 46–78.9 and 62.2–77.2 μg/mL respectively, exceeding the values obtained by the control (40.2 μg/mL of xylose and 31.3 μg/mL of glucose). Similarly, the percentages of total xylose and glucose produced in regard to biomass quantities were quantified. It was found that on the third day, these percentages ranged from 4.3 to 5.6% in the control, and on the fifth day they were between 6.4 and 8.1%, while for the evaluated treatments they were between 4.7 and 7.9% on the third day and 3.4 and 9.7% on the fifth day.

4. DISCUSSION

Algae play a vital role in the biogeochemical carbon cycle. Although many are able to use different sources of organic carbon, CO_2 is the main source of carbon for microalgae. However the use of other carbon sources can improve not only biomass production, but also the production of specific metabolites. The stress generated by nitrogen shortage compared to the carbon resource can affect the cells' response to nutrient availability, consequently affecting growth rates and the consumption of the carbon source, mainly CO_2 fixation (Flynn, 1991). This relates to the cell's prior nutrition history. Previous studies report that some cells that suffered stress because of a slight decrease in nitrogen availability (which affects transport and nitrate assimilation functions although not necessarily limiting the growth rate) were not able to use nitrate at night, while cells that had high nitrate limitations were able to do so (Caperon, 1968). This likely reflects changes in the metabolism of carbon and nitrogen at higher stress levels.

The results of this study are in line with those obtained by Chinnasamy, Ramakrishnan, Bhatnagar and Das (2009), since by increasing the concentration of the organic carbon source it was possible to increase biomass productivity in *C. vulgaris*. Likewise, it has been found that acetate stimulates the production of carbohydrates and lipids and promotes chlorophyll synthesis in *C. vulgaris* (Laliberté & de la Noüie, 1993). According to the results presented in Figures 5 and 6, high acetate concentrations promoted an increase in the amount of carbohydrates

produced. This is due to the metabolism of this and other organic sources of carbon through the activity of the isocitrate lyase enzyme through the glyoxylate cycle (Laliberté & Hellebust, 1989), which increases as a function of the concentration acetate between 0 and 20 mM (Combres, Laliberté, Reyssac & de la Noüe, 1994). Finally, the acetate is metabolized to acetyl *CoA*, then converted into succinate and later used as a basis for the synthesis of different metabolites, such as carbohydrates.

In addition to carbon, nitrogen is a determining factor in biomass production (Xiong, Li, Xiang & Wu, 2008). However, the carbon/nitrogen relation, and the carbon source in particular, in this case sodium acetate, is essential for cell growth and xylose and glucose production in *C. vulgaris*. The provision of a source of carbon and nitrogen in the correct ratio (20 mM sodium acetate, 0.97 mM sodium nitrate) causes both biomass and xylose and glucose productivity to increase considerably in just three days of culture, as shown in Figures 8a and b. Although the results show that for the case of these microalgae, when sodium acetate is used as a carbon source, it is possible to increase the concentration of carbohydrates as compared to the control (T0), but it is not possible to obtain more than 78.9 μg/mL of xylose and 77.2 μg/mL of glucose, which corresponds to 7.6 and 7.5%, respectively. On the other hand, the changes employed not only alter the concentration of specific metabolites, but also significantly increase total biomass productivity, so it is possible to obtain, every three days, 1.04 g/L of biomass (T3) with a xylose concentration of 78.9 μg/mL and a glucose concentration of 77.2 μg/mL equivalent to 7.6 and 7.5% of biomass, respectively. Alternatively, it is possible to obtain, every five days, 0.79 g/L of biomass with a concentration of 76.9 μg/mL of xylose and 73.7 μg/mL of glucose equivalent to 9.7 and 9.3% of the produced biomass, respectively. On the other hand, phosphate is essential for microalgae growth and metabolism and it is also one of the essential elements that constitute DNA, RNA, ATP; in addition, is an important constituent of lipid portion, many coenzymes and some cell membrane materials, among others (Barsanti & Gualtieri, 2006). In the case of phosphorus, the limitation of algal growth can be at least twofold. First, there is a limitation of nucleic acid synthesis. This limitation can affect photosynthetic, due to the requirement of large s large amounts of proteins (mainly Rubisco), which are

synthesized by ribosomes rich in phosphorus (Agren, 2004). However in this study the cultures were not limited by phosphate, it should be noted that although on treatment 3 (T3) with 70% phosphate consumption, 15% of its biomass corresponds to glucose and xylose; T5 (with 19% of these carbohydrates) consumed only 20% of the total available phosphate, indicating that phosphate consumption is not directly proportional to the concentration of carbohydrates.

It is important to highlight that the presence of glucose and xylose in the culture media is due to the formation of mucilage (extracellular polymeric substance), which consists of hydrated biopolymers (polysaccharides, proteins, nucleic acids and lipids) and forms a capsule around the algae, thereby producing a biofilm that enables them to cover and immobilize the microbial aggregates (Flemming & Windeger, 2010). The thickness of this polysaccharide capsule varies depending on the growth phase and culture conditions. Additionally, during cell growth in discontinuous cultures both the capsules and the sludge may be released to the surrounding environment from the polysaccharide material, causing a gradual increase in viscosity (De–Philippis & Vincenzini, 1998).

The commercial interest at an industrial level of pigments present in *C. vulgaris*, lies not only in their viability as coloring agents for food, but also in its antioxidant and antimutagenic properties. In this study, it was observed that during the extraction of these pigments, they always dragged 2.3 to 35.1% of the xylose and 2.9 to 47.5% of produced glucose. Taking in consideration the biorefinery concept, if some would like to take advantage of these pigments, the amount of carbohydrates described above may be lost.

During the development of this research it was shown that the microalgae store various carbohydrates (mainly xylose and glucose) into different parts, not only in the cell wall, but also in the chloroplasts inside structures called pyrenoids, which are sometimes expelled to the culture media. In the media, their concentration might be of up to 51.3% for xylose and 55.7% for glucose, suggesting the possibility of their recovery directly from it and not from the produced biomass, in a more economical the process that does not need the biomass concentration and separation phases.

In addition, the treatments with the highest concentrations of extracellular glucose and xylose showed low phosphate consumption. Likewise, the viability of *C. vulgaris* culture in semi–continuous processes where the carbohydrate–rich (glucose and xylose) media is recirculated and serves as inoculum for new cultures could be verified.

Others studies like the one of Rodjaroen, Juntawong, Mahakhant and Miyamoto (2007) not induce a nutritional stress, on the other hand Dragone *et al.* (2011) induce an increase in the concentration of starch by nitrogen starvation, but in this work does not change the concentration of carbon added to the culture medium (in this case a fixed concentration of 2% v/v CO_2), and the culture time is extended until it reaches the stationary growth phase. although these authors obtained starch concentrations above 40% of the produced biomass, this cultures requires first and stage of enriched urea–iron followed by a free urea culture process, this increases the time of the hole process, in contrast to these results, this paper proposes a process that takes up to 5 days in culture; however, like the work of Dragone *et al.* (2011), these process is not continuous.

Finally, the intersection of all these results may give insights on the viability of microalgae–based biofuels, because the reduction in the time required culture time for obtained the concentration of carbohydrates (up to 0.080 g/L every 3 days) is a key factor that would develop industrial scale batch cultures using the found concentrations of sodium acetate and nitrate. However, as the results obtained in this study show, the choice of the culture method that allows for maximum exploitation must be strongly linked both to the productivity of the different added–value metabolites (proteins, pigments, lipids, etc.) and to nutrient intake.

5. CONCLUSIONS

- The adequate adjustment between the amount of carbon and nitrogen can not only increase biomass production, but also promote the deposition of certain metabolites. In this study, a concentration of 20 mM sodium acetate and 0.97 mM sodium nitrate (T3) were able to improve biomass concentration

by up to two times (from 0.45 g/L up to 1.04 g/L), xylose (from 40.1 μg/mL up to 78.9 μg/mL) and glucose (from 31.2 to 77.2 μg/mL) as compared to the control (0 mM sodium acetate; 2.94 mM sodium nitrate) in three days of culture. However, unlike this treatment, T5 (carbon/nitrogen ratio of 10 mM sodium acetate: 1.94 mm sodium nitrate) 0.8g/L of biomass with a concentration of 76.9 μg/mL of xylose and 73.7 μg/mL of glucose were obtained after five days of culture.

- Although both treatments mentioned above are significant both for biomass and carbohydrate production, it does not seem to be clear which one is more efficient in the production of xylose and glucose, since time and the consumption of certain nutrients (in this case phosphate), may also be important decision factors. Even though concentrations similar to those produced with T5 can be obtained with T3 in only 3 days of culture, the phosphate consumption of the latter is almost three times greater than in treatment 5 (from 23 to 76%).

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NOTATION			
T0	Control treatment, Bold Basal culture media	T5	Treatment 5
T1	Treatment 1	T6	Treatment 6
T2	Treatment 2	T7	Treatment 7
T3	Treatment 3	T8	Treatment 8
T4	Treatment 4	T9	Treatment 9

ANNEX A. CALCULATION OF THE MASS EXTINCTION COEFFICIENT OF XYLOSE

Table A1. Absorbance variation with xylose concentration.

Mass extinction coefficient (Xilose)	
Concentration (μg/mL)	Absorbance (480nm)
100	2.589
80	2.385
60	1.625
40	1.212
20	0.641
0	0

$y = 21.176x^5 - 134.66x^4 + 298.85x^3 - 266.62x^2 + 111.2x - 7E\text{-}09$
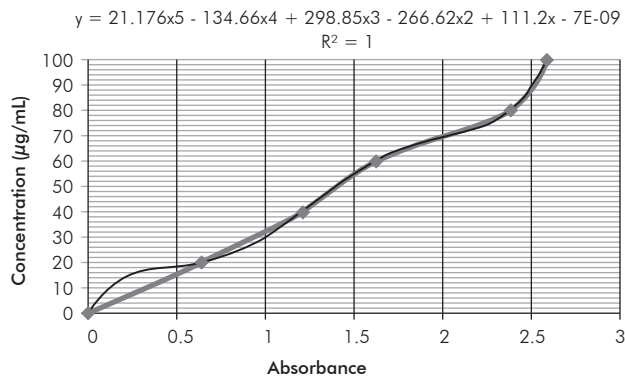$R^2 = 1$

Figure A1. Graph of absorbance data as a function of xylose concentration for calculation of the mass extinction coefficient.

ANNEX B. CALCULATION OF THE MASS EXTINCTION COEFFICIENT OF GLUCOSE

Table B2. Absorbance variation with glucose concentration.

Mass extinction coefficient (Glucose)	
Concentration (μg/mL)	Absorbance (480nm)
100	2.569
80	1.866
60	1.469
40	0.960
20	0.346
0	0

$y = -0.497x^5 - 7.257x^4 + 44.81x^3 - 74.16x^2 + 78.40x - 8E\text{-}10$
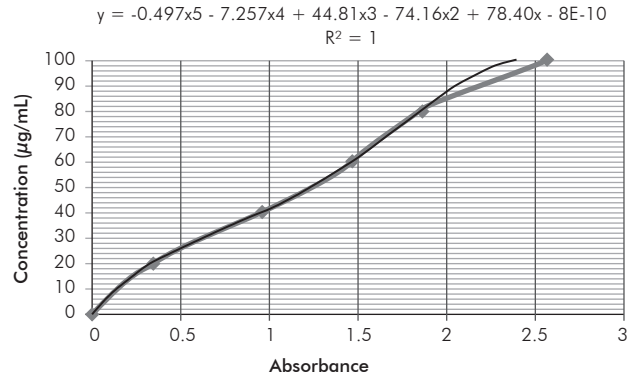$R^2 = 1$

Figure A2. Graph of absorbance data as a function of glucose concentration for calculation of the mass extinction coefficient.