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SYNTHESIS OF NEUTRAL LIPIDS IN *CHLORELLA* SP. UNDER DIFFERENT LIGHT AND CARBONATE CONDITIONS

Jazmín-Vanessa Pérez-Pazos[1] and Pablo Fernández-Izquierdo[1*]

[1] Microbial Biotechnology Research Group, Universidad de Nariño, Pasto, Nariño, Colombia

e-mail: pabfdez@gmail.com

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ABSTRACT

Lipids are biomolecules of great scientific and biotechnological interest due to their extensive applications. Microalgae are potential biological systems used in the synthesis of lipids, particularly *Chlorella* sp., which is characterized by its high lipid content and for having the right profile for the obtainment of biofuel. Lipid production in microalgae is influenced by several physical and chemical factors. Any modification thereof can cause a stress response represented by changes in synthesized lipid composition, varying from one species to another. This paper evaluates the effect of different light wavelengths, photoperiods and calcium carbonate ($CaCO_3$) supply in lipid synthesis in *Chlorella* sp. In order to do so, the microalgae was grown in Bold's Basal Medium (BBM) at 20°C with constant aeration and subject to low blue (470 nm) and red (700 nm) light wavelengths, 0,5 $g \cdot L^{-1}$ and 1,5 $g \cdot L^{-1}$ concentrations of $CaCO_3$ and 6-hour light, 18-hour darkness (6:18) and 18-hour light, 6-hour darkness (18:6) photoperiods. The results indicate a higher growth rate for microalgae under red light, 0,5 $g \cdot L^{-1}$ of $CaCO_3$ and a photoperiod of 6:18. On the other hand, lipid production is higher under blue light, 1,5 $g \cdot L^{-1}$ of $CaCO_3$ and an18:6 photoperiod. Analysis by gas chromatography indicate that the fatty acids in the samples are oleic, linoleic and palmitoleic, which are of recognized importance in the biodiesel industry. This suggests that neutral lipid synthesis can be optimized in two stages: first, by promoting growth and subsequently, by inducing lipid production.

Keywords: *Fatty acids, Microalgae, Lipids, Photosynthesis, Photobioreactor.*

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**To whom correspondence should be addressed*

RESUMEN

Los lípidos son biomoléculas de gran interés científico y biotecnológico por sus amplias aplicaciones, las microalgas constituyen materia prima con potencial para síntesis de lípidos, particularmente *Chlorella* sp., es una de las más representativas debido al elevado contenido de lípidos y perfil idóneo para la obtención del biocombustible. La producción de lípidos en las microalgas se encuentra influenciada por varios factores físicos y químicos, la modificación de estos provoca una respuesta de estrés que se manifiesta por variaciones en la composición de lípidos sintetizados, que varía de una especie a otra. En esta investigación se evaluó el efecto de diferentes longitudes de onda de luz, fotoperiodos y suministro de carbonato de calcio ($CaCO_3$) en la síntesis de lípidos en la microalga *Chlorella* sp., para esto se cultivó la microalga en medio basal de Bold (Bold's Basal Medium, BBM) a 20°C y aireación constante, sometidas bajo longitudes de onda de luz azul (470 nm) y roja (700 nm), concentraciones $CaCO_3$ de 0,5 $g{\cdot}L^{-1}$ y 1,5 $g{\cdot}L^{-1}$y fotoperiodos con fases de 6 horas luz y 18 horas oscuridad (6:18) y 18 horas luz y 6 horas oscuridad (18:6). Los resultados obtenidos indican que microalgas bajo luz roja, concentración de 0,5 $g{\cdot}L^{-1}$ de $CaCO_3$ y fotoperiodo 6:18 presentaron mayor tasa de crecimiento, por otra parte, la producción de lípidos es mayor bajo luz azul, 1,5 $g{\cdot}L^{-1}$ de $CaCO_3$ y fotoperiodo 18:6. Los resultados de los cromatrogramasmuestranácidos grasos como oleico, linoleico y palmitoleicode gran importancia en la industria del biodiesel. Estos resultados sugieren que es posible optimizar la síntesis de lípidos neutros en dos fases, primero promoviendo el crecimiento y posteriormente induciendo la producción de lípidos.

Palabras claves: *Ácidos grasos, Microalgas, Lípidos, Fotosíntesis, Fotobiorreactor.*

RESUMO

Os lipídios são biomoléculas de grande interesse científico e biotecnológico por suas amplas aplicações, as microalgas constituem matéria-prima com potencial para síntese de lipídios. *Chlorella* sp., particularmente, é uma das mais representativas devido ao elevado conteúdo de lipídios e perfil idôneo para a obtenção do biocombustível. A produção de lipídios nas microalgas é influenciada por vários fatores físicos e químicos, a modificação destes provoca uma resposta de estresse que é manifestada por variações na composição de lipídios sintetizados, que varia de uma espécie para outra. Nesta pesquisa foi avaliado o efeito de diferentes longitudes de onda de luz, fotoperíodos e fornecimento de carbonato de cálcio ($CaCO_3$) na síntese de lipídios na microalga *Chlorella* sp., para isto foi cultivada a microalga em meio basal de Bold (Bold's Basal Medium, BBM) a 20°C e aeração constante, submetidas sob longitudes de onda de luz azul (470 nm) e vermelha (700 nm), concentrações $CaCO_3$ de 0,5 $g{\cdot}L^{-1}$ e 1,5 $g{\cdot}L^{-1}$ e fotoperíodos com fases de 6 horas luz e 18 horas escuridão (6:18) e 18 horas luz e 6 horas escuridão (18:6). Os resultados obtidos indicam que microalgas sob luz vermelha, concentração de 0,5 $g{\cdot}L^{-1}$ de $CaCO_3$ e fotoperíodo 6:18 apresentaram maior taxa de crescimento, por outra parte, a produção de lipídios é maior sob luz azul, 1,5 $g{\cdot}L^{-1}$ de $CaCO_3$ e fotoperíodo 18:6. Os resultados dos cromatrogramas mostram ácidos graxos como oleico, linoleico e palmitoleico de grande importância na indústria do biodiesel. Estes resultados sugerem que é possível aperfeiçoar a síntese de lipídios neutros em duas fases, primeiro promovendo o crescimento e posteriormente induzindo a produção de lipídios.

Palavras-chaves: *Ácidos graxos, Microalgas, Lipídios, Fotossíntese, Fotobiorretor.*

1. INTRODUCTION

Lipids are a group of biomolecules that biologically have two important functions: serving as an energy source and as building blocks of the membranes in organisms (Segré, Ben-Eli, Deamer, & Lancet, 2001). However, they have now taken on great biotechnological interest, because they play a major role in products such as cosmetics, pharmaceuticals, fuels, among others (Rutz & Janssen, 2007). The lipids of a plant origin contain fatty acids and triglycerides, which are susceptible to esterification for use as a source of energy (Fahy *et al.,* 2005; Villanueva, 2005; Hernandez & Quintana, 2010). Up until now, in order to produce lipids from vascular plant species, extensive areas of land are used to grow the plants. In many cases this system of production leads to deterioration in soil quality and pollution of ecosystems with by-products from the extraction of lipids (Dismukes, Carrieri, Bennette, Ananyev & Posewitz, 2008). Similarly, this type of agriculture has contributed to the deforestation of natural ecosystems and the substitution of crops for human consumption (Escudero, Cid & Escudero, 2009). In order to minimize these disadvantages, the utilization of microalgae can be an efficient alternative for producing lipids (Xua, Miaoa & Wu, 2006; Trösch & Trick, 2008; Barajas *et al.,* 2009; Hirth, 2009; Trösch, Mertsching & Hirth, 2009).

Microalgae synthesize intracellular lipids in the form of neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLS). These substances have a variable composition and concentration, reflecting the nature of the organism, the influence of culture conditions and the physiological state there of (Tokusoglu & Ünal, 2003). The NLs are used as raw material for the production of biodiesel and they consist mainly of wax esters (WEs), triacylglycerols (TAGs), diacylglycerols and monoacylglycerols, (Borowitzka, 1995; Chen, Jiang & Chen, 2007; Wältermann & Steinbüchel, 2007). Due to this characteristic, this type of micro-organisms are a potential source of lipid synthesis for biofuel (Lee, Whitledge & Kang, 2008; Jacob-Lopes, Gimenes-Scoparo, Ferreira-Lacerda & Teixeira-Franco, 2009; Yoo, Jun, Lee, Ahn & Oh , 2010).

The output of neutral lipid synthesis in microalgae is based on the variation of farming conditions, such as nutrient type and concentration (Yingying & Changhai, 2009; Yeesang & Cheirsilp, 2011), CO_2 availability (Mendes, Nobre, Cardoso, Pereira & Palavra, 2003) temperature (Zepka, Jacob-Lopes & Queiroz, 2007; Converti, Casazza, Ortiz, Perego & Del Borghi, 2009), light type and intensity, photoperiod (Lee *et al.,* 2008; Rosenberg, Oyler, Wilkinson & Betenbaugh, 2008; Jacob-Lopes *et al*., 2009), among others. Light and carbon dioxide are essential factors that affect the physiological response in microalgae, because as phototrophic organisms, they use light photons as a source of energy and absorb carbon dioxide to synthesize organic compounds (Moheimani, 2005; Schulze, Beck & Müller-Hohenstein, 2005; Lee *et al*., 2008). Therefore, variations in light intensity and carbon dioxide supply cause variations in the synthesis of neutral lipids (De Castro-Araújo & Tavano-García, 2005; Rodríguez, Canales & Borrás-Hidalgo, 2005; Sharkey, 2005; Bertoldi, Sant-Anna, Da-Costa & Barcelos, 2006; Sharma, Kumar-Singh, Panda, Mallick, 2006; Chen *et al.*, 2007)

It has been established that when the microalgae are grown with low light intensity, they assimilate carbon preferentially in the direction of the synthesis of amino acids and other essential cell components. However, under conditions of saturated light, they form sugars, lipids and starch through the pentose pathway, which involves phosphate reduction (Hoff & Snell, 2004; Jacob-Lopes *et al*., 2009; Zak, *et al*., 2001). Furthermore, carbon is also a factor that determines lipid accumulation. Continuous carbon assimilation at a high concentration promotes the synthesis of fatty acids under this condition, and at a high light intensity, the lipids become a protective factor of the body against photo-oxidative stress (Grossman & Takahashi, 2001; Hu *et al*., 2008; Rosenberg *et al*., 2008; Meng *et al*., 2009; Rodolfi *et al.*, 2009).

Knowledge of the micro-organism's metabolism and control of environmental variables helps establish systems focused on obtaining micro-algae biomass with a high lipid content (Derner, Ohse, Villela, Matos-de Carvalho & Fett, 2006; Chisti, 2007; Eriksen, 2008; Marinho, *et al.,* 2009; Yingying & Changai, 2009; Rogenski, 2010; Souza, 2010). However, the effect of each of the factors varies from one species to another. Therefore, in order to develop a technological strategy for biomass production and lipid synthesis, the effect of environmental factors in the micro-organism under study has to be assessed. Therefore, the effect of light and the CO_2 supply in the growth and synthesis of the wild microalga *Chlorella* sp. was evaluated. IBUN 0016.

2. EXPERIMENTAL DEVELOPMENT

Strain and Culture

For this study, the wild microalgae *Chlorella* sp. was used. The microorganism LAUN0016 was grown in Bold's Basal Medium (BBM) (Derner *et al*., 2006), supplemented with 1200000 UI penicillin G sodium. The sample was incubated in a photobioreactor with cool white light at a temperature of 20ºC and a photoperiod of 12 hours light and 12 hours darkness, until growth was observed.

Effect of Light and CO_2 On the Synthesis of Neutral Lipids

The pilot phase was conducted in a serpentine photobioreactor with closed fermentation driven by motor pumps with a range of 1m, in order to provide the system with constant agitation, with different colored lamps of cold light, red or blue, installed in each of the fermenters.

The photoperiod was controlled by a timer. Calcium carbonate ($CaCO_3$) was used as a carbon source and system temperature was 20°C. A 2^3 factorial design with 3 repetitions was applied. The factors of light wavelength, $CaCO_3$ concentration and photoperiod, with their respective levels of variation, are shown in Table 1.

The response variables to be measured are: microalgae growth and lipid production. Growth was measured in units of absorbance at 750 nm every 24 hours using a Jenway Genoa spectrophotometer.

Neutral lipids were extracted according to a method described by Yellore and Desai (1998) and Braunnegg *et al.* (2007) with certain modifications described by Fernández, Ortiz, Guerrero, Burbano & España (2006). Neutral lipids are extracted by adding 1,5 mL of hypochlorite at 5%. After that, they are placed in a water bath at 60°C for 2 hours, and washed with distilled water followed by the addition of cold methanol. After that, they are centrifuged at 33000 g for 20 min, to obtain the pellets, which represent neutral lipids.

3. CHARACTERIZATION OF NEUTRAL LIPIDS

The neutral lipids were characterized by gas chromatography using a method of pre-column derivatization of samples with *MeOH/HCl* 5%. After that, in a separating funnel, the fatty acid methanol esters (FAMEs) are extracted with hexane and 1 mL of the sample is injected into the chromatograph (Christie 2003). The run conditions were 150°C for 4 minutes @ 250°C for 5 min, 4°C/minute. The detector, FID, 280°C, the DB-5 column (30 m, 0,25 µm, 0,25 mm) on a Shimadzu GC 17A computer (Chen *et al*., 2007)

The fatty acids were quantified based on *Equation 1*.

$$FA\ (\%) = \frac{Ai}{\Sigma A} \times 100\ \% \qquad (1)$$

Where Ai is the area of the peak corresponding to component i, and ΣA is the sum of the areas of all the peaks.

4. RESULTS

The effects of the variables light wavelength, $CaCO_3$ supply and photoperiod on growth, chlorophyll production and lipid synthesis in the microalgae *Chlorella* sp. were evaluated through factorial design 2^3. Results are shown in Table 2.

Table 1. Description of the levels of each of the factors used in factorial design 2^3.

Level	Light Wavelength	Concentration of $CaCO_3$ ($g \cdot L^{-1}$)	Photoperiod (Hourslight: HoursDarkness)
1	700 nm	1,5	18:6
-1	500 nm	0,5	6:18

Table 2. Results factorial design 2^3.

Experiment					
Light Wavelength	Concentration of $CaCO_3$ ($g \cdot L^{-1}$)	Photoperiod (Hourslight: HoursDarkness)	Growth (μ)	Total Chlorophyll ($g \cdot L^{-1}$)	Lipids ($g \cdot L^{-1}$)
500 nm	0,5	6:18	0,0105 +/- 0,0003	0,0057 +/- 0,0006	0,2481 +/- 0,0799
700 nm	0,5	6:18	0,1090 +/- 0,0014	0,0317 +/- 0,0009	0,2261 +/- 0,0700
500 nm	1,5	6:18	0,0112 +/- 0,0003	0,0129 +/- 0,0010	0,5631 +/- 0,0438
700 nm	1,5	6:18	0,0119 +/- 0,0001	0,0115 +/- 0,0015	0,4334 +/- 0,0106
500 nm	0,5	18:6	0,0100 +/- 0,0004	0,0024 +/- 0,0001	0,1714 +/- 0,0092
700 nm	0,5	18:6	0,0102 +/- 0,0001	0,0103 +/- 0,0005	0,1540 +/- 0,0042
500 nm	1,5	18:6	0,0112 +/- 0,0003	0,0066 +/- 0,0008	0,7783 +/- 0,0219
700 nm	1,5	18:6	0,0135 +/- 0,0001	0,0443 +/- 0,0027	0,6539 +/- 0,0170

Growth of the microalgae Chlorella sp.

For the growth response variable, the analysis of the results of the experimental design indicates that all interactions of the first order are significant (p-value<0,05), with a reliability of 95%. $CaCO_3$ - photoperiod (p-value = 0,0085) showed positive interaction. On the other hand, light - photoperiod (p-value = 0,0107) and Light - $CaCO_3$ (p-value = 0,0113) interactions are negative.

Growth in the microalgae *Chlorella* sp., under laboratory conditions is influenced by the interaction of all the variables. Figure 1a illustrates that the interaction of the factors $CaCO_3$ - Photoperiod favors the growth of the microalgae when $CaCO_3$ concentration is 0,5 $g \cdot L^{-1}$ and a 6:18 photoperiod. On the other hand, growth decreases significantly when calcium carbonate is found at a concentration of 1,5 $g \cdot L^{-1}$, regardless of the photoperiod. The interaction of the variables light and photoperiod favors the growth of micro-algae when the light presents a wavelength of 700 nm and a 6:18. On the other hand, when light is at a wavelength of 500 nm under any photoperiod, it reduces the growthof the microalga *Chlorella* sp., Figure 1b, while with a wavelength of 700 nm and a $CaCO_3$ concentration of 0,5 $g \cdot L^{-1}$, Figure 1c, the growth of *Chlorella* sp.increases.

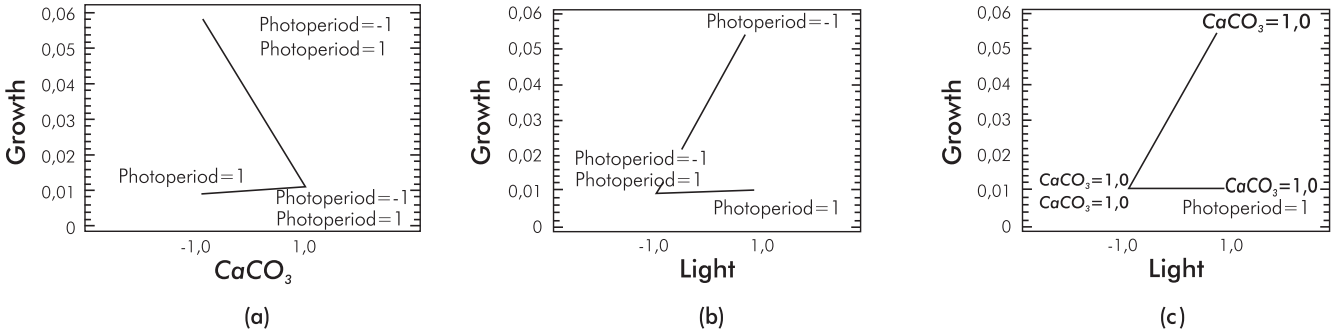

Figure 1. Interaction charts of the variables that affect growth.

(a) $CaCO_3$ - Photoperiod; (b) Light - Photoperiod and (c) Light - $CaCO_3$.

Synthesis of Neutral Lipids

The Pareto Chart (Figure 2) shows that all the main effects and $CaCO_3$- Photoperiod interaction were significant. However, light has a negative effect on lipid synthesis when grown at a length of 700 nm. On the other hand, carbonate-photoperiod interaction promotes the lipid synthesis when the carbonate concentration is 1,5 g·L^{-1} and when the photoperiod is 18:6 (Figure 3).

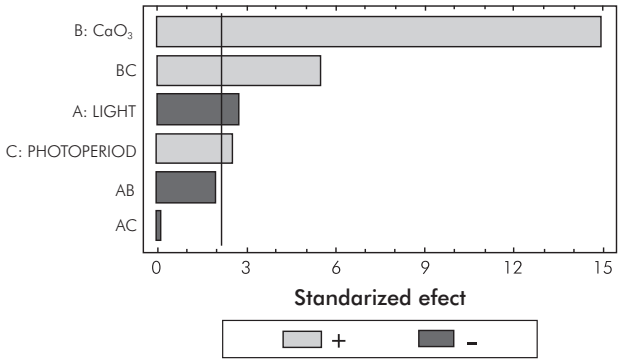

Figure 2. Standardized paretochart for lipids.

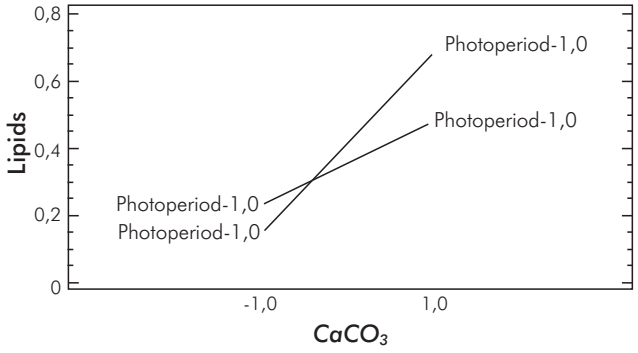

Figure 3. Chart Showing $CaCO_3$ - Photoperiod interaction forlipids.

Characterization of the fatty acids synthesized by Chlorella sp., by gas chromatography.

The analysis of the lipid profile of the sample of *Chlorella* sp. by gas chromatography, under the best conditions of lipid synthesis with blue light; $CaCO_3$ concentration of 1,5 g·L^{-1} and an 18:6, reported 10 compounds, three of which were identified: linoleic fatty acid, with a percen-tage of 6,45%, palmitoleic fatty acid with a percentage of 4,01% and oleic fatty acid with a percentage of 2,75% (Figure 4).

For the sample of *Chlorella* sp., under the best conditions of lipid synthesis with red light, a $CaCO_3$ concentration of 1,5 g·L^{-1} and an 18:6 photoperiod, 18 compounds were reported, one of which was identified, corresponding to linoleic fatty acid (Figure 5).

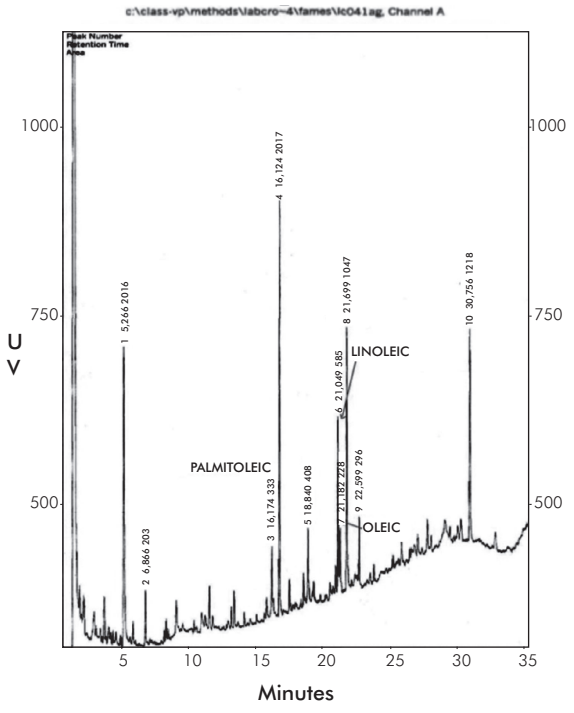

Figure 4. Analysis by gas chromatography of lipids produced by *Chlorella* sp. (Blue Light; $CaCO_3$ 1,5 g·L^{-1}; FP 18:6).

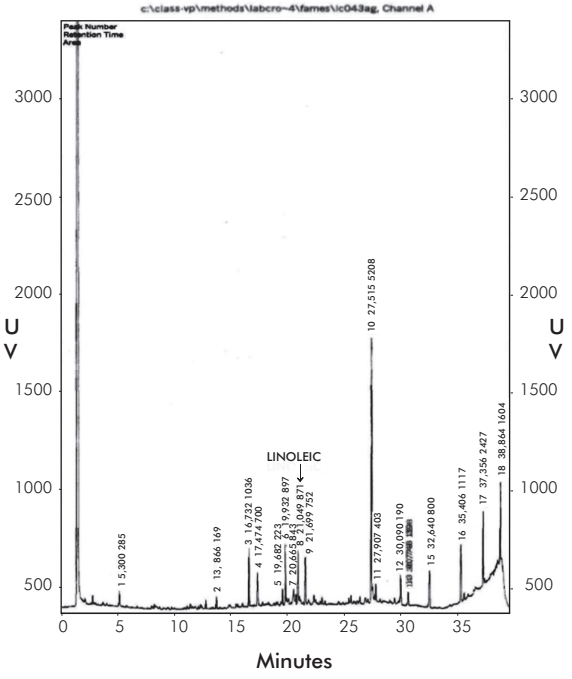

Figure 5. Analysis by gas chromatography of lipids produced by *Chlorella* sp. (Red Light; $CaCO_3$ 1,5 g·L^{-1}; FP 18:6).

5. DISCUSSION

All the factors and their interactions have significant effects on lipid synthesis as well as on microbial growth. In order to promote the growth,the most appropriate conditions are a wavelength of 700 nm, a calcium carbonate concentration of 0,5 $g \cdot L^{-1}$ and a 6:18 photoperiod. Red light provides a higher level of excitement in the chlorophyll electrons, which causes a significant increase in the effectiveness of these pigments. The electrons produce water hydrolysis, which leads to the synthesis of ATP that is used for the synthesis of biomolecules that promote the growth of micro-algae (Rosemond, Mulholland & Brawley, 2000; Piippo *et al*., 2006). With the excited chlorophylls and exposure to light, after 6 hours, there is enough energy and reduction power to sequester carbon and synthesize compound organisms through the Calvin cycle performed during the dark phase of photosynthesis, which in this case is 18 hours, so there is more time for the synthesis of organic compounds other than lipids. This behavior is probably due to the activation of the phytochrome by the red light that regulates the expression of some nuclear genes that produce chloroplastic proteins related to photosynthesis (Hill, 1996; Neff, Fankhauser & Chory, 2000; Rosemond, *et al*., 2000; Piippo, *et al*., 2006). On the other hand, growingthe microalga *Chlorella* sp., LAUN0016, under the blue light, probably affects the expression of genes in the cell nucleus associated with lipid synthesis.

This study established that microalgae growth is favored at a calcium concentration of 0,5 $g \cdot L^{-1}$ because under these conditions, the carbonate is solubilized and is available to meet the carbon demand required for cell growth (Medadro & Flexas, 2003; Massol-Deyá, Muñiz, Colón, Graulau & Tang, 2005). But when the carbonate concentration is high, the solubility constant is exceeded and tends to precipitate; therefore there is not enough carbon available for microbial growth.

This study showed that for lipid synthesis, the best conditions are a wavelength of 500 nm corresponding to blue light, a calcium carbonate concentration of 1,5 $g \cdot L^{-1}$ and an 18:6 photoperiod.

The high lipid content seems to be an initial response to the exposure of microalgae in blue light, which has high energy content (Sánchez-Saavedra & Voltolina, 2002; Gupta & Agrawal, 2006). Other studies have shown that the energy from blue light is captured by the pterin and transferred to the flavin, which probably intercedes in cryptochrome phosphorylation. This can cause a chain of signal transduction, which can affect the regulation of genes in the cell nucleus (Neff *et al.,* 2000).

In this paper, the largest concentration of lipids with *Chlorella* sp. was obtained when using an 18:6 photoperiod. This condition can be considered a stress factor because, in the tropics, the organisms have photoperiods of 12:12. It can be assumed that microalgae exposed to 18 hours of light have an imbalance in oxide-reduction potential with accumulation of reduction power, which has to transfer the hydrogen ions to the reserve organic compounds such as the lipids to restore balance. This condition of stress is accentuated when the calcium carbonate concentration is 1,5 $g \cdot L^{-1}$ because at high concentrations, the carbonate is not available since it exceeds the solubility constant (Medadro & Flexas, 2003; Massol-Deyá *et al.,* 2005). However, there are some molecules available that can be assimilated pre-ferentially for lipid synthesis.

Altogether, these conditions can be considered a stress factor for *Chlorella* sp., which favors lipid synthesis. This state is characterized by the modification of the basic physiological functions causing the activation of defensive or response mechanisms that lead to the adjustment of cell metabolism to the new conditions (Piippo, *et al*., 2006; Tadeo, 2003). The microalgae grown photoautotrophically under conditions of severe stress assimilate carbon preferentially in the direction of the synthesis of amino acids and other special cell components, such as neutral lipids (Zak *et al*., 2001), since they require the increase in lipid composition, which is a determining factor in the restoration of photosynthetic machinery (Mendes & Wagener; 2001; Medadro & Flexas, 2003). It is important to point out that variation in *Chlorella* sp. growth conditions also affected lipid composition and concentration, but this type of response varies from one species to another (Sanchez, Martinez & Espinola, 2006).

6. CONCLUSIONS

- These findings underscore the importance of controlling light wavelength, carbonate concentration and photoperiod because these factors affect both growth

and the synthesis of neutral lipids in the microalgae *Chlorella* sp.

- The levels of light wavelength, carbonate concentrations and photoperiod that favor growth inhibit lipid synthesis; in the same sense, the conditions that favor the synthesis of neutral lipids produce an inverse response in the growth of *Chlorella* sp.

- The lipid profile obtained under a light wavelength of 500 nm is different from that obtained whenthe microalgae *Chlorella* sp., is grown at 700 nm. This suggests that the type and concentration of lipids synthesized by manipulating light wavelength can be controlled.

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