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Lipid content of marine microalgae *Chaetoceros muelleri* Lemmermann (Bacillariophyceae) grown at different salinities

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Resumo

Teor lipídico da microalga marinha *Chaetoceros muelleri* Lemmermann (Bacillariophyceae) cultivada em diferentes salinidades. A produção e armazenamento de lipídios por microalgas em resposta a variações nos fatores ambientais são específicos para cada espécie de microalga. O presente trabalho teve como objetivo avaliar o crescimento e a concentração de lipídios totais da microalga *Chaetoceros muelleri* cultivada em três diferentes salinidades (15, 25 e 35). O experimento foi realizado em triplicatas, utilizando frascos de nove litros com volumes úteis de 7 L, sendo cultivo do tipo estacionário ou "batch". A temperatura da sala de cultivo e a intensidade luminosa foram mantidas em 28±1ºC e 20 $\mu.mol.m^{-2}.s^{-1}$, respectivamente. Os resultados mostraram que no presente trabalho, o rendimento lipídico (10,41±1,89, 10,87±2,10, 12,33±1,81% nas salinidades 15, 25 e 35, respectivamente) não foi influenciado pela concentração salina ($p>0,05$). No entanto, a concentração celular máxima sofreu esta influência e foi significativamente maior ($p<0,05$) nas salinidades de 15 e 25 as quais obtiveram 16,3±1,50 e 17,6±2,90 x 10^5 $cel.mL^{-1}$, respectivamente.

Key words: Cultivo; Lipídeos; Microalgas

Abstract

The production and storage of lipids by microalgae in response to variations in environmental factors are specific for each microalgal species. The present study aimed to evaluate the growth and concentration of total lipids of the microalga *C. muelleri* Lemmermann cultivated at three different salinities (15, 25 and 35). The experiment was conducted in triplicate, using 9-L containers (7-L working volume), with the culture being stationary or batch type. The temperature and light intensity of the cultivation room was kept at 28±1ºC and 20 $\mu.mol.m^{-2}.s^{-1}$, respectively. The results showed that the lipid yield (10.41±1.89, 10.87±2.10 and 12.33±1.81%

at salinities of 15, 25 and 35, respectively) was not influenced by the salt concentration ($p>0.05$), but the maximum cell concentration was significantly higher ($p<0.05$) at salinities of 15 and 25, which was 16.3±1.50 and 17.6±2.90 x 10^5 cells.mL^{-1}, respectively.

Key words: Culture; Lipids; Microalgae

Introduction

Microalgae are considered potentially useful in applications in the food, cosmetic and pharmaceutical industries (OLAIZOLA, 2003; SOARES et al., 2006). At present, they are considered potential raw material for biodiesel production with physical and chemical characteristics similar to those currently used. The productivity of microalgae can surpass that of biofuel plant products worldwide, such as soybean and corn (CHISTI, 2007; MATA et al., 2010) and African palm oil (LORA; ANDRADE, 2009). These algae can be cultivated in industrial installations by using photobioreactors, avoiding any conflict with food production (CHISTI, 2007; JANAUN; ELLIS, 2010).

Marine species, especially diatoms, are even more promising due to the high lipid content (LOURENÇO, 2006). The species *Chaetoceros muelleri* Lemmermann has chorophyll *a*, *c*1 and *c*2, xanthophylls (fucoxanthin) and carotenes as the major pigments, which usually give it a yellowish-brown color. This species has a cell wall formed by two parts that fit within each other, mainly composed of silica, and uses chrysolaminarin and lipids as energy reserve substances (DE STEFANO et al., 2009).

Microalgae produce more oil under unfavorable culture conditions (HU et al., 2008). According to the cited authors, under optimal growth conditions, these organisms synthesize fatty acids, especially for esterification into membrane lipids, which constitute about 20-50% of their dry weight. But under unfavorable environmental or stress conditions, many microalgae change their lipid content through biosynthetic pathways for the formation and accumulation of neutral lipids, such as the triacylglycerols (TAG).

According to Alyabyev et al. (2007), salt stress causes a series of bioenergetic and biochemical changes in photosynthetic organisms. Among them, the most important are the increase in biopolymer and lipid catabolism, changes in the energy rate that supports biochemical processes, and changes in the permeability of the plasma membrane with disruption of ion homeostasis. These alterations are related to bioenergetic aspects and are essential for understanding the adaptive mechanisms of microalgae to salinity (ILLMAN et al., 2000; RAO et al., 2007).

Additional expenditure of metabolic energy under stress conditions is required to maintain ion balance and electrochemical gradients, to permit the biosynthesis of organic compounds that play a key role in the protection, osmoregulation, and maintenance of cell structure. Accordingly, the increase in energy release rate can ensure the rapid and effective acclimation of microalgae to stress (RODOLFI et al., 2008).

Microalgae are an attractive alternative to fossil fuels, due to their potential to achieve a high productivity using photosynthesis to convert solar energy into chemical energy, the short life cycle, high growth rate, high oil yield, and ability to absorb nutrients from waste waters (PATIL et al., 2008; HUANG et al., 2010).

This study aimed to evaluate the growth and total lipid content of the microalga *C. muelleri* cultivated at three different salinities (15, 25 and 35).

Material and Methods

The microalga *C. muelleri* was obtained from the strain bank of the Laboratory of the Center for Technology in Aquaculture of the Department of Fishing Engineering from the Federal University of Ceará. Guillard f/2 culture medium was used for the maintenance of inocula and to carry out the experiments (GUILLARD, 1975).

The cultures were subjected to constant aeration (4 L air.min^{-1}), by using a diaphragm pump (Hydor Bomba Ario 4). The temperature and light intensity in

the cultivation room were kept at 28±1ºC and 20 µmol.m^{-2}.s^{-1}, respectively. Irradiance was adjusted by regulating the distance between the light and the culture vessels, which was evaluated with the aid of a digital light meter (DIGITAL LUX TESTER YF-1065).

Constant illumination was provided by using two 40 W fluorescent lamps to obtain a higher photosynthetic rate, and hence higher productivity. All the material used in the experiment, including test tubes, Erlenmeyer flasks, and 9-L containers, were previously washed with water and detergent, rinsed and then autoclaved at 120°C for 15 min. The cultures were performed at salinities 15, 25 and 35, in triplicate and continuous, using 9-L containers with a working volume of 7 L; they consisted of 4.5 L of Guillard medium and 2.5 L of a pre-culture of *C. muelleri* produced in the same medium (inoculum). After microalgae inoculation, there was no addition of fresh medium throughout the growth of the culture, representing a stationary or batch type culture.

After the dilution of the inocula, the microalgal culture, at the three salinities and triplicates, were started with an optical density at 700 nm (diatoms) (OD_{700nm}) of 0.100, this value being used to represent the adaptation phase (lag). The cultures were maintained constant under the same conditions of light, nutrients, temperature, aeration and pH (8.0) (HANNA HI 221).

The monitoring of the cultures was carried out reading the optical density and cell counting. The OD_{700nm} was determined with a spectrophotometer (HACH DR 2000). The initial optical density was determined using the equation 1.

$$V = v - (D_F/D_I) \times v \quad (1)$$

where,

V – volume of the culture to be replaced by the culture medium,

v – volume of the culture, and

D_F and D_I – desired optical density and initial optical density of the inoculum, respectively.

Cell counting was done with the aid of a light microscope (TNE-10BN), using a mirrored Neubauer chamber. For counting, an aliquot of 5 mL was taken and fixed with formaldehyde neutralized with sodium tetraborate (borax); the results were expressed as cells.mL^{-1}.

The mean cell counts, obtained from the three replicates for each salinity, were used to construct growth curves. Accordingly, the growth stages were identified throughout the cultures and used to determine the maximum cell concentrations (X_{max}) and the growth rates in divisions per day (K), which were obtained for the days with higher productivity (OHSE et al., 2008). The growth rate (K) was calculated by using equation 2:

$$K = \log_2 (N_f - N_o)/D_t, \quad (2)$$

where,

K – is the growth rate,

N_0 and N_f – optical densities at the start and on the day when the culture reached the maximum cell concentration, respectively, and

D_t – the duration of the culture, in days.

These two variables were subjected to linear correlation analysis and the linear regression equation was determined using equation 3 (XU et al., 2006).

$$Y = a.X+b, \quad (3)$$

where,

Y – corresponds to cell density (cells.mL^{-1}),

X – optical density (OD_{700nm}),

a – angular coefficient or slope, and

b – linear coefficient.

At the end of the exponential growth phase, identified by spectrometry and cell count, the cultures were flocculated by adding 2 N NaOH. After the sedimentation of flakes, the culture medium was separated from the algal biomass by siphoning, and the biomass was then washed with distilled water to remove excess salt. Afterwards, the biomass of *C. muelleri* was oven-dried with circulating air at 60°C for 24 h.

The lipids were extracted by placing 5 g of dry biomass of *C. muelleri* into an 250 mL-Erlenmeyer flask with 25 mL of ethanol, 12.5 mL of chloroform and 5 mL of distilled water. The Erlenmeyer was sealed and immersed for 40 min in an ultrasonic bath

(CRISTÓFOLI-1014-127 V) set at 40 kHz and 80 W. Next, 12.5 mL of chloroform and 12.5 mL of distilled water were added to the mixture, which was sonicated for another 20 min. The solid fraction was obtained by vacuum filtration and oven-dried for 24 h at 105°C to recover the biomass. After the formation of two distinct phases in the filtered liquid, the nonpolar chloroform layer containing the lipids and the polar layer formed by methanol and water. The lipids were recovered and after evaporation of the chloroform, and the lipid content was quantified by weighing. The total lipid content in the biomass was determined by the weight difference of the solid mass before and after the extraction process. Three extractions of the biomass for each salinity were performed. The total percentage of lipid (PL) was calculated by equation 4:

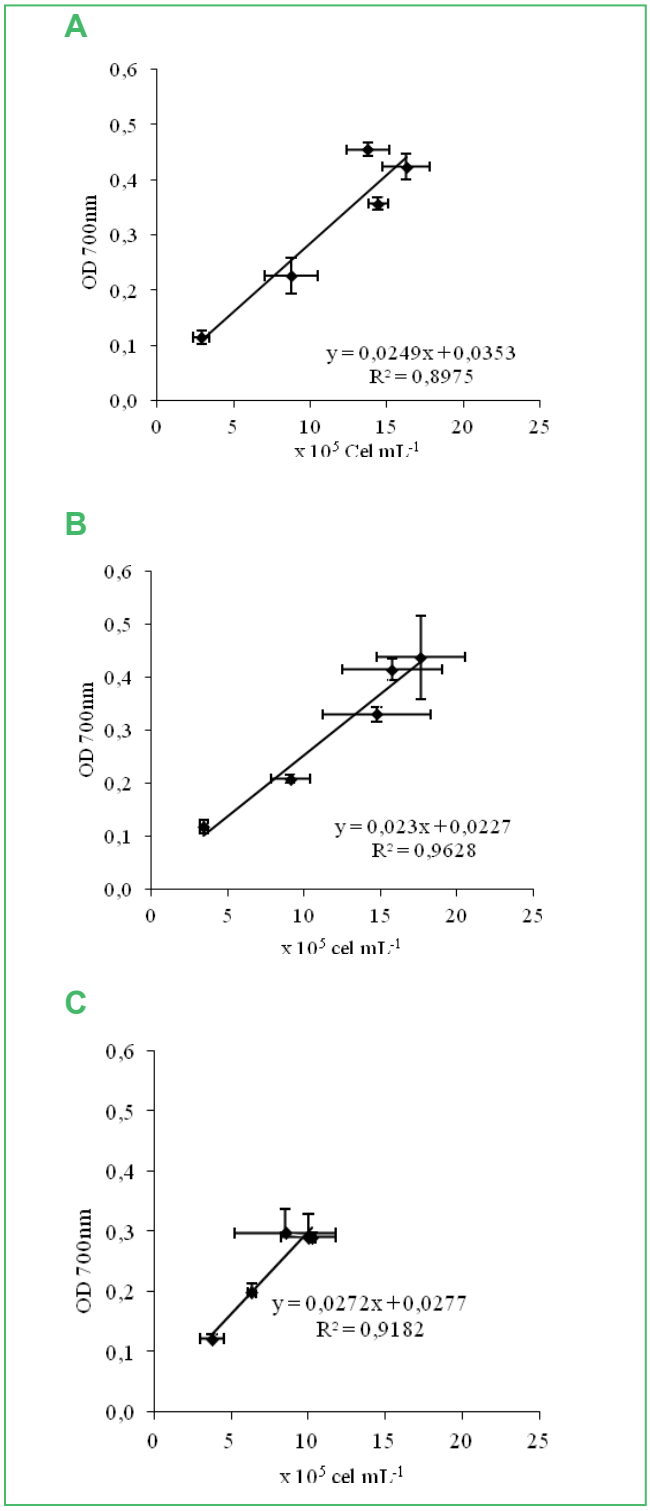
$$PL(\%) = (\text{weight before the extraction} - \text{weight after the extraction}) \times 100)/5\ g \quad (4)$$

To test for possible statistical differences between the lipid concentration, growth rate and maximum productivity of the cultures at the different salinities, the data were subjected to one-factor analysis of variance (ANOVA), and in case of significant differences, means of the treatments were compared by Tukey's test. In all analyses, the significance level adopted was 5%.

Results

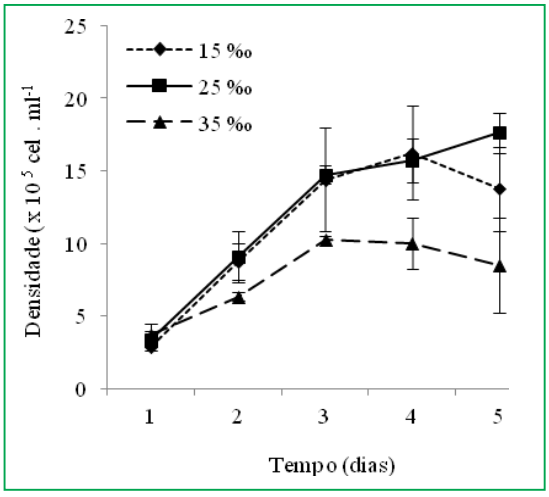
The daily determinations of OD_{700nm} and cell counts (cells.mL^{-1}) of the cultures at the three salinities showed strong and positive linear correlations (Figure 1). The growth curves (Figure 2) obtained from the cultures grown at salinities of 15 and 25 were fairly similar, with very sharp exponential growth phases, only differing on the last culture day, when cell density at a salinity of 25 was significantly higher. In the cultures performed at a salinity of 35, the growth curve demonstrated a lower development of algal population throughout the culture.

FIGURE 1: Linear correlations between OD_{700nm} and cell counts (cells.mL^{-1}) of the cultures of *Chaetoceros muelleri* and respective linear regression equations, at salinities of 15 (A), 25 (B) and 35 (C). n=3. Each point on the curves refers to the mean of three replicates ± standard deviation. The coefficient of determination (R2) and the regression lines are also indicated in each diagram.



The exponential growth phase, or log phase, was observed from the first to the third day in the cultures carried out at the three salinities. Afterwards, the cultures at salinities of 15 and 25 entered a slow growth phase, which lasted until the fifth day only at a salinity of 25. No induction phase of growth was observed for the three treatments. The culture grown at a salinity of 35 reached the stationary phase on the third day, and the cells tended to show senescence after the fourth day. On the other hand, after the reduction in relative growth on the fourth day, microalgal cultivation performed at the lowest salinity showed a trend toward senescence.

FIGURE 2: Growth curves of *Chaetoceros muelleri* grown at three salinities, 15, 25 and 35, for 5 days. Each point on the curves refers to the mean of three replicates ± standard deviation.



A significant variation ($p<0.05$) was observed in the maximum cell concentration (MCC) between the salinities studied. The cultures at salinities of 15 and 25 reached higher MCC values, without significant difference between them. The highest growth rate was observed at a salinity of 15, reaching the maximum concentration around 73 h (Table 1).

In the present study, the lipid yield was not influenced by salinity, while the maximum cell concentration was significantly higher ($p<0.05$) at salinities of 25 and 15 (Table 1). Thus, a greater production of total lipids could be achieved, especially in large scale, considering the total cells produced at these salinities.

The lipid yield values at the three salinities were not statistically different ($p<0.05$) in relation to the dry weight of the microalgae, where content values of 10.41±1.89, 10.87±2.10 and 12.33±1.81% were obtained for salinities of 15, 25 and 35, respectively.

Discussion

Microalgal lipid content seems to be highly variable and is related to environmental conditions. The influence of light intensity, temperature, nutrients and the stage of culture growth on the content of lipids and fatty acids in microalgae have been extensively studied. The production and storage of lipids by microalgae in response to variations in environmental factors are specific for each species, hindering generalization. Nevertheless, according to the literature, it appears that the lipid content of diatoms increases dramatically when the cultures reach the stationary phase due to growth-limiting factors, such as the reduction in the levels of silicate and nitrogen (PERNET et al., 2003).

Oshe et al. (2008) cultivated the same species at a salinity of 30 and observed a decrease in growth rate, requiring around seven days to reach the stationary phase. Khatoon et al. (2010), in analyzing the effect of salinity on three diatom species (*Navicula, Cymbella* and *Amphora*) grown in Conway medium at 28ºC and light intensity of 31.9 µmol.photons $m^{-2}.s^{-1}$, 12:12 h light-dark cycle, obtained a biomass increase from

TABLE 1: Kinetic yield parameters of *Chaetoceros muelleri* cultures at three different salinities.

Salinity	TMC[1] (hours)	GR[2] (divisions day^{-1})	MCC[3] (x 10^5 cells.mL^{-1})	LY[4] (%)
15	73 ± 1[b 5]	0.90 ± 0.12[a]	16.3 ± 1.50[a]	10.41 ± 1.89[a]
25	96 ± 1[a]	0.55 ± 0.11[b]	17.6 ± 2.90[a]	10.87 ± 2.10[a]
35	48 ± 1[c]	0.63 ± 0.18[ab]	10.3 ± 0.30[b]	12.33 ± 1.81[a]

[1] Time of maximum concentration; [2]Growth rate; [3]Maximum cell concentration; [4]Lipid yield; [5]Different letters represent significant difference ($p<0.05$).

the eighth day of culture, reaching a maximum on the twelfth day at all salinities tested (15, 20, 25, 30 and 35). The same authors found that biomass increased with salinity, in accordance with Ghezelbash et al. (2008), who showed that microalgae cultivated at a salinity of 40 $g.L^{-1}$ had the highest biomass. This result was similar to the findings of the present study. The same authors described that among several environmental factors, salinity also limits the growth and productivity of microorganisms.

The microalgae cultivated at the three salinities were flocculated to obtain dry biomass on the fifth day, by which time there was a trend toward stagnation in population growth, probably resulting from decreased availability of nutrients in the medium, among other factors. For an optimal performance in microalgal culture, several nutrients are essential for algal growth, such as nitrogen, hydrogen, phosphorus, calcium, magnesium, potassium and sulfur, as well as micronutrients such as iron, boron, copper, zinc, vanadium, molybdenum and sodium, usually present in culture media (SIPAÚBA-TAVARES; ROCHA, 2003).

Renaud et al. (2002) examined the growth of 18 microalgal species at a salinity of 25±1, temperature of 25±1ºC. The authors found a density of 28.9 $x10^5$ cells. mL^{-1} at salinities of 15 and 25 and obtained growth rates of 0.87 and 0.56 divisions.day^{-1}, respectively. The culture of the diatom *C. muelleri* in Guillard f/2 medium at 19ºC, with injection of CO_2, resulted in a MCC of 30 to 120 x 10^5 cells.mL^{-1}. Krichnavaruck et al. (2005) cultured *C. calcitrans* in a photobioreactor and obtained a maximum cell density of 88 x 10^5 cells.mL^{-1} in stationary phase, showing the efficiency of these structures.

The culture of the diatoms *C. wighami* and *Amphora coffeaeformis* in Guillard f/2 culture medium, at a salinity of 30, temperature of 28°C, and irradiance of 40 µmol.$m^{-2}.s^{-1}$ resulted in growth rates of 0.95 and 0.72 divisions.day^{-1} (RAJADURAI et al., 2005). For the diatom *Thalassiosira weissflogii*, Reinfelder et al. (2000) observed growth rates ranging from 1 to 1.5 divisions. day^{-1}, which were not significantly different from the growth rate obtained for *C. muelleri* at a salinity of 15 in our study.

Pacheco-Vega and Sánchez-Saavedra (2009) obtained a lipid content of 19.70±1.99 and 22.80±3.74% for *C. muelleri* in the Guillard f/2 medium and in a new medium, respectively. This new culture medium was prepared with 72% phosphate, phosphoric acid as phosphorus source and a liquid fertilizer, with 32% of total nitrogen supplied as urea (16.4%) and ammonium nitrate (15.6%), added to provide concentrations of P and N equal to those in Guillard medium. These cultures were maintained at 21ºC with constant light (100 µmol photons.$m^{-2}.s^{-1}$). Both media were prepared with sea water filtered (1-µm mesh size) and enriched with 0.107 $mol.L^{-1}$ Si. In another study with the same microalga cultivated in Guillard medium, with a photoperiod of 18:6, lipid yields of 25.25 and 11.67% were obtained for salinities of 25 and 35, respectively (ARAUJO et al., 2011). Wahlen et al. (2011) attained a lipid content of 44±0.87 mg.100 mg^{-1} biomass for the diatom *C. gracilis*.

Renaud et al. (2002) investigated the influence of temperature on growth and chemical composition of fatty acids of *Chaetoceros* sp. at temperatures around 25ºC and found about 16.8% lipids for this species. The fatty acids of microalgae can be influenced by several factors, including temperature (MORAIS; COSTA, 2008). The cell membrane fluidity depends on the degree of unsaturation of fatty acids, with the membrane being more fluid with increasing unsaturation (MARSZALEK; LODISH, 2005). Yeesang and Cheirsilp (2010) observed the effect of light when culturing *Botryococcus* spp. using different light intensities. The lipid content was greater when the culture was exposed to light of 33 to 49.5 µmol photons.$m^{-2}.s^{-1}$.

Takagi et al. (2006) cultured the microalga *Dunaliella tertiolecta* and found that cell concentration decreased with increase in NaCl concentration in the culture medium, but an increase in intracellular lipid content and a high percentage of triglycerides were seen in the exponential growth phase. However, increasing the NaCl concentration was not a good strategy, due to the sharp decrease in cell concentration for this species.

Converti et al. (2009) asserted that the growth and lipid content of *Chlorella vulgaris* can be affected by the temperature of the culture. The authors found a 17% reduction in growth rate at 30ºC, when

compared to a temperature of 25°C, and a significant reduction in the amount of lipids (from 14.71±0.30 to 5.90±0.42 g.100 g dry weight^{-1}). For the microalga *Nannochloropsis oculata*, the lipid content doubled from 7.90±0.21 to 14.92±0.82 g.100 g dry weight^{-1} under the same conditions of temperature. When the nitrogen concentration in the medium was reduced by 75%, there was an increase in lipid content from 7.90 to 15.31% in *N. oculata*, and from 5.90 to 16.41% in *C. vulgaris*. In microalgal cultures, each species has an ideal temperature, which is usually between 20 and 30°C (CHISTI, 2007).

The use of photobioreactors is also an alternative for a greater and faster productivity, as well as the use different techniques to recover the microalgae from the culture medium and the use of the biomass after lipid extraction to obtain other compounds with biological activity. Besides, the modulation of other parameters such as temperature, photoperiod, irradiance, composition of the culture medium, and type of culture can be done for possible increase in lipid yield.

In conclusion, the variation of salinity in the culture medium had no influence on the lipid production of the microalga *C. muelleri*. However, this variation resulted in a significant increase in productivity and greater cell concentration at salinities of 15 and 25, demonstrating the greater advantage of growing this microalga under these salinity conditions.

In addition, these results indicate that a higher yield of total lipids can be obtained in *C. muelleri*, especially on a large scale, considering the total number of cells produced at these salinities. Further and more detailed studies are needed, such as the characterization and qualification of lipids found in the species.

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