EFFECT OF NITRATE DEPLETION ON LIPID ACCUMULATION BY THE MARINE MICROALGA *Nannochloropsis oculata*

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ABSTRACT

Nannochloropsis oculata is a marine algae with high lipid content, being a promising species for biofuel production. It is also used as food in aquaculture for larval fish and crustaceans due to its high nutritional value and compatible size with the larvae feeding. This study evaluated the lipid content in the biomass of *N. oculata* under nitrate depletion. The cultivation was carried out in 8 L bottles with three replications, at salinity 27, illuminance of 60 µE cm^{-2} s^{-1} and temperature 28 °C. The levels of sodium nitrate used were 15; 30; 45; 60 and 75 mg L^{-1}, while maintaining constant the levels of all other nutrients of the Guillard f/2 medium. The growth curve was monitored every two days by spectrophotometry at 680 nm (DO_{680nm}) and cell counting in a Neubauer chamber. For separation of the culture medium from the cells, we used chemical flocculation method, adding 2N NaOH. The higher lipid content in the biomass was obtained at the lowest level of nitrate in the culture medium.

Keywords: biomass; lipid content; cells yield

EFEITO DA DEPLEÇÃO DE NITRATO NO ACÚMULO LIPÍDICO DA MICROALGA MARINHA *Nannochloropsis oculata*

RESUMO

Nannochloropsis oculata é uma microalga marinha que possui alto teor lipídico, sendo uma promissora espécie para a produção de biocombustível. É utilizada também como alimento na aquicultura na fase larval de peixes e crustáceos devido ao seu elevado valor nutricional e por possuir tamanho compatível com o mecanismo alimentar das larvas. O objetivo do trabalho foi avaliar o rendimento lipídico da *N. oculata* com depleção de nitrato. Os cultivos foram realizados em frascos de 8 L com três repetições, na salinidade de 27, iluminância de 60 µE cm^{-2} s^{-1} e temperatura de 28 ºC. As quantidades de nitrato de sódio utilizadas foram 15; 30; 45; 60 e 75 mg L^{-1}, mantendo constantes as quantidades dos outros nutrientes do meio Guillard f/2. A curva de crescimento foi acompanhada a cada dois dias por espectrofotometria a 680 nm (DO_{680nm}) e contagem celular em uma câmara de Neubauer. Para separação do meio de cultivo das células foi utilizado o método de floculação química, adicionando NaOH 2N. O melhor rendimento lipídico foi obtido na menor quantidade de nitrato no meio de cultura.

Palavras chave: biomassa; conteúdo de lipídios; rendimento celular

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INTRODUCTION

Nannochloropsis oculata is a unicellular marine microalgae that can absorb sunlight and carbon dioxide in the presence of inorganic nutrients, to produce organic matter through photosynthesis. It has high lipid content, being a promising species for the production of biodiesel; it is also used as food in aquaculture, especially for the larval stage of fish and crustaceans due to its easy and rapid cultivation and small size compatible with the feeding mechanism of larvae (WEI *et al*., 2013).

The main physical and chemical factors affecting the growth of *N. oculata* include light, temperature, salinity, availability and quality of nutrients (LIN *et al*., 2012; TAMBURIC *et al.*, 2014). The interactions of microalgae with the culture medium and with the physical environment result in changes in cell density, which tends to increase exponentially after the inoculation. On the other hand, the concentrations of dissolved nutrients in the culture medium tend to decline with algal abundance increase, reaching the complete depletion, depending on the time of the culture development, leading to microalgae stress (LI *et al*., 2011).

The selection of culture medium is extremely important for mass production of microalgae. According to ELLIS *et al.* (2012), the choice of the medium should not consider only the operating costs involved, because often low-cost culture media may be deficient in some components and do not allow maximum production of algal biomass. The inappropriate use can affect the growth rate and biochemical composition of cells (CONVERTI *et al*., 2009). Indeed, for each microalgal species, productivity and the biochemical composition of the cells depend strongly on the type of culture and the nutrient profile of the medium (GUEDES *et al*., 2011).

Growth rate and oil production are also directly related to the concentration of nutrients present in the culture medium of microalgae. Nitrogen plays a key role in controlling the productivity of these organisms and there is a certain concentration of nitrogen at which biomass and lipid production can be maximized (DE LA HOZ *et al*., 2011). CONVERTI *et al.* (2009) studied the effect of nitrogen and temperature on growth and lipid content of *N. oculata* and found that the decrease of nitrogen concentration in the medium from 0.30 to 0.08 g $NaNO_3$ L^{-1} can increase the lipid fractions from 7.90 to 15.31% of biomass dry weight. GRIS *et al.* (2013) reported a relationship between $NaNO_3$ concentration and lipid content of *N. oculata* was inversely proportional, as well as they obtained a maximum lipid content around 30%. DRAGONE *et al.* (2011) argued that the nutrient limitation in the culture medium causes the starch to rise and may become feasible the production of bioethanol.

CHIU *et al.* (2009) reported that the production of biofuels from microalgae depends on the growth rate of the cultured species and the oil content therein. Microalgae with high lipid production are the most desirable for obtaining biodiesel. Depending on the species, microalgae produce different types of lipids, hydrocarbons and other complex oils.

In this context, the present study determined the lipid content (%) of the *N. oculata* dry weight (DW) cultivated with decreasing levels of sodium nitrate in the culture medium.

MATERIAL AND METHODS

The strain of *N. oculata* used in this study was obtained from the strain bank of the Laboratory of the Center for Aquaculture Technology (CTA), Department of Fisheries Engineering, Federal University of Ceará, where it is maintained at 22 ± 2 °C in test tubes, with artificial light and photoperiod of 8 h/16 h light-dark cycle. The medium used for inoculum maintenance and for the experiment was Guillard f/2 (GUILLARD, 1975).

The levels of sodium nitrate used during cultivation were 15; 30; 45; 60 and 75 mg L^{-1}, while maintaining constant the levels of monobasic sodium phosphate, sodium silicate, vitamins and trace metals of the Guillard f/2 medium (GUILLARD, 1975). It is noteworthy that the level of sodium nitrate commonly used in standard Guillard f/2 medium is 75 mg L^{-1}. Culture media were prepared with seawater with salinity 30 previously autoclaved at 121 °C for 15 minutes.

A culture of *N. oculata* pre-established with 300 mL was used as inoculum in a 3 L Erlenmeyer. Subsequently, the volume of the container was completed with Guillard f/2 medium containing 75 mg L^{-1}. After the development of the new inoculum, accompanied through optical densities (DO_{680nm}) using a HACH 2000 spectrophotometer and cell counting (cells mL^{-1}) in a Neubauer chamber, aliquots (300 mL) were used to start the cultivations with different levels of sodium nitrate.

Batch cultures were performed in triplicate in 3 L containers and subjected to constant aeration by means of diaphragm pumps to ensure the movement of the culture and increase the photosynthetic rate. Light intensity was 60 µE cm^{-2} s^{-1} determined by a digital light meter and temperature of 28 ± 1°C, measured by a thermometer. The experiment lasted for five days and during this period the culture optical density was measured daily using a spectrophotometer at 680 nm and cell counting conducted using a Neubauer chamber. At the end of the cultivations, we used a 2N NaOH flocculating solution to separate microalgae from the culture medium (ARAUJO *et al.*, 2011), washed the biomass with distilled water and dried in a forced air circulation oven at 60 °C for 24 h (RODRIGUES *et al.*, 2009).

The concentration of nitrate-N in culture media was determined at the beginning and end of the experiment by spectrophotometry at 500 nm. For this, 100 mL samples of each repetition were taken and centrifuged at 3,000 x g for 5 min. Then, to 25 mL of each sample we added NitraVer 5 Nitrate reagent, and after five minutes, samples were read in the spectrophotometer.

Lipid extraction was carried out by the method of BLIGH and DYER (1959), in which we added to the dry weight of the microalgae (5 g), in triplicate, in a 250 mL-Erlenmeyer flask, 25 mL methanol, 12.5 mL chloroform and 5 mL water. The flask was capped and sonicated for 40 min in an ultrasonic bath with a frequency of 40 kHz and power of 80 W. Then, we added 12.5 mL chloroform and 12.5 mL 1.5% sodium sulfate solution and sonicated again for 20 min. The solid was vacuum filtered and oven-dried at 105 °C for 24 hours.

Values referring to final concentrations of nitrate, biomass recovery and lipid content from microalgae grown under different levels of sodium nitrate were subjected to analysis of variance (ANOVA) and, in the case of a significantdifference, subjected to the t-test for mean values at 5% using the MicroCal Origin 6.0 software.

RESULTS

The culture grown with the highest level of sodium nitrate in the medium (75 mg L^{-1}) showed significantly higher cells yield (cells mL^{-1}) compared with the other cultures (Table 1). Similarly, differences in the growth phases were observed among treatments. For instance, the cultures with 45, 60 and 75 mg L^{-1} sodium nitrate in the culture medium started the exponential growth phase after the second day; the cultures with 15 and 30 mg L^{-1} started this phase after the third cultivation day (Figure 1).

Table 1. Means (± standard deviation) of final cell density (cells mL^{-1}) of microalgae *Nannochloropsis oculata* grown under different levels of sodium nitrate and respective lipid content (%) in the dry weight.

Parameter	Sodium nitrate (mg L^{-1})				
	15	**30**	**45**	**60**	**75**
Cell density yield (cells mL^{-1})	80.96 ± 12.54^{a}	130.96 ± 9.95^{b}	169.56 ± 9.67^{c}	173.36 ± 11.74^{c}	202.17 ± 8.44^{d}
Lipid content(%)	49.41 ± 0.14^{a}	35.68 ± 0.12^{b}	31.21 ± 0.58^{c}	26.77 ± 0.36^{d}	23.36 ± 0.15^{e}

Different lowercase letters indicate significant differences between cell density and lipid content in the cultivation with varying nitrate concentrations.

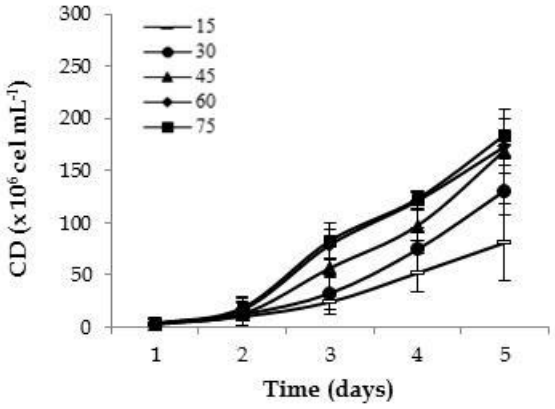

Figure 1. Growth curves of *Nannochloropsis oculata* maintained at different sodium nitrate levels (mg L^{-1}) expressed in terms of cell density (cells mL^{-1}) per cultivation day.

The removal of sodium nitrate was accompanied at the beginning and end of the cultivation. The results showed significant differences, where crops with 45, 60 and 75 mg L^{-1} sodium nitrate presented a higher removal percentage, followed by the level of 30 mg L^{-1} and finally the level of 15 mg L^{-1}, which had the lowest removal percentage of this nutrient at the end of the study (Table 2).

The cell density was significantly higher with increasing levels of sodium nitrate in the culture medium, unlike the oil content per biomass weight, where, with the gradual depletion of sodium nitrate, lipid content increased.

The culture grown with the lowest level of sodium nitrate (15 mg L^{-1}) presented the lowest population growth, but yielded the greatest lipid content in the dry weight. The lipid content in the biomass of *N. oculata* was significantly lower with increasing levels of nitrates in the medium (Table 1).

Table 2. Means (± standard deviation) of the initial and final sodium nitrate levels and their removal rates (%) in the cultivation of microalgae *Nannochloropsis oculata* grown under different levels of sodium nitrate.

Cultivation phase	Sodium nitrate (mg L^{-1})				
	15	**30**	**45**	**60**	**75**
Initial	2.42 ± 0.22^{a}	3.25 ± 0.68^{ab}	4.50 ± 0.72^{b}	6.34 ± 0.53^{c}	7.41 ± 0.60^{c}
Final	0.380 ± 0.020^{a}	0.400 ± 0.030^{ab}	0.450 ± 0.024^{b}	0.517 ± 0.013^{cd}	0.533 ± 0.011^{d}
Nitrate removal (%)	84.29^{a}	87.69^{b}	90.00^{c}	91.84^{c}	92.80^{c}

Different lowercase letters indicate significant differences between the initial and final concentrations of nitrate and removal percentage of this nutrient.

DISCUSSION

Sodium nitrate medium concentration was determinant for growth and lipid cell storage of *N. oculata*. The population growth and lipid content are also directly related to the concentration of nutrients present in the culture medium of microalgae. Nitrogen plays an essential role in controlling the productivity of these organisms and there is a certain concentration of nitrogen at which biomass and lipid production can be maximized and accumulate energy in the form of lipids, under stress of reducing nitrogen source (DE LA HOZ *et al*., 2011).

Several studies such as LI *et al*. (2011), KONG *et al*. (2010), WANG *et al*. (2010) have reported a deceleration of the daily growth rate in cultures of *Chlorella* sp. according to of the nutrients uptake especially phosphate and nitrate, which are essential for the growth of algae. In this study, with increasing algal population, sodium nitrate depletion was quite evident over time, independent of the level of this nutrient in the culture medium.

RODOLFI *et al*. (2009) cultivated the microalgae *Nannochloropsis* sp. in 110 L photobioreactors under direct sunlight with varying nitrogen concentration. The authors observed a decrease of 16.7% in biomass when the microalgae was grown under nitrogen depletion, similar to our results for *N. oculata*.

CHEN *et al*. (2011) evaluated the effects of varying nutrient concentration on the growth of the microalgae *Dunaliella tertiolecta* and also found an increase in algal density with tenfold

increased level of sodium nitrate in the culture medium. In turn, JIANG-MING *et al*. (2010) cultivated *Chlorella vulgaris* at various concentrations of potassium nitrate (5, 3, 1 and 0.2 mg L^{-1}) in the culture medium and verified an increase from 0.4 to 1.20 g L^{-1} in biomass concentration with the increase in potassium nitrate concentration from 0.2 to 5.0 mg L^{-1}. These results are similar to those registered in the present study, where higher levels of sodium nitrate caused biomass to rise.

In the present study, *N. oculata* reached higher oil content at lower levels of sodium nitrate (15 mg L^{-1}) in the culture medium. CONVERTI *et al*. (2009) analyzed the effects of nitrogen concentration on lipid content of a *N. oculata* strain and when the initial nitrogen concentration in the medium was reduced by 75% the authors reported an increase of approximately 48% in the lipid content of this species. This result indicates that nitrogen depletion increases lipid content of microalgae. Similar relationship was observed by GRIS *et al*. (2013), which also found a lipid content of 30% (of dry weight) in *N. oculata* cells cultivated in 45 mg L^{-1} of sodium nitrate.

Algae generally responded to nitrogen deprivation as a command to store the energy and then reacted to this response by accumulating the lipids. In the cultivation of the green algae *Neochloris oleoabundans* in an anaerobic effluent digester and in batch type cultivation, LEVINE *et al*. (2011) achieved a lipid content of 10 to 30% methyl esters of fatty acids in relation to the dry weight. Furthermore, the content of polyunsaturated fatty acids (C16: 3, C18: 2 and C18: 3) decreased with increasing deprivation of up to 90-95% ammonium nitrate from the medium over six days of culture, while the content of C18: 1 increased. The removal of sodium nitrate by *N. oculata* from the growth medium ranged between 84 and 93%. Similarly CHARITY *et al*. (2009) cultivated *Scenedesmus* sp. in water from a fish farming effluent to evaluate the removal of nutrients, growth and yield of biomass through natural sedimentation. Cultures were performed in outdoor tanks of 150 L with constant aeration and natural photoperiod and temperature. Microalgae removed 94.44% (23.80 mg L^{-1}) ammonia, 77.54% (7.04 mg L^{-1}) phosphate and 35.59% (26.09 mg L^{-1}) organic matter. LEVINE *et al*. (2011) observed that the microalgae *N. oleoabundans* assimilated 90-95% ammonium nitrate from the medium after six days in batch type cultivation.

The marine microalgae *D. tertiolecta* was cultivated by MASSART *et al.* (2010) to determine the growth rate and the oil content at different levels of nutrients in the culture medium. The authors used the fluorescence technique to determine the oil content inside the cells, and evidenced its reduction with increasing population growth of microalgae. In the present study, the stress caused by nitrate depletion in the medium reduced the growth of cultures, but increased oil production thereof.

CONCLUSIONS

The microalgae *N. oculata* produces more lipids with declining level of sodium nitrate in the culture medium, but this reduction can significantly decrease the cell density.

REFERENCES

ARAUJO G.S.; MATOS L.J.B.L.; GONÇALVES L.R.B.; FERNANDES, F.A.N. 2011 Bioprospecting for oil producing microalgal strains: Evaluation of oil and biomass production for ten microalgal strains. *Bioresource Technology*, *102*(8): 5248-5250.

BLIGH, E.G. and DYER, W.J. 1959 A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8): 911-917.

CHARITY, E.A.R.; ALEXANDRA, L.V.B.; CARMEN, H.C.L.; EVER, D.M.A. 2009 Biomass production of microalga *Scenedesmus* sp. with wastewater from fishery. *Revista Tecnica de la Facultad de Ingenieria Universidad del Zulia, 32*(2): 126-134.

CHEN, M.; TANG, H.; MA, H.; HOLLAND, T.C.; SIMON, K.Y.; SALLEY, S.O. 2011 Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. *Bioresource Technology*, *102*(2): 1649-1655.

CHIU, S.; KAO, C.; TSAI, M.; ONG, S.; CHEN, C.; LIN, C. 2009 Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. *Bioresource Technology*, *100*(2): 833-838.

CONVERTI, A.; CASAZZA, A.A.; ORTIZ, E.Y.; PEREGO, P.; DEL BORGHI, M. 2009 Effect of temperature and nitrogen concentration on the

growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification*, *48*(6): 1146-1151.

DE LA HOZ, S.; BEN-ZVI, A.; BURRELL, R.E.; McCAFFREY, W.C. 2011 The dynamics of heterotrophic algal cultures. *Bioresource Technology*, *102*(10): 5764-5774.

DRAGONE, G.; FERNANDES, B.D.; ABREU, A.P.; VICENTE, A.A.; TEIXEIRA, J.A. 2011 Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Applied Energy*, *88*(10): 3331-3335.

ELLIS, J.T.; HENGGE, N.N.; SIMS, R.C.; MILLER, C.D. 2012 Acetone, butanol, and ethanol production from wastewater algae. *Bioresource Technology*, *111*: 491-495.

GRIS, L.R.S.; PAIM, A.C.; FARENZENA, M; TRIERWEILER, J.O. 2013 Influence of $NaNO_3$ concentration and incident light intensity on *Nannochloropsis oculata* lipid accumulation. *Brazilian Archives of Biology and Technology*, *56*(4): 673-678.

GUEDES, A.C.; AMARO, H.M.; MALCATA, F.X. 2011 Microalgae as sources of high added-value compounds - a brief review of recent work. *Biotechnology Progress*, *27*(3): 597-613.

GUILLARD, R.R.L. 1975 Culture of phytoplankton for feeding marine invertebrates. In: SMITH, W.L. and CHANLEY, M.H. *Culture of marine invertebrate animal*. New York: Plenum Publishing. p.29-60.

JIANG-MING, L.; LI-HUA, C.; XIN-HUA, X.; LIN, Z.; HUAN-LIN, Z. 2010 Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. *Bioresource Technology*, *101*(17): 6797-6804.

KONG, Q.; ZHU, L.; SHEN, X. 2010 The toxicity of naphthalene to marine *Chlorella vulgaris* under different nutrient conditions. *Journal of Hazardous Materials*, *178*(1-3): 282-286.

LEVINE, R.B.; COSTANZA-ROBINSON, M.S.; SPATAFORA, G.A. 2011 *Neochloris oleoabundans* grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. *Biomass and Bioenergy*, *35*(1): 40-49.

LI, Y.; CHEN, Y-F.; CHEN, P.; MIN, M.; ZHOU, W.; MARTINEZ, B.; ZHU, J.; RUAN, R. 2011 Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology*, *102*(8): 5138-5144.

LIN, Q.; GU, N.; LI, G.; LIN, J.; HUANG, L.; TAN, L. 2012 Effects of inorganic carbon concentration on carbon formation, nitrate utilization, biomass and oil accumulation of *Nannochloropsis oculata* CS 179. *Bioresource Technology*, *111*: 353-359.

MASSART, A.; AUBRY, E.; HAMTSON, A.L. 2010 Study of culture strategies of *Dunaliella tertiolecta* combining high cell density and accumulation of lipids to produce biodiesel. *Biotechnology, Agronomy and Society and Environment*, *14*(2): 567-572.

RODOLFI, L.; ZITTELLI, G.C.; BASSI, N.; PADOVANI, G.; BIONDI, N.; BONINI, G.; TREDICI, M.R. 2009 Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, *102*(1): 100-112.

RODRIGUES, J.A.G.; SOUSA JR., J.; LOURENÇO J.A.; LIMA P.C.W.C.; FARIAS, W.R.L. 2009 Cultivo de camarões tratados com polissacarídeos sulfatados da rodofícea *Halymenia pseudofloresia* mediante uma estratégia profilática. *Ciência Agronômica*, *40*(1): 71-78.

TAMBURIC, B.; SZABÓ, M.; TRAN, N.T.; LARKUM, A.W.D.; SUGGETT, D.J.; RALPH, P.J. 2014 Action spectra of oxygen production and chlorophylla fluorescence in the green microalga *Nannochloropsis oculata*. *Bioresource Technology*, *169*: 320-327.

WANG, H.M.; PAN, J.L.; CHEN, C.Y.; CHIU, C.C.; YANG, M.H.; CHANG, H.W.; CHANG, J.S. 2010 Identification of anti-lung cancer extract from *Chlorella vulgaris* C-C by antioxidant property using supercritical carbon dioxide extraction. *Process Biochemistry*, *45*(12): 1865-1872.

WEI, L.; HUANG, X.; HUANG, Z.; ZHOU, Z. 2013 Orthogonal test design for optimization of lipid accumulation and lipid property in *Nannochloropsis oculata* for biodiesel production. *Bioresource Technology*, *147*: 534-538.