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Influence of $NaNO_3$ Concentration and Incident Light Intensity on *Nannochloropsis oculata* Lipid Accumulation

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ABSTRACT

This study aimed at evaluating the best conditions for lipid accumulation in the marine microalga Nannochloropsis oculata *in an airlift photobioreactor. Experiments were carried out following a central composite design with the following variables: temperature (19 to 29°C), sodium nitrate concentration ($NaNO_3$) in the culture medium (f/2) (25 to 125 mg.L^{-1}) and incident light intensity (49 to 140 µE.$m^{-2}s^{-1}$). The maximum lipid production was 132.4 mg.L^{-1} under the following conditions: 27°C, $NaNO_3$ concentration 105 mg.L^{-1} and 122 µE.m^{-2}.s^{-1}, which was 30% of dry weight of the biomass.*

Key words: *Nannochloropsis oculata*, Airlift Photobioreactor, Central Composite Design, Lipid

INTRODUCTION

The global demand for energy is expanding quickly and, according to the U.S. Energy Information Administration (2011), this expansion will continue over the next 25 years. Total global energy consumption will double by 2035, compared to 1990. Developing countries such as Brazil, Russia, China, and India have been highlighted as potentially high energy consumers. The development of new renewable energy sources is, thus, a significant option to ensure the safe growth of a nation, leading to fewer limitations in energy availability.

Among different sources of renewable energy, microalgae biofuels have emerged as a technically viable alternative, mainly due to their high efficiency as oil producers and given that, compared to oil crops, they require less fertile land and less water for their cultivation and their production is possible on non-arable land. Furthermore, there is the appeal of CO_2 bio-mitigation and wastewater treatment, since the nutrients for microalgae cultivation (especially nitrogen and phosphorus) may be obtained from the organic effluent from agri-food industries (Chisti 2007; Brennan and Owende 2010).

Despite their great potential, the microalgae alternative is not currently viable for energy applications, principally for economic reasons. In order to optimize microalgae production, some of the most effective improvements that need to be implemented are: increased CO_2 fixation and light utilization efficiency; development of improved biofuel precursor components and high value-added products selectivity; studies on the application of appropriate biomass processing technologies; and improvements in separation processes (Chisti 2007; Brennan and Owende 2010; Gallagher 2011). A number of reviews have

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discussed the generation of biofuels from different microalgae, which include *Nannochloropsis oculata* - mainly because of its high growth rate and lipid content (Roncarati et al. 2004).

Several studies have been made on the effects of cultivation variables on *N. oculata* production. Flynn et al. (1993) studied the effect of carbon-nitrogen on algae growth; Converti et al. (2009) studied the effect of nitrogen and temperature on the growth and lipid content; Spolaore et al. (2006) studied the effects of pH, temperature, aeration rate, and incident light intensity; and Rodolfi et al. (2009) achieved a *Nannochloropsis* sp lipid content of around 60% under the conditions of nutrients limitations. The aim of this work was to study the effect of temperature, nitrogen, and incident light intensity on the growth and lipid content of *N. oculata* using a central composite design.

MATERIALS AND METHODS

Algal Strain and Inoculum Preparation

The strain of *N. oculata* was obtained from the Laboratory of the Ecology of Phytoplankton and Marine Microorganisms (Federal University of Rio Grande, Brazil). Cultures were maintained in an autoclaved f/2 culture medium using artificial seawater (marine salt Red Sea, salinity 33 ± 1 $g.L^{-1}$) and enriched with nutrients (Lourenço 2006). They were kept in 6L Erlenmeyer flasks (working volume 4L), with aeration (filtered air without CO_2 enrichment, 0.25 VVM, i.e., 1L.min^{-1}) in a refrigerated incubator. Incident light intensity was fixed at approximately 98 $\mu E.m^{-2}.s^{-1}$. The cells were maintained at a controlled temperature (25 ± 1°C) and photoperiod (12 h light/12 h dark). Every 10 days, the cultures were sub-cultured and re-inoculated into a fresh medium (1/10 dilution).

Experimental System and Experimental Design

Experiments were conducted in batch mode with 12 rectangular internal loop airlift photobioreactors (optical path 6 cm, height 35 cm and working volume 3.2 L). Heat transfer was carried out by pumping a controlled temperature water stream through stainless steel tubes (1/2″), located at the downcomer. Photoperiod was kept constant (12 h light/12 h dark) and photobioreactors were illuminated from one side (riser) by two fluorescent cool lamps (Universal Duramax Super Daylight 20W, Brazil). Incident light intensity was measured from the light-attached surface of the photobioreactor using a luximeter (Instrutherm LD-200, Brazil).

Agitation was provided by continuous air injection (without additional CO_2) into two porous stones placed at the bottom of the riser of each system. An aeration rate of 800 mL.min^{-1} (0.25 vvm) was measured with gas flow meters (rotameters) (Dwyer Instruments, Inc). The overall volumetric mass transfer coefficient of the liquid phase (k_La) was determined using the Dynamic method (Chisti 1989) at 19°C and an aeration rate of 750 ml.min^{-1}, where k_La was equal to 28.62 h^{-1}.

A central composite design (divided into two blocks) was performed to evaluate the effects of temperature, sodium nitrate concentration in the f/2 medium and incident light intensity on final biomass and lipid concentration. Each batch of photoautotrophic cultivation was carried out for 11 days. Independent variable values are shown in Table 1. The central values were chosen on the basis of data reported in the literature (Spolaore et al. 2006; Chiu et al. 2009; Converti et al. 2009). The pre-cultured *N. oculata* was inoculated in photobioreactors at an initial biomass concentration (calculated dry weight of microalgal biomass per liter) of 26.84 ± 1.00 and of 21.46 ± 0.77 mg.L^{-1} in the experimental blocks 1 and 2, respectively.

Table 1 - Quantitative values of the encoded factor level.

Factor	-α	-1	0	+1	+α
Temperature (°C)	19	21	24	27	29
$NaNO_3$ concentration in f/2 medium (mg.L^{-1})	25	45	75	105	125
Incident light intensity ($\mu E.m^{-2}.s^{-1}$)	49	68	95	122	140

Analytical Methods

Samples (4.0 mL) were withdrawn daily at the same time to monitor the cell growth. Biomass density was evaluated by measuring the optical density at 570 nm. (Pró-Análise UV-1600 spectrophotometer, Brazil).

Each sample was diluted in order to reach an absorbance measurement between 0.1 and 1.0,

when required. For each block of the experiments, a calibration curve of cell dry weight (filtration on Macherey-Nagel GF-3 filters, Germany) versus absorbance was established. Therefore, for each batch experiment, growth curves (Fig. 1) and final biomass concentration were determined according to culture absorbance. The pH was monitored daily using indicator paper (Merck, range 0-14, USA) and remained between 7.0 and 8.0 throughout the entire experimental period.

Lipid extraction was conducted according to a modified Folch method, combined with ultrasound (Brum et al. 2009; Lee et al. 2010). Dry biomass was obtained by centrifugation (3044g, 5 min, 10°C) and lyophilization (to a constant weight). A sample (500 mg) was macerated with a chloroform/methanol solution (2:1, v/v, total 30 ml) in a porcelain crucible and subsequently sonicated for 90 min (Ultrasonic Bath Unique 1400A MaxiClean 40 kHz, Brazil). The solid content of the sample was removed by centrifugation (2214 g, 8 min, 4°C) and the phase containing the lipids was subjected to additions of 6.0 mL 0.88% KCl solution, followed by chloroform/methanol (1:1, v/v, total 4.0 mL), and finally by hand agitation (in triplicate). The remaining lipid phase was filtered with anhydrous Na_2SO_4, recovered in a previously weighed balloon and submitted to solvent evaporation in a rotary evaporator (70°C, vacuum). Then, the lipid-containing balloon was kept in a desiccator for 10 min and then weighed to determine the lipid mass.

Statistical analysis was performed using *Statistica 10.0* (*StatSoft Incorporation*) software. The linear, quadratic and interaction factors of the variables were calculated. The significance level (α) was equal to 0.05%, which confirmed the significance of the results for the response variables with 95% confidence. A p-value of less than 0.05 was considered to indicate the significance. *F-tests* were carried out to evaluate the significance of the fitted models at 5% (Montgomery 2001).

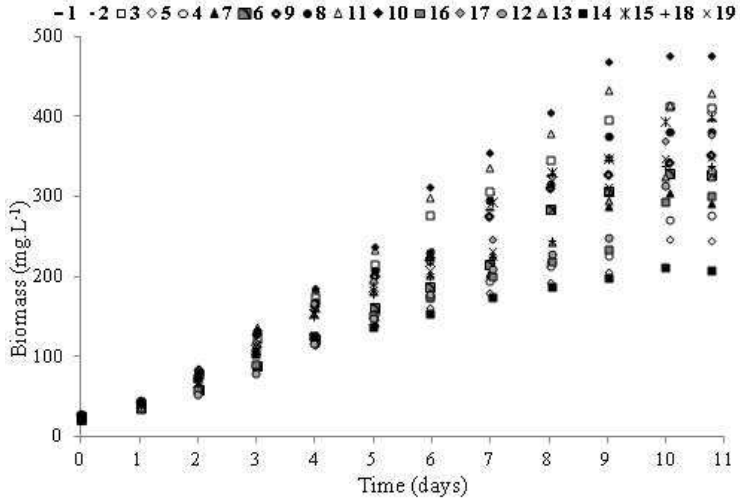

Figure 1 - Growth curves for experiments 1 to 19.

RESULTS AND DISCUSSION

Two equations were created, one for each block, which related *N. oculata* final biomass concentration (*y*, in mg.L^{-1}) to optical density (*x*), with R^2 = 99.2% and 97.9%, respectively:

$$y = 247.70x - 9.04 \quad (1)$$

$$y = 259.10x - 8.76 \quad (2)$$

Dry weight determinations were made from the tests coded as experiments 10 and 11 (Table 2) in Block 1, and as experiments 15 and 17 in Block 2, since these experiments achieved the highest biomass concentration values in each block. The similarity between the coefficients of linear regression (i.e., 274.47 and 259.10) was consistent with the work of Spolaore et al. (2006), in which different experimental conditions did not affect the linear relationship between the absorbance and dry weight. Table 2 presents the design matrix of the central composite design and the experimental results.

Table 2 - Central composite design parameter levels (encoded values).

	Experiment	Independent			Dependent variables		
		T	N	I	Final biomass concentration (mg.L^{-1})	Lipid content (%)	Lipid concentration (mg.L^{-1})
Block 1	1	0	0	0	390.6	24.42	95.4
	2	0	0	0	397.3	24.35	96.7
	3	0	0	0	433.7	23.47	101.8
	4	-1	-1	-1	274.7	30.36	83.4
	5	+1	-1	-1	246.0	28.54	70.2
	6	-1	+1	-1	333.2	21.38	71.2
	7	+1	+1	-1	299.5	18.73	56.1
	8	-1	-1	+1	381.7	26.17	99.9
	9	+1	-1	+1	360.1	29.00	104.4
	10	-1	+1	+1	482.7	24.86	120
	11	+1	+1	+1	453.0	29.22	132.4
Block 2	12	-α	0	0	328.1	18.15	59.6
	13	+α	0	0	338.0	18.13	61.3
	14	0	-α	0	218.2	25.86	56.4
	15	0	+α	0	404.3	16.53	66.8
	16	0	0	-α	309.5	21.42	66.3
	17	0	0	+α	383.1	26.65	102.1
	18	0	0	0	353.5	22.28	78.8
	19	0	0	0	366.0	18.79	68.8

* T = temperature, N = $NaNO_3$ concentration in f/2 medium, I = incident light intensity.

The reduced models fitted for final biomass concentration (Equation 3) and lipid content (Equation 4) satisfactorily explained the effect of $NaNO_3$ concentration in the f/2 medium (N) and incident light intensity (I), with an explained variation of data equivalent to 82.18 and 88.62%, respectively. The positive values of the linear regression coefficients associated with N and I (Equation 3) indicated that an increase in the final biomass concentration was favored by increasing the values of these variables. The negative value of the quadratic regression coefficient associated with N gave a parabolic data profile. The positive values of the regression coefficients associated with I (Equation 4) indicated that an increase in lipid content wasis favored by increasing the value of this variable. The same applied to the interaction effect between N and I, which showed that the lipid content increased in proportion to the combined effect of both variables. The negative value of the linear regression coefficient associated with N, however, demonstrated that the relationship between this variable and lipid content was inversely proportional, which was similar to the results obtained by Converti et al. (2009).

This inverse proportionality was consistent, since algae generally responded to nitrogen deprivation as a command to store the energy and then reacted to this response by accumulating the lipids. This happened naturally at the end of the exponential growth phase of batch culture when nutrients were scarce (Brown et al. 1997).

$$biomass\ (mg.L^{-1}) = 366.6 + 45.4 \cdot N - 19.1 \cdot N^2 + 47.4 \cdot I \quad (3)$$

$$lipid\ (\%) = 22.14 - 2.61 \cdot N + 1.39 \cdot I + 1.52 \cdot I^2 + 2.21 \cdot N \cdot I \quad (4)$$

$$lipid\ (mg.L^{-1}) = 89.25 - 5.38 \cdot T^2 - 4.97.N^2 - 17.28.I - 5.65.T.I + 9.30.N.I \quad (5)$$

The best experimental result obtained was 132.4 mg.L^{-1} for final lipid concentration under the following conditions: 27°C, $NaNO_3$ concentration 105 mg.L^{-1} and 122 µE.m^{-2}.s^{-1} (experiment number 11 in Table 2). In some Block 1experiments, the maximum lipid content reached was around 30%, which could be associated with the stress, and was an important result compared to those obtained by Converti et al. (2009), where the maximum lipid content reached was around 16%.

In the region bounded by the experimental points (Fig. 2), experimental conditions that favored the production of biomass did not correspond exactly to those that favored the accumulation of lipids,

which was a challenge for the optimization of these two variables together. However, if one analyzes the region above the N and I upper experimental limits, an extrapolation of data suggested it as possible to obtain satisfactory results for both the responses with higher values for these variables, as suggested in Figure 3, provided by equation 5. In this case, only significant factors were considered, with an explained variation of data equivalent to 90.94%. This information should be taken into consideration when planning future studies.

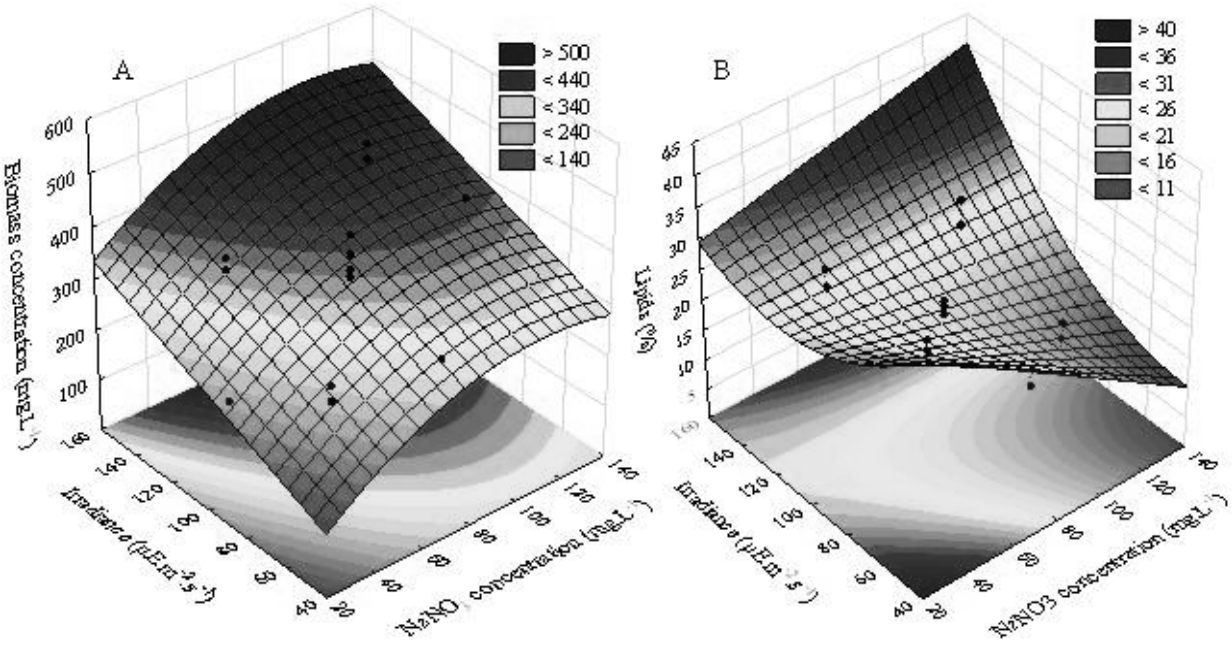

Figure 2 - Response surfaces for biomass concentration (a) and lipid content (b) in the reduced fitted models. Dots represent experimental data.

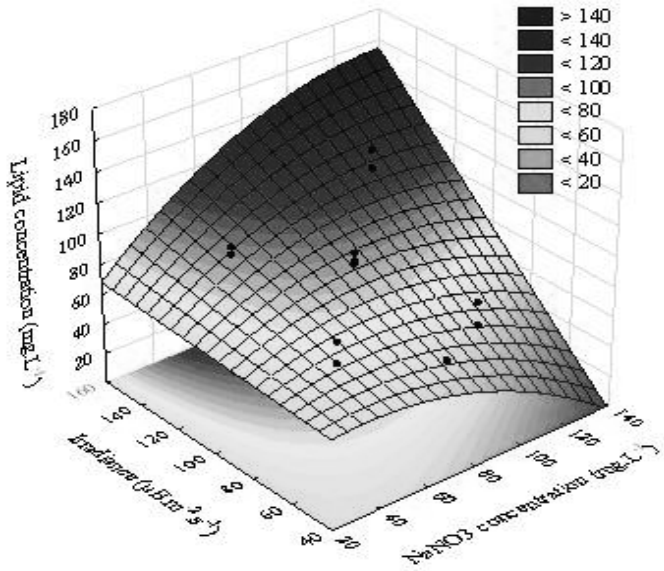

Figure 3 - Response surfaces for lipid concentration. Dots represent experimental data.

CONCLUSION

In this work the influence of temperature, incident light intensity and $NaNO_3$ medium concentration on *N. oculata* lipid accumulation was studied. The best experimental result was 132.4 $mg.L^{-1}$ for final lipid concentration, under the following conditions: 27°C, 122 $\mu E.m^{-2}.s^{-1}$ and 105 $mg.L^{-1}$, respectively, corresponding to ca.30% of dry weight biomass. In the experimental design, the interaction between $NaNO_3$ medium concentration and incident light intensity was particularly important for lipid storage. Further studies could increase the biomass and lipids productivity, making *N. oculata* a promising microalga for energy purposes.

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