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Microalgae Biomass and Bioactive Compounds Change According to the Medium's N and pH

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HIGHLIGHTS

- Variation of N levels and the pH of the medium improves growth of *N. oleoabundans*.
- N is an important factor on the lipid content of *N. oleoabundans*.
- *N. oleoabundans* produces higher chlorophyll content at pH 9.
- *N. oleoabundans* produces lipids with saponification values similar to soybean oil.
- There is interaction of pH and sodium nitrate on the response of *N. oleoabundans*.

Abstract: Microalgae have been widely studied as raw materials for biofuels, food supplements and several high value products. The hypothesis is that the pH and N levels in the culture medium are important factors to increase microalgae biomass production and its by-products. Thus, this study aimed to evaluate response of *Neochloris oleoabundans* to sodium nitrate concentration and pH levels by assessing biomass production, pigments, total lipid content and lipid profile of cells. The experimental design was a complete factorial with two factors: sodium nitrate ($NaNO_3$) using the doses of 0.25, 1.12 and 2.5 g L^{-1} and pH 5.0, 7.0 and 9.0, resulting in nine treatments and three replicates. Differences in the N concentration and pH of the in Bold's basal medium increased up to 21.1% the production of dry biomass but also up to 36% of lipids and up to of 0.81% of carotenoid concentration in the *N. oleoabundans* cell. The nuclear magnetic resonance (NMR) analysis of bio-oil showed changes in the lipid profile. Oil extracted from *N. oleoabundans* cells growing in medium at pH 9 and in 2.5 g L^{-1} of $NaNO_3$ showed fatty acids with molecular weights similar to crude soybean oil, while the oil from treatment 3 (2.5 $NaNO_3$ g L^{-1}) showed a slight increase in molecular weight. Overall, the best adjustment of the liquid medium to grow *N. oleoabundans* is at pH 9.0 and with addition of 2.5 g of sodium nitrate.

Keywords: carotenoids; chlorophyll; lipid profile; *Neochloris oleoabundans*; sodium nitrate.

INTRODUCTION

Microalgae biomass is an excellent raw material for food and pharmaceutical products in the forms of tablets and capsules, and their extracts are also included in noodles, wine, beverages, breakfast cereals, and the cosmetic and pharmaceutical industries [1-6]. A classic example of application of microalgal products in the in the food and supplements and health industry is astaxanthin, which is one of the most valued co-products [7]. An additional advantage is that it does not compete with food crops for arable land. Currently, in addition to the these traditional applications, there is a wide range of emerging microalgae applications such as wastewater treatment from agro-industries with high biomass production [8, 9], biofuels, biofertilizers and production of chemicals in a biorefinery approach [10], for which the market is huge [11].

Although, like any new biotechnology, it needs to be investigated and improved on an industrial scale [11-13]. After carbon, nitrogen is one of the most important inorganics limiting nutrients required for growth of microalgal cells [14], which is essential for the synthesis of proteins and chlorophyll [15-17]. Microalgal biomass may comprise from 1% to 10% of nitrogen in organic and inorganic form, which can be assimilated from the culture medium in the ammoniacal form or as nitrate; moreover, differences in absorption may occur depending on the species [18]. According to Menegol et al. [19], the biomass, protein and eicosapentaenoic acid increases when *Heterochlorella luteoviridis* is grown at higher concentrations of nitrogen.

Microalgae have great potential to produce enzymes, such as beta-galactosidase [6], whose production can be increased when using cheese whey as a carbon source [20]. Stimulating the biosynthesis of compounds that influence cell growth through changes in culture conditions is an option to reduce microalgae biomass production costs [21]. Sampathkumar and Gothandam [22] suggested that sodium bicarbonate can be used as an inorganic carbon source for enhancing lutein and lipid production of *Chlorella pyrenoidosa*.

The pH and nitrogen (N) content may have influence on microalgal production and lipid composition due to their direct effects on growth. Control of pH is an essential variable in the cultivation of microalgae because it affects directly the availability and the absorption of several chemical elements in the culture medium [23]. By comparing different hydrogen ion concentration (pH) on the growth of microalgae, it was found that the optimum pH value for biomass yield and productivity of *Chlorella vulgaris* was between pH 6.5 and 7.5 [24]. In a study combining soybean wastewater in CO_2 absorption-microalgae hybrid system, pH control of the hybrid systems was also useful method to mitigate ammonia toxicity and accelerate the growth of *Chlorella* sp. [25]. These author suggested that to warranty better growth of microalgae, it seems to be needed to adjust pH to neutral condition.

In this point of view, the hypothesis is that there is an interaction between the pH and N levels in the growth medium and this has an effect on biomass production and its composition. Hence, this study aimed to evaluate response of *N. oleoabundans* to sodium nitrate amounts and pH levels by assessing biomass production, pigments, total lipid concentration and lipid profile of cells. In this study, response surface methodology was used as a statistical tool that simulated different combination of factors because it saves time and reagents. This methodology has been employed before for optimization of nutrients in culture media, e.g., for *C. pyrenoidosa* [26]. The novelty the study is to report findings on the relationships between pH and nitrate levels of medium on *N. oleoabundans* biomass production, pigments, total lipid content and lipid profile, which will subsidize more knowledge to microalgae cultivation.

MATERIAL AND METHODS

Microalgae strain and growth conditions

The *N. oleoabundans* strain (UTEX#1185) was purchased from the Culture Collection of Algae at the University of Texas in Austin, Austin, TX, USA and kept in Bold's basal medium (BBM) [27] in the Microbial Collection (IPR) at Institute Agronomic of Paraná in Londrina, Paraná, Brazil. Microalgae were grown without external aeration and without agitation in clear glass bottles containing 2 L of sterilized BBM medium in a growth chamber with a 12:12 h light:dark photoperiod at 28.0 ± 2.0°C in the light phase and 22.0 ± 2.0°C in the dark phase. The photon flux density of photosynthetically active radiation was 100 ± 20 μE m^{-2} s^{-1}.

The experiment was performed with a complete factorial design with three replicates as follows: three encoded levels (−1, 0, 1) in nine trials and two factors (A and B, which corresponded to sodium nitrate concentrations of 0.25, 1.12 and 2.5 g L^{-1} and the pH values of 5.0, 7.0 and 9.0) of the BBM based on response surface methodology using STATISTICA 7.0 software [28] as described in Table 1.

N. oleoabundans was inoculated using 10% (v/v) of a culture of 6.83 x 10^7 cells mL^{-1}. The pH was adjusted with hydrochloric acid or potassium hydroxide according to the experimental design. All procedures were performed in a laminar flow hood (Veco, Bioseg-12) to avoid contamination.

Analytical determination

The pH of the culture medium was measured every day using a pH metre (Metrohm, 827), and when necessary, the pH was adjusted with hydrochloric acid or potassium hydroxide according to the experimental design. On the 14th day of *N. oleoabundans* cultivation, samples were collected to determine cell count, the optical density, dry biomass production, pigment contents (chlorophyll a and carotenoids), percentage (%) content of lipids and physical chemical characterization of lipids.

Cell count and biomass production

Cell counting was determined using an improved Neubauer hemocytometer and an optical microscope (Nikon, Eclipse E200) with a 40× objective and a visual magnification of 400×.

Dry biomass yield was determined as described elsewhere [29] using 30-mL aliquots of each sample, which were centrifuged (Hermle, Z 383 K) at 25 °C at 16,600 × g for 10 min and dried at 60 °C for 48 h.

Pigments

The pigment extraction procedures were conducted as described by Maroubo et al. [30] with slight modifications. Briefly, an aliquot of 3 mL of *N. oleoabundans* culture was centrifuged for 10 min at 9000 x g in a refrigerated centrifuge (Hermle, Z 383 K) at 25 °C. The supernatant was discarded, and the tubes were kept in an ultrafreezer at -86ºC (Freeztec, mod. ftv342) for 24 h to disrupt the cells. Afterwards, the samples were thawed and macerated with glass stick, 3 mL of 90% acetone was added, and the pellet and solution were mixed with a vortex (Daiger, Genie 2). After adding acetone, all procedures were performed without light in the room. The tubes were kept in the refrigerator for 1 to 4 h, shook every 15-20 min. The samples were centrifuged for 10 min at 9000 × g and immediately read in a spectrophotometer at the following wavelengths: 480, 510, 665 and 750 nm. Then, an aliquot of 20 µL of hydrochloric acid (HCl) at 1 N was added to the samples, the samples were shaken for 1 min, and the absorbance was read at the wavelengths of 665 and 750 nm.

The contents of chlorophyll a and carotenoids were calculated according to Lorenzen [31] and Strickland and Parsons [32] following Equation 1 and Equation 2, respectively:

$$Chlorophyll\ a: \frac{F \text{ x } [(A_{665} - A_{750}) - (A_{665ac} - A_{750ac}) \text{x } v]}{\text{V x C}} \quad (1)$$

$$Total\ carotenoids: \frac{\{7.6 \text{ x } A_{480} [(3.0 \text{ x } - A_{750}) - (1.49 \text{ x } A_{510}) - (2.0 \text{ x } A_{750})]\} \text{x } v}{\text{V x C}} \quad (2)$$

where chlorophyll a = µg L^{-1}), total carotenoids = µg L^{-1}, F= 26.73 (result factor of the coefficient of light absorption and its comportment after solubilization and extraction with 90% acetone), *v*= volume of acetone used in the extraction (mL), V = volume of centrifuged sample (L), C = optical path (1 cm), A_{665} = absorbance at 665 nm, A_{750} = absorbance at 750 nm, A_{665ac} = absorbance at 665 nm after adding hydrochloric acid, A_{750ac} = absorbance at 750 nm after adding hydrochloric acid, A_{480} = absorbance at 480 nm, A_{510} = absorbance at 510 nm, and A_{750} = absorbance at 750 nm.

Total lipid content, profile and NMR

An aliquot of 1.5 L of *N. oleoabundans* culture was centrifuged at 9000 x g for 10 min in a refrigerated centrifuge at 25ºC (Hermle, Z 383 K), and the biomass pellet was lyophilized for total lipid determination and the lipid profile analyze by nuclear magnetic resonance (NMR). Lipid determination was based on the gravimetry method [33], as described by Ryckebosch et al. [34], in which total lipids were extracted from 50 mg of lyophilized biomass with chloroform:methanol:water at a ratio of 1:1:0.8.

For the analysis of the lipid profile, we chose treatments 3 and 9 because they had higher levels of lipid in the biomass and biomass production. The samples were analyzed by NMR at the Spectroscopy Laboratory, State University of Londrina, Londrina, Brazil. The samples were dissolved in CDCl3 solvent and analyzed using a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm double resonance broadband inverse (BBI) probe at 303 K. All 1H-NMR experiments were performed at 400.13 MHz with the standard pulse sequences as described by Braun and collaborators [35]. MW- molecular weight, Iodine and

saponification values were calculated by the 1H NMR data of integrated spectra following procedures described by Reda et al. [36].

Statistical analyses

The experiment was carried out using a full factorial design with nine trials and two factors (Table 1), which corresponded to the sodium nitrate concentrations (0.25, 1.12 and 2.5 g L^{-1}) and the pH (5.0, 7.0 and 9.0) using software STATISTICA v7.0 [37]

Analysis of variance was used to determine the individual and combined effects of sodium nitrate concentration and pH on the dependent variables, and Tukey's test (α=0.05) was applied to compare means using Software SISVAR v5.3 [38].

RESULTS

Growth and biochemical composition

N. oleoabundans growing in medium containing 2.5 g $NaNO_3$ L^{-1} at pH 9.0 had the highest lipid concentration (37.5%) and dry biomass (104.4 mg L^{-1}). The highest total carotenoid value was 2.98% at pH 7.0 in treatment 5 with 0.12 g $NaNO_3$ L^{-1}. The number of cells ranged from 0.50 x 10^7 cells mL^{-1} at a pH of 5.0 to 1.71 x 10^7 cells mL^{-1} at a pH of 9.0 (Table 1).

Table 1 Experiment runs according to design in complete factorial scheme for two variables (N and pH) with the three levels and data based on experimental design of growth and biochemical composition of *N. oleoabundans*.

Runs	$NaNO_3$ g L^{-1}	pH	lipids	Chl	Carotenoids	Biomass	Cells
			%			mg L^{-1}	10^7 mL^{-1}
			20.0	0.33	0.22	56.0	0.50
1	-1 (0.25)	-1 (5.0)	20.8	0.30	0.21	60.0	0.61
			19.9	0.26	0.17	70.3	0.40
			33.3	0.67	0.27	67.2	0.83
2	-1 (0.25)	0 (7.0)	35.5	0.84	0.17	59.8	0.71
			33.0	0.88	0.28	51.2	0.77
			36.5	0.93	0.19	99.8	1.58
3	-1 (0.25)	1 (9.0)	34.3	0.93	0.23	94.2	1.25
			36.0	0.85	0.22	103.6	1.31
			20.6	1.30	0.28	53.2	1.30
4	0 (1.12)	-1 (5.0)	21.0	1.31	0.38	59.0	0.85
			21.5	1.37	0.32	56.1	1.02
			20.5	2.49	0.60	47.2	0.87
5	0 (1.12)	0 (7.0)	21.0	2.98	0.45	51.0	1.23
			20.4	2.73	0.41	57.6	0.85
			19.5	1.42	0.28	82.8	1.69
6	0 (1.12)	1 (9.0)	22.0	1.44	0.37	85	1.85
			22.7	1.60	0.28	88.2	1.58
			23.8	1.72	0.9	46.4	0.80
7	1 (2.5)	-1 (5.0)	24.6	1.86	0.92	47.2	0.83
			26.1	1.82	0.91	48.4	0.77
			26.2	1.80	0.86	53.2	1.11
8	1 (2.5)	0 (7.0)	26.6	2.62	0.88	51.0	1.06
			30.7	2.16	0.70	59.4	1.23
			37.0	1.53	0.45	104.4	1.11
9	1 (2.5)	1 (9.0)	37.5	1.43	0.46	104.2	1.15
			35.5	1.89	0.47	101.4	1.15

The cell count results on the 14th day of culture showed that both variables, $NaNO_3$ concentration ($p=0.000$) and pH, exhibited a significant ($p<0.05$) effect (Figure 1).

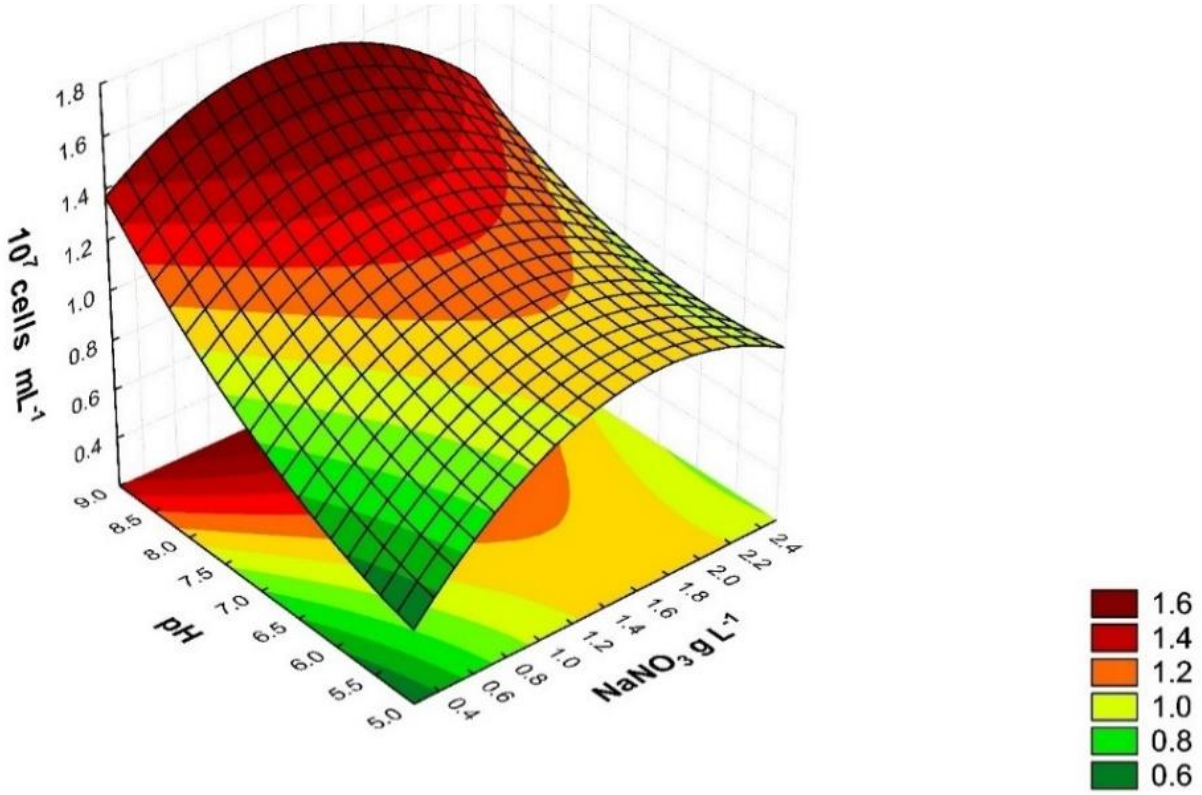


Figure 1. Response surface graphs for the interactive effect of initial concentration $NaNO_3$ and pH variation on number of cells (10^7 cells mL^{-1}) of *N. oleoabundans* on the 14th day of cultivation.

No experimental adjustment was significant (lack of fit = 0.89), and the equation coefficient was R^2=0.89. Equation 3 describes the obtained response surface.

$$z = 0.654 + 1.186x - 0.258x^2 - 0.241y + 0.033y^2 - 0.059xy \quad (3)$$

The analysis of the dry biomass values (mg L^{-1}) indicated a significant effect ($p<0.05$) on the 14th day of *N. oleoabundans* cultivation for both variables studied, g L^{-1} of $NaNO_3$ (p=0.003) and pH (p=0.00). *N. oleoabundans* cultivated with culture medium with a pH of 9.0 and $NaNO_3$ concentrations of 0.25 or 2.5 g L^{-1} showed the highest dry biomass production (Table 2). Notably, a good strategy to increase biomass production and the number of cells is to make the culture medium alkaline by adjusting the pH to 9.0.

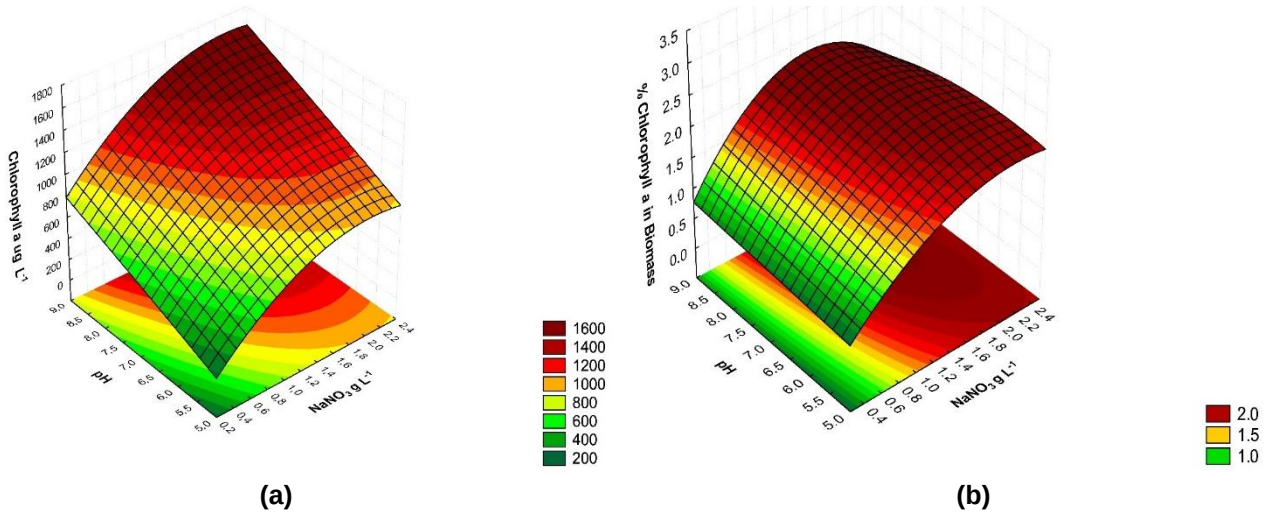
Table 2. Dry biomass (mg L^{-1}) of *N. oleoabundans* on the 14th day in culture medium BBM with variation of pH and N ($NaNO_3$) concentration. Data from experiment using optimized conditions.

	pH		
$NaNO_3$ (g L^{-1})	5.0	7.0	9.0
	mg L^{-1}		
0.25	[1]62.1Ba	59.4Ba	99.2Aa
1.12	56.1Bab	51.9Ba	85.3Ab
2.50	47.3Bb	54.5Ba	103.3Aa
CV (%) = 7.02			

[1]Means followed by the uppercase letter at line and lower case at column not differ by Tukey test ($p<0.05$). CV=coefficient of variation.

Pigments

The chlorophyll a content of the cells from the treatment group with a pH of 9 (average of 1675 µg L^{-1}) was higher than that of treatment 1 with a pH of 5, and the chlorophyll a content at the $NaNO_3$ concentration of 0.25 g L^{-1} decreased, with an average of 186 µg L^{-1} (Figure 2a).



(a) **(b)**

Figure 2. Response surface graphs for the interactive effect of initial concentration $NaNO_3$ and pH variation on (a) contents of Chlorophyll *a* (µg L^{-1}) and (b) percentage of Chlorophyll *a* in dry biomass of *N. oleoabundans* on the 14th day of cultivation.

Chlorophyll a content had a significant effect ($p<0.05$) of both variables, g L^{-1} of $NaNO_3$ ($p=0.00$) and pH ($p=0.00$); no experimental adjustment was significant ($p=0.22$), and the regression coefficient was $R^2=0.88$. Equation 4 describes the response surface.

$$z = -850 + 818x - 220x^2 + 179y - 1.96y^2 + 15.5xy \quad (4)$$

The lowest percentage of chlorophyll a in biomass was 0.30 ± 0.03%, obtained in treatment 1 with the pH adjusted to 5.0 and a $NaNO_3$ concentration of 0.25 g L^{-1} (Figure 2b). This treatment exhibited low average values of optical density and cell count on the 14th day of culture.

The percent chlorophyll a in microalgal biomass showed a significant effect ($p<0.05$) of both studied variables, g L^{-1} of $NaNO_3$ ($p=0.00$) and pH ($p=0.00$). No experimental adjustment was significant ($p=0.13$), the regression coefficient was $R^2=0.93$, and response surface is described in Equation 5:

$$z = 0.18 + 0.48x - 0.19 - 0.0002y^2 + 0.23xy - 0.007x^2y^2 \quad (5)$$

For carotenoid concentration (Figure 3a), there was a significant effect ($p<0.05$) of both studied variables, $NaNO_3$ ($p=0.00$) and pH ($p=0.00$), and no experimental adjustment was significant ($p=0.32$), validating the model and the regression coefficient ($R^2 = 0.95$)

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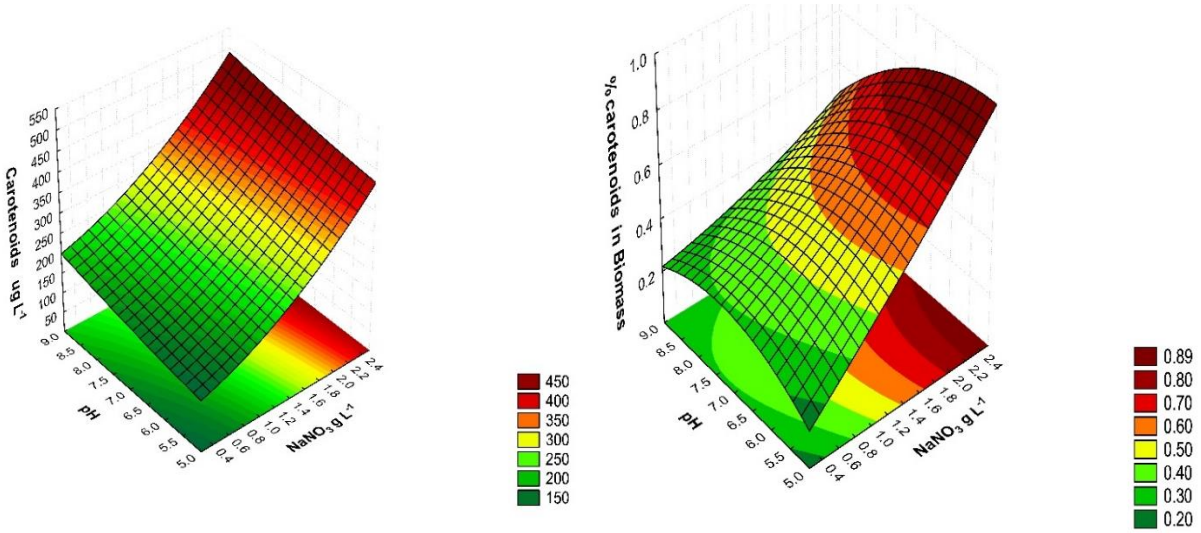

(a) **(b)**

Figure 3. Response surface graphs for the interactive effect of initial $NaNO_3$ concentration and pH variation on (a) total carotenoids content in µg L^{-1} and, (b) percentage (%) of total carotenoids in dry biomass of *N. oleoabundans* on the 14th day of cultivation.

The response surface is described in Equation 6:

$$z = 80.85 + 42.21x + 31.56x^2 + 0.60y + 1.23y^2 \quad (6)$$

The percentage of carotenoid in dry biomass showed a significant effect ($p<0.05$) of both studied variables, g L^{-1} of $NaNO_3$ ($p=0.00$) and pH ($p=0.00$); no experimental adjustment was significant, and the regression coefficient was $R^2=0.94$ (Figure 3b).

The analysis of dry biomass and carotenoids in g L^{-1} together showed that the high contents of these variables occurred in both treatments 7 and 8, with values of 0.91±0.01% and 0.81±0.09%, respectively, according to Equation 7:

$$z = -1.34 + 0.60x + 0.41y - 0.02y^2 - 0.06xy + 0.002x^2y \quad (7)$$

Total lipid content, profile and NMR

The statistical analysis of lipid values indicated a significant effect ($p<0.05$) of both studied variables, sodium nitrate ($p=0.00$) and pH ($p=0.00$). No experimental adjustment was significant ($p=0.45$), and the regression coefficient was $R^2=0.96$, according to the response surface described by Equation 8.

$$z = -39.00 + 4.22x + 12.11x^2 + 16.70y - 0.86y^2 - 0.40xy^2 - 4.07x^2y + 0.43x^2y^2 \quad (8)$$

N. oleoabundans had the highest lipid content an average of 36% of the dry biomass when growing at pH of 9.0 and with a $NaNO_3$ concentration of 0.25 or 2.5 g L^{-1} (Figure 4).

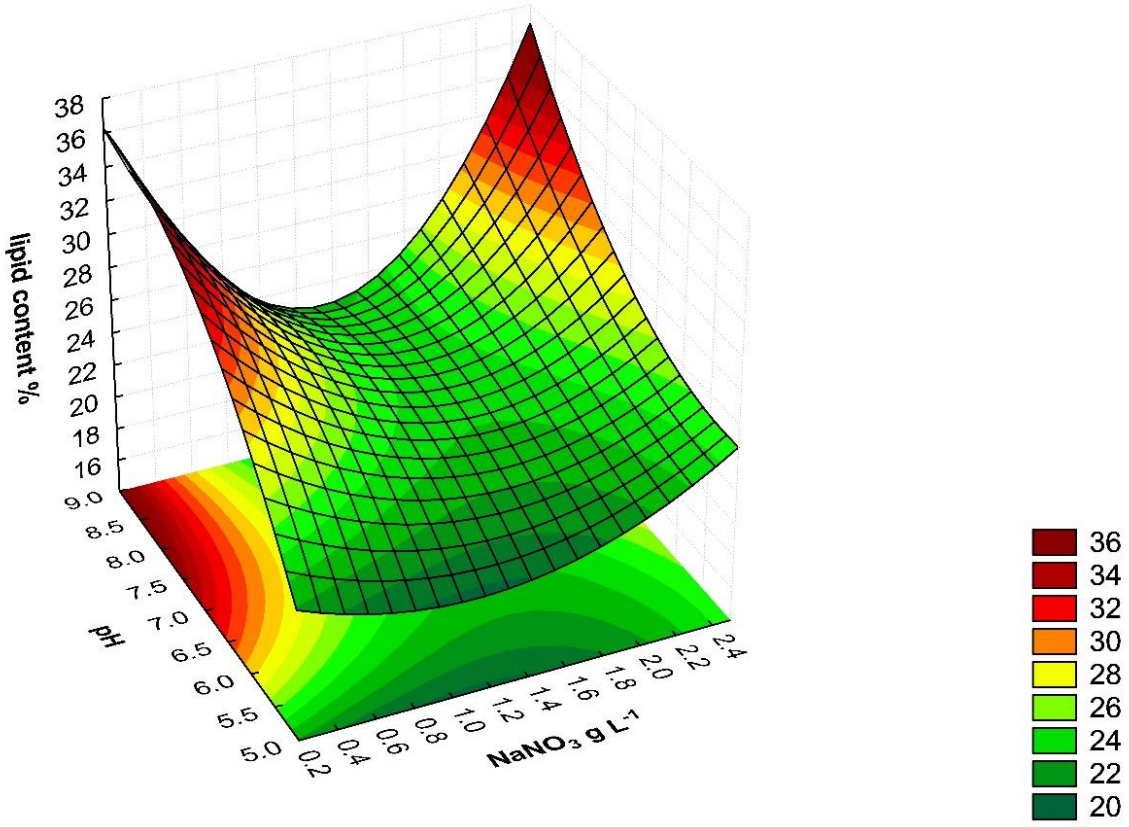

Figure 4. Response surface graphs for the interactive effect of initial $NaNO_3$ concentration and pH variation on total lipid (%) of *N. oleoabundans* on the 14th day of cultivation.

Cultivation at pH 9.0 and containing 1.25 g of sodium nitrate resulted in the lowest lipid value in biomass, representing 22% of the biomass. The statistical analysis of lipid values indicated a significant effect ($p<0.05$) of both studied variables, sodium nitrate ($p=0.00$) and pH ($p=0.00$).

The physico-chemical analysis of the lipid extract of *N. oleoabundans* growing in the culture medium at pH 9.0 and containing two levels of nitrogen (0.25 and 2.5 g L^{-1} of $NaNO_3$) are shown in Table 3.

Table 3. Physico-chemical analysis of the lipid extract (values calculated by the data from ^{1}H NMR of integraded spectres) from *N. oleoabundans* growing in BBM medium at pH 9.0 and N concentration of 0.25 and 2.5 of $NaNO_3$ (g L^{-1}), comparison with soybean oil.

Parameters	Culture medium treatments		
		0.25 $NaNO_3$	2.5 $NaNO_3$
	Soybean oil*	Microalgae oil	
MW- molecular weight	870.2	896.6	875.1
Iodine value	118.5	172.2	153.9
Saponification value (mg KOH g^{-1})	195.0	187.0	192.1
Free acidity index (%)	1.5	0.7	0.9

*Reda et al. [36].

The spectra obtained by ^{1}H NMR analysis of lipid extracted from cells growing in medium at a pH of 9.0 and in 2.5 g L^{-1} of $NaNO_3$ showed fatty acids with molecular weights similar to crude soybean oil, while the oil from treatment 3 (0.25 $NaNO_3$ g L^{-1}) showed a slight increase in molecular weight.

DISCUSSION

The highest total Chl value was 2.98% at pH 7.0 in treatment 5 with 1.12 g $NaNO_3$ L^{-1}, for total carotenoids value was 0.9% at pH 5.0 in treatment 7 with 2.5 g $NaNO_3$ L^{-1} and for lipid content an average of 36% of the dry biomass when growing at pH of 9.0 and with a $NaNO_3$ concentration of 0.25 or 2.5 g L^{-1} (Table 1).

There was an increase in the number of *N. oleoabundans* cells at pH 9.0 with addition of 1.12 g $NaNO_3$ L^{-1}, reaching the highest density of 1.85 10^7 cell mL^{-1}. The hypothesis is that the alkalization of the medium and increase the concentration of $NaNO_3$ may result in an increased number of *N. oleoabundans* cells compared with other culture media. For *Dunaliella viridis*, there was an increase in the number of cells when the sodium nitrate concentrations increased up to 5.0 mM in a mixotrophic culture medium [21]. The best growing of *Spirulina* sp. was with 1.25 g L^{-1} $NaNO_3$ [39].

The pH is a crucial factor that determines the growth of microalgae, because to having direct effects on nitrogen availability for example on NH_3/NH_4^+ equilibrium [40-42]. According to Khalil et al. [43], growth media with pH values adjusted to 9, 10 and 11 were more suitable for biomass production by *C. eliopsoidea* than growth media at a pH 7.5, while using acidic growing media biomass production was decreased. Likewise, Bartley et al. [44] studied *Nannochloropsis salina* and suggested that a pH between 8.0 and 9.0 is the most appropriate to for microalgal biomass production. In contrast, for *Scenedesmus* sp., the highest specific growth rate and biomass productivity was observed at a pH of 7.5 [45]. Studies have reported sodium nitrate as a nitrogen source widely used in the cultivation of green microalgae and its concentration positively influences the production of biomass, e.g., for *N. oleoabundans* [46-49], for *C. vulgaris* [50] and, for *Heterochlorella luteoviridis* [19].

Chlorophylls are tetrapyrroles, a large and diverse family of biosynthetically related molecules in which nitrogen is a major component [17, 51]. Chlorophyll is directly involved in capturing light energy and converting it into chemical energy through photosynthesis [52], therefore higher chlorophyll content indicates that the microalgae are actively photosynthesizing and utilizing light for growth and biomass production. Chlorophyll content also changes under the nutrient conditions that may serve as an indicator of stress in microalgae which is the indicator of photosynthesis and photochemical processes during which the energy accumulated in ATP is generated [53]. The chlorophyll content is one growth parameter to estimate biomass and indicator of physiological state in microalga [54].

In this study, increasing sodium nitrate content in the culturing medium increases the concentrations of chlorophyll *a* and carotenoids. A decrease in chlorophyll content is described as a general response to nitrogen deprivation in the medium of cultured microalgal chlorophytes [46, 55-59]. Urreta et al. [60] finding that nitrate increases chlorophyll (*a* and *b*) and carotenoids of *N. oleoabundans* that similar to this study. In this study, the highest quantity of total carotenoids was 500 µg L^{-1} and 0.89% of the biomass of *N. oleoabundans*, growing at the photon flux density of photosynthetically active radiation (PAR), 100 ± 20 µE m^{-2} s^{-1}. In contrast, *N. oleoabundans* cultivated in BBM with 0.3 g L^{-1} of KNO_3 and at the high light intensity of 400 µmol photons m^{-2} s^{-1} produced approximately 29 mg carotenoids per g of dry biomass, or 2.9%, which suggests that this microalgae is a source of natural carotenoids [61]. The accumulation of carotenoids by microalgae can be upregulated in response to oxidative stress in cells caused by factors such as high light irradiance, high salt, high temperature, and nutrient deficiency [55, 62].

The lipid content of *Scenedesmus* sp. and *Coelastrella* sp. microalgae was affected by factors such as pH and nitrogen source concentration in the culture medium. After all the nitrogen is consumed by cells, and nitrogen is consequently exhausted in medium culture, the high pH, resulted in greater triacylglycerol accumulation, especially in buffered systems [63]. In *N. oleoabundans* microalgae cultured in Bristol medium at 26ºC and 30ºC with and without nitrate supply, the highest lipid productivity of 38.78 mg L^{-1} day^{-1} was observed at 26ºC and with nitrate [47]. The sodium nitrate concentration in the medium culture had an important influence on the lipid content of *N. oleoabundans* (HK-129), especially the lack of sodium nitrate at the end of the growing period, which may increase the lipid percentage in biomass [49]. *Coccomyxa subellipsoidea* cultivated in one stage continuous N-sufficiency (1.0 g L^{-1} KNO_3) augmented the lipid productivity by 232.37 mg L^{-1} day^{-1} [64].

In this study, it observed similar saponification values in the lipid extract from the *N. oleoabundans* and crude soybean oil. The saponification value is defined as the number of milligrams of potassium hydroxide (KOH) required to saponify one gram of oil or fat [65]. This index shows the amount of iodine in grams that is consumed by 100 grams of sample under the given conditions [66]. According to the authors, the higher the iodine value, the greater the degree of unsaturation, or the number of double bonds between fatty acids. In this study, a high iodine value reflects the degree of unsaturation of the samples; the values obtained in lipid extract from treatment 3 (172.2) and treatment 9 (153.9) were slight superior to soybean oil (118.5). For the calculation of such parameters according to Carneiro et al. [65] the most relevant ^{1}H NMR signals are those from the methylene groups adjacent to unsaturated carbon atoms ($RO(O=C)CH_2$-(CH_2)x-CH_2-CH=CH-CH_2-(CH_2)yCH_3 and/or $RO(O=C)CH_2$-(CH_2)x-CH_2-CH=CH-CH_2-CH=CH-CH_2-(CH_2)yCH_3). The ^{1}H NMR spectra for the lipid from *N. oleoabundans* (treatment 3) 0.25 $NaNO_3$ showed those signals proportionally higher than in the other treatment.

Working with wet biomass of a green microalgae *Monoraphidium* sp., it was extracted on average approximately 10% bio-oil, which was quite similar to the composition of the petroleum, except for the oxygen content which was much higher [67]. High levels of unsaturation in vegetable oils such as monounsaturated fatty acids and polyunsaturated fats are associated with greater tendency for oxidative changes. The degree of oil unsaturation has been considered for a long time to be one of the most important factors due to the different reactivity of unsaturated fatty acids as a result of chemical changes, increasing the free fatty acids, carbonyl compounds, and high molecular weight products and decreasing saturated fatty acids [68]. By comparing bio-oil from microalgae and vegetable, a study by Waghmare et al. [69] revealed that the microalgal oil had the highest physical and chemical stability during the frying process compared to sunflower and palm oils.

CONCLUSION

Alteration of sodium nitrate levels and the pH of the culture medium improves the growth of the microalgae *N. oleoabundans*, increasing biomass production, concentrations of chlorophyll *a*, carotenoids and lipid content. The lipids obtained from the biomass of *N. oleoabundans* have chemical characteristics similar to natural crude soybean oil. The best adjustment of the factors, pH and nitrogen, in liquid medium to grow *N. oleoabundans* is at pH 9.0 and with 2.5 g of sodium nitrate.

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