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The effect of nitrogen limitation on carbohydrates and β-glucan accumulation in *Nannochloropsis oculata*

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ABSTRACT

Nitrogen (N) is the second most abundant element in microalgae biomass, following carbon. Microalgae have a high carbon and nitrogen metabolism system, making nitrogen limitation (N-limitation) a crucial factor that modifies their biochemical composition. In *Nannochloropsis* genus cultures, N-limitation can cause lipid or carbohydrate accumulation. This research aims to evaluate the effect of N-limitation on carbohydrate and β-glucan accumulation in *Nannochloropsis oculata* during maximum biomass production (i.e., finished logarithmic phase and early start stationary phase in batch culture) in the two-stage process context. To achieve this goal, we tested the effect of five levels of N availability (treatments 100, 75, 50, 25, and 0 % of N, concerning F medium) in batch culture using sodium nitrate ($NaNO_3$) as the N source. The initial availability of N in each treatment was 1.7, 1.3, 0.8, 0.4, and 0 mmol•l^{-1}. The results showed that the highest concentration of *N. oculata* biomass, carbohydrate, and β-glucans content was produced during the logarithmic phase at 0.8 and 1.32 mmol•l^{-1} N initial batch culture concentration (50 and 75 % N treatments), without a significant difference ($P > 0.05$) from the F medium used. Total carbohydrates obtained were 15.2 %, 19.7 %, 17.2 %, 12.7 %, and 14.2 % (dry weight) for the 100 %, 75 %, 50 %, 25 %, and 0 % N treatments, respectively. Similarly, the β-glucans in microalgal biomass were 5.0 %, 7.4 %, 6.7 %, 4.4 %, and 1.7 % (dry weight) for treatments 100 %, 75 %, 50 %, 25 %, and 0 % N, respectively. The 25 % and 0 % N treatments had a negative effect ($P < 0.05$) on cell population growth, carbohydrates, and β-glucans accumulation. Based on these results, we recommended conducting studies on the effect of N-limitation in *N. oculata* culture primarily at 50 % and 75 % N treatments, during the stationary phase and/or second stage of the two-stage process context, such as a chronic or and long-term effect, along with a partial supply of N or testing other inductors might also be explored. This knowledge can help to optimize the industrial production of microalgae β-glucans.

1. Introduction

In microalgae, carbohydrates are synthesized to produce glucose and starch-like energy storage products, which serve as both structural components of their cell walls and an energy reserve [1]. Glucose is the primary carbon-containing product formed during photosynthesis, which provides energy for the metabolic processes of microalgae, and helps them survive temporary adverse conditions [2]. The content of carbohydrates in microalgal biomass ranges from 5 to 23 %, with an average of protein 6–52 % and lipids 7–23 % [3]. However, the carbohydrate content varies depending on microalgae species and culture methods [4]. For example, in the genus *Nannochloropsis*, carbohydrate content ranges from 6 to 53 % dry weight (DW, Table 1). Controlling the composition of the culture medium and the environmental conditions such as nutrient limitation, saline stress, light, and temperature, makes is possible to increase the accumulation of carbohydrates in microalgal biomass [5–7].

Microalgae undergo different growth phases, including lag, logarithmic, stationary, and death phase, and each species has specific nutrient requirements in each phase, with common nutrients used for all

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species [5]. One practical technique to enrich microalgal biomass with different macronutrients is nutrient limitation, which involves controlling the composition of the culture medium [7]. Microalgae have a versatile metabolism, which can be photoautotroph, mixotroph, or heterotroph. However, like all organisms, their metabolism fundamentally depends on the availability of nutrients, with nitrogen (N) being particularly crucial. N is essential for the basic building blocks of life, including proteins, enzymes, chlorophylls, energy-transfer molecules (ATP and ADP), and genetic materials (RNA and DNA), which are required for survival [8]. In chloroplasts, N is absorbed as a nitrate ion and reduced to nitrite by nitrate reductase enzyme. The nitrite is then transported directly to the chloroplast and further reduced to ammonium by nitrite reductase. These transformations consume a significant amount of energy [9]. The ammonium produced by the assimilation of nitrate generated by photorespiration is then converted into amino acids, which form proteins. When N is limited in the culture medium, photosynthesis tends to decrease due to the catabolism of the protein components of photosynthesis to scavenge nitrogen needed for other metabolic processes. Consequently, nitrogen limitation (N-limitation) causes a significant decrease in total cell protein and drives metabolism toward higher lipid and carbohydrate production [10].

This response is common among microalgae and enables them to adapt to unfavorable environments by using carbohydrates as a sink for excessive carbon and electrons when protein and polar lipid synthesis are reduced under stress conditions [11]. High biosynthesis of carbohydrates in N-limitation generates oxidative stress that is the consequence of carbohydrate accumulation in microalgae [12]. Nutrient depletion conditions can lead to reactive oxygen species (ROS) being generated under nutrient depletion conditions, impairing the photosynthetic apparatus and causing declined photosynthesis [13].

In nutrient limitation studies, elementary nutrients can be omitted at the start of the culture [14]. However, this can lead to low growth rates and low biomass production. Therefore, optimizing nutrient concentrations at the beginning of the culture is relevant. Optimization of limited nutrients has two main outcomes: supporting adequate biomass production while also acting as a limiting factor that drives the metabolic route of interest. Several researchers suggest a two-stage process; the first stage involves biomass production under optimum conditions until the logarithmic phase is finished, and in the second stage (after the start of the stationary phase), the cells must be exposed to stressed conditions, to alter their composition [7,14]. The importance of microalgal carbohydrates for biotechnological applications has increased, particularly in high-value compound production, such as biofuels [15,16] and immunostimulants [17]. Therefore, strategies are necessary to improve microalgal biomass carbohydrate levels at a reasonable cost.

Studies on β-glucans from *Nannochloropsis* genus are scarce (see Table 1). It is important to highlight that β-glucans from microalgae of the *Nannochloropsis* genus are considered the most promising functional food ingredients due to their molecular characteristics known as "Pathogen-associated molecular patterns", but with better characteristics than the β-glucans obtained from other sources, such as β-1,3/1,6 structure, low molecular weight, and low polymerization and branching degree (e.g. *N. gaditana* CCMP526) [18]. Studies have reported high volumetric productivity of β-glucans (0.08 g/L) in *Nannochloropsis* batch culture [19]. Microalgal β-glucans can be obtained through simple extraction techniques because covalent bonds do not bind them to other structural components of cells, which facilities the purification of these polysaccharides from biomass. Therefore, it is necessary to conduct more studies to make β-glucans production from *Nannochloropsis* spp. more efficient compared to other sources (e.g. yeast) already used in animal and human food production. Microalgae β-glucan has been shown to possess similar anti-oxidation, immunomodulation, and anti-tumor properties, as well as regeneration-promoting activities, as other sources of β-glucan indicating its potential application in multiple commercial fields including food and pharmaceutical production [17].

The objective of our laboratory is to develop improvements in larvae-culture biotechnologies for marine fish. In our larvae-culture protocols, microalgae *Nannochloropsis oculata* (Fig. 1) play an important role as they are food for the prey of small larvae and provide a relevant nutritional contribution, creating a more natural environment when they are introduced into larviculture tanks. Additionally, we are evaluating β-glucans from *N. oculata* [19] as promising immunostimulatory agents in the early development of teleost fish, which will help to understand the mechanisms of trained immunity [20]. For these reasons, we consider carbohydrates (β-glucans) and the mechanisms of their accumulation in the biomass of *N. oculata* to have great importance in the aquaculture field.

The advantages of *Nannochloropsis* as a potential source of β-glucans include: I) Freshwater is not required to culture most of its species. II) Biomass can be obtained on an industrial scale from photobioreactors. III) It is possible to modify the biochemical composition of biomass to increase the fraction of carbohydrates. IV) High volumetric productivity of β-glucan. V) Microalgal β-glucan can be obtained through simple extraction techniques from biomass. VI) Present a water-soluble bioactive β-glucan and molecular structure such as yeasts.

N. oculata is a marine, immotile, unicellular, and green-yellow microalgae belonging to the Class Eustigmatophyceae. This species is oleaginous and can produce valuable compounds, such as polysaccharides, lipids, proteins, pigments, vitamins, and enzymes which can be used for biotechnological and pharmaceutical applications [21]. *N. oculata* has a high potential for producing water-soluble bioactive

Table 1
Total carbohydrates and β-glucans content in microalgae biomass cultivated in a traditional medium and culture medium with N-limitation (% DW, percentage dry weight).

Microalgae	Total carbohydrates (% dw)		β-Glucans (% dw)		Reference
	Traditional medium	Nitrogen limitation	Traditional medium	Nitrogen limitation	
Nannochloropsis sp.	16	53	–	–	[50]
Nannochloropsis oculata	23	29	–	–	[53]
Nannochloropsis oceanica	6	19	–	–	[52]
Nannochloropsis oculata	16	17.8	6.8	7.9	[a]
Nannochloropsis oculata	23.5	–	16.8	–	[19]
Nannochloropsis sp.	22.7	–	15.8	–	[19]
Nannochloropsis sp.	30.9	–	23.4	–	[19]
Odontella aurita	–	–	63.1	–	[48]
Microchloropsis salina	17.2	–	8.0	–	[49]
Isochrysis zhanjiangensis	–	–	31.9	–	[12]
Spirulina maxima	13	60	–	–	[28]
Chlorella vulgaris	15	55	–	–	[16]
Tetraselmis suecica	15	50	–	–	[50]
Dunaliella tertiolecta	7	46	–	–	[51]

[a] Present study.

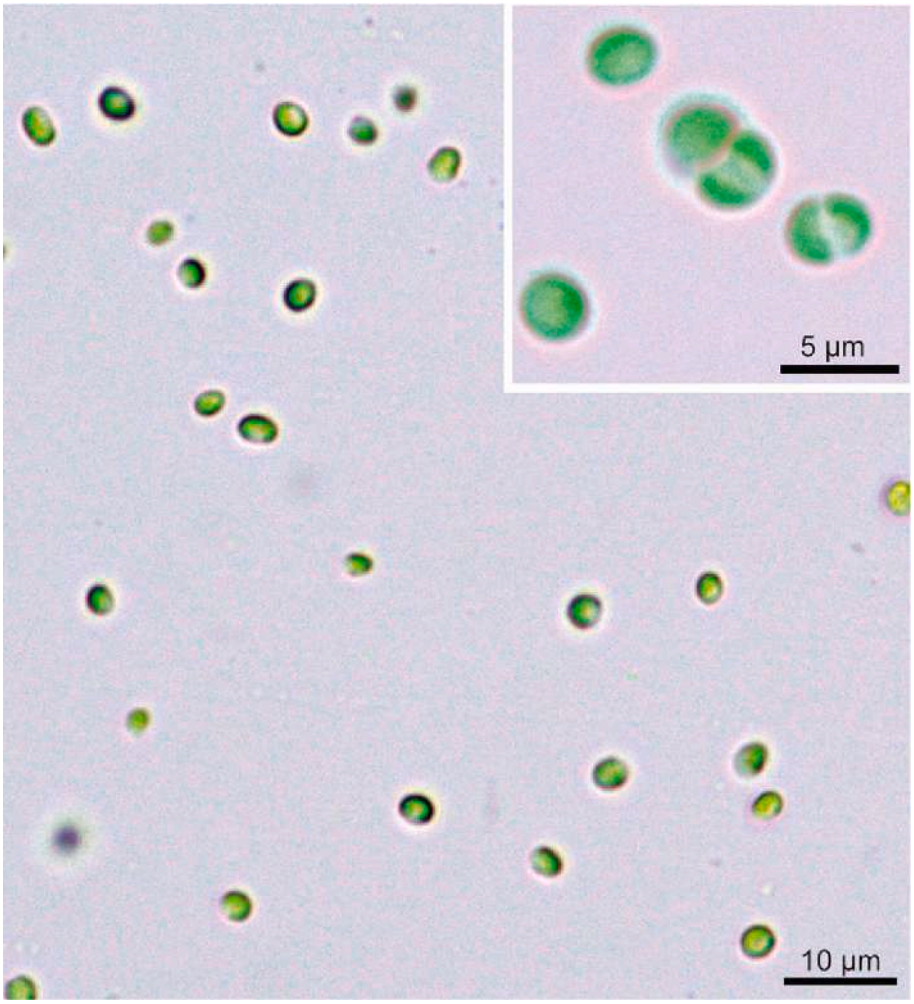

Fig. 1. Photomicrographs of *Nannochloropsis oculata* (CIB76) in the exponential growth phase (40× and 100×).

β-glucans similar in molecular structure to those found in yeast [19,22], however, it is currently unknown how N-limitation affects the accumulation of β-glucans in microalgae. Therefore, this study aims to investigate the effect of N-limitation on the accumulation of carbohydrates and β-glucans in *N. oculata* biomass in the first stage of the two-stage process.

2. Materials and methods

2.1. Strain and starter batch cultures

We used the *N. oculata* (CIB76) strain from the Collection of Microalgae Laboratory of the Centro de Investigaciones Biológicas del Noroeste (CIBNOR, S.C.; México). The experimental microalgae batch culture was conducted in the Laboratorio de Ecofisiología de Organismos Acuáticos y Cultivos de Apoyo de la Facultad de Ciencias del Mar de la Universidad Autónoma de Sinaloa. For microalgae batch cultures, we followed the seawater treatment sequence of filtering seawater (35 PSU) by 10, 5, 1 μm cartridges and activated carbon, disinfecting it with 1 ml of commercial 5 % sodium hypochlorite per liter of seawater, with neutralizing with sodium thiosulfate after 24 h before use.

We started the microalgae cultures using a successive transfer technique from a 15-ml tube. The initial cultures (200 and 3000 ml) were obtained from a batch system using F medium [23]. We set the light intensity at 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuously through six daylight white fluorescence lamps that together use 450 watts and emit 6000 Luxes, located on one side and very close to the microalgae container. Subsequently, we transferred the microalgae cultures to a 16-L carboy for the N-limitation experiment, with vigorous and constant aeration but no CO_2 supply. We did not supplement CO_2 to avoid possible antioxidant reactions that promote carbohydrate biosynthesis, independent of N-limitation. We recorded the temperature (27.5 ± 0.7 °C) and pH of water cultures daily.

2.2. Experimental design

We implemented a random experimental design to investigate the effect on microalgae growth of five levels of nitrogen availability at the start of batch culture. Specifically, the treatments include 100 %, 75 %, 50 %, 25 %, and 0 % of N (sodium nitrate, $NaNO_3$) available in normal F medium [23], following the methods of Voltolina et al. [24]. We used four replicates for each treatment. Nutrients were added at a volumetric rate of 1 $ml \bullet l^{-1}$ of seawater until the proper concentration levels of the culture medium were obtained. The initial availability of N for each treatment was 1.76, 1.32, 0.88, 0.44, and 0 $mmol \bullet l^{-1}$ for the 100 %, 75 %, 50 %, 25 %, and 0 % treatments, respectively. We did not modify any other nutrients from the F medium formula. The experiment was conducted until the transition from the lag to the stationary growth phase (the first stage in the two-stage process), at which point biomass sampling was conducted. We evaluated the growth rate, residual nitrogen, biomass composition, and β-glucan determination at least three times for each treatment.

2.3. Growth rate evaluation

To measure the cell density in each culture we used an improved Neubauer hemocytometer (0.1 mm depth, Brighline Optik Labor) under a light microscope (Leica model CME) [25]. We defined the cell division rate in each culture using the equation $\mu = (\ln Nt_1 - ln\, Nt_0) / (t_1 - t_0)$, where Nt_1 is the number of cells at the sampling time, and Nt_0 is the number of cells at the beginning of the experiment. The cumulative rate of cell division Σμ was calculated as $\Sigma\mu = \Sigma\mu_i = \mu_1 + \mu_2 \ldots \mu_t$ [26]. We used a logistic model to analyze the cell density growth, we used a logistic model, where $N = B_\infty/1 + be^{-ct}$. Here, N refers to the cell density at the sampling time, B_∞ is load capacity, b is the density coefficient, e is the base of natural logarithms, and ct is the growth rate [27].

2.4. Biomass determination

We monitored the density of microalgae every 24 h until the transition between the logarithmic and stationary growth phases, which we determined based on the maximum value of the cumulative cell division rate per day (Σμ) at different times for each treatment [26]. During this phase, microalgae experience faster biomass production, nutrient depletion, and consequently the highest carbohydrate productivity. In batch cultures, the transition from the logarithmic to stationary growth phase is when cultures start to *crash*. A key characteristic before the *crash* is a large aggregation of cells at the bottom of the tank that significantly decreases suspended cells. Therefore, we used the transition as an indicator for the sampling time [28].

To obtain microalgae samples, we filtered them through glass fiber filters (Whatman GF/C 47 mm). We determined the dry weight by subtracting the non-variable dry weight obtained after drying at 60 °C for 48 h from the wet weight. For inorganic content measurements, we calcined samples at 450 °C and later weighed them until they reached a constant weight. We calculated the organic weight by subtracting the inorganic weight from the dry weight [29]. We calculated biomass weight by dividing the dry weight or organic weight by the cells' density, expressed as unit dry weight (UDW, pg•cel^{-1}) and unit organic weight (UOW, pg•cel^{-1}), respectively.

2.5. β-Glucan determination

To determine β-glucan, we collected the microalgae biomass through centrifugation (Eppendorf 5810R) at 3600 rpm and 10 °C, for 10 min. We froze the obtained biomass at −70 °C (Arctiko, model ULF 650) and then lyophilized it at −50 °C and 0.050 mmHg (Lyophilizer Labconco, 208220) before analyzing it using the Megazyme® enzyme kit (CAT# K-YBGL; Lot: 171205-6). Additionally, we quantified β-glucans using a yeast β-glucans standard with 49 % purity supplied by the kit. We determined the total glucans through controlled acid hydrolysis with H_2SO_4 and measured the released glucose using glucose oxidase/peroxidase reagent (GOPOD). Finally, we determine β-glucans by subtracting the α-glucans from total glucans.

2.6. Residual nitrogen

To evaluate the residual concentrations of nitrates (NO_3^-), nitrites (NO_2^-), ammonium (NH_4^+), and phosphates (HPO_4^{2-}) in the batch microalgae cultures, we used the water samples obtained after filtering the microalgae biomass. We measured the residual nitrogen concentration using the following methods: UV spectrophotometry for nitrates, the sulfanilamide method and N-(1-naphthyl)-ethylenediamine dihydrochloride in the formation of a red azo compound for nitrites, and the indophenol blue method based on the formation of monochloramine in the presence of phenol and sodium nitroprusside for ammonium ions. We used the ascorbic acid method to determine the phosphates concentration by reacting to a mixture of molybdic, ascorbic acid, and trivalent antimony as a catalyst. We follow the methodology described by APHA [30] for these determinations.

2.7. Lipids, proteins, and total carbohydrates

Quadruplicate samples of lipids, proteins, and total carbohydrates were filtered through 47 mm Whatman GF/C glass-fiber filters and stored at −80 °C until processing. Proteins were extracted by heating for 15 min with 0.1 N sodium hydroxide, and their concentration was determined using Bradford's method [31]. Carbohydrates were extracted with sulfuric acid and measured according to the method described by Dubois [32], while lipids were extracted following the protocol of Bligh and Dyer [33].

2.8. Statistical analysis

To analyze volumetric productivity, final density, α-glucans, lipids, dry weight, and organic weight, no parametric Kruskal-Wallis tests were conducted, and multiple comparisons were made using Tukey's method. One-way ANOVA tests followed by Tukey's posthoc tests were conducted to analyze total glucans, β-glucan, β-glucan unitary, carbohydrates, proteins, ash weight, dry weight/organic weight, carbohydrates cell, and carbohydrates volumetric [34]. For percentage data, an arcsine transformation was performed before conducting statistical analyses [35]. The SigmaStat 3.5 software was used for all statistical analyses, and the logistic model was applied to analyze cell density using CurveExpert Basic 1.4 software.

3. Results

3.1. Microalgae cell growth

In the logistic model, the highest load capacity (B_∞) of 44.0 × 10^6 cells•ml^{-1} was observed at the start of the batch culture with 75 % N available, while the treatments with 100 %, 50 %, and 25 % N available at the start of the batch culture treatments reached 41.2, 35.1, and 25.7 × 10^6 cells•ml^{-1} respectively. The logarithmic growth phase was more extensive at the usual N concentration (medium F), and in all cases, the data fit with the logistic model (r = 0.99) (Fig. 2).

The maximum growth rate (1.34, $\mu_2 \cdot d^{-1}$) was observed in the 75 % N treatment at the start of the batch culture and decreased with respect to N availability. Growth rates of 1.31, 1.32, and 1.28 were observed for 100 %, 50 %, and 25 % N treatment respectively, and no significant differences were found between treatments ($P > 0.05$). The transition between logarithmic and stationary growth phases (maximum Σμ) occurred on days 6, 8, 9, and 10; for 25 %, 50 %, 75 %, and 100 % N, respectively, and biomass sampling was conducted (Fig. 2). No data were obtained from microalgae growth with 0 % N available at the start of the batch culture, as it did not increase density concerning the initial cell density and *crashed* at day 2 (Table 2).

3.2. Cellular biomass

The treatments with 100 % and 75 % N available at the start of the batch culture showed the highest dry, organic, and inorganic weights (DW, OW, and IW), and were significantly higher than the rest of the treatments ($P < 0.05$, except for IW). The percentage of organic matter (OW/DW) did not show significant differences between treatments ($P > 0.05$). Furthermore, unitary dry weight (UDW) and unitary organic weight (UOW) show higher values in treatments with 100 % and 75 % and were significantly different from the rest of the treatments ($P < 0.05$) (Table 2).

3.3. Lipids, proteins, and carbohydrates

The highest averages of proteins, carbohydrates, and lipids were recorded in the 75 % N treatment at the start of the batch culture.

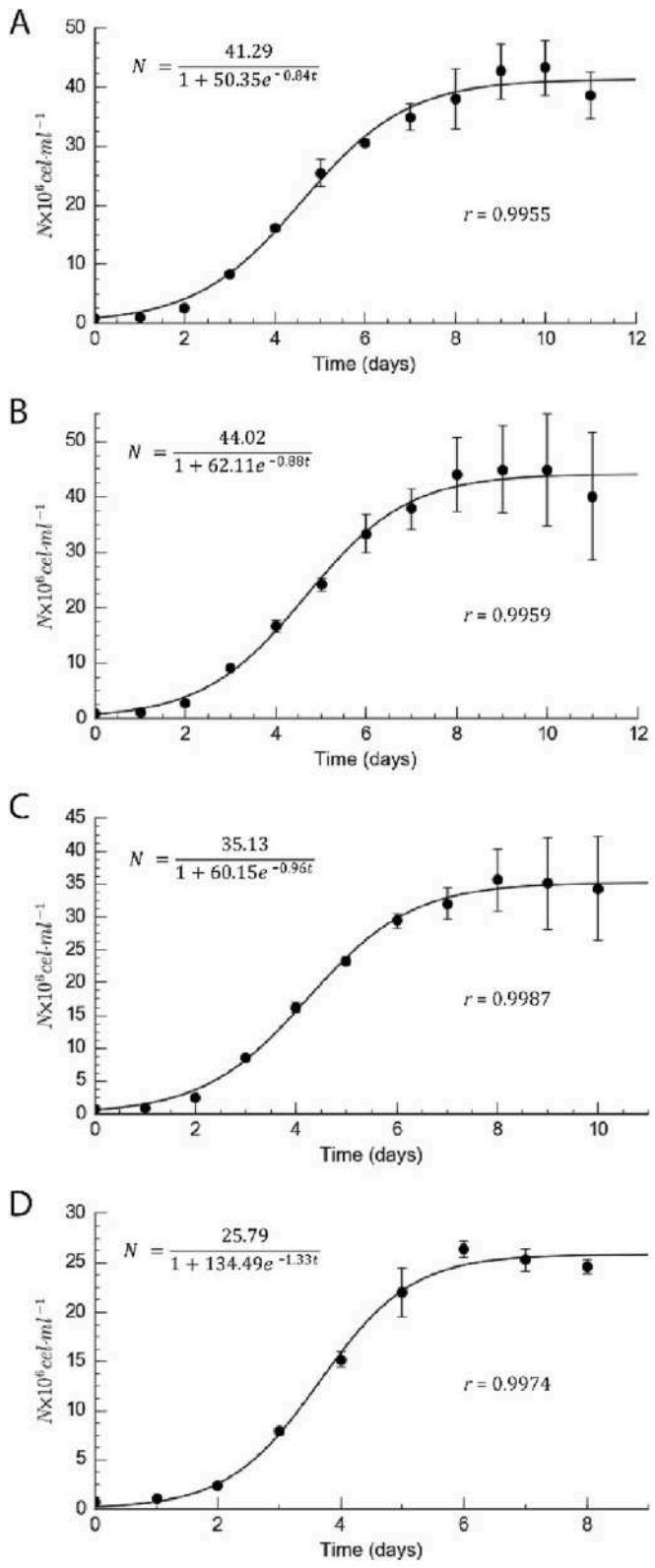

Fig. 2. Population growth of *N. oculata* with 100 % (A), 75 % (B), 50 % (C), and 25 % (D) N available concerning the F medium at the start of the batch culture.

Proteins in the 75 % N treatment were significantly different ($P < 0.05$) from those in the 0 % N treatment, and carbohydrates in the 75 % N treatment were significantly different ($P < 0.05$) from those in the 25 % N treatment. Lipids and total composition did not show significant differences ($P > 0.05$) between treatments (Fig. 3).

Table 2
Biomass (dry weight) of *N. oculata* obtained in a culture medium at different N concentrations. Nt = cells density, N = nitrogen, DW = dry weight, OP = organic weight, UDW = unit dry weight and UOW = unit organic weight. Different letters indicate significant differences ($P < 0.05$).

	%N			
	100	75	50	25
N_t ($\times 10^6$ cel•ml^{-1})	33.9 ± 3.6^a	40.1 ± 11.5^a	34.3 ± 7.9^a	24.6 ± 0.7^a
Cell division (μ_2•d^{-1})	1.31 ± 0.0	1.34 ± 0.0	1.32 ± 0.0	1.28 ± 0.0
DW (mg•l^{-1})	0.61 ± 0.1^a	0.57 ± 0.0^a	0.39 ± 0.0^b	0.30 ± 0.0^b
OW (mg•l^{-1})	0.37 ± 0.04^a	0.37 ± 0.04^a	0.26 ± 0.02^b	0.19 ± 0.01^c
IW (mg•l^{-1})	0.21 ± 0.10	0.19 ± 0.05	0.12 ± 0.03	0.10 ± 0.07
OW/DW (%)	63.9 ± 8.3	67.9 ± 4.9	69.9 ± 5.7	69.1 ± 8.8
UDW (pg•cel^{-1})	18.3 ± 4.6^a	15.3 ± 5.0^a	11.9 ± 3.0^b	12.2 ± 3.2^b
UOW (pg•cel^{-1})	11.0 ± 1.1^a	10.1 ± 3.1^a	8.1 ± 1.4^b	7.9 ± 0.4^b

3.4. Content and volumetric production of β-glucans and carbohydrates

The percentage of β-glucans in *N. oculata* biomass was not significantly different ($P > 0.05$) between 100 %, 75 %, and 50 % of the available nitrogen at the start of the batch culture. However, a significantly difference ($P < 0.05$) was detected between 0 % and 25 % of the available nitrogen at the start of the batch culture. In terms of α-glucans and total glucans, the lowest percentage was achieved under the 0 % N condition compared to the other treatments ($P < 0.05$) (see Table 3).

Carbohydrate productivity showed significant differences ($P < 0.05$) in 100 % and 75 % N (0.10 ± 0.02 and 0.10 ± 0.03 g•l^{-1}, respectively) compared to the rest of the treatments. Moreover, β-glucans per cell in the 25 % N treatment were lower (0.5 ± 0.2 pg•cel^{-1}) and significantly different ($P < 0.05$) from the other treatments. The 75 % N treatment showed the highest average of β-glucan productivity (4.3 ± 1.3 mg•l^{-1}), which was significantly different ($P < 0.05$) from the 0 %, 25 %, and 50 % N treatments. Only 100 % N treatments showed no significant difference ($P > 0.05$) (see Table 4).

3.5. Residual N and P

The residual concentration of nitrates (NO_3^-) tended to be higher as the N supply increased, and significant differences ($P < 0.05$) were observed between treatments (see Table 5). The highest nitrate concentration occurred in the 100 % N treatment, while the lowest concentration was observed in the 25 % N treatment. The nitrites (NO_2^-) concentration was not significantly different ($P > 0.05$) between the 100 %, 75 %, and 50 % N treatments, but the 100 % N treatment tended to have the highest residual concentration of nitrites. Ammonium (NH_4^+) concentrations were much higher in the 100 %, 50 %, and 25 % N treatment compared to the 0 % N treatment, but the ammonium concentration in the 75 % N treatment was not statistically different ($P > 0.05$) from the 0 % treatment, despite a lower average concentration. The pH of the medium was significantly lower ($P < 0.05$) in the 0 % N treatment (7.9), while the pH in the other treatments was close to 9.6. The phosphate concentration was higher and statistically different ($P < 0.05$) in the 0 % N treatment compared to the other treatments, indicating a similar phosphate consumption in all treatments (see Table 5).

4. Discussion

Microalgae are naturally exposed to nitrogen limitation, which triggers various responses including ultrastructural rearrangements, carbon reallocation, transcriptome reprogramming, metabolome remodeling, and lipid restructuring [36]. Understanding the molecular details of these responses is important for learning how microalgae thrive in their natural environment and critical for optimizing biomass

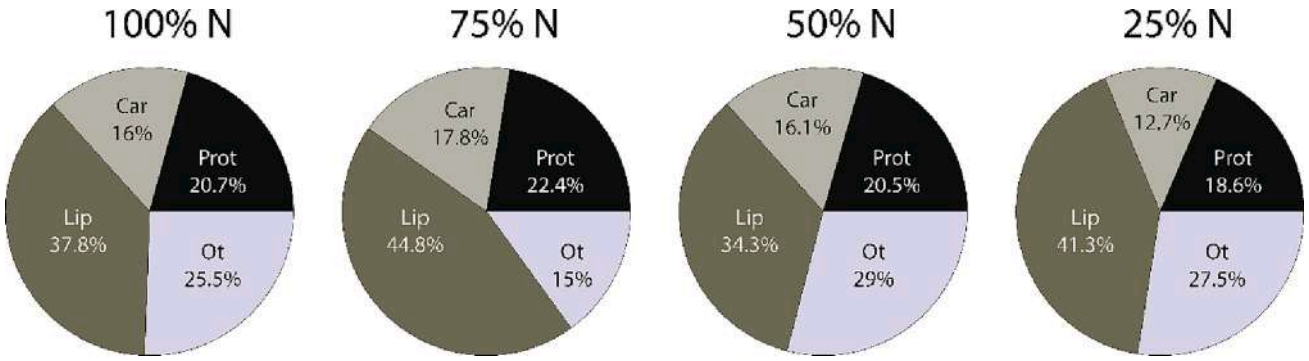

Fig. 3. Organic composition (%) on *N. oculata* biomass cultivated in N-limitation concerning F medium. Different letters indicate significant differences ($P < 0.05$).

Table 3
β-Glucans content (%) in *N. oculata* biomass at different N concentrations concerning F medium. Different letters indicate significant differences ($P < 0.05$).

		%N				
	βG-yeast*	100	75	50	25	0
β-Glucans (%)	50.0 ± 1.5	5.0 ± 1.8^{a}	7.4 ± 1.5^{a}	6.7 ± 1.3^{a}	4.4 ± 0.7^{b}	1.7 ± 0.1^{c}
α-Glucans (%)	0.9 ± 0.0	1.8 ± 0.8^{a}	0.5 ± 0.1^{a}	0.6 ± 0.0^{a}	1.3 ± 0.2^{a}	0.2 ± 0.0^{b}
Total glucans (%)	50.9 ± 1.6	6.8 ± 0.9^{a}	7.9 ± 1.3^{a}	7.3 ± 1.3^{a}	5.7 ± 0.7^{a}	1.9 ± 0.1^{b}

* Yeast β-glucan control.

Table 4
Cellular content of β-glucans (average ± d.e.), volumetric productivity of β-glucans and carbohydrates in *N. oculata*. Different letters indicate significant differences ($P < 0.05$).

	%N				
	100	75	50	25	0
Carbohydrates productivity ($g \bullet l^{-1}$)	0.10 ± 0.02^{a}	0.10 ± 0.03^{a}	0.06 ± 0.01^{b}	0.04 ± 0.01^{bc}	0.003 ± 0.000^{c}
β-Glucans ($pg \bullet cel^{-1}$)	1.0 ± 0.5^{a}	1.1 ± 0.2^{a}	0.8 ± 0.1^{a}	0.5 ± 0.2^{b}	1.2 ± 0.3^{a}
β-Glucans productivity ($mg \bullet l^{-1}$)	3.2 ± 1.6^{ba}	4.3 ± 1.3^{a}	2.6 ± 0.6^{b}	1.4 ± 0.6^{bc}	0.04 ± 0.00^{c}

Table 5
Residual concentrations of nitrates (NO_3^-), nitrites (NO_2^-), ammonium (NH_4^+), and phosphates (HPO_4^{2-}) in the stationary phase of *N. oculata* cultures at different N concentrations concerning F medium. n. d. = no determinate. Different letters indicate significant differences ($P < 0.05$).

	%N				
	100	75	50	25	0
Temperature (°C)	27.5 ± 0.8	27.4 ± 0.4	27.6 ± 0.7	27.6 ± 0.7	27.6 ± 0.7
pH	9.6 ± 0.6^{a}	9.6 ± 0.6^{a}	9.6 ± 0.6^{a}	9.6 ± 0.7^{a}	7.9 ± 0.1^{b}
Nitrates ($mg \bullet l^{-1}$)	7.4 ± 1.6^{a}	4.5 ± 2.0^{a}	2.6 ± 0.3^{a}	1.1 ± 0.1^{b}	1.4 ± 0.1^{b}
Nitrites ($mg \bullet l^{-1}$)	3.4 ± 0.6	2.8 ± 0.4	2.6 ± 0.3	n.d.	n.d.
Ammonium ($mg \bullet l^{-1}$)	82.7 ± 30.3^{ab}	10.7 ± 12.1^{a}	78.7 ± 15.6^{ab}	73.9 ± 13.9^{ab}	19.6 ± 2.1^{b}
Phosphates ($mg \bullet l^{-1}$)	0.1 ± 0.0^{b}	0.4 ± 00^{b}	0.2 ± 0.0^{b}	0.1 ± 0.0^{b}	5.0 ± 0.0^{a}

composition and yield for large-scale cultivations [37]. This study investigates how N-limitation at the beginning of the batch culture affects the growth of carbohydrates accumulation (β-glucans) in *N. oculata* during the logarithmic phase as a prelude to the two-stage process context.

However, strategies such as nutrient limitation have drawbacks [7,14]. For example, the lack of nitrogen in microalgae culture can result in slowed growth rates, reduced photosynthetic efficiency, and the remobilization of nitrogen-rich metabolites, such as protein and chlorophyll, to transiently support survival. Microalgae respond to these conditions by channeling carbon and energy from photosynthesis into energy-storage metabolites, such as carbohydrates and lipids (primarily TAG) [38]. Carbohydrates sink excessive carbon and electrons when protein and polar lipid synthesis are diminished under stress conditions, enabling microalgae to acclimate to unfavorable environments [11].

Optimum N-limitation is important because incorrect N-limitation can decrease microalgae biomass harvest, as reported in *Dunaliella tertiolecta* [39], *Chlorella vulgaris* [7], *Phaeodactylum tricornutum*, *Isochrysis aff. galbana* clone T-Iso, *Rhodomonas baltica*, and *Nannochloropsis oceanica* [40]. Over N-limitation in *N. oculata* negatively affects the volumetric productivity of microalgae biomass (Fig. 2) and, consequently, carbohydrate and β-glucan production in *N. oculata* during the present experiment (25 % and 0 % N treatments). However, treatments with 100 % and 75 % N available at the start of the batch culture showed the highest unitary, dry, and organic weights (UW, DW, and OW), and were significantly different from the other treatments ($P < 0.05$). The highest volumetric productivity was observed in the 100 % N treatment (0.61 $mg \bullet l^{-1}$, DW). In the 0 % N treatment, microalgae cell density did not increase, and the population *crashed* early. These results also indicate that the contribution of seawater N used was close to zero. Therefore, it is important to supplement the necessary N in the culture medium to develop highly productive microalgae cultures and obtain desired metabolites such as β-glucans. Markou et al., [14] propose that prioritizing microalgal biomass generation (the first stage in the two-stage process) can assist in developing a strategy to harvest the desired composition of microalgal biomass.

Microalgae use photosynthesis to transform light energy into chemical energy, comprising two main reactions: light and dark. During light reactions, photosynthetic antennas absorb light energy, which is used to split water into protons, electrons, and oxygen. Then electrons and protons are used to generate energy carriers (NADPH and ATP), supporting the cell's metabolic needs. During dark reactions, CO_2 is reduced to carbohydrates via the Calvin cycle inside the chloroplast, using energy derived from NADPH and ATP [41]. The first step in the Calvin cycle, is the assimilation of CO_2, catalyzed by ribulose-1,5-bisphosphate carboxylase oxygenase. CO_2 is used to carboxylate five-carbon compounds (ribulose-1,5-bisphosphate) into two three-carbon compounds (3-phosphoglycerate), one of which is a substrate for carbohydrates, while the other is used in the next Calvin cycle [41]. We do not supplement CO_2 in batch cultures to avoid possible antioxidant reactions that promote carbohydrate biosynthesis,

independent of nitrogen limitation. Studies on the application of inorganic carbon sources to microalgae culture, like bicarbonate or CO_2 gas in microalgae cultivation, revealed that these sources were both a carbon source and oxidative stress. Supplementation of bicarbonate reduces the oxidative stress caused by ROS, lowers lipid peroxidation damage, and improves the activities of antioxidant enzymes (SOD, CAT, and APX) in *D. salina* cultures under nutrient stress [13]. Several studies have been carried out on supplementing of inorganic carbon in the form of gas, but only a few microalgae can directly take up gaseous CO_2 for its growth. In contrast, others convert gaseous carbon into bicarbonate through chemical disequilibrium and utilize it for photosynthesis [13]. However, we consider *N. oculata* a promising candidate for carbohydrates and β-glucans from supplemental CO_2, with the possibility of regulating the activity via adjusting cultivation conditions, especially in the second stage of the two-stage process.

Previous studies on the *Nannochloropsis* genus using traditional culture media have reported a total carbohydrate content ranging from 6 % DW, and accumulation by the effect of N-limitation of up to 53 % (as shown in Table 1). This study reveals that in a batch culture during the first stage in the two-stage process, an initial available N (sodium nitrate, $NaNO_3$) culture concentration of 0.8 and 1.32 mmol•l^{-1} (50 and 75 % N treatments, respectively) produces *N. oculata* biomass with high carbohydrate and β-glucans content. Furthermore, no significant difference was observed with F medium ($P > 0.05$). The 25 % and 0 % N treatments negatively affect cell population, carbohydrate, and β-glucans accumulation (see Fig. 2 and Table 3).

However, research on a sustained limitation over long timescales that distinguishes the biological response to short-term acute depletion from long-term chronic depletion is still lacking. Microalgae harvest time is a key factor in batch culture because microalgae can adapt rapidly to transient changes in nutrient availability within the environment, such as harvest time after the logarithmic phase has finished [42]. Although several studies investigate the effect of nutrients on microalgae by applying short-term or long-term starvation in batch cultures [36] they do not explore sustained limitation over long timescales. For example, high volumetric productivity of the *Nannochloropsis* genus has been reported [64.8 mg•l^{-1}, 47] [81.5 mg•l^{-}1, 16]. However, β-glucans volumetric productivity of *N. oculata* in the present study (as shown in Table 4) is lower than in previous reports, ranging from 0.04 (0 % N) to 4.3 mg•l^{-1} (75 % N). This could be due to the moment of biomass sampling that was carried out, just at the transition from logarithmic to stationary phase in batch culture (the first stage in the two-stage process), when cells are not exposed long enough to N-limitation to change their metabolic pathways and reach maximum values of carbohydrate accumulation, or β-glucans. However, at the end of the logarithmic phase, the highest amount of biomass of *N. oculata* was obtained, and the effect of N-limitation on the accumulation of carbohydrates and β-glucans was described. This allows the development of a strategy to modify the biochemical composition of the biomass prior to harvest (acute effect) or prelude to semi-continuous production (chronic effect).

N. gaditana cells can acclimate to chronic nitrogen limitations while maintaining photosynthetic activity and accumulating lipids [36]. Both nitrogen and phosphorus limitations increase triacylglycerols content by modulating the fluxes of reduced carbon molecules toward lipid biosynthesis rather than by inducing fatty acid synthesis. Under phosphorus limitation photosynthetic activity is maintained, while under nitrogen depletion, where proteins of the photosynthetic apparatus are reduced in number but still functional and capable of achieving half of the biomass productivity with 30 % of the nitrogen supply [36]. In this study, we found that lipids were not significantly different between treatments ($P > 0.05$) but were statistically different ($P < 0.05$) from protein and carbohydrates in every treatment. Only protein in the 75 % N treatment differed significantly ($P < 0.05$) from the 0 % N treatment. Therefore, in all treatments, the basal metabolic pathways in *N. oculata* were not modified during logarithmic phase culture (see Fig. 3). We consider it necessary to evaluate nitrogen and phosphorus limitation over a long period by adding CO_2 with the response surface methodology to optimize carbohydrate production in microalgae of the genus *Nannochloropsis*.

Furthermore, the β-glucan content in *N. oculata* biomass ranged from 1.7 % (0 % N) to 7.4 % (75 % N) (as shown in Table 3). However, our previous research (see Table 1) reported higher β-glucans (21.7 %, 15.4 %, and 14.3 % DW) in three *Nannochloropsis* sp. Strains were grown in batch culture using F/2 medium, and samples were taken (NN-X-1: day 13, *N. oculata*: day 15; and NpUNAM: day 22) before the culture *crashed* in the late stationary phase [19]. In the present study, biomass samples were taken on days 6, 8, 9, and 10; for 25 %, 50 %, 75 %, and 100 % N treatments, respectively. However, we point out that the production potential of β-glucans is based on volumetric productivity generated by each species and in culture protocol [19].

Regarding residual concentrations of N and phosphates, the 0 % N treatment serves as the reference point. Nitrates (NO_3^-) tend to reach higher concentrations as the N supply increases (Table 5). Nitrates in the 0 % and 25 % N treatments were statistically different from the rest of the treatments ($P < 0.05$). Nitrites (NO_2^-) were not determined due to their low concentration in the 0 % and 25 % N treatments, and the resting treatments not statistically different ($P > 0.05$). Ammonium (NH_4^+) concentrations were much higher in 100 %, 50 % and 25 % N treatments compared to the 0 % N treatment.

Interestingly in the 75 % N treatment, ammonium was not statistically different ($P > 0.05$) from the 0 % treatment, even with a lower average concentration. This suggests that microalgae in 75 % N treatment consumed ammonium (see Table 5). Kumar and Bera [5] examined nitrogen utilization in microalgae and pointed out that ammonium is necessary for the formation of nucleic acids, proteins, and other organic molecules, as well as a product of their catabolism. Nevertheless, ammonium can be toxic and even cause cell death at high concentrations [43]. While 0 % N treatment had a statistically significant difference ($P < 0.05$) in phosphate compared to other treatments, indicating similar phosphate consumption in *N. oculata* batch cultures (Table 5).

In classical carbohydrate biosynthesis, glucose-1-phosphate requires activation by ATP to form ADP-glucose catalyzed by ADP-glucose pyrophosphorylase (AGPase), the committed step in glucose moiety production. ROS can accumulate under oxidative stress conditions [44]. Typically, AGPase is activated by 3-phosphoglyceric acid, the first product of photosynthetic CO_2 fixation, and inhibited by inorganic phosphate, which indicates a low cell energy state [45]. AGPase activity is regulated under nutrient stress conditions such as nitrogen, phosphorus, and sulfur deprivation. During short-term nitrogen starvation, AGPase activity generally increases with carbohydrate accumulation [46]. However, recent studies suggest that AGPase may be dispensable or even impair nutrient-induced carbohydrate accumulation in some microalgae [47]. It is crucial to evaluate AGPase activity in future studies on optimizing nutrient limitation for microalgal carbohydrate production.

Therefore, we recommended studying the effect of N-limitation (50 %, 75 %, and 100 % N treatments, principally) during the early stationary phase, for a chronic long-term effect, along with the partial N supply or other stressors (e.g., salinity, light, temperature) during late stationary phase, to complete the two-stage process proposal. This is especially important for industrial applications, where cultures must be maintained long-term (semi-continue biomass production), and an optimal balance between biomass productivity and carbohydrate accumulation is necessary to achieve maximum yield. Additionally, this knowledge can improve the industrial production of microalgae β-glucans.

5. Conclusions

Over N-limitation in *N. oculata* culture (0.4 and 0 mmol•l^{-1} to 25 and 0 % N, F medium, respectively) affects biomass production, carbohydrates, and β-glucans accumulation. A correct N-limitation in *N. oculata*

culture are 0.8 and 1.32 mmol•l^{-1} N initial concentration (50 and 75 % N, F medium) until the logarithmic phase finishes, to obtain biomass with the best concentration of carbohydrates and β-glucans before the second stage in the two-stage process.

Author statement

These results provide valuable information to researchers and private initiatives that are interested in the use of microalgal biomass in the field of biofuels or immunostimulants.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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