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Research Article

Alternative culture medium for large-scale production of *Nannochloropsis oculata*

[Medio de cultivo alternativo para la producción a gran escala de *Nannochloropsis oculata*]

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Abstract

Microalgae of the genus *Nannochloropsis* are highly valued in aquaculture due to their protein, fatty acid, and carotenoid content, offering potential as additives or substitutes for fish meal or oil. However, large-scale production encounters challenges, particularly concerning the costs of culture medium. Thus, this study aimed to assess the impact of substituting artificial sea salt with common salt and standard medium with agricultural fertilizer in *Nannochloropsis oculata* production to reduce production costs. Initially, the effects of reduced salinity were evaluated at salinities of 30, 10, 5, and 1 psu, followed by an examination of the effects of replacing artificial sea salt with common salt and using agricultural fertilizer on microalgae growth. Salinities of 30 and 10 psu exhibited the highest growth parameters. The salt source had no significant impact on culture growth, while the agricultural fertilizer enhanced it. Common salt increased the relative concentration of eicosapentaenoic acid compared to artificial sea salt, although the total lipid concentration was higher in microalgae cultured with artificial sea salt. The developed medium was validated through semi-continuous cultures in 100 L flat vertical bags, showing to be an economically viable alternative without hindering *N. oculata* growth.

Keywords: *microalgae, aquaculture, salinity, fertilizer, fatty acids.*

Resumen

Las microalgas del género *Nannochloropsis* son altamente valoradas en la acuicultura debido a su contenido de proteínas, ácidos grasos y carotenoides, ofreciendo potencial como aditivos o sustitutos de la harina o el aceite de pescado. Sin embargo, la producción a gran escala enfrenta desafíos, particularmente en lo que respecta al medio de cultivo. Por lo tanto, este estudio tuvo como objetivo evaluar el impacto de sustituir la sal marina artificial por sal común y el medio de cultivo estándar por fertilizante agrícola en la producción de *Nannochloropsis oculata*, con el fin de reducir los costos de producción. Inicialmente, se evaluaron los efectos de la reducción de salinidad a salinidades de 30, 10, 5 y 1 psu, seguido de un examen de los efectos de reemplazar la sal marina artificial por sal común y usar fertilizante agrícola en el crecimiento de las microalgas. Las salinidades de 30 y 10 psu exhibieron los parámetros de crecimiento más altos. La fuente de sal no tuvo un impacto significativo en el crecimiento del cultivo, mientras que el fertilizante agrícola lo mejoró. La sal común aumentó la concentración relativa de ácido eicosapentaenoico en comparación con la sal marina artificial, aunque la concentración total de lípidos fue mayor en las microalgas cultivadas con sal marina artificial. El medio desarrollado fue validado a través de cultivos semicontinuos en bolsas verticales planas de 100 L, demostrando ser una alternativa económicamente viable sin obstaculizar el crecimiento de *N. oculata*.

Palabras clave: *microalgas, acuicultura, salinidad, fertilizante, ácidos grasos.*

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1. Introduction

With global population growth, the demand for food has been increasing, including marine animal derivatives. It is estimated that by 2030, the total global fish production will reach 209 million tons, with 103 million originating from aquaculture (FAO, 2018). With the advancement and growth of this sector, the demand for raw materials for feed production will inevitably increase in parallel, including fish meal and oil. It is assumed that about 16% of capture fisheries will be allocated to produce feed ingredients (FAO, 2018). Unfortunately, the demand for these ingredients cannot be met. Therefore, there is a need for more sustainable resources that can reduce the use of fish meal and oil in aquaculture (Nishshanka *et al.,* 2022).

In this scenario, microalgae emerge as a viable alternative. Microalgae are microscopic photosynthetic organisms capable of growing in different environmental conditions using sunlight, carbon, nitrogen, and phosphorous. These microorganisms are considered superfoods as they are sources of vitamins, carotenoids, antioxidants, essential amino acids, lipids, polysaccharides, and other bioactive compounds (Eze *et al.,* 2023). Due to their high nutritional value and richness in bioactive compounds, they not

only promote the nutrition of aquatic organisms but also enhance immunity and disease resistance (Nagappan *et al.,* 2021). Additionally, as a primary source of fatty acids, they can improve the lipid profile in aquatic animals, increasing their nutritional value. Thus, microalgae can be used for the feeding of zooplankton, crustaceans, mollusks, shrimp, and fish (Dineshbabu *et al.,* 2019). Also, they constitute a beneficial strategy for enhancing water quality and aiding in the biocontrol of diseases (Harpeni *et al.,* 2024).

Microalgae of the genus *Nannochloropsis* are among the most used and important in aquaculture due to their composition rich in proteins, long-chain polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA), and carotenoids (Ferreira *et al.,* 2021). Studies show the potential of this microalga as an alternative to fish-derived components or as a feed additive. The partial replacement of fish oil with *Nannochloropsis* sp. in the form of pure powder biomass or its lipids in feed has led to improvements in the growth and fatty acid profile of shrimp larvae, post-larvae, and juveniles (Oswald *et al.,* 2019; Oswald *et al.,* 2020). Also, the partial replacement of fish meal with the microalga in turbot feed increased the concentration of essential amino acids in the meat and improved the organism's antioxidant capacity (Qiao *et al.,* 2019). Gbadamosi and Lupatsch (2018) also observed that using *N. salina* as a total replacement for fish meal did not affect the performance and health of tilapia compared with traditionally fed fish. Used as a feed additive, a preparation of *Nannochloropsis* spp. has been shown to enhance the immunity and resistance of shrimp subjected to thermal shock (Guimarães *et al.,* 2021).

Despite the advantages of using microalgae as feed in aquaculture, a bottleneck in large-scale microalgae production is the high production cost, including costs related to the culture medium (Carvalho *et al.,* 2019). The application of analytical-grade nutrients as used on laboratory scale is impractical. Therefore, more economical alternative nutrient sources are employed, such as commercial fertilizers. However, since these differ from laboratory culture media, the impact of new sources must be assessed (Nayak *et al.,* 2016; Neto *et al.,* 2018; Carvalho *et al.,* 2019).

For marine microalgae, another important factor to consider is salinity. Salinity influences the osmotic and ionic balance of cells in relation to the environment and affects energy expenditure. High or very low salinity values can inhibit the growth of some species and induce significant physiological changes, as well as alter the content of lipids, carbohydrates, proteins, and pigments (Cañavate and Fernández-Díaz, 2022; Haris *et al.*, 2022). In this regard, the salt source must be evaluated by producers. Many researchers use natural seawater as the base for the culture medium, known as a semi-defined medium, as they cannot control the water composition at the time of collection. However, the imprecision of the composition, water quality variation, and risks of water contamination are negative aspects of its use, besides being impractical for non-coastal locations. Artificially salinized water, therefore, is an interesting alternative that can lead to similar growth (Lelekov *et al.,* 2016), and commercial formulations are available.

Unfortunately, the high cost of these artificial sea salts may limit their use in massive-scale cultivation.

In this context, the objective of this study was to assess the impact of substituting artificial sea salt with common salt and standard medium with agricultural fertilizer in *Nannochloropsis oculata* production, developing a financially viable culture medium. The study was developed in three stages: (i) salinity reduction, (ii) substitution of commercial sea salt with common salt, and standard nutrient medium with agricultural fertilizer, and (iii) evaluation of the new culture medium on a pilot scale.

2. Materials and methods

2.1 Microalga, culture conditions and reagents

The microalga *Nannochloropsis oculata* was obtained from the culture collection of the Laboratory of Algae Cultivation and Biotechnology of the State University of Santa Catarina and maintained in the microalgae cultivation room in F/2 culture medium (Guillard 1975) and artificial sea salt (salinity 30 psu). All presented experiments, including the validation in pilot scale, were conducted in the cultivation room with a controlled temperature of 23ºC, continuous aeration with filtered atmospheric air, and 24-hour illumination with a light intensity of 66.6 µmol photons m^{-2} s^{-1}. Physicochemical parameters such as salinity and pH were regularly monitored. All chemicals used were of analytical reagent grade, except for the commercial products such as the artificial sea salt, common salt, agricultural fertilizer and, $(NH_4)_2SO_4$.

2.2 Evaluation of Nannochloropsis oculata growth at low salinity

Aiming for the reduction of the amount of salt in the cultivation medium of *N. oculata*, the effects of concentrations of salt below the traditional level (30 psu) on culture growth were assessed. Salinities of 30 (control), 10, 5, and 1 psu were applied (represented by Sal 30, Sal 10, Sal 5, and Sal 1, respectively). Prior to the experiment, the microalga was precultured to each tested salinity (lower than 30 psu). Every two days, it was subcultured from the current salinity (initially 30 psu) medium to a new one with a salinity 2 ups lower. This procedure was repeated until obtaining cultures with the desired salinities for each treatment. For the experiment, commercial artificial sea salt was used. According to the supplier, it provides 0.3 g g^{-1} of sodium, 9 mg g^{-1} of calcium, 27 mg g^{-1} of magnesium, 9 mg g^{-1} of potassium, 0.51 g g^{-1} of chlorides, 0.06 g g^{-1} of sulfates, 3.15 mg g^{-1} of carbonates, and trace elements (unspecified). F/2 medium was used, and the cultivation was conducted in Erlenmeyer flasks containing 1.5 L of culture. The experiment was initiated with approximately 300 x 10^4 cells mL^{-1} and lasted for 9 days, when all the treatments had reached the stationary phase. The treatments were conducted in parallel and triplicate.

2.3 Selection of salt and nutrient sources

To assess the feasibility of using a more economical cultivation medium, the use of common table salt (CS) was compared to artificial sea salt (AS) in two different culture media: the standard F/2

medium (F2) and with agricultural fertilizer plus $(NH_4)_2SO_4$ (AF). The agricultural fertilizer used was a NPK type, claiming to contain 18% N, 6% P_2O_5, and 18% K_2O (w/w). $(NH_4)_2SO_4$ was added to give a N:P molar ratio equal to 16:1. They were used in a concentration providing the same amount of N as F/2 medium.

An experimental design with two factors was applied, resulting in four treatments: ASF2, ASAF, CSF2, and CSAF. Each treatment was conducted in parallel and in triplicate with a volume of 1.5 L in Erlenmeyer flasks with a salinity of 20 psu. The experiment was initiated with approximately 1,700 x 10^4 cells mL^{-1} and lasted for 7 days, as it was observed in the previous experiment that this was the time for the culture to begin entering the stationary phase. At the end, the total lipid content was determined using the Bligh and Dyer method (Bligh and Dyer, 1959), and the fatty acid profile was analyzed by gas chromatography-mass spectrometry (GC/MS, 5977B, Agilent, USA) in the ASAF and CSAF cultures.

2.4 Validation of the cultivation medium on a pilot scale

The results from the laboratory scale experiment were replicated on a pilot scale using the culture medium with adjustments determined in the previous experiments. The microalgal cultivation was conducted in 100 L flat vertical bags (FVAs) with a culture medium containing agricultural fertilizer and $(NH_4)_2SO_4$ (giving a N:P molar ratio equal to 16:1), salinized with common salt (20 psu). The cultivation was carried out in batch mode until the culture reached between 4,000 x 10^4 and 5,000 x 10^4 cells mL^{-1}, after which harvests of 45% of the volume were performed every 7 days in a semi-continuous system. Harvesting was done by flocculation followed by filtration. The supernatant from flocculation process was returned to the culture and fertilized again. Three replicates were conducted, each lasting 20-30 days.

2.5 Monitoring of microalgal growth

For the growth analysis of the cultures, cell density and, dry biomass was regularly monitored. Cell density (CD) was determined by cell counting using a Neubauer chamber under an optical microscope. Dry biomass (DB) was estimated using a gravimetric method. A known volume of the culture was filtered through a pre-dried and weighed fiberglass filter. The filter was then dried in an oven at 50 ºC until a constant weight was achieved. The dry biomass is determined by the difference between the final and initial weights of the filter.

From these data, the maximum cell density (MCD) was determined, and the specific growth rate (μ, d^{-1}) and biomass productivity (g L^{-1} d^{-1}) were calculated using the respective formulas:

$$\mu = \frac{ln\ CD_f - ln\ CD_i}{\Delta t}$$

where CD_i is the initial cell density, CD_f is the final cell density and Δt is the time interval between CD_i and CD_f. It is important to highlight that CD_f and Δt corresponded to those when the culture reached the MCD, and not necessarily to those of the last day of the experiment.

$$Productivity = \frac{DB_f - DB_i}{\Delta t}$$

where DB_i is the initial dry biomass, DB_f is the final dry biomass and Δt is the time interval between DB_i and DB_f.

2.6 Statistical analysis

For the experiment with different salinities, the data were analyzed through one-way ANOVA, while for the experiment involving different salt and nutrient sources, a factorial ANOVA was applied. To identify statistical differences between the treatments, the Tukey post-hoc test was used, considering a significance level of 0.05. The data are presented as mean ± standard deviation.

3. Results

3.1 Effect of salinity

The salinity of each treatment remained constant according to its respective concentration. Sal 30, Sal 10, and Sal 5 maintained an average pH value equal to or above 9.0 (9.0 ± 0.12, 9.5 ± 0.07, and 9.0 ± 0.13, respectively), while Sal 1 remained at 8.8 ± 0.08. The growth of the CD of *N. oculata* at different salinities is presented in Figure 1. It can be observed that the growth of the cultures over time was directly proportional to the increase in salinity, with Sal 10 and Sal 30 exhibiting the highest cell densities from day 2 onwards ($p < 0.05$), concluding the experiment with an average of 1,611.67 x 10^4 ± 385.46 x 10^4 and 2,125.00 x 10^4 ± 354.29 x 10^4 cells mL^{-1}, respectively.

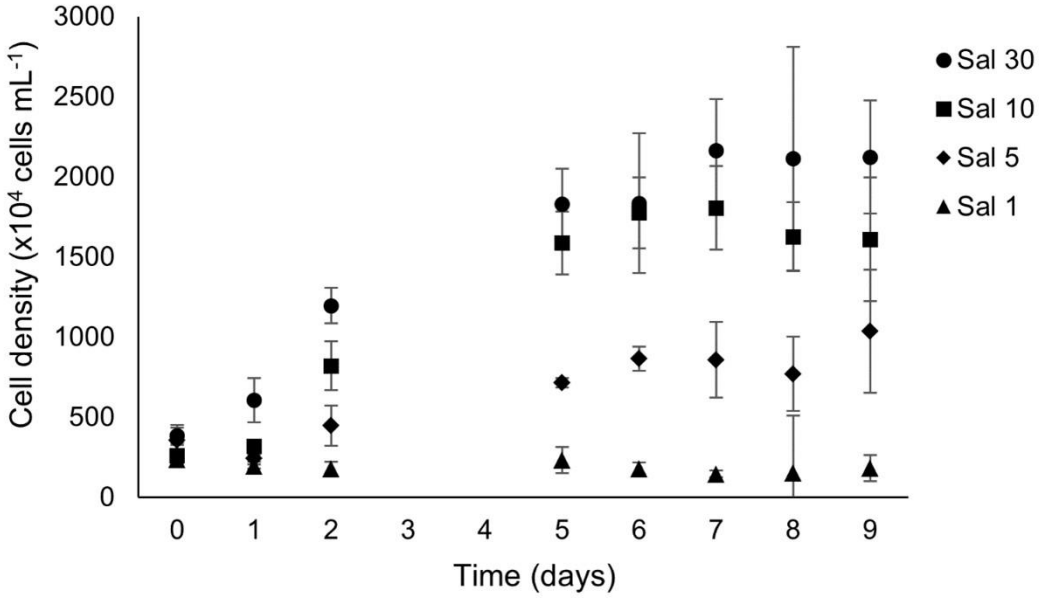

Figure 1. Cell growth curve of *Nannochloropsis oculata* culture at different salinities (30, 10, 5 and 1). Data points represent the means and error bars indicate the standard deviation (n = 3).

The same trend occurred regarding DB, which showed higher values in Sal 30 (0.29 ± 0.04 g L^{-1}) on the 8th day, followed by Sal 10 (0.16 ± 0.02 g L^{-1}), Sal 5 (0.14 ± 0.01 g L^{-1}), and Sal 1 (0.03 ± 0.00 g L^{-1}). Consequently, the productivity of Sal 30 (0.027 ± 0.006 g L^{-1} d^{-1}) was higher than the other treatments ($p < 0.05$), while the productivity of Sal 10 was similar to Sal 5 ($p = 0.885$) and higher than Sal 1 ($p = 0.006$) (Table 1).

However, even though Sal 30 showed higher values for biomass gain and productivity, the MCD and μ were not statistically different from Sal 10 ($p = 0.290$ and $p = 0.852$, respectively), both reaching MCD on the 7th day of cultivation. Cultures cultivated in Sal 5 showed reduced growth, with MCD, productivity, and μ values approximately 40%, 48%, and 52% lower, respectively, compared with the control. Clearly, in Sal 1, there was no growth of the microalga, resulting in a negative μ (-0.07 ± 0.02 d^{-1}) and productivity equivalent to zero (Table 1).

Table 1. Growth parameters of *Nannochloropsis oculata* cultivation at different salinities.

Salinity	MCD (x10^4 cells mL^{-1})	Biomass gain (g L^{-1})	μ (d^{-1})	Productivity (g L^{-1} d^{-1})
30	2,319.17 ± 482.29^a	0.23 ± 0.04^a	0.25 ± 0.03^{ab}	0.027 ± 0.006^a
10	1,901.67 ± 146.32^a	0.12 ± 0.02^b	0.28 ± 0.05^a	0.017 ± 0.003^b
5	945.83 ± 162.89^b	0.11 ± 0.01^b	0.13 ± 0.07^b	0.013 ± 0.002^b
1	195.00 ± 28.83^c	0.00 ± 0.01^c	-0.07 ± 0.02^c	0.000 ± 0.001^c

Data are shown as mean ± s.d. (n = 3). Different letters (a, b, and c) represent statistically significant differences among the different treatments ($p < 0.05$).

3.2 Evaluation of alternative sources of salt and nutrients

The cell growth curves in Figure 2 demonstrate that, until the last day of cultivation, the treatments showed an increase in their CD, except for CSF2, which reached a plateau on the 3rd day. The averages of ASAF and CSAF at the end of the cultivation reached the highest cell concentrations (8,200.00 x 10^4 ± 1,025.00 x 10^4 and 7,812.50 x 10^4 ± 137.50 x 10^4 cells mL^{-1}, respectively), followed by ASF2 (6,616.67 x 10^4 ± 1,169.46 x 10^4 cells mL^{-1}) and CSF2 (5,075.00 x 10^4 ± 800.00 x 10^4 cells mL^{-1}).

All treatments maintained a salinity of 20 psu throughout the cultivation. The growth parameters MCD, productivity, and μ showed no significant difference between the type of salt or nutrient source ($p > 0.05$). However, the final DB showed significant differences between nutrient sources, with AF treatments yielding higher results than those of F2 ($p = 0.023$) (Table 2). Regarding pH values, cultures with AS (AF: 8.59 ± 0.15; F2: 8.48 ± 0.12) were higher than those with CS (AF: 8.15 ± 0.15; F2: 8.33 ± 0.10) ($p = 0.005$).

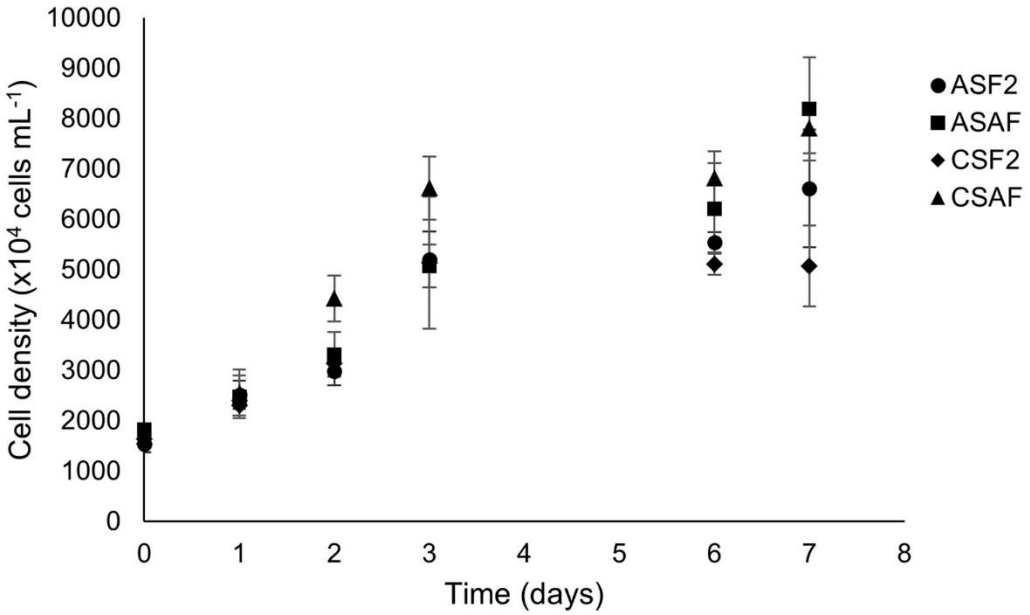

Figure 2. Cell growth curve of *Nannochloropsis oculata* cultivation in different culture media: ASF2 (artificial salt + F/2 medium), ASAF (artificial salt + agriculture fertilizer medium), CSF2 (common salt + F/2 medium), and CSAF (common salt + agriculture fertilizer medium). Data points represent the means and error bars indicate the standard deviation (n = 3).

Table 2. Growth parameters of *Nannochloropsis oculata* cultivation in different culture media: ASF2 (artificial salt + F/2 medium), ASAF (artificial salt + agriculture fertilizer medium), CSF2 (common salt + F/2 medium), and CSAF (common salt + agriculture fertilizer medium).

Treatment	MCD ($x10^4$ cells mL^{-1})	Final DB ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ d^{-1}$)	μ (d^{-1})
ASF2	$6{,}616.67 \pm 1432.29^{Aa}$	0.51 ± 0.04^{Aa}	0.06 ± 0.01^{Aa}	0.21 ± 0.04^{Aa}
ASAF	$7{,}425.00 \pm 1688.93^{Aa}$	0.62 ± 0.02^{Ab}	0.07 ± 0.00^{Aa}	0.21 ± 0.03^{Aa}
CSF2	$5{,}366.67 \pm 488.83^{Aa}$	0.41 ± 0.08^{Aa}	0.04 ± 0.01^{Aa}	0.19 ± 0.02^{Aa}
CSAF	$7{,}291.67 \pm 912.53^{Aa}$	0.53 ± 0.12^{Ab}	0.06 ± 0.01^{Aa}	0.22 ± 0.01^{Aa}

Data are shown as mean ± s.d. (n = 3). Different letters represent significant differences between the factors of the treatments, with uppercase letters (A) referring to the types of salts (AS, CS), and lowercase letters (a and b) referring to the types of nutrients (F2 and AF).

In Table 3 it is possible to verify if there is a significant effect of the isolated factors or their interaction on the response variables based on the p-value. A significant effect was observed only for the nutrient medium on the final biomass and

for the type of salt on pH, with a variation rate of 62.30% and 78.84% for their respective factors. The other variables were not influenced by the type of salt or nutrient source.

Table 3. Factorial ANOVA of the effect of salt type and nutrient medium on growth parameters and pH of *Nannochloropsis oculata*. Significant differences are represented by the p-value, and the influence of each factor on the responses is indicated by the percentage of variation.

Factors	Response variable					
	MCD	**Productivity**	**μ**	**Final biomass**	**Biomass gain**	**pH**
Salt	9.25% p=0.657	61.83% p=0.060	3.03% p=0.576	37.39% p=0.061	40.00% p=0.098	78.84% p=0.004
Medium	87.54% p=0.313	37.41% p=0.128	37.41% p=0.118	62.30% p=0.023	57.99% p=0.054	1.00% p=0.668
Salt*Medium	3.21% p=0.313	0.76% p=0.814	0.76% p=0.697	0.31% p=0.848	2.02% p=0.686	20.16% p=0.080

Regarding lipids content, the cultivation of ASAF showed 17.8 ± 0.2% (w/w) of total lipids, while CSAF obtained a ratio of 12.4 ± 0.2%. However, the fatty acid profile showed a higher ($p < 0.001$) relative quantity of EPA in CSAF than that obtained by ASAF. Significant differences were also observed in the production of hexadecanoic acid ($p = 0.046$) and 9-hexadecenoic acid ($p = 0.012$), with ASAF showing higher quantities (Table 4).

Table 4. Fatty acid profile of *Nannochloropsis oculata* cultivated in the culture media ASAF and CSAF.

Fatty acid	Symbology	Relative quantity (%)	
		ASAF	**CSAF**
Tetradecanoic acid	C14:0	6.70 ± 0.33[a]	6.05 ± 0.65[a]
Hexadecanoic acid	C16:0	36.72 ± 0.97[a]	30.41 ± 2.98[b]
9-Hexadecenoic acid	C16:1 ω-7	25.34 ± 0.39[a]	18.30 ± 2.24[b]
Octadecanoic acid	C18:0	0.87 ± 0.63[a]	0.85 ± 0.66[a]
9-Octadecenoic acid	C18:1 ω-9	3.76 ± 0.86[a]	8.73 ± 1.81[a]
9,12-Octadecadienoic acid	C18:2 ω-6	6.15 ± 0.27[a]	6.56 ± 0.08[a]
5,8,11,14-Eicosatetraenoic acid	C20:4 ω-6	0.71 ± 0.52[a]	1.98 ± 1.93[a]
5,8,11,14,17-Eicosapentaenoic acid	C20:5 ω-3	20.05 ± 0.36[a]	31.04 ± 0.23[b]

Different letters (a and b) represent a significant difference between the different culture media ($p < 0.05$).

3.3 Validation of the cultivation medium on a pilot scale

The cultivations carried out in FVAs maintained a salinity of 20 psu and an average pH of 8.4 ± 0.62. They were initiated with an average initial CD of 2,443.33 x 10^4 ± 109.11 x 10^4 cells mL^{-1}, and the batch cultivation lasted an average of 9 days, after which harvests and dilutions began. These harvests occurred every 6-8 days, and μ was calculated during these intervals, resulting in 0.12 ± 0.03 d^{-1}. The cultures were maintained for an average of 23 days, and the MCD achieved was 5,031.67 x 10^4 ± 429.74 x 10^4 cells mL^{-1}. The average of DB and CD obtained in the harvests were 0.34 ± 0.01 g L^{-1} and 4,350.00 x 10^4 ± 849.79 x 10^4 cells mL^{-1}, respectively.

4. Discussion

Salinity is one of the crucial factors in the cultivation of marine microalgae, and there are numerous studies evaluating its effects on the growth and biochemical composition of these microorganisms (Gu *et al.,* 2012; Ishika *et al.,* 2018; Pugkaew *et al.,* 2019). Changes in salinity induce osmotic stress on organisms, requiring them to undergo physiological adjustments to the new environment (Guo *et al.,* 2019).

Most studies on this topic assess the effect of high salinities on the growth of *Nannochloropsis*. However, in the present experiment, the aim was to understand the impact of lower-than-usual salt concentrations, considering that artificial seawater contributes to increased cultivation costs.

No difference in the growth of *N. oculata* was observed between salinities of 30 and 10 psu, except for the dry biomass productivity. Similar conclusions were drawn in studies conducted with *Nannochloropsis* sp., where reducing NaCl concentration from 27 to 13/13.5 g L^{-1} did not negatively impact cultivation growth (Martínez-Roldán *et al.,* 2014) and even increased cell density after 7 days of cultivation (Pal *et al.,* 2011). The species *N. salina* also exhibited higher maximum cell density at lower salinity (22 psu) compared to the traditionally used 34 psu (Bartley *et al.,* 2013).

Cultures at salinities of 5 and 1 psu exhibited a significant reduction in growth. In this study, the microalga *N. oculata* had its growth rate halved at a salinity of 5 psu; however, it was not completely inhibited, as observed at a salinity of 1 psu. This characteristic is species-specific. For instance, according to Bartley *et al.* (2013), *N. salina* does not grow at salinities below 8 psu. In a study conducted by Zulkifli *et al.* (2018), *N. oculata* was able to grow in a culture medium with zero salinity comparable to cultures with saline water, but only up to the 8th day. On the other hand, the strain *N. oceania* CCALA 804 demonstrates efficient growth in a medium with zero salinity, indicating tolerance to drastic osmotic reductions (Pal *et al.,* 2013; Solovchenko *et al.,* 2014).

Considering the similarity between the cultures of the treatments with salinities of 30 and 10 psu, with a reduction only in productivity (0.027 vs. 0.017 g L^{-1} d^{-1}, respectively), an intermediate salinity (20 psu) was chosen for the subsequent experiments.

In the cultivation of marine microalgae, seawater is commonly used as the base of the culture medium by researchers and producers. However, some

factors make its use impractical, such as variations in water quality due to tides, pollution, and climate, and especially the geographic and economic limitations for locations far from the sea (Lelekov *et al.,* 2016; Venteris *et al.,* 2013). It should be considered that coastal land is of high value and subject to greater climatic fluctuations. Additionally, seawater intake requires the installation and maintenance of equipment on the coast or offshore. An assessment conducted by Venteris *et al.* (2013) in the United States concluded that the use of saline waters is a costly alternative compared to freshwater. These disadvantages and complications make artificial salinization an attractive option.

For this purpose, there are several artificial sea salts in the market that fulfill the function of simulating seawater, providing salinity, trace elements, and buffers that contribute to water quality. However, these products have a high cost, and consequently, their use becomes impractical in large-scale microalgae production. In this context, after reducing the salt concentration in the culture medium of *N. oculata*, the substitution of artificial sea salt with common table salt was evaluated.

The achieved values of maximum cell density, specific growth rate, final biomass, and biomass gain showed no significant difference between the types of salt. Therefore, replacing artificial sea salt with common table salt is not detrimental to the cultivation of *N. oculata*, maintaining the same levels of cell growth. The only parameter that showed a significant difference was pH, which likely remained higher in artificial sea salt due to the presence of carbonate salts. Nevertheless, it did not influence growth. Therefore, common salt becomes a viable alternative to seawater intake and, especially, a more economical option compared to artificial sea salt in massive production. While common salt costs around US$ 0.16/kg, sea salt is priced at US$ 2.43/kg, 15 times higher.

The use of agricultural fertilizer as a nutrient source is a strategy for reducing the costs of the culture medium in large-scale microalgae production, including *Nannochloropsis* species (Camacho-Rodríguez *et al.,* 2013; Liu and Bangert, 2015; Neto *et al.,* 2018). In the present study, only the nutrient source (medium) affected the final biomass, with the highest concentrations obtained with agricultural fertilizer (AF). The maximum cell density averages for AF were also higher than those achieved by F2, although not showing a significant difference. Therefore, the application of agricultural fertilizer is advantageous compared to the standard F2 medium. It is also worth noting that the interaction between salt and nutrient medium showed no effect on any microalgal growth parameter.

Regarding the production of lipids and fatty acids, the culture medium with sea salt showed a higher lipid content but presented a lower relative quantity of EPA compared to the medium with common salt. From the ASAF to the CSAF treatment, the fatty acid profile showed a reduction of 6.31% and 7.04% in the relative quantity of 16:0 and 16:1 fatty acids, respectively, while an 11% increase in EPA (C20:5) was observed. The sea salt applied in this study contains trace elements that possibly enhance lipid accumulation by *Nannochloropsis oculata*. It has been demonstrated that different levels of iron, zinc, manganese, and molybdenum in the culture medium significantly interfere with lipid storage in

some species of microalgae, but their requirements are species-specific (Ghafari *et al.,* 2018). In contrast, the depletion of calcium and magnesium has been shown to increase lipid content in other species such as *Chlorella vulgaris* and *Scenedesmus obliquus* (Gorain *et al.,* 2013).

Similar to the total lipids, micronutrients influence the fatty acid profile. Savvidou *et al.* (2020) observed that the depletion of iron and manganese decreased lipid content but increased the levels of polyunsaturated fatty acids in *Nannochloropsis oceanica*, increasing the amount of EPA (C20:5) by 3.63% and 4.91%, while decreasing some saturated fatty acids. These results align with the findings of the present study. Therefore, it is essential to identify the micronutrients that favor the production of desired lipids and fatty acids by *Nannochloropsis oculata* to subsequently add them strategically to the medium with common salt.

When validating the cultivation medium in FVBs, the obtained data were lower than those presented earlier on a smaller scale. It is noticeable that there was a reduction in both the specific growth rate and the achieved dry biomass productivity. This change was expected since the configuration of the 2 L photobioreactor differs from the 100 L flat vertical bags. Changes in shape, aeration, and luminosity, among other aspects, influence cultivation productivity. According to (Borowitzka and Vonshak, 2017), scaling up microalgae production introduces important hydrodynamic changes, especially regarding the homogeneity of the culture, which is more challenging to achieve on a larger scale. Thus, light and nutrients are not uniformly distributed to all cells, leading to a reduction in productivity.

Despite the cultivation reaching lower growth in this stage of the study, other studies with the same genus show similar and even lower results than those found in the present experiment. When using inorganic fertilizers, *N. gaditana* was able to achieve a higher biomass concentration (0.4 g L^{-1}), but with a specific growth rate equal to 0.15 d^{-1} on the 6th day of cultivation (Riveros *et al.,* 2018), a value close to that found in the present work. Neto *et al.* (2018) obtained lower biomass concentrations of *N. oculata* using fertilizers, with a range from 0.12 to 0.20 g L^{-1}. In contrast, the optimization of the culture medium by the response surface methodology led to the production of up to 0.58 g L^{-1} of *N. oculata* UTEX 2164 in 9 days (Mehra and Jutur 2022), a higher value than that found in the present study. However, it is important to note that different scales and nutrient sources make it difficult to compare results.

Given the potential demonstrated by the CSAF medium for *N. oculata* production, some adjustments are necessary for larger-scale cultivation to achieve productivities close to those observed on a smaller scale. Therefore, more in-depth studies on agitation mode, luminosity (width of the flattened bag), cultivation operation, and the addition of micronutrients should be conducted.

5. Conclusions

In the present study, it was observed that the marine microalga *N. oculata* can grow in a cultivation medium with a salinity of 20 psu, which is lower than the typically applied salinity. Additionally, it was possible to replace artificial sea salt and the standard nutrient

medium with common salt and agricultural fertilizer without negatively impacting its growth. Thus, it was possible to develop a medium requiring less and cheaper resources in relation to the standard one, making the production of *N. oculata* more economically viable. It is worth noting that studies on the addition of micronutrients should be conducted later to ensure a good lipid composition of the microalga. Adjustments in the configuration of the flat vertical bags photobioreactor of 100 L are also necessary to achieve growth parameters closer to those obtained on a small scale.

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