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Growth Kinetic and Biodiesel Lipid Extraction of *Nannochloropsis oculata* Microalgae in a Photobioreactor under Varying Salinity Conditions

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ABSTRACT

Microalgae are microorganisms that in recent years have become protagonists in research because they are potential candidates for use in obtaining compounds of interest such as lipids, which may be transformed into bioenergy compounds like biodiesel. *Nannochloropsis oculata* is a marine microalga whose main characteristic is its high lipid content. In this work, the effect of salinity intensity on the growth of *N. oculata* was investigated in the photobioreactor batch cultures incubated with a salinity ranging from 20 to 40 ppt to analyze its growth profile and chlorophyll pigment to obtain dry biomass and biofuel produced as lipid extraction. The results indicated specific growth rate maximum values of 0.343 day^{-1}, obtained at 35 ppt salinity. Chlorophyll pigment increases with salt concentration between 25 and 35 ppt. The total lipid extracted increases considerably at moderate salinities condition (25–35) ppt, the maximum dry biomass harvest and productivity, accomplished after the microalgae cultivation salinity at 30 ppt was 0.623 g/l and 62.3 mg/l respectively. Same applies to the maximum total lipid content and productivity, which was 221 mg/l and 22.1 mg/l. day, respectively. These findings show that a variety of salinities support optimal biomass yield and biochemical composition in *N. oculata* cultivation. Salinity monitoring is crucial for successful cultivation. Furthermore, the advantages of *N. oculata* microalga, including its large cell size (facilitating harvest and grazer tolerance) and its salinity resilience, should be considered.

Keywords: biofuel, chlorophyll pigment, biomass, lipid production, specific growth.

INTRODUCTION

Today, climate change is a problem that has affected the environment, health, and even society at alarming levels. This phenomenon has evolved on a large scale, causing different associations and countries to be forced to take action and create plans to mitigate pollution (Kiehbadroudinezhad et al., 2024). Due to advances in engineering and technology, renewable energy has been an excellent alternative to counteract the negative effects that climate change has reflected in the world, with solar energy among the most prominent, wind and biomass energy, through the development of biofuels (Amjith and Bavanish, 2022). Biofuels resemble conventional fuels but are derived from renewable sources, resulting in significantly lower carbon emissions, which can even reach zero due to the biomass cycle (Srivastava et al., 2021). In addition, biofuels, are capable of partially or fully supplying demand for conventional fuels such as gasoline and diesel. Biodiesel, is a biofuel obtained from oilseed sources, and this is combined with conventional diesel in different proportions, ranging from B5 of 95% diesel, 5% biodiesel to B100 present 100% biodiesel, this will depend on the engine to which the mixture is submitted (Cihan, 2024). On the other hand, this biofuel, when obtained from oilseeds (rich in lipids), has a wide spectrum of sources that can be grown to obtain the oil. Currently, materials such as soy, palm, and Jatropha have been studied to obtain biodiesel. However, these have disadvantages that compromise resources such as

farmland and water for their growth, entering a social conflict. This is why alternative sources of lipids are sought, which do not compete directly with these resources; and among the most attractive sources are microalgae (Srivastava et al., 2021; Amjith and Bavanish, 2022).

In recent years the use of microalgae has now attracted the interest of research and various industries, including the food, pharmaceuticals, and cosmetics industries because it is considered a promising source for the development of various byproducts in sectors such as nutrition, cosmetics, and energy; this is due to their properties of high growth rates, accumulation of interest compounds (such as lipids that can be transformed into biofuels), and that large amounts of biomass can be obtained in relatively small areas; without compromising resources such as farmland and freshwater (Barot, 2022).

Algae are a vital and prolific resource, attracting scientific and industrial interest due to their rapid growth and production of diverse bioactive compounds. A key benefit of using these microorganisms is their diverse range of species, allowing for targeted studies based on specific needs (Javed et al., 2022; Chanana et al., 2023). For a bioenergetic approach, especially for cultivating biofuels like biodiesel in photobioreactors under salinity stress, microalgae species with high lipid accumulation potential are preferred. These include genera like *Dunaliella, Isochrysis, Scenedesmus, Tetraselmis, and Chlorella*, among others (Duan et al., 2012; Francavilla et al., 2015). Among the different types of microalgae, the genus *N. oculata* has been extensively studied for its potential applications in aquaculture due to its high content of essential fatty acids, pigments, and other valuable metabolites. Furthermore, these strains are presently between the most promising for the production of biodiesel (Krishnan et al., 2015).

Salinity plays a crucial role in lipid accumulation, as its initial maintenance followed by gradual increases can enhance lipid production (Yang et al., 2024). Salt in the medium encourages microalgae growth and nutrient absorption, while also influencing fatty acid metabolism to enhance lipid synthesis. An increase in Na+ levels due to salinity stress can enhance lipid accumulation in microalgae while also inducing oxidative stress. In addition, increasing salinity can enhance the palmitic and oleic acid compositions in microalgae. Moreover, during salinity stress, microalgae tend to produce more neutral lipids, which enhance cell membrane rigidity and help regulate mineral ions in the cells (Elloumi et al., 2020).

The increase in lipid production in microalgae at different salinity levels involves complex processes such as osmotic stress responses, metabolic shifts, changes in photosynthesis and growth, gene expression alterations, membrane remodelling, and ion homeostasis. The specific mechanisms and their contributions vary by microalgal species, salinity stress intensity, and exposure duration (Song et al., 2022). Understanding these factors is essential for optimizing lipid production in microalgae for biofuel and other commercial applications. *N. oculata*, can cultivate in a high range of salinities and temperatures, making it a versatile and resilient organism for aquaculture applications. The growth rate, biomass production, and pigment composition of *N. oculata* can be significantly influenced by environmental conditions like salinity, temperature, and light-intensity (Ammar et al., 2018). Salinity significantly influences the growth and metabolism, in addition to the productivity of marine algae. High salinity significantly restricts the microalgae growth of *Nannochloropsis* and *Chlorella* species (Martínez-Roldán et al., 2014; Pugkaew et al., 2019).

Altering lipid metabolism may enhance algal cell survival. Salinity tension reduces neutral lipid contented but increases polar lipids in *Nitzschia laevis*. Additionally, some marine species, such as *Navicula, Dunaliella*, and *Nannochloropsis*, exhibit increased total lipids and triacylglycerols under salinity stress (Martínez-Roldán et al., 2014). Algal cells employ various mechanisms to tolerate high salinity, often producing compounds like mannitol and glycerol to regulate osmotic balance. On the other hand, non-vacuolated, halotolerant marine microalgae maintain cytoplasmic Na^+ homeostasis under high salinity by limiting Na^+ influx and actively releasing it out across the plasma membrane (Banik and Dutta, 2023). *Nannochloropsis* species respond differently to salinity changes, depending on their species, growth stage, and environmental conditions (Ma et al., 2014). Nevertheless, the impact of salinity on the biochemical content of *Nannochloropsis*, specifically its chlorophyll and lipid levels, remains poorly understood. This is crucial, as these components are vital for its potential as a biodiesel feedstock.

The primary objective of the present study is to investigate the effects of salinity fluctuations on the growth, biomass production, chlorophyll pigment, and lipid content of *N. oculata* cultivation

investigated under photobioreactor (FBR) with monitoring and control of parameters, to analyze whether their biomass yields and lipid extracted are feasible for potential use in the biofuel industry and understanding how salinity variations impact these parameters. The outcomes from this research will deliver valuable insights into the optimal conditions for culturing *N. oculata* and enhancing its applications in aquaculture and other industries.

MATERIAL AND METHODS

Microalgae culture media

The marine microalgae *Nannochloropsis oculata* was used in this study and achieved from the Algae Research Supply (Carlsbad, USA). The inoculum of 5 ml of the microalgae strain was provided. The growth medium F/2 was used for nutrients media, medium consisted of filtered seawater and sterilized, with a nutrient solution whose final composition (per liter): 4.36 mg EDTA, 5 mg NaH_2PO_4 $2H_2O$, 75 mg $NaNO_3$, 3.15 mg $FeCl_3$ $6H_2O$, 0.0098 mg $CuSO_4$ $5H_2O$, 0.022 mg $ZnSO_4$, 0.01 mg $CoCl_2$ $6H_2O$, 0.180 mg $MnCl_2$ $4H_2O$, 30 mg Na_2SiO_3, 0.0063 mg Na_2MoO_4 $2H_2O$, 0.01 mg Vitamin B12, 0.2 mg Vitamin B1 and 0.1 mg Vitamin H (Chaisutyakorn et al., 2018). In the initial stage, *N. oculata* was cultured by adding 5 ml of inoculation in an Erlenmeyer flask 500 ml having 245 ml of F/2 nutrient media, inoculation lasting (10–15) days (Ammar et al., 2018). The microalgae that were used as inoculum were collected at its exponential growth stage through the technique of successive transfers (Ammar et al., 2018), where it grew from 500 mL flasks, to reach an operating volume. Next, the culture was carried out in 10 L bubble column photobioreactor FBR cultivation was maintained in a controlled laboratory environment under different salinity variation conditions (20, 25, 30, 35 and 40 g/L), a temperature (30 °C), initial pH 8, and the illumination of four fluorescent lump, for 18/6 h light to dark cycle intensity for 10 days. In addition, air was supplied to the continuous and constant cultivation (12 L/minute nominal flow) (Fig. 1).

Growth kinetics measurement

Daily measurements were made in triplicate over 10 days, corresponding to the exponential growth phase (determined in preliminary internal trials of the *N. oculata* microalgae); for this, developed cell density measurement methodologies; measuring cell density using a hemocytometer under a microscope. In addition, determination of spectrophotometric absorbance, measuring the absorbance of the suspended cells, using a UV/Vis spectrophotometer (LAMBDA 950 PerkinElmer), at two wavelengths, according to a spectrophotometric scan from 400 to 700 nm, where 685 nm peaks were chosen, being at the wavelengths was greater absorbance (Navarro-Peraza et al., 2017). Using the growth kinetics data, the specific growth rate (μ), Equation 1 was calculated, and the doubling time (Td) Equation 2, using the following equations (Ammar et al., 2018; Pugkaew et al., 2019).

$$\mu = \frac{\ln X - \ln X_o}{t} \quad (1)$$

$$T_d = \frac{\ln 2}{\mu} \quad (2)$$

where: μ – the specific rate of growth, X – the final concentration X_o – the initial concentration, T_d – time of duplication and t is the time elapsed in days.

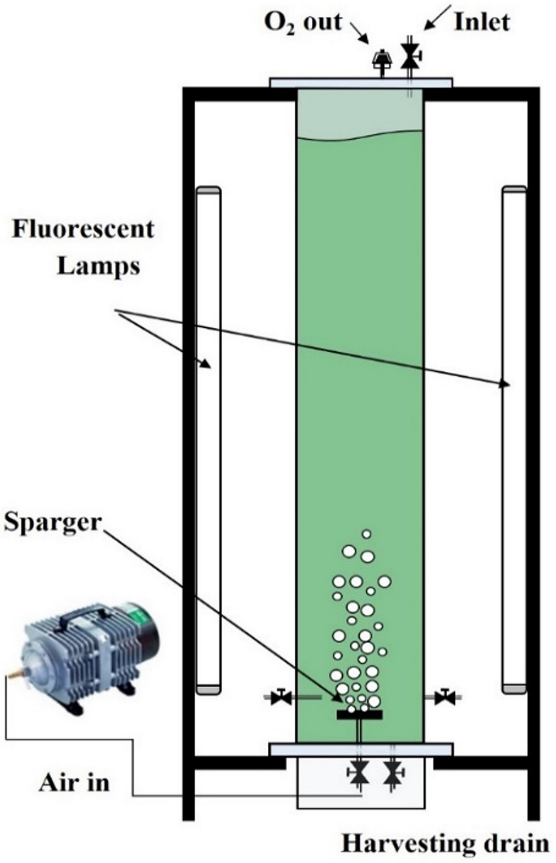

Figure 1. Schematic of FBR used in experimental wor

Chlorophyll content

The total Chlorophyll content was specified using spectrophotometric methods, and 10 ml of the culture suspension was centrifuged for 10 minutes at 5000 rpm, the supernatant was discarded, and the precipitate was suspended in 90% acetone and stored in the dark at 4 °C for 24 hours. Thereafter, the absorbance of the extract was measured using a spectrophotometer at 665 and 649 nm. Chlorophyll content (mg/l) was calculated using the formula (3) provided by Sabzi et al. (2018) and Wang et al. (2024).

$$\text{Chl.} = 6.1\ OD665 + 20.04\ OD649 \quad (3)$$

where: OD665 and OD649 – absorbances at wavelength 665 and 649 respectively

Microalga dry biomass

The microalga biomass was collected through a flocculation-sedimentation process. The pH of the cultivation was raised to 11 with NaOH (1N) and allowed to stand for a period of 24 h at 25 °C temperature. Subsequently, to recover the microalga paste, it was centrifuged at 5000 rpm × 5 min, and stored under -20 °C. Yields were calculated according to the total microalga paste obtained during the experiment (Ammar et al., 2018).

Lipid production

The lipid extraction from the biomass dried samples was carried out by mixing with methanol-chloroform solution (2:1 v/v) and extracted using a Soxhlet extractor for 6 hrs. The resulting solution was centrifuged for 10 minutes at 9000 rpm to extract and preserve the organic lipid-containing bottom layer (Egesa and Plucinski, 2024). According to Egesa and Plucinski (2024), the yield of lipid production LE is determined by the following Equation 4:

$$LE\ (\%) = \frac{LW}{DW} \times 100\% \quad (4)$$

where: *LE* – the total lipid extraction (%w/w), *LW* – the weight of lipid extracted (g/L) and *DW* – is the weight of dry biomass (g/L).

The productivity of biomass and lipid extraction of *N. oculata* microalgae were calculated by Equations 5 and 6. Finally, the flowchart scheme shown in Figure 2 illustrates the steps involved in *N. oculata* cultivation under different salinity condition procedures.

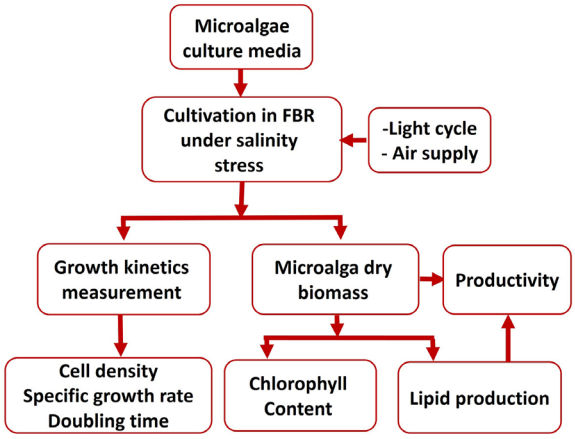

Figure 2. Flowchart illustrating the steps involved in *N. oculata* cultivation under different salinity conditions

$$BP = \frac{DW}{T} \times 1000 \quad (5)$$

$$LP = \frac{LW}{T} \times 1000 \quad (6)$$

where: *BP* – the biomass productivity (mg/L. day), *LP* – the total lipid extraction productivity (mg/L.day), and *T* – the time of cultivation (day).

RESULTS AND DISCUSSION

Effect of salinity on *N. oculata* growth

Cell concentration and optical density were regularly employed to observe the *N. oculata* cultures' growth. It should be emphasized that when cell-size and pigment content vary within the salinity stress. Consequently, the optical density and the dry biomass concentration were used to determine growth performance in the present study. Measurements were made in triplicate with each of the cell concentration determination methodologies. The effect of salinity intensity on the growth of marine microalga *N. oculata* was investigated in the PBR batch cultures incubated with a salinity ranging from 20 to 40 ppt. Cell density concentration was determined by sampling from the cultivation system daily. Figure 3a describes the growth curves of the microalgae under different salinity intensities over 10 days of cultivation. The results show had little effect on the *N. oculata* growth, where maximum biomass concentration was obtained while cultured at 30 ppt compared with other different salinity,

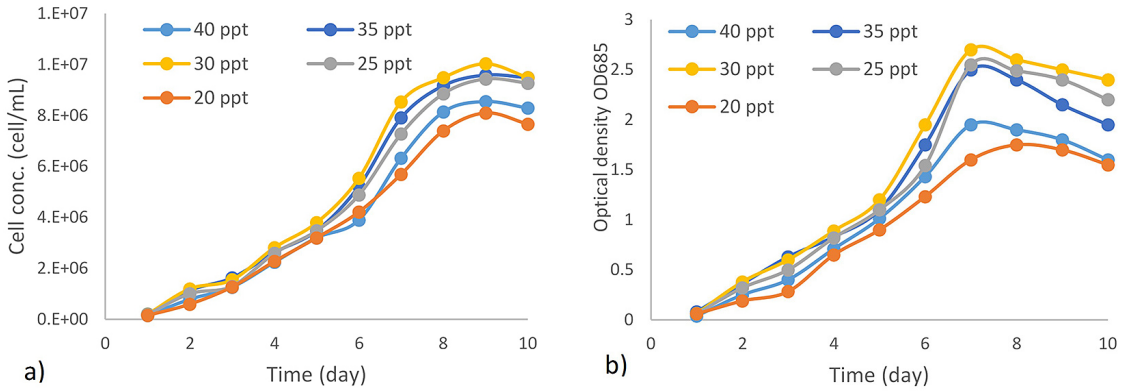

Figure 3. Salinity changes affect a growth profile, (a) cell concentration count cell/mL (b) absorbance OD685

the initial cell concentration of 2×10^5 cells/ mL, increasing to 1×10^7 cells/mL by nine days of cultivation. On the other hand, in the spectrophotometric method, the maximum absorbance was presented on day 7, with 2.7 OD, for the wavelength of 685 nm as shown in Figure 3b. Likewise, Pugkaew et al. [2019], obtained the same behavior as in this research for the marine microalgae *Tetraselmis suecica*, which reached a cell density of less than 1 OD at 680 for maximum growth at salinity intensity (20–30) ppt at six days of cultivation where the medium treatment F/2 to 25 °C. A typical microalgae conduct was observed, with the maximum specific growth rate (μ) values of 0.343 per day, and a doubling time (*Td*) of 2.2 days obtained at 35 ppt salinity (Figure 4a). These results can be compared with the results obtained by different authors, Navarro-Peraza et al., [2017] obtained a μ of 0.165 d^{-1}, for *N. ocluta*. A 35 °C in half F/2 media, a value very similar to that obtained in this work. Moreover, the study by Peng et al., [2020] obtained a μ around 0.30 d^{-1}. The study reported by Kawaroe et al., [2015], found a μ within the range of 0.19–0.30 d^{-1}, and a Td of 2.27–3.51 day, however, it is important to note that these microalgae were not subjected to salinity stress, so these efficiency parameters are not affected, because, by increasing salinity, there may be a decrease in cell concentration (Navarro-Peraza et al., 2017; Sabzi et al., 2018).

Algal growth at 20 and 40 ppt salinity showed slightly lower optical density throughout the cultivation period. However, algae grown in the 25–35 ppt salinity range recovered and displayed a similar specific growth rate during the log-phase compared to the last salinity concentrations. Conversely, the 20 ppt salinity led to poor growth throughout the experiment. Under these conditions, the cultivation culture entered the stationary-phase about day 7, exhibiting slightly varying peak optical densities (OD_{685}). On the other hand, the final dry biomass harvests remained largely unaffected by the salinity stress levels, excepting for the lowermost salinity (20 ppt) and the highest (40 ppt). At these extremes, the final biomass decreased significantly, by approximately 25% and 58% for 20 and 40 ppt respectively compared to the maximum cell concentration observed at 30 ppt salinity. On the other hand, dry biomass harvested decreased under both high and low salinity conditions (Fig. 4b).

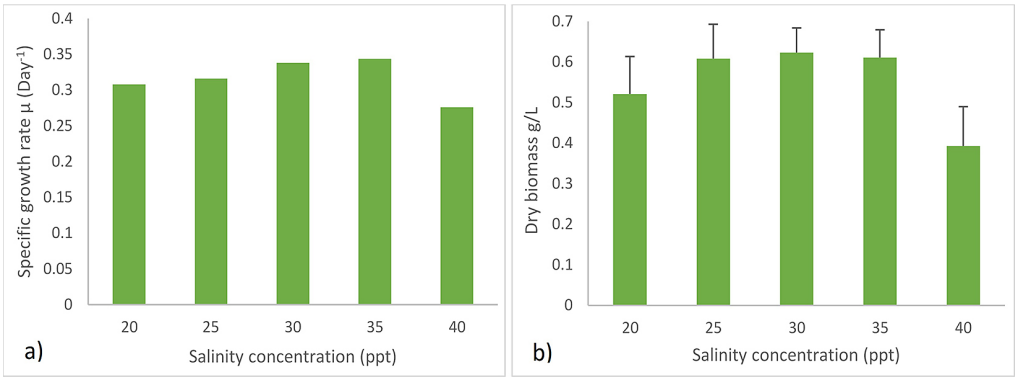

Figure 4. Effect of salinity fluctuations of *N. oculata*, (a) extreme specific growth rate, (b) microalgae dry biomass

Effect of salinity on N. oculata photosynthesis and lipid production

Chlorophyll pigment increases with salt concentration between 25 and 35 ppt, boosting photosynthesis. However, salinity of 40 ppt negatively impacts microalgae cells, causing stress and hindering photosynthesis. Conversely, low salt concentrations restrict nutrient availability, hindering growth, the same trend can noticed for chlorophyll pigment were varies from 3.56% to 4.18% of dry-weight *N. oculata* biomass. While the total chlorophyll content of the microalgae varied only slightly with salinity (Fig. 5a). Suboptimal photosynthesis rates were not due to cell chlorosis. However, the most striking effect was the increased chlorophyll levels at 30–35 ppt salinity, suggesting larger light-harvesting complexes associated with the photosystems. Salinity plays a crucial role in lipid accumulation, as its initial maintenance followed by gradual increases can enhance lipid production. Total lipid extracted varied from 23 to 34.86 % of dry-weight *N. oculata* biomass. The lowest lipid extracted was found in the highest salinity conditions (40 ppt). The total lipid extracted increases considerably under moderate salinities conditions (25–35) ppt (Fig. 5b).

To assess the potential of *N. oculata* microalgae as a biofuel feedstock, yield production of the cultures under different salinity cultivation was determined. The maximum biomass harvest, accomplished when the culture were cultivated as 30 ppt salinity was 0.623 g/L. The same applies to the highest total lipid yield content, which was 210 mg/L. The data on growth rate specified that the *N. oculata* microalgae have wide-ranging salinity tolerance. A comparable salinity tolerance has been described previously for *N. oculata* (Wei and Huang, 2017; Chaisutyakorn et al., 2018; Ishika et al., 2018).

Effect of salinity on N. oculata biomass and lipid productivity

The productivity of *N. oculata* cultures under various salinity levels was evaluated to assess its potential as a biodiesel feedstock (Table 1). Maximum biomass and lipid productivity observed at 30 ppt salinity, reached 62.3 mg/L. day and 22.1 mg/L. day, respectively. Concerning the yields obtained at work, biomass productivity was obtained at the end of each batch (10 days) at 62 mg/L. day. This can be compared to the report by Şirin and Sillanpää, [2015] obtained productivity of biomass was 0.49 g/L. day, for *N. oculata*, higher than that found in this investigation. In addition, conversely, the study of Gouveia and Oliveira, [2009] obtained productivity for the *N. oculata*, which achieved much lower biomass productivity, with results of 0.003 g/L day. The high cost of producing microalgae biodiesel is a significant obstacle to its commercialization, however, this cost

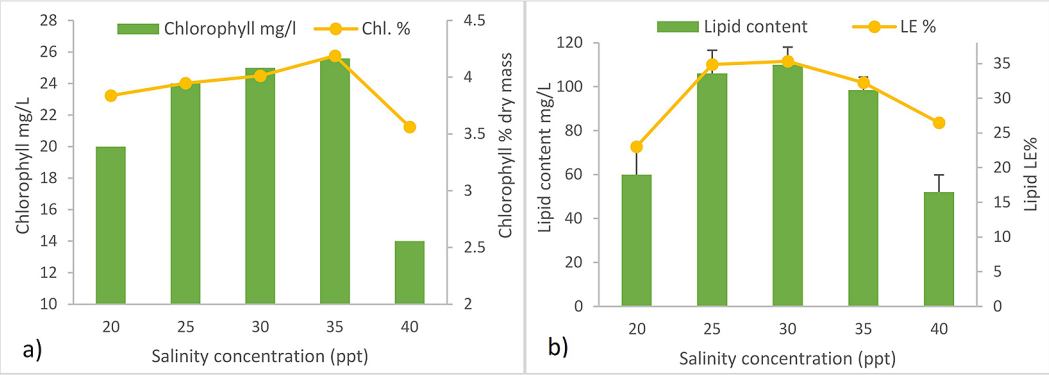

Figure 5. Effect of salinity fluctuations of *N. oculata*, (a) Chlorophyll pigment and percentage per dry biomass, (b) lipid extracted content and percentage per dry biomass

Table 1. Productivity of cell biomass and total lipid, of *N. oculata* cultured at different salinity

Productivity	Salinity concentration ppt				
	20	25	30	35	40
Biomass BP mg/L. day	52.1	60.8	62.3	61.1	39.3
Lipid LP mg/L. day	12.3	21.2	22.1	19.7	10.4

may be reduced with increased cultural productivity. Optimizing both biomass production and biochemical content within that biomass will enhance productivity (Griffiths and Harrison, 2009). The biochemical composition in terms of total lipid would more correctly indicate the quantity of fatty acid that might be produced from the microalgae dry biomass, which would be useful for comparing the biodiesel feedstock potential. Direct comparisons of productivity between this and other studies on batch cultures should be viewed with caution due to variations in growth rates and the impact of the cultivation period. However, the maximum biomass harvest and productivity of *N. oculata* in the present study were compared to those reported previously by Sabzi et al., [2018]. Optimizing bioenergy potential requires a balance between biomass and lipid productivity, ensuring their synergy. Salinity monitoring and control are crucial for successful cultivation, as variations can negatively impact biomass and lipid productivity. Before realistic comparisons can be made, outdoor and large-scale cultivation of these microalga cultures must be established and evaluated. Furthermore, the advantages of *N. oculata* microalga, including its large cell size (facilitating harvest and grazer tolerance) and its salinity resilience, should be considered.The study of salinity's impact on *Nannochloropsis oculata* in a photobioreactor faces several limitations that may influence result interpretation, including experimental design constraints like the tested salinity range, study duration, and laboratory conditions. Factors like photobioreactor design, measurement methods, strain-specific responses, and nutrient interactions must be considered to avoid confounding results. Addressing these limitations in future studies could yield more comprehensive findings, especially for industrial microalgal cultivation.

CONCLUSIONS

The findings of this research on *N. oculata* culture under salinity effect in a controlled FBR mirrored previous observations. Cell concentration and biomass yields were even higher than those reported in earlier studies. This may indicate that the microalga, in addition to being a candidate of interest for application in bioenergy sectors (specifically for the creation of biofuels such as biodiesel), under a salinity variation of 20–40 ppt, opens a scenario for this species to be able to scale it to crops abroad, thus being a step to optimize resources and that this technology has better scope and greater technical-economic feasibility. Based on these results, it can be concluded that *N. oculata* can tolerate high salinity and can grow and produce lipids over a wide range of salinity levels.

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