

Research article

Influence of nitrogen, phosphorus and sulfur concentration on bioplastic and FAMES production in *Scenedesmus* sp.

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ABSTRACT

Microalgae can fix CO₂ into valuable molecules; these products are expected to sustainably replace petroleum-derived plastics and oils. The biorefinery strategies are implemented to simultaneously generate high-value products by extracting a fraction of the microalgae. This research evaluated the growth kinetics of *Scenedesmus* sp. under different macronutrients such as nitrogen, phosphorus, and sulfur (low, medium and high concentration). The correlation of cell growth behavior with biochemical characterization was determined with emphasis on the potential of the microalgae to use as raw material for bioplastic production. The results showed.

that a high sulfur concentration has influenced and increased the cell growing reaching 1.42 g L⁻¹ at 15th cultivation day compared with the nitrogen and phosphorus variation. The content of carbohydrates in the biomass was enhanced under limitation of nitrogen and phosphorus. The same statement was proved for the PHA accumulation reaching 9.5 ± 0.83 % and 6.4 ± 0.32 % DW (dry weight) respectively. Deprivation of nitrogen, phosphorus and sulfur improved the microalgae culture for lipids production. In the present study the higher lipid content (273 mg g⁻¹) was observed under phosphorus limitation. The monitoring of FAMES considering former condition in the medium revealed high content of palmitic [30 ± 1.5 %_{TFA}], stearic (C18:0) [1.8 ± 0.11 %_{TFA}], and oleic acid. Therefore, these results lead to observing the impacts of macronutrients (N, P, S) on the microalgae growth and determined the better conditions for the accumulation of macromolecules that can be extracted under biorefinery concepts valorizing the biomass for bioplastic production and other biomolecules for future industrial applications.

1. Introduction

The essential use of plastics in contemporary life is due to their versatility, economy, and durability. The United Nations Environment Assembly warns that the current global production of plastics exceeds 430 million tons, with 40 % lasting less than a month and only a third being recyclable, generating large amounts of waste (UN, 2023). These plastic wastes threaten biodiversity, water

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quality, and human health due to their resistance to biodegradation [1]. Conventional plastics are made of petrochemical polymers, encouraging dependence on non-renewable resources, and contributing to elevated greenhouse gas emissions [2]. Therefore, replacing petroleum plastics with bioplastics from renewable sources (biological origin) is a sustainable and green alternative. Nevertheless, the use of agricultural resources as raw materials for bioplastics presents a challenge with the demands of the food industry [3]. A promising alternative is microalgae biomass, which does not need cropland for its growth and requires fewer nutrients than terrestrial plants [4]. There are multiple techniques to generate biodegradable bioplastics from microalgae, including blending microalgal biomass with synthetic polymers or bioplastics, using biomass to recover or polymerize PHA, using microalgae cellulose as reinforcement in matrixes plastic, and the fermentation of microalgal biomass to produce monomers such as PLA [5,6].

Microalgae are photosynthetic microorganisms that generally inhabit aquatic ecosystems, capture CO₂ and are used in different biotechnological areas. They can grow through different metabolic pathways (photoautotrophic, photoheterotrophic, or mixotrophic), making them a valuable research topic [7]. Microalgae, under the concept of circular economy, can use wastewater and CO₂ from exhaust gases as a source of nutrients while accumulating macromolecules, such as proteins that range from 6 % to 52 %, carbohydrates from 5 to 23 %, and lipids between 7 and 23 % of its total composition [8–11]. Moreover, micro algal biomass can contain industrial and commercial interest bioactive molecules including minerals, pigments, vitamins, fatty acids, antioxidants, and PHA (biopolymer) [12]; [93]. Regarding the PHA content, some microalgae strains, such as *Scenedesmus* sp., can accumulate >8.6 % DW (dry weight) under normal growth conditions. However, the PHA accumulation can rise 3.4-fold (until 29.9 % DW) when medium conditions during cultivation are deficient [93]. Nitrogen limitation in the culture media is correlated to the storage of carbonaceous compounds, such as PHAs and FAMES [13]. Furthermore, when applying various cultivation techniques, such as mixotrophic cultivation using an organic carbon source, certain microalgae can accumulate up to 13.15 % by weight [14], which allows the utilization of wastewater as an alternative media.

Some microalgae strains have been identified by synthesizing and storing intracellular PHA as a secondary metabolite under the limitation of nutrients [15]. However, there are challenges regarding cell productivity and the expense of the bioprocess. The relatively low rates of PHA accumulation in microalgal biomass could be overcome by exploring potential routes for fractionation and the valorization of complete microalgal biomass through biorefinery processes [16,17]. An example is the fractionation of lipids from microalgal biomass and the conversion into biodiesel through transesterification [18].

Microalgae biomass is a potent source of omega-6 and omega-3, accumulating up to over 50 % of DW under the influence of abiotic factors [19]. These fatty acids have multiple double bonds in their structure and are liquid at room temperature. Also, FAMES are obtained employing the lipid transesterification technique, which converts lipids into FAME. FAMES are less polar than fatty acids. Obtaining FAME from microalgae lipids is an excellent matrix for the manufacture of biodiesel, bioplastic, and bio-lubricants [20–22].

Following lipid extraction, the biomass defatted can undergo processing; in this way carbohydrates could be used in the food and fuel industries. Furthermore, the protein fraction can serve as an ingredient or additive for feed in livestock, aquaculture, and poultry farming [23] and as biofertilizers [24]. Also, the fatty acids and pigments found in microalgae can function as food additives, nutraceuticals, and pharmaceuticals [25,26]. In this sense, the objective is to use the all-biomass fractions.

Considering that the current studies on nutrient deprivation to enhance specific microalgae metabolites production are focused on the variation of nitrogen and phosphorus. The aim of the present study is to validate the behavior of *Scenedesmus* sp. under three different macronutrients conditions (nitrogen, phosphorus and adding the effect of sulfur) to demonstrate the multifactorial influence that macronutrients have on the same microalgae strain growth and their specific biomolecules synthesis such as proteins, carbohydrates and lipids. Furthermore, the monitoring accumulation of PHAs and FAMES enhance the developing sustainable processes to produce bioplastics and biofuels simultaneously, especially varying the concentrations of macronutrients in the culture media, and due to that there is a lack of reports on PHAs and FAMES production under these conditions, particularly regarding sulfur concentration, which has a relevant role in metabolism and photosynthesis.

2. Materials and methods

2.1. Equipment and reagents

A microplate spectrophotometer (BGM Labtech, Fluorstar omega, 415–0470) was used to measure the absorbance. Reagent for growth medium preparation, biomass biochemical composition, FAMES and PHA quantification were acquired from Sigma-Aldrich. The Lowry Protein Assay Kit was acquired from Thermo Fisher Scientific.

2.2. Seed culture conditions

The *Scenedesmus* sp. strain (UTEX, Austin, TX, USA) was used in this study. The growth medium used to carry all the experiments was BG11. The culture medium BG11 was prepared as it was mentioned in Ref. [27]. In each culture, it was established continuous aeration at 1.5 L m⁻² min⁻¹ with 0.04 % CO₂ (atmospheric air) enrichment was achieved by an OPTIMA 4.5-Watt pump, coupled with a 0.20 µm air filter. Aeration in each culture was carried out using a sterilized silicone tubing, connected to an air filter and pump. Constant illumination (100 µmol photons m⁻² s⁻¹) and temperature of 21 ± 1 °C further optimize culture conditions for microalgae growth.

2.3. Growth behavior of *Scenedesmus* sp. under different macronutrient concentrations

For carried out each experiment, 100 mL of pre-washed *Scenedesmus* sp. seed culture (1 g L⁻¹) was established in a culture glass

Table 1

Factorial design used to determine the macronutrients concentration (N, P, S) effect on cell growth and general characterization of biomass in *Scenedesmus* sp. cultures.

RunEst	Run	Blocks	Concentration	Nutrient
11	1	2	Low	S
17	2	2	High	S
18	3	2	High	P
12	4	2	Low	P
15	5	2	Medium	P
16	6	2	High	N
13	7	2	Medium	N
10	8	2	Low	N
14	9	2	Medium	S
24	10	3	Medium	P
20	11	3	Low	S
19	12	3	Low	N
22	13	3	Medium	N
25	14	3	High	N
26	15	3	High	S
23	16	3	Medium	S
21	17	3	Low	P
27	18	3	High	P
8	19	1	High	S
6	20	1	Medium	P
3	21	1	Low	P
4	22	1	Medium	N
1	23	1	Low	N
5	24	1	Medium	S
7	25	1	High	N
2	26	1	Low	S
9	27	1	High	P

Macronutrients: Nitrogen as NaNO_3 (Low [N-]: 500 mg L^{-1} , Medium [Control]: 1500 mg L^{-1} , High [N+]: 4000 mg L^{-1}). Phosphorus as K_2HPO_4 (Low [P-]: 20 mg L^{-1} , Medium: 40 mg L^{-1} , High [P+]: 100 mg L^{-1}). Sulfur as BG11 (Low [S-]: 30 mg L^{-1} , Medium: 75 mg L^{-1} , High [S+]: 200 mg L^{-1}). Other macronutrients in the culture medium were maintained at standard concentrations: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 36 mg L^{-1} , Citric Acid- H_2O 6 mg L^{-1} , $\text{C}_6\text{H}_5\text{FeNO}_7$ 6 mg L^{-1} , $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ 1 mg L^{-1} , Na_2CO_3 20 mg L^{-1} , H_3BO_3 2.86 mg L^{-1} , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.81 mg L^{-1} , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.22 mg L^{-1} , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.39 mg L^{-1} , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.079 mg L^{-1} and $\text{Co}(\text{zNO}_3)_2 \cdot 6\text{H}_2\text{O}$ 0.04 mg L^{-1} . Due to the capacities of the laboratory, the experiments were carried out in blocks. The temperature, aeration, and light conditions were maintained at the same in all blocks. Each block consists of 9 variations, with the conditions detailed previously. Each block containing 9 variations was established as a replicate of the experiment, thus being 3 blocks.

flask with 900 mL of BG11 medium. All experiments were maintained under the identical conditions detailed previously. For this study a factorial design was used. Biomass composition and cell growth of *Scenedesmus* sp. were determined under three different N, P, and S concentrations over 15 days. In all experiments only one macroelement was changed at the same time, the other two elements were maintained under standard conditions in the culture medium (Table 1). The control condition was established considering BG11 medium concentrations for all macroelements (NaNO_3 1.5 g L^{-1} , K_2HPO_4 40 mg L^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 75 mg L^{-1}).

Before carried out all the experiments, a correlation equation between DW (g L^{-1}) of microalgae biomass with absorbance at 750 nm has been developed as it was mentioned in Ref. [27]. This relationship is expressed in Equation (1). For cell growth monitoring, 1 mL of each culture was sampled each day to determine the biomass concentration curve by absorbance measurements.

$$\text{Scenedesmus sp. } \text{g L}^{-1} = 1.4954 (\text{Abs}_{750}) - 0.0584 \quad R^2 = 0.981 \quad (1)$$

2.4. Biochemical characterization of *Scenedesmus* sp. biomass grown under different macronutrients concentrations

All experiments were sampling on days 5, 10, and 15 by taking 15 mL of each culture. Every sample was stored at 4°C . To carried out the protein, carbohydrate, and lipids analysis, each sample was centrifuged at 7000 rpm for 20 min at 4°C . The supernatant was discarded, and the biomass concentrated was washed twice and resuspended it in deionized water.

2.5. Total protein measure of *Scenedesmus* sp. grown with different macronutrients concentrations

For determined the total protein content in microalgae cultures was used Modified Lowry Protein Assay Kit. Briefly, 1000 μL of Lowry's reagent was mixed with 200 μL of biomass (previously washed), followed by thorough vortex. Folin reagent (100 μL) was incorporated to the sample after an incubation step of 10 min, and the sample was vortexed again. The samples absorbances were measured at 750 nm after 30 min of dark reaction. At the end, BSA calibration previously established was used to quantify the protein concentration in each sample. The concentrations used were from 0 to $1500 \text{ } \mu\text{g mL}^{-1}$, and it was run by triplicate.

2.6. Total carbohydrates quantification of *Scenedesmus* sp. grown under different macronutrients concentrations

Total carbohydrates production of *Scenedesmus* sp. was measured using the sulfuric acid-UV assay previously established by López-Legarda et al. [28]. Shortly, concentrated sulfuric acid (1000 μL) was mixed with 300 μL of the previously washed biomass and incubated in an ice bath for 5 min. After this, using a microplate reader BGM Labtech absorbance at 315 nm was measured. Finally, glucose calibration curve previously established was used to determine the carbohydrates concentration (mg L^{-1}) in each sample. The concentrations used were from 0 to 100 mg mL^{-1} , and it was run by triplicate.

2.7. Total lipid quantification of *Scenedesmus* sp. grown under different macronutrients concentrations

Total lipid synthesized of *Scenedesmus* sp. was measured using a modified assay of Sulfo-phospho-vanillin established by Mishra et al. [29]. Firstly, the washed microalgae obtained was concentrated ten times to carry out the analysis. In this method, to 100 μL of the concentrated microalgae was added 2 mL of 98 % H_2SO_4 . The sample was incubated 10 min at 100 °C and then was established in a cold bath with ice for 15 min followed by the addition of 5 mL of phospho-vanillin solution. After 30 min incubation at 37 °C, the absorbance was reading at 530. A canola oil standard curve previously determined was used for quantified lipid concentration in each sample. The concentrations used were from 0 to 1000 $\mu\text{g mL}^{-1}$, and it was run by triplicate.

2.8. FAMES of *Scenedesmus* sp. grown different macronutrients concentrations

For lipids extraction, a reagent mixture consisting of 4 mL of methanol, 2 mL of chloroform, and 0.8 mL of distilled water was used. Briefly, 4 mL of this solvent mixture was put in 200 mg of lyophilized microalgae, vortexed for 1 min, and centrifuged at 6000 rpm for 15 min at 4 °C. The supernatant was discarded, and the lower phase was carefully recovered through a 0.45 μm PTFE syringe filter (Thermo Fisher Scientific, Waltham, MA, USA). The recovered lower phase was transferred to a pre-weighed tube. Then, the sample was dried using a concentrator at 50 °C and 15 mm Hg for 3 h. Total lipid content was then determined based on the Bligh and Dyer method [30] with modifications.

Fatty acids presence was identified by derivatization to FAMES in glass vial. For this, it was used a mixture of 9 mL of methanol and 1 mL of sulfuric acid solution, 2 mL per sample. It was used 1 mL of triundecanoin (C11:0) as internal standard. Then, it was incubated for 1.5 h at 80 °C. At once, after cooling to room temperature, each sample was recuperated using pure hexane (3 mL) by shaking for 3 min. The top layer generated was recuperated, then the lower layer was again extracted with pure hexane. The recovered phases were arranged in a 10 mL volumetric flask.

Fatty acid profiles were determined using a PerkinElmer Clarus 600/560D Gas Chromatograph coupled with a Mass Spectrometer (GC-MS), equipped with an HP-88 capillary column (100 m, 0.25 mm $\times$ 0.20 μm). Helium served as the carrier gas at a constant flow rate of 1 mL min^{-1} . The injection volume was 1 μL . The final oven temperature reached 240 °C, rising from an initial temperature of 140 °C at a rate of 4 °C min^{-1} . The injector temperature was 265 °C and split ratio was 1:10. Mass Spectrometer analysis was performed with an electron ionization source at 70 eV. The source and interface temperatures were both set to 210 °C, and the mass range scanned was $m/z = 30\text{--}550$. FAME identification was based on retention times compared to a standard 37- components FAME mixture chromatogram, and the quantification was performed using the standard internal method (AOAC Official Method 996.06).

2.9. PHA of *Scenedesmus* sp. grown with different macronutrients concentrations

PHA extraction was carried out by chloroform extraction method with some modifications described by Ref. [31]. Dry microalgae biomass (100 mg) was washed with 10 mL of ethanol and placed it in an ultrasonic bath for 30 min. The sample was centrifuged at 4500 rpm for 4 min at 4 °C, and the supernatant was discharged. The pellet was incubated with 10 mL of 4 % commercial hypochlorite solution for 1 h at 37 °C and centrifuged again at 8000 rpm for 15 min at 10 °C. The supernatant was discharged, and the pellet was washed twice with 5 mL each of ethanol and bi-distilled water, followed by centrifugation as described previously. The recovered pellet was transferred to pre-weighed lab glass tubes and treated with 10 mL of boiling chloroform. The mixture was then filtered through Whatman filters with pore size of 0.45 μm . The chloroform solution was evaporated using a SpeedVac Concentrator SPD1010 (Thermo Scientific Savant) for 2 h at 37 °C and 15 mmHg pressure. The DW of the samples was measured to quantify PHA production.

FT-IR analysis was carried out for each biopolymer sample to determine the presence of polyhydroxyalkanoates within the spectral range of 400–4000 cm^{-1} using FT-IR spectrophotometer (PerkinElmer Inc., Waltham, MA, USA).

2.10. Data analysis

The statistical analysis was performed using Minitab 21 (Minitab Inc.). A multi-factor ANOVA with Tukey pairwise comparisons were employed for each culture condition at the $\alpha = 0.05$ significance level. R-studio using ggplot2 package was used for generating heatmaps for FAMES expression. μ_{max} values were calculated using a logit model.

3. Results and discussion

3.1. Growth behavior of *Scenedesmus* sp. on different macronutrients concentrations

Scenedesmus sp. had a greater cell growth in the treatments related to sulfur variation (Fig. 1). In medium concentration [Control], it was obtained a cell growth of 0.22 g L^{-1} , 0.29 g L^{-1} , and 0.70 g L^{-1} at 5th, 10th, and 15th culture day ($\mu_{\max} = 0.13$), respectively. These data are similar to the reported by Ref. [32], where *Scenedesmus* sp. reached approximately 0.64 g L^{-1} at 15th cultivation day.

In the experiments with nitrogen variation (Fig. 1a), it was obtained a high biomass in low nitrogen concentration [N-], where it was obtained 0.78 g L^{-1} at 10th and 0.95 g L^{-1} at the end of the experiment (15th culture day) ($\mu_{\max} = 0.44$). In this variation it could be shown an increase in cell growth against to the control condition, this result is similar to the found with *Scenedesmus obliquus* where under nitrogen deprivation greater cell growth was obtained (15 % more) [33]. Also, in previous studies it was found that low nitrogen could increase growth in some species of microalgae such as *Chlorella emersonii* and *Isochrysis galbana* [34,35]. This behavior may be due to the internal consumption of some molecules as a source of nitrogen such as chlorophyll, reducing by this way the color intensity of the cultures. Also, other biomolecules that could be reduced for this internal consumption are the proteins [33,36]. Furthermore, in high nitrogen concentration [N+], also it was obtained greater microalgae growth compared to control condition. In this condition, it was obtained 0.86 g L^{-1} at 10th and 0.83 g L^{-1} at the end of the experiment (15th culture day) ($\mu_{\max} = 0.45$).

Regarding, the phosphorus variation in culture medium (Fig. 1b), it was observed an increase in cell growth in low phosphorus concentration [P-], it was 0.79 g L^{-1} at 10th and 0.95 g L^{-1} on the 15th day of cultivation ($\mu_{\max} = 0.37$). Similar to these results, with *Scenedesmus obliquus* FACHB-417 under low phosphorus concentrations obtained a slightly greater microalgae growth compared to the control [37].

In the case of high phosphorus concentration [P+], it was obtained slightly greater cell growth compared to control condition at 5th and 10th culture day, where it was obtained 0.45 g L^{-1} and 0.69 g L^{-1} ($\mu_{\max} = 0.45$). These results probably be related to the found in *Chaetoceros muelleri* culture, where it was observed a slight increases in cell growth under high phosphorus conditions [38]. The above could be related to the increase of mitochondrial activity via poly-P storage energy, such as in the case of *Chlorella regularis* where it was obtained 25 % more of mitochondrial activity under high phosphorus condition compared to control conditions [39]. Both high and low concentrations of phosphorus can negatively affect cell growth [40], therefore, finding the ideal concentration of phosphorus for each strain of microalgae can be very important to produce a good amount of biomass.

In the experiments with sulfur variation (Fig. 1c), under low sulfur concentration [S], it was obtained greater microalgae growth compared to control condition. In this condition, it was obtained 0.40 g L^{-1} at 5th, 0.82 g L^{-1} at 10th, and 0.93 g L^{-1} at 15th cultivation

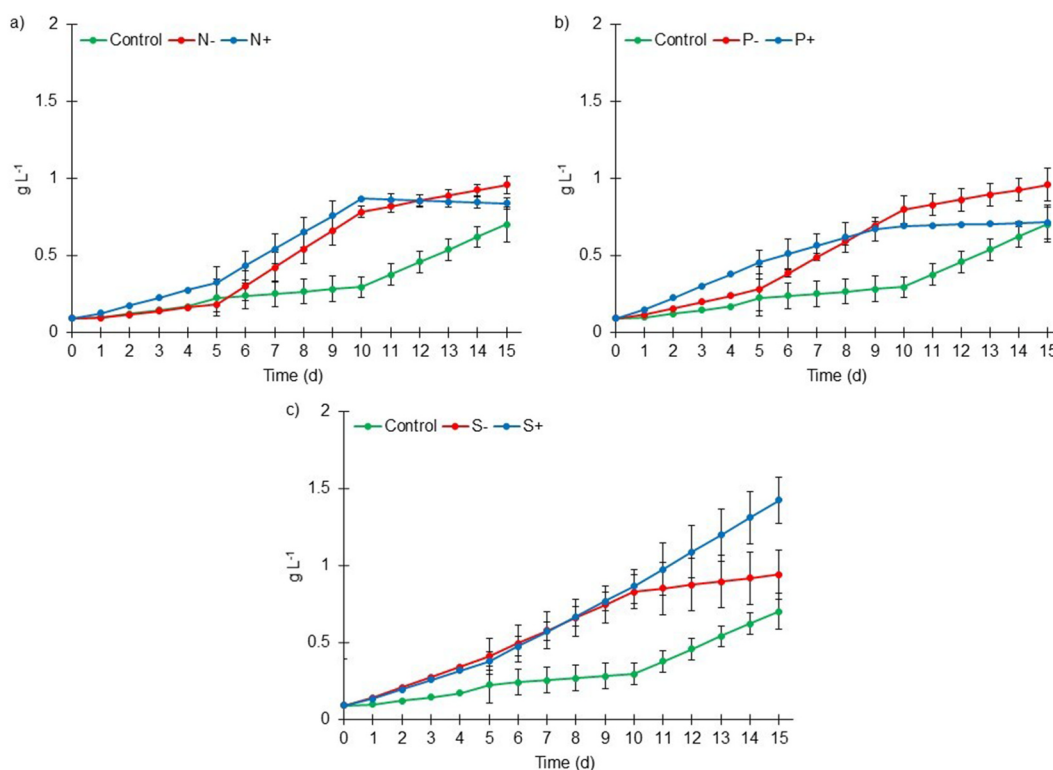


Fig. 1. Growth behavior of *Scenedesmus* sp. grown under different a) nitrogen, b) phosphorus, and c) sulfur concentrations (g L^{-1}). All the samples were taken every day and measured by triplicate.

day ($\mu_{max} = 0.36$). Under low sulfur concentrations, the cell size and biomass production of the microalga *Galdieria sulphuraria* increase compared to standard conditions [41]. In the other hand, it was obtained a better cell growth in high sulfur concentration [S+], where it was obtained a cell growth of 0.37 g L^{-1} at 5th, 0.86 g L^{-1} at 10th, and 1.42 g L^{-1} at 15th cultivation day ($\mu_{max} = 0.24$). *Scenedesmus acuminatus* also can improved its growth under high sulfur conditions, where it was produced 7.6 g L^{-1} in contrast to what was produced in control conditions when obtaining 6 g L^{-1} [42]. The results are similar to the report by Ref. [43], who conclude that microalgae cultures under sulfur variations could have increased biomass production. From the statistical analysis (ANOVA-Tukey pairwise) of microalgae cell growth, it was found that at highest microalgae biomass production (1.42 g L^{-1}) was produced at both high sulfur [S+] and low nitrogen [N-] concentration throughout the experiment, culminating at the 15th day of culture with 0.98 g L^{-1} of biomass.

The biomass can be treated and used for the bioplastic production, considering it under sustainable processes. It was carried out some bioplastic production using unfractionated biomass, mainly for its carbohydrate content. For example, *Chlamydomonas reinhardtii* was plasticized using Twin-screw extrusion process with glycerol as plasticizer [44]. There are additional reports on the use of microalgae biomass as an additive to some thermoplastic starch films (e.g., corn and cassava). In addition, it was demonstrated that its incorporation not affect the rigidity of bioplastics [45,46]. At the end, the high quality of the bioplastic obtained can be used for various purposes such as food packaging [47]. The results above demonstrate the potential of usefulness of the microalgae biomass generated.

3.2. Protein content of *Scenedesmus* sp. grown with different macronutrients concentrations

Scenedesmus sp. produced a higher concentration of proteins in control condition, however, in the treatments related to nitrogen and phosphorus limitation it was observed an increase in proteins production (Fig. 2). In medium macro elements concentration [Control], it was obtained 158 mg g^{-1} at 5th, 186 mg g^{-1} at 10th, and 170 mg g^{-1} at 15th culture day. These data are comparable to the obtained by Ref. [32], where *Scenedesmus* sp. reached 130 mg g^{-1} at 15th cultivation day.

In the experiments with nitrogen variations (Fig. 2a), it was obtained a better protein production in low nitrogen concentration [N-], where it was obtained 260 mg g^{-1} at 10th culture day (approximately 35 % greater than control condition). This behavior was observed in previous reports in other microalgae strains, for example, *Chlamydomonas reinhardtii* cultured under nitrogen limitation show an increase in its protein content ($\leq 13\%$). This observed behavior response might be associated to the utilization of intracellular nitrogen storage in specific biomolecules (e.g., chlorophyll) [48].

Also, it was determined that under nitrogen limitation or starvation conditions, microalgae can overproduce proteins associated

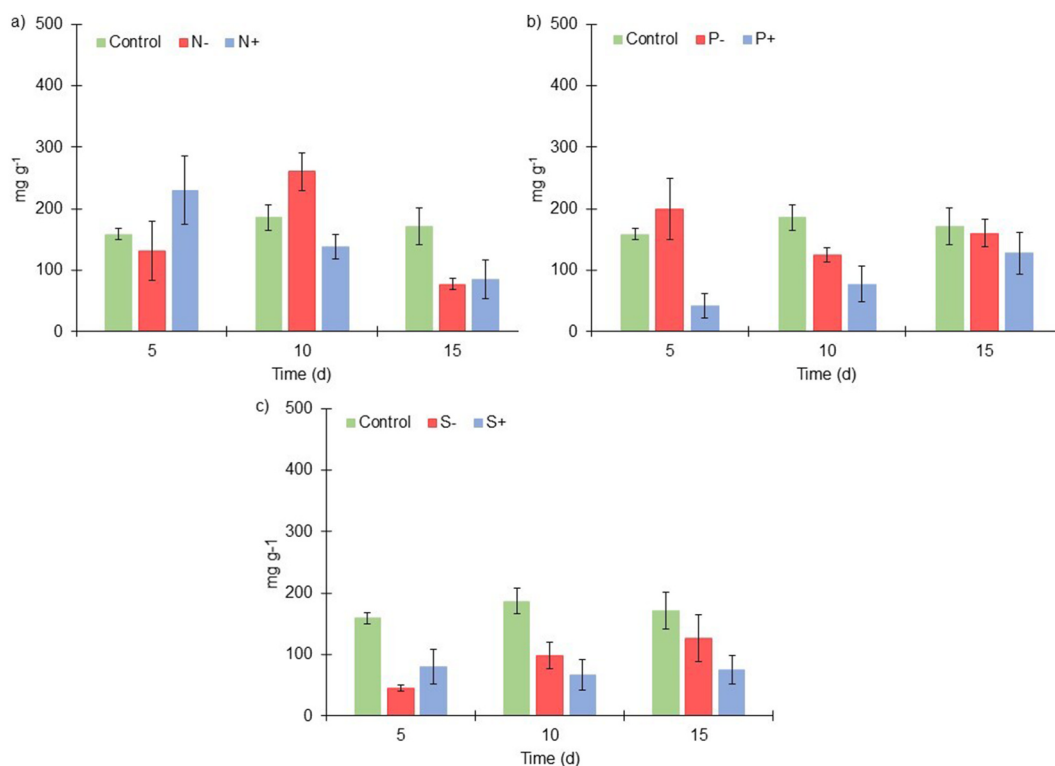


Fig. 2. Protein production of *Scenedesmus* sp. grown under three different concentrations of a) nitrogen, b) phosphorus, and c) sulfur (mg g^{-1}). Samples were collected at five-day intervals and measured in triplicate.

with lipids and carbohydrates generation such as Citrate synthase and Glycerol-3-phosphate O-acyltransferase among others [49]. In the other way, in high nitrogen concentration [N+], it was obtained 230 mg g^{-1} at 5th cultivation day (approximately 50 % greater than control condition).

Primary assimilation of nitrogen to synthesize amino acids requires carbon structures such as keto-acids and energy in form of ATP and NADPH, which are provided by the TCA cycle. Ammonium is the most preferred nitrogen source for algae and its assimilation under autotrophic or heterotrophic behavior is mediated by glutamine synthetase to produce glutamine and glutamate synthase to produce 2 molecules of glutamate from glutamine and one molecule of α -ketoglutarate. Alternative pathways are used to incorporate ammonium into glutamate, this reaction is catalyzed by glutamate dehydrogenase (Khan, M. et al., 2016)

Regarding, the phosphorus variation in culture medium (Fig. 2b), it was observed a slightly increase in protein content in low phosphorus concentration [P-], where it was obtained 200 mg g^{-1} at 5th cultivation day. *Scenedesmus obliquus* FACHB-417 cultured under phosphorus limitation had the same protein production behavior as the control, which was like what was obtained in this study on day 5 and 15 of the culture [37]. Similar to this study, *Chlorella* sp. cultured under phosphorus limitation could accumulate a higher protein concentration than when it is cultivated high phosphorus concentrations [50]. These results could be associated to the microalgae capability to maintain high levels of some proteins related in phosphorus storage and its assimilation [51].

In the case of high phosphorus concentration [P+], all sampling days obtained lower protein concentration than control condition. These could be related to some organelle damage, in *Chlorella regularis* cell it could be shown ruptures and disorganization in chloroplasts and cell surfaces damage under phosphorus excess conditions [52].

In the experiments with sulfur variation (Fig. 2c), in all treatments (high and low concentration) it was observed a lower protein content compared to control condition. Sulfur variations could reduce protein content in microalgae biomass. Previously, it was determined that sulfur in microalgae behavior is involved in nuclear transcripts encoding some proteins related to photosynthetic activity, a very important process in microalgae metabolism [53]. In the other hand, these variations could also affect the biosynthesis of sulfur amino acids (methionine and cysteine) [41]. Regarding the statistical analysis (ANOVA-Tukey pairwise) of proteins in this experiment, it was found that low nitrogen [N-] condition led to higher proteins production at 10th day of cultivation (260 mg g^{-1}). Sulfur depletion induces in some organisms like higher plants and microalgae the capacity of the cell to assimilate exogenous sulfur, restricting cellular and reproductive processes, and modulating metabolic processes. However, there is a solid interconexión between sulfur, nitrogen and carbon assimilation. Particularly, in some strains like *D. salina*, sulfur limitation declines the total proteins. This behavior was also confirmed in *Scenedesmus acuminatus*, it seems that the sulfur limitation increase the amino acids pools, which means reduce the amount of soluble protein. After 2–6 days, the total free amino acids decreased gradually. This may be because free amino acids can be used in pyruvate or succinyl-CoA/acetyl-CoA conversion, an intermediate in the citric acid (TCA) cycle, and after that could be used to produce lipids [42].

Although proteins have not had a direct application in bioplastics production. However, they can be used to produce other by-products of medium. For example, microalgae proteins could be used as stabilizers of emulsions and foams and have the potential to replace commonly surfactants and animal-based proteins using for this purpose [54]. It is well known that proteins from microalgae are a good source of essential amino acids, increasing protein digestibility, quality and nutritional values for use as human food or protein supplements and particularly in population with vegans or vegetarians practices (Yanwen et al., 2021). The demand of protein is growing and livestock protein-based became less sustainable. Therefore, microalgae are frequently recognized and compared with protein animal sources adding beneficial effects in human health and environment (Eilam, Y. et al., 2023).

3.3. Carbohydrates content of *Scenedesmus* sp. grown with different macronutrients concentrations

Scenedesmus sp. produced a greater carbohydrates concentration in lower macro nutrients concentrations especially in nitrogen and phosphorus treatments (Fig. 3). In medium macro elements concentration [Control], it was obtained 326 mg g^{-1} at 5th, 289 mg g^{-1} at 10th, and 386 mg g^{-1} at 15th culture day. Our results are related with those of Chandra et al. [55], who reported *Scenedesmus acuminatus* reached approximately 250 mg g^{-1} of carbohydrates under usual conditions.

In the experiments with nitrogen variation (Fig. 3a), it was obtained a better carbohydrate production in low nitrogen concentration [N-], where it was obtained 361 mg g^{-1} and 374 mg g^{-1} (approximately 29 % greater than control condition) at 5th and 10th, respectively. It was established in previous reports that nitrogen deprivation enhances carbohydrate accumulation in several microalgae strains. For example, in *Scenedesmus rotundus* MG910488, it was obtained 50 % more concentration of carbohydrates under low nitrogen concentrations compared to standard conditions [56]. Also, under nitrogen limitation, *Eustigmatos calamaris* produced >80 % of carbohydrates [57], and *C. reinhardtii* CC-124 exhibit a 4-fold increase in carbohydrates content [58].

It is commonly that microalgae cultures under nitrogen deprivation or limitation seek to accumulate energy in the form of carbohydrates, since its generation consumes a smaller amount of energy compared to the needed to produce lipids [59], in this form, microalgae cell ensures the necessary energy for general metabolism and DNA replication [60]. In the case of high nitrogen concentration [N+], in all the sampling days it was obtained a lower carbohydrates concentration than control condition. These results are according to a finding in our previous study, where *C. reinhardtii* was affected in the production of carbohydrates in nitrogen high concentrations [27].

Regarding, the phosphorus variation in culture medium (Fig. 3b), it was observed a slightly increase in carbohydrates content in low phosphorus concentration [P-], where it was obtained 357 mg g^{-1} at 5th, and 327 mg g^{-1} at 10th (approximately 29 % higher than control condition). In other microalgae it has also been observed that at a low phosphorus concentration, microalgae can accumulate a higher amount of carbohydrates against to standard culture conditions. As in the case of *Chlorella* spp., where almost 50 % DW of carbohydrates was obtained under phosphorus limitation [61]. It was determined previously that high accumulation of

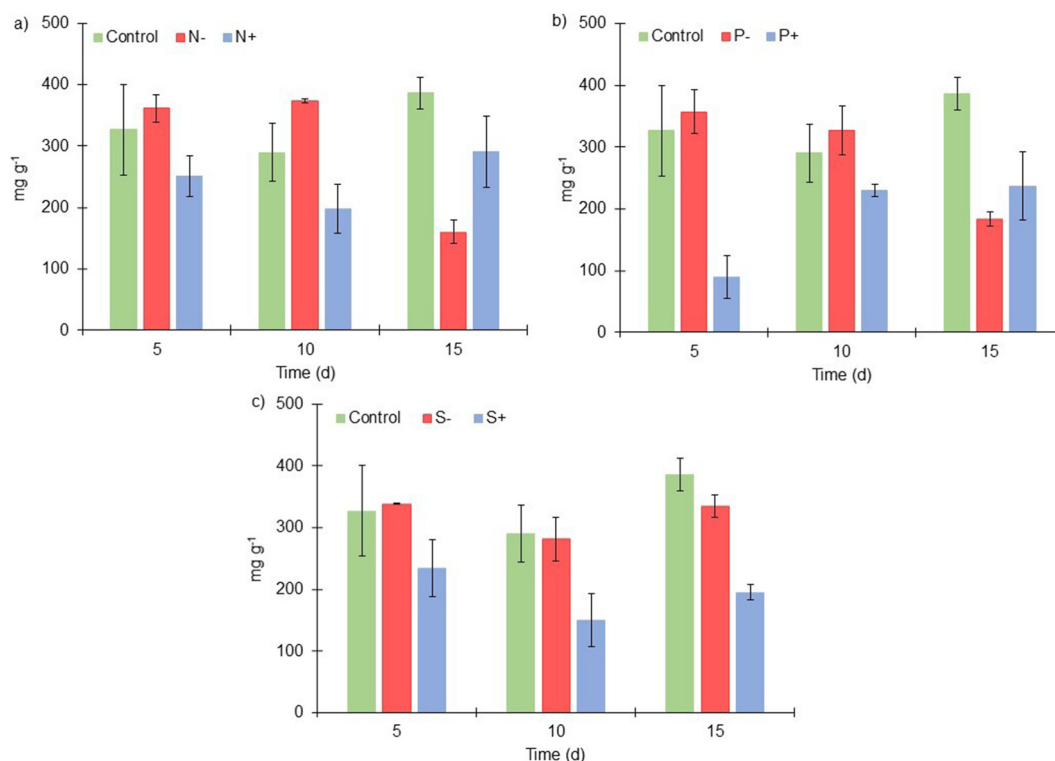


Fig. 3. Total carbohydrates production of *Scenedesmus* sp. grown at three different concentrations of **a)** nitrogen, **b)** phosphorus, and **c)** sulfur (mg g⁻¹). Samples were collected at five-day intervals and measured in triplicate.

glucose-6-phosphate known as a precursor of starch synthesis can be achieved under phosphorus starvation conditions by the reduction of the exchange of triose phosphate, generating a greater carbohydrate production in microalgae cultures [62].

In the case of high phosphorus [P+], in all sampling days it was obtained a lower carbohydrates concentration than control condition. These results are similar to the found with *Scenedesmus* LX1 grown under nitrogen and phosphorus limitation, where it was obtained higher content of carbohydrates (at least 20 % more) compared to when the microalgae were grown with high concentrations of phosphorus and nitrogen [63].

In the experiments with sulfur variation (Fig. 3c), it was obtained a slightly better carbohydrate production in low sulfur concentration [S-], where it was obtained 338 mg g⁻¹ at 5th culture day. These results are similar to the found in *Chlorococcum humicola* microalga grown under low sulfur concentration, where it was obtained a higher accumulation of carbohydrates (up to 10 %) compared to when it was grown under standard conditions [64]. Regarding high sulfur concentration [S+], all sampling days obtained lower carbohydrates concentration than control condition. It was shown previously that microalgae cultured in sulfur starvation conditions could improve carbohydrates accumulation by up regulated some enzymes related to starch production such as starch synthase and glycogen branching enzyme [65]. Omics science, along with enzymatic and genetic validations may reveal a partial view of the pathway regarding to starch and lipid biosynthesis as well as carbon allocation under nutrient depletion conditions. Therefore, genetic manipulation tools, like CRISPR system and synthetic biology could improve genetic engineering to understand the biosynthesis pathway and regulatory mechanism of microalgal in starch and lipids production [66]. Regarding the statistical analysis (ANOVA-Tukey pairwise) of carbohydrate concentration in whole experiment, it was concluded that at medium macroelements condition [Control] and low nitrogen [N-] was generated a higher carbohydrate concentration at 15th and 10th culture day (386 mg g⁻¹ and 374 mg g⁻¹, respectively).

Carbohydrates from microalgae are used for bioplastic production, especially starch. This polymer has proper characteristics to be mixed with some compounds such as cellulose and fibers (e.g., laver flack) to improve some proprieties of bioplastics. These additives are able to reduce the gas permeability of bioplastics, making it optimal for use in food packaging[67,68]. Glycerol added to bioplastic-based carbohydrates is able to reduce material degradation and improve mechanical properties of plastics [69,70].

3.4. Lipid content of *Scenedesmus* sp. grown with different macronutrients concentrations

Scenedesmus sp. produced a higher lipids content mainly in the treatments with nitrogen and phosphorus limitation (Fig. 4). In medium Control, the concentration of lipids was obtained 137 mg g⁻¹ at 5th, 176 mg g⁻¹ at 10th, and 163 mg g⁻¹ at 15th culture day. These results are similar according to Vinitha et al. [71], who reported that *Scenedesmus obtusus* reached approximately 200 mg g⁻¹ of lipids under standard conditions. Also, *Scenedesmus obliquus* could produce from 50 mg g⁻¹ to 160 mg g⁻¹ at similar conditions [72,73].

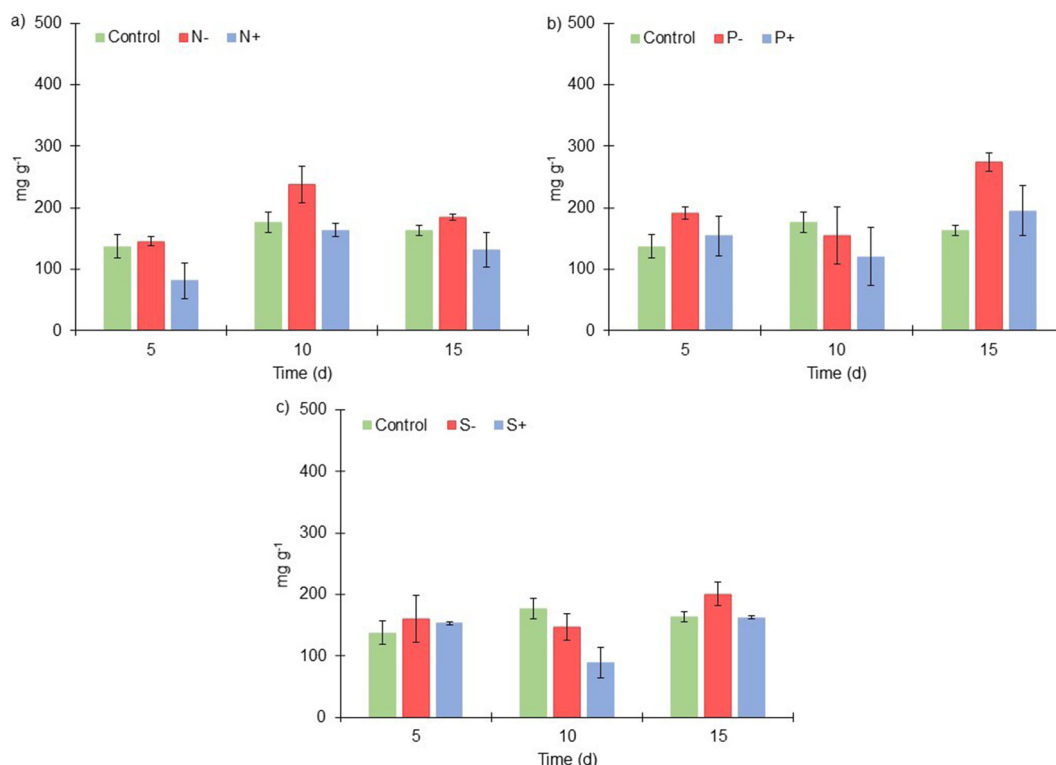


Fig. 4. Lipid content of *Scenedesmus* sp. grown at three different concentrations of a) nitrogen, b) phosphorus, and c) sulfur (mg g^{-1}). Samples were collected at five-day intervals and measured in triplicate.

In the experiments with nitrogen variation (Fig. 4a), it was obtained a better lipids production in low nitrogen concentration [N-], where it was obtained 237 mg g^{-1} at 10th culture day (approximately 35 % greater than control condition). This behavior was observed in previous reports, for example, *Scenedesmus obliquus* improved its lipids content up to 35 % by reducing nitrogen by at least one third in the culture medium after being five days in these conditions [33].

In microalgae cultures (e.g., *Tetrademus bernardii* and *Scenedesmus acutus*) grown under nitrogen limitation conditions was observed an upregulation of genes related with TAG biosynthesis such as genes encoding diacylglycerol acyltransferase (DGAT2 and DGAT1), acetyl-coenzyme A carboxylase and palmitoyl-acyl carrier protein [74,75], this could be an explanation to the results obtained in this study. Also, it is possible that with total deprivation of nitrogen an improvement in lipids production could be observed. In the other way, high nitrogen concentration [N+], in all the sampling days it was obtained lower lipids production than control condition.

Regarding, the phosphorus variation in culture medium (Fig. 4b), it was noticed an increase in lipids concentration in low phosphorus content [P-], especially at 15th culture day where it was obtained 273 mg g^{-1} at 15th culture day (approximately 65 % greater than control condition). This result is similar to the found with *Scenedesmus* sp. previously, since at lower phosphorus concentration the microalgae produced more lipids (approximately 50 % more compared to standard conditions) where it was observed genes overexpressed involved in biosynthesis of fatty acid (e.g., 3-ketoacyl-CoA synthase and 2-enoyl-CoA reductase) after six days of cultivation[76]. The observed high lipid production may be associated with lower carbohydrate production (Fig. 3b), especially in 15th culture day. In the case of high phosphorus [P+], it was observed a slightly increase in lipids content at 5th (154 mg g^{-1}) and 15th (194 mg g^{-1}) culture day.

In the experiments with sulfur variation (Fig. 4c), an increase in lipids production in low sulfur concentration [S-] at 5th and 15th culture day was observed, where it was obtained 160 mg g^{-1} (16 % higher than control condition) and 200 mg g^{-1} (22 % higher than control condition), respectively. This could be because the upregulation of some genes involved to fatty acids desaturase and phosphatidic acid phosphatase, both involved in acetyl-CoA metabolomics flow [65]. In addition, it was determined that some microalgae cultures such as *Chlamydomonas reinhardtii* grown under sulfur limitation conditions could over expressed genes involved in TAG biosynthesis such as acyltransferases (e.g., diacylglycerol acyltransferase [DGTT1]) [77]. Unfortunately, there are not extensive studies regarding the biosynthetic carbon partitioning pathways of microalgal cells under varying conditions of sulfur concentrations [42]. Regarding the statistical analysis (ANOVA-Tukey pairwise) of lipids production, it was found that low phosphorus [P-] condition produced the highest lipid content (273 mg g^{-1}) in whole experiment at 15th cultivation day.

In general, several studies on the microalgae growing reveled that different factors influenced the lipid production, many of them have illustrated that gene expression from a series of metabolic pathways controlled the lipid accumulation depending on the changing

environment. For example of RNA processing, ribosome biosynthesis, photosynthesis, protein metabolism, energy generation, TCA (tricarboxylic acid) cycle, carbon fixation, among others (Xin, Y; et al., 2024)

Microalgae lipids represent a promising feedstock for sustainable industries, with oil productivity of up to 136,900 L ha⁻¹. Among these, triacylglycerols (TAGs) are the main lipid class in microalgae biomass, making them particularly suitable for bioplastic production [78]. Plastic films derived from TAGs and glycerol have been resulted promising for the high demand of new renewable plastics [79]. However, the industrial scaling of microalgae-based bioplastics and products requires an integrated approach that combines process optimization and life cycle analysis to evaluate both environmental and economic impacts [80]. The market for lipids is projected to grow at a compound annual rate of 6–8 % until 2030, driven primarily by the demand for omega fatty acids in food supplements. Nevertheless, this application faces high operational costs due to the reliance on synthetic media. In order to address and make a balance in cost-benefit the use of wastewater as culture medium could enhance the biodiesel, bioplastics, and bio lubricants production, because is simple to monitor the macronutrients content that influence in the macroalgae metabolism. Currently productions costs range around €6.7 per kg of biomass containing 24 % lipids (w/w). (Benvenuti et al., 2017). The scaling demand needs high lipids production however the major challenge associated with is low microalgae biomass, resulting in the high cost of cultivation and downstream processing. Thus, it is necessary to develop approaches for improving the cultivation and energy-saving downstream processing of lipids (Xin, Y; et al., 2024)

3.5. FAMES production of *Scenedesmus* sp. grown with different macronutrients concentrations

Scenedesmus sp. in control condition at 15th culture day produced 29 mg g⁻¹ [28 ± 0.6 % of total fatty acids] of palmitic acid (C16:0), 27 mg g⁻¹ [27 ± 2.7 %_{TFA}] of linoleic acid (C18:2n6c), 22 mg g⁻¹ [21 ± 2.7 %_{TFA}] of α-linoleic acid (C18:3n3), and 12 mg g⁻¹ [12 ± 1.6 %_{TFA}] of oleic acid (C18:1n9c) (Fig. 5a, b, c). These results demonstrate consistency with the observations reported by Ref. [81], where *Scenedesmus* sp. DDVG I obtained 29 %_{TFA} of palmitic acid, 19 %_{TFA} of oleic acid, and 34 %_{TFA} of linoleic acid.

Regarding the experiments of nitrogen variation, in low nitrogen concentration [N-], it has been produced 22 mg g⁻¹ [33 ± 2.2 %_{TFA}] of palmitic acid, 10 mg g⁻¹ [16 ± 3.8 %_{TFA}] of oleic acid, 17 mg g⁻¹ [25 ± 3.4 %_{TFA}] of linoleic acid, and 11 mg g⁻¹ [17 ± 3.1 %_{TFA}] of α-linoleic acid, although a greater amount of fatty acids was not produced, an greater concentration of some fatty acids could be observed such as the case of palmitic acid and oleic acid. *Scenedesmus obliquus* culture under nitrogen limitation conditions produced higher percentage of oleic acid, results compared to what was obtained in this study [82]. Also, *Phaeodactylum tricornutum* culture that under nitrogen limitation conditions produced higher percentage of palmitic acid [83]. In addition, *Eustigmatos calamaris* grown under nitrogen limitation condition produced higher amounts of C16 - C20 fatty acids [57].

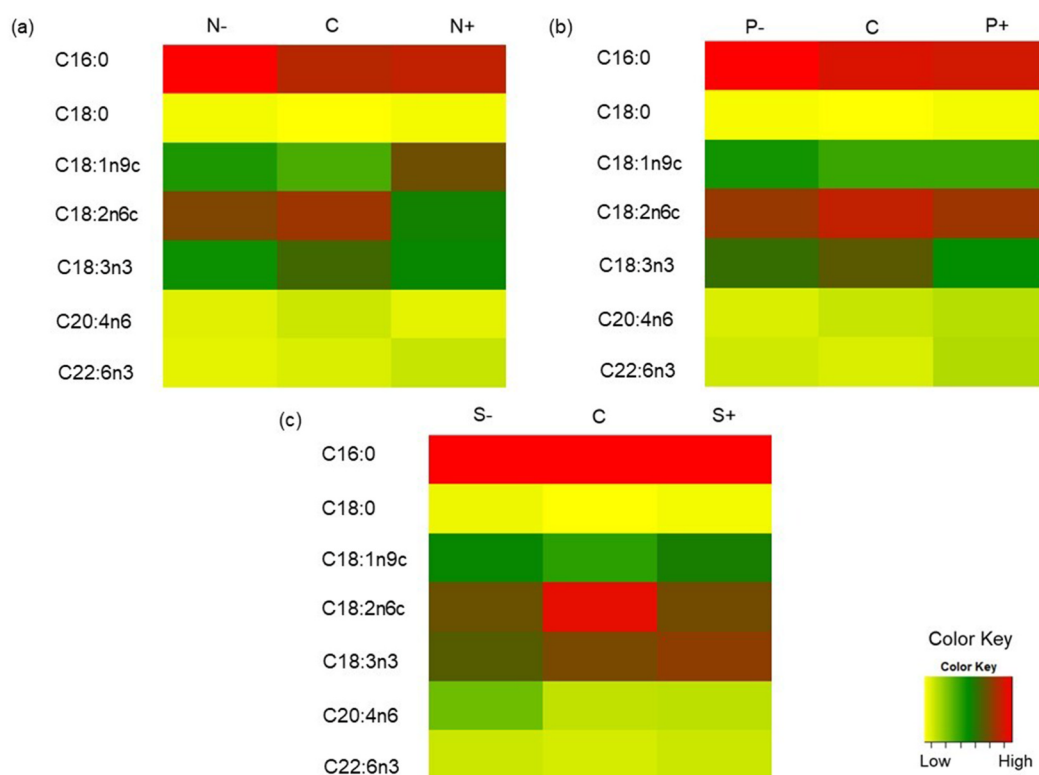


Fig. 5. Heatmap of FAMES profile (as % of total lipids) in *Scenedesmus* sp. grown under three different concentrations of a) nitrogen, b) phosphorus, and c) sulfur. The samples were taken at 15th culture day and measured by triplicate.

In high nitrogen concentration [N+], it has been produced 20 mg g^{-1} [$29 \pm 3.7 \%_{\text{TFA}}$] of palmitic acid, 16 mg g^{-1} [$24 \pm 4.2 \%_{\text{TFA}}$] of oleic acid, 14 mg g^{-1} [$18 \pm 4.3 \%_{\text{TFA}}$] of linoleic acid, and 15 mg g^{-1} [$17 \pm 3.6 \%_{\text{TFA}}$] of α -linoleic acid at 15th cultivation day. Compared to the control treatment, this condition increased the content of some fatty acids, particularly palmitic and oleic acids.

Under low phosphorus [P-] conditions, the production of palmitic [$30 \pm 1.5 \%_{\text{TFA}}$], stearic (C18:0) [$1.8 \pm 0.11 \%_{\text{TFA}}$], and oleic acid [$15 \pm 2.7 \%_{\text{TFA}}$] was slightly higher than in the control. *Scenedesmus obliquus* culture under nitrogen limitation conditions produced higher percentage of oleic acid, results compared to what was obtained in this study [82]. Microalgae grown under phosphorus limitation could produce a higher concentration of longer chain fatty acids compared to control condition [84]. These results could be linked to the upregulation of genes involved in fatty acids synthesis such as 3-oxoacyl-ACP reductase, 3-ketoacyl-CoA synthase, particularly related to the production of palmitic acid, consistent with findings by Yang et al. [76]. Under high phosphorus [P+] conditions, no significant increase in fatty acid content was observed compared to the control.

Regarding sulfur variations, *Scenedesmus* sp. under low concentration [S-] produced in $2.3 \pm 0.18 \%_{\text{TFA}}$ of stearic acid (50 % more than control) and $15 \pm 3.8 \%_{\text{TFA}}$ of oleic acid (22 % more than control). Also, arachidic acid (C20:4n6) was produced 2-fold greater than control condition, these results are like to the found previously with microalgae *Chlamydomonas reinhardtii* [27]. It was determined previously that microalgae could enhance FAMES production under sulfur limitation conditions by upregulation of some genes involved in fatty acids biosynthesis such as 3-ketoacyl-ACP reductase, acetyl-CoA carboxylase, and 3-ketoacyl-ACP synthase production [43]. Conversely, high sulfur concentration [S+] conditions resulted in greater oleic acid concentration (28 % more), α -linoleic acid (5 % more), arachidonic acid (6 % more), and docosahexaenoic acid (C22:6n3) [12 % more] compared to the control.

Specific production of FAMES by varying the macronutrients in the culture medium improve the bioplastics production. Previous reports have demonstrated that oleic acid, can improve the characteristics of some bioplastics[85]. In this sense, the fractionation of biomass can be suggested for obtain different biomolecules to produce bioplastics and in the same process valorizing the FAMES to improve the properties of the material.

3.6. PHA of *Scenedesmus* sp. grown with different macronutrients concentrations

PHA, is known as a biopolymer, was analyzed in *Scenedesmus* sp. grown under different concentrations of nitrogen, phosphorus, and sulfur (Fig. 6).

It was found that under low nitrogen and phosphorus conditions could be produced a higher concentration of PHA, where it was produced $9.5 \pm 0.83 \%$ and $6.4 \pm 0.32 \%$ DW respectively. These results are according to those found other microalgae species such as *Oscillatoria* sp. (14 %) [86], and *Synechocystis* sp. (3 %) [87], as well as *Chlorella* sp (1.5 %)[88]. However, the results obtained are lower than those previously obtained with the same species (*Scenedesmus* sp.) where it was obtained 29 % w w⁻¹ of PHA under phosphorus starving conditions(García et al., 2020). PHA production is encouraged under stress conditions, since they are energy reserve molecules. There are different metabolic routes to produce PHA, including the use of molecules obtained from the Calvin cycle and TCA cycle. In this sense, changing the macronutrients of the culture medium can particularly impact PHA production, however, as has been seen, this also has implications to produce lipids and carbohydrates. Under conditions of deficiency of these nutrients, there is a decrease in the synthesis of nitrogenous biomolecules (proteins, nucleotides) and phosphorylated (phospholipids), which reduces cellular energy demand and conducts the accumulation of NADPH and NADH. This situation leads to a reorganization of cellular metabolism, slowing down the tricarboxylic acid cycle and directing carbon towards the formation of acetyl-CoA, a precursor of PHA [89,90].

It was shown that the nitrogen variations can positively impact both low and high nitrogen conditions. It could also be observed

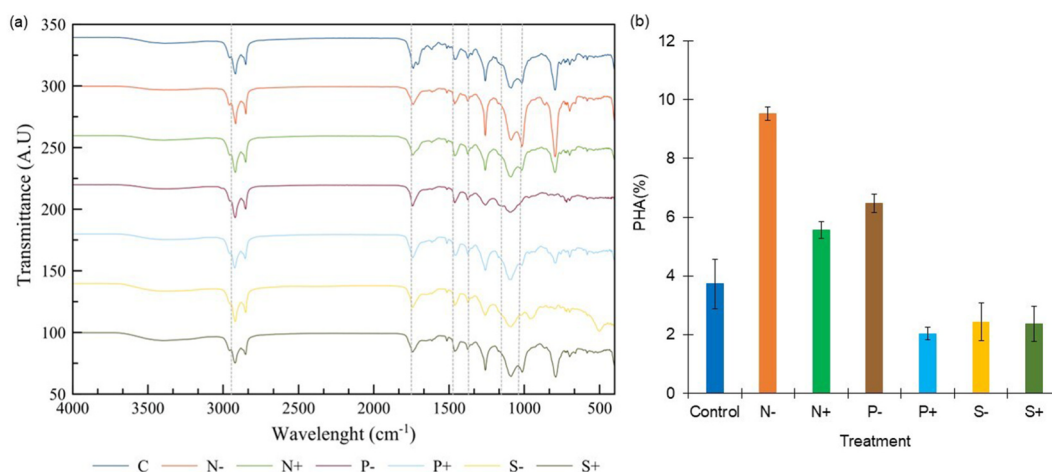


Fig. 6. Analysis of the PHA production of *Scenedesmus* sp. cultures grown at three different macronutrients concentrations. **a)** FTIR spectra of the PHA produced by *Scenedesmus* sp. cultures **b)** Analysis of the PHA production by *Scenedesmus* sp. cultures grown. The samples were taken at 15th culture day and measured by triplicate.

Table 2Cell growth and target metabolites production on *Scenedesmus* sp. (mg g⁻¹) under different macronutrients conditions.

Target interest	Condition	Maximum production (mg g ⁻¹)	Culture Day
Whole cell	High S	1.42 ^a	15
Proteins	Low N	260	10
Carbohydrates	Low N	374	10
Total lipids	Low P	273	15
Palmitic acid (C16:0)	Control	30	15
Oleic acid (C18:1n9c)	High N	16	15
Linoleic acid (C18:2n6c)	Control	28	15
α-linoleic acid (C18:3n3)	Control	22	15
Stearic acid (C18:0)	High N	2	15
Docosahexaenoic acid (C22:6n3)	Control	4	15
PHA	Low N	10	15

FAMES production is expressed as milligrams of FAMES per gram of microalgae biomass processed.

^a g L⁻¹.

that the variation of sulfur in the media has a very negative influence on the production of PHA since it was produced 2.4 ± 0.63 % and 2.3 ± 0.61 % DW in low and high sulfur concentrations, respectively.

FTIR spectra was used for confirming PHA presence in microalgae cultures. It was shown some peaks related to PHA presence, such as C=O (1746 cm⁻¹) [91], -CH₃ (1370 cm⁻¹), -CH₂ (1460 cm⁻¹), and -CH (2930-2950 cm⁻¹) groups. Also, it can be observed a strong band at 1020 cm⁻¹ and 1050 cm⁻¹, this could be related to C-O stretching and PHB production (García et al., 2020). In addition, it can be observed -C-O-C and -C-C groups at 1250 cm⁻¹ and 790 cm⁻¹ [92], respectively.

Table 2. summarize the macronutrient's conditions (N, P and S) that enhance the biomolecules production and cell growth. Except for proteins, the table shows the most optimal cultivation conditions to produce biomolecules that can be used for bioplastics production. It can conduct future research and biorefinery processes based on the changes of nutrient conditions with interest on proteins, lipids, and carbohydrates production. FAMES production (was expressed per milligram of biomass) is disclosed considering the current relevant role of fatty acids on nutrition and its food industrial applications.

4. Conclusions

In this work, the influence of three macronutrients (N, P, and S) variation in *Scenedesmus* sp. growth, proteins, carbohydrates and lipids production was analyzed. Our results have demonstrated that S (1.42 g L⁻¹) and N (0.98 g L⁻¹) variations enhanced the microalgae growth. Interesting, low nitrogen levels had higher impact in the production of proteins, carbohydrates and lipids at 10th day of culture. These results suggest that one single change in the nitrogen could increase the production of the valuable biomolecules. Complementary, FAMES profile was monitored based on its application, it was observed a clear influence of these macronutrients remarking that high concentrations of nitrogen enhanced the oleic acid production. Supportive results have shown the relevance of sulfur concentration in the biomolecules production since the amount of proteins and PHAs. It was highlighted in this study that the reduction of nitrogen and phosphorus can encourage the synthesis of PHA, contrary to the variation of sulfur can trigger a negative impact on its production. Further investigation to evaluate the mechanisms in which these P, N and S impacted the metabolic pathways for the production of proteins, carbohydrates and lipids are need. However, the present study releases proper conditions for the biorefinery of *Scenedesmus* sp. and the sustainable valorization of the biomass for the recovery of biomolecules, considering them for further bioplastic applications.

CRedit authorship contribution statement

Itzel Y. López-Pacheco: Formal analysis, Data curation, Writing – original draft, Conceptualization, Methodology. Luis Cornelio Callejas-Correales: Methodology. Georgia María González-Meza: Formal analysis, Writing – original draft. José Rodríguez-Rodríguez: Methodology, Data curation. Reyna Berenice González-González: Formal analysis, Writing – review & editing. Elda M. Melchor-Martínez: Project administration, Writing – review & editing, Funding acquisition, Validation, Formal analysis, Supervision. Roberto Parra-Saldívar: Project administration, Conceptualization, Writing – review & editing, Supervision.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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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.

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