Article

Enhancement of Metabolite Production in High-Altitude Microalgal Strains by Optimized C/N/P Ratio

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Featured Application: Two strains from high-altitude lakes showed an exciting capacity to produce carbohydrates, proteins, and lipids under an optimized C/N/P ratio.

Abstract: This study evaluated the role of C/N/P in the increase in the synthesis of carbohydrates, proteins, and lipids in two high-mountain strains of algae (*Chlorella* sp. UFPS019 and *Desmodesmus* sp. UFPS021). Three carbon sources (sodium acetate, sodium carbonate, and sodium bicarbonate), and the sources of nitrogen ($NaNO_3$) and phosphate (KH_2PO_4 and K_2HPO_4) were analyzed using a surface response (3 factors, 2 levels). In *Chlorella* sp. UFPS019, the optimal conditions to enhance the synthesis of carbohydrates were high sodium carbonate content (3.53 g/L), high KH_2PO_4 and K_2HPO_4 content (0.06 and 0.14 g/L, respectively), and medium-high $NaNO_3$ (0.1875 g/L). In the case of lipids, a high concentration of sodium acetate (1.19 g/L) coupled with high KH_2PO_4 and K_2HPO_4 content (0.056 and 0.131 g/L, respectively) and a low concentration of $NaNO_3$ (0.075 g/L) drastically induced the synthesis of lipids. In the case of *Desmodesmus* sp. UFPS021, the protein content was increased using high sodium acetate (2 g/L), high KH_2PO_4 and K_2HPO_4 content (0.056 and 0.131 g/L, respectively), and high $NaNO_3$ concentration (0.25 g/L). These results demonstrate that the correct adjustment of the C/N/P ratio can enhance the capacity of high-mountain strains of algae to produce high concentrations of carbohydrates, proteins, and lipids.

Keywords: sodium carbonate; sodium acetate; sodium nitrate; carbon-nitrogen-phosphate ratio; lipids; carbohydrates

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1. Introduction

Rapid population growth has caused a rise in demand for food and energy [1], thus keeping greenhouse gas emissions (CH_4, CO_2, CO, NO_X, SO_X, and others) at high levels [2,3] and adding to the severe threat of climate change [4,5]. Therefore, to achieve the sustainable development of human society, it is necessary to identify and exploit new renewable sources that will contribute to the development of food and energy processes [6–8].

In recent years, microalgae and cyanobacteria have been established as sustainable sources of carbohydrates, proteins, lipids, secondary metabolites, and minerals of great interest to the cosmetic, energy, pharmaceutical, and food industries [9–14]. Microalgae cultivation is a promising alternative to combine anthropogenic emissions with the production of raw materials for the energy, food, pharmaceutical, and cosmetic industries [15].

The use of microalgae presents significant advantages over terrestrial crops, including high content of target products: up to 60% protein [16–19], between 20–40% carbohydrates (mainly starch) [20–22], and between 20–50% lipids [23–26]. Similarly, the ability of these systems to use wastewater as a nutrient source, along with their low soil requirements and high cell growth rates, have positioned them as promising alternatives to producing multiple feedstocks [27–31]. This is due to the rapid maturation of production technologies and the international market for specialized metabolites [32].

The number of industrially produced strains is limited to a handful of genera and species such as *Chlorella* sp. (Chlorophyta) [22,33–36], *Dunaliella salina* (Chlorophyta) [37–39], *Haematococcus lacustris* (formerly *Haematococcus pluvialis*) (Chlorophyta) [40–43], *Nannochloropsis* sp. (Ochrophyta, Eustigmatophyceae) [44–48], *Phaeodactylum tricornutum* (Bacillariophyta) [49–56], *Porphyridium* sp. (Rhodophyta) [57–59], *Scenedesmus* sp. (Chlorophyta) [60–63], *Spirulina* (*Arthorspira*) sp. (Cyanobacteria) [62–70], *Tisochrysis lutea* (Haptophyta, Coccolithophyceae) [71–75], and *Tetraselmis* sp. (Chlorophyta) [72–80]. However, the most popular genus is *Spirulina* (*Arthorspira*) sp. According to Araújo et al. [81], in Europe, 222 different companies (57% of the total worldwide) are devoted to the production of *Spirulina* biomass, with an annual production of up to 143 tons.

The proper selection of microalgae strains is a key aspect in the utilization and cost reduction of biotechnological processes since native strains present greater adaptation to the environmental conditions of local ecosystems [82,83]. High mountain lakes are remote and extreme ecosystems subject to harsh climatic conditions, characterized by low temperatures and high solar and ultraviolet radiation (UVR) [84]. These extreme conditions can give them unique characteristics such as tolerance to high radiation, high plasticity to sudden temperature changes, and the ability to deposit high concentrations of metabolites (carbohydrates, proteins, lipids, etc.). Studies investigating species diversity in continuous water bodies such as rivers, wetlands, and high mountain lakes have increased, reflecting the interest in and recognition of these habitats [85–87]. However, these types of strains are poorly represented in the scientific literature.

Culture parameter optimization is a key point in the improvement of industrial algal production. Both culture media and operational conditions must be "tailored-made" for individual strains to maximize their productivity [88]. One available tool is the application of the design of experiments (DoE) [88–92] to improve concentrations of specific metabolites. This type of experiment has been successfully applied to improve the deposition of specific metabolites in algae and cyanobacteria before [93–98], especially in the selection of the carbon and nitrogen sources and their specific concentrations. However, to the best of our knowledge, there is no scientific literature on the optimization of culture media for strains from high-mountain environments. Therefore, the present study aimed to determine the optimal C/N/P ratio that enhances the concentration of carbohydrates, proteins, lipids, and carotenoids in two algal strains isolated from high-mountain lakes.

2. Materials and Methods

2.1. Strains

Chlorella sp. (CHLO_UFPS019) and two strains of *Desmodesmus* sp. (DESM_UFPS020 and UFPS021) were isolated from lakes located at 3300–3900 M.A.S.L. in the region of Norte de Santander (Colombia) and have shown an interesting capability to produce large concentrations of carbohydrates, proteins, lipids, and carotenoids (data not shown).

The identities of the strains were determined by internal transcribed spacer (ITS) gene analysis. DNA was extracted according to the CTAB-NaCl protocol [99]. The ITS gene was amplified by polymerase chain reaction (PCR) using ITS1F ITS4R primers [100] following the conditions described by Fei et al. [101]. The sequences were compared with ITS gene sequences available in GenBank databases, showing that all the strains had a high percentage of identity with their respective genera (99.2% for *Desmodesmus* sp. UFPS16, 99.46% for *Desmodesmus* sp. UFPS18, and 98.85% for *Chlorella* sp. UFPS14).

The strains are kept in the INNOValgae collection (Universidad Francisco de Paula Santander, Colombia), and were cultured in a 2 L tubular glass flask with 1.3 L of Bold's basal medium [102] at pH 7.0. Each flask was mixed through the injection of filtered air with 1% (*v*/*v*) CO_2 at a flow rate of 0.78 L min^{-1}, with a light:dark cycle of 12:12 h at 100 µmol m^{-2} s^{-1}, at 27 °C for 30 days.

2.2. Experimental Design

The optimization of carbohydrates (analyzed in *Chlorella* sp. UFPS019), proteins (analyzed in *Desmodesmus* sp. UFPS021), lipids (analyzed in *Chlorella* sp. UFPS019), and carotenoids (analyzed in *Desmodesmus* sp UFPS020) through the adjustment of the carbon/nitrogen/phosphate ratio was evaluated (in triplicate) using a central non-factorial response surface design with three central points and three blocks in the software STATISTICA 7.0 (Statsoft). The central points permitted a statistical check for the goodness-of-fit of the factorial model. Sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), and sodium acetate ($NaC_2H_3O_2$) were tested to identify the carbon source that maximized the production of the metabolites. The variables and their levels can be found in Table 1. The concentrations of $NaNO_3$ (0.25 g/L), K_2HPO_4 (0.075 g/L), and KH_2PO_4 (0.175 g/L) were adjusted following the content of each nutrient in Bold's basal medium [102]. The final concentration of each carbon source was calculated according to its relative carbon content.

Table 1. Variables evaluated with their respective levels.

Factor	Units	Low Level (−1)	High Level (+1)
Carbon source	g/L	0.2	0.5
$NaNO_3$	g/L	0.125	0.25
K_2HPO_4	g/L	0.0375	0.075
KH_2PO_4	g/L	0.0875	0.175

For each experiment, the C/N/P ratio was adjusted in 300 mL of Bold's basal medium [103] at pH 7.0 according to the resolved design (Table 2). Each flask was attached to a gas line with an airflow of 0.18 L min^{-1} (Resun, LP-100, Aarau, Switzerland) without the addition of extra CO_2, and a light:dark cycle of 12:12 h at 100 µmol m^{-2} s^{-1} at 27 °C. To produce carbohydrates and proteins, the strains were grown for 20 days, while to produce lipids and carotenoids, the strains were cultured for up to 40 days. In the case of carotenoids, the light intensity was increased up to 250 µmol m^{-2} s^{-1}, with a light:dark cycle of 12:12 h at 27 °C. The pH was monitored twice a day and adjusted to pH 7.0 ± 0.5 on a laminar flow cabinet using sterile solutions of either NaOH (1 M) or HCl (1 M).

2.3. Quantification of Biomass and Metabolites

The biomass produced was concentrated using a lab-scale electroflotation device [104], washed thrice with distilled water, reconcentrated by centrifugation (2876× *g*, 20 °C, 20 min), lyophilized, and stored (4 °C) until use. Finally, the different components of the strains were measured in triplicate.

For carbohydrates, 5 mg of grounded biomass were mixed with 5 mL of 1 M H_2SO_4 in acid-resistant test tubes, vortexed (Multi Reax, Heidoplh, Schwabach, Germany) at 1500 rpm for 10 min, and incubated (100 °C, 60 min). The mixture was cooled down at room temperature (30 min) and centrifuged (2876× *g*, 20 min). Two milliliters of the supernatant was mixed with 1 mL of phenol solution (5% *w*/*v*) and 5 mL of concentrated H_2SO_4 and cooled down at room temperature (30 min). The sample was measured at 485 nm, and the final content was calculated in % *w*/*w*. [103].

Table 2. Resolved design of experiments.

Standard Run	Block	$NaNO_3$	K_2HPO_4	KH_2PO_4	Carbon Content
		g/L	g/L	g/L	(g/L)
1	1	0.125	0.0375	0.0875	0.20
2		0.125	0.075	0.175	0.50
3		0.25	0.0375	0.0875	0.50
4		0.25	0.075	0.175	0.20
5 (Central point)		0.1875	0.056	0.131	0.35
6	2	0.125	0.0375	0.0875	0.50
7		0.125	0.075	0.175	0.20
8		0.25	0.0375	0.0875	0.20
9		0.25	0.075	0.175	0.50
10 (Central point)		0.1875	0.056	0.131	0.35
11	3	0.083	0.056	0.131	0.35
12		0.292	0.056	0.131	0.35
13		0.1875	0.0249	0.0581	0.35
14		0.1875	0.0876	0.2044	0.35
15		0.1875	0.056	0.131	0.099
16		0.1875	0.056	0.131	0.61
17 (Central point)		0.1875	0.056	0.131	0.35

The total lipids were measured using the method described by Mishra et al. [105]. Briefly, 5 mg of ground biomass were mixed with 2 mL of concentrated H_2SO_4 in acid-resistant test tubes and vortexed (Multi Reax, Heidoplh) at 1500 rpm for 5 min. The sample was then incubated (100 °C, 10 min) and cooled down in an ice bath for 5 min. Five milliliters of fresh phospho-vanillin reagent was added to the sample and incubated for a second time (37 °C, 15 min). The final sample was centrifuged ($2876\times g$, 20 min), the supernatant was measured at 530 nm, and the final content was calculated in % w/w.

The total content of proteins was measured using the method described by Mota et al. [106]. Briefly, 5 mg of grounded biomass was mixed with 0.5 mL of distilled water and 0.5 mL of 10% w/v of SDS solution and vortexed (Multi Reax, Heidoplh) at 1500 rpm for 10 min. The sample was centrifuged ($8000\times g$, 5 min), and 1 mL of the supernatant was diluted in 5 mL of reagent C using the Lowry method. Finally, 0.5 mL of Folin reagent (diluted 3 times) was added to the mixture and incubated at room temperature for 30 min. The sample was measured at 750 nm, and the final content was calculated in % w/w.

Finally, the content of total carotenoids was measured according to Hynstova et al. [107]. Briefly, 100 mg of grounded biomass was mixed with 1 mL of acetone and 10 mg of $MgCO_3$. The mixture was vortexed (Multi Reax, Heidoplh) at 2000 rpm for 10 min. The sample was then centrifuged ($8000\times g$, 20 min). The supernatant was diluted 4 times in acetone and measured at 470 nm, and the final content was calculated in % w/w.

3. Results

3.1. Production of Carbohydrates under Different C/N/P

The experimental data obtained from the C/N/P ratio on the production of carbohydrates were fitted on two models (linear (L) and quadratic (Q) with a p-value of 0.05 ($p = 0.05$)). The statistical software provided a number to identify them; in this case $NaNO_3$ was tagged with the number 1, followed by the phosphate buffer and carbon source: ($NaNO_3$ (1), K_2HPO_4 + KH_2PO_4 (2), and carbon source (3). This tag is used by the software to identify possible interactions between two or more variables (example: 1L_by_2L represents and interaction between the lineal model of $NaNO_3$ and K_2HPO_4 + KH_2PO_4). In the Y-axis of each Pareto chart can be found the analysis for every single factor adjusted by the two models (Q and L) with the numbers of the three variables evaluated (1, 2 or 3), and on the right side of each row, the assigned number allows us to understand which level has

the most effect. Lower numbers (-) indicate that lower levels of that specific variable are positively or negatively affecting the response.

According to the Pareto chart (Figure 1a), when sodium carbonate (Sc) is used as a carbon source, the carbohydrate synthesis is significantly affected by all three factors of the C/N/P ratio (p = 0.05). The surface response (Figure 1b) shows that high concentrations of $NaNO_3$ (0.15–0.25 g/L) and high concentrations of the carbon source (2.64–4.41 g/L of Na_2CO_3) positively affect the synthesis of carbohydrates up to 50% w/w of the total biomass. In the case when sodium bicarbonate (Sb) is employed, the phosphate buffer and the carbon source significantly affect the synthesis of carbohydrates (Figure 1c). The surface response (Figure 1d) shows that high concentrations of K_2HPO_4 and KH_2PO_4 (0.075 and 0.175 g/L, respectively), and high concentrations of the carbon source (2.1–3.49 g/L of $NaHCO_3$) positively affect the synthesis of carbohydrates up to 60% (w/w) of the total biomass. Finally, the Pareto chart of sodium acetate (Sa) (Figure 1e) shows that none of the analyzed nutrients in the designated concentrations significantly affects (p = 0.05) the synthesis of carbohydrates; however, the surface response (Figure 1f) shows that high concentrations of K_2HPO_4 and KH_2PO_4 (0.075 and 0.175 g/L, respectively), and high concentrations of the carbon source (0.85–1.36 of $C_2H_3NaO_2$) can increase the concentration of carbohydrates up to 45% (w/w) of the total biomass in *Chlorella* sp. UFPS019.

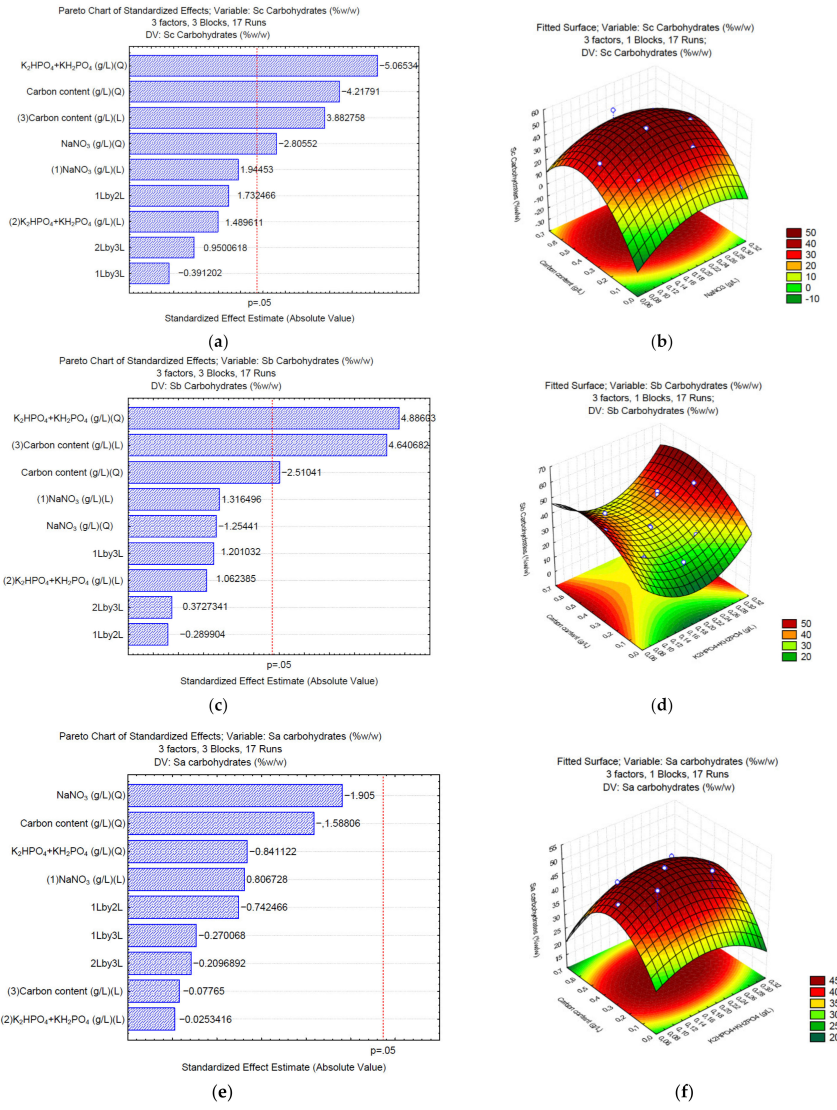

Figure 1. Pareto charts and surface response of carbohydrate production using Na_2CO_3 (**a**,**b**), $NaHCO_3$ (**c**,**d**), and $C_2H_3NaO_2$ (**e**,**f**).

3.2. Production of Proteins under Different C/N/P

In the case of protein synthesis, when sodium carbonate (Sc) is used, the Pareto chart (Figure 2a) shows no significant effect for any of the analyzed variables; however, according to the surface response (Figure 2b), high $NaNO_3$ (>0.25 g/L) and low concentrations of the carbon source (<1.76 g/L of Na_2CO_3) might increase the total content of proteins. In the case of sodium bicarbonate (Sb), the phosphate buffer and the carbon source significantly affect the synthesis of proteins (Figure 2c). The surface response (Figure 2d) shows that medium concentrations of K_2HPO_4 and KH_2PO_4 (0.0375 and 0.0875 g/L, respectively), and medium concentrations of the carbon source (1.39–3.49 g/L of $NaHCO_3$) positively affect the synthesis of proteins up to 40% *w*/*w* of the total biomass. Finally, the Pareto chart of sodium acetate (Sa) (Figure 2e) shows that the phosphate buffer, sodium nitrate, and the interaction between $NaNO_3$ and sodium acetate affect the synthesis of proteins. The surface response (Figure 2f) shows that high $NaNO_3$ (>0.25 g/L) and low concentrations of the carbon source (<1.5 g/L of $C_2H_3NaO_2$) might increase the total content of proteins in *Desmodesmus* sp UFPS021.

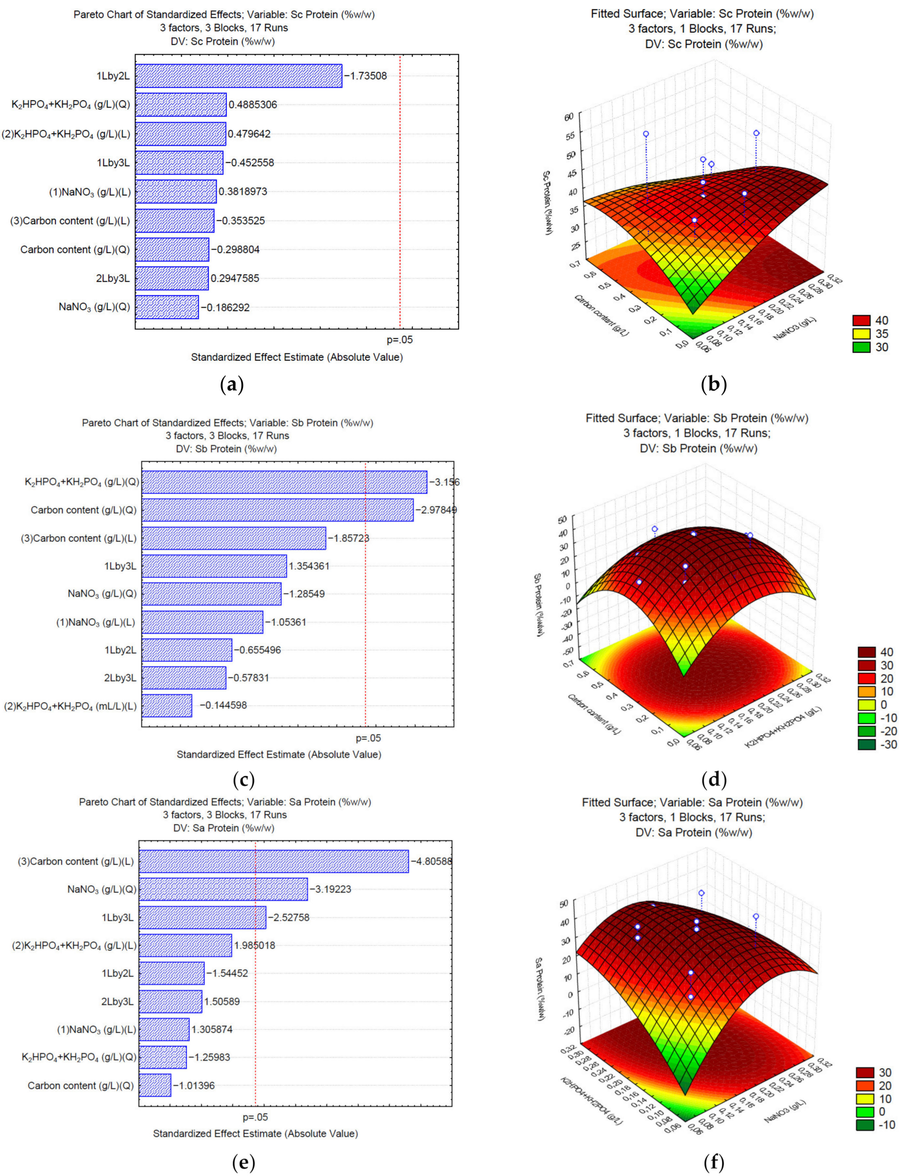

Figure 2. Pareto charts and surface response of protein production using Na_2CO_3 (**a**,**b**), $NaHCO_3$ (**c**,**d**), and $C_2H_3NaO_2$ (**e**,**f**).

3.3. Production of Lipids under Different C/N/P

To produce lipids, the use of sodium carbonate (Sa) as a carbon source shows that none of the analyzed variables significantly (p = 0.05) affects the final content of lipids (Figure 3a). The surface response (Figure 3b) shows that medium concentrations of K_2HPO_4 and KH_2PO_4 (0.0375 and 0.0875 g/L, respectively) and the carbon source (3 g/L of Na_2CO_3) might increase the total content of lipids. In the case of sodium bicarbonate (Sb), $NaNO_3$, and the interaction between the phosphate buffer and the carbon source significantly affect the synthesis of lipids (Figure 3c). The surface response (Figure 3d) shows that high concentrations of K_2HPO_4 and KH_2PO_4 (>0.075 and 0.175 g/L, respectively), and low concentrations of the carbon source (<0.7 g/L of $NaHCO_3$) positively affect the synthesis of lipids up to 30% w/w of the total biomass. Finally, the Pareto chart of sodium acetate (Sa) (Figure 3e) shows that sodium nitrate affects the synthesis of lipids. The surface response (Figure 3f) shows that low $NaNO_3$ (<0.075 g/L) and medium concentrations of the carbon source (0.68–1.7 g/L of $C_2H_3NaO_2$) might increase the total content of lipids in *Chlorella* sp. UFPS019.

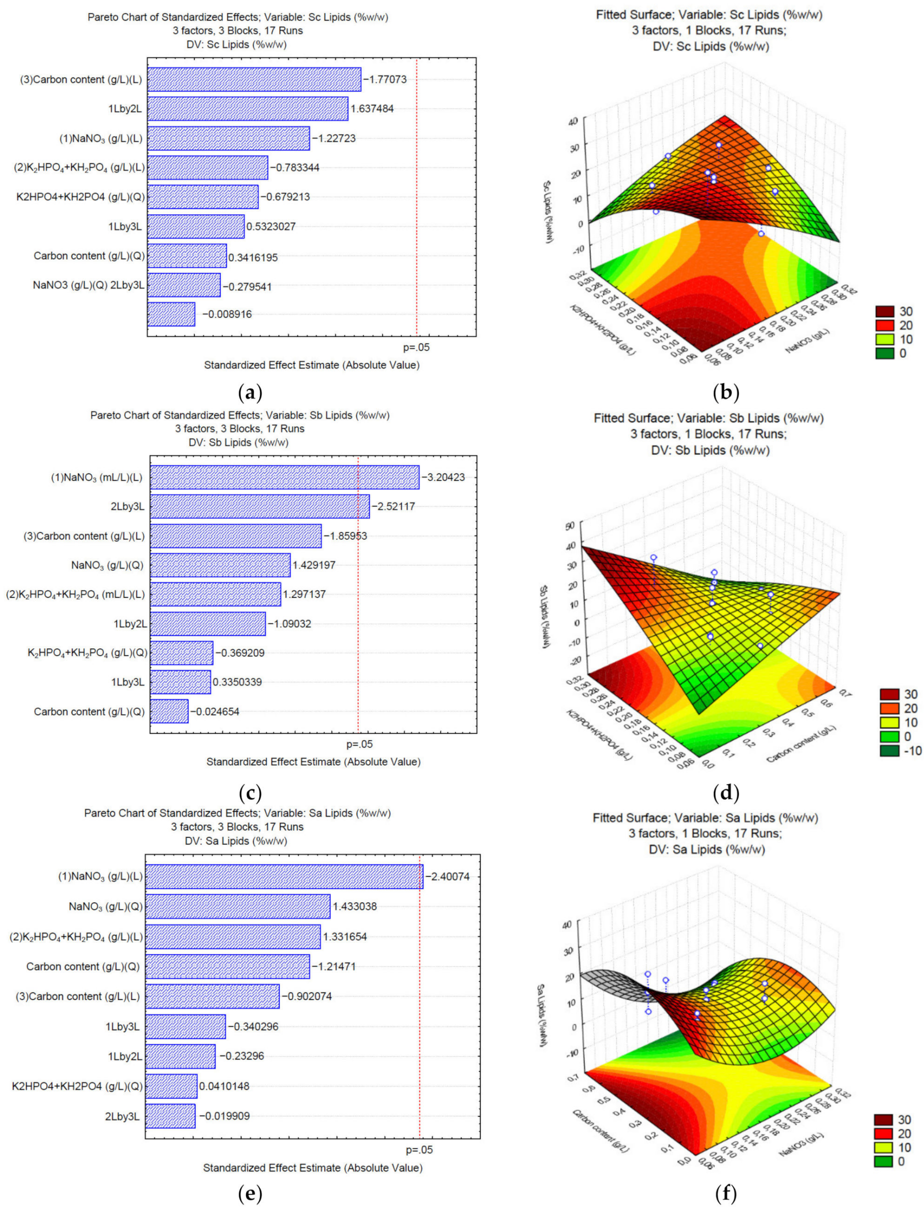

Figure 3. Pareto charts and surface response of lipid production using Na_2CO_3 (**a**,**b**), $NaHCO_3$ (**c**,**d**), and $C_2H_3NaO_2$ (**e**,**f**).

3.4. Production of Total Carotenoids under Different C/N/P

In the case of total carotenoids, none of the analyzed variables in any of the different carbon sources studied (Figure 4a,c,e) showed a significant effect. In the case of Na_2CO_3 (Sa), the surface response (Figure 4b) shows that lower concentrations of the carbon source (<0.9 g/L of Na_2CO_3) and K_2HPO_4, and KH_2PO_4 (<0.0375 and 0.0875 g/L, respectively) might increase the content of total carotenoids. In the case of $NaHCO_3$ (Sb) (Figure 4d), lower content of carbon source (<0.7 g/L of $NaHCO_3$) and high levels of K_2HPO_4, and KH_2PO_4 (>0.075 and 0.175 g/L, respectively) can increase the concentration of total carotenoids. Finally, when sodium acetate (Sa) is used (Figure 4f), high levels of $NaNO_3$ (>0.25 g/L) and K_2HPO_4 and KH_2PO_4 (>0.075 and 0.175 g/L, respectively) can increase the final content of total carotenoids in *Desmodesmus* sp. UFPS020.

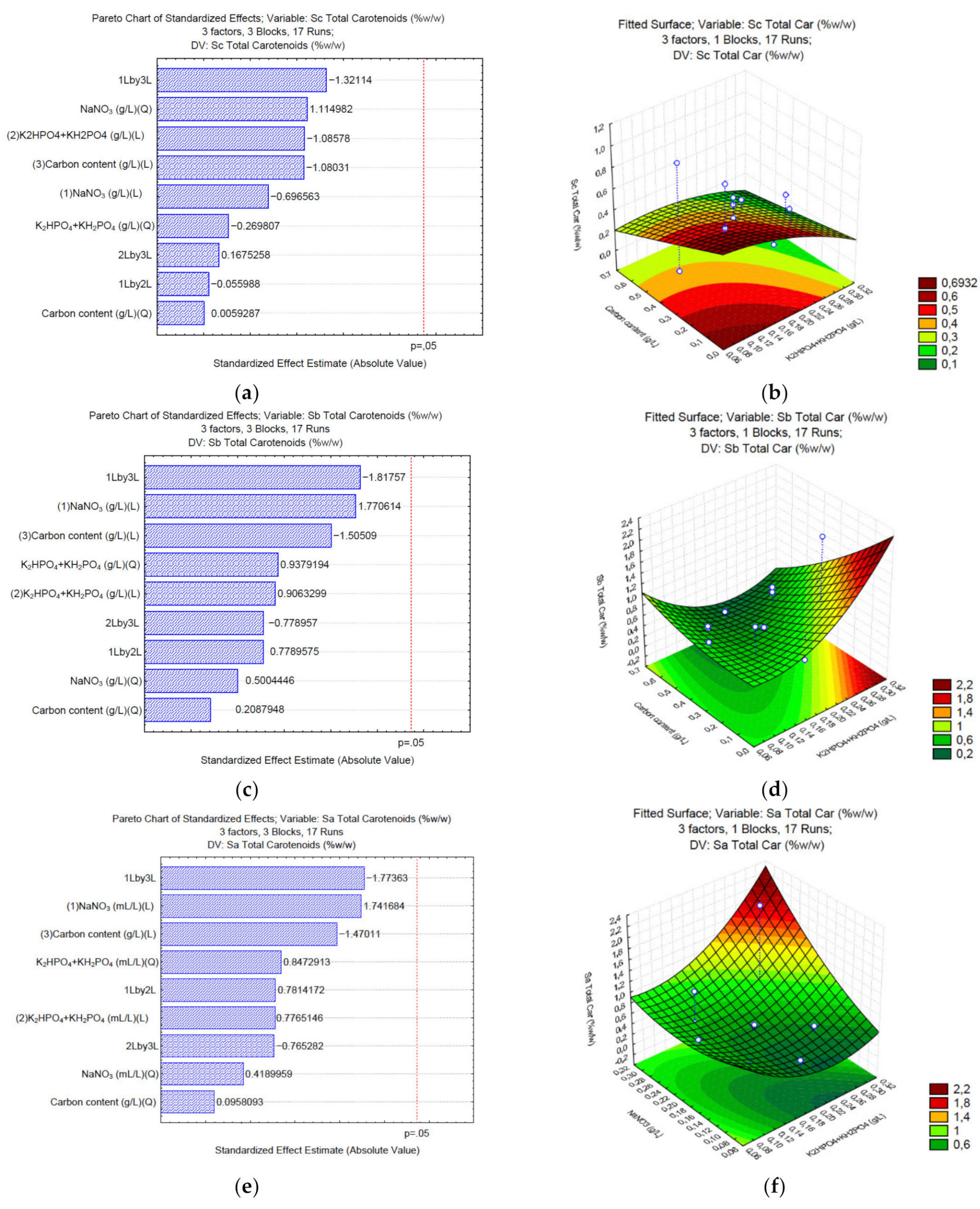

Figure 4. Pareto charts and surface response of total carotenoids production using Na_2CO_3 (**a**,**b**), $NaHCO_3$ (**c**,**d**), and $C_2H_3NaO_2$ (**e**,**f**).

By analyzing the results from the interactions in the C/N/P ratio, the values of the three variables were adjusted to increase the production of carbohydrates, proteins, and

lipids in *Chlorella* sp. UFPS019 and *Desmodesmus* sp. UFPS021. Table 3 represents the highest scenarios for increasing each metabolite in the designated strains. Both algae were grown in a 10 L flask (0.6 L/min of filtered air, 12:12 h light/dark cycle at 100 μmol·m^{-2}·s^{-1}) for 20 days for carbohydrates and proteins, and 40 days for lipids. As a control, both strains were grown in Bold's basal medium without any modification. The biomass produced was concentrated by electroflocculation [104]. The culture media were filtered on 0.45 μm GF-C (Sartorius, Germany) to remove excess cells, and the media were analyzed for the final content of NO_3 and PO_4 using HANNA test kits (HI 93728-01, and HI 93713-01).

Table 3. Variables for optimal biomass concentration in both strains were studied.

Strain	Variable	Units	Value
Chlorella sp. UFPS019	Na_2CO_3	g/L	3.53
	KH_2PO_4		0.06
	K_2HPO_4		0.14
	$NaNO_3$		0.1875
	carbohydrates	% *w*/*w*	50
	$C_2H_3NaO_2$	g/L	1.19
	KH_2PO_4		0.056
	K_2HPO_4		0.131
	$NaNO_3$		0.075
	Lipids	% *w*/*w*	27.5
Desmodesmus sp. UFPS021	$C_2H_3NaO_2$	g/L	2
	KH_2PO_4		0.056
	K_2HPO_4		0.131
	$NaNO_3$		0.25
	Proteins	% *w*/*w*	38.6

The experimental data for each of the metabolites were adjusted to the exponential or logarithmic phase of microalgal growth, in which the maximum specific speed (μmax), saturation, and affinity constants for NO_3 and PO_4 (K_s), as well as the yield coefficient, define the biomass production rate from a mass measure ($Y_{\frac{X}{S}}$). Table 4 presents the data obtained for each strain under optimal conditions.

Table 4. Growth parameters for production of carbohydrates, proteins, and lipids.

Chlorella sp. UFPS019 (Carbohydrates)		
μ	0.0029 h^{-1}	
dt	120 h^{-1}	
	NO_3	PO_4
$Y_{\frac{X}{S}}$	0.2819	0.0698
K_s	0.1885	0.0768
Chlorella sp. UFPS019 (Lipids)		
μ	0.00647 h^{-1}	
dt	120 h^{-1}	
	NO_3	PO_4
$Y_{\frac{X}{S}}$	0.1566	0.0318
K_s	0.004	0.001209
Desmodesmus sp. UFPS021 (Proteins)		
μ	0.00567 h^{-1}	
dt	120 h^{-1}	
	NO_3	PO_4
$Y_{\frac{X}{S}}$	0.36	0.0775
K_s	0.0284	0.0772

Figure 5 shows that to produce both carbohydrates and proteins, the two strains were still in the exponential growth phase, while the algae had to be in the late stationary phase to produce lipids. On the other hand, it was shown that for all experiments, NO_3 was consumed entirely after 20 days, while the concentration of PO_4 varied. In the case of the optimized medium for carbohydrate production, 55% of the phosphate was consumed, while protein and lipid consumption was higher (70%).

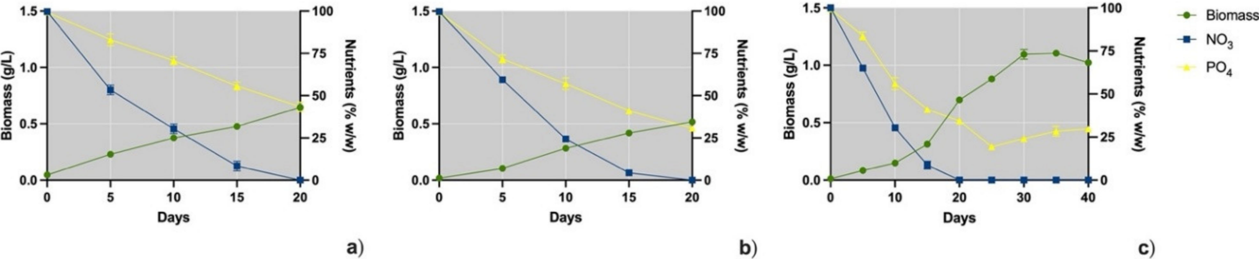

Figure 5. Biomass production and consumption of NO_3 and PO_4 in the optimized C/N/P ratio for production of carbohydrates (**a**), proteins (**b**) and lipids (**c**).

The results obtained for the different metabolites were analyzed using one-way ANOVA (Figure 6). This result shows that the concentrations of carbohydrates, proteins, and lipids under the optimized conditions were statistically significant in comparison with the results expected from the surface response and the control. The latter indicated that the proposed method effectively enhances the production of the different metabolites in the studied strains.

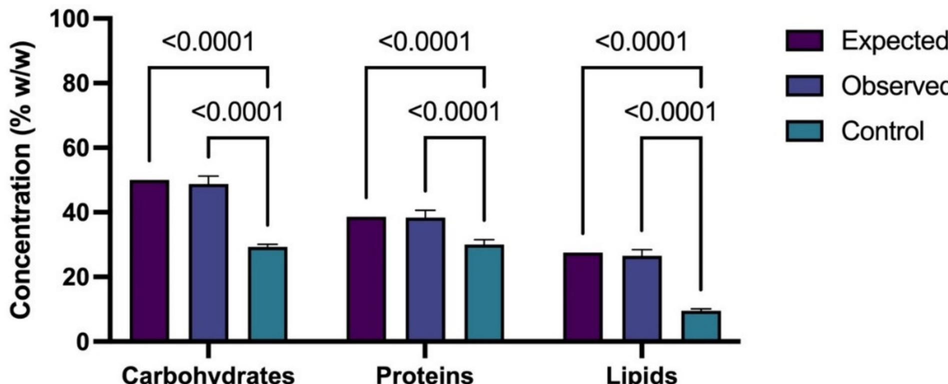

Figure 6. One-way ANOVA between the expected and obtained results to produce carbohydrates and lipids in *Chlorella* sp. UFPS019 and proteins in *Scenedesmus* sp. UFPS021.

4. Discussion

The optimization of culture media is considered one of the key processes to improve the production of biomass and target metabolites, whereby it is possible to adjust the concentrations of certain nutrients (both macro and micronutrients) depending on the strain. The enhancement of the different metabolites can be manipulated through growth conditions; therefore, the main strategies include nutritional factors such as nutrient limitation/supplementation (nitrogen, phosphorus, sulfur, iron), and continuous addition of carbon source, as well as factors such as light intensity, pH, temperature, and salinity [108]. In recent years, different researchers have demonstrated the efficiency of this approach, especially using one-factor-at-a-time (OFAT) experiments, which involve the adjustment of one factor while the others are kept constant. Examples of this approach can be found in the scientific literature with a wide range of strains including (but not limited to) *Botryococcus braunii* [109], *Chlorella vulgaris* [110], *Chlorococcum* sp. (Chlorophyta) [111], *Vischeria vischeri* (formerly *Eustigmatos vischeri*) (Ochrophyta, Eustigmato-

phyceae) [112], *Isochrysis galbana* (Haptophyta, Coccolithophyceae) [113], *Lobosphaera incisa* (Chlorophyta) [114], and *Picochlorum soloecismus* (Chlorophyta) [115]. However, OFAT is considered time-consuming and labor-intensive when several variables may affect the outcome. In this case, due to their intrinsic nature of combining multiple salts in different concentrations, algal culture media are a perfect candidate for more robust and advanced methodologies. In recent years, the optimization of culture media and extraction of different metabolites through the application of design of experiments (DoE) has become more common [116–120]. Table 5 present a list of the most recent papers where the improvement of a specific metabolite was achieved through the application of DoE.

The most evaluated metabolites are lipids (including hydrocarbons) [23,93,97,121–130]. In the second position can be found the optimization of carbohydrate synthesis [93,131,132], followed by the enhancement of protein synthesis [97,98]. The latter has occurred due to the international interest in the application of algal biomass as a replacement for biofuel production, which remains a strong alternative within the international scientific community [32]. In the quest to improve the synthesis of each of the algal metabolites, the most studied components are the nitrogen and carbon sources [121–129]. According to de Farias Silva et al. [133], nitrogen limitation causes an interruption in amino acid synthesis, and photosynthetically fixed carbon in the Calvin cycle is then converted to carbohydrates. Another nutrient of great importance is phosphate, which is essential for starch and sucrose synthesis; however, high concentrations of P inhibit the action of enzymes such as ADP-glucose phosphorylase [134].

Table 5. List of reported algal strains with culture media optimized via design of experiments.

<table>
<tr><th>Strain</th><th>Biomass (g/L)</th><th>Productivity (g/L·d^{-1})</th><th>Carbohydrates (% w/w)</th><th>Proteins (% w/w)</th><th>Lipids (% w/w)</th><th>References</th></tr>
<tr><td>Ankistrodesmus falcatus KJ671624</td><td>1.74</td><td>0.124</td><td colspan="2">n/a</td><td>59.6</td><td>[121]</td></tr>
<tr><td>Auxenochlorella protothecoides (formerly Chlorella protothecoides)</td><td>1.06</td><td>0.71</td><td colspan="2">n/a</td><td>43.9</td><td>[122]</td></tr>
<tr><td>Auxenochlorella pyrenoidosa (formerly Chlorella pyrenoidosa)</td><td>0.89</td><td></td><td colspan="2">n/a</td><td></td><td>[98]</td></tr>
<tr><td>Botryococcus sp.</td><td>0.28</td><td colspan="3">n/a</td><td>73</td><td>[123]</td></tr>
<tr><td>B. braunii</td><td>2</td><td>0.133</td><td colspan="2">n/a</td><td>70</td><td>[24]</td></tr>
<tr><td>B. braunii LB572</td><td>4.57</td><td>0.18</td><td colspan="2">n/a</td><td>64.9</td><td>[124]</td></tr>
<tr><td>Chlorella sp.</td><td>0.44</td><td colspan="3">n/a</td><td>55</td><td>[123]</td></tr>
<tr><td>Chlorella protothecoides UTEX 250</td><td>1.19</td><td colspan="3">n/a</td><td>12.9</td><td>[125]</td></tr>
<tr><td>C. sorokiniana 211-32</td><td>1.18</td><td>0.039</td><td colspan="2">n/a</td><td>38</td><td>[126]</td></tr>
<tr><td>C. sorokiniana UTEX 1602</td><td>0.68</td><td colspan="3">n/a</td><td>9</td><td>[127]</td></tr>
<tr><td>C. sorokiniana UTEX 2805</td><td>0.66</td><td>n/a</td><td>52</td><td colspan="2">n/a</td><td rowspan="2">[131]</td></tr>
<tr><td>C. vulgaris UTEX 2714</td><td>0.24</td><td>n/a</td><td>59</td><td colspan="2">n/a</td></tr>
<tr><td>C. vulgaris UTEX 1803</td><td>3.7</td><td colspan="2">n/a</td><td>60</td><td>n/a</td><td>[95]</td></tr>
<tr><td>C. vulgaris FSP-E</td><td>5.51</td><td></td><td>51.3</td><td></td><td></td><td>[132]</td></tr>
<tr><td>Chlorococcum oleofaciens</td><td>1.6</td><td></td><td></td><td></td><td>20</td><td>[97]</td></tr>
<tr><td>Dunaliella salina</td><td>n/a</td><td>0.035</td><td colspan="2">n/a</td><td>22</td><td rowspan="2">[128]</td></tr>
<tr><td>D. tertiolecta</td><td>n/a</td><td>0.044</td><td colspan="2">n/a</td><td>23</td></tr>
<tr><td>D. parva</td><td>n/a</td><td>0.045</td><td colspan="2">n/a</td><td>39</td><td>[129]</td></tr>
<tr><td>Desmodesmus armatus</td><td>1.65</td><td colspan="2">n/a</td><td>53.6</td><td>n/a</td><td>[96]</td></tr>
<tr><td>Scenedesmus sp. ASK22</td><td>4.67</td><td></td><td>31.06</td><td></td><td>37.1</td><td>[130]</td></tr>
<tr><td rowspan="4">S. obliquus</td><td colspan="2">n/a</td><td>10–17</td><td>50–56</td><td>12–14</td><td>[128]</td></tr>
<tr><td>2.63</td><td>n/a</td><td>40</td><td colspan="2">n/a</td><td>[132]</td></tr>
<tr><td>0.685</td><td colspan="3">n/a</td><td>39.6</td><td>[93]</td></tr>
<tr><td></td><td colspan="2">n/a</td><td></td><td>66</td><td>[23]</td></tr>
<tr><td>S. vacuolatus</td><td>2</td><td colspan="3">n/a</td><td>4%</td><td>[90]</td></tr>
<tr><td rowspan="2">Chlorella sp. UFPS019</td><td>0.72</td><td>0.036</td><td>48.8</td><td colspan="2">n/a</td><td rowspan="3">This research</td></tr>
<tr><td>1.5</td><td>0.048</td><td colspan="2">n/a</td><td>26.5</td></tr>
<tr><td>Desmodesmus sp. UFPS021</td><td>0.95</td><td>0.038</td><td>n/a</td><td>38.4</td><td>n/a</td></tr>
</table>

In microalgae, carbohydrates are generally found in different concentrations depending on the species and culture conditions; however, few species can synthesize a high carbohydrate content [134]. *Porphyridium cruentum* can accumulate carbohydrates up to 57% of its dry weight, while *Chlamydomonas* sp. normally does not exceed 17% [135]. According to Dragone et al. [136], *C. vulgaris* can accumulate between 9 and 41% of its total weight (in dry weight), while *S. obliquus* can accumulate between 10 and 47% (in dry weight). According to Muthuraj et al. [137], in *Chlorella* sp., nitrogen reduction in the culture medium enhanced the accumulation of carbohydrates (up to 66% *w*/*w*). Under sufficient inorganic phosphate levels, *Chlorella* sp. FC2 IITG presented high carbohydrate content (up to 47.35% *w*/*w*), whereas under deficient conditions, the content was significantly reduced (32.21% *w*/*w*). The results obtained in this paper are in accordance with Tourang et al. [138], where the interaction between the carbon (low to medium content) and phosphate source (medium to high content) can effectively enhance the final concentration of carbohydrates in *Chlorella* sp. UFPS019.

In the case of lipids, a method focused on the enhancement of lipid and TAG biosynthesis through the selective deficiency of specific nutrients (such as phosphorus and nitrogen) coupled with salinity stress is the most widely used protocol [128], since N and P availability in the media will play an essential role in the synthesis of amino acids and proteins [139]. Examples such as the optimization of *Dunaliella parva* via response surface methodology prove that a high content of $NaNO_3$ (0.63 g/L), NaCl (1.61 M), and low KH_2PO_4 (0.02 g/L) will increase the final content of lipids up to 1.4 times [128]. In the specific context of this research, the concentration of nitrate employed in the optimization was 0.075 g/L of $NaNO_3$, while the concentrations of KH_2PO_4 and K_2HPO_4 were 0.056, and 0.131 g/L, respectively. These results are similar to those reported using *Selenastrum* sp. GA66 (0.06 g/L of $NaNO_3$ and 2-fold increase in total lipids) [140]. However, the evaluation of the C-N-P ratio is a rare approach that has not been studied in-depth.

Since CO_2 is the most common carbon source, the analysis of other inorganic sources and even some organic carbon sources has been limited over the years. Our results prove that the proper adjustment of not only N and P, but also the specific addition of an organic source, will effectively increase the final content of total lipids in this *Chlorella* sp. UFPS019 strain. However, in the long run, this method (especially when nitrogen is reduced) will substantially reduce the final content of proteins and the overall biomass produced in the selected strains. Regarding carotenoids, the results demonstrated that all the different experiments were successful in maximizing carotenogenesis. The latter may be due to the lack of other stressful conditions such as light and salinity [141,142]. Finally, to produce proteins, both algae and cyanobacteria require adequate concentrations of carbon, nitrogen, and phosphate sources, since proteins and amino acids are the building blocks for the synthesis of multiple enzymes and different cellular processes [143,144]. Therefore, excesses of N and P will allow the fast synthesis of different proteins, which is reflected in the optimization conditions obtained in this research (0.25 g/L of $NaNO_3$ and 1.875 g/L of KH_2PO_4 + K_2HPO_4).

5. Conclusions

The optimal C/N/P ratios for the synthesis of carbohydrates, proteins, and lipids in two high-mountain algal strains, *Chlorella* sp. UFPS019, and *Desmodesmus* sp. UFPS021, were determined. In *Chlorella* sp. UFPS019, the optimal conditions to enhance the synthesis of carbohydrates were high sodium carbonate content (3.53 g/L), high KH_2PO_4 and K_2HPO_4 content (0.06 and 0.14 g/L, respectively), and medium-high $NaNO_3$ (0.1875 g/L). In the case of lipids, a high concentration of sodium acetate (1.19 g/L) coupled with high KH_2PO_4 and K_2HPO_4 content (0.056 and 0.131 g/L, respectively) and a low concentration of $NaNO_3$ (0.075 g/L) drastically improved the synthesis of lipids. In the case of *Desmodesmus* sp. UFPS021, the protein content was increased using high sodium acetate (2 g/L), high KH_2PO_4 and K_2HPO_4 content (0.056 and 0.131 g/L, respectively), and a high $NaNO_3$ concentration (0.25 g/L). Further studies should focus on the possible interactions

between other stress-inducing nutrients (Na, Mg, Ca, etc.) and LEDs that can eventually maximize the final content of these metabolites.

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