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Fatty Acid Biosynthesis from *Chlorella* in Autotrophic and Mixotrophic Cultivation

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HIGHLIGHTS

- Microalgae biomass can be used as a food, pharmaceuticals, and energy production.
- *Chlorella* is a microalga whose composition is influenced by cultivation conditions.
- We evaluated seven different variables in autotrophic and mixotrophic cultures.
- Photoperiod and sodium bicarbonate showed the greatest influence on growth and PUFA.

Abstract: Microalgae are photosynthetic microorganisms whose composition and biomass production can be influenced by manipulating the cultivation conditions employed. However, few studies have evaluated the effects of various cultivation conditions in autotrophic and mixotrophic conditions. The present work aimed to evaluate the effects of cultivation conditions on the cell growth and biosynthesis of fatty acids (FAs) by microalgae of the genus *Chlorella* in autotrophic and mixotrophic cultivation. Evaluation of the effects of the conditions was performed using an experimental design methodology. The highest values of maximum biomass concentration (X_{max}) and maximum biomass productivity (P_{max}) were obtained in autotrophic cultures. Palmitic acid was the FA obtained at the highest concentration in both cultivation modes. The concentrations of polyunsaturated FAs (PUFAs) ranged from 12.2 to 41.2% in autotrophic cultures and from 11 to 34.3% in the mixotrophic cultures. The variables photoperiod and sodium bicarbonate concentration showed the greatest influence on the X_{max}, P_{max}, and PUFA concentration in autotrophic and mixotrophic cultivations, respectively. This study verified that the selection of conditions and mode of cultivation contribute to the production of microalgal biomass and FA biosynthesis.

Keywords: cultivation conditions; experimental design; lipids; microalgae; polyunsaturated fatty acids.

INTRODUCTION

Microalgae are microorganisms capable of efficiently converting solar energy and nutrients through photosynthesis into biomass and oxygen [1]. The biomass produced is composed of biomolecules such as proteins, lipids, and carbohydrates that can present potential applications in food [2], pharmaceuticals, cosmetics and energy production [3]. Among the biocompounds produced by microalgae, fatty acids stand out as they have high application potential in the production of biofuels and in the food industry [4]. Fatty acids are mostly found as 18-carbon atom molecules with a vegetable oil-like composition and are classified into saturated, unsaturated, or polyunsaturated types [5].

Production of microalgal biomass requires nutrients such as carbon, nitrogen, and phosphorous for its cultivation. Carbon is one of the main constituents of microalgae, representing between 30 and 50% (w w^{-1}) of dry biomass [6]. Microalgae have the ability to convert inorganic [7,8] and organic carbon from different biomass sources [9-11]. In autotrophic conditions, microalgae fix carbon dioxide (CO_2) and/or soluble carbonates by photosynthesis in biomass rich in reserve compounds such as lipids. In the mixotrophic conditions, these microorganisms have their cultivation supplemented with an organic carbon source in addition to CO_2, which may result in higher biomass productivities.

Several studies have reported the application of organic waste in microalgae cultivation. In this sense, these organic sources appear as an alternative to reduce process costs, as well as contribute to the production of high value-added products such as fatty acids [9,12-15]. In microalgae cultivation, besides the carbon source, aspects such as strain species, nutritional conditions (deprivation and increase of nutrient concentration), and physicochemical changes (pH, temperature and light) may promote stress conditions for cultivation. Changes in metabolism may occur in response to these conditions, which may contribute to the biosynthesis of compounds such as lipids and carbohydrates [16-20]. In this context, selection of cultivation conditions can contribute to the determination of the best biomass production parameters and the biosynthesis of high value compounds. Considering the above, this work aimed to evaluate the effects of cultivation conditions on the growth and fatty acids biosynthesis in autotrophic and mixotrophic cultivation of microalgae of the genus *Chlorella*.

MATERIAL AND METHODS

Microorganisms and Culture Medium

The microalgae used in this study were *Chlorella vulgaris* and *Chlorella kessleri*, obtained from the Collection of Cultures of the Laboratory of Biochemical Engineering (LEB) of the Federal University of Rio Grande (FURG). The inoculum of every strain was maintained in MBM (Modified Bristol's Medium) culture medium [21].

Experimental Conditions

Screening variables using plackett-burman design

In the present study, a Plackett-Burman statistical experimental design (Screening Design) was used to select variables of the process and nutritional factors that significantly affect the responses of microalgal cultures. Plackett-Burman (PB) experimental design is considered a useful tool to determine the effects of a large number of variables on the evaluated responses [22,23].

For each cultivation condition (autotrophic and mixotrophic), 8 assays were conducted (in duplicate) and the effects of 7 independent variables at two levels (–1 low level; +1 high level) on growth responses and lipid profiles in the microalgal cultures were examined.

In the first batch of assays (autotrophic condition) (Table 1), the effects of 7 independent variables were studied: microalgae species, photoperiod, carbon dioxide concentration (CO_2), sodium nitrate concentration ($NaNO_3$), iron concentration (Fe), culture medium, and initial biomass concentration (X_0).

The $CO_{2(g)}$ was mixed with compressed air according to the concentrations established in the experimental design, and the mixture was supplied to the cultures for 15 min at every 1 h during the light period [24].

Table 1. Plackett-Burman experimental design matrix for the evaluation of the variables in autotrophic cultivation.

Assay	Microalgae	Photoperiod	CO_2 (%, v v^{-1})	$NaNO_3$ (mg L^{-1})	Fe (mg L^{-1})	Culture Medium*	X_0 (g L^{-1})
A1	*C. vulgaris*	12h/12h	6	75	8	F/2	0.2
A2	*C. kessleri*	12h/12h	6	10	4	F/2	0.4
A3	*C. vulgaris*	24h/0h	6	10	8	MBM	0.4
A4	*C. kessleri*	24h/0h	6	75	4	MBM	0.2
A5	*C. vulgaris*	12h/12h	12	75	4	MBM	0.4
A6	*C. kessleri*	12h/12h	12	10	8	MBM	0.2
A7	*C. vulgaris*	24h/0h	12	10	4	F/2	0.2
A8	*C. kessleri*	24h/0h	12	75	8	F/2	0.4

MBM composition (g L^{-1}): KNO_3 (0.25); $CaCl_2$ (0.01); $MgSO_4.7H_2O$ (0.075); K_2HPO_4 (0.075); KH_2PO_4 (0.175); NaCl (0.025); $FeSO_4.7H_2O$ (0.02); A5 Solution (1.0 mL L^{-1}); A5* Solution (g L^{-1}) - H_3BO_3 (2.9); $MnCl_2.4H_2O$ (1.81); $ZnCl_2$ (0.11); $CuSO_4.5H_2O$ (0.08); $3(NH_4)_2O.7MoO_3.4H_2O$ (0.018) [21].
*F/2 composition to one liter of the seawater: Major Nutrients: $NaNO_3$ (75 mg); $NaH_2PO_4.H_2O$ (5 mg); Vitamins: Thimine-HCl (0.1 mg); Biotin (0.5 µg); B12 (0.5 µg); Trace metals: Na_2EDTA^+ (4.36 mg); $FeCl_3.6H_2O^+$ (3.15 mg); $CuSO_4.5H_2O$ (0.01 mg); $ZnSO_4.7H_2O$ (0.022 mg); $CoCl_2.6H_2O$ (0.01 mg); $MnCl_2.4H_2O$ (0.18 mg); $Na_2MoO_4.2H_2O$ (0.006 mg) [25].

In the second batch of assays (mixotrophic condition), the effects of 7 independent variables were studied: microalgae species, organic carbon source (glucose and industrial oleaginous residue -IOR), organic carbon concentration (C_O), sodium nitrate concentration ($NaNO_3$), sodium bicarbonate ($NaHCO_3$), culture medium, and initial biomass concentration (X_0) (Table 2). The photoperiod used in mixotrophic cultures was 12 h light/dark [26]. The industrial oleaginous residue (IOR) used as an organic carbon source was composed of 28.4% sucrose, 18.6% stachyose, 9.7% raffinose, 0.25% galactose, 0.24% glucose, and 0.13% fructose.

The assays conducted under autotrophic and mixotrophic conditions were carried out in Erlenmeyer photobioreactors (2 L), with a working volume of 1.5 L and under controlled conditions at 30 °C and illuminance of 41.6 $\mu mol_{photons}$ m^{-2} s^{-1} for 15 d. The cultures were stirred continually by supplying compressed air at a flow rate of 0.3 vvm (volume of air per volume of medium per minute) (450 mL_{air} min).

Table 2. Plackett-Burman experimental design matrix for the evaluation of the variables in mixotrophic cultivation.

Assay	Microalgae	Carbon source	C_O (g L^{-1})	$NaNO_3$ (mg L^{-1})	$NaHCO_3$ (g L^{-1})	Culture Medium	X_0 (g L^{-1})
M1	*C. vulgaris*	Glucose	1.0	75	16.8	F/2	0.2
M2	*C. kessleri*	Glucose	1.0	10	1.0	F/2	0.4
M3	*C. vulgaris*	IOR*	1.0	10	16.8	MBM	0.4
M4	*C. kessleri*	IOR*	1.0	75	1.0	MBM	0.2
M5	*C. vulgaris*	Glucose	5.0	75	1.0	MBM	0.4
M6	*C. kessleri*	Glucose	5.0	10	16.8	MBM	0.2
M7	*C. vulgaris*	IOR*	5.0	10	1.0	F/2	0.2
M8	*C. kessleri*	IOR*	5.0	75	16.8	F/2	0.4

*IOR - industrial oleaginous residue

The culture medium employed as variables were F/2 (medium formulated with half the concentration of the nutrients of Medium F) [25] and MBM (Modified Bristol's Medium) [21]. These culture mediums were prepared without the original nutrients that were varied in the experimental design (carbon and nitrogen). Nutrient supplementation was carried out with concentrated solutions followed by directly adding to each assay. Evaporation was controlled by maintaining the volume of the cultures by daily replacement with sterile distilled water.

Analytical determinations

Biomass concentration

The biomass concentration of the cultures was determined in duplicate (n = 2) by measuring optical density at 670 nm in a digital spectrophotometer (Shimadzu UV/VIS Mini-1240, Japan) every 24 h, from a standard curve that correlates optical density with biomass dry weight [27].

Quantification of total lipids

At the end of the experiments, the biomass was recovered from the liquid medium by centrifugation (15200 *g*, 20 ºC, 10 min) and then dried in an oven with forced air circulation at 40 ºC for 24 h. Total lipids were extracted and quantified in duplicate (n = 2) according to the method proposed by Bligh and Dyer [28].

Esterification of lipids and fatty acid profile

The esterification of lipids to obtain esters of fatty acids was carried out in duplicate (n = 2) according to the method proposed by Metcalfe and Schmitz [29]. Determination of the fatty acid profile in every assay was performed by gas chromatography on a gas chromatograph (Varian 3400CX), equipped with a flame ionization detector and fused silica column containing a stationary phase of polyethylene glycol (30 m in length and 0.32 mm diameter). Nitrogen was used as the carrier gas at a flow rate of 0.5 mL min^{-1}. The injector and detector temperatures were 250 °C and 280 °C, respectively. The initial column temperature was 100 °C and then there was an increase of 8 °C min^{-1} up to 230 °C, remaining at this temperature for 20 min. Fatty acids were identified by comparing sample retention times with standards (Sigma, USA) and were quantified by normalization of peak areas.

Evaluated growth responses

The maximum biomass concentration (X_{max}, g L^{-1}) of every assay was obtained by the microalgae growth profile. The biomass productivity (Px, mg L^{-1} d^{-1}) was calculated according to the equation $Px = (X_t - X_{t-1})\ t^{-1}$, where X_t (mg L^{-1}) is the biomass concentration at time t (d). The maximum biomass productivity (P_{max}) was considered the highest response obtained in each assay.

Statistical analysis

The mean results obtained in each assay were taken as responses and were evaluated by experimental design methodology using Statistica 6.0 software. Standard error was calculated through the global analysis of results with a confidence level of 95%.

RESULTS AND DISCUSSION

Biomass concentrations and maximal biomass productivity (Table 3) were higher in autotrophic cultures. Analyzing the effects presented in Table 4 for autotrophic cultures, the independent variables photoperiod, initial biomass concentration, iron, and carbon dioxide concentration had a significant effect ($p < 0.05$) on the maximum biomass concentration. The variables photoperiod and initial biomass concentration had a positive effect on X_{max}, confirming that exposure to higher light periods and higher initial concentration may provide an increase in X_{max}.

Table 3. The mean results of maximum biomass concentration (X_{max}, g L^{-1}) and maximum biomass productivity (P_{max}, mg L^{-1} d^{-1}) for autotrophic and mixotrophic cultures.

Assay	Autotrophic cultivation		Mixotrophic cultivation	
	X_{max}	P_{max}	X_{max}	P_{max}
1	0.51	256.4	0.19	22.0
2	0.87	106.0	0.70	122.3
3	0.97	157.8	0.42	74.9
4	0.90	112.4	0.45	80.3
5	0.70	135.3	0.80	176.8
6	0.36	32.8	0.29	99.5
7	0.82	124.2	0.63	180.7
8	0.81	94.8	0.53	38.1

Light is essential and often limiting for the growth of microalgae and is considered a determinant factor for the photosynthetic rate [30]. According to Wahidin and coauthors [17], the light regime, which includes luminous intensity and photoperiod, is considered one of the main factors that influence the growth and biochemical composition of microalgae.

According to Gonçalves and coauthors [18], exposure of microalgae cultures to high periods and light intensities promotes higher growth rates and biomass productivity. Using assays carried out with the microalgae *Chlorella vulgaris*, exposure of the cells to high light intensity (180 µE m^{-2} s^{-1}) and a photoperiod of 24:0 h (light: dark) resulted in an increase in the biomass concentration (1.35 g L^{-1}). In the current study, similar behavior to that verified by Gonçalves and coauthors [18] was observed, with an increase of 0.27 g L^{-1} in the X_{max} response when the assays were exposed to a 24-h light photoperiod.

CO_2 concentration and Fe concentration exerted a negative effect, and in a lower modulus in relation to X_{max}. Therefore, this effect demonstrates that the increase in CO_2 and Fe concentrations may cause X_{max} reduction by 0.14 and 0.16 g L^{-1}, respectively. Liu and coauthors [31] in cultures with *C. vulgaris* in F/2 medium supplemented with 0.67 mg L^{-1} of Fe, did not observe an increase in cell concentration, but the biomass obtained presented a high concentration of total lipids (56.6%).

Table 4. Effects estimate of independent variables about the responses of X_{max} and P_{max} in autotrophic cultivation.

Factor	X_{max}			
	Effect	***s. e**	**t (8)**	**p**
Mean	0.74	0.02	29.6	< 0.001
Microalgae	-0.02	0.05	-0.31	0.763
Photoperiod	0.27	0.05	5.34	< 0.001
CO_2	-0.14	0.05	-2.77	0.024
$NaNO_3$	-0.03	0.05	-0.56	0.590
Fe	-0.16	0.05	-3.13	0.014
Culture Medium	0.02	0.05	0.42	0.690
X_0	0.19	0.05	3.79	0.005
	P_{max}			
	Effect	***s. e**	**t (8)**	**p**
Mean	127.4	10.4	12.2	< 0.001
Microalgae	-81.9	20.9	-3.92	0.004
Photoperiod	-10.3	20.9	-0.49	0.634
CO_2	-61.3	20.9	-2.94	0.019
$NaNO_3$	44.5	20.9	2.13	0.066
Fe	15.9	20.9	0.76	0.467
Culture Medium	35.8	20.9	1.71	0.125
X_0	-8.0	20.9	-0.38	0.711

*Standard Error

The microalgae species and CO_2 concentration had a significant ($p < 0.05$) and negative effect on P_{max}. The microalgae species promoted the reduction in this response in 81.9 mg L^{-1} d^{-1} when the strain of *C. vulgaris* was used. Increase in the CO_2 concentration from 6 to 12% (v v^{-1}) in the gas stream also led to a reduction of 61.3 mg L^{-1} d^{-1} in the maximum productivity of the microalgal biomass.

As reported in several studies that use carbon dioxide in microalgal cultures, tolerance to CO_2 levels and cell growth responses and biomass composition depends on each species [32], and may vary between the same depending on the cultivation conditions applied [33].

Chiu and coauthors [34] have verified that feeding the cultures with CO_2 above 5% resulted in cell growth inhibition of the microalgae *Chlorella* sp. and *Nannochloropsis oculata*. However, Morais and Costa [35] reported that the highest values of X_{max} for *Spirulina* sp. (4.13 g L^{-1}) and *Scenedesmus obliquus* (2.12 g L^{-1}) were observed when using concentrations of 6% and 12% CO_2.

In mixotrophic cultures (Table 5), the $NaHCO_3$, C_O, and X_0 variables significantly influenced ($p < 0.05$) the maximum biomass concentration. The C_O and X_0 concentrations had a positive effect whereas $NaHCO_3$ had a negative effect and a higher modulus (-0.29) on the X_{max} response. For the maximum productivity response, the C_O, $NaNO_3$, and $NaHCO_3$ variables showed a significant effect ($p < 0.05$).

The organic carbon source concentration presented a positive effect (48.9), whereas the $NaNO_3$ (-40.0) and $NaHCO_3$ (-81.4) variables had a negative and superior effect. In this sense, the increase in inorganic carbon ($NaHCO_3$) concentration from 1 to 16.8 g L^{-1} resulted in reduced cell growth and biomass productivity, demonstrating that addition of a high concentration of this nutrient caused an inhibitory effect on microalgae growth. Use of the highest concentrations of glucose and IOR favored the increase in both growth responses, indicating that the application of mixotrophic cultures may favor the production of microalgal biomass.

Table 5. Effects estimate of independent variables about the responses of X_{max} and P_{max} in mixotrophic cultures.

Factor	**X_{max}**			
	Effect	***s. e**	**t (8)**	**p**
Mean	0.50	0.01	38.8	< 0.001
Microalgae	-0.02	0.03	-0.61	0.561
Carbon source	0.01	0.03	0.47	0.651
C_O	0.12	0.03	4.70	0.002
$NaNO_3$	-0.02	0.03	-0.65	0.543
$NaHCO_3$	-0.29	0.03	-11.1	< 0.001
Culture medium	0.02	0.03	0.85	0.421
X_0	0.22	0.03	8.5	< 0.001
	P_{max}			
	Effect	***s. e**	**t (8)**	**p**
Mean	99.3	7.68	12.9	< 0.001
Microalgae	-28.6	15.4	-1.86	0.099
Carbon Source	-11.7	15.4	-0.76	0.469
CO	48.9	15.4	3.18	0.013
$NaNO_3$	-40.0	15.4	-2.60	0.031
$NaHCO_3$	-81.4	15.4	-5.30	< 0.001
Culture medium	-17.1	15.4	-1.11	0.299
X_0	7.41	15.4	0.48	0.643

*Standard Error

Several authors consider mixotrophic cultivation as a promising method for cultivating microalgae, because it uses light and an organic carbon source, resulting in high microalgal biomass productivities, as well as increased lipid biosynthesis [5,36]. In this form of cultivation, several sources of organic carbon have been used including glycerol [37], glucose [38], and acetate [39]. Sforza and coauthors [37] used glycerol (1% w v^{-1}) as an organic carbon source in the mixotrophic cultivation of *Nannochloropsis salina*, and verified an X_{max} of 0.43 g L^{-1} in 16 d of cultivation. Andrade and Costa [40] verified that the organic carbon source (molasses) was the variable that had the greatest influence on the X_{max} response of *Spirulina platensis* (2.94 g L^{-1}) when used at a concentration of 0.75 g L^{-1}.

The MBM medium is used for the cultivation of the microalga *Chlorella* [11,20], but different species of this microalga have also been cultivated in saline medium and have proved resistant to them [41]. The F/2 medium, which is a saline medium [25], was used in the present work as an alternative to increase the cellular concentration and concentration of fatty acids in the biomass [31]. However, the variable culture medium had no significant effects on cell growth or fatty acid synthesis.

In both autotrophic and mixotrophic cultivations, the fatty acids palmitic (C16:0), followed by linoleic (C18:2) stand out, with the latter being an essential fatty acid belonging to the ω-6 family (Table 6 and 7). The concentrations of C16:0 and C16:1 for both cultivation modes are above the values found by Přibyl and coauthors [42] for *C. vulgaris* CCALA-256 (C16:0 -20.5 and C16:1 - 0.52%, respectively), in experiments with autotrophic cultivation in an outdoor system.

Table 6. Concentration of the main fatty acids (%), polyunsaturated fatty acids (PUFA) (%) and ω3+ω6 (%) present into microalgae biomass *C. vulgaris* and *C. kessleri* in autotrophic cultivation.

Assay	C16:0	C16:1	C17:1	C18:0	C18:1 ω9ct	C18:2 ω6t	C18:3 ω6	C18:3 ω3	PUFA	(ω3+ω6)
A1	35.6	6.7	0.7	2.1	16.3	21.1	4.9	6.7	34.4	33.8
A2	31.3	1.0	3.8	2.0	15.6	31.6	0.1	8.4	41.2	41.2
A3	30.4	6.4	1.4	1.8	19.3	21.8	2.3	10.4	37.0	36.8
A4	41.0	1.8	1.2	2.4	12.7	25.0	0.9	2.8	33.2	33.2
A5	21.2	17.5	1.1	1.7	12.3	21.8	4.1	13.3	39.4	39.3
A6	46.0	0.5	1.9	4.8	18.5	18.0	0.5	3.3	22.4	22.2
A7	53.8	1.5	0.4	3.4	21.9	7.0	1.4	2.2	12.2	11.3
A8	47.9	1.1	1.3	2.6	12.4	16.7	0.3	2.0	25.0	25.0

C16:0 - Palmitic acid; C16:1 - Palmitoleic acid; C17:1 - Margaroleic acid; C18:0 - Stearic acid; C18:1 - Oleic acid; C18:2 - Linoleic acid; C18:3ω3 - α-linolenic acid; C18:3ω6 - γ-linolenic acid. c: cis; t: trans

Table 7. Concentration of the main fatty acids (%), polyunsaturated fatty acids (PUFA) (%) and ω3+ω6 (%) present into microalgae biomass *C. vulgaris* and *C. kessleri* in mixotrophic cultivation.

Assay	C16:0	C16:1	C17:1	C18:0	C18:1 ω9ct	C18:2 ω6t	C18:3 ω6	C18:3 ω3	PUFA	(ω3+ω6)
M1	29.6	3.8	1.5	3.2	25.6	7.1	4.4	18.6	30.9	30.9
M2	44.8	1.1	2.0	3.6	16.4	21.7	0.4	3.7	25.9	25.9
M3	25.4	7.6	0.9	3.1	25.2	9.8	5.5	17.9	33.6	33.5
M4	46.0	1.5	2.2	3.9	16.9	15.7	0.6	3.6	22.3	22.3
M5	36.3	4.0	1.4	3.4	23.4	15.4	2.7	6.7	24.9	24.9
M6	36.2	2.5	2.7	4.1	17.1	20.5	1.0	2.3	28.6	28.2
M7	33.3	3.4	0.9	2.5	25.9	16.4	4.9	0.7	11.0	11.0
M8	31.5	3.2	1.8	5.4	15.4	21.7	2.7	2.5	34.3	34.3

C16:0 - Palmitic acid; C16:1 - Palmitoleic acid; C17:1 - Margaroleic acid; C18:0 - Stearic acid; C18:1 - Oleic acid; C18:2 - Linoleic acid; C18:3ω3 -α-linolenic acid; C18:3ω6 - γ-linolenic acid. c: cis; t: trans

The results with palmitic and palmitoleic acid verified in that study are also higher than those found by Lam and Lee [13] (25 and 2%, respectively) in the biomass of *C. vulgaris* in mixotrophic cultivation, using organic fertilizer as the source of nutrients. Li and coauthors [43] when using *Chlorella minutissima* UTEX2341 used glycerin as an organic carbon source, and obtained 14.91% of palmitic acid (C16:0) and 0.98% of palmitoleic acid (C16:1) at 25°C.

Costa and coauthors [44] cultivated the microalgae *C. vulgaris* and *C. minutissima*, between 30 °C and 35 °C with 1% CO_2 (v v^{-1}) and 16.8 g L^{-1} of $NaHCO_3$ as carbon sources, and presented 26.5% of C16:0. The authors obtained a concentration of PUFA ranging from 18.0% to 35.6% for *C. vulgaris* and 2.4% to 13.0% for *C. minutissima*. Radmann and Costa [45] analyzed the fatty acid profile of *Spirulina* sp. LEB 18, *Scenedesmus obliquus* LEB-22, *Synechococcus nidulans* LEB-25, and *Chlorella vulgaris* LEB-106, cultivated

with CO_2, SO_2, and NO and obtained linoleic acid concentrations (C18:2) of 2.7, 4.0, 3.5, and 6.3%, respectively. These values were lower than those found in the present study, in which the concentration of C18:2 ranged from 7% to 31.6% in autotrophic cultures and from 7.1% to 21.7% in mixotrophic cultivation. In the work performed by these authors, α-linolenic acid (C18:3ω-3) was not detected in the biomass of *C. vulgaris*.

In autotrophic cultivation, the CO_2, X_0, and photoperiod variables significantly influenced ($p < 0.05$) the PUFA concentration in the biomass (Table 8). The CO_2 concentration (-11.7) and photoperiod (-7.52) had a negative effect, whereas the variable X_0 had a positive effect (10.1) on the PUFA concentration. In autotrophic cultivation, increase in CO_2 concentration from 6 to 12% (v v^{-1}) caused a significant reduction of 11.7% in the PUFA concentration. However, an increase in the concentration of saturated fatty acids (C16:0 and C18:0) was observed with increasing CO_2 concentration supply to the A6, A7, and A8 assays.

Table 8. Effects estimate of independent variables on the concentration of PUFAs.

Factor	**Autotrophic PUFAs**			
	Effect	***s. e**	**t (8)**	**p**
Mean	30.6	1.16	26.4	< 0.001
Microalgae	-0.30	2.32	-0.13	0.899
Photoperiod	-7.52	2.32	-3.24	0.012
CO_2	-11.7	2.32	-5.02	0.001
$NaNO_3$	4.80	2.32	2.07	0.072
Fe	-1.82	2.32	-0.78	0.455
Culture Medium	-4.81	2.32	-2.07	0.072
X_0	10.1	2.32	4.35	0.002
	Mixotrophic PUFAs			
	Effect	***s. e**	**t (8)**	**p**
Mean	27.8	0.59	47.2	< 0.001
Microalgae	-0.06	1.18	-0.05	0.958
Carbon source	0.44	1.18	0.38	0.717
Co	-0.75	1.18	-0.63	0.544
$NaNO_3$	0.58	1.18	0.49	0.634
$NaHCO_3$	8.10	1.18	6.85	< 0.001
Culture Medium	0.88	1.18	0.75	0.477
X_0	3.71	1.18	3.15	0.014

*Standard error

In the mixotrophic cultivations, it was verified that the increase in $NaHCO_3$ concentration showed a significant effect and a positive modulus on the PUFA production. In contrast to the negative effect on growth parameters, this increase in inorganic carbon concentration promoted an increase of 8.1% in the content of PUFAs in the *Chlorella* biomass. According to Peng and coauthors [46], the high availability of inorganic carbon in the culture medium promotes an increase in the enzymatic activity of Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco), which is the key enzyme for the conversion of 3-phosphoglycerate, a substrate for the biosynthesis of fatty acids and carbohydrates in plants and microalgae.

Microalgae are the primary natural producers of LC-PUFA. These organisms offer a promising vegetative and non-polluted resource for biotechnology and bioengineering of LC-PUFA production as an alternative to fish oil. The PUFAs are important for a variety of nutraceutical and pharmaceutical purposes. An appropriate ratio of PUFA of the ω-3 and ω-6 groups is vital for healthy nutrition, and adequate dietary intake has strong health benefits in humans [47]. High PUFA biomass can be used directly in the formulation of food [2,48] and animal feed [49].

CONCLUSION

In autotrophic cultures, the photoperiod had a significant and superior effect on the X_{max}, P_{max}, and PUFA responses. In the mixotrophic assays, the sodium bicarbonate concentration had a significant and superior effect on the evaluated responses. Palmitic acid was the saturated FA obtained at higher concentrations in

all assays and PUFA concentrations varied between 12.2% and 41.2% in autotrophic cultures and between 11% and 34.3% in the mixotrophic mode. Therefore, it was verified that selection of conditions and the mode of cultivation contribute to the production of microalgal biomass and FA biosynthesis.

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