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Natália Santana Carvalho
Federal University of Bahia
Kricelle Mosquera Deamici
GreenCoLab
Luiggi Cavalcanti Pessôa
Senai Cimatec University
Bianca Bomfim Andrade
Federal University of Bahia
Renata Quartieri Nascimento
Federal University of Bahia
Jânia Betânia Alves da Silva
Universidade Federal do Recôncavo da Bahia
Carolina Oliveira de Souza
Federal University of Bahia
Lucas Guimarães Cardoso
Universidade Salvador
Denilson de Jesus Assis
denilson.assis@animaeducacao.com.br
Universidade Salvador

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Valorization of crude glycerol from heterotrophic microalgae cultivation: optimization and characterization of bioproducts.

Natália Santana Carvalho[a,b], Kricelle Mosquera Deamici[c], Luiggi Cavalcanti Pessôa[b], Bianca Bomfim Andrade[d,b], Renata Quartieri Nascimento[d], Jânia Betânia Alves da Silva[a,e], Carolina Oliveira de Souza[d], Lucas Guimarães Cardoso[a,f], Denilson de Jesus Assis[a,f*]

[a] Graduate program in Chemical Engineering (PPEQ), Polytechnic School, Federal University of Bahia, Salvador, Bahia, Brazil

[b] Senai Cimatec University, Salvador, Bahia, Brazil

[c] GreenCoLab – Associação Oceano Verde

[d] Department of Bromatological Analysis, College of Pharmacy, Federal University of Bahia, Salvador, Bahia, Brazil

[e] Center of Science and Technology, Mechanical Engineering Collegiate, Federal University of Recôncavo of Bahia, Cruz Das Almas, Bahia, Brazil

[f] Department of Engineering, Salvador University, Salvador, Bahia, Brazil

[g] Graduate program in Biotechnology-Northeast Biotechnology Network (RENORBIO), Federal University of Bahia, Salvador, Bahia, Brazil

* Corresponding author

Email: denilson.assis@unifacs.br (Denilson Assis)

Abstract

Crude glycerol, the main by-product of biodiesel production, represents a promising low-cost substrate for heterotrophic microalgae cultivation. Despite its potential for bioconversion into high-value products, knowledge regarding the optimization of process parameters governing biomass and extracellular polymeric substance (EPS) production remains scarce.

In this study, the effects of agitation speed and concentrations of crude glycerol and sodium nitrate on cell biomass and extracellular polymer (EPS) production by *Chlorella vulgaris* IBL-C105 under heterotrophic cultivation were evaluated. A Doehlert Matrix for three factors was employed to generate combinations of these variables, enabling statistical modeling and simultaneous optimization of process parameters. The optimal condition (1.6 g L^{-1} $NaNO_3$, 2.6 g L^{-1} glycerol, and 165 rpm) resulted in the production of 1.18 g L^{-1} biomass within 24 h, with complete substrate consumption. Under this condition, the oil produced exhibited a fatty acid profile suitable for biodiesel production, meeting all analyzed biodiesel quality standards. Furthermore, the supernatant from this cultivation showed an emulsification index above 50%, indicating potential application as an emulsifying agent. The EPS extracted from the supernatant displayed pseudoplastic rheological behavior, with properties comparable to commercial xanthan gum, highlighting their potential as rheological modifiers. Overall, the results demonstrate that heterotrophic cultivation of *C. vulgaris* using crude glycerol constitutes a promising strategy for the integrated production of bioproducts, contributing to the valorization of biodiesel industry residues.

Keywords: Biomass. Extracellular polymers. Heterotrophic. *Chlorella vulgaris*.

Introduction

The growing demand for sustainable sources of energy and materials, associated with the energy crisis, environmental impacts, and the high cost of natural products, has stimulated global interest in the development of bioproducts obtained through economically viable and environmentally sustainable biotechnological routes (MANDAL *et al*., 2025). In this context, microorganisms stand out as promising platforms for the production of various compounds of industrial interest, including proteins, carbohydrates, pigments, antioxidants, nanoparticles, extracellular polymers, and polyhydroxyalkanoates (PHAs), as well as lipids. These metabolites can subsequently be processed to obtain biofuels and other high-value-added bioproducts (BRENNAN & OWENDE, 2010; *HAN et al.*, 2018; BYRTUSOVÁ *et al*., 2021; KATAGI *et al*., 2024). Moreover, microbial production offers important advantages over conventional sources, such as reduced dependence on seasonality, absence of direct competition with the food market, and potential reduction of operational costs (JONES *et al*., 2019; RUSSO *et al*., 2025).

Microalgae have emerged as microorganisms of industrial interest due to their metabolic versatility and ability to accumulate high levels of intracellular lipids, which can range from 15 to 75% of dry mass, in addition to producing extracellular metabolites such as extracellular polymers (EPS) with diverse industrial applications (CHISTI, 2007; SUPARMANIAM *et al*., 2025; WINGENDER *et al*., 1999; SURYAWANSHI *et al*., 2022). These microorganisms can grow under different cultivation modes—autotrophic, heterotrophic, and mixotrophic—with heterotrophic cultivation being advantageous as it does not depend on light and enables high productivities of biomass and lipids for biodiesel and other bioproduct production (KURNIAWAN *et al*., 2025; JOHNSON *et al*., 2025; UDAYANTHA *et al*., 2025). Furthermore, cultivation conditions directly influence cell composition

and productive performance, allowing the metabolism to be directed toward the production of specific compounds (EL-SHEEKH *et al*., 2024; TIAN *et al*., 2025).

In recent years, the use of industrial residues as substrates for microalgae cultivation has emerged as a promising strategy to reduce production costs and promote the valorization of waste streams. However, few studies employ residues as nutrient sources in microalgae cultivation for the generation of high-value bioproducts, with most research in this area focusing primarily on wastewater bioremediation (DIAS *et al*., 2019), autotrophic or mixotrophic cultivation (DA SILVA *et al*., 2021; GHAFFAR *et al*., 2023), or biomass production for animal nutrition (ANTONELO *et al*., 2019). Despite the growing interest in microalgal bioprocesses, studies focused on heterotrophic cultivation using industrial residues as carbon sources remain limited, particularly regarding the simultaneous production of intracellular lipids and extracellular biopolymers under optimized process conditions. Among industrial residues with potential for bioprocess applications, crude glycerol stands out as a co-product generated in large quantities during biodiesel production, which can be used as a carbon source in heterotrophic and mixotrophic microalgae cultivation, promoting cell growth and the synthesis of metabolites of interest (KOROZI *et al*., 2023). In this context, its utilization contributes to waste valorization and reduction of environmental impacts, in line with the principles of the circular economy. Furthermore, the performance of bioprocesses involving microalgae can be influenced by the combination of different operational parameters that regulate cellular metabolism, with the use of statistical experimental design being an important strategy to systematically evaluate the influence of these variables and identify optimal cultivation conditions to maximize target bioproduct production. Considering the metabolic potential of microalgae, the availability of industrial residues as alternative carbon sources, and the need for more sustainable processes for bioproduct production, it is hypothesized that heterotrophic cultivation of microalgae using crude glycerol as substrate, combined with process optimization, may simultaneously favor cell growth and the production of metabolites

of interest, such as lipids and extracellular polymers, contributing to the development of biotechnological strategies aligned with the concepts of biorefinery and circular economy.

Thus, this study aims to evaluate and optimize the heterotrophic cultivation of microalgae using crude glycerol as a carbon source, investigating its effects on biomass production and the recovery of biomolecules of interest, with a view to waste valorization and the development of sustainable processes for bioproduct generation.

2. Materials and Methods

2.1 Microorganisms and culture media

In this study, the microalgae *Chlorella vulgaris* IBL-C105 were cultivated, donated by LAPESCA/LAPBIM – Laboratory of Applied Chromatography and Biomaterials, located at the School of Pharmacy, UFBA.

The inocula were propagated in Erlenmeyer flasks containing BG-11 medium, which consists of the following components (g L^{-1}): $NaHCO_3$ (0.4); $NaNO_3$ (1.5); $K_2HPO_4 \cdot 3H_2O$ (0.04); $MgSO_4 \cdot 7H_2O$ (0.075); $CaCl_2 \cdot 2H_2O$ (0.036); $C_6H_8O_7 \cdot Fe \cdot NH_3$ (0.006); $C_{10}H_{16}N_2O_8$ (0.001); Na_2CO_3 (0.02); $C_6H_8O_7 \cdot H_2O$ (0.006); and A5+Co solution (1 mL L^{-1}). The A5+Co solution contains (g L^{-1}): H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.222), $NaMoO_4$ (0.015), $CuSO_4 \cdot 5H_2O$ (0.079), and $Co(NO_3)_2 \cdot 6H_2O$ (0.049) (RIPPKA *et al*., 1979). The culture media and materials used were sterilized in an autoclave at 121 °C for 20 min.

Maintenance cultures were carried out in a BOD (Biochemical Oxygen Demand) incubator at 28 °C, with a light intensity of 41.60 µmol photons m^{-2} s^{-1}, a 12 h light/dark photoperiod, and aeration with compressed air filtered through glass wool.

2.2 Proximate Composition of Glycerol

Crude glycerol from biodiesel production was provided by Petrobras, located in the city of Candeias-BA. The proximate composition was determined through the quantification of total lipids, volatiles, crude protein, ash, and total carbohydrates.

Methods from the Association of Official Analytical Chemists (AOAC, 1997) were used to determine protein, volatiles, lipids, and ash content. Crude protein content was determined from total nitrogen using the Kjeldahl method (conversion factor 6.25); volatiles were measured by drying at 105 °C in a forced-air oven; total lipids were quantified using the Bligh-Dyer method (1959); and ash content was determined by incineration in a muffle furnace at 700 °C. Carbohydrate content was estimated by difference.

2.3 Biomass concentration and pH

Biomass concentration was monitored daily by optical density using a spectrophotometer (PerkinElmer Lambda 35 UV/VIS) at a wavelength of 670 nm. Optical density values were converted into dry biomass concentration through a calibration curve (COSTA *et al*., 2018). The pH of the cultures was measured daily using a digital pH meter (Tecnal, Brazil).

2.4 Acclimatization to crude glycerol

Microalgae were previously acclimatized to crude glycerol in a TE-424 shaker incubator (Tecnal, Brazil), in 250 mL Erlenmeyer flasks containing 200 mL working volume. Cultures were conducted in BG-11 medium containing 1.5 g L^{-1} $NaNO_3$ and different concentrations of crude glycerol (2, 5, 10, and 50 g L^{-1}), under heterotrophic conditions at 140 rpm. The acclimatization condition was selected based on the highest cell biomass productivity (COSTA *et al*., 2018).

2.5 Cultivation conditions

Heterotrophic cultures were conducted in 250 mL Erlenmeyer flasks containing 200 mL of medium, inoculated with 30% (v/v) of previously acclimatized culture and incubated in a TE-424 shaker

(Tecnal, Brazil) at 25 °C. Agitation speed and concentrations of glycerol and sodium nitrate were defined by experimental design. Biomass was monitored throughout cultivation as described in section 2.4. The cultivation period varied according to the duration of the microalgae growth phase, with cultivation time determined by the onset of the stationary phase, influenced by process conditions.

2.6 Experimental design (Doehlert Matrix)

Optimization of cultivation conditions was performed using a Doehlert matrix for three independent variables, totaling 15 trials, of which 3 were central point replicates. Three levels were adopted for agitation speed, in coded values (Z, -0.817, 0, 0.817) and real values (Z, 80, 140, 200 rpm); five levels for sodium nitrate concentration, in coded values (X, -1.0, -0.5, 0, 0.5, 1.0) and real values (X, 0.5, 1, 1.5, 2, 2.5 g L^{-1}); and seven levels for glycerol concentration, in coded values (Y, -0.866, -0.577, -0.289, 0, 0.289, 0.577, 0.866) and real values (Y, 0.5, 1.1, 1.7, 2.3, 2.8, 3.4, 4 g L^{-1}).

2.7 Recovery of microalgal biomass

At the end of cultivation, biomass was recovered by centrifugation (Eppendorf 5702 R) at 4400 rpm for 7 min. The pellet was washed with distilled water and centrifuged again to remove residual salts from the culture medium. The biomass obtained was frozen in an ultrafreezer (ColdLab CL580-86V) at -80 °C, lyophilized (Liotop L101), and stored at -20 °C. Lyophilized biomass was subjected to quantification of total lipids by the method of Folch *et al*. (1957) and crude protein by the Kjeldahl method (AOAC, 1997), using a conversion factor of 5.22, specific for microalgae (LOURENÇO, 2006).

The supernatant was reserved for investigation of extracellular polysaccharides (EPS).

2.8 Kinetic growth parameters

Biomass concentration values obtained during cultivation under optimized conditions were used to calculate the following kinetic parameters: maximum productivity (Pmax), maximum specific growth rate (μmax, h^{-1}), maximum biomass concentration (Xmax), and generation time (tg). Biomass productivities (P, g L^{-1} h^{-1}) for each cultivation day were calculated using Equation 1, with the highest value corresponding to Pmax.

$$P = (X_t - X_0)/(t - t_0) \qquad \text{Eq. 1}$$

Where:

X_t = biomass concentration (g L^{-1}) at time t (d);

X_0 = biomass concentration (g L^{-1}) at time t0 (d).

Maximum specific growth rate (μmax, h^{-1}) was calculated by linear regression of the logarithmic phase of the ln X (g L^{-1}) versus t (h) curve. Generation time (t_g, h), the time required for cell doubling, was determined during the exponential growth phase of each culture, using Equation 2 (BAILEY & OLLIS, 1986; COSTA *et al*., 2002).

$$t_g = \ln 2/ \mu_{máx} \qquad \text{Eq. 2}$$

2.9 Identification and quantification of fatty acids

Fatty acids were transesterified to fatty acid methyl esters (FAMEs) by saponification of lipids with NaOH in methanol, followed by methylation catalyzed with BF_3 (12% in methanol). FAMEs were extracted with iso-octane and analyzed using a Clarus 680 gas chromatograph (PerkinElmer) equipped with a flame ionization detector (FID) and a DB-FFAP fused silica capillary column (0.25 mm internal diameter). Column temperature ranged from 150 to 210 °C, with a heating rate of 5 °C min^{-1}. Injector and detector temperatures were maintained at 250 °C and 280 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Fatty acid identification was performed by

comparing relative retention times of sample peaks with commercial standards (Sigma-Aldrich, USA) (DO PRADO *et al*., 2025). Peak areas were determined using the Clarus Chromatography workstation program to normalize percentage areas of total fatty acids (ANDRADE *et al*., 2025).

2.10 Biodiesel quality parameters

Biodiesel quality parameters were estimated based on molecular structures, size, quantity, and/or position of double bonds of fatty acids composing the lipids. Cetane number, iodine value, cold filter plugging point, and oxidative stability were calculated according to Andrade *et al*. (2025). Results were compared with limits established for Brazil, the United States, and Europe.

2.11 Emulsification index (EI_{24})

The emulsification index was used to identify the presence of biosurfactants in the supernatant of the optimized culture. The analysis was conducted according to the methodology described by Sivakumar *et al*. (2013) and Cooper & Goldenberg (1987). Supernatants from microalgae cultures were mixed with different oils (mineral, sunflower, soybean, and olive) in test tubes at a ratio of 2:3 (v/v). Tubes were vortexed for 2 min and left to stand for 24 h. The emulsification index was determined by the ratio between the height (cm) of the emulsified layer (EL) and the total height (TH) of the liquid layer in the tube, according to Equation 3.

$$EI_{24}\,(\%) = (EL)/(TH)*100 \qquad \text{Eq. 3}$$

2.12 Extraction of extracellular polymers

EPS were extracted from biomass-free microalgal supernatants by addition of absolute ethanol at a ratio of 3:1 (ethanol:medium) (PESSÔA *et al*., 2024). The mixture was stored at 4 °C for 24 h. The precipitated material was filtered, dried in an oven (35 °C), and quantified in g L^{-1}.

2.13 Rheological parameters of EPS

Apparent viscosity was measured using a Haake Rheotest 2.1 concentric cylinder rheometer coupled to a water bath. To evaluate apparent viscosity behavior, biopolymer solutions (0.4% w/v) were prepared and refrigerated for 12 h prior to analysis. Measurements were obtained as a function of shear rate variation (25 to 1000 s^{-1}) at 25 °C (ASSIS *et al*., 2014; FRANÇA *et al*., 2025). Rheological data were fitted to the Ostwald-de Waele model according to Equation 4.

$$\eta = \frac{T}{\gamma} = k\gamma^{n-1}\ \eta = \frac{T}{\gamma} = k\gamma^{n-1}\eta = \frac{T}{\gamma} = k\gamma^{n-1} \quad \text{Eq. 4}$$

Where:

η = apparent viscosity (Pa·s);

T = shear stress (Pa);

γ = shear rate (s^{-1});

k = fluid consistency index ($Pa \cdot s^n$);

n = flow behavior index.

2.14 Glycerol consumption

Glycerol consumption was monitored during optimized cultivation using a colorimetric method with an enzymatic kit for triglyceride quantification (Triglycerides Liquiform, Labtest, Brazil), with readings taken in a spectrophotometer at 505 nm (DIVINO, 2015).

For quantification, a standard curve was constructed using glycerol diluted in BG-11 medium at concentrations ranging from 0.025 to 0.45 g L^{-1} (CORREIA, 2015; DIVINO, 2015). Samples were incubated at 37 °C for 10 min, and absorbance values were converted into glycerol concentration by interpolation on the calibration curve (CORREIA, 2015; DIVINO, 2015; PEREIRA & RIBEIRO, 2018).

2.15 Statistical treatment

All analyses were performed in triplicate, and results were expressed as means ± standard deviations. Statistical treatment of the data was carried out using Statistica 7.0 software, considering a confidence level of 95%. The significance of independent variables and the adequacy of the models were evaluated by ANOVA and Fisher's F-test (ASSIS *et al*., 2014). Optimization of responses was defined through analysis of the stationary point of the response surfaces of the adjusted models.

3. Results

3.1 Proximate composition of crude glycerol

Crude glycerol derived from biodiesel production presents variable physicochemical composition, mainly related to the raw material used, transesterification conditions, type of catalyst employed, and efficiency of the purification process. As a consequence, the impurities present in this co-product may vary in nature and concentration (UPRETY *et al*., 2017; GUERFALI *et al*., 2020).

The proximate composition of the crude glycerol used in this study is shown in Table 1.

Table 1 – Proximate composition of crude glycerol

Volatiles + Moisture (%)	**Total lipids (%)**	**Proteins (%)**	**Glycerol + Carbohydrates (%)**	**Ash (%)**
36.89 ± 0.01	15.46 ± 0.02	0.12 ± 0.01	44.99 ± 0.01	2.56 ± 0.01

The impurities present are typically associated with oilseed residues, free fatty acids, unconverted triglycerides, mineral salts, and by-products of the biodiesel production process (THOMPSON & HE, 2006; HUDHA & LAKSMANA, 2018).

3.2 Inoculum acclimatization

Acclimatization of microalgae to crude glycerol and absence of light was carried out to promote metabolic adaptation of the culture to heterotrophic growth. During this stage, different glycerol concentrations (2, 5, 10, and 50 g L^{-1}) were evaluated. It was observed that higher glycerol concentrations (5, 10, and 50 g L^{-1}) were not favorable for microalgae growth.

3.3 Optimization of microalgal biomass and extracellular polymer production

The influence of sodium nitrate concentration (X), glycerol concentration (Y), and agitation speed (Z) on biomass production (W1) and extracellular polymer (W2) synthesis by *Chlorella vulgaris* was investigated through an experimental design based on the Doehlert matrix. The experimental matrix and results obtained for the analyzed responses are presented in Table 2. Significant variation was observed in both biomass production and EPS synthesis under the different conditions evaluated, indicating that the investigated variables exert a direct influence on microalgal metabolism under heterotrophic conditions.

Thus, an experimental design was carried out for each strain, consisting of 15 experiments expressed in a matrix with three factors, including 12 experiments and 3 central point replicates, as shown in Table 2.

Table 2 – Doehlert matrix composed of independent variables (real values) and responses for biomass and extracellular polymer production by *Chlorella vulgaris* IBL-C105

Trial	X (gL^{-1})	Y (gL^{-1})	Z (gL^{-1})	W1 (gL^{-1})	W2 (gL^{-1})

1	2.5	2.3	140	0.09	0.26
2	2.0	4.0	140	0.55	0.14
3	2.0	2.8	200	1.20	0.20
4	0.5	2.3	140	0.40	0.09
5	1.0	0.5	140	0.00	0.00
6	1.0	1.7	80	0.00	0.07
7	2.0	0.5	140	0.02	0.00
8	2.0	1.7	80	0.01	0.13
9	1.0	4.0	140	0.68	2.31
10	1.5	3.4	80	0.00	0.18
11	1.0	2.8	200	0.43	0.21
12	1.5	1.1	200	0.68	0.42
13*	1.5	2.3	140	1.16	0.12
14*	1.5	2.3	140	1.20	0.14
15*	1.5	2.3	140	1.26	0.12

*Central point.

X = sodium nitrate concentration, Y = glycerol concentration, Z = agitation speed, W1 = dry biomass production by *Chlorella vulgaris*, W2 = extracellular polymer production by *Chlorella vulgaris.*

The data obtained were used to study the influence of sodium nitrate concentration (X), glycerol concentration (Y), and agitation speed (Z) on dry biomass production (W1) and extracellular polymer production (W2) by *Chlorella vulgaris*.

Regarding the effects of agitation speed and concentrations of glycerol and sodium nitrate on biomass production by *Chlorella vulgaris* IBL-C105, the combinations of the independent variable levels X

(0.5 to 2.5 g L^{-1}), Y (0.5 to 4 g L^{-1}), and Z (80 to 200 rpm) influenced cell biomass production, with variation from 0 to 1.26 g L^{-1} for *C. vulgaris*, depending on the cultivation conditions (Table 2).

The individual and interaction effects of the independent variables on biomass production by *C. vulgaris* can be estimated from the data presented in Table 3.

Table 3 – Estimation of the effects of X (sodium nitrate concentration), Y (glycerol concentration), and Z (agitation) on biomass production (W1) and EPS production (W2) by *Chlorella vulgaris***.**

	W1		W2	
Term	**Coefficient**	**p-value**	**Coefficient**	**p-value**
Constant	1.22000	0.000018	0.09300	0.058589
X	0.00625	0.815096	-0.22325	0.005625
X.X	-0.48750	0.000180	0.04100	0.226522
Y	0.48625	0.001426	0.88350	0.000498
Y.Y	-1.32750	0.000241	0.99800	0.001155
Z	0.76648	0.000310	0.15166	0.058781
Z.Z	-1.04674		-0.03111	0.678189

		0.000289		
X.Y	-0.07500	0.223289	-1.08400	0.000416
X.Z	0.40503	0.004318	0.32475	0.015948
Y.Z	0.18602	0.128999	-0.00610	0.960658

The coefficients showed that the individual quadratic effects of glycerol concentration, sodium nitrate concentration, and agitation were negative ($p < 0.05$) on biomass production by *C. vulgaris*. This indicates that, within the experimental domain, the lower levels of these variables individually contributed to increased biomass production, since biomass production increases with rising levels of these variables until reaching the inflection point (the maximum of the parabola). At this point, the trend is reversed, and further increments of these variables trigger a decrease in biomass production. On the other hand, the linear effect of glycerol concentration, linear agitation, and the interaction between sodium nitrate concentration and agitation showed positive effects ($p < 0.05$), indicating that, within the experimental domain, higher levels of these variables contribute to increased biomass production. The linear effect of glycerol concentration was 1.5 times lower than that of agitation.

Regression of the data generated a second-order polynomial model (Equation 5) for biomass production (W1) by C. vulgaris.

$$\mathbf{W1 = -4.58 + 2.08X - 0.97X^2 + 1.05Y - 0.22Y^2 + 0.03Z + 0.01XZ} \quad \text{(Eq. 5)}$$

Where: $NaNO_3$ (X); Glycerol (Y); Agitation (Z)

The results of the analysis of variance (ANOVA) for W1 (Table 4) showed that the calculated F-value of the regression was approximately 3.4 times higher than the tabulated F-value, which confirms the predictive capacity of the mathematical model generated. For the model to be predictive, in the statistical significance test of the regression, the calculated F-value must be greater than the tabulated F-value (Ribeiro *et al*., 2018).

Table 4 – Analysis of variance (ANOVA) for statistical evaluation of the biomass production model (W1) and extracellular polymer production model (W2) by *Chlorella vulgaris*.

	Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F		R^2
					Calculated	Tabulated	
	Regression	4.597	9	4.597	13.95	4.1	
	Residual	0.267	6	0.089			
W1	**Lack of Fit**	0.259	3	0.086	36.03	9.28	0.933
	Pure Error	0.007	3	36.031			
	Total	3.986	15	0.002			
	Regression	3.145	9	3.145	2.13	4.10	
	Residual	1.463	6	0.488			
W2	**Lack of Fit**	1.452	3	0.484	124.22	9.28	0.681
	Pure Error	0.012	3	124.219			
	Total	4.584	15	0.004			

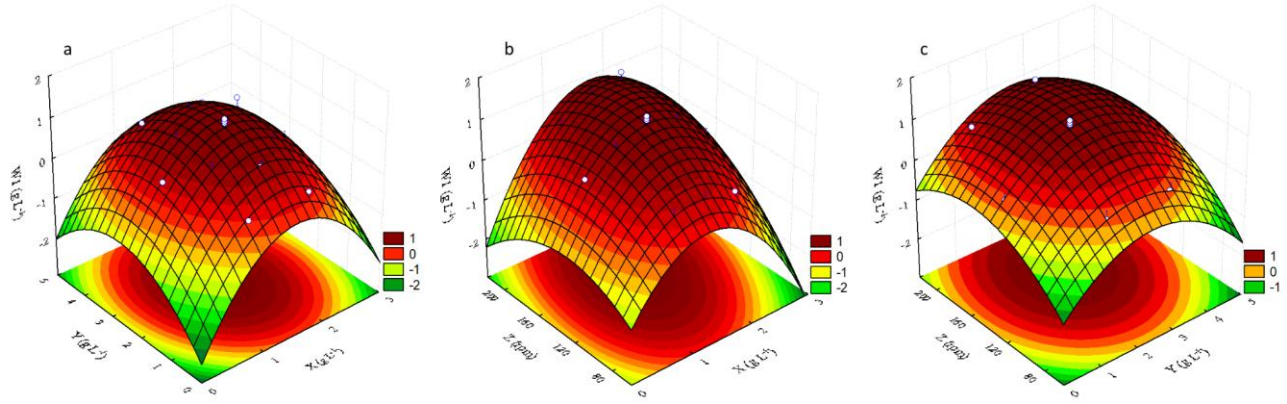
Residual = Lack of Fit + Pure Error

The data in Table 4 also allow us to infer that the biomass production model (W1) generated presents a good fit, since the determination coefficient (R^2) of the model was 0.933, indicating that the variations not explained by the proposed model account for only 6.69% of the total.

Figure 1 represents the response surfaces described by the mathematical model generated, by fixing Z, Y, and X, respectively. In all representations, it is possible to observe that the maximum biomass production by *C. vulgaris* (W1) occurred in the region of the central point of the matrix (X, 1.5 g L^{-1}; Y, 2.3 g L^{-1}; and Z, 140 rpm).

The optimal combination of the variables X, Y, and Z to generate the maximum W1 response, within the experimental domain, was 1.6 g L^{-1}, 2.6 g L^{-1}, and 165 rpm, respectively.

Fig. 1 – Response surface of biomass production by *C. vulgaris* (W1) as a function of: a) sodium nitrate concentration (X) and glycerol concentration (Y); b) sodium nitrate concentration (X) and agitation (Z); and c) glycerol concentration (Y) and agitation (Z).



Production under the optimized conditions yielded 1.20 g L^{-1} of *C. vulgaris* biomass, a value very close to that obtained at the central point.

Regarding the effects of agitation speed and concentrations of glycerol and sodium nitrate on extracellular polymer production by *Chlorella vulgaris* IBL-C105, the combinations of the independent variable levels X (0.5 to 2.5 g L^{-1}), Y (0.5 to 4 g L^{-1}), and Z (80 to 200 rpm) influenced EPS production, with variation from 0 to 2.31 g L^{-1} for C. vulgaris, depending on the cultivation conditions (Table 2).

From the individual and interaction effects of the independent variables on extracellular polymer production by *C. vulgaris*, shown in Table 3, the coefficients indicated that the individual linear and quadratic effects of glycerol concentration and the interactive effect of sodium nitrate concentration with agitation were positive ($p < 0.05$) on EPS production by the microalga, suggesting that higher levels of these variables contributed to increased EPS production. Conversely, sodium nitrate concentration and the interaction between sodium nitrate and glycerol concentrations exhibited negative linear effects ($p < 0.05$), indicating that lower levels of these variables individually favored increased extracellular polymer production. The linear effect of glycerol concentration was four times greater than that of sodium nitrate concentration, in absolute values.

Regression of the data generated a second-order polynomial model for extracellular polymer production (W2) by C. vulgaris. However, the analysis of variance data shown in Table 4 indicate that the model is not predictive, since the calculated F-value < tabulated F-value.

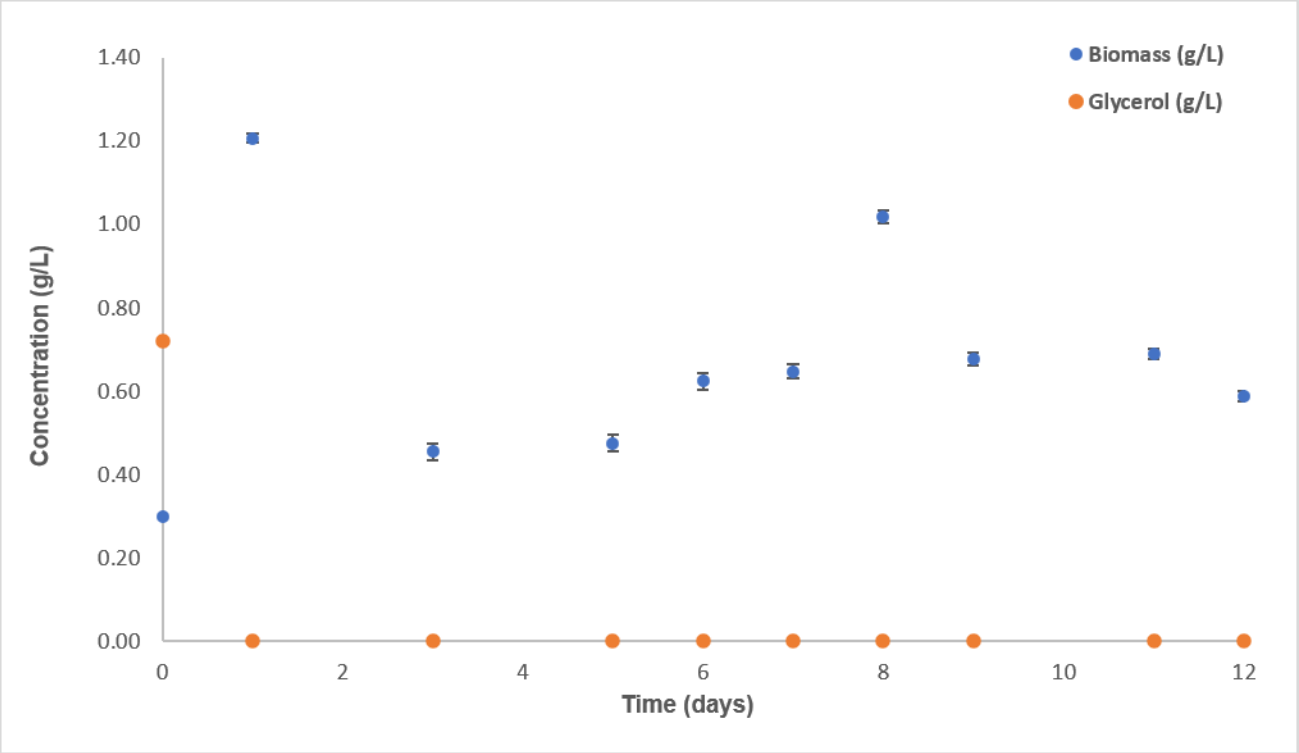
The regression presented a determination coefficient (R^2) of 0.681, evidencing that the model did not have a good fit, as 31.92% of the total variation could not be explained by the proposed model.

3.4 Characterization of bioproducts under optimized conditions

3.4.1 Biomass of *C. vulgaris* IBL-C105

Heterotrophic cultivation under the optimized condition (1.6 g L^{-1} sodium nitrate, 2.6 g L^{-1} glycerol, and 165 rpm) resulted in rapid cell growth and high substrate-to-biomass conversion efficiency, as shown in Figure 2.

Fig. 2 – Growth kinetics of *Chlorella vulgaris* throughout cultivation under optimized conditions.

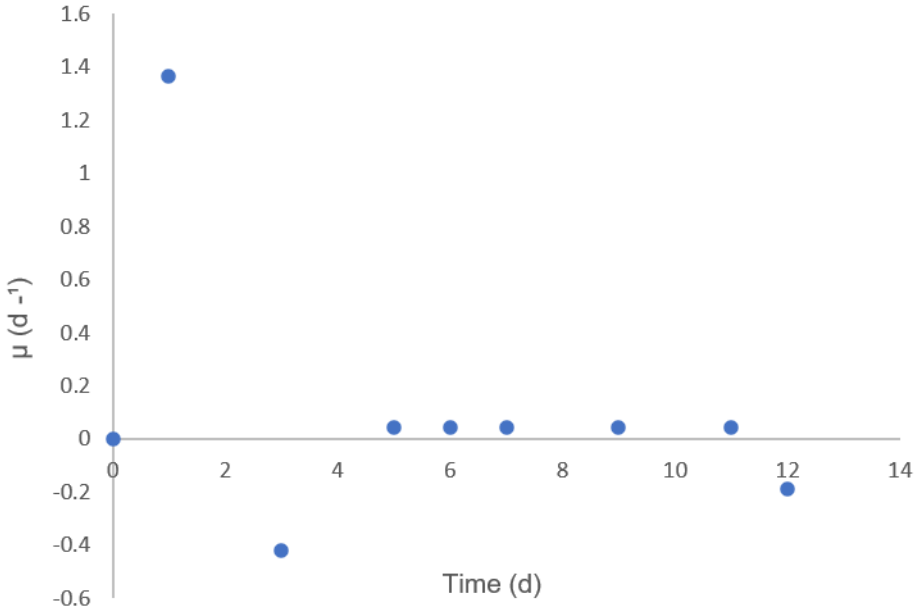


The maximum biomass concentration (Xmax) was 1.18 g L^{-1}, reached after 24 h of cultivation, coinciding with the complete consumption of glycerol present in the medium. The growth parameters found during this cultivation period were maximum productivity (Pmax) of 0.875 g L^{-1} d^{-1}, maximum specific growth rate (μmax) of 1.37 d^{-1}, and generation time (tg) of 12.24 h. The control cultivation, without glycerol but under the same sodium nitrate and agitation conditions, produced a lower biomass concentration of 0.24 g L^{-1} and did not produce EPS.

During the growth phases in microbial cultivation, two processes are associated: nutrient consumption from the medium and metabolite release (ALTERTHUM *et al*., 2020). In the cultivation of *C. vulgaris* in the present study, under optimized conditions, the lag phase was not observed, since prior to the start of the assays, the inoculum was acclimated to the medium containing glycerol and absence of light. The greater the similarity between the medium from which the inoculum was taken and the medium to which it was added for cultivation, the shorter the duration of the lag phase (GUIMARÃES, 2016). The exponential phase, shown in Figure 3, lasted 1 day (day 0–1), during which complete glycerol consumption occurred and the specific growth rate (μ) was constant and

maximum (1.37 d^{-1}). According to Guimarães (2016), this parameter is proportional to the cell concentration in the medium.

Fig. 3 – Specific growth rate during the cultivation days of *C. vulgaris* under optimized conditions.



According to Alterthum *et al*. (2020), the exponential phase, in addition to the maximum specific growth rate, is also characterized by the generation time (tg), the time required for the cell concentration to double. The tg found in the optimized cultivation of *C. vulgaris* was 12.24 h.

The first decline phase lasted 2 days (day 3–5). In this phase, the concentration of dead cells became greater than that of new cells (μ negative), which may occur due to unfavorable conditions, such as nutrient depletion in the medium. This explains the onset of this phase immediately after complete glycerol consumption. During this decline phase, despite the absence of glycerol, cells attempted to absorb other nutrients present in the medium, causing a slight increase in cell concentration (μ ceased to be negative), but not enough to significantly increase μ, since nutrient and metabolite concentrations decreased, reducing cell growth. Thus, the number of new cells equaled the number of dying cells, making the growth rate null (zero) and indicating the beginning of the stationary phase (GUIMARÃES, 2016). In the present study, the stationary phase began on the 5th day of cultivation and lasted until the 11th day.

On the 12th day of cultivation, the second decline phase began, as the scarcity of glycerol and other nutrients in the medium caused the cell concentration to decrease at a faster rate than cell production, making the specific growth rate negative (ALTERTHUM *et al*., 2020). This decline phase marked the end of cultivation.

The phases presented in this study indicate positive results for the cultivation of *C. vulgaris* under optimized conditions, since according to Alterthum *et al*. (2020), processes should aim to minimize the lag phase, maximize growth rate, extend the exponential phase, and delay the onset of the stationary phase. To achieve these objectives and maximize cell concentration, it is essential to evaluate how microbial growth is influenced by each variable.

The composition of biomass from optimized cultivation of *C. vulgaris* was identified by the percentage of total lipids (41.01 ± 0.01).

The fatty acid composition of biomass produced by microalgae is a fundamental characteristic for biodiesel quality (HE *et al*., 2015). The fatty acid concentrations obtained from *C. vulgaris* under optimized conditions, and from the same species in the study by Silva (2021), are shown in Table 5.

Table 5 – Fatty acid composition (%) in biomass of C. vulgaris cultivated under the optimal combination in the present study and reported by another author.

Fatty acid	***C. vulgaris* (SILVA. 2021)**	***C. vulgaris***
C4:0	1.29	-
C11:0	0.24	-
C13:0	3.16	0.54
C14:0	0.17	0.82
C14:1	0.85	-
C15:0	0.52	-
C15:1	1.20	2.32
C16:0	12.50	28.44
C16:1	1.05	2.76

C17:1	-	3.00
C18:0	16.65	11.66
C18:1 cis (n9)	1.20	14.19
C18:1 trans (n9)	2.96	-
C18:2 cis (n6)	-	16.10
C18:3 n6	13.09	
C18:3 n3	-	2.28
C18:4	-	-
C20:0	31.90	1.58
C20:4 n6	-	-
C20:5 n3	-	-
C22:0	-	2.80
C:22:4 n6	-	-
C22:5 n3	-	-
C22:6 n3	-	-
C24:1	-	-
Saturated	66.43	45.84
Monounsaturated	7.26	22.27
Polyunsaturated	13.09	18.38
Not determined	13.22	13.51

Biodiesel from microalgal oil obtained through heterotrophic cultivation can be an alternative to conventional diesel (XU *et al*., 2006). Regardless of the technological route used, biodiesel must ensure market acceptance by meeting internationally accepted specifications for its use. These characteristics and/or properties, which determine biodiesel identity and quality standards, include cetane number, iodine value, and cold filter plugging point (RAMOS *et al*., 2003).

Quality parameters were calculated based on the fatty acid composition of lipids obtained from *C. vulgaris*, to evaluate the possible application of this microalgal oil in biodiesel production. These parameters and the reference values are presented in Table 6.

Table 6 – Reference values of parameters for biodiesel quality.

Property	**Reference value by country**	***C. vulgaris***
Cetane number	> = 45 (ANP Brazil) > = 51 (European Union) > = 47 (United States)	62.77
IV (g iodo/100 g)	<= 120g/100g	59.70
CFPP (°C)	<= 19°C	15.74
SV	-	182.37
DU (weight % %)	-	60.03
LSCF (weight % %)	-	10.25

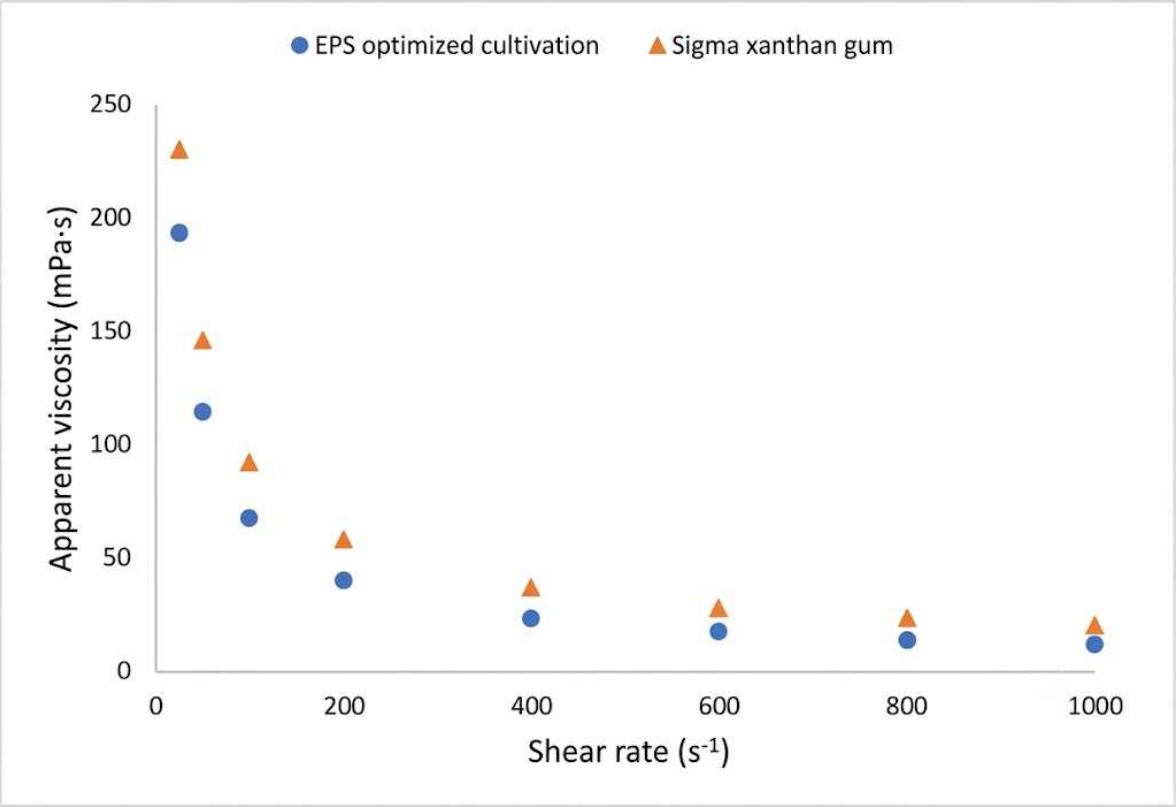
IV = Iodine value; CFPP = Cold filter plugging point; SV = Saponification value; DU = Degree of unsaturation and LSCF = Long-chain saturation factor

3.4.2 Rheological and emulsifying properties of extracellular polymers

The extracellular polymer (EPS) produced by *C. vulgaris* under the optimal combination of variables X, Y, and Z was extracted from the supernatant and solubilized in water for analysis of its rheological behavior.

Figure 4 shows the similarity of the effect of shear rate on the apparent viscosity of EPS solutions from *C. vulgaris* produced under optimal conditions and xanthan gum (Sigma), a bacterial exopolymer widely used as a viscosifier, thickener, and emulsifier in sectors such as food, chemical, and petrochemical industries (MENEZES *et al*., 2012; ANDRADE *et al*., 2021).

Fig. 4 – Effect of shear rate on the viscosity of aqueous solutions (0.4%, w/v) of extracellular polymer from *C. vulgaris* and Sigma xanthan gum.



For comparative purposes, the rheological behavior of the polymer generated under the optimal combination for biomass production by *C. vulgaris* was analyzed, and similarity was identified with the polymer synthesized by *C. vulgaris*. Table 7 shows the apparent viscosity value and rheological parameters of polymer solutions (0.4%, w/v) produced by *C. vulgaris* and Sigma xanthan gum.

Table 7 – Apparent viscosity of aqueous solutions (0.4%, w/v) of extracellular polymer from *C. vulgaris* and Sigma xanthan gum.

Parameters	***C. vulgaris* polymer**	**Sigma xanthan gum**
Apparent viscosity (mPa.s)*	193.17	230.17
K	2200.4	1928.2
n	0.24	0.34
R^2	0.97	0.95

* 25°C e 25 s^{-1}

Beyond the possibility of extracting extracellular polymers, the biomass-free medium can also be fully utilized, especially as an emulsifying agent in processes that do not require agent purity. The strategy is based on the direct application of the medium composed of metabolites and unconverted components, thereby reducing operational costs associated with bioproduct extraction and waste generation. Thus, emulsification indices were determined for the supernatant sample from *C. vulgaris* cultivation under optimized conditions.

Santos (2021) presented emulsification index results for commercial xanthan gum (Sigma Aldrich G1253). These results were then compared with the emulsification index values obtained from optimized *C. vulgaris* cultivation in the present study, as shown in Table 8.

Table 8 – Emulsification indices of samples obtained from *C. vulgaris* cultivation and commercial xanthan gum (Sigma) reported in Santos (2021), in different oils.

	Emulsification Index (EI24) (%)			
Sample	**Mineral oil**	**Sunflower oil**	**Soybean oil**	**Olive oil**
Xanthan gum	50	63	55	62
C. vulgaris	53.16	54.32	55.17	52.63

4. Discussion

4.1 Proximate composition of crude glycerol

The percentage of glycerol found (Table 1) was higher than the values reported by Valerio *et al*. (2015), who observed between 15.4 and 30.2% in crude glycerol samples of different origins, but lower than that reported by Guerfali *et al*. (2020), approximately 64.5%. These variations reflect differences in raw materials and purification steps adopted in industrial biodiesel processes.

From a biotechnological perspective, the composition of this residue is particularly relevant for microbial cultivation, since carbon (C) and nitrogen (N) elements play a central role in cellular metabolism and biomolecule synthesis (ASSIS *et al*., 2019). In microbial systems, the relative availability of these nutrients can direct metabolism toward different biosynthetic pathways,

including lipid and polymer production (SILVA *et al*., 2023; ROMERO-FRASCA & BUITRÓN, 2024; YANG *et al*., 2024). In the case of microalgae, variations in nutrient availability in the culture medium may result in significant changes in biomass productivity and synthesis of compounds of interest (ESTÉVEZ-LANDAZÁBAL *et al*., 2013).

In addition to acting as an organic carbon source in microalgae cultivation, crude glycerol may contain trace amounts of nutritional components such as phosphorus, magnesium, nitrogen, calcium, sodium, and sulfur, which can contribute to microbial metabolism and cell growth. In this context, glycerol can be assimilated by microorganisms as a metabolic intermediate, being converted into central compounds of cellular metabolism such as dihydroxyacetone phosphate and glycerol-3-phosphate, which participate in metabolic pathways related to energy generation and lipid biosynthesis (PYLE *et al*., 2008; SMYTHERS *et al*., 2019).

Despite these advantages, the composition of the crude glycerol used showed low protein content, indicating limited nitrogen availability. According to Uprety *et al*. (2017), nitrogen content in different crude glycerol samples is generally reduced, ranging from 0.03 to 1.2% by mass. Interestingly, nitrogen-limiting conditions may favor lipid accumulation in oleaginous microorganisms, since excess available carbon is redirected to energy storage pathways (RATLEDGE, 2004), which broadens the possibilities of using the crude glycerol analyzed.

Although the residual glycerol analyzed is practically devoid of nitrogen sources, its high carbon content makes it a potentially interesting substrate for heterotrophic microalgae cultivation aimed at bioproduct production. In such systems, supplementation of the medium with additional nitrogen sources may allow adjustment of the C/N ratio, simultaneously favoring cell growth and synthesis of metabolites of interest.

4.2 Inoculum acclimatization

Compared to the results found during acclimatization, similar results were reported by Bonini (2012), who observed reduced growth of *C. vulgaris* at higher glycerol concentrations (4.6, 9.2, and 46 g L^{-1}).

This behavior has been associated with the inhibitory effect of high substrate concentrations, possibly related to increased osmotic pressure in the culture medium due to glycerol presence.

In the present study, satisfactory cell growth was observed at a concentration of 2 g L^{-1} glycerol, which was then selected for inoculum acclimatization. The acclimatization process was conducted for 15 days.

The concentration used during acclimatization also served as a reference for defining the levels of variables evaluated in the experimental design, ensuring that the studied ranges remained within a physiologically tolerated range for microalgae.

4.3 Optimization of microalgal biomass and extracellular polymer production

The inherent variability of biological systems constitutes a significant source of experimental error. Recent studies with microalgae have demonstrated that growth and metabolite production are highly sensitive to small variations in environmental conditions, such as light intensity, temperature, pH, and nutrient availability, which can increase data dispersion and compromise the predictive performance of statistical models (ZHANG *et al*., 2021; ZHANG *et al*., 2022; DO & DU, 2024).

The possible exclusion of relevant variables from the experimental design may have contributed to the low predictive capacity of the model. Contemporary approaches highlight that complex biotechnological systems often involve multiple interactions and nonlinear effects, and the omission of critical variables can significantly reduce the quality of the fit (ZHANG *et al*., 2024; ZHANG *et al*., 2023; FREITAS *et al*., 2025).

4.4 Characterization of bioproducts under optimized conditions

4.4.1 Biomass of *C. vulgaris* IBL-C105

De Ahmad *et al*. (2025) conducted autotrophic cultivation of *C. vulgaris* for 12 days in a chamber with continuous illumination and obtained a maximum biomass concentration of 0.61 g L^{-1}. Thus, it can be observed that the heterotrophic cultivation of *C. vulgaris* under optimized conditions in the

present study resulted in a higher biomass concentration, with shorter duration and reduced costs, since the use of alternative carbon sources eliminates expenses with nutrients, artificial lighting, and aeration (PEREZ-GARCIA *et al*., 2011; ROSSI *et al*., 2024).

De Morais *et al*. (2020) evaluated mixotrophic cultivation of *Spirulina sp*. LEB 18 in medium containing glycerol (2.5 g L^{-1}) for 15 days, and in all conditions studied, the highest biomass concentration (1.94 g L^{-1}) and productivity (0.43 g L^{-1} d^{-1}) were found in the period from 0 to 2 days, when the microalgae consumed glycerol and stored energy. This increase in productivity occurred during the same period in which the available glycerol was completely consumed by the microalgae (2 days). After this period, a decrease in biomass yield was observed in all assays supplemented with this organic substrate, since depletion of the organic source can reduce biomass productivity (DE MORAIS *et al*., 2019).

Katiyar *et al*. (2017), in heterotrophic cultivation of *Chlorella sp*. for 7 days with crude glycerol (4 g L^{-1}), obtained nearly twice the biomass productivity (0.45 g L^{-1} d^{-1}) compared to the control cultivation in basal medium. Ma *et al*. (2016), in mixotrophic cultivation of *C. vulgaris* for 7 days with crude glycerol (5 g L^{-1}), observed that the maximum biomass concentration (1.82 g L^{-1}), maximum biomass productivity (0.26 g L^{-1} d^{-1}), and maximum specific growth rate (1.65 d^{-1}) occurred within the first three days of cultivation, the same period in which glycerol was almost completely consumed. From the fourth day of cultivation, the specific growth rate (μ) and nutrient removal rates were low.

Regarding the phases represented in Figure 3, Bonini (2012) compared heterotrophic and mixotrophic cultivation of *C. vulgaris* in medium supplemented with glycerol (0.92 g L^{-1}), and in heterotrophic cultivation obtained a generation time of 7.7 h and a maximum specific growth rate of 0.09 h^{-1}. In mixotrophic cultivation, the generation time and maximum specific growth rate were 16.1 h and 0.043 h^{-1}, respectively, demonstrating that heterotrophic growth was more favorable for biomass production, as it reduced the time required for cell concentration to double. Moreover, prolonged

heterotrophic cultivation of microalgae often leads to contamination problems (PEREZ-GARCIA *et al*., 2011; BONINI, 2012).

Although microalgae naturally synthesize lipids, it is possible to induce and intensify this synthesis through control of nutritional stress, pH, CO_2 injection, light intensity and quality, and photoperiod (CHO *et al*., 2013; SUTHAR & VERMA, 2018). Some supplied nutrients, such as nitrogen and phosphorus, can affect growth and lipid content, since nutrient limitation alters carbon dynamics and diverts it toward lipid accumulation, i.e., induces triacylglycerol metabolism instead of protein and carbohydrate synthesis. Therefore, through nutrient control, it is possible to stimulate adaptive mechanisms without impairing the production of specific metabolites (IDRIS *et al*., 2017; GOUGOULIAS *et al*., 2021). This mechanism may explain the lipid accumulation observed in this study.

Cho *et al*. (2016) conducted autotrophic cultivation of *C. vulgaris* and, according to their results, suggested that inorganic carbon, phosphorus, and light intensity are the main limiting factors in microalgal biomass production and lipid productivity. Idris *et al*. (2017), also with autotrophic cultivation of *C. vulgaris,* studied the effect of nitrogen stress on biomass and lipid production, and found maximum lipid content of 58.98% ± 0.02 in freshwater and 49.87% ± 0.01 in seawater. It can be observed that under nitrogen stress, lipid productivity increased, but growth rate decreased, and the lipid content values reported in that study are similar to those found in the present work.

In the present study, optimized cultivation of *C. vulgaris* resulted (Table 5) in 45.84% saturated fatty acids (SFA), 22.27% monounsaturated fatty acids (MUFA), and 18.38% polyunsaturated fatty acids (PUFA). Silva (2021), in mixotrophic cultivation of *C. vulgaris*, reported 66.43%, 7.26%, and 13.09% for SFA, MUFA, and PUFA, respectively.

Medina *et al*. (2017) conducted heterotrophic cultivation of *Chlorella sp*. in medium containing glucose, and the fatty acid composition obtained was 30.04% saturated, 54% monounsaturated, and 16.74% polyunsaturated. In the present study, the percentage of saturated fatty acids presented by *C. vulgaris* was higher, and these acids are of interest for biodiesel production, since the higher the

saturation of the chain, the higher the cetane number, which is one of the quality parameters of biodiesel. In addition, the presence of these acids enables the production of an oil with greater resistance to fuel oxidation and higher stability (ZUÑIGA *et al*., 2011; CASTRO & BARAÑANO, 2019; DE MORAIS *et al*., 2020).

Katiyar *et al*. (2017), in heterotrophic cultivation of *Chlorella sp.* with crude glycerol (4 g L^{-1}), reported 42.91% saturated fatty acids and 11.92% polyunsaturated fatty acids. In the present study, *C. vulgaris* also showed a reduced percentage of unsaturated fatty acids (18.38%), and the low content of these acids favors biodiesel quality, as it reduces vulnerability to polymerization, thereby increasing the shelf life of this biofuel (ZHANG *et al*., 2009; KATIYAR *et al*., 2017).

In biodiesel, the fatty acid profile corresponds to that of the raw material used. The fatty acid profiles of the most common oilseed sources are mainly composed of five C16 and C18 fatty acids: palmitic (C16:0), stearic (C18:0), oleic (C18:1 cis (n9)), linoleic (C18:2 cis (n6)), and linolenic (C18:3 n3) (KNOTHE, 2009).

The main fatty acids identified in microalgal oils obtained from *C. vulgaris* under optimized conditions were those containing 16 and 18 carbons. The action of acetyl-CoA carboxylase favors the high concentrations of these acids, as it converts carbon present in the medium into malonyl-CoA, through elongation and desaturation, mainly forming C16 and C18 fatty acids (CARDOSO *et al*., 2020).

Ge et al. (2018), in heterotrophic cultivation of *Chlorella vulgaris* in medium containing glycerol (2.0 g L^{-1}), found mainly C16, C18, and C20 fatty acids, reporting C16:0 (13.9%), C18:1 (19.2%), and C20:3 (14.9%). In heterotrophic cultivation of *Chlorella sp*. by Medina *et al*. (2017), C16:0 (7.24%) and C18:2 (11.86%) levels were lower than those obtained by *C. vulgaris* in the present study, 28.44% and 16.10%, respectively.

Oleic acid (C18:1) is considered an indicator of biodiesel quality and was found in the fatty acid composition of *C. vulgaris* (14.19%) cultivated under optimized conditions in medium containing

crude glycerol. To improve biodiesel properties, such as oxidative stability and melting point, the addition of methyl oleate is suggested (KNOTHE, 2008; MORAIS *et al*., 2005).

Arachidic acid (C20:0) showed a reduced percentage in *C. vulgaris* (1.58%), which may be related to partial supplementation of BG-11 medium with glycerol, which has lower nitrate (NO_3^-) concentration than BG-11 medium without supplementation. In the study by Costa *et al*. (2006), with cultivation of *Chlorella minutíssima* in BG-11 medium, the C20:0 content was 22.62%, and it was observed that increasing NO_3 concentration raised the C20:0 content.

When analyzing the quality parameters of biodiesel, it becomes clear that the oil obtained from the cultivation of *C. vulgaris* presented a cetane number in accordance with the reference values of Brazil, the European Union, and the United States. This demonstrates that it will have good combustion in engines, since the cetane number is related to the ignition delay (the period between the start of fuel injection and the start of combustion) of fuel and biofuel. High-quality combustion should occur with rapid ignition, followed by smooth and complete fuel combustion. Thus, the cetane number is inversely proportional to ignition delay; in other words, the higher the cetane number, the shorter the ignition delay, and the better the combustion in diesel engines (BAMGBOYE & HANSEN, 2008).

The structure of the methyl esters of fatty acids that compose biodiesel determines ignition quality, and the cetane number depends on how fatty acids are distributed in the original oil or fat used for biodiesel production (BAMGBOYE & HANSEN, 2008). This value is related to saturation and carbon chain length of fatty acids: the more saturated and longer the carbon chains, the higher the cetane number. Conversely, the cetane number decreases with increased unsaturation and increases with longer chain length (ZUÑIGA *et al*., 2011; CASTRO & BARAÑANO, 2019).

Regarding the cold filter plugging point (CFPP), the oil obtained from *C. vulgaris* presented a value corresponding to the reference parameter, which facilitates its use for biodiesel production. CFPP is the temperature at which fuel flow is interrupted when cooled, and it is an important parameter for biodiesel use, especially in countries with low temperatures. One of the limitations of biodiesel use

on a larger scale is its tendency to solidify or lose fluidity at higher temperatures than diesel, which can cause filter clogging in engines (GIRARDI, 2019).

CFPP is defined by the fatty acid profile, i.e., it is related to the chain length of esters present in biodiesel and to the saturation level of molecules. The higher the content of unsaturated fatty acids in the oil used for biodiesel production, the lower the CFPP value, meaning better cold flow properties (LÔBO *et al*., 2009; LIU, 2015; OLIVEIRA JR. *et al*., 2015).

The iodine value of the oil obtained from *C. vulgaris* is in accordance with the reference value, indicating that this oil is stable and less susceptible to oxidation. This parameter indicates the tendency of oil or fat to undergo oxidation, polymerization, and deposit formation in engines. It influences several relevant biodiesel properties, such as CFPP and oxidative stability (BAPTISTA *et al*., 2008; RAMOS *et al*., 2009; ADEWALE *et al*., 2014).

This parameter is a measure of total unsaturation within a fatty acid mixture, determined by the formal addition of iodine to double bonds, expressed in g iodine/100 g sample. The iodine value of biodiesel is almost equal to that of the oil used in its production (RAMOS *et al*., 2009; KNOTHE *et al*., 2010). In vegetable oils, it is one of the identity parameters, meaning different types of biodiesels may present iodine values similar to those of their triglyceride sources. However, oils with similar iodine numbers can have considerably different oxidative stabilities. Therefore, predicting the oxidative stability of a given oil requires knowledge of its fatty acid composition, which can only be obtained through chromatographic analysis methods (RAMOS *et al*., 2003).

Thus, it is clear that different factors can influence biodiesel quality. Nevertheless, in the present study, the fatty acid compositions obtained and the biodiesel properties evaluated, which were in accordance with reference values, suggest the potential application of *C. vulgaris* biomass in biodiesel production.

4.4.2 Rheological and emulsifying properties of extracellular polymers

According to the Figure 4, the behavior of the curves is similar and demonstrates that the fluids are non-Newtonian and pseudoplastic, since viscosities decrease as shear rate increases (FOX *et al*., 2010). In different industrial sectors, pseudoplastic behavior can be observed in various products and is of great importance in the chemical industry (DE CAMARGO, 2020). This type of fluid, when at rest, presents a disordered state of its molecules; however, when subjected to shear stress, its molecules tend to align in the direction of the applied force. The greater the force, the greater the alignment, and consequently, the lower the apparent viscosity (SAMPAIO, 2014).

Pseudoplastic behavior is desirable in processes where flow viscosity needs to be lower than viscosity at rest, enabling better control on application surfaces, ease of spreading, and flow of materials for storage in tubes or containers. This behavior is suitable for topical products, in which the initial resistance to emulsion flow decreases after shear, facilitating use (MORAIS *et al*., 2005; COUTINHO *et al*., 2018).

Pseudoplastic fluids are found in pharmaceutical products; in healthcare (blood, sunscreen, toothpaste); in food (yogurt, ketchup, mayonnaise); in paint industries, emulsions, polymer solutions; colloidal suspensions; and in the petroleum industry (drilling fluids) (CHHABRA & RICHARDSON, 2008; FOX *et al*., 2010).

The analysis of the apparent viscosity and rheological parameters of the polymer solutions (0.4% w/v) produced by *C. vulgaris* and xanthan gum sigma (Table 7) shows that the fluid behavior index (n) showed that the three solutions present pseudoplastic behavior, characterized by $n < 1$ (FOX *et al*., 2010). The extracellular polymer from *C. vulgaris* exhibited apparent viscosity similar to that of xanthan gum, but was considered more pseudoplastic, since its consistency index (k) was higher than that of Sigma xanthan gum, and it also presented a lower flow index (n). In addition to having more significant pseudoplastic behavior, this polymer is produced by bacteria, has a high degree of purity, and is industrially synthesized from conventional energy and carbon sources.

De Jesus *et al*. (2019) evaluated the apparent viscosity of extracellular polymer solution at 0.05% (w/v), produced by *Spirulina sp*. LEB-18 in synthetic medium (Zarrouk) over 30 days of cultivation,

finding an apparent viscosity of 18.34 mPa·s. In this study, the biopolymer from *C. vulgaris* presented results closer to those of commercial gum, using residues as partial substitutes for synthetic medium and requiring less cultivation time (12 days), which demonstrates the quality of this biopolymer and its potential for applications in various industrial sectors.

The emulsification indices found for samples from optimized *C. vulgaris* cultivation were above 50%, as shown in Table 8. This indicates that the polymer can be considered a good emulsifying agent for all tested oils, since emulsions are considered stable when the emulsification index is equal to or greater than 50% after 24 h at rest (RAMOS *et al*., 2020; SANTOS, 2021).

Bacterial polysaccharides, such as xanthan gum, are bioemulsifiers applied in the food and cosmetics industries, capable of emulsifying various hydrophobic compounds due to their pseudoplastic nature, which provides excellent stability to oil-in-water emulsions (DÁRIO *et al*., 2011; HAIDAR *et al*., 2020).

The results obtained from *C. vulgaris* under optimized conditions showed values close to those of Sigma xanthan gum, evidencing a high production of biosurfactants. Biosurfactants are surfactants produced by microorganisms, with specific properties (detergency, emulsification, foaming, phase dispersion, lubrication, solubilization, and wettability) for applications in agriculture, detergent production, pharmaceutical, cosmetic, food, and petrochemical industries, among others (BANAT *et al.*, 2000; SCHMITZ *et al*., 2012; SANTOS *et al*., 2018). Thus, this bioproduct obtained from the studied microalgae species demonstrates potential for use as an emulsifying agent in specific industrial sectors with lower purity requirements, such as the petrochemical industry.

In the optimized cultivation of *C. vulgaris*, the highest emulsification index was found in the analysis using soybean oil (Table 8). This indicates that the ability to form stable emulsions depends not only on the emulsifying properties of the biosurfactant but also on the type of oil used in the analysis (FREITAS *et al*., 2022).

4 CONCLUSION

The heterotrophic cultivation of *Chlorella vulgaris* IBL-C105 using crude glycerol as a carbon source demonstrated efficiency in the synthesis of multiple high-value bioproducts. The optimization of agitation speed and sodium nitrate and glycerol concentrations enabled rapid biomass production (1.18 g L^{-1} in 24 h), associated with complete substrate consumption, evidencing the potential of this system for bioremediation, integrated waste valorization, and process intensification (or coupled with high productivity). In addition, the optimized condition favored lipid accumulation with a suitable profile for biodiesel production, highlighting the energetic potential of the biomass. Extracellular polymers exhibited pseudoplastic behavior, with properties similar to commercial xanthan gum, indicating application as a rheology-modifying agent, and the biomass-free medium presented an emulsification index above 50%, suggesting the presence of compounds with surface activity and potential application as an emulsifying agent in industrial formulations.

DECLARATIONS

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