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Original Research Article

1 # Microalgae as a source of functional PUFAs: a
2 green low-cost pathway via enzymatic
3 hydrolysis

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16 ## ABSTRACT
17

Microalgal oil is an alternative source of polyunsaturated fatty acids (PUFAs) that can replace conventional ones such as vegetable and fish oils. In this work, *Nannochloropsis gaditana* oil was investigated as a source of high-value polyunsaturated fatty acids.
The cultivation conditions were optimized using a 2^2 full factorial design to simultaneously assess the influence of $NaNO_3$ (from 75 to 225 mg L^{-1}) and CH_3COONa (from 2 to 6 g L^{-1}) concentrations on the lipid productivity. All cultivations were done in 4 L tank photo bioreactors for 7 days, yielding 21.87 mg L^{-1} day^{-1} maximum lipid productivity when using 225 mg L^{-1} of nitrate and 6 g L^{-1} of acetate. The obtained microbial oil was further characterized revealing palmitic (22.1%), oleic (22.3%), and linoleic (17.0%) as the main fatty acids. The resulting microbial oil was employed in the hydrolysis reactions and different sources of lipases as biocatalysts. The highest performance was achieved by the lipases *Burkholderia cepacia* (86%), *Candida rugosa* (84%), and *Rhizopus oryzae* (82%).

18
19 *Keywords: Nannochloropsis gaditana, full factorial design, $PUFA_S$, enzymatic hydrolysis.*
20
21 ## 1. INTRODUCTION
22

23 Microalgae have been an important subject of study at universities throughout the world due
24 to their possibilities of product generation. For instance, microalgae cultivation has been
25 documented to be a vital source of food generation for aquaculture and a promising source of
26 lipids for biodiesel production [1–3]. This importance arises from several aspects of microalgae
27 cultivation such as high intracellular lipid accumulation, content. In order to improve the
28 microalgae oil productivity, a good possibility is to optimize the nutrient concentrations in the
29 culture media. The nitrate source (sodium nitrate used in the present work) is widely discussed
30 and reported to have strong influence over lipid production [4–7]. The carbon source has also
31 a great influence in microalgae growth and lipid accumulation. CO_2, for instance, is largely
32 used. However, great amounts of CO_2 is lost because of its low solubility in water [8,9]. Sodium
33 acetate, otherwise, is an organic carbon source not yet largely employed. Acetate, though
34 have been reported as good influence increasing lipid production [4].

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Another aspect that further attracts strong attention is the possibility of growing microalgae even in lands that are not fertile or suitable for agriculture, hence reducing the competition with food production [10–13]. Microalgae are a promising source of lipids to produce PUFAs, which are high-value products in pharmaceutical, cosmetic, food, and feed industries due to their various associated health benefits. For example, the omega 3 (ω3) fatty acids (FAs), specifically eicosapentaenoic acid (EPA, C20:5), docosahexaenoic acid (DHA C22:6), and α–linoleic acid (ALA C18:3), are not synthesized by the human body and thus need to be ingested in the diet. The ω3 FAs have been demonstrated to exhibit anti-inflammatory (EPA/DHA), antithrombotic (EPA), antiarrhythmic (EPA/DHA), vasodilatory (EPA), and anti-tumor activities (DHA). Additionally, their roles in avoiding oxidative stress (DHA) and improving the bone density (DHA) have been reported [14–17]. ALA, in turn, can help in brain development and insulin resistance. ALA is also known as a precursor in the synthesis of EPA and DHA. Therefore, the inclusion of ALA in the diet is of high importance for the maintenance of good health [16]. Many microalgae species have been described in the literature as good ω3 producers. These species include *Nannochloropsis gaditana*, *Nannochloropsis oculata*, *Pavlova lutheri*, *Isochrysis galbana*, *Scenedesmus* sp., *Isochrysis* sp., *Dunaliella salina*, among others [1,14,18]. In this context, it has been described that microalgae produce not only high amounts of lipids but also considerably good proportions of ω3, and thus they could replace fish oil and be used as the main ω3 sources [16,19].

A reasonable process used to obtain PUFAs from *Nannochloropsis gaditana* oil is enzymatic hydrolysis. Indeed, lipase enzymatic hydrolysis has several advantages over the chemical procedure. The advantages include the high fatty acid selectivity that is of critical importance for the proposed application and the mild reaction conditions in terms of pH, pressure, and temperature, which are essential for processes that involve highly labile polyunsaturated fatty acids [20,21]. In addition, the enzymatic hydrolysis can be performed with samples containing high amounts of free fatty acids or water, which allow to avoid the costly drying procedures and to produce high quality glycerol [20,22].

The choice of the lipase is one of the most decisive variables in the enzymatic hydrolysis, especially due to its high impact on the overall cost of this process. The fundamental function of lipases is to catalyze the hydrolysis of ester bonds such as the conversion of triglycerides into fatty acids. However, these enzymes can be also used to catalyze transesterification, esterification, and interestification reactions depending on the existing media. Lipases can be basically obtained from three different sources; namely animal, vegetal, or microbial source. Among these origins, microbial lipases have been widely studied in the literature and gained the most interest due to their higher resistance and lower production cost [20]. *Candida rugosa* lipase, for instance, is a valuable lipase in enzymatic hydrolysis for many reasons. It is very active and versatile enzyme that can tolerate the presence of a great range of free fatty acids as well as water in the oil sample, realizing good conversions [20,22,23].

Considering these factors, the objective of this work was to screen grade commercially available lipases to be used in hydrolysis reactions from *Nannochloropsis gaditana* microalgae oil for PUFAs production. The microalgae were cultivated under different nutrient concentrations (sodium nitrate and sodium acetate) according to full factorial design 2^2 to enhance the oil production. The cultivation conditions that yielded the highest oil productivity were used to perform several batch runs in order to accumulate sufficient microbial oil that permits to carry out the enzymatic hydrolysis tests.

2. MATERIAL AND METHODS

2.1 Microalgae strain and biocatalysts

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83 Marine microalga Nannochloropsis gaditana (BMAK 130) came from Seaweed Culture
84 Collection (Oceanographic Institute - University of São Paulo) and was kindly provided by the
85 Department of Biological Oceanography (São Paulo, Brazil). Five commercial lipases in a
86 crude form were used in this study as catalyst for the hydrolysis enzymatic: Candida rugosa
87 (LipomodTM 34P) and Rhizopus oryzae (Lipase L036P) acquired from Biocatalysts (Cardiff,
88 United Kington), Burkholderia cepacia (Lipase BLC); pancreatic (lipase type II), Thermomyces
89 laguginosus (Lipolase) bought from Sigma-Aldrich (St. Louis, MO, USA). All other reagents
90 were of analytical grade.

91 2.2 *Nannochloropsis gaditana* growth conditions: experimental design and data 92 analysis

93 The effect of concentrations of $NaNO_3$ (75 to 225 mg L^{-1}) (X_1) and CH_3COONa (2 to 6 g L^{-1})
94 (X_2) on the culture of *Nannochloropsis gaditana* were studied using a 2^2 full factorial design.
95 The microalgae culture was inoculated at 10% (v/v) in tank photobioreactors (15 cm wide and
96 33 cm height), with 4 L working volume, sparged with sterile air at 1.4 L min^{-1} aeration rate
97 and maintained at 24 ± 1 °C under 150 klux light intensity for 7 days. The cultivation media
98 was a modified, without silica, Guillard f/2 medium [24]. The center point was repeated six
99 times in order to improve the error determination. Microalgae cells were recovered by
100 flocculation using 1 mol L^{-1} $FeCl_3$ solution, in order to reduce the working volume to 3-5% of
101 its original, saving time and energy in the filtration step [7]. Biomass productivity was obtained
102 dividing the total amount of dried biomass obtained in a single cultivation run by the working
103 volume of the photobioreactor (4 L) and the cultivation period (7 days). The 'Design expert'
104 (version 6.0 - Stat-Ease Corporation, USA), 'Statistica' (version 8.0 - Stat Soft Inc., USA) and
105 'Minitab' (version 18.0 – Minitab Inc., USA) software were used for regression and graphical
106 analyses of the obtained data. The lipid productivity was taken as response variable. Design
107 expert' software was used to obtain graphical and numerical analysis based on the criterion
108 of desirability.

109 2.3 Microalgal oil extraction

110 Microbial oil was extracted from biomass according to a modified Folch method, performed
111 under ultrasound, with a mixture of chloroform: methanol (3:1 v/v). The mixture was sonicated
112 for 10 min. This procedure was repeated three times [25]. In this process, the ultrasound
113 promotes cavitation throughout the extraction media, causing cell rupture, whilst the solvent
114 mixture effectively dissolves and recovers the microbial lipids [26,27]. Extracted lipids were
115 dried in a rotatory evaporator to remove the residual solvent and subsequently dried at 60°C
116 until constant weight was attained. The production efficiency of the microbial lipids was
117 calculated based on the biomass concentration (X), lipid concentration (P), lipid yield (% P)
118 and lipid specific yield ($Y_{P/X}$) obtained in the culture cultivations. The results were also
119 analyzed considering the following parameters: volumetric productivity in relation to biomass
120 (Q_X) and volumetric productivity in relation to lipid (Q_P) [28]. The lipid productivity was obtained
121 multiplying the biomass productivity by the lipid content obtained in the oil extraction.

122 2.4 Microalgal oil characterization

123 AOCS's method [29] was used for total free fatty acids (FFA) determination, which was
124 expressed in terms of free oleic acid (%). Kinematic viscosity was determined with a Brookfield
125 viscometer (Brookfield Viscometers Ltd, England) using a CP 52 cone [30]. Iodine value was
126 determined by [29].

127 The fatty acid methyl esters (FAMEs) were synthesized according to [29] and identified by gas
128 chromatography (CG). The CG analyses were performed by a PerkinElmer® - Clarus 580

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129 chromatograph, equipped with a flame-ionization detector (250°C, 40 mL min^{-1} H_2, and
130 400 mL min^{-1} synthetic air). A 30 m capillary column with 0.25 mm internal diameter and 5%
131 diphenyl 95% dimethylpolysiloxane stationary phase (non-polar) was employed during the
132 analysis. The oven ramp temperature rate was 3°C min^{-1} from 120 to 235°C and 1°C min^{-1}
133 until 255°C, during the total 60 min of the analysis. Nitrogen was the carrier gas (5 mL min^{-1}).
134 The external patterns used were MIX Supelco®, with 37 fatty acids methyl ester (Sigma-
135 Aldrich®), C4-C24.

136 **2.5 Hydrolysis reactions**

137 Batch hydrolytic reactions were performed in 25 mL spherical glass reactor under magnetic
138 stirring (400 rpm) at 40 °C for 8 h, containing the microalgae oil emulsion at 1:2 oil/water ratio
139 (1 g of microalgae oil), 1 wt% soy lecithin emulsifier, 3 mL of a buffer solution with pH = 7.0
140 and hexane as a solvent (2.5 mL added into the reaction vessel). The reaction was started by
141 the addition of 10 wt% of each lipase: *Candida rugosa*, *Rhizopus oryzae*, *Burkholderia*
142 *cepacia*, pancreatic and *Thermomyces laguginosus*. At intervals, aliquot of the reaction
143 medium (0.2 g) was taken at various time intervals and analyzed by titration. Fifty milliliters of
144 50:50 (v/v) mixture of acetone in ethanol were added to the sample to dissolve the oil and to
145 denature the enzyme, thus effectively freeze the reaction. The mixture was titrated with
146 standard 0.02 mol L^{-1} potassium hydroxide solution. The hydrolysis percentage (H%) was
147 calculated by equation 1. H% is defined as the percentage weight of free fatty acids in the
148 sample divided by its maximum theoretical amount [21].

149 $$\% H = \frac{V_{KOH} \times M_{KOH} \times \overline{\mathrm{MW}}}{W \times f} \times 100 \qquad (1)$$

150 Where: V_{KOH} is the volume of potassium hydroxide solution (KOH) required during titration;
151 M_{KOH} is the KOH molarity (0.02 mol L^{-1}); $\overline{\mathrm{MW}}$ is the average molecular weight of fatty acids (g
152 mol^{-1}); W is the weight of the sample taken and f is the fraction of oil at start of reaction.

153 The initial rate of reaction was calculated using the equation 2 [31].

154 $$r_0 = \frac{10^4 S_0}{MW}\left(\frac{dX}{dt}\right) \qquad (2)$$

155 Where: r_0 = is initial rate of hydrolysis (μmol L^{-1} min^{-1}); S_0 is initial concentration of oil (g L^{-1});
156 (dX/dt) is slope of the degree of hydrolysis (X) versus time curve.

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158 **3. RESULTS AND DISCUSSION**
159
160 **3.1 Experimental design for the biomass production**
161

162 The influence of the sodium nitrate concentration (ranging from 75 to 225 mg L^{-1}) and the use
163 of sodium acetate as the carbon source (with concentrations ranging from 2 to 6 g L^{-1}) on
164 *Nannochloropsis gaditana* biomass and lipid productivities were studied using a 2^2 full factorial
165 design with a central point. The results are summarized in Table 1.

166 **Table 1. Experimental results from the 2^2 full factorial design study of *Nannochloropsis***
167 ***gaditana* cultivation media. The independent variables are: sodium acetate**
168 **concentration (X_1) and sodium nitrate concentration (X_2), in their natural and coded**
169 **values. The two last columns show the dependent variables: cell and lipid**
170 **productivities. The corner points (1-4) were made in duplicate, and the center point (5)**
171 **was repeated six times.**
172

Exp.	Nitrate X_1(mgL^{-1})	Acetate X_2(g L^{-1})	Cell productivity (g L^{-1} day^{-1})	Lipid productivity (mg L^{-1} day^{-1})
1	75 (-1)	2 (-1)	88.26 ± 2.40	12.76 ± 1.24
2	75 (-1)	6 (+1)	97.01 ± 4.94	14.01 ± 2.27
3	225 (+1)	2 (-1)	111.03 ± 2.02	9.48 ± 0.03
4	225 (+1)	6 (+1)	188.93 ± 10.78	21.87 ± 1.77
5	150 (0)	4 (0)	147.62 ± 3.67	18.63 ± 0.79

173

174 Cell productivities ranged from 88.26 to 144.21 mg L^{-1} day^{-1}, and the oil productivities varied
175 between 9.48 to 21.87 mg L^{-1} day^{-1}. From the statistical analysis of these results, a Pareto
176 chart, depicted in Figure 1, was obtained. The Pareto chart showed that both nitrate and
177 acetate had relevant influence over the lipid productivity. Acetate, however, had the stronger
178 observed impact in the microalgae oil production.

179 From the Pareto chart, it was concluded that both the acetate and the nitrate had strong
180 influence over the *Nannochloropsis gaditana* lipid productivity. The interaction effect, also
181 influent to the lipid productivity, can only be observed when the two independent variables,
182 nitrate and acetate, are simultaneously adjusted. In the present study, a positive and strong
183 interaction factor means that the simultaneous increase of acetate and nitrate concentrations
184 have a higher impact on the lipid productivity. This can be related to the fact that increasing
185 the sodium nitrate concentration raises the cell production, while increasing the sodium
186 acetate influences the lipid content. Therefore, increasing both concentrations would have a
187 good impact on both of cell production and lipid content.

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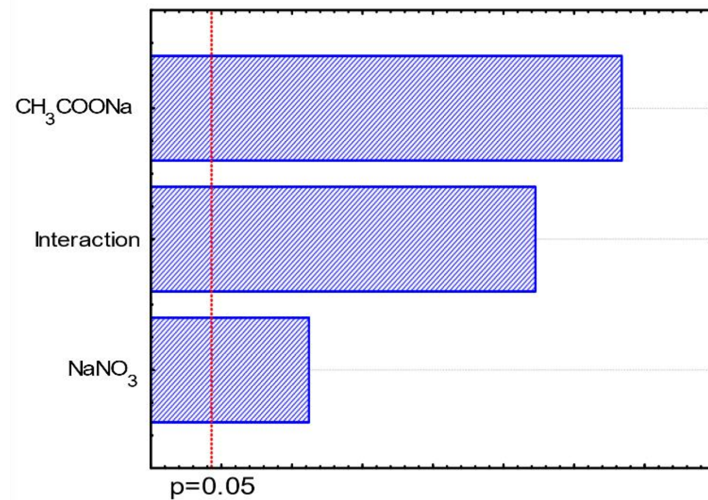

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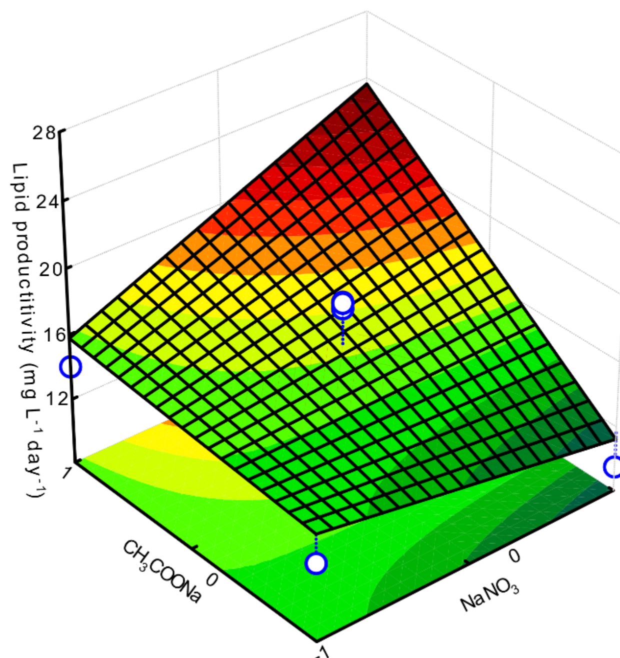
190 **Figure 1. Pareto chart indicating the standardized effect of the nitrate, acetate and**
191 **their interaction over the *Nannochloropsis gaditana* lipid productivity.**

192 The modeling of the lipid production, obtained from the 2^2 full factorial analysis, is shown in
193 Figure 2. In this graphic the acetate and nitrate are presented in their coded variables, from
194 - 1 to 1, and the lipid productivity is represented in terms of mg l^{-1} day^{-1}. The obtained modeling
195 of lipid productivity reveals that the optimum point in the studied area is the one where both
196 variables are at the highest levels (225 mg L^{-1} of nitrate and 2g L^{-1} of acetate - +, +).
197 Furthermore, the "upward bending" of the surface near the optimum point as a result of the
198 strong interaction effect of the factors is noticeable.



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200 **Figure 2. Modeling of *Nannochloropsis gaditana* lipid productivity as a function**
201 **of nitrate and acetate concentrations.**

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202 Table 2, in turn, illustrates the results obtained in this study with respect to biomass
203 concentration (X), volumetric biomass production rate (Q_X), lipid concentration (P), lipid
204 specific yield ($Y_{P/X}$), specific rate of lipid production (qP), and volumetric lipid production rate
205 (Q_P) obtained in the (+, +) cultivation condition.

206

207 **Table 2. Cultivation parameters obtained for *Nannochloropsis gaditana* in the optimum**
208 **studied condition (+, +).**

Parameters	Values
Biomass (X, g L^{-1})	1.32 ± 0.40
Lipid concentration (P, mg L^{-1})	185 ± 0.30
Lipid specific yield ($Y_{P/X}$, mg lipids mg $biomass^{-1}$)	0.14 ± 0.10
Volumetric lipid production rate (Q_P, mgL^{-1} day^{-1})	26.43 ± 0.30
Volumetric biomass production rate (Q_X, mg L^{-1} day^{-1})	188.93 ± 0.10
Specific rate of lipid production (qP, mg lipid mg $biomass^{-1}$ day^{-1})	0.02 ± 0.08

209

210 The results obtained in the present work are adequate especially when compared with the
211 literature regarding the microalgae *Nannochloropsis gaditana*, such as the work of Pedro et
212 al. [32] for example. In their studies, the cultivation was done in 7.2 m^3 outdoor raceways
213 pounds using natural sea water supplemented with agricultural fertilizers under batch and
214 continuous cultivation modes. They reported maximum biomass and oil productivities of 190
215 mg L^{-1} day^{-1} and 30.4 mg L^{-1} day^{-1} [32], slightly greater than the results presented here. The
216 differences might stem from the higher nitrate concentration (10 mM NO_3^{-2}, i.e., approximately
217 892 mg L^{-1}) utilized in their work as compared to the nitrate concentrations employed here.
218 Similarly, [33] used 10 mM of NO_3^{-2} in outdoor flat-panel photobioreactors obtaining a
219 maximum lipid yield of 38 mg L^{-1} day^{-1}. On the other hand, Mitra et al. [15] studied factors like
220 salinity, light intensity, and photoperiod in a 1 L Erlenmeyer containing a culture media with
221 100 mg L^{-1} of KNO_3 as the nitrogen source. Despite of the relatively low concentration of
222 nitrate, the best oil productivity reported was 14.63 mg L^{-1} day^{-1} [15], lower than the best result
223 in the current work. However, their results are slightly higher than those obtained here in
224 experiment 1 (-,-), which is reasonable with respect to the nitrogen source. Matos et al. [34]
225 worked using a desalination concentrate, residue from a desalination plant, in various
226 proportions with regular Guillard f/2 media. Their best result (75% of desalination concentrate)
227 showed lipid productivities of 16.8 mg L^{-1}, smaller than the 21.87 mg L^{-1} achieved in this work.
228 Such difference is probably related to the presence of acetate, which was absent in their work.
229 Matos et al. [35] investigated the autotrophic, mixotrophic, and heterotrophic conditions also
230 using 75% of desalination concentrated. For the autotrophic and mixotrophic cultivations, the
231 reactors were illuminated with different photoperiods (heterotrophic condition is not
232 illuminated), and 2 g L^{-1} glucose was added under the mixotrophic and heterotrophic
233 conditions. Matos et al. [35] concluded good cell productions particularly with the mixotrophic
234 conditions, reaching a maximum biomass productivity of 142 mg L^{-1} day^{-1} with a photoperiod
235 of 16 h of light and 8 h of dark (16L:8D). In addition, their maximum oil productivity was 15.9
236 mg L^{-1} day^{-1}, obtained with a 16L:8D photoperiod and autotrophic cultivation [35]. Curiously,
237 the employment of glucose as organic carbon source by Matos et al. [35] did not lead to an
238 increase in oil productivity. However, the data in Table 1 clearly parades that an increase in

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239 the sodium acetate concentration would enhance the lipid production of *Nannochloropsis*
240 *gaditana*.

241

242 **3.2 Characterization of *Nannochloropsis gaditana* microbial oil**

243

244 Table 3 summarizes the results of the oil characterization: free fatty acids (%), viscosity (mm^2
245 s^{-1}), acid value (mg KOH g^{-1}), and iodine value (gI_2 $100g^{-1}$).

246 **Table 3. Oil characterization obtained for *Nannochloropsis gaditana* in the optimum**
247 **studied condition (+, +) obtained with 2^2 full factorial design study.**

Properties	Value
Free fatty acids (%)	18.40 ± 1.20
Acid value (mg KOH g^{-1})	36.62 ± 0.90
Iodine value (gI_2 $100g^{-1}$)	78 ± 0.70
Viscosity (mm^2 s^{-1})	58.4 ± 0.50

248

249 The present study shows high IV, 78 ± 0.70 gI_2 $100g^{-1}$, due to amounts of PUFAs on
250 *Nannochloropsis gaditana* oil. Minhas et al.[36] reported some IVs, estimated from oil
251 extracted from 22 different microalgae, obtaining IV in the range of 14.6 to 162.7 gI_2 $100g^{-1}$.
252 The results in Table 3 do not show any disagreement with their results but reveal to be similar
253 in some cases. The IV of the microalgae *Scenedemus* sp. V11, for example, showed 77.08
254 gI_2 $100g^{-1}$. Other publications as the ones depicted in the sequence found out that
255 *Nannochloropsis gaditana* oil can provide different IVs. Carrero et al [37] reported an IV of 161
256 gI_2 $100g^{-1}$. Despite the fact that Carrero et al. [37] did not describe the cultivation process, the
257 most reasonable explanation for the lower IV obtained in this work can be related to the use
258 of acetate as an organic carbon source in the cultivation (*vide infra*). Mitra et al. [15], otherwise,
259 obtained IVs ranging from 49.94 to 79.71 gI_2 $100g^{-1}$, close to the values in this study. Woong
260 et al. [38] also obtained similar IVs ranging from 51 to 72 gI_2 $100g^{-1}$.

261 The kinematic viscosity of the *Nannochloropsis gaditana* oil was 58.4 mm^2 s^{-1} and the
262 rheological tests showed that the viscosity decreased when the shear rate increased (results
263 not shown), characterizing a non-Newtonian fluid as expected [28,30].

264 Figure 3 shows the main identified FAMEs obtained in this study using CG to analyze
265 *Nannochloropsis gaditana* lipids. The graphical representation combines only the FAMEs with
266 concentrations higher than 2% in the sample. Figure 4 describes a set of results obtained from
267 the literature for *Nannochloropsis gaditana* microalgae in comparison to the present results.

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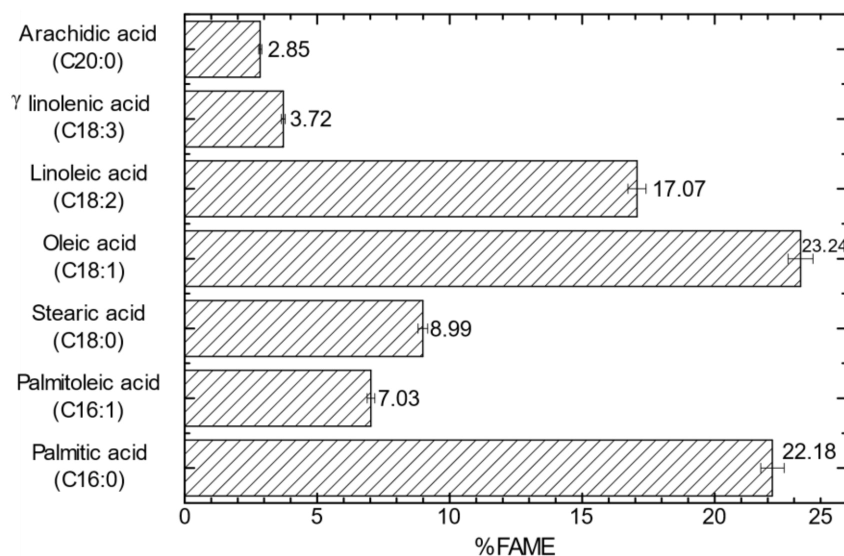

268

269 **Figure 3: Main identified FAMEs profile (concentrations higher than 2%) in the oil**
270 **extracted from *Nannochloropsis gaditana* cultivated with 225 mg L^{-1} of nitrate and 6 g**
271 **L^{-1} acetate.**

272 In addition to the main FAMEs obtained in the CG analysis, depicted in Figure 3, other ones
273 were observed but in smaller amounts such as: C12:0-Lauric acid (0.85%); C14:0-Myristic
274 acid (0.78%); C17:0- Margaric acid (1.58%); and C21:0-Heneicosanoic acid (1.32%).

275 From the CG analysis, it was observed great amounts of C18 FAMEs, as can be clearly
276 noticed from the results in Figure 3. The combined amount of C18:1, C18:2, and C18:3 is
277 44.03%. The majority of previous studies reported lower amounts of C18 with the exception
278 of few conditions such as in the work of Matos et al. [35]. Their study described the cultivation
279 in a dark, heterotrophic condition (using glucose as the carbon source, 2 g L^{-1}) obtaining 12.2%
280 C18:1, 26.6% C18:2, 13.8% C18:3 (52.6% total) [35]. They also reported low concentrations
281 of C20:5 like the results presented in the current study (i.e., less than 1% of this FAME) Matos
282 et al. [34], on the other hand, obtained high amounts of C18:1 when using glucose, glycerol,
283 and glycerin as the carbon sources. Thus, it is possible to correlate the presence of carbon
284 organic sources with the FAMEs profile by observing these results. In contrast, the work of
285 Mitra et al. [15] and Pedro et al. [32,33] did not employ any organic carbon source obtaining
286 satisfactory amounts of C20:5. Pedro et al. [33] observed a significant fall in the C20:5
287 concentration with an increase of C18:2 when the cultivation temperature was risen to 33°C.
288 Therefore, it is interesting to examine the effect of the cultivation condition on how
289 *Nannochloropsis gaditana* can produce a high amount of C18 or C20:5 FAME.

290 Another factor that should be highlighted in this study (Figure 3) is the low proportion of C16:1
291 (only 7.03%) in comparison to the literature, which generally manifested good amounts of this
292 FAME. In line with our results, Matos et al. [34] obtained low C16:1 when using glycerin as the
293 carbon source. Moreover, the herein obtained C16:0 content is similar to the results
294 documented by Pedro et al. [32,33] who used natural sea water and agricultural fertilizers as
295 nutrient supplementation without organic carbon sources.

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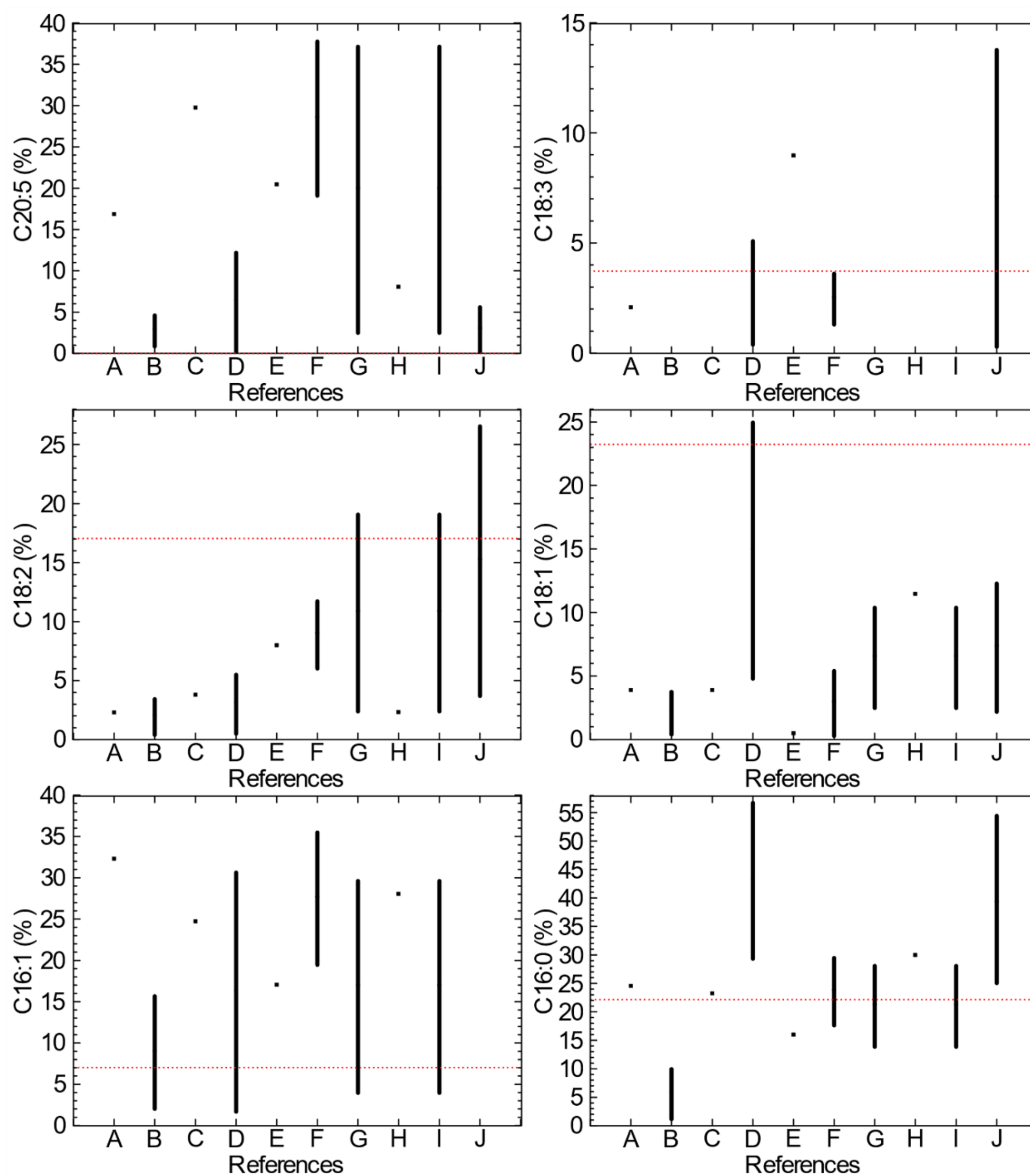

297

298 **Figure 4: Comparative graphics of some FAMEs % (C20:5, C18:3, C18:2, C18:1, C16:1**
299 **and C16:0) obtained in the oil extracted from *Nannochloropsis gaditana* biomass. The**
300 **results obtained in the present work are represented as horizontal dashed lines. If the**
301 **studied reference had only one CG analysis of PUFAs the result is shown as a single**
302 **dot. If the reference had many PUFAs analysis the results are shown as a vertical line**
303 **which starts with the lowest % FAME obtained in the reference and ends with the**
304 **highest % FAME achieved by the authors. References are as follow: A:** [37]**; B:** [39]**; C:**
305 [40]**; D:** [34]**; E:** [41]**; F:** [15]**; G:** [32]**; H:** [42]**; I:** [33]**; J:** [35]**.**

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307 The C20:0 was not mentioned in the references of Figure 4, but it was previously described
308 for *Chlorella vulgaris* by Abedini Najafabadi et al. [4] when studying different carbon sources.
309 Curiously, it is possible to conclude, by analyzing the results in their work, that the use of CO_2
310 (3% of aeration), sodium acetate, sodium bicarbonate, or molasses did not have a significant
311 effect on the C20:0 amount, which persisted to be around 1.6%, slightly lower than the result
312 in this study (2.85%).

313

314 ## 3.3 Enzymatic hydrolysis

315

316 Figure 5 demonstrates the results of the *Nannochloropsis gaditana* lipid hydrolysis catalyzed
317 by different lipases. As can be observed, all lipases were able to catalyze the hydrolysis from
318 microalgal oil, although at different rates. The best performance was achieved by using the
319 lipases from *Burkholderia cepacia*, *Candida rugosa*, and *Rhizopus oryzae* in the order of 86%,
320 84%, and 82%, respectively in 8-hour reactions. On the other hand, the reactions catalyzed
321 by pancreatic and *Thermomyces laguginosus* lipases provided lower hydrolysis percentages
322 of 67% and 58%, respectively during the same reaction times.

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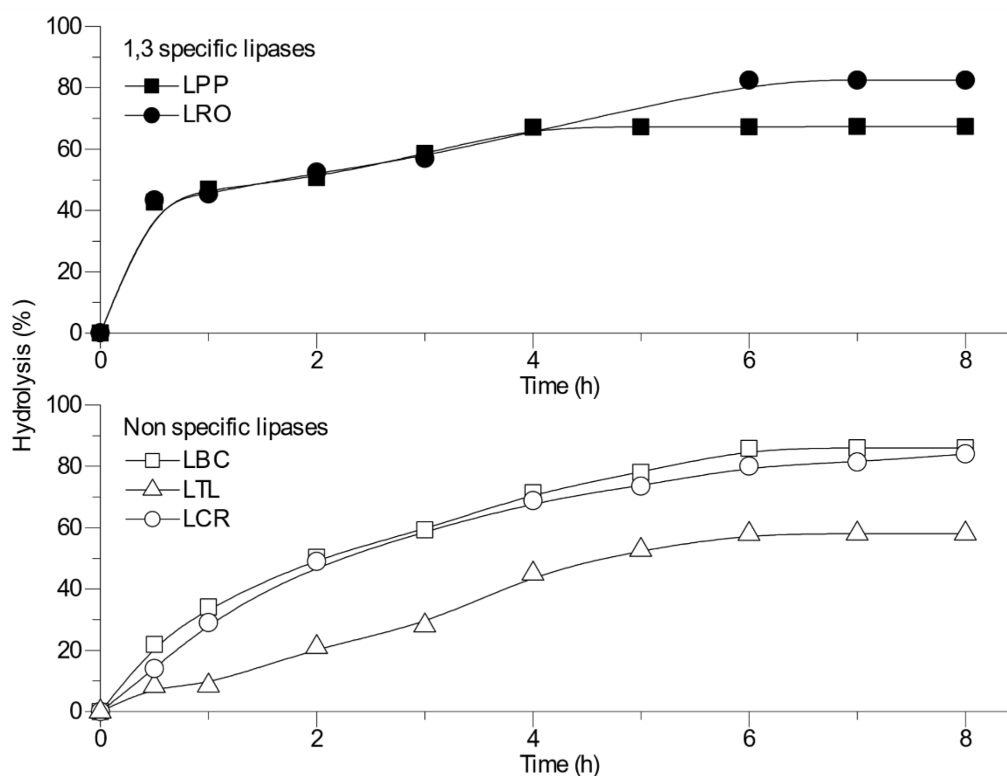

324

325 **Figure 5: Hydrolysis progress of the *Nannochloropsis gaditana* oil using different lipase**
326 **sources as catalysts (conditions: 400 rpm at 40 °C for 8 h, containing the microalgae**
327 **oil emulsion at 1:2 oil/water ratio, 1 wt% soy lecithin emulsifier, 3 mL of a buffer solution**
328 **with pH = 7.0 and hexane as a solvent).**

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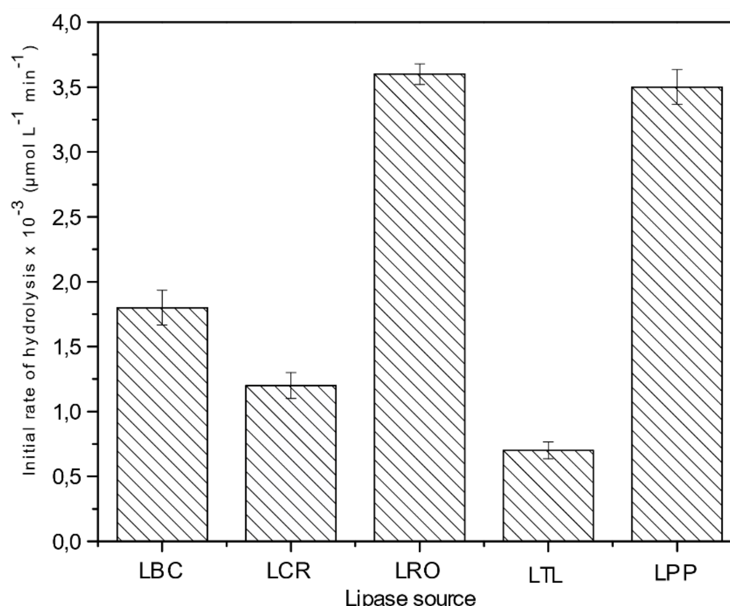
329 Figure 6 shows the effect of lipase source on the initial rate of hydrolysis from microbial oil.
330 Experiments were carried at 40 ºC and an agitation speed of 400 rpm. The initial substrate
331 concentration was 20 g L^{-1}. The values of the initial rates of hydrolysis varied from 0.70 x 10^{-3}
332 to 3.60 x 10^{-3} µmol L^{-1} min^{-1}, depending on the lipase source employed as the catalyst. The
333 best performance was attained using *Rhizopus oryzae* and pancreatic lipases with an initial
334 rate of 3.60 x 10^{-3} and 3.50 x 10^{-3} µmol L^{-1} min^{-1}, respectively (Figure 6). The other lipases
335 resulted in initial rates that are lower than 1.8 x 10^{-3} µmol L^{-1} min^{-1}, with the lowest rate (0.70
336 x 10^{-3} µmol L^{-1} min^{-1}) realized by the lipase from *Thermomyces laguginosus*. This lipase also
337 provided the lowest % hydrolysis (58%).
338



339

340 **Figure 6: Initial rate of the hydrolysis of *Nannochloropsis gaditana* oil using different**
341 **lipase sources as catalysts.**

342

343 The results here obtained, see Figures 5 and 6, are similar to those found by Noor at al. [31]
344 using palm oil and lipase SP398 (Novo Nordisk A/S – Denmark), which accomplished initial
345 rates of hydrolysis in the range of 1.3 to 3.5 x 10^{-3} µmol L^{-1} min^{-1}. The rates were found to vary
346 with the different investigated factors such as surfactant concentration, speed of agitation, and
347 oil-aqueous phase ratio.

348 It is important to emphasize that each of the assessed biocatalysts has different specificity.
349 Lipases can be classified as specific and non-specific according to the position of the fatty
350 acid cleavage on triacylglycerol molecules [43]. The tested lipases in the hydrolysis reactions
351 in this work are classified as 1,3 specific (pancreatic, *Thermomyces laguginosus*, and
352 *Rhizopus oryzae*) and non-specific (*Burkholderia cepacia* and *Candida rugosa*) [44,45]. This
353 can explain the improved performance of *Burkholderia cepacia* and *Candida rugosa* lipases,
354 being non-specific in terms of cleavage at any position on the triacylglycerol. In addition, a
355 similar performance was observed for pancreatic and *Rhizopus oryzae* lipases (classified as
356 1,3 specific) during the first three hours of the reaction. On the other hand, *Rhizopus oryzae*

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357 lipase proved to have a higher efficiency after 6 h of the reaction, revealing a similar
358 performance to non-specific lipases at the end of the reaction.

359 The most comparable results in the literature are the ones that utilized lipases in the hydrolysis
360 of vegetable or waste oils, since few investigations on microalgae oil have been reported to
361 date. Most papers have dealt with biodiesel production where the hydrolysis step was followed
362 by esterification in a process known as hydroesterification. In this case, the lipase was used
363 in the hydrolysis subsequently followed by esterification that could be enzymatically or
364 chemically catalyzed, attaining high hydroesterification yields (≅90%) in both cases [20,23,46].
365 Freitas et al. [21] worked with soybean oil and examined lipases from different sources in the
366 enzymatic hydrolysis, concluding a maximum % hydrolysis around 65% after 6 h and 70%
367 after 24 h when using *C. rugosa* lipase.

368 Therefore, enzymatic hydrolysis has been demonstrated to be highly effective in obtaining free
369 fatty acids from *Nannochloropsis gaditana* oil. Nevertheless, further studies should be
370 performed in order to assess the effect of the different factors on the hydrolysis degree of
371 microalgal oil, such as the lipase (wt%) and emulsifier (wt%) concentrations.

372 **4. CONCLUSION**

373

374 Important data concerning the productivity and hydrolysis of *Nannochloropsis gaditana* oil
375 were concluded from the described investigations. Sodium acetate can be pointed out as a
376 proper *Nannochloropsis gaditana* carbon source for lipid productivity, particularly when the
377 sodium nitrate concentration is high enough. This is due to the positive effect of the acetate
378 on the lipid content of cells and the influence of the nitrate on the cell productivity. The best
379 achieved oil productivity was 21.87 mg L^{-1} day^{-1}, using cultivation media with 6 gL^{-1} of acetate
380 and 225 mg L^{-1} of nitrate (+, + condition). *Nannochloropsis gaditana* oil revealed that the main
381 fatty acids consisted of γ linolenic acid (3.72%), linolenic acid (17.07%), oleic acid (23.24%),
382 stearic acid (8.99%), palmitoleic acid (7.03%), and palmitic acid (22.18%). This is probably
383 resulting from the employment of sodium acetate as the carbon source. Regarding the
384 hydrolysis of the microbial oil from *Nannochloropsis gaditana*, the highest levels of free fatty
385 acids were attained by lipases from *B. cepacia*, *C. rugosa*, and *R. oryzae*. Further studies are
386 still needed to determine the effect of other variables that may affect the hydrolysis
387 performance.

389

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