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

**Eduardo Henrique Bredda[1], Messias Borges Silva [1,2], Heizir Ferreira de Castro[2], Ana Paula Teixeira da Silva[2] and Patrícia Caroline Molgero da Rós[*2]**

*1 Engineering Faculty of Guaratingueta, State University Julio de Mesquita Filho-UNESP, Dr. Ariberto Pereira da Cunha Avenue, 333, 12.516-410, Guaratingueta, São Paulo, Brazil.*

*2 Engineering School of Lorena, University of São Paulo, Campinho Road, 12.602-810, Lorena, São Paulo, Brazil.*

## ABSTRACT

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 $NaNO_3$ (0.075 to 0.225 $mg.L^{-1}$) and $CH_3COONa$ (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%).

*Keywords: Nannochloropsis gaditana, full factorial design, $PUFA_S$, enzymatic hydrolysis.*

## 1. INTRODUCTION

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

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

* E-mail address: patriciadaros@usp.br.

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 [16]. Therefore, the inclusion of ALA in the diet is of high importance for the maintenance of good health. 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 [20,23,24]. 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 [20]. 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 [20,22,25].

Considering these factors, the objective of this work was to study the influence of the sodium acetate, as carbon source, and the sodium nitrate in the cultivation of the *Nannochloropsis gaditana* microalgae, aimed enhancing the lipid productivity ($P_L$) and to screen grade commercially available lipases to be used in hydrolysis reactions from *Nannochloropsis gaditana* microalgae oil for PUFAs production.

* E-mail address: patriciadaros@usp.br.

## 2. MATERIAL AND METHODS

### 2.1 Microalgae strain and biocatalysts

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

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

The effect of concentrations of $NaNO_3$ (0.075 to 0.225 $g.L^{-1}$) ($X_1$) and $CH_3COONa$ (2 to 6 $g.L^{-1}$) ($X_2$) on the culture of *Nannochloropsis gaditana* were studied using a $2^2$ full factorial design. The microalgae culture was inoculated at 10% (v/v) in tank photobioreactors (15 cm wide and 33 cm height), with 4 L working volume, sparged with sterile air at 1.4 $L.min^{-1}$ aeration rate and maintained at 24 ± 1 °C under 150 klux light intensity for 7 days. The cultivation media was a modified, without silica, Guillard f/2 medium [26]. The center point was repeated six times in order to improve the error determination. Microalgae cells were recovered by flocculation using 1 $mol.L^{-1}$ $FeCl_3$ solution, in order to reduce the working volume to 3-5% of its original, saving time and energy in the filtration step [7]. Biomass productivity was obtained dividing the total amount of dried biomass obtained in a single cultivation run by the working volume of the photobioreactor (4 L) and the cultivation period (7 days). The 'Design expert' (version 6.0 - Stat-Ease Corporation, USA), 'Statistica' (version 8.0 - Stat Soft Inc., USA) and 'Minitab' (version 18.0 – Minitab Inc., USA) software were used for regression and graphical analyses of the obtained data. The lipid productivity was taken as response variable. Design expert' software was used to obtain graphical and numerical analysis based on the criterion of desirability.

### 2.3 Microalgal oil extraction

Microbial oil was extracted from biomass according to a modified Folch method, performed under ultrasound, with a mixture of chloroform: methanol (3:1 v/v). The mixture was sonicated for 10 min. This procedure was repeated three times [27]. In this process, the ultrasound promotes cavitation throughout the extraction media, causing cell rupture, whilst the solvent mixture effectively dissolves and recovers the microbial lipids [28,29]. Extracted lipids were dried in a rotatory evaporator, for approximately 30 min, to remove the residual solvent and subsequently dried at 60°C until constant weight was attained. The production efficiency of the microbial lipids was calculated based on the biomass concentration (X), lipid concentration (P), lipid yield (% P) and lipid specific yield ($Y_{P/X}$) obtained in the culture cultivations. The results were also analyzed considering the following parameters: volumetric productivity in relation to biomass ($Q_X$) and volumetric productivity in relation to lipid ($Q_P$) [30]. The lipid productivity was obtained multiplying the biomass productivity by the lipid content obtained in the oil extraction.

* E-mail address: patriciadaros@usp.br.

## 2.4 Microalgal oil characterization

AOCS's method [31] was used for total free fatty acids (FFA) determination, which was expressed in terms of free oleic acid (%). Kinematic viscosity was determined with a Brookfield viscometer (Brookfield Viscometers Ltd, England) using a CP 52 cone [32]. Iodine value was determined by [31].

The fatty acid methyl esters (FAMEs) were synthesized according to [31] and identified by gas chromatography (CG). The CG analyses were performed by a PerkinElmer® - Clarus 580 chromatograph, equipped with a flame-ionization detector (250°C, 40 $mL.min^{-1}$ $H_2$, and 400 $mL.min^{-1}$ synthetic air). A 30 m capillary column with 0.25 mm internal diameter and 5% diphenyl 95% dimethylpolysiloxane stationary phase (non-polar) was employed during the analysis. The oven ramp temperature rate was 3 $°C.min^{-1}$ from 120 to 235°C and 1 $°C.min^{-1}$ until 255°C, during the total 60 min of the analysis. Nitrogen was the carrier gas (5 $mL.min^{-1}$). The external patterns used were MIX Supelco®, with 37 fatty acids methyl ester (Sigma-Aldrich®), C4-C24.

## 2.5 Hydrolysis reactions

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

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

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

The initial rate of reaction was calculated using the equation 2 [33].

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

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

* E-mail address: patriciadaros@usp.br.

## 3. RESULTS AND DISCUSSION

### 3.1 Experimental design for the biomass production

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

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

| Exp. | Nitrate $X_1(g.L^{-1})$ | Acetate $X_2(g.L^{-1})$ | Cell productivity ($g.L^{-1}.day^{-1}$) | Lipid productivity ($mg.L^{-1}.day^{-1}$) |
|---|---|---|---|---|
| 1 | 0.075 (-1) | 2 (-1) | 88.26 ± 2.40 | 12.76 ± 1.24 |
| 2 | 0.075 (-1) | 6 (+1) | 97.01 ± 4.94 | 14.01 ± 2,27 |
| 3 | 0.225 (+1) | 2 (-1) | 111.03 ± 2.02 | 9.48 ± 0,03 |
| 4 | 0.225 (+1) | 6 (+1) | 188.93 ± 10.78 | 21.87 ± 1,77 |
| 5 | 0.150 (0) | 4 (0) | 147.62 ± 3.67 | 18.63 ± 0,79 |

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

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

* E-mail address: patriciadaros@usp.br.

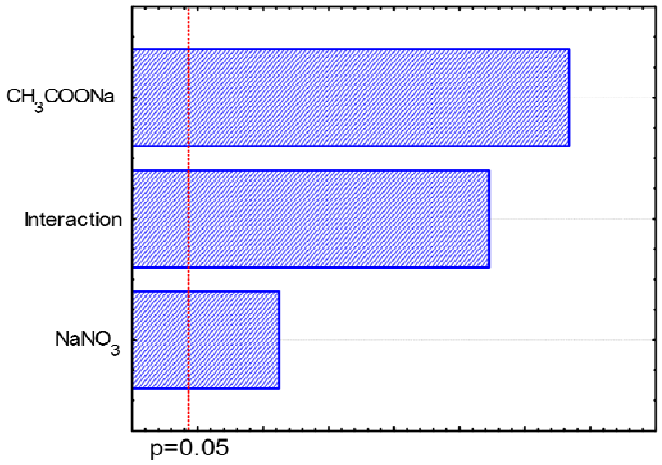



**Figure 1. Pareto chart indicating the standardized effect of the nitrate, acetate and their interaction over the *Nannochloropsis gaditana* lipid productivity.**

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

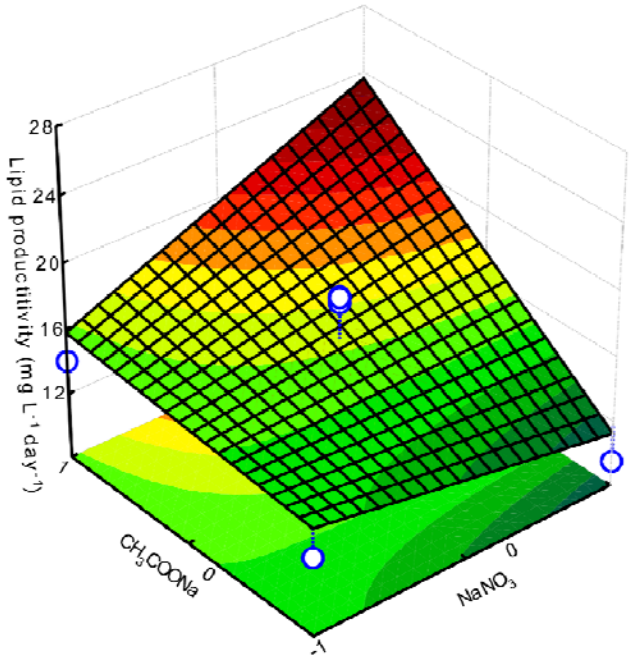



**Figure 2. Modeling of *Nannochloropsis gaditana* lipid productivity as a function of nitrate and acetate concentrations.**

* E-mail address: patriciadaros@usp.br.

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

**Table 2. Cultivation parameters obtained for *Nannochloropsis gaditana* in the optimum 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 ($Q_P$, $mg.L^{-1}.day^{-1}$) | 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 |

The results obtained in the present work are adequate especially when compared with the literature regarding the microalgae *Nannochloropsis gaditana*, such as the work of Pedro et al. [34] for example. In their studies, the cultivation was done in 7.2 $m^3$ outdoor raceways pounds using natural sea water supplemented with agricultural fertilizers under batch and continuous cultivation modes. They reported maximum biomass and oil productivities of 190 $mg.L^{-1}.day^{-1}$ and 30.4 $mg.L^{-1}.day^{-1}$ [34], slightly greater than the results presented here. The differences might stem from the higher nitrate concentration (10 mM $NO_3^{-2}$, i.e., approximately 892 $mg.L^{-1}$) utilized in their work as compared to the nitrate concentrations employed here. Similarly, [35] used 10 mM of $NO_3^{-2}$ in outdoor flat-panel photobioreactors obtaining a maximum lipid yield of 38 $mg.L^{-1}.day^{-1}$.

Mitra et al. [15] studied factors like salinity, light intensity, and photoperiod in a 1 L Erlenmeyer containing a culture media with 100 $mg.L^{-1}$ of $KNO_3$ as the nitrogen source. Despite of the relatively low concentration of nitrate, the best oil productivity reported was 14.63 $mg.L^{-1}.day^{-1}$ [15], lower than the best result in the current work. However, their results are slightly higher than those obtained here in experiment 1 (-,-), which is reasonable with respect to the nitrogen source. Matos et al. [36] worked using a desalination concentrate, residue from a desalination plant, in various proportions with regular Guillard f/2 media. Their best result (75% of desalination concentrate) showed lipid productivities of 16.8 $mg.L^{-1}$, smaller than the 21.87 $mg.L^{-1}$ achieved in this work. Such difference is probably related to the presence of acetate, which was absent in their work.

Matos et al. [37] investigated the autotrophic, mixotrophic, and heterotrophic conditions also using 75% of desalination concentrated. For the autotrophic and mixotrophic cultivations, the reactors were illuminated with different photoperiods (heterotrophic condition is not illuminated), and 2 $g.L^{-1}$ glucose was added under the mixotrophic and heterotrophic conditions. Matos et al. [37] concluded good cell productions particularly with the mixotrophic conditions, reaching a maximum biomass productivity of 142 $mg.L^{-1}.day^{-1}$ with a photoperiod

* E-mail address: patriciadaros@usp.br.

of 16 h of light and 8 h of dark (16L:8D). In addition, their maximum oil productivity was 15.9 $mg.L^{-1}.day^{-1}$, obtained with a 16L:8D photoperiod and autotrophic cultivation [37]. Curiously, the employment of glucose as organic carbon source by Matos et al. [37] did not lead to an increase in oil productivity. However, the data in Table 1 clearly parades that an increase in the sodium acetate concentration would enhance the lipid production of *Nannochloropsis gaditana*.

Moraes et al. [38] worked with $CO_2$ as carbon source in the *Nannochloropsis gaditana* cultivation. In their studies the $CO_2$ injection was used to control the pH of the culture medium. So, it was added in the moments the microalgae absorbed more of it though photosynthesis, to avoid waste. The lipid productivity ranged from 27.7 to 39.5 $mg.L^{-1}.day^{-1}$. The carbon source used in this work, sodium acetate, presents some advantages in relation to the use of $CO_2$, such as: easier to operate, especially in large-scale cultivation and does not need special pressurized tanks.

Onay [39] also worked with *Nannochloropsis gaditana* microalgae in Guillard f/2 medium, however mixed with municipal wastewaters. In their work the maximum biomass obtained was 167 $mg.L^{-1}.day^{-1}$, slightly lower than the presented result in this work.

## 3.2 Characterization of *Nannochloropsis gaditana* microbial oil

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

**Table 3. Oil characterization obtained for *Nannochloropsis gaditana* in the optimum 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 |

The present study shows high IV, 78 ± 0.70 $gI_2.100g^{-1}$, due to amounts of PUFAs on *Nannochloropsis gaditana* oil. Minhas et al.[40] reported some IVs, estimated from oil extracted from 22 different microalgae, obtaining IV in the range of 14.6 to 162.7 $gI_2.100g^{-1}$. The results in Table 3 do not show any disagreement with their results but reveal to be similar in some cases. The IV of the microalgae *Scenedemus* sp. V11, for example, showed 77.08 $gI_2.100g^{-1}$. Other publications as the ones depicted in the sequence found out that *Nannochloropsis gaditana* oil can provide different IVs. Carrero et al [41] reported an IV of 161 $gI_2.100g^{-1}$. Despite the fact that Carrero et al. [41] did not describe the cultivation process, the most reasonable explanation for the lower IV obtained in this work can be related to the use of acetate as an organic carbon source in the cultivation (*vide infra*). Mitra

* E-mail address: patriciadaros@usp.br.

et al. [15], otherwise, obtained IVs ranging from 49.94 to 79.71 $gI_2.100g^{-1}$, close to the values in this study. Woong et al. [42] also obtained similar IVs ranging from 51 to 72 $gI_2.100g^{-1}$.

The kinematic viscosity of the *Nannochloropsis gaditana* oil was 58.4 $mm^2\ s^{-1}$ and the rheological tests showed that the viscosity decreased when the shear rate increased (results not shown), characterizing a non-Newtonian fluid as expected [30,32].

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

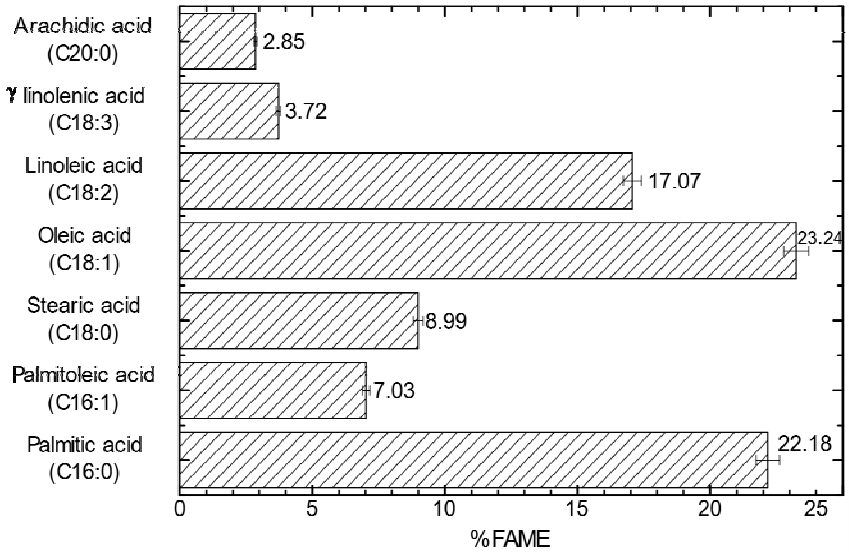



**Figure 3: Main identified FAMEs profile (concentrations higher than 2%) in the oil extracted from *Nannochloropsis gaditana* cultivated with 225 $mg.L^{-1}$ of nitrate and 6 $g.L^{-1}$ acetate.**

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

From the CG analysis, it was observed great amounts of C18 FAMEs, as can be clearly noticed from the results in Figure 3. The combined amount of C18:1, C18:2, and C18:3 is 44.03%. The majority of previous studies reported lower amounts of C18 with the exception of few conditions such as in the work of Matos et al. [37]. Their study described the cultivation in a dark, heterotrophic condition (using glucose as the carbon source, 2 $g.L^{-1}$) obtaining 12.2% C18:1, 26.6% C18:2, 13.8% C18:3 (52.6% total) [37]. They also reported low concentrations of C20:5 like the results presented in the current study (i.e., less than 1% of this FAME) Matos et al. [36], on the other hand, obtained high amounts of C18:1 when using glucose, glycerol, and glycerin as the carbon sources. Thus, it is possible to correlate the presence of carbon organic sources with the FAMEs profile by observing these results. In contrast, the work of Mitra et al. [15] and Pedro et al. [34,35] did not employ any organic carbon source obtaining satisfactory amounts of C20:5. Pedro et al. [35] observed a significant fall in the C20:5 concentration with an increase of C18:2 when the cultivation

* E-mail address: patriciadaros@usp.br.

temperature was risen to 33°C. Therefore, it is interesting to examine the effect of the cultivation condition on how *Nannochloropsis gaditana* can produce a high amount of C18 or C20:5 FAME.

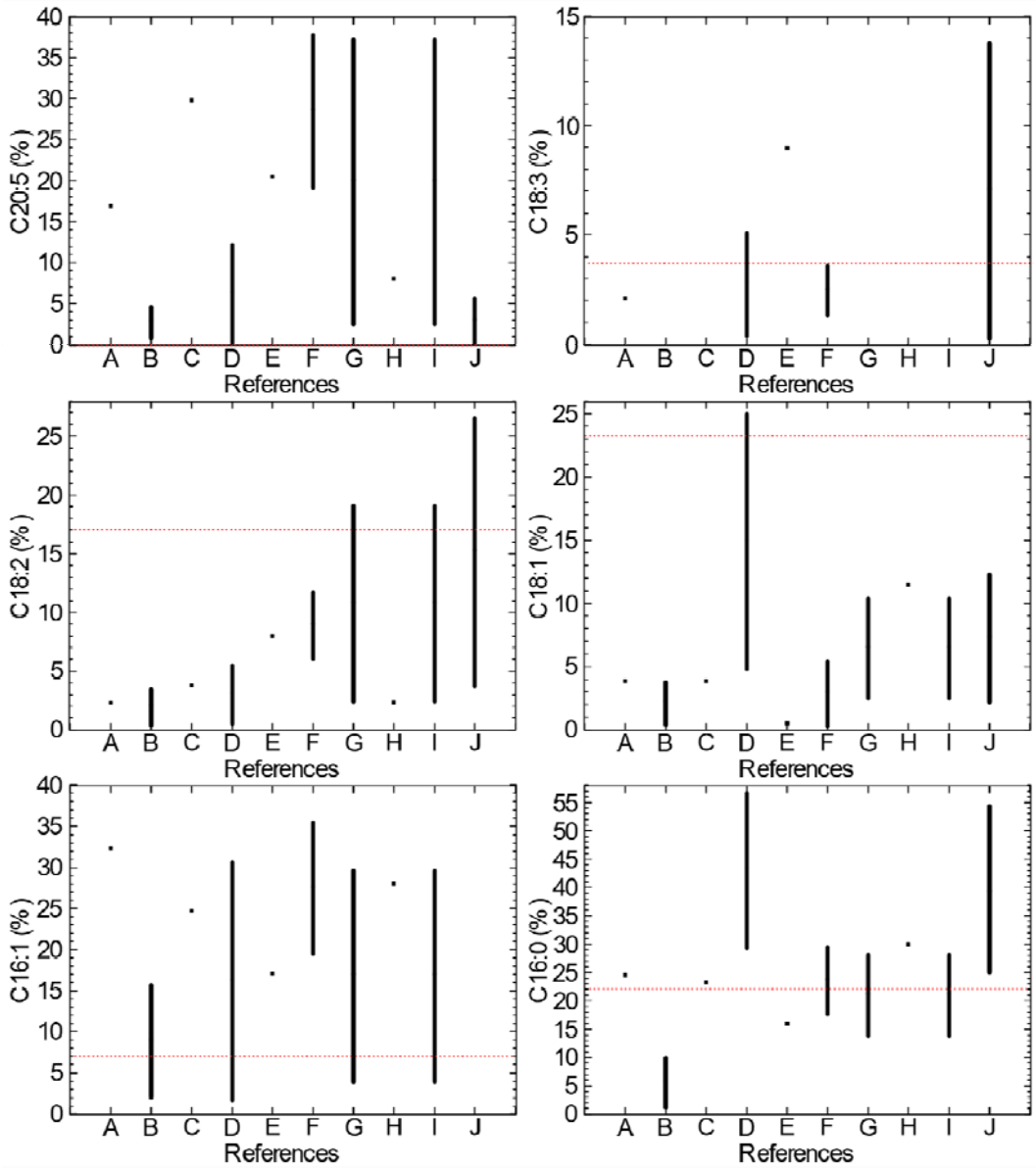



**Figure 4: Comparative graphics of some FAMEs % (C20:5, C18:3, C18:2, C18:1, C16:1 and C16:0) obtained in the oil extracted from *Nannochloropsis gaditana* biomass. The results obtained in the present work are represented as horizontal dashed lines. If the studied reference had only one CG analysis of PUFAs the result is shown as a single dot. If the reference had many PUFAs analysis the results are shown as a vertical line which starts with the lowest % FAME obtained in the reference and ends with the highest % FAME achieved by the authors. References are as follow: A:** [41]**; B:** [43]**; C:** [24]**; D:** [36]**; E:** [44]**; F:** [15]**; G:** [34]**; H:** [45]**; I:** [35]**; J:** [37]**.**

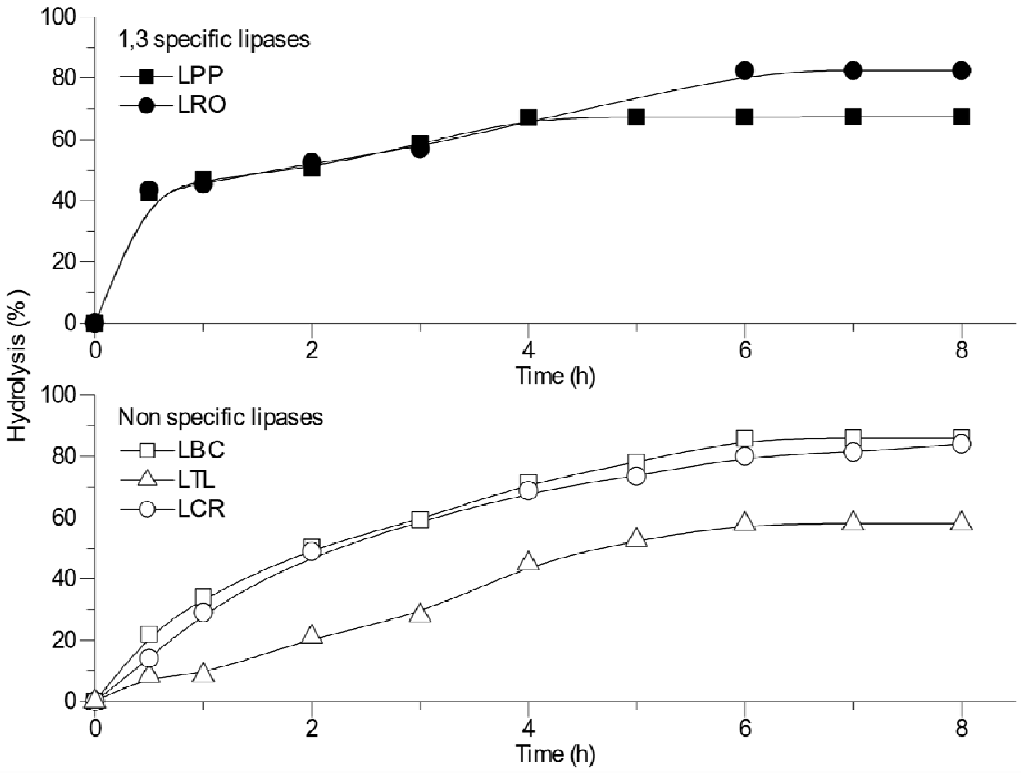
* E-mail address: patriciadaros@usp.br.

Another factor that should be highlighted in this study (Figure 3) is the low proportion of C16:1 (only 7.03%) in comparison to the literature, which generally manifested good amounts of this FAME. In line with our results, Matos et al. [36] obtained low C16:1 when using glycerin as the carbon source. Moreover, the herein obtained C16:0 content is similar to the results documented by Pedro et al. [34,35] who used natural sea water and agricultural fertilizers as nutrient supplementation without organic carbon sources.

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

## 3.3 Enzymatic hydrolysis

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




* E-mail address: patriciadaros@usp.br.

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

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

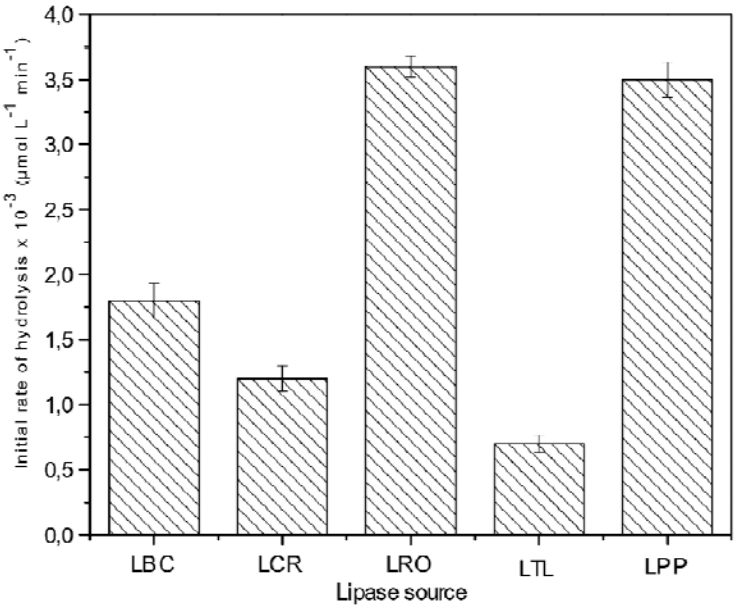



**Figure 6: Initial rate of the hydrolysis of *Nannochloropsis gaditana* oil using different lipase sources as catalysts.**

The results here obtained, see Figures 5 and 6, are similar to those found by Noor at al. [33] using palm oil and lipase SP398 (Novo Nordisk A/S – Denmark), which accomplished initial rates of hydrolysis in the range of 1.3 to 3.5 x $10^{-3}$ $\mu mol.L^{-1}.min^{-1}$. The rates were found to vary with the different investigated factors such as surfactant concentration, speed of agitation, and oil-aqueous phase ratio.

It is important to emphasize that each of the assessed biocatalysts has different specificity. Lipases can be classified as specific and non-specific according to the position of the fatty acid cleavage on triacylglycerol molecules [46]. The tested lipases in the hydrolysis reactions in this work are classified as 1,3 specific (pancreatic, *Thermomyces laguginosus*, and *Rhizopus oryzae*) and non-specific (*Burkholderia cepacia* and *Candida rugosa*) [47,48]. This

* E-mail address: patriciadaros@usp.br.

can explain the improved performance of *Burkholderia cepacia* and *Candida rugosa* lipases, being non-specific in terms of cleavage at any position on the triacylglycerol. In addition, a similar performance was observed for pancreatic and *Rhizopus oryzae* lipases (classified as 1,3 specific) during the first three hours of the reaction. On the other hand, *Rhizopus oryzae* lipase proved to have a higher efficiency after 6 h of the reaction, revealing a similar performance to non-specific lipases at the end of the reaction.

The most comparable results in the literature are the ones that utilized lipases in the hydrolysis of vegetable or waste oils, since few investigations on microalgae oil have been reported to date. Most papers have dealt with biodiesel production where the hydrolysis step was followed by esterification in a process known as hydroesterification. In this case, the lipase was used in the hydrolysis subsequently followed by esterification that could be enzymatically or chemically catalyzed, attaining high hydroesterification yields (≅90%) in both cases [20,25,49]. Freitas et al. [21] worked with soybean oil and examined lipases from different sources in the enzymatic hydrolysis, concluding a maximum % hydrolysis around 65% after 6 h and 70% after 24 h when using *C. rugosa* lipase.

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

## 4. CONCLUSION

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

## COMPETING INTERESTS

The authors have declared that no competing interests exist.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* E-mail address: patriciadaros@usp.br.

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* E-mail address: patriciadaros@usp.br.

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* E-mail address: patriciadaros@usp.br.