**Fatty acid methyl ester production from wet microalgal biomass by lipase-catalyzed direct transesterification**

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**Abstract**

The aim of this work was to optimize the production of fatty acid methyl ester (FAME, biodiesel) from wet Nannchloropsis gaditana microalgal biomass by direct enzymatic transesterification. This was done in order to avoid the high cost associated with the prior steps of drying and oil extraction. Saponifiable lipids (SLs) from microalgal biomass were transformed to FAME using the lipase Novozyme 435 (N435) from Candida antarctica as the catalyst, and finally the FAME were extracted with hexane. t-Butanol was used as the reaction medium so as to decrease lipase deactivation and increase mass transfer velocity. A FAME conversion of 99.5% was achieved using wet microalgal biomass homogenized at 140 MPa to enhance cell disruption, a N435:oil mass ratio of 0.32, methanol added in 3 stages to achieve a total of 4.6 cm3 g−1 of oil and 7.1 cm3 g−1 oil of added t-butanol, with a reaction time of 56 h. The FAME conversion decreased to 57% after catalyzing three reactions with the same lipase batch. This work shows the influence of the polar lipids contained in the microalgal biomass both on the reaction velocity and on lipase activity.

Key words: Biodiesel; Microalga; *Nannochloropsis gaditana*; Lipase, Direct transesterification; Polar lipid

1. **Introduction**

Microalgae are currently considered one of the most promising alternatives for biodiesel production. Microalgae are photosynthetic organisms capable of converting (under light conditions) water and carbon dioxide into macromolecules such as oils, polysaccharides and proteins; some of them can give oil yield (liter/hectare) a minimum of 10 times higher than the best vegetable oil crop and achieve high growth rates compared to agricultural crops [1]. Unlike plant oils, which are mostly neutral lipids (NLs), microalgal oils are rich in polar lipids (glycolipids, GLs, and phospholipids, PLs) and thus have a higher viscosity, which may lead to reduced transesterification velocity and FAME conversion [2,3]. For this and other reasons, the neutral saponifiable lipids (NSLs) (free fatty acids, tri-, di- and monoacylglycerols), which are also found in microalgae [2,4,5], are the most interesting lipids to produce FAMEs. The lipid composition depends on the microalgal species, culture conditions and time of harvesting. It is known that nitrogen concentration in the culture medium is the main factor that upsets lipid synthesis in microalgae [6]. San Pedro et al. [7] found that the lipid profile and content changed when the microalga *Nannochloropsis gaditana* was cultured under nitrogen starvation conditions - the saponifiable lipids (SLs) greatly increased (21.6 % of biomass dry weight) compared to that in continuous culture (12.0 %). Moreover, the former is richer in NSLs (17.2 % of biomass dry weight) than that cultured without nitrogen limitations (7.9 %). However, biomass productivity is lower under nitrogen starvation conditions and such culture conditions are far more laborious and difficult to implement on an industrial scale [7]. Furthermore, algae harvesting in the exponential growth phase (usually when production is in continuous operation mode) will give microalgae with more polar lipids than those harvested in the late stationary growth phase (usually in batch cultures), which contain more NSLs [8].

 The production of biodiesel from microalgal biomass has been widely reported in the literature using the conventional method, which involves the extraction of lipids from microalgal biomass followed by their conversion to FAMEs [3,9]. However, these procedures have the inconvenience of the multiple process steps necessary to produce biodiesel, such as: drying, cell disruption, oil extraction and the transesterification reaction. For this reason, direct (in-situ) transesterification has been studied as a promising alternative to other methods since the oil extraction and transesterification steps are carried out simultaneously; hence, both energy consumption and process costs can be reduced [5,10,11].

Also, it is possible to apply a prior treatment to the wet microalgal biomass in order to enhance cell disruption, oil extraction and biodiesel conversion. In recent years, various cell disruption techniques have been studied by several authors [12,13,14] such as microwaving, bead beating, sonication and autoclaving. However, the scaling up of some of these is difficult. Consequently, cell disruption by high pressure homogenization (HPH) is one of the most widely used methods since it has proven effective in aqueous environments and is easily scalable [2,15,16].

Microalgal oils must be transesterified using acid or [enzymatic catalysts](https://www.sciencedirect.com/topics/engineering/enzymatic-catalyst%22%20%5Co%20%22Learn%20more%20about%20enzymatic%20catalysts%20from%20ScienceDirect%27s%20AI-generated%20Topic%20Pages). [Alkaline catalysts](https://www.sciencedirect.com/topics/engineering/alkaline-catalysts%22%20%5Co%20%22Learn%20more%20about%20Alkaline%20catalysts%20from%20ScienceDirect%27s%20AI-generated%20Topic%20Pages) cannot be used because microalgal oils contain free fatty acids [[17]](https://www.sciencedirect.com/science/article/pii/S0961953416302203?via%3Dihub#bib17). Lipases work at lower temperatures than acids and the separation and purification of biodiesel and glycerol is easier. Moreover, acid catalysts require a large amount of water to be removed [17,18]. However, lipases have yet drawbacks that should be overcome, such as its high cost, low stability and low reaction velocity. Perhaps for these reasons, almost all of the authors carried out direct transesterification using acid [19,20], basic [21] or heterogeneous catalysts [12]. Only a few authors performed this process using enzymatic catalysis, as was the case with Sivaramakrishnan and Muthukumar [22], who achieved a maximum FAME yield of 82 % in the direct transesterification of the macroalga *Oedogonium* sp. oil using a *Bacillus* sp. Lipase; and Tran et al. [23], who attained a conversion of saponifiable lipids to FAMEs of 95.7 % by direct transesterification of *Chlorella vulgaris* lipids using immobilized *Burkholderia* lipase as the catalyst.

The aim of this work was to produce biodiesel by lipase-catalyzed direct transesterification of SLs in wet *Nannochloropsis gaditana* microalgal biomass, taking both the FAME yield and lipase stability into account. t-Butanol was used as the reaction medium to help oil extraction within the microalgal cell, to decrease the reaction mixture viscosity and to preserve lipase stability.

**2. Materials and methods**

*2.1 Microalgal biomass, lipases and chemicals*

The strain *Nannochloropsis gaditana* B-3 was used as the oil-rich substrate in this study. It was obtained from the Marine Culture Collection of the Institute of Marine Sciences of Andalucía (CSIC, Cádiz, Spain). It was grown in an outdoor tubular photobioreactor in “*Las Palmerillas, Cajamar*” Research Center (El Ejido, Almería, Spain, latitude and longitude 36.7741 and -2.8153, respectively). The mass fraction of biomass in the wet biomass was 25 %, 31.1 ± 0.1 % of total lipids (TLs or oil) of biomass dry weight and a total fatty acid mass fraction (or saponifiable lipids, SLs, as an equivalent to fatty acids) of 12.1 ± 0.1 % in the dry biomass. The transesterification reactions were catalyzed by the lipase Novozym 435 (N435) from *Candida antarctica* (kindly donated by Novozymes A/S, Bagsvaerd, Denmark)*,* which is supplied immobilized on a macroporous acrylic resin and in the conditions used in this work the lipase does not show positional specificity.

The chemicals used were analytical grade hexane (95 % purity, synthesis quality) (Panreac S.A, Barcelona, Spain), methanol (99.9 % purity, Carlo Erba Reagents, Rodano, Italy) and tert-butanol (analytical grade, Fluka, Barcelona, Spain). All reagents used in the analytical determinations were of analytical grade. Standards were obtained from Sigma-Aldrich (St Louis, Mo, USA) and used without further purification.

*2.2. Homogenization of the wet microalgal biomass, direct transesterification of the saponifiable lipids (SLs) and the fatty acid methyl esters (FAMEs) extraction procedure*

The homogenization of wet microalgal biomass was carried out in a laboratory homogenizer (PandaPlus 2000 S.N. 8983 model, purchased from Gea Niro Soavi S.p.A, Parma, Italy). This equipment can operate at a feed rate of 9 L h-1 and up to 200 MPa pressure; although in this case it was not possible to operate above 140 MPa because it proved difficult to pass the wet solid (75 % water mass fraction) through the homogenizer. Therefore, wet microalgal biomass was homogenized at 140 MPa and stored at -24 ºC until use.

Figure 1 shows the method proposed for carrying out the “in situ” transesterification. In a typical experiment, 3 g of homogenized biomass (0.23 g oil) was mixed with 0.074 g of lipase N435 (N435: oil mass ratio of 0.32) and 3.3 cm3 of t-butanol (14.2 cm3 g-1 oil). Then, 1.07 cm3 of methanol (4.6 cm3 methanol g-1 oil) was added in three steps at 0, 10 and 24 h, adding 0.29 cm3 (1.25 cm3 methanol g-1 oil) in the first step and 0.39 cm3 (1.68 cm3 methanol g-1 oil) in the others. The direct transesterification was carried out in 50 cm3 Erlenmeyer flasks with silicone-capped stoppers. The mixture was incubated at 40 ºC and stirred in an orbital shaking air-bath (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany) at 21 Hz for 56 h.

After the reaction, 8 cm3 of hexane was added and the resultant slurry was stirred for 10 min (first extraction). After that, the mixture was centrifuged at 1640 × g for 5 min to remove the residual biomass and separate the lipase; and the biphasic t-butanol-methanol-water-hexane system was separated in a decantation funnel. 5 cm3 of water and a small amount of NaCl were added in order to facilitate the separation of the extract’s hydrophilic components and break the emulsion that had formed. Subsequently, a second extraction was carried out of the FAMEs remaining in the t-butanol-methanol-water phase by adding 8 cm3 of hexane. Aliquots of both hexane phases were taken and analyzed by gas chromatography, as described in Section 2.3.

Furthermore, consecutive reactions were carried out to produce FAMEs catalyzed by the same lipase batch. In these experiments, lipase was washed with t-butanol before each new reaction cycle, using a t-butanol: lipase ratio of 50 cm3 g-1, and this mixture was agitated at 21 Hz for 10 min. All experiments were carried out in duplicate and their corresponding analyses in triplicate; and each value recorded is therefore the arithmetic mean of six experimental data sets (data shown as mean value ± standard deviation).

* 1. *Determination of total lipids (TLs), saponifiable lipids (SLs) and the transesterification reaction conversion*

TLs comprise both SLs and unsaponifiable lipids. SLs comprise neutral saponifiable lipids (NSLs, such as acylglycerols and free fatty acids) and polar lipids (glycolipids, GLs, and phospholipids, PLs). The TL content of the microalgal biomass was determined by the Kochet method [24], which is based on the extraction of lipids from lyophilized biomass with chloroform/methanol 1/1 (v/v).

The saponifiable lipid (SL) content of *N. gaditana* biomass was determined by direct methylation of the lyophilized microalgal biomass and methyl esters determination was by gas chromatography (GC), following the procedure described in Jiménez Callejón et al. [2]. This GC analysis was carried out using an Agilent Technologies 6890 gas chromatograph (Avondale, PA, USA), using a capillary column of fused silica, OmegawaxTM (0.25 mm × 30 m, 0.25 µm standard film, Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID) [2,3].

The conversion of SLs to FAMEs by lipase-catalyzed direct transesterification was calculated by the equation:

$$FAME conversion \left(\%\right)= \frac{Fatty acid transformed to FAMEs}{Total fatty acid amount }\*100 (1)$$

To determine the SL mass transformed to FAMEs in the transesterification reaction (the numerator of Eq.1), a volume of sample containing about 1 mg of fatty acids was taken and mixed with 50 µL of internal standard (19:0 methyl ester) and 1 cm3 of hexane - this mixture was analyzed directly by GC. To determine the total SL mass as FAMEs, the mixture of sample, internal standard and hexane was methylated by direct transesterification with acetyl chloride/methanol and analyzed again by GC to obtain the denominator of Eq.1 (total SL amount convertible to FAMEs).

* 1. *Fractionation of microalgal lipids*

 The homogenized biomass was lyophilized and then two samples containing about 10 mg of dry biomass were suspended in 0.5 cm3 of chloroform and fractionated by chromatography using single-use silica gel cartridges (Sep-pack plus WAT020520, Waters Corporation, Milford, MA), into neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLs); this fractionation was carried out following the procedure of Kates [25], which is based on the elution of the microalgal lipids in the silica gel cartridges with 30 cm3 of chloroform, 30 cm3 of acetone along with 20 cm3 of chloroform:methanol 85:15 v/v and 30 cm3 of methanol to collect the NLs, GLs and PLs, respectively, from each of these mobile phases. The solvents were evaporated in a rotary evaporator (Buchi, R210, with a V-700 vacuum pump and V-850 controller; Switzerland) and each fraction’s SLs were converted into FAMEs by direct methylation and then analyzed by GC (Section 2.3) for the quantitative determination of the SLs contained in each fraction.

1. **Results and discussion**

*3.1. Lipidic composition of N. gaditana biomass*

The total lipid (TLs) (i.e. oil) and saponifiable lipid (SLs) mass fractions of wet *N. gaditana* biomass were 31.1 % and 12.1 % of dry biomass, respectively. This means that the microalgal biomass has a relatively high mass fraction of unsaponifiable lipids (19.1 %) which are not convertible to FAMEs. This lipidic composition is largely the result of the culture conditions (see Section 1) [2,3,7]. Table 1B shows the percentages of neutral saponifiable lipids (NSLs), GLs and PLs obtained by lipid fractionation into lipidic classes. As can be observed, 30 % of SLs are NSLs and the remaining 70 % are polar lipids. NSLs (acylglycerols and FFAs) are part of the neutral lipid fraction, which also contains unsaponifiable lipids like pigments and hydrocarbons. GLs and PLs are also SLs, since they contain fatty acids. For the production of biodiesel from microalgae, NSLs are more interesting since they generally have a lower degree of unsaturation than polar lipids and therefore the biodiesel produced from NSLs has greater oxidation stability. Microalgae with higher NSL percentages and lower polar lipid contents can be produced growing the microalgae under nitrogen starvation conditions. However, these culture conditions are much more laborious and difficult to implement on an industrial scale and the biomass productivity is lower [7].

*3.2. Influence of microalgal biomass high-pressure homogenization (HPH)*

The objective of this work was to determine the transesterification conditions to maximize FAME conversion, aiming for a conversion rate above 90 % so as to minimize the acylglycerol content in the final biodiesel. In order to preserve lipase stability, experiments were carried out using an immobilized lipase (Novozym 435), t-butanol as the reaction medium and by adding the methanol in steps thus reducing its concentration and avoiding or decreasing lipase deactivation caused by the methanol [26,27,28].

To determine whether pretreatment of the wet microalgal biomass using high-pressure homogenization (HPH) could improve the FAME conversion, experiments were carried out comparing the results achieved both with and without this pretreatment. Figure 2 shows that a higher initial reaction velocity and conversions were attained when the wet biomass was pretreated by HPH (67.5 % in 24 h instead of 58.5 % obtained using wet biomass without homogenizing). This conversion increase is caused by the HPH pretreatment disrupting the microalgal cells, facilitating the access of the acyl-acceptor and the solvent (methanol and t-butanol, respectively) to the SLs contained in the cells - thus increasing the FAME conversion. A similar conversion increase was obtained by Macías-Sánchez et al. [20] in the production of FAMEs by direct transesterification of lipids in the microalga *N. gaditana* by acid catalysis. In addition, Jimenez Callejón et al. [2] used this pretreatment for wet *N. gaditana* microalgal biomass in order to improve the lipid extraction yield using hexane, and to demonstrate that both extraction velocity and lipid extraction yield increase with HPH pretreatment and the pressure applied (1700 bar). So, although HPH is energetically costly, taking the above results into account, wet *N. gaditana* microalgal biomass was submitted to a HPH pretreatment to enhance cell disruption and increase the FAME conversion.

*3.3. Influence of the N435:oil ratio*

Figure 3 shows that, as expected, the reaction velocity and the conversion both increase with the lipase amount. Maximal conversions of 69.5, 77.4, 79.4 and 82.3 % were obtained at 52 h with lipase/oil mass ratios of 0.11, 0.23, 0.32 and 0.43, respectively. In an attempt to use the least amount of enzyme possible, and taking into account the small differences between the results obtained using 0.23, 0.32 and 0.43, a mass ratio of 0.32 was chosen. However, most researchers use amounts lower than 10 %, although these researchers use vegetable oils with very high NSL percentages. Tran et al. [23] demonstrated, in the production of FAMEs by direct transesterification of the microalga *Chlorella vulgaris,* that higher lipase loading is needed to achieve high FAME conversions as the lipid content lowers. In this work, the lipids that are transformable to biodiesel are mixed with water (75 % water mass fraction) and the total cell components (12 % SL of biomass dry weight); for this reason, a high lipase amount is required. In this respect, authors such as Sangaletti-Gerhard et al. [29] obtained a FAME conversion of 57 % in the direct transesterification of wet greasy sludge in hexane medium using 10 % of N435 with respect to wet sludge (which contains 44.4 % of oils and fats as dry weight). Other authors, such as Lee et al. [30], carried out the conversion of oil extracted from *Chlorella* (with more than a 95 % TAG content) using 50 % of N435; obtaining a yield of 75.5 % in 6 h using dimethyl carbonate as the reaction medium and acyl-acceptor. Alavijeh et al. [31] employed 40 % of N435 (based on the crude oil weight) to obtain a maximum conversion of 66.7 % in 72 h, for the transformation of lipids extracted from the microalga *Chlorella vulgaris*, using ethyl acetate as the acyl-acceptor. Therefore, the N435/oil ratio used in this work is of the same order than the used by other researchers who transform complex oils (direct transesterification of microalgal oils [23], wet greasy sludge [29] or oil extracted from microalgae [30, 31]) to biodiesel.

*3.4. Influence of methanol:oil ratio*

[Fig. 4](https://www.sciencedirect.com/science/article/pii/S0961953416302203?via%3Dihub#fig4) shows that the higher the methanol/oil ratio, the higher the FAME conversion – this is logical given that, as the [methanol concentration](https://www.sciencedirect.com/topics/engineering/methanol-concentration%22%20%5Co%20%22Learn%20more%20about%20methanol%20concentration%20from%20ScienceDirect%27s%20AI-generated%20Topic%20Pages) increases, the transesterification equilibrium will be more displaced towards the formation of FAME. This figure shows that both the FAME conversion and the reaction velocity were maximal when a methanol/oil ratio of 4.6 cm3 g−1 was used. In this case, the FAME yield attained was 94.8%. This methanol/oil ratio is similar to that achieved by Tran et al. [[23]](https://www.sciencedirect.com/science/article/pii/S0961953416302203?via%3Dihub#bib23), who used methanol/oil ratios between 22 and 3.5 cm3 g−1 in the direct transesterification of lipids contained in dried sonicated [slurry](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/slurries%22%20%5Co%20%22Learn%20more%20about%20slurry%20from%20ScienceDirect%27s%20AI-generated%20Topic%20Pages) from the microalga Chlorella vulgaris, using hexane as the reaction medium. These researchers observed that the optimal methanol/oil ratio must be higher, the lower the biomass lipid content [[23]](https://www.sciencedirect.com/science/article/pii/S0961953416302203?via%3Dihub#bib23).

*3.5. Influence of the t-butanol:oil ratio or substrate concentration*

Lipase-catalyzed transesterification can be performed in the presence or absence of a solvent as the reaction medium. The lipase-catalyzed direct methanolysis of lipids contained in microalgal biomass, in solvent-free medium, can suffer mass transfer limitations and lipase inactivation due to the non-solubilized methanol [26]. The use of an organic solvent gives rise to the extraction and solubilization of microalgal oil and methanol, which facilitates contact between both reactants, decreasing the reaction mixture viscosity and preserving the lipase stability [17,23,32,33]. However, the addition of a large amount of solvent can cause excessive dilution of the reactants and a decrease in the reaction velocity; for this reason, it is necessary to optimize the solvent/oil ratio or the substrate concentration. t-Butanol was chosen as the reaction medium because it is a solvent with intermediate polarity, in which all the reactants (neutral, polar lipids and methanol) and products (FAMEs and glycerol) are soluble; this facilitates the reactant and product transfer and decreases lipase inactivation. In previous works, we determined that t-butanol is a more suitable reaction medium than hexane to convert SLs extracted from *N. gaditana* to FAMEs, catalyzed by N435 and the *R. oryzae* intracellular lipase; this is because the FAME conversions and the lipase reusability were always higher in t-butanol than in hexane [3,34]. Tran et al. [23], in the lipase-catalyzed direct transesterification of *Chlorella vulgaris* lipids, obtained higher FAME conversions using hexane than with t-butanol. This result may be because this microalga mainly contains NSLs (acylglycerols and free fatty acids), which are highly soluble in a non-polar solvent such as hexane; whereas *N. gaditana* lipids have high polar lipid percentages (Table 1), which are more soluble in a more polar solvent such as t-butanol [3]. Moreover, hexane is not an appropriate solvent when using wet biomass with a high water content as the substrate, since water and hexane are totally immiscible and the former forms a hydrophilic barrier for hydrophobic substrates thus decreasing the reaction velocity.

Therefore, experiments were carried out at different t-butanol/oil ratios (7.1, 14.3, 21.3 and 28.3 cm3 t-butanol g-1). Lower t-butanol/oil ratios were not tested due to the high viscosity of the reaction mixture and, therefore, the difficulty of attaining correct homogenization. Figure 5 shows that both reaction velocity and FAME conversion increase with the substrate concentration, although these differences are small in the range tested, and the final conversions attained at 52 h using 14.3, 21.3 and 28.3 cm3 t-butanol g-1 oil are similar. The highest reaction velocity and FAME conversion were achieved with the lowest t-butanol/oil ratio (7.1 cm3 g-1) or the highest substrate concentration. Thus, a maximum conversion of 99.5 % was obtained using 7.1 cm3 t-butanol/g oil, appreciably higher than that obtained with 14.3 cm3 t-butanol g-1 (94.8 %). In a previous work, a lower t-butanol/oil ratio (3.9 cm3 g-1) was required in the transesterification of the oil extracted from *N. gaditana* biomass, catalyzed also by N435, for a 94.7 % conversion at 48 h [3]; this is because, in the direct transesterification, all the biomass is present in the reaction medium and the viscosity is higher. Moreover, most of the oil remained inside the microalgal biomass, which adsorbs some of these lipids during transesterification; and the solvent is particularly important to enhance the mass transfer of reactants and products [18]. So, these authors (in the direct transesterification of *Chlorella vulgaris* lipids catalyzed by *Burkholderia* lipase in hexane) did not observe conversion to biodiesel when the solvent mass fraction was less than 50 %. These results indicate that, in this methodology, the solvent plays a crucial role in dissolving microalgal oil for effective transesterification.

Therefore, the optimal conditions for carrying out the direct transesterification of *N. gaditana* SLs, obtaining the maximal FAME conversion (99.5 %) were: a N435/oil mass ratio of 0.32, 4.6 cm3 methanol g-1 (added in three steps), 7.1 cm3 t-butanol g-1, 40 ºC, 21 Hz, over 52 h. Table 1A shows that the fatty acid profiles of the microalgal lipids are very similar to the FAMEs that are finally produced; this shows that the process does not alter the fatty acid composition of the original microalgal biomass.

* 1. *Lipase stability under optimal conditions*

Figure 6 shows the conversion attained in three consecutive transesterification reactions using the same batch of lipase N435. Experiments were carried out under the conditions previously established as optimal and using 7.1 and 14.3 cm3 t-butanol g-1 to determine if the t-butanol amount influences lipase stability. After each reaction cycle, the lipase was washed with t-butanol following the procedure described in Section 2.2. Figure 6 shows that the final FAME conversion decreased with the number of uses of lipase. Using the lowest t-butanol/oil ratio (7.1 cm3 g-1), the FAME conversion obtained in cycle 1 was higher (99.5 % versus 94.7 % with 14.3 cm3 t-butanol/g oil) but the final FAME conversion decreased faster; since with 7.1 cm3 t-butanol g-1, the final conversion decreased by almost 30 % in the second reaction cycle (99.5 % in cycle 1 and 67.5 % in cycle 2), whilst the decrease was 18 % in the first two reaction cycles using 14.3 cm3 t-butanol g-1. These results seem to indicate that, under the conditions established as optimal, there is a constant loss of lipase activity, which is more pronounced the lower the t-butanol amount. These results contrast with those obtained by Royon et al. [33], who described how the conversion yield obtained in the methanolysis of cotton seed oil catalyzed by N435 did not decrease significantly after 500 h (equivalent to 20.8 reaction cycles) in a t-butanol system (32.5 % v/v). However, this research was performed using vegetables oils which only contained NSLs and no polar lipids. The conversion decrease observed in this work might be due to several factors, such as lipase deactivation due to the presence of methanol as well as the high polar lipid content [3]. Table 1 shows that the polar lipid content of the homogenized biomass was 70 %, which is far higher than that used in Navarro López et al. [35]. In this case, we proved that the FAME conversions achieved when using the same lipase bath to catalyze successive reactions greatly decreased, the higher the polar lipid content of the microalgal SLs - indicating that polar lipids were responsible, along with the presence of methanol, for lipase inactivation [35]. Other authors reported similar results as those obtained in this work, such as Tran et al. [18], who observed a FAME conversion decrease of almost 40 % after six uses of the same *Burkholderia* lipase batch (288 h) in the direct transesterification of *Chlorella vulgaris* microalgal oil. Tran et al. [23] observed that the activity diminution rate of recycled *Burkholderia* lipase increased as the lipid content of the *Chlorella vulgaris* microalgae decreased - and this activity always decreased with the number of uses. Jiménez Callejón et al. [2] showed that microalgal biomass with low lipid content also has low acylglycerol and high polar and unsaponifiable lipid percentages. These results mean that polar or unsaponifiable lipids could also be the cause of the loss in lipase activity. Besides, also the presence of moisture, methanol and biodiesel contributes to the degradation of the polymethylmethacrylate, that is the lipase immobilization support in N435 [36].

1. **Conclusions**

Direct transesterification of saponifiable lipids in wet microalgal biomass is a promising alternative to produce biodiesel since no biomass drying is required and the oil extraction and transesterification are carried out in the same step. Prior homogenization of the *N. gaditana* biomass at high pressureenhances cell disruption and improves FAME conversion. Thus, homogenized wet microalgal biomass was directly transesterified and a maximum FAME conversion of 99.5 % was obtained using a N435:oil mass ratio of 0.32, methanol/oil and t-butanol/oil ratios of 4.6 and 7.1 cm3 g−1, respectively, at 40 °C and 56 h. FAME conversion decreased by 40 % when a lipase batch was used to catalyze three successive transesterification reactions. Both the reaction velocity and the reusability of the lipase over several reaction cycles seem to depend on the polar lipid content of the microalgal biomass.

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Fig. 1. Direct transesterification of SLs from wet *N. gaditana* biomass and extraction of FAMEs with hexane.

**Fig. 2**. Influence of high-pressure homogenization (HPH) of microalgal biomass on the FAME conversion. Reaction conditions: 3 g of wet microalgal biomass (25 % dry biomass weight and 31.1 % of oil weight in the dry biomass), N435:oil mass ratio of 0.32, t-butanol:oil and methanol:oil ratios of 14.3 and 1.45 cm3 g−1, respectively, two additions of methanol at 0 and 10 h, 40 °C, 21 Hz.

**Fig. 3**. Influence of reaction time and the Novozym 435/oil mass ratio on the FAME conversion. Conditions: 3 g of wet microalgal biomass (25% dry biomass weight and 31.1% of oil weight in the dry biomass), t-butanol:oil and methanol:oil ratios of 14.3 and 2.27 cm3 g−1, respectively, three additions of methanol at 0, 10 and 24 h, 40 °C, 21 Hz.

**Fig 4.** Influence of reaction time and the methanol/oil (v:w) ratio on the FAME conversion. Conditions: 3 g of wet microalgal biomass (0.23 g oil), 14.3 cm3 t-butanol/g oil, N435:oil mass ratio of 0.32, 40 ºC, 21 Hz, and three additions of methanol at 0, 10 and 24 h. (●) 1.04 cm3 g−1 in three additions of 0.21, 0.41 and 0.41 cm3 g−1, respectively; (○) 2.3 cm3 g−1 in three additions of 0.62, 0.83 and 0.83 cm3 g−1; (▼) 3.3 cm3 g−1 in three additions of 0.83, 1.25 and 1.25 cm3 g−1; (Δ) 4.6 cm3 g−1 in three additions of 1.25, 1.67 and 1.67 cm3 g−1, respectively.

**Fig 5**. Influence of reaction time and the t-butanol/oil ratio (v:w) on the FAME conversion. Conditions: 3 g of wet microalgal biomass (0.23 g oil), N435:oil mass ratio of 0.32, methanol:oil ratio of 4.6 cm3 g−1, three additions of methanol at 0, 10 and 24 h, 40 °C, 21 Hz.

**Fig. 6.** Reuse of the same lipase batch to catalyze three consecutive reactions (cycles) under the following conditions: t-butanol/oil ratios of 7.1 and 14.3 cm3 g−1, 3 g of wet microalgal biomass, N435:oil mass ratio of 0.32, methanol:oil ratio of 4.6 cm3 g−1, three additions of methanol at 0, 10 and 24 h, 40 °C, 21 Hz.

**Table 1**

A) Fatty acid composition (weight percentage of total fatty acids) of *N. gaditana* wet paste biomass and FAMEs obtained by direct transesterification. B) Weight percentage of neutral saponifiable lipids (NSLs), glycolipids (GLs) and phospholipids (PLs) obtained by fractionation of homogenized microalgal lipids (Section 2.4).

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| (A) |  |  |
| Fatty acids | Biomass, %  | FAMEs, % |
| 14:00 | 7.3 ± 0.9 | 3.8 ± 0.6 |
| 16:00 | 23.3 ± 1.6 | 20.7 ± 1.3 |
| 16:1n7 | 24.8 ± 1.7 | 21.3 ± 1.0 |
| 18:1n9 | 3.9 ± 0.4 | 4.6 ± 0.4 |
| 18:2n6 | 3.8 ± 0.7 | 8.8 ± 0.4 |
| 20:4n6 | 6.8 ± 0.5 | 7.3 ± 0.4 |
| 20:5n3 | 29.8 ± 2.8 | 30.3 ± 0.3 |
| (B) |  |  |

 |
| Lipid species | % |
| NSLs | 29.9 ± 0.6 |
| GLs | 57.4 ± 1.4 |
| PLs | 12.7 ± 0.8 |

Recovery of Novozym 435

*N.gaditana*

12 % SLs; 25 % dry biomass

High-pressure homogenization (HPH*)*

Direct transesterification

First FAME extraction

Hydroalcoholic phase

Hexanic phase containing FAMEs (1)

Hydroalcoholic phase (residue)

Hexanic phase containing FAMEs (2)

Fig. 1.



**Fig. 2**.



**Fig. 3**.



**Fig 4.**



**Fig 5**.



**Fig. 6.**