**Extraction of microalgal lipids and the influence of polar lipids on biodiesel production by lipase-catalyzed transesterification**

Elvira Navarro López, Alfonso Robles Medina\*, Pedro Antonio González Moreno, Luis Esteban Cerdán, Emilio Molina Grima

*Area of Chemical Engineering, University of Almería, 04120 Almería, Spain*

*Corresponding author: Alfonso Robles Medin. Tel: +34 950 015065; Fax: +34 950 015484. E-mail address:* *arobles@ual.es**. Address: Area of Chemical Engineering, Department of Engineering, University of Almería, 04120 Almería, Spain.*

Article history:

Received 19 April 2016

Received in revised form 9 June 2016

Accepted 10 June 2016

**DOI 10.1016/j.biortech.2016.06.035**

**Abstract**

This work demonstrates the influence of polar lipids on the transformation of *Nannochloropsis gaditana* saponifiable lipids (SLs) to fatty acid methyl esters (FAMEs, biodiesel) by lipase-catalyzed transesterification. In order to obtain microalgal SL fractions containing different polar lipid (glycolipids and phospholipids) contents, lipids were extracted from wet microalgal biomass using seven extraction systems, and the polar lipid contents of some fractions were reduced by low temperature acetone crystallization. We observed that the polar lipid content in the extracted lipids depended on the polarity (log P value) of the first solvent used in the extraction system (ethanol, hexane, isopropanol or ethyl acetate). Lipid fractions with polar lipid contents between 75.1% and 15.3% were obtained. Some of these fractions were transformed into FAMEs by methanolysis, catalyzed by the lipases Novozym 435 and *R. oryzae* in tert-butanol medium. We observed that the reaction velocity was higher the lower the polar lipid content, and that the final FAME conversions achieved after using the same lipase batch to catalyze consecutive reactions decreased in relation to an increase in the polar lipid content. This indicates that these lipids affect transesterification velocity, FAME conversion and lipase stability - even in the presence of tert-butanol.

Key words: Biodiesel; Microalga; *Nannochloropsis gaditana*; Lipase stability; Lipid extraction; Polar lipid

1. **Introduction**

Biodiesel has received much attention as it seems to be a promising alternative to conventional diesel. Producing it from vegetable oils or animal fats is inconvenient given that these raw materials can also be used for feed and, furthermore, are in limited supply. Microalgae are currently considered one of the most likely candidates for biodiesel production. They are photosynthetic organisms capable of converting (under light conditions) water and carbon dioxide into macromolecules such as oils, polysaccharides and proteins; some of which can produce between 7 and 31 times more lipids than the best vegetable oil crops and achieve high growth rates compared to agricultural crops (Chisti, 2007). However, while plant oils are made up mostly of neutral lipids (NLs), microalgal oils are rich in polar lipids - glycolipids, (GLs) and phospholipids (PLs) - having a higher viscosity that can lead to reduced transesterification velocity and FAME conversion (Jiménez Callejón et al., 2014; Navarro López et al., 2015). For this and other reasons, the neutral saponifiable lipids (NSLs) - free fatty acids, tri-, di- and monoacylglycerols - which are also found in microalgae (Nielsen et al., 2008; Hidalgo et al., 2013) are the most interesting lipids for biodiesel production. The microalgal lipid composition used to produce biodiesel depends on the microalgal species, the culture conditions, the time of harvesting and also the solvent and method used to extract the lipids from the microalgae. San Pedro et al. (2013) 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 wt% of biomass dry weight) compared to that in continuous culture (12.0 wt%). Moreover, the former is richer in NSLs (17.2 wt% of biomass dry weight) than that cultured without nitrogen limitations (7.9 wt%). However, biomass productivity is lower under nitrogen starvation conditions and culture conditions such as these are far more laborious and difficult to implement on an industrial scale (San Pedro et al., 2013). Furthermore, algae harvesting in the exponential growth phase (usually when production is in continuous operation mode) will produce microalgae with more polar lipids than those harvested in the late stationary growth phase (usually in batch cultures), which contain more NSLs (Molina Grima et al., 2013).

The most-used method for producing biodiesel is by the transesterification of acylglycerols with short-chain alcohol (methanol or ethanol) in the presence of various catalysts (Stamenkovik et al., 2011). In the industrial processes so far proposed, alkali catalysts have generally been used. However, they cannot be used if the oil contains free fatty acids (FFAs > 0.5%), as occurs with microalgal oils (Robles Medina et al., 2009). Microalgal oils with high FFA contents can be transesterified using acid and enzymatic catalysts; nonetheless, lipases work at lower temperatures (25-50ºC) and the subsequent separation and purification of biodiesel and glycerol is easier when using lipases (Khor et al., 2010). To overcome these drawbacks, many authors have studied the use of lipases as catalysts for biodiesel production. Some of the extracellular lipases most commonly used are from species such as *Candida antarctica* (Novozyme® 435), *Thermomyces lanuginosus* (Lipozyme® TL IM) or *Rhizomucor miehei* (Lipozyme® RM IM). However, the use of extracellular lipases requires complicated and expensive recovery, purification and immobilization processes for industrial applications and there has been considerable interest in the use of intracellular lipase whole-cell biocatalysts, which have the advantage of avoiding isolation operations, purification and extracellular lipase immobilization (Li et al., 2008; Ban et al., 2002). Consequently, the filamentous fungus from *Rhizopus oryzae* is one of the most widely used for biodiesel production via enzymatic transesterification. However, lipases have other drawbacks for use in biodiesel production such as their rapid deactivation due to methanol. If the methanolysis or ethanolysis are carried out in solvent-free systems, some methanol or ethanol can be non-solubilized due to the limited solubility of these alcohols in the oil, and this non-solved alcohol causes lipase deactivation. To overcome the problem, some authors proposed the stepwise addition of methanol (Shimada et al., 1999) with t-butanol as the solvent, which solves the methanol and reduces lipase deactivation (Li et al., 2008; Li et al., 2010).

In previous works carried out at our laboratory on lipase-catalyzed transesterification of microalgal lipids to produce biodiesel (Navarro López et al., 2015, 2016), we observed that the polar SLs (GLs and PLs) contained in these microalgal lipids could be responsible for the decrease both in reaction velocity and in lipase stability. The aim of the present work was to study this influence. Hence, we extracted microalgal SLs from wet *N. gaditana* microalgal biomass using different solvents. In this way, SL fractions with different polar lipid contents were obtained and these fractions were then transformed to FAMEs by lipase-catalyzed transesterification, using both the extracellular lipase Novozym 435 and the intracellular lipase *R. oryzae*. Consequently, we were able to study the influence of the lipidic composition of microalgal lipids on the reaction velocity, FAME conversion and lipase stability.

1. **Materials and methods**
	1. *Microalga, lipases and chemicals*

Wet paste biomass from the marine microalga *Nannochloropsis gaditana* was used as the oil-rich substrate. Cells were grown in an outdoor tubular photobioreactor at “Las Palmerillas, Cajamar” research centre (El Ejido, Almería, Spain). The wet biomass supplied was harvested and centrifuged (Centrifuge Brand Supelco 4-15,Sartorius, Germany) to 85 wt% moisture content. The dry weight content of the wet biomass samples was determined from the weight difference between the wet biomass samples and the same samples following lyophilisation. This wet biomass contained 31.1 ± 0.1 wt% of total lipids (TLs) in the dry biomass. The total fatty acid content (or saponifiable lipids, SLs, as equivalent fatty acids) in the biomass was 12.0 ± 0.1 wt% in the dry biomass. The fatty acid composition of the wet paste biomass from *N. gaditana* used in this study is shown in Navarro López et al. (2015).

The transesterification reactions were catalyzed by the lipase Novozyme 435® (N435) from *Candida antarctica* (Novozymes A/S, Bagsvaerd, Denmark) and by the fungus *R. oryzae* IFO 4697. N435 is supplied immobilized on a macroporous acrylic resin and usually this lipase does not show any positional specificity. The fungus *R. oryzae*, which produces a 1,3-positional specific lipase, was provided by the NITE Biological Resource Center (NBRC, Chiba, Japan). The chemicals used for culturing the fungus, and the culture conditions and methodology used for producing this catalyst, are described in Navarro López et al. (2016).

The chemicals used were analytical grade ethanol (96% v/v), hexane (95% purity, synthesis quality), 2-propanol, ethyl acetate, chloroform and acetone (analytical quality) - all from 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.

* 1. *Lipid extraction from wet microalgal biomass and purification by crystallization in acetone*

Lipid extraction from the wet *N. gaditana* biomass was carried out using seven extraction systems: ethanol (96% v/v)-hexane, hexane, hexane:isopropanol (3:2 v/v), ethyl acetate, hexane:ethyl acetate (3:2 v/v), hexane-ethanol (50% water) and hexane-ethanol (5% water). The extraction of lipids with ethanol followed by purification with hexane (Fig. 1) is described in detail by Navarro López et al. (2015). Using this procedure, an extract with only 31 wt% of SLs was obtained, but this purity was increased by crystallization in acetone following the procedure also described by Navarro López et al. (2015).

The extraction of SLs with hexane (Fig. 2) is based on a similar method proposed by Jiménez Callejón et al. (2014). In this method, wet *N. gaditana* microalgal biomass was firstly homogenized at 1500 bar in a laboratory homogenizer (Panda Plus 2000 S.N. 8983 model, Gea Niro Soavi, Parma, Italy) and then extracted using 10 mL of hexane/g biomass at room temperature for 20 h with a constant agitation of 200 rpm. The hexanic phase was analyzed by gas chromatography (GC, Section 2.4) to determine the SL extraction yield. All the hexanic extracts were evaporated in a rotary evaporator (Buchi, R210, with a vacuum pump V-700, Switzerland) to recover the hexane. These lipids were also subjected to acetone crystallization treatment to increase the SL purity and decrease the phospholipid (PL) content. The extraction of microalgal lipids with ethyl acetate, hexane:isopropanol (3:2 v/v) and hexane:ethyl acetate (3:2 v/v) was carried out using the same procedure as that used with hexane (Fig. 2).

The extraction of microalgal lipids with the hexane-ethanol (50% water) and hexane-ethanol (5% water) systems was carried out following the procedure shown in Figure 3. With these solvent systems, microalgal lipids were firstly extracted with hexane and then this hexanic phase was extracted with ethanol:water (50:50 v/v) in one case and with ethanol:water (95:5 v/v) in the other, to decrease the polar lipid content of the lipids dissolved in the hexanic phase.

*2.3. Transesterification of microalgal saponifiable lipids (SLs)*

Microalgal SLs were transformed to fatty acid methyl esters (FAMEs) by lipase-catalyzed methanolysis using the optimized conditions obtained in previous works (Navarro López et al., 2015, 2016). In a typical experiment, 1.4 g of extracted microalgal lipids (95% of SL) was mixed with 13.3 mL of t-butanol (10 mL/g SLs), 0.30 g of N435 (0.225 g N435/g SL) or 0.93 g of *R. oryzae* fungus (0.7 g of *R. oryzae*/g SLs) and 2.1 mL of methanol (a methanol/SL molar ratio of 11:1). In the experiments catalyzed by N435, methanol was added in three steps at the reaction times of 0, 10 and 24 h, adding a molar ratio of 3:1 in the first step and 4:1 in the other two. In the experiments catalyzed by *R. oryzae,* both the fungus and the methanol were added in three steps at the reaction times of 0, 24 and 48 h, adding 0.31 g of *R. oryzae* at each step and 573 μL of methanol in the first step (3 mol methanol/mol SLs) and 764 μL of methanol in the other two (4 mol of methanol/mol SLs per step). SLs are expressed as equivalent fatty acids (mean molecular weight 277 g/mole) and the molar ratios are expressed as moles of methanol per mole of fatty acid in the microalgal SLs. The esterification reaction was carried out in 50 mL Erlenmeyer flasks with silicone-capped stoppers. In the experiments catalyzed by N435, the mixture was incubated at 40 oC and stirred in an orbital shaking air-bath (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany) at 200 rpm for 48 h; while the experiments catalyzed by *R. oryzae,* the temperature, stirring rate and reaction time were 35 ºC, 130 rpm and 72 h, respectively. The reactions were stopped by lipase separation using filtration (glass plate, pore size range 16-50 μm). The final reaction mixture was conserved at -24 ºC until analysis. All reactions were carried out in duplicate and their corresponding analyses in triplicate; thus each value recorded is the arithmetic mean of six experimental data sets (data shown as mean value ± standard deviation).

*2.4. Determination of total lipids (TLs) and saponifiable lipids (SLs), analysis of products and fractionations of extracted microalgal lipids*

TLs comprise both SLs and unsaponifiable lipids. SLs comprise neutral saponifiable lipids (NSLs) such as acylglycerols and free fatty acids, and polar lipids such as glycolipids (GLs) and phospholipids (PLs). The TL content of the microalgal biomass was determined by the Kochet method (1978).

The SL content of *N. gaditana* microalgal biomass was quantified by direct transesterification of the microalgal biomass with acetyl chloride/ methanol (1:20 v/v) to transform all the SLs into FAMES (Jimenez Callejón et al., 2014). The FAMEs were then analyzed by gas chromatography (GC) using an Agilent Technologies 6890 gas chromatograph (Avondale, PA, USA). Further details of the analysis method are described in Navarro López et al. (2015).

The SL yield (wt%) in the crude lipidic extracts from the microalgal biomass is the percentage of extracted SLs with respect to the total amount of SLs contained in the original biomass, both determined as equivalent fatty acids by GC. To determine the fatty acid amount in the extracts, a known volume was dried under an N2 stream, and methylation was carried out by direct transesterification with acetyl chloride/ methanol (1:20 v/v) (Jiménez Callejón et al., 2014). The SL purity (wt%) is the weight percentage of SLs (determined by GC) with respect to the total amount of extracted lipids; this was determined by weighing after complete removal of the solvent contained in the lipid extract. In the lipid purification experiments by crystallization in acetone, a known volume of supernatant samples was taken and acetone was removed in the N2 stream. Then, 1 mL of hexane and 50 µL of internal standard (prepared weighing 25 mg of nonadecanoic acid, 19:0, in 10 mL of hexane) were added. Samples were also methylated by the procedure described previously (Jiménez Callejón et al., 2014).

 The conversion of SLs to FAMEs by transesterification, catalyzed by the N435 and *R. oryzae* lipases, was determined following the procedure described in Martín et al. (2012). This conversion was calculated by the equation:

To determine the SL amount transformed to FAMEs (the equation numerator (1)), we took a known sample volume containing about 1 mg of fatty acids (around 20 μL) and mixed it with 50 µL of internal standard solution and 1 mL of hexane. This mixture was directly analyzed by GC. Then, these samples were methylated by direct transesterification with acetyl chloride/methanol and analyzed again by GC to obtain the denominator of Equation (1). The internal standard solution was prepared dissolving 25 mg of 19:0 methyl ester in 10 mL of hexane.

 The lipid extracts obtained from the wet microalgal biomass by the procedures described in Section 2.2 were fractionated into neutral lipids (NLs), GLs and PLs following the procedure described in Jiménez Callejón et al. (2014), which is based on the elution of lipids in Sep-pack classic cartridges (Waters Corporation, Milford, MA) with chloroform and acetone, along with chloroform:methanol 85:15 v/v and methanol to collect the NLs, GLs and PLs from each of these mobile phases, respectively (Kates, 1986). The GC analysis of all fractions gave the percentage of NSLs, GLs and PLs with respect to SLs (Table 1).

1. **Results and discussion**

*3.1. Extraction of saponifiable lipids (SLs) from the microalga N. gaditana*

 The wet biomass used in this work contained 15 wt% of dry biomass, 31.1 ± 0.1 wt% of total lipids (TLs) and 12.0 ± 0.1 wt% of saponifiable lipids (SLs) from the dry biomass. It was therefore a microalgal biomass with a low SL content and a high unsaponifiable lipids (19.1 wt%) content, which are not convertible to fatty acid methyl esters (FAMEs, biodiesel). This biomass was cultivated in continuous mode, with standard algal medium (8.0 mM nitrate) at a dilution rate of 0.3 day-1. Under these conditions maximum biomass productivity was achieved but this biomass has a low SL content and these SLs contain high percentages of polar lipids (GLs and PLs) (Jiménez Callejón et al., 2014; San Pedro et al., 2013). Table 1 (first line) shows the percentages of NSLs, GLs and PLs in the *N. gaditana* biomass, and, effectively, highlights the high percentage of polar lipids, above all GLs (57.4%). This lipidic profile is quantitatively different, for example, from that obtained for the same microalga by Ryckebosch et al. (2014a) (40%, 31% and 29% of NL, GLs and PLs, respectively), because this lipidic profile is dependent on the culture conditions.

 With the goal of obtaining microalgal lipid fractions with different polar lipid contents, different extraction systems and procedures were used for extracting the SLs from the wet *N. gaditana* microalgal biomass. Table 1 shows the SL extraction yields, SL purities and the percentages of NSLs, GLs and PLs obtained with each of the extraction systems and procedures tested. The extraction with ethanol (96% v/v) and the posterior purification of this extract with hexane has been used in previous works (Navarro López et al., 2015, 2016), giving high SL yields (around 85%, Table 1) but low SL purities (38%). This result was also observed by Ryckebosch et al. (2014a) in the extraction of lipids from the same microalga, since only 60% of the extract consisted of lipids - this is because polar solvents extract larger amounts of both unsaponifiable lipids and non-lipid substances given that the polar solvents are capable of extracting more polar materials, such as proteins and carbohydrates, from the microalgal biomass (Ryckebosch et al., 2014b). A low SL purity was also achieved by Ramluckan et al. (2014a), who extracted lipids from the microalga *Chlorella* sp. using ethanol in the Soxhlet extraction method (at the ethanol boiling temperature) and only 52% of the extracted lipids were SLs. This low purity (38%) was increased to 95% by crystallization in acetone (AC) at a low temperature, and this treatment also reduced the polar lipid content from 75.1 to 49% (Table 1). Using this procedure, gums (PLs) and waxes are precipitated out because of their insolubility in acetone at low temperature and therefore SL purity increases because these lipids remain mostly solubilized in acetone (Rajam et al., 2005).

 Hexane was used as the extraction solvent to directly extract a lipidic fraction with a lower polar lipid content (Table 1). However, in this case, a low SL yield was obtained (37.7%), similar to that obtained by Ryckebosch et al. (2014a) with this solvent (36%). Hexane is a non-polar solvent and lower polar SLs were extracted (35.4%, Table 1) compared to ethanol (75.1%). When the acetone crystallization treatment was applied to the lipids extracted with hexane, the polar lipid content did not decrease and the SL purity only increased from 56% to 61%. This result indicates that the lipids previously extracted with hexane contained a low or negligible amount of waxes and gums susceptible to precipitation in acetone at low temperature.

 The hexane:isopropanol (3:2), hexane:ethyl acetate (3:2) and ethyl acetate solvent systems were also used to obtain SLs with low polar lipid contents (Ryckebosch et al., 2014a). With hexane:isopropanol (3:2), a higher SL yield, and therefore a higher polar lipid content, was achieved due to the relatively high polarity of isopropanol (log P = 0.05, Sangster, 1989). With this system, a GL content was obtained higher than that achieved with hexane, but with a similar PL yield. Using hexane:ethyl acetate (3:2) and ethyl acetate, slightly higher SL yields and a similar polar lipid content were obtained to that using only hexane. The polarity of ethyl acetate (log P value) is between that for hexane and isopropanol (Table 1). These yields are also similar to the ones obtained by Ryckebosch et al. (2014a).

 Additionally, the hexane-ethanol:water (95:5 v/v) and hexane-ethanol:water (50:50 v/v) solvent systems were tested to try to further decrease the polar lipid content. In this case, the SLs were firstly extracted with hexane and then the hexanic solution was extracted with ethanol:water (95:5 v/v) or ethanol:water (50:50 v/v) (Fig. 3) to extract the polar lipids and increase the NSL content of the hexanic phase. The minimum polar lipid content was achieved byextracting the polar lipids from the hexanic phase with ethanol:water (95:5 v/v). This last liquid-liquid extraction reduced the polar lipid content from 35.4% to 15.3%; although the SL recovery yield was also reduced from 37.7% to just 15.8% (Table 1).

 Table 1 shows that the SL yields were not high, which, among other reasons, might be because *Nannochloropsis* species possess a thick rigid cell wall that contain an aliphatic, non-hydrolysable biopolymer called algaenan (Gelin et al., 1997). The table shows that, in all the extracting systems tested, except for ethanol (96%)-hexane, the extracts were richer in NSLs, or poorer in polar lipids, than the microalgal biomass, due to the low polarity of the first solvent used in these extraction systems. Table 1 also shows that, in general, the SL yield was higher the higher the polarity was of the first solvent used in the extraction process. Thus, the highest extraction yield was obtained using ethanol (log P = - 0.30, Sangster, 1989) whilst the lowest was using hexane (log P = 4), as the first extraction solvent. This also had an influence on the profile of the extracted lipids because the higher the polarity of the solvent, the higher the percentage of polar lipids and the lower the NSL content (Table 1). In microalgae cells (eukaryotic) neutral lipids (NLs) interact through weak van der Waals forces to form lipid globules in the cytoplasm. These free NLs can be extracted from the cell using nonpolar solvents such as hexane; this is because similar van der Waals forces are formed between the NLs and the nonpolar solvent. However, some NLs form complexes with polar lipids, which create hydrogen bonds with proteins in the cell membrane. Nonpolar solvents are not capable of disrupting these hydrogen bonds and therefore can extract neither the complexed NLs nor the polar lipids from the cells. In contrast, polar organic solvents, such as ethanol or isopropanol, can form hydrogen bonds with the lipids and hence break the lipid-protein complexes leading to extraction of both more complexed NLs and polar lipids from the cells (Halim et al., 2012; Shahidi and Wanasundara 2002; Ryckebosch et al., 2014a). Therefore, in the case of ethanol (96%)-hexane extraction, it seems that the polar lipids, once extracted from the microalgal biomass using a polar solvent, such as ethanol, can be extracted into a non-polar solvent, such as hexane, because the lipid-protein complexes were already broken.

*3.2. Influence of the polar lipid content on the enzymatic transesterification of microalgal SLs*

 In order to observe the influence of the polar lipid content on the reaction velocity and final conversion of the transesterification of microalgal SLs, some of the previously extracted SL fractions from the wet *N. gaditana* microalgal biomass were transformed to fatty acid methyl esters (FAMEs, biodiesel) by alcoholysis catalyzed using the lipases N435 (extracellular) and *R. oryzae* (intracellular).

 The five SL fractions extracted with the ethanol (96% v/v)-hexane (75.1% of polar lipids, Table 1), ethanol (96% v/v)-hexane + AC (49% of polar lipids), hexane (35.5% of polar lipids), hexane + AC (37.4% of polar lipids) and hexane-ethanol/water (95/5 v/v) (15.3% of polar lipids) systems were subjected to enzymatic transesterification catalyzed by lipase N435. Figure 4A shows that the final conversions achieved using the four SL fractions with lower polar lipid contents were very high (around 95%) and non-significant differences between these final conversions were observed. This FAME conversion is similar to that obtained by other authors who transformed microalgal SLs into biodiesel using enzymatic catalysis under similar conditions; for example, Tran et al. (2013) obtained a 91-96% FAME conversion in the transesterification of *Chlorella vulgaris* lipids, catalyzed by the *Burkoholderia* lipase. However, the final conversion achieved with the ethanol (96% v/v)-hexane SL fraction was appreciably lower (86.3%, Fig. 4A); this SL fraction is no less pure than the others (38%, Table 1) but contains the highest polar lipid percentage (75.1%, Table 1). Figure 4A also shows that the highest and the lowest initial reaction velocities correspond to the SL fractions with, respectively, the lowest and the highest polar lipid contents; these are, again respectively, the SL fractions extracted with the hexane-ethanol:water (95/5 v/v) (15.3% polar lipids, Table 1) and ethanol (96% v/v)-hexane (75.1% polar lipids) systems. Non-significant differences were observed between the initial reaction velocities obtained with the other SL fractions, which contained closer polar lipid contents (49.0, 37.4 and 35.4%, Table 1). These results show that the higher the polar lipid content, the lower both the final conversion and the reaction velocity; although these differences are not high. Such results could be explained by the polar lipids being bound on the immobilized lipase preparation and interfering with the interaction between the lipase molecule and the substrates (Watanbe et al., 2002). Nonetheless, Figure 4A shows that the influence of the polar lipid content is relatively low, which might be because the lipase immobilization support of N435 is hydrophobic (at least compared to the hydrophilic supports used for lipases such as Lipozyme RM IM and TL IM), and this hydrophobic support could limit the adsorption of polar compounds present in the medium (such as polar lipids and glycerol) (Séverac et al., 2011).

 Figure 4B shows the variation in FAME conversions with the reaction time obtained in the *R. oryzae*-catalyzed transesterification of the *N. gaditana* microalgal SLs extracted with the ethanol (96% v/v)-hexane (75.1% polar lipids, Table 1), ethanol (96% v/v)-hexane + AC (49% polar lipids), hexane + AC (37.4% polar lipids) and hexane-ethanol:water (95:5 v/v) (15.3% polar lipids) systems. This figure again shows that the final conversions achieved using the three SL fractions with lower polar lipid contents were relatively high (around 82%) and non-significant differences between these final SL fraction conversions were observed. In addition, this figure shows that the final conversion achieved with the ethanol (96% v/v)-hexane fraction was appreciably lower (70.2%) because the SL fraction contained the highest polar lipid content (75.1%). The final conversion achieved with the ethanol-hexane + AC fraction was similar to that obtained with the hexane-ethanol:water and hexane+AC fractions although the latter had lower SL purity (59.8% and 61% versus a 95% purity for the former, Table 1), which demonstrated that the SL content did not influence the FAME conversion. These FAME conversions were lower than those obtained by other authors who transformed SLs into biodiesel under similar conditions using *R. oryzae* intracellular lipase as the catalyst: for example, Arumugan and Ponnusami (2014) attained a 92% FAME yield; however, these authors used a vegetable oil. Indeed, few works exist regarding microalgal lipid biodiesel production using *R. oryzae* as the catalyst. Figure 4B also shows that the highest initial reaction velocities corresponded to the SL fractions with the lowest polar lipid contents (the hexane –ethanol:water and hexane + AC systems). Li et al. (2014), in the transesterification of soybean oil with phospholipid contents higher than 5%, catalyzed by the free lipase NS81006 from *Aspergillus niger*, also observed that the higher the phospholipid content in the oil, the lower the reaction velocity and biodiesel yield. All these results demonstrate that, in effect, polar lipids decrease the reaction velocity and FAME conversions due to the increase of reaction medium viscosity and because these lipids bind to the lipase, thus interfering with the lipase molecule’s interaction with the substrates (Watanabe et al., 2002; Wang et al., 2014; Navarro López et al., 2015, 2016).

*3.3. Influence of the polar lipid content on the FAME conversions achieved after repeated use of the lipases*

 Figure 5 shows the variation in the FAME conversions with reaction time achieved in three consecutive transesterification reactions catalyzed by the same N435 batch. These reactions were carried out under the conditions established as optimal for this lipase in a previous work (Navarro López et al., 2015). After each reaction cycle, the lipase was filtrated and washed with 50 mL of t-butanol/g lipase before being used. Figure 5a shows that both the reaction velocity (lipase activity) and the final conversion decreased with the number of lipase uses. Figures 5a-e show that the conversion attained at 48 h decreased by about 23%, 10%, 4.6%, 2.7% and 0.63% after three uses of the same lipase batch, and these SL fractions contained 75.1%, 49%, 37.4%, 35.4% and 15.3% of polar lipids (Table 1) respectively; that is to say, the higher the polar lipid content, the higher the conversion loss. Therefore, the results seem to indicate that under these experimental conditions and using lipids with a high polar lipid content, there is a constant loss in lipase activity (Figure 6a- d); whereas using microalgal SLs with a low polar lipid content (15.3%), the conversion loss is far lower or even negligible (0.63%, Fig. 6e) - at least when it is used for 144 h (48 h × 3 uses) under the operational conditions used in this work (see the Figure 4 caption). This last result is in agreement with those obtained by other authors who observed that N435 did not deactivate in the presence of t-butanol, even though it did in the presence of methanol, when refined vegetable oils were used as substrates (Li et al., 2006; Royon et al., 2007). Wang et al. (2014) carried out methanolysis on lipids extracted from the microalga *Nannochloropsis oceanica,* catalyzed by lipase N435 over 165 h in t-butanol observing some conversion losses; although the conversion efficiency remained greater than 90% after this period (165 h), which is a similar result to the one obtained in this work. These authors used crude algal oils extracted from microalgal biomass cultivated under nitrogen-depleted conditions, and therefore with higher triacylglycerols (NSLs) contents than polar lipid contents.

 Figure 6 shows the variation in the FAME conversion with reaction time achieved in three consecutive transesterification reactions catalyzed by the same *R. oryzae* batch. These reactions were carried out under the conditions established as optimal for this lipase in a previous work (Navarro López et al., 2016), and after each reaction cycle the lipase was filtrated and washed with t-butanol before use, using 50 mL of t-butanol/g lipase. Figures 6a-d show that both reaction velocity (lipase activity) and final conversion attained at 72 h decrease with the number of lipase uses. This figure shows that, after three uses of the same lipase batch, these conversion losses were 74%, 58%, 15% and 16% for polar lipid contents of 75.1%, 49%, 37.4% and 15.3%, respectively; i.e. the higher the polar lipid content, the greater the conversion losses. These conversion losses are greater than those observed for lipase N435 in the transesterification of the same SL fractions (Fig. 5), which shows that N435 is much more stable than *R. oryzae* under similar conditions, perhaps because lipase N435 is immobilized on a hydrophobic support that adsorbs small amounts of polar lipids and reaction products (Séverac et al., 2011). A similar result had already been observed by Li et al. (2014) in the transesterification of soybean oil, with 5% and 10% of PLs catalyzed using the free lipase NS81006 from *A. niger*. For example, after three uses the FAME yield for a 10% PL content dropped from 75.3% to 22.2% (a conversion loss of 70.5%). These authors found that the coexistence of methanol and PLs was responsible for the lower catalytic performance and the conversion loss following the lipase’s repetitive use. PLs act as surfactants and generate reverse micelles in which free lipase and methanol are solubilized giving higher local methanol concentration around the lipase and, consequently, causing a toxic effect on the lipase’s catalytic performance. This might also explain the high activity losses observed in this work when SLs with a high polar lipid content were used as feedstock; although in this case no free lipase was used, rather an immobilized lipase (N435) and a whole cell intracellular lipase (*R. oryzae*).

1. **Conclusions**

In this work, we extracted the SLs and polar lipids from wet *Nannochloropsis gaditana* microalgal biomass and found that the recovery yields were higher, the higher the polarity of the extraction solvent. In the lipase-catalyzed transesterification of these SLs using different polar lipid contents, we observed that the reaction velocity was higher when using SLs with low polar lipid contents, and that the FAME conversions achieved when using the same lipase batch to catalyze successive reactions greatly decreased in percentage terms, 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.

**Acknowledgements**

This research was supported by grants from the *Ministerio de Economía y Competitividad* (Spain), Project CTQ2010-16931. This project was co-funded by FEDER (The European Fund for Regional Development).

**References**

1. Arumugan, A., Ponnusami, V., 2014. Biodiesel production from *Calophyllum inophyllum* oil using lipase producing *Rhizopus oryzae* cells immobilized within reticulated foams. Renew. Energy 64, 276–282.
2. Ban, K., Hama, S., Nishizuka, K., Kaieda, M., Matsumoto, T., Kondo, A., 2002. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. J. Mol. Catal. B Enzym. 17, 157-165.
3. Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.
4. Gelin, F., Boogers, I., Noordeloos, A., Damste, J., Riegman, R., De Leeuw, J. 1997. Resistant biomacromolecules in marine microalgae of the classes *Eustigmatophyceae* and *Chlorophyceae*: Geochemical implications. Organ. Geochem. 26, 659-675.
5. Halim. R., Danquah, M.K., Webley, P.A., 2012. Extraction of oil from microalgae for biodiesel production: a review. Biotechnol Adv. 30, 709–732.
6. Hidalgo, P., Toro, C., Ciudad, G., Navia, R., 2013. Advances in direct transesterification of microalgal biomass for biodiesel production. Rev. Environ. Sci. Biotechnol. 12, 179-199.
7. Jiménez Callejón, M.J., Robles Medina, A., Macías Sánchez, M.D., Hita Peña, E., Esteban Cerdán, L., González Moreno, P.A., Molina Grima, E., 2014. Extraction of saponifiable lipids from wet microalgal biomass for biodiesel production. Bioresour. Technol. 169, 198-205.
8. Kates, M., 1986. Definition and classification of lipids. In: Burdon, R.H., van Knippenber, P.H. (Eds.), Techniques of Lipidology, Isolation, Analysis and Identification of Lipids. Elsevier Science Publishers, Amsterdam.
9. Khor, G.K., Sim, J.H., Kamaruddin, A.H., Uzir, M.H., 2010. Thermodynamics and inhibition studies of Lipozyme TL IM in biodiesel production via enzymatic transesterification. Bioresourc. Technol. 101, 6558-6561.
10. Kochet, G., 1978. Quantitation of the macromolecular components of microalgae. In: Hellebust, J., Cragie, S. (Eds.), Handbook of Phycological Methods. Physiological and Biochemical Methods. Cambridge University Press, London.
11. Li, W., Du, W., Li, Q., Li, R-W., Liu, D., 2010. Dependence of the properties of organic solvent: study on acyl migration kinetics of partial glycerides. Bioresourc. Technol. 101. 5737-5742.
12. Li, W., Du, W., Liu, D., 2014. Exploration of the effect of phospholipids on free lipase-mediated biodiesel production. J. Mol. Catal. B: Enzym. 102, 88-93.
13. Li, W., Du, W., Liu, D., Yao, Y., 2008. Study on factors influencing stability of whole cell during biodiesel production in solvent free and t-butanol system. Biochem. Eng. J. 41, 111-115.
14. Li, L., Du, W., Liu, D., Wang, L., Li, Z., 2006. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. J. Mol. Catal. B: Enzym. 43, 58-62.
15. Martín, L., González, P., Rodríguez, A., Hita, E., Jiménez, M., Esteban, L., Molina, E., Robles, A., 2012. Concentration of docosahexaenoic acid (DHA) by selective alcoholysis catalyzed by lipases. J. Am. Oil Chem. Soc. 89, 1633–1645.
16. Molina Grima, E., Ibáñez González, M.J., Giménez Giménez, A., 2013. Solvent extraction from microalgal lipids. IN: Borowitzka Michael, A., Moheimani, Navid R. (Eds.). Algae for Biofuel and Energy. Springer, Heidelberb/ New York/ London, pp 187-206.
17. Navarro López, E., Robles Medina, A., González Moreno, P.A., Jiménez Callejón, M.J., Esteban Cerdán, L., Martín Valverde, L., Castillo López, B., Molina Grima, E. 2015. Enzymatic production of biodiesel from *Nannochloropsis gaditana* lipids: influence of operational variables and polar lipid content. Bioresour. Technol. 187, 346-53.
18. Navarro López, E., Robles Medina, A., González Moreno, P.A., Esteban Cerdán, L., Martín Valverde, L., Molina Grima, E., 2016. Biodiesel production from *Nannochloropsis gaditana* lipids through transesterification catalyzed by *Rhizopus oryzae* lipase. Bioresour.Technol. 203, 236-244.
19. Nielsen, P.M., Brask, J., Fjerbaek, L., 2008. Enzymatic biodiesel production: Technical and economical considerations. Eur. J. Lipid Sci. Technol. 110, 692-700.
20. Rajam, L., Kumar, D.R.S., Sundaresan, A., Arumughan, C., 2005. A novel process for physically refining rice bran oil through simultaneous degumming and dewaxing. J. Am. Oil Chem. Soc. 82, 213–220.
21. Ramluckan, K., Moodley, K.G., Bux, F., 2014. An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. Fuel 116, 103–108.

Robles Medina, A., Gónzalez Moreno, P.A., Esteban Cerdán, L., Molina Grima, E., 2009. Biocatalysis: Towards ever greener biodiesel production. Biotechnol. Adv. 27, 398-408.

1. Royon, D., Daz, M., Ellenrieder, G., Locatelli, S., 2007. Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent. Bioresour. Technol. 98, 648-53.
2. Ryckebosch, E., Cuéllar Bermúdez, S.P., Termonte-Verhalle, R., Bruneel, C., Muylaert, K., Parra-Saldivar, R., Foubert, I. 2014a. Influence of extraction solvent system on the extractability of lipid components from the biomass of *Nannochloropsis gaditana*. J. Appl. Phycol., 26, 1501-1510.
3. Ryckebosch, E., Bruneel, C., Termonte-Verhalle, R., Muylaert, K., Foubert, I. 2014b. Influence of extraction solvent system on extractability of lipid components from different microalgae species. Algal Research, 3, 36-43.
4. San Pedro, A., González, C., Acién, G., Molina, E., 2013. Marine microalgae selection and culture condition optimization for biodiesel production. Bioresour. Technol. 134, 353-61.
5. Sangster, J., 1989. Octanol-water partition coefficients of simple organic compounds. J. Phys. Chem. Ref. Data, 18, 1111-1227.
6. Séverac, E., Olivier, G., Turon, F., Monsan, P., Alain, M., 2011. Continuous lipasecatalyzed production of esters from crude high-oleic sunflower oil. Bioresour. Technol. 102, 4954–4961.
7. Shahidi, F., Wanasundara, P.K.J.P.D., 2002. Extraction and analysis of lipids. In: Akoh, CC,Min, DB (eds) Food lipids.Marcel Dekker, New York, pp 133–168.
8. Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H., Tominaga, Y.1999. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc. 76, 789-93.
9. Stamenkovik, O.S., Veličković, A.V., Velijković, V.B., 2011. The production of biodiesel from vegetables oils by ethanolysis: Current states and perspectives. Fuel. 90, 3141-3155.
10. Tran, D.T., Chen, C.-L., Chang, J.-S., 2013. Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst. Bioresour. Technol. 135, 213-221.
11. Wang, Y., Liu, J., Gerken, H., Zhang, C., Hu, Q., Li, Y., 2014. Highly-efficient enzymatic conversion of crude algal oils into biodiesel. Bioresour. Technol. 172, 143–149.
12. Watanabe, Y., Shimada, Y., Sugihara, A., Yoshio, T., 2002. Conversion of degummed soybean oil to biodiesel fuel with immobilized *Candida antárctica* lipase. J. Mol. Catal. B Enzym. 17, 151–155.

|  |
| --- |
| **Table 1.** Extraction of SLs from wet *N. gaditana* microalgal biomass: SL yields, purities and percentages of neutral saponifiable lipids (NSLs), glycolipids (GLs) and phospholipids (PLs) of the extracted fractions. |
| Extraction system | SL yield (%) | SL purity (%) | NSL (%) | GL (%) | PL (%) |
| Homogenized biomass | - | 12± 2.4 | 29.9 ± 0.6 | 57.4 ± 1.4 | 12.7 ± 0.8 |
| Ethanol (96% v/v)-hexane | 85± 6.7 | 38.1±1.8 | 24.9 ± 0,0 | 61.8 ± 0.3 | 13.3 ± 0.3 |
| Ethanol (96% v/v)-hexane + ACa | 75± 5.7 | 95±3.2 | 51.0 ± 1.8 | 43.5 ± 0.2 | 5.5 ± 1.9 |
| Hexane:isopropanol (3:2) (v/v) | 63.4±1.4 | 32.2±1.1 | 49.7 ± 1.4 | 47.2 ± 1.8 | 3.1 ± 0.6 |
| Hexane:ethyl acetate (3:2) (v/v) | 49.7±0.3 | - | 65.7 ± 7.2 | 31.6 ± 8.7 | 2.7 ± 0.2 |
| Ethyl acetate | 44.1±1.0 | - | 64.9 ± 1.7 | 34.6 ± 1.9 | 0.5 ± 0.2 |
| Hexane  | 37.7± 3.5 | 56±2.4 | 64.6 ± 0.8 | 32.2 ± 2.6 | 3.2 ± 0.1 |
| Hexane + ACa | 34.8± 2.6 | 61±3.6 | 62.5 ± 0.5 | 34.9 ± 1.6 | 2.5 ± 0.3 |
| Hexane-ethanol:water (50% water) | 27.5± 3.4 | - | 69.1± 8.7 | 29.4± 2.5 | 1.5± 0.1 |
| Hexane-ethanol:water (5% water) | 15.8± 2.4 | 59.8±4.6 | 84.7± 8.7 | 13.2± 1.6 | 2.1± 0.2 |
| Log P values: ethanol -0.03, isopropanol 0.05, ethyl acetate 0.73, hexane 4 (Sangster, 1989). a AC: acetone crystallization treatment. |



**Figure 1.** Extraction of lipids from microalgal biomass using the ethanol (96% v/v)-hexane system.



**Figure 2**. Extraction of lipids from microalgal biomass using hexane. Extraction with the solvents ethyl acetate, hexane:isopropanol (3:2 v/v) and hexane:ethyl acetate (3:2 v/v) was also carried out using this procedure. ‘db’ signifies dry biomass.



**Figure 3.** Extraction of lipids from microalgal biomass using the hexane-ethanol:water (95:5 v/v) and hexane-ethanol:water (50:50 v/v) solvent systems.

Figure 1. Extraction of lipids from microalgal biomass using the solvent system Hexane:Ethanol:Water.

Figure 2. Extraction of lipids from microalgal biomass using the solvent system Hexane:Ethanol:Water.

|  |
| --- |
| a) |
| b) |
| **Fig. 4.** Variations in FAME conversions with reaction time in the N435 (a) and *R. oryzae* (b) catalyzed transesterification of *N. gaditana* microalgal SLs extracted using different extraction systems. Operational conditions: (a) 0.225 w/w N435/SL ratio, 10 mL of t-butanol/g SLs, 11:1 methanol/SL molar ratio, methanol added in three steps at 0, 10 and 24 h, 40ºC, 200 rpm. (b) 0.7 w/w *R. oryzae*/SL ratio, 10 mL of t-butanol/g SLs, 11:1 methanol/SL molar ratio, 72 h, 3 additions of methanol and lipase at 0, 24 and 48 h, 35ºC, 130 rpm. a AC: acetone crystallization treatment.

|  |  |
| --- | --- |
| a)  | b) |
| c)  | d) |
| e)   |
| **Fig. 5.** Reuse of the same batch of N435 lipase to catalyze three consecutive reactions (cycles) with the SL fractions extracted using: a) ethanol (96% v/v)-hexane (75.1 wt% polar lipids); b) ethanol (96% v/v)-hexane + AC (49 wt% polar lipids); c) hexane + AC (37.4% polar lipids); d) hexane (35.4% polar lipids); e) Hexane-ethanol:water (95:5%) (15.3% polar lipids). Operational conditions: see Figure 4 caption.

|  |  |
| --- | --- |
| a) |  |
| c)  | d)  |
| **Fig. 6.** Reuse of the same *R. oryzae* lipase batch to catalyze three consecutive reactions (cycles) with the SL fractions extracted using the following systems: a) ethanol (96% v/v)-hexane (75.1 wt% polar lipids); b) ethanol (96% v/v)-hexane + AC (49 wt% polar lipids); c) hexane + AC (37.4% polar lipids); d) Hexane-ethanol:water (95:5%) (15.3% polar lipids). Operational conditions: see Figure 4 caption. |

 |

 |