**Optimization of biodiesel production from wet microalgal biomass by direct transesterification using the surface response methodology**

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ABSTRACT

The production of fatty acid methyl esters (FAMEs, biodiesel) was optimized from wet *Nannochloropsis gaditana* microalgal biomass (81.8 wt% water and 28.1 wt% saponifiable lipids, SLs, from the dry biomass). FAME production was carried out by direct acid-catalyzed methylation of the SLs from the microalgal biomass and FAME extraction with hexane. A three-variable, three-level Box-Behnken design (BBD) was applied to optimize the sulphuric acid (catalyst) concentration, the methanol/SL ratio and the hexane/SL ratio. The best FAME yield was obtained with a methanol/SL ratio of 254 mL/g, a sulphuric acid concentration of 3.7 % (v/v) and a hexane/SL ratio of 107.7 mL/g at 100ºC and for 105 min. Under these conditions, 100% of the SLs were transformed into FAMEs. Finally, the FAME purity was increased from 78.7 to 86.8 wt% using an adsorption treatment with bentonite.

Keywords: Biodiesel; Wet microalga; *Nannochloropsis gaditana*; Direct transesterification; Surface response methodology; Adsorption.

1. **Introduction**

The production of biodiesel from microalgal biomass has been widely reported in the literature by extracting lipids from microalgal biomass followed by their conversion to FAME [1, 2]. However, these procedures have the inconvenience of the multiple process steps necessary to produce biodiesel, such as: cell harvest, drying, oil extraction, the transesterification reaction and purification.

The physiological properties of most microalgal species, such as their small cell size (3-20 m) and their low concentration in culture, result in energy intensive harvesting processes such as centrifugation. However, the energy demand would be dramatically reduced if microalgal cells could be aggregated spontaneously by means of flocculation [3]. The development of efficient bioflocculation methods for microalgae could significantly reduce the energy and cost requirements in the large-scale production of microalgal biomass. Several research groups have recently explored a variety of bioflocculation strategies for microalgae. Bioflocculation using bacteria, fungi, or other microalgae is promising, especially in the context of biofuel production. At this point, however, most approaches are only validated at the lab scale. Results in large-scale setups are highly variable and hard to control because a fundamental understanding of the flocculation mechanisms is lacking [4].

Drying microalgae after harvesting consumes lots of energy, which accounts for 20-30% of the total cost of biodiesel production [5].

Lipid extraction and conversion steps are important obstacles to overcome for the commercialization of microalgal biodiesel because of the high cost and energy input required. The extraction step takes more than 50% of total energy consumption [6].

For these reasons, direct or “in situ” transesterification is an efficient way to convert oil-bearing microalgal biomass to biodiesel directly, thus eliminating the extraction step which is required in the conventional method. This can be performed by contacting the wet algal biomass directly with the required alcohol and catalyst [7, 8].

The lipid composition of microalgae is qualitatively different from that of common vegetable oilseeds. Microalgal lipids can generally be classified as neutral (NLs) and polar, based on the polarity of the molecular head group. NLs include neutral saponifiable lipids (NSLs), such as acylglycerols and free fatty acids (FFAs), as well as unsaponifiable lipids, such as hydrocarbons, sterols, waxes and pigments (carotenes and chlorophylls). Polar lipids can be further sub-categorized into phospholipids (PLs) and glycolipids (GLs) (both are also saponifiable lipids as they contain fatty acids) [9, 10]. NSLs (triacylglycerols, TAGs; diacylglycerols, DAGs; monoacylglycerols, MAGs; and FFAs) from microalgae are more interesting for biodiesel production than polar lipids because the former generally have a lower degree of unsaturation than the latter, which are richer in polyunsaturated fatty acids (PUFAs). For this reason, the biodiesel (FAMEs) produced from NSLs has a greater oxidation stability [11] and cetane number; this increases as the degree of unsaturation decreases [12]. The microalgae’s fatty acid composition can also be affected by the culture conditions, such as different nutritional and environmental factors, cultivation conditions and growth phases [13].

In this paper, the methanol volume, catalyst concentration (direct transesterification variables) and hexane volume used in the later extraction were optimized using the response surface methodology (RSM), employing a three-variable, three-level Box-Behnken design (BBD). The goal was to maximize the FAME (biodiesel) yield obtained from the SLs contained in wet *Nannochloropsis gaditana* biomass, cultivated under nitrogen-starvation conditions (28.1 wt% of SLs of the dry biomass). Temperature and reaction time were optimized in a previous study [8]. Lastly, these FAMEs were purified by adsorption with bentonite.

*N. gaditana* belongs to the species *Nannochloropsis* (Eustigmatophyceae), which is currently considered a potential species for biodiesel production due to its high lipid content (although this content depends on the growing conditions). It is also characterized by a high content of FFAs (approximately 9 wt% of total lipids) [14].

1. **Materials and Methods**

*2.1. Microalgal biomass and chemicals*

The microalga used in this study was the Eustigmatophyceae *Nannochloropsis gaditana*, cultured outdoors at the pilot scale in a set of three fence-type tubular photobioreactors with an average impinging irradiance and temperature values of 2137 E m-2 s-1 and 28.6ºC, respectively. Each photobioreactor had a working volume of 340 L. These were located at the University of Almería facility, Spain (+36º 49ꞌ 43.13ꞌꞌ, -2º 24ꞌ 9.39ꞌꞌ). Firstly, it was cultivated in continuous mode with standard medium (8.0 mM nitrate) at a dilution rate of 0.21 day-1. Once steady state was reached, the whole volume of each reactor was centrifuged and the biomass was resuspended in medium lacking nitrate (nitrogen-starvation conditions). Afterwards, the reactors were operated in batch mode for 12 days [15, 16]. The wet biomass supplied had an 81.8 ± 0.01 wt% water content. The dry weight content of the wet biomass samples was determined in triplicate using the weight ratio between samples after their lyophilization and the same wet biomass samples after their centrifugation.

The chemicals used were methanol (99.9 % purity, Carlo Erba Reagents, Rodano, Italy), sulphuric acid (96 % purity), n-hexane (95 % purity), chloroform, acetone (all of analytical grade, Panreac, SA, Barcelona, Spain) and bentonite (Guinama, Alboraya, Spain). Likewise, all of the analytical reagents were of analytical grade (Panreac, SA, Barcelona, Spain).

*2.2. Direct transesterification of microalgal saponifiable lipids (SLs) and extraction of fatty acid methyl esters (FAMEs)*

The operating variables studied during the “*in situ*” transesterification were the methanol/SL ratio (expressed as mL/g SL) and the catalyst concentration (expressed as a volume percentage with respect to the methanol volume). The temperature and reaction time (100ºC and 105 min) were optimized in a previous study [8] and were kept constant in all the experiments. In order to ensure that there was no variation in the stirring power per unit volume, the volume of the methanol-catalyst mixture was maintained constant (40 mL) while the biomass amount was varied to modify the methanol/SL ratio. The operation method was as follows: between 2.1 and 12.2 g of wet biomass (81.8 wt% moisture), approximately, were added to a methanol volume, 38.1-39.6 mL, and a sulphuric acid volume, 1.9-0.4 mL (5%-1% v/v of the catalyst concentration). The reaction was carried out in a 500 mL glass reactor (Duran Schott, Mainz, Germany) heated and agitated (200 rpm) using a magnetic stirrer with a heating element (Multimix Heat D, Ovan, Spain). Each reaction was performed in duplicate. After the reaction, the reactor was cooled at room temperature and then the effect of the hexane/SL ratio on the FAME extraction was studied; the hexane volume varied between 2.9 mL and 67.4 mL, approximately, and the resultant slurry was stirred for 10 min. Subsequently, the mixture was centrifuged (Supelco 4-15 centrifuge, Sartorius, Germany) at 7000 rpm for 13 min to remove residual biomass, and then the methanol-water and hexane phases were separated in a decantation funnel. Aliquots of the hexane phase (which contained the FAMEs) were taken and analyzed by GC according to the procedure described in Section 2.3. Residual biomass was not removed before the hexane extraction step since preliminary tests showed that the FAME extraction yield decreased significantly. It is possible that FAMEs were distributed between the methanol and the solvent present within the biomass. Furthermore, this did not include a centrifugation step that would increase the biodiesel production costs at the large scale (results not shown).

Once the optimal conditions were determined, the scale of the above-mentioned process was increased by approximately 74 fold. Thus, the wet biomass paste (222 g, 81.8 wt% moisture) was mixed with 2883 mL of methanol and 106.7 mL of sulphuric acid. The resulting slurry was placed in a thermostatic stirred tank pressure reactor (Berghof, Germany) equipped with a blade impeller, with a nominal volume of 5 L, where the slurry was heated at 100 ºC for 105 min at a stirring speed of 200 rpm. The pressure reached a maximum value of 2.5 atm. Both at the large and small scale, and due to the high temperature used, we repeatedly checked that no methanol losses occurred by measuring the methanol volume in the reactors after completing the transesterification reaction. The reactor was subsequently cooled to room temperature and 1834 mL of hexane was added to the biomass slurry, which was mixed for 10 min. A previous scaling up study demonstrated that the optimal hexane/SL ratio must be increased by 50% in order to compensate the hexane losses from the extraction step. The mixture was then centrifuged (Supelco 4-15 centrifuge, Sartorius, Germany) at 7000 rpm for 13 min to remove residual biomass and the methanol-water and hexane phases were separated in a decantation funnel. The hexane extract was then evaporated under a nitrogen atmosphere (Buchi R210 rotary evaporator with a V-700 vacuum pump and a V-850 controller, Switzerland) at 45ºC to obtain the crude biodiesel.

*2.3. Determination of the biomass lipid content and the FAME yield of the direct transesterification process*

The total lipid content of dry biomass was determined using the method described by Richardson et al. [17]. A 100 mg amount of lyophilized biomass was milled with 100 mg of alumina and the mixture was extracted three times with 2 mL of chloroform-methanol 2:1 (v/v). The lipid extract and the residue were separated by centrifugation at 3500 rpm for 5 min. Then, 3 mL of HCl (0.1 mol/L) and 0.15 mg of MgCl2 were added to precipitate the proteins. After centrifugation, the chloroform lower phase was collected and evaporated until constant weight was reached.

The saponifiable lipid (SL) content of the biomass was determined in an Agilent Technologies 6890N Series Gas Chromatograph (Santa Clara, CA, USA) after subjecting a dry biomass sample to direct transesterification, following the method described by Rodríguez Ruiz et al. [18]. Nonadecanoic acid (C19:0, Sigma, USA) was used as the internal standard. The weight percentage of SLs present in the biomass was determined by the equation:

 (1)

The chromatograph was equipped with a capillary column of fused silica OmegawaxTM (0.25 mm × 30 m, 0.25 μm standard film, Supelco, Bellefonte, PA) and a flame ionization detector (FID). Nitrogen was the carrier gas at a flow rate of 58.1 mL/min and a split ratio of 1:40. The injector and detector temperatures were set at 250 and 260 ºC, respectively. The oven temperature was initially set at 150 ºC for 3 min, then programmed to increase to 240 ºC at a rate of 7.5 ºC/min and then set at 240 ºC for 12 min.

The FAME percentage obtained after the transesterification reactions (with respect to dry biomass) was determined by equation (2), taking aliquots of the hexane phase containing these FAMEs and analysing them by GC.

 (2)

The transesterification reaction yield was determined by equation (3), based on the results obtained with equations (1) and (2).

 (3)

*2.4. Determination of lipid classes by fractionation*

Lipid fractionation was performed by chromatography on single-use silica gel cartridges (Sep-Pak classic, Waters Corporation, Milford, MA) following the procedure described in Macías-Sánchez et al. [8]. Using this procedure, the total lipids extracted by the Richardson et al. [17] method (Section 2.3) were separated into three fractions of increasing polarity: neutral lipids, glycolipids and phospholipids. The neutral lipid fraction was fractionated by preparative thin-layer chromatography (TLC) to determine the percentage of neutral saponifiable lipids (acylglycerols and free fatty acids) using the procedure described by Hita et al. [19].

*2.5. Purification of FAMEs by adsorption with bentonite*

FAME samples obtained in the scaled process (crude biodiesel, Section 2.2) were purified by adsorption with bentonite. The operating variables studied were: the bentonite/crude biodiesel ratio (w/w) (ratios of 1:1, 2:1 and 4:1 were tested), the solvent/crude biodiesel ratio (5, 10 and 15 mL/g) and the temperature (30, 40 and 50 ºC). In all these experiments, a mixture containing 0.5 g of crude biodiesel, solvent and bentonite was agitated at 250 rpm in the orbital shaking air-bath (Heidolph unimax 1010-inkubator 1000, Germany) for 24 h at a determined temperature. Bentonite was then removed by centrifugation at 3900 rpm for 10 min (Centrifuge Selecta Mixtasel, Spain) and washed with the same solvent type used in the adsorption step to increase the FAME recovery yield. The FAMEs recovered in both steps were analysed separately. The purity of the bentonite-treated biodiesel was determined by equation (4):

 (4)

The amount of FAMEs present in the biodiesel treated with bentonite (the numerator) was determined by taking aliquots at each extraction step and analyzing them by GC, and the denominator was the total amount of crude biodiesel treated with bentonite, which was determined by weighing.

*2.6. Experimental design*

A three-variable, three-level Box-Behnken design (BBD) was applied to optimize the direct transesterification of microalgal SLs (from the wet microalgal biomass) and the extraction of FAMEs in order to obtain the highest FAME yield. The BBD is a second-order multivariate technique based on a three-level incomplete factorial design that is applied widely for the assessment of critical experimental conditions; that is to say, the maximum or minimum response function. The number of experiments (N) needed for the development of the Box-Behnken matrix is defined as N= 2k (k-1)+C0, where k is the factor number and C0 is the replicate number of the central point [20-25]. The BBD was specifically selected since it requires fewer runs in cases where there are three variables.

The three independent variables that were set were the catalyst concentration, the methanol/SL ratio and the hexane/SL ratio. The three levels of independent variables are presented in Table 1, which shows that the whole design consisted of 15 experimental points carried out in a random order. Three replicates at the centre of the design were used to allow us to estimate the pure error sum of squares. The response value for each trial was the duplicate average.

Based on the variance analysis, the regression coefficients of the individual linear, quadratic and interaction terms were determined. In order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimal conditions, the regression coefficients were then used to make a statistical calculation for generating 3-D surface plots from the fitted polynomial equation. The STATGRAPHICS Plus 5.1 (1994–2001, Statistical Graphics Corp.) software package was used to analyze the experimental data. *P*-values below 0.05 were considered statistically significant.

**3. Results and discussion**

*3.1. Lipid content of microalgal biomass*

Table 2 shows that the total lipid (TL) and saponifiable lipid (SL) contents of *N. gaditana* biomass were 29.4 wt% and 28.1 wt% of dry biomass, respectively; meaning that this biomass contained only 4.4% of unsaponifiable lipids, which could not be transformed into fatty acid methyl esters (FAMEs). Table 2 also shows that 76.8% of the total fatty acids were saturated and monounsaturated, whereas PUFAs made up 19.1% of the total fatty acids. The fractionation of total lipids into lipidic classes showed that 64 wt% of the SLs were neutral saponifiable lipids and the remaining 36 wt% were polar lipids. Neutral lipids are made up of SLs, such as acylglycerols and free fatty acids (FFAs), as well as unsaponifiable lipids such as hydrocarbons, sterols, waxes and pigments. Polar lipids also contain SLs such as phospholipids and glycolipids [9, 10]. This high SL percentage (95.6% of total lipids), the relatively high percentage of saturated and monounsaturated fatty acids (76.8% of total fatty acids) and also the relatively high NSL percentage (64% of SLs) contrasted with the same three contents in the microalga used by Macías-Sánchez et al. [8] (49.3% of total lipids being SLs, 42% saturated and monounsaturated fatty acids and 52.5% NSLs). These differences were because the microalga used in this work was cultured under nitrogen-starvation conditions, which allowed the percentages of SLs, saturated and monounsaturated fatty acids and NSLs to increase, in turn decreasing the content of unsaponifiable lipids (not transformable to FAMEs), PUFAs and polar lipids, respectively [15, 26]. This lipidic composition and fatty acid profile allowed a purer biodiesel to be obtained (richer in FAMEs), which is more stable in terms of oxidation and has a larger cetane number - since these latter parameters increase with the degree of fatty acids or FAME saturation [11, 12].

An analysis by TLC of the neutral saponifiable lipid fraction showed that FFAs and acylglycerols represented 13.4 and 86.6 wt%, respectively. This high FFA content (8.6 wt% of SLs and 2.4 wt% of total lipids) indicated that the transesterification reaction could not be catalyzed by an alkaline catalyst [27].

*3.2. Box-Behnken analysis*

In a previous work [8], biodiesel was also obtained by direct transesterification of wet *N. gaditana* biomass that had a lower water content (75%; because two centrifugation steps were carried out) and a lower SL content (11.1% of biomass dry weight; because the biomass was not cultured under nitrogen-starvation conditions). In that work, the influence of the catalyst (acetyl chloride) concentration, methanol/SL ratio, temperature and transesterification time were studied. A 100% FAME yield was attained using 171.1 mL methanol/g SL, 5% (v/v) acetyl chloride, with respect to the methanol, at 100 ºC and for 105 min. The FAMEs formed were extracted using 104 mL of hexane/g SLs; however, the influence of this variable was not studied. In the present study, the optimized temperature and reaction time were kept constantand we used wet *N. gaditana* biomass with a higher water content (81.8%; because only one centrifugation step was carried out) and a higher SL content (28.1%). In addition, we used sulphuric acid instead of acetyl chloride as the catalyst due to its lower price. As a result of using biomass with a higher water and SL content, we could expect a different methanol/SL ratio and catalyst concentration to be required.

A three-variable, three-level Box-Behnken design (BBD) was applied to optimize the influence of the catalyst concentration (% v/v, coded variable X1), the methanol/SL ratio (mL/g, coded variable X2) and the hexane/SL ratio (mL/g, coded variable X3) on the FAME yield. The value ranges chosen for these variables (Table 1A) were established taking into account the values obtained or used in the previous work [8]. The experimental FAME yields obtained in each of the 15 experiments carried out are shown in Table 1B. The maximum FAME yield (103.3%) under the experimental conditions was recorded with a catalyst concentration of 5% (v/v), a methanol/SL ratio of 212 mL/g and a hexane/SL ratio of 107.7 mL/g.

The estimated effects and interactions within the range of variables studied and the variance analysis of both the direct transesterification and extraction process are given in Table 3. The sign associated with each of the effects indicated a positive or negative influence of the dependent variable on the yield. A positive value for the estimated effect indicated an increase in the FAME yield if the variable increased to its high level. A negative value indicated that a better FAME yield was obtained at low variable levels. With regards to the interactions, a positive value indicated that the response would increase if both variables change to the same level, whether high or low. A negative value indicated an increase in the response if the variables changed in opposite directions (one variable increasing to a high level and the other decreasing to a low level [28]). The degree of significance for each factor is represented in Table 3 by its *P*-value - when a factor had a *P*-value smaller than 0.05, it significantly influenced the process with a confidence level of 0.95. Table 3 shows that the three factors (catalyst concentration, X1; methanol/SL ratio, X2; and hexane/SL ratio, X3) were significant on their own and an increase in the three factors increased the FAME yield in the studied levels; the hexane/SL ratio being the most influential and the methanol/SL ratio the least. Furthermore, the interactions, above all between the methanol/SL and the hexane/SL ratios, were significant and an increase of both factors to the same level increased the FAME yield. This is logical because an increase in the amount of methanol also requires an increase in the amount of hexane to maintain a high FAME extraction yield.

An empirical correlation was obtained between the FAME yield and the input variables using the experimental data (Table 1B) and the STATGRAPHICS Plus 5.1 program (1994–2001, Statistical Graphics Corp.). This correlation is given by Eq. (5):

Y= 89.23 + 14.55·X1 + 7.04·X2 + 28.35·X3 – 13.54·X12 + 9.48·X1·X2 – 7.65·X1·X3 – 45.27·X22 + 14.95·X2·X3 – 12.47·X32 (5)

The coefficients of determination (R2) and adjusted-R2 were calculated to check the adequacy and fitness of the model. The R2 gives the proportion of total variation in the response predicted by the model. A high R2 ensured a satisfactory adjustment of the quadratic model to the experimental data. The coefficient of determination value obtained (R2 = 0.9779) indicated that only 2.21% of the total variations were not explained by the model. The adjusted-R2 was used to evaluate the model’s adequacy and fitness. The adjusted-R2 value corrected the R2 value for the sample size and for the number of terms in the model. The value of the adjusted-R2 (0.9680) was also high and indicated a high correlation between the observed and the predicted values, which confirmed that the model was highly significant.

To determine the optimal variable levels for the FAME yield, the three-dimensional surface plots were constructed according to Eq. (5). Fig. 1A shows the effect of the catalyst concentration and the methanol/SL ratio at a fixed hexane/SL ratio of 67.3 mL/g. At a constant catalyst concentration, the FAME yield increased along with the methanol/SL ratio to an average value for this variable of 212 mL/g, above which the FAME yield decreased. If the methanol/SL ratio remained constant, three tendencies were observed: at a methanol/SL ratio of 62 mL/g SL, an increase in the catalyst concentration up to 3% (v/v) increased the FAME yield; this yield then decreased when the catalyst concentration reached 5% (v/v). When the methanol/SL ratio was 212 mL/g, an increase in catalyst concentration favored the FAME yield, remaining constant above 3% (v/v). Finally, at a methanol/SL ratio of 362 mL/g, the FAME yield increased throughout the catalyst concentration range, but the maximum FAME yield was not attained; the maximum yield (90% in this case) was attained using a catalyst concentration of 3% (v/v) at a methanol/SL molar ratio of 212 mL/g.

Fig. 1B shows the effect on FAME yield of the catalyst concentration and the hexane/SL ratio at a fixed methanol/SL ratio of 212 mL/g. For a constant hexane/SL ratio, three similar tendencies to that shown in Fig. 1A were observed; for a hexane/SL ratio of 26.9 mL/g, the FAME yield increased in line with the catalyst concentration. For a hexane/SL ratio of 67.3 mL/g, the FAME yield increased up to a catalyst concentration of 3% (v/v), remaining constant up to the 5% (v/v). Finally, for the maximum hexane/SL ratio used (107.7 mL/g), the FAME yield attained a maximum of 100% for a catalyst concentration of approximately 3% (v/v), slowly decreasing as this concentration increased up to 5% (v/v).

Fig. 1C shows the effect of the hexane/SL and methanol/SL ratios at a fixed catalyst concentration of 3% (v/v). For a constant methanol/SL ratio, we observed that generally an increase in the hexane/SL ratio increased the FAME yield, and a maximum 100% yield was attained for an approximate hexane/SL ratio of 107.7 mL/g and a methanol/SL ratio of 212 mL/g (see also Fig. 1B). If the hexane/SL ratio remained constant, the FAME yield increased up to an average methanol/SL ratio of 212 mL/g; then the FAME yield decreased considerably if the methanol/SL ratio increased.

The optimal conditions obtained using the response surface methodology (RSM) were as follows: catalyst concentration, 3.7% (v/v); methanol/SL ratio, 254 mL/g; and hexane/SL ratio, 107.7 mL/g. To compare the predicted result (109.4%) with the practical value, a rechecking experiment was performed using these deduced optimal conditions. The mean value of 106.3 ± 1.5% (n=2), obtained from real experiments, demonstrated the validity of the RSM model. The strong correlation between the real and the predicted results confirmed that the response model was adequate to reflect the expected optimization.

*3.3. Comparison with recent studies of direct transesterification with wet microalgal biomass*

In the direct transesterification process, the alcohol performs a vital role, acting both as a solvent, extracting the lipids and FAMEs from the biomass, and as a reactant, converting the SLs to FAMEs [29]. The volume of methanol to use depended on the water content in the reaction medium, i.e., water increased the amount of alcohol required to achieve complete FAME synthesis [30]. The higher the methanol/SL ratio, the higher the FAME yield; this is logical since the transesterification equilibrium was more displaced toward the formation of product as the methanol concentration increased. Table 4 shows this trend; although *C. gracilis* [29] and *N. salina* [6] had a similar moisture contents, the FAME yield in the latter was considerably higher when a higher methanol concentration was used.

It is well documented that in direct transesterification, the acid catalyst (microalgal lipids are characterized by a high FFA content) [31,32] also helps to break the microalgal cell walls, allowing the methanol to access the oil in the cell. This allows the synthesis and extraction of FAMEs from both nonpolar and polar lipids (mainly phospholipids present in the cell membrane) [33]. The breaking of the cell wall by sulphuric acid will depend on its characteristics. Table 4 shows the results obtained with three *Nannochloropsis* species (*N. oceanica* [34], *N. salina* [6] and *N. gaditana* [8, this study]) observing different catalyst concentrations, as well as different temperature and reaction time conditions to obtain high FAME yields. Beachman et al. [35] carried out a study on the cell wall ultrastructure of the mentioned species concluding that there were differences in thickness. The observed differences were not phenotypic fluctuations due to some transient environmental condition but instead were a distinct genetic trait of each species. *N. oceanic* was the species with the greatest cell wall thickness (111.3± 3.9 nm). *N. gaditana* had a thickness of 86.5 ± 3.2 nm. In the case of *N. salina*, the study was carried out with two strains that showed different thicknesses; CCAP 849/3 with 65.5 ± 2.8 nm and CCAP 849/6 with 108.1 ± 4.7 nm. These differences could explain the high catalyst concentration used in the direct transesterification of SLs contained in *N. oceanica* [34]. In the case of *N. salina* [6], it could be that its thickness was less than *N. gaditana* [this study] species since the reaction time was one hour (with a similar catalyst concentration and the same reaction temperature).

If the results obtained are compared with *N. gaditana* [8] (Table 4), in this study the catalyst concentration could be reduced to 40%, but methanol/SL ratio had to increase to 49%, possibly because the wet biomass used in this work had a higher water content (81.8% versus 75%). The optimum hexane/SL ratio was similar to that used in the previous work [8]. A decrease in this ratio appreciably reduced the FAME yield; as an example of this, the yield decreased to 89% when the hexane/SL ratio decreased to 67.3 mL/g, almost maintaining optimal values for the catalyst concentration and the methanol/SL ratio (3% and 212 mL/g, respectively).

*3.4. Purification of biodiesel by adsorption with bentonite*

The microalgal biomass contained 29.4 wt% of TLs and 28.1 wt% of SLs (Table 2); i.e.~~,~~ the microalga contains 4.4 wt% of non-saponifiable lipids (such as sterols, tocopherols, carotenoids, etc.) and possibly other non-lipidic compounds. These and other compounds were also extracted by hexane from the microalgal biomass and subsequently contaminated the FAMEs [36] - given that the FAME purity in the biodiesel obtained after direct transesterification and extraction with hexane was 78.7 wt%. Bentonite is an adsorbent commonly used in the decolouration of oils and, for this reason, was used to clarify the biodiesel and increase its purity. This adsorption treatment was carried out with bentonite using hexane as the solvent [36, 37]. Table 5 shows the FAME yields and purities obtained at different bentonite/biodiesel ratios. The bentonite/biodiesel ratios of 1:1 and 2:1 (w/w) were tested and the best results regarding both purity and FAME recovery were obtained with a bentonite/biodiesel ratio of 2:1. This treatment increased the biodiesel purity from 78.7 wt% up to a maximum value of 86.8 wt%. The FAME recovery yield increased to 90.5 wt% when a washing step was carried out to recover some of the FAMEs adsorbed on the bentonite. This washing step appeared to be necessary since the FAME recovery yield increased from 78.9 wt% to 90.5 wt% while the purity remained constant. An increase in the bentonite/biodiesel ratio up to 4:1 did not improve the purity, and three washing steps were necessary to recover 91.5% of the initial FAMEs (Table 5).

Once the bentonite/biodiesel ratio was chosen, the solvent/crude biodiesel ratio (v/w), temperature and time were studied to try and increase the biodiesel purity (Tables 6-7). Table 6 shows that the biodiesel recovery yield in the liquid phase was higher at the highest hexane/biodiesel ratios, at least in the first adsorption step. Moreover, this table shows that the biodiesel purity was lower at the highest hexane/biodiesel ratio (15 mL/g),while it was similar at the two lowest hexane/biodiesel ratios (5 and 10 mL/g); this is logical given that impurities are adsorbed more by the bentonite when less solvent is used. For this reason, hexane/biodiesel ratios of around 10 mL/g seem optimal in achieving a compromise between yield and purity. At this ratio, experiments were also carried out at 30, 40 and 50 ºC - it was observed that at 30ºC, both yield and purity were lower (88.7% and 81%, respectively) than at 40ºC (Table 6); while at 50ºC, both yield and purity were similar (91.5% and 85.7%, respectively). Therefore, 40ºC is a suitable temperature to carry out the bentonite adsorption process.

Finally, experiments were carried out to try and decrease the adsorption time. Table 7 shows that no differences in the biodiesel recovery yield were observed between 1 and 24 h. Purities were likewise similar between 1 and 14 h, and only a slight increase was observed when the adsorption time was 24 h. We also observed that, while the purity did not change when washing the adsorbent, the biodiesel recovery did increase, and therefore at least one washing step is required.

The best results were thus obtained using a 2:1 (w:w) bentonite/biodiesel ratio, 10 mL of hexane/g crude biodiesel, at 40 ºC for 24 h. Under these conditions, over 90% of the FAMEs were recovered with an 86.8% purity. The amount of bentonite used in each experiment was not reused since it was not possible to recover all the retained FAMEs after the washing steps.

As is mentioned in Section 1, it is interesting that the lipid composition of the microalgae had a low content of PUFAs and a high content of NSLs since the biodiesel (FAMEs) produced from NSLs had greater oxidation stability [11] and cetane number; this increased as the degree of unsaturation decreased [12].

If the biodiesel purity obtained in this study (86.8% FAME, biomass with 19% PUFAs of the total fatty acids) is compared with that obtained by Macías-Sánchez et al. [8] (*N. gaditana*; 82.7% FAME, biomass with 44.8% PUFAs of the total fatty acids) and Cerón-García et al. [38] (*C. protothecoides*; 97% FAME, biomass with 12.9% PUFAs of the total fatty acids), one can observe that the lower the PUFAs content in the biomass, the higher the purity of the biodiesel obtained by direct transesterification of wet biomass and the subsequent purification stage. In the case of *C. protothecoides*, a purification step was not necessary since it complied with the EN 14214 standard- biodiesel should have a 96.5% FAME [39].

**4.** **Conclusions**

The direct transesterification of saponifiable lipids (SLs) from wet microalgal biomass for the production of microalgal biodiesel is an alternative of considerable interest since the removal of the drying and lipid extraction steps reduces the cost of biodiesel production [40]. A three-variable, three-level Box-Behnken design (BBD) was applied to optimize the direct transesterification of microalgal SLs and the extraction of FAMEs from wet *N. gaditana* microalga biomass (cultured under nitrogen-starvation conditions) with 28.1 wt% SLs and a moisture content of 81.8 wt% - this was done in order to obtain a high FAME yield. A high methanol/SL ratio (254 mL/g) was necessary to achieve 100% FAME yields given the high water content of the biomass. This yield was attained with a catalyst (sulphuric acid) concentration of 3.7% v/v (with respect to methanol), at 100ºC and for a reaction time of 105 min (both parameters were optimized in a previous study [3]). The FAMEs formed in the transesterification reaction were extracted by carrying out a single extraction with 107.7 mL hexane/g SLs. Using this process, biodiesel with 78.7 wt% of FAMEs was obtained. This FAME purity was increased up to 86.8% wt% using an adsorption treatment with bentonite.

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**Figure captions**

**Figure 1**: Response surface plots of the FAME yield affected by the catalyst concentration, methanol/SL ratio and hexane/SL ratio.

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| **Table 1. Experimental domain (A) and Box-Behnken design: natural and coded variables for each experiment and the experimentally-observed responses for the FAME yield (B).** |
| (A) |
| Independent variables (unit) | Coded symbols | Levels |
| X | -1 | 0 | 1 |
| Catalyst concentration (% v/v) | X1 | 1 | 3 | 5 |
| Methanol/SL ratio (mL/g) | X2 | 62 | 212 | 362 |
| Hexane/SL ratio (mL/g) | X3 | 26.9 | 67.3 | 107.7 |
| (B) |
| Test | X1 (catalyst concentration) | X2 (methanol/SL ratio) | X3 (hexane/SL ratio) | Extraction yield, Yexp (% ± SD) |
| 1 | 0 (3)  | 1 (362) |  1 (107.7) | 78.0±8.0 |
| 2 | -1 (1) | 0 (212) | -1 (26.9) | 7.9±6.4 |
| 3 | 1 (5) | 0 (212) | -1 (26.9) | 51.5±1.1 |
| 4 | -1 (1) | 0 (212) | 1 (107.7) | 90.3±7.8 |
| 5 | 0 (3) | -1 (62) | 1 (107.7) | 31.3±4.9 |
| 6 | 0 (3) | 0 (212) | 0 (67.3) | 84.5±6.4 |
| 7 | 0 (3) | -1 (62) | -1 (26.9) | 15.0±4.2 |
| 8 | 1 (5) | -1 (62) | 0 (67.3) | 30.2±3.7 |
| 9 | 0 (3) | 1 (362) | -1 (26.9) | 1.8±0.5 |
| 10 | 1 (5) | 0 (212) | 1 (107.7) | 103.3±4.7 |
| 11 | -1 (1) | 1 (362) | 0 (67.3) | 11.8±4.0 |
| 12 | 1 (5) | 1 (362) | 0 (67.3) | 60.6±2.8 |
| 13 | 0 (3) | 0 (212) | 0 (67.3) | 91.0±4.4 |
| 14 | 0 (3) | 0 (212) | 0 (67.3) | 92.3±1.2 |
| 15 | -1 (1) | -1 (62) | 0 (67.3) | 19.3±0.9 |

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| **Table 2. Fatty-acid composition (wt% of total fatty acids in the biomass) of wet biomass from the microalga *N.* *gaditana* along with the total lipid (TL), saponifiable lipid (SL) (wt% of biomass dry weight) and NSL contents (wt% on total SLs).** |
| Fatty acids | Composition (wt%) |
| 14:0 | 7.7 ± 0.0 |
| 16:0 | 30.7 ± 0.0 |
| 16:1n7 | 24.6 ± 0.0 |
| 18:0 | 1.4 ± 0.0 |
| 18:1n9 | 11.8 ± 0.1 |
| 18:1n7 | 0.6 ± 0.0 |
| 18:2n6 | 6.2 ± 0.0 |
| 18:3n3 | 1.0 ± 0.0 |
| 20:4n6 | 3.6 ± 0.0 |
| 20:5n3 | 8.3 ± 0.1 |
| Others | 4.1 ± 0.1 |
| ∑ Saturated + monounsaturated | 76.8 ± 0.0 |
| ∑ PUFAs | 19.1 ± 0.0 |
| TLs | 29.4 ± 0.5 |
| SLs | 28.1 ± 0.2 |
| NSLs | 64.0 ± 0.2 |

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| **Table 3. Estimated effects and the analysis of variance of the process studied** |
| **Variable** | **Effects** | ***P*-value** |
| X1 | 29.10 | 0.0000 |
| X2 | 14.08 | 0.0004 |
| X3 | 56.70 | 0.0000 |
| X12 | -27.08 | 0.0000 |
| X1·X2 | 18.95 | 0.0007 |
| X1·X3 | -15.30 | 0.0039 |
| X22 | -90.53 | 0.0000 |
| X2·X3 | 29.90 | 0.0000 |
| X32 | -24.93 | 0.0001 |

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| **Table 4. Conditions and results obtained in recent studies regarding the production of FAMEs by direct transesterification of SLs contained in wet microalgal biomass.**  |
| Microalga[Reference] | Moisture content(wt%) | SL content(wt% of dry biomass) | Methanol/SL ratio (mL/g) | H2SO4 concentration(% v/v) | Temperature(ºC) | Reaction time(min) | FAME yield (%) |
| *C. gracilis* [29] | 75 | 36 | 111 | 1.8 | 80 | 20 | 84 |
|  |  |  |  |  |  |  |
| *N. oceánica* [34] | 65 | 19.2 | 40 | 10 | 95 | 120 | 82.8 |
|  | 65 | 19.2 | 40 | 30 | 95 | 60 | 84.7 |
| *N. salina* [6] | 76.5 | 18.6 | 228.9 | 1.8 | 90 | 60 | 94.2 |
|  | 76.5 | 18.6 | 171,7 | 3.3 | 100 | 60 | 99.8 |
| *N. gaditana* [8] | 75 | 11.1 | 171.1 | 5 (acetyl chloride) | 100 | 105 | 100 |
|  |  |  |  |  | 90 | 180 |  |
|  |  |  |  |  | 80 | 300 |  |
| *N. gaditana* [this study] | 81.8 | 28.1 | 254 | 3.7 | 100 | 105 | 100 |
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| **Table 5. Purification of crude biodiesel (initial purity 78.7 wt%) by adsorption with bentonite: influence of the bentonite/biodiesel ratio.** |
| **Bentonite/biodiesel ratio (w/w)** | **FAME yielda****and purityb** | **Adsorption step** | **1st washing**  | **2nd washing**  | **3rd washing** | **Purification + washing** |
| 1:1 | Yield | 88.9±2.5 | 3.6±0.6 | ― | ― | 92.5±1.9 |
| Purity | 82.1±4.2 | 92.3±10.9 | ― | ― | 82.4±4.3 |
| 2:1 | Yield | 78.9±0.4 | 11.6±0.2 | ― | ― | 90.5±0.2 |
| Purity | 86.0±3.2 | 92.9±1.4 | ― | ― | 86.8±3.0 |
| 4:1 | Yield | 85.7±1.686.8±0.6 | 5.8±0.3 | 91.5±1.2 |
| Purity | 85.1±0.9 | 86.7±0.6 |
| a FAMEs recovered as purified FAMEs (wt%) with respect to the initial FAMEs submitted to this adsorption treatment. b FAME purity (wt%) determined by eq. (5). Operational conditions: 40ºC, 24 h, 250 rpm, 10 mL hexane/g crude biodiesel in the adsorption step and 5 mL solvent/g crude biodiesel in each washing step. |

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| **Table 6. Purification of crude biodiesel (initial purity 78.7 wt%) by adsorption with bentonite: influence of the solvent (hexane)/biodiesel ratio.** |
|  | **Solvent (hexane)/biodiesel ratio (v/w)** |
| **FAME yieldsa and puritiesb** | **5** | **10** | **15** |
| Adsorption stepc,  Yield Purity | 64.3±1.084.4±0.4 | 78.9±0.486.0±3.2 | 81.0±0.577.8±3.0 |
| Washingc,  Yield Purity | 15.6±1.790.7±0.6 | 11.6±0.292.9±1.4 | 9.0±0.277.8±0.4 |
| Purification + washing, Yield Purity | 79.9±1.284.9±2.5 | 90.5±0.286.8±3.0 | 90.0±0.377.8±2.8 |
| a FAMEs recovered as purified FAMEs (wt%) with respect to the initial FAMEs submitted to this adsorption treatment. b FAME purity (wt%) determined by eq. (5). Operational conditions: 2:1 bentonite/biodiesel (w/w) ratio, hexane as solvent, 40ºC, 24 h, 250 rpm. c In the adsorption and washing step, 7.5 and 5 mL hexane/g crude biodiesel were used, respectively.  |

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| **Table 7. Purification of crude biodiesel (initial purity 78.7 wt%) by adsorption with bentonite: influence of time**. |
| Time (h) | FAME yielda and purityb | Adsorption step | 1st washing  | 2nd washing  | Purification + washing |
| 1 | Yield | 77.1±1.6 | 10.7±0.1 | 3.3±0.3 | 91.1±2.0 |
| Purity | 81.3±0.2 | 87.5±1.1 | 68.0±11.7 | 81.3±0.8 |
| 2 | Yield | 77.6±0.9 | 11.0±0.1 | 3.7±0.0 | 92.3±1.0 |
| Purity | 82.3±0.8 | 89.6±3.3 | 93.1±8.2 | 83.5±1.3 |
| 4 | Yield | 77.6 | 9.1 | 3.3 | 90.0 |
| Purity | 81.2 | 79.9 | 82.3 | 81.1 |
| 8 | Yield | 77.3±1.9 | 11.6±0.3 | 3.7±0.2 | 92.6±1.4 |
| Purity | 81.7±1.8 | 78.3±2.1 | 100.0±0.1 | 81.8±1.3 |
| 14 | Yield | 76.4±0.8 | 11.4±0.5 | 3.8±0.0 | 91.6±1.4 |
| Purity | 80.7±1.0 | 84.1±3.1 | 90.4±5.6 | 81.5±1.4 |
| 24 | Yield | 78.9±0.4 | 11.6±0.2 | ― | 90.5±0.2 |
| Purity | 86.0±3.2 | 92.9±1.4 | ― | 86.8±3.0 |
| a FAMEs recovered as purified FAMEs (wt%) with respect to the initial FAMEs submitted to this adsorption treatment.b FAME purity (wt%) determined by eq. (5).Operational conditions: 2:1 bentonite/biodiesel (w/w), pure hexane, 40ºC, 250 rpm, 10 mL solvent/g crude biodiesel in the adsorption step and 5 mL solvent/g crude biodiesel in each washing step. |

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**Figure 1**