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Production of biodiesel from vegetable oil and microalgae by fatty acid extraction and enzymatic esterification

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The aim of this work was to obtain biodiesel (methyl esters) from the saponifiable lipids (SLs) fraction of the microalga *Nannochloropsis gaditana*, whose biomass dry weight contains 12.1 wt% of these lipids. SLs were extracted from the microalga as free fatty acids (FFAs) for subsequent transformation to methyl esters (biodiesel) by enzymatic esterification. Extraction as FFAs rather than as SLs allows them to be obtained with higher purity. Microalgal FFAs were obtained by direct saponification of lipids in the biomass and subsequent extraction-purification with hexane. Esterification of FFAs with methanol was catalysed by lipase Novozym 435 from *Candida antarctica*. Stability studies of this lipase in the operational conditions showed that the esterification degree (ED) attained with the same batch of lipase remained constant over six reaction cycles (36 h total reaction time). The optimal conditions attained for 4 g of FFAs were 25°C, 200 rpm, methanol/FFA molar ratio of 1.5:1, Novozym 435/FFA ratio of 0.025:1 w/w and 4 h reaction time. In these conditions the ED attained was 92.6%, producing a biodiesel with 83 wt% purity from microalgal FFAs. Several experimental scales were tested (from 4 to 40 g FFAs), and in all cases similar EDs were obtained.

[**Key words:** Biodiesel; Microalga; *Nannochloropsis gaditana*; Lipase; Novozym 435; Free fatty acid; Used vegetable oil]

Biodiesel is defined as the monoalkyl esters of long chain fatty acids from vegetable oils and animal fats, for use in ignition-compression engines (diesel). It is a renewable, biodegradable and non-toxic biofuel. The depletion of fossil fuel reserves, together with heightened awareness of climate change and contaminant emissions, have led to increased interest in biodiesel as a potential alternative to fossil fuels (1,2).

Compared to the two previous generations of biodiesel feedstocks (i.e., food and non-food crops), the third generation, microalgae, has appeared to be a suitable energy source for biodiesel production. Algae use sunlight to produce oils, but they do so more efficiently than crop plants due to their higher productivity (3). Moreover, some species can accumulate large amounts of lipids and they do not need high quality agricultural land or even high quality water to grow biomass. However, the production cost of high grade algae oils constitutes an obstacle in the short term. The main reason is that the operational conditions leading to high grade oil in microalgae are usually those providing low growth rates (low temperature, low light intensity and nitrogen deficiency) (4). Use of the biorefinery concept will help to lower the cost of production. Like a petroleum refinery, a biorefinery uses every component of the biomass raw material to produce useable products (3).

Now biodiesel is commonly produced by using basic catalysts, such as sodium or potassium hydroxides, since these catalysts give high yield in short reaction times. However, in the case of oils with high contents of free fatty acids (FFAs), such as microalgal oils, alkaline catalysis presents some problems due to the formation of soaps, which decreases the biodiesel yield and increases the purification costs. Acid catalysts transform both FFAs and acylglycerols into methyl esters (5). However, the reaction velocity of transesterification with an acid-catalyst is much slower than with an alkaline one and more methanol is required (6). In addition, acid catalysts are highly corrosive and so are seldom used on an industrial scale.

Lipases also catalyse the transformation of both FFAs and acylglycerols to methyl esters, but at lower temperatures than in alkaline and acid catalysis (30–40°C). Moreover, the purification of biodiesel is easier, cheaper and entails a lower consumption of water, because neither alkalis nor acids need to be separated, only the excess of alcohol and the glycerol. This glycerol is also easily recuperated at a high degree of purity (without mixing with alkalis or acids), and it can be used in nobler applications. On the other hand, if the lipase is immobilized, the separation of the reaction mixture and the catalyst is straightforward. Many works on the enzymatic production of biodiesel have studied the effect of methanol on the stability of the lipase, because when methanol exceeds the solubility limit in the reaction mixture the lipase is irreversibly deactivated. To address this problem several alternatives have been put forward, such as the addition of methanol by steps (7), the immobilization of lipase (8) and the utilization of solvents, such as tert-butanol (9).

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Biodiesel production from algae can be carried out by different methods. The most common one is a two-step method in which algae oil is extracted with organic solvent and then converted to biodiesel. Also, some authors use the direct extraction-transesterification of lipids in algae, catalysed by an acid or an enzyme (10). Tran et al. (10) compared the transesterification of the extracted microalgal oil and the direct enzyme transesterification of oil in previously disrupted biomass. Their results show that the latter procedure achieved higher biodiesel conversion (72.1% and 97.3% wt oil, respectively).

The goal of this work was to produce biodiesel from microalgae by an alternative two-step enzymatic procedure: (i) extraction of purified saponifiable lipids (SLs) as FFAs by direct saponification in the microalgal biomass, and (ii) enzymatic esterification of the extracted FFAs with methanol. Due to the difficulty of producing sufficient amounts of microalgal FFAs, the optimization experiments were carried out with FFAs from used vegetable oil and the optimal conditions were then applied to FFAs from the microalga *Nannochloropsis gaditana*. The findings show that similar results were attained with both FFAs.

MATERIALS AND METHODS

Microalga, oil, lipases and chemicals Wet paste biomass of the marine microalga *N. gaditana* was used as an oil-rich substrate. This biomass was facilitated by the Estación experimental de Las Palmerillas (Cajamar, El Ejido, Spain). Cells were grown in an outdoor tubular photobioreactor, centrifuged at 7000 rpm for 10 min, and then stored at −20°C until use. This wet biomass contained 24.2% ± 0.7% w/w of dry biomass and 29.1 ± 0.0 wt% of total lipids on dry biomass. The total fatty acid content (or saponifiable lipids as equivalent fatty acids) in the biomass was 12.1 ± 0.2 wt% on dry biomass. Free fatty acids (FFAs) from used vegetable oil (UVO) were also used. This oil was provides by the biodiesel manufacturer company Albabío (Nijar, Spain). Table 1 shows the fatty acid composition of both oils. The esterification reaction was catalysed by the lipase Novozym 435 from *Candida antarctica* (Novozymes A/S, Bagsvaerd, Denmark). This lipase is immobilized on a macroporous acrylic resin and it is positionally non-specific.

Ethanol (96%, analysis quality) and hexane (95% purity, synthesis quality), both of Panreac (Barcelona, Spain), were used to extract FFAs from the microalga and to saponify the UVO. HCl (37%, Panreac S.A., Barcelona, Spain), methanol (99.9% purity, Carlo Erba Reagents, Rodano, Italy), NaOH and KOH (85% purity, J.T. Baker, Deventer, Holland), all of analytical quality, were also used. All reagents used in the analytical determinations were also of analytical grade.

Obtaining of FFAs from UVO and microalgal biomass FFAs from UVO were obtained by saponification of the oil. The reaction mixture contained 350 mL of oil and 700 mL of a hydroalcoholic solution of NaOH, which was prepared dissolving 120 g of NaOH in 400 mL of water and 400 mL of ethanol (96% v/v). The saponification was carried out shaking this mixture at 200 rpm, at 60°C for 30 min. The mixture was then cooled at room temperature, 140 mL of water were added and the pH was adjusted to values between 1 and 2 using 37% HCl. FFAs were extracted with 300 mL of hexane, shaking at 200 rpm for 30 min. Finally the hydroalcoholic and hexane phases were separated by decantation and the hexanic phase was washed twice with water.

FFAs from the microalga *N. gaditana* were obtained following a modification of the three-step procedure developed by Ibáñez González et al. (11) (Fig. 1). 0.50 kg of wet biomass was treated with 3.63 L of ethanol (96% v/v) (30 mL ethanol (96%)/g dry biomass), containing 24.2 g of KOH (85% purity) in a 10 L reactor, which was jacketed for temperature control. Direct saponification was carried out at 60°C for 1 h with constant stirring at 200 rpm with a propeller stirrer (Eurostar digital, IKA Staufen, Germany), in argon atmosphere and covered with aluminium foil to protect from light. The biomass residue was then separated from the hydroalcoholic solution by filtration using a porous glass plate (pore diameter 100 μm, Pobel, Madrid, Spain). This biomass residue was washed with 1.4 L of ethanol (96% v/v) (14 mL ethanol (96%)/g dry biomass), to increase the fatty acid recovery yield and this washing solution was added to the hydroalcoholic fatty acid solution from the main extraction. In the following step, water (674 mL) was added to the hydroalcoholic phase to reach 30% wt in water, to increase the immiscibility of the hydroalcoholic solution and hexane, and the unsaponifiable contents were extracted with 4.30 L hexane (hexane/hydroalcoholic solution ratio 1:1 v/v). This extraction was carried out stirring at 200 rpm for 10 min. Subsequently, the solution was decanted for 30 min. The unsaponifiable hexanic solution was removed, leaving the lower hydroalcoholic phase in the extractor. Next, concentrated HCl was added to the hydroalcoholic phase to pH 5, to form the FFAs. These FFAs were extracted with hexane (4.3 L), and the mixture was stirred at 200 rpm for 10 min. It was then allowed to decant for 30 min and two phases were obtained, a bottom one that was discarded and the upper hexanic phase containing the FFAs. This FFA solution was evaporated in a rotary evaporator (Buchi, R210, with vacuum pumpV-700 and controller V-850, Switzerland) to remove and recover the solvent and the FFAs.

TABLE 1. Fatty acids composition (percentage with respect to the total fatty acids of biomass) of the wet paste biomass from *Nannochloropsis gaditana* and from the used vegetable oil (UVO).

Fatty acid	Microalgal oil (wt %)	UVO (wt %)
14:0	6.0 ± 0.1	–
16:0	23.8 ± 0.0	9.1 ± 0.1
16:1n7	22.2 ± 0.2	0.3 ± 0.0
18:0	0.5 ± 0.1	4.1 ± 0.0
18:1n9	5.9 ± 0.1	49.5 ± 0.3
18:1n7	–	0.9 ± 0.0
18:2n6	4.8 ± 0.0	34.6 ± 0.4
20:4n6	6.9 ± 0.2	–
20:5n3	27.5 ± 0.5	0.6 ± 0.0
Others	2.5 ± 0.4	0.9 ± 0.0

Esterification of FFAs catalysed by lipase Novozym 435 In this reaction FFAs react with methanol to produce methyl esters (biodiesel) and water in the presence of the lipase Novozym 435 which acts as catalyst. Experiments were carried out with 4 g of FFAs, 0.86 mL of methanol (methanol/FFA molar ratio 1.5:1) and different Novozym 435/FFA ratios. The esterification reaction was carried out in 50 mL Erlenmeyer flasks with silicone-capped stoppers. In a typical experiment the mixture was incubated at 40°C and stirred in an orbital shaking air-bath (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany) at 200 rpm for 24 h. The reactions were stopped by separation of Novozym 435 by filtration. The final reaction mixture was conserved at 4°C until analysis. Experiments were carried out modifying the following variables: reaction time, temperature, stirring velocity, methanol/FFA molar ratio and Novozym 435 amount. All reactions and their corresponding analyses were carried out in duplicate, and each value recorded is therefore the arithmetic mean of four experimental data (data shown as mean value ± standard deviation).

Analysis of reaction products The identification of the reaction products (FFAs, methyl esters and to lesser extent acylglycerols) was carried out by thin layer chromatography (TLC), followed by gas chromatography (GC), to determine quantitatively the species present. The fatty acid profile of the oils was also determined by GC. TLC was carried out on plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey–Nagel, Sigma–Aldrich), activated by heating at 105°C for 30 min. The samples were spotted directly on the plate by adding 0.2 mL of reaction product mixture. The mobile phase used was chloroform/acetone/methanol (95:4.5:0.5 v/v/v). Spots of each lipid were visualized by spraying the plate with iodine vapour in a nitrogen steam. The position of the spot corresponding to each lipidic species is characterized by the response factor. Each lipid type was scraped from the plates and methylated according to the method of Rodriguez-Ruiz et al. (12). Fatty acid methyl esters were analysed by GC with an Agilent Technology 6890 gas chromatograph (Avondale, PA, USA) using a capillary column of fused silica OmegawaxTM (0.25 mm × 30 mm, 0.25 μm standard film, Supelco, Bellefonte, PA, USA), and a FID (flame-ionization detector). Nitrogen was the carrier gas. Nonadecanoic acid (19:0) (Sigma–Aldrich) was used as internal standard for quantitative determination of fatty acids. Matreya (Pleasant Gap, PA, USA) n-3 PUFAs (polyunsaturated fatty acids) standard (catalogue number 1177) was used for the quantitative determination of fatty acids.

The esterification degree (ED) represents the percentage of initial FFAs consumed by methylation. ED was determined by acid-base titration after checking that the results were similar to those obtained by TLC and GC, which take longer. The samples were diluted with acetone (ratio 1:1 v/v) and phenolphthalein was used as indicator. The samples from esterification of microalgal FFAs were washed with alumina, diluted with acetone (fatty acid/acetone ratio 1:20 v/v) and titrated, using thymol blue as indicator. Previously the acidity of the initial reaction mixture had been determined under the same conditions in which the acidity was measured, once the reaction had finished. ED was calculated by the equation:

$$ED\ (\%) = \frac{V_0 - V}{V_0} 100 \qquad (1)$$

where V_0 and V are the volumes of 1 or 0.1 N NaOH solution used in the titration of the initial mixture and esterification product, respectively. To determine the purity of the FFAs and methyl ester from the FFA microalgal extract and esterification reaction products, respectively, a sample of known weight was analysed by GC. Purity (wt%) was determined as the ratio between the methyl ester weight obtained by GC and the sample weight.

RESULTS AND DISCUSSION

Extraction of FFAs from the microalga *N. gaditana* FFAs from the microalga *N. gaditana* were extracted by the procedure