



Article

Biodiesel Production (FAEEs) by Heterogeneous Combi-Lipase Biocatalysts Using Wet Extracted Lipids from Microalgae

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Abstract: The production of fatty acids ethyl esters (FAEEs) to be used as biodiesel from oleaginous microalgae shows great opportunities as an attractive source for the production of renewable fuels without competing with human food. To ensure the economic viability and environmental sustainability of the microbial biomass as a raw material, the integration of its production and transformation into the biorefinery concept is required. In the present work, lipids from wet *Isochrysis galbana* microalga were extracted with ethyl acetate with and without drying the microalgal biomass (dry and wet extraction method, respectively). Then, FAEEs were produced by lipase-catalyzed transesterification and esterification of the extracted lipids with ethanol using lipase B from *Candida antarctica* (CALB) and *Pseudomonas cepacia* (PC) lipase supported on SBA-15 mesoporous silica functionalized with amino groups. The conversion to FAEEs with CALB (97 and 85.5 mol% for dry and wet extraction, respectively) and PC (91 and 87 mol%) biocatalysts reached higher values than those obtained with commercial Novozym 435 (75 and 69.5 mol%). Due to the heterogeneous nature of the composition of microalgae lipids, mixtures with different CALB:PC biocatalyst ratio were used to improve conversion of wet-extracted lipids. The results showed that a 25:75 combi-lipase produced a significantly higher conversion to FAEEs (97.2 mol%) than those produced by each biocatalyst independently from wet-extracted lipids and similar ones than those obtained by each lipase from the dry extraction method. Therefore, that optimized combi-lipase biocatalyst, along with achieving the highest conversion to FAEEs, would allow improving viability of a biorefinery since biodiesel production could be performed without the energy-intensive step of biomass drying.

Keywords: FAEEs; biodiesel; mixed biocatalysts; lipases; microalgae

1. Introduction

Nowadays, we are facing a major energy crisis not only caused by the decline of fossil reserves but also by the problems caused by their use. Therefore, there is a need to look for new sources of cleaner, safer and renewable energy. Currently, the research is focuses in obtaining advanced biofuels to ensure the economic viability and environmental sustainability of biomass as a raw material [1]. Oleaginous microalga are promising species that can accumulate large amount of lipids (>20 wt% of their biomass). They constitute an attractive source for the production of biofuels such as biodiesel without competing with human food [2].

Biodiesel can be generated from the lipids in microalgae by the esterification of their free fatty acids (FFA) and the transesterification of their saponifiable lipids with methanol or ethanol into their corresponding methyl or ethyl esters (FAMES or FAEEs, respectively) in the presence of a catalyst. Due to its high content of free fatty acids in the lipids extracted from microalgae, the most common way to carry out the biodiesel process is by using acid catalysts. This generates certain disadvantages not only in the process, such as high energy consumption, corrosion of materials and the difficulty of transesterifying triglycerides, but also in the post-reaction treatments, such as the recovery of the catalyst [3]. In recent years, new catalysts based on enzymes (biocatalysts) are being developed to avoid these problems [4], achieving better selectivity and specificity. Immobilized lipases can be an alternative because they are capable of carrying out the transesterification and esterification reactions at lower temperatures energy cost, facilitating the recovery of the catalyst and the purification of glycerol [5]. However, the main problem of biocatalysts is the presence of water in the reaction medium that can cause the hydrolysis of esters. In addition, the use of certain solvents such as methanol can lead to inactivation of the catalyst [6,7].

Due to the high cost of enzymes, the use of heterogeneous biocatalysts is required for the economic viability of the whole process since they can be reused. In addition, the immobilization of enzymes in solid carriers can increase thermal and chemical stability and protects enzyme molecules from denaturation. There are different techniques of enzymatic immobilization: binding to the support, confinement or encapsulation and cross-linking [8]. The most common technique is to bind the enzyme onto the support by covalent or ionic attachment or by physical adsorption [9]. To carry out this immobilization, materials such as mesoporous silicas (SBA-15, SBA-16, MCM-41, FDU-12) [4,10–13] or carbon nanotubes [14,15] have been used. The above materials have large pores or cavities where the enzymes can be housed. However, it is usually necessary to modify the surface of support to achieve a better anchorage by functionalization with amine, chloride, sulfur or phenol groups [10,13,16–19].

Because of the high specificity of lipases for different substrates, catalytic activity of lipases depends on the source organism producing the enzyme. Most of biodiesel production from microalgal oil has been done with lipases from *Pseudomonas cepacia* [15,20], *P. fluorescens* [15,21], *Thermomyces lanuginosus* [15,22], *Candida rugosa* [15,23] and *C. antarctica* [4,15]. The last one is frequently used in the form of the commercial catalyst Novozym 435 [6,15,24,25]. Although most of studies reported the use of single lipases, some authors showed that the yield of FAEE could increase when some lipases are used in combination [5,20]. Therefore, the concept of combi-lipase biocatalysts have been recently developed to improve the biodiesel production processes [26–28]. However, these studies reported the lipase-catalyzed synthesis of biodiesel using vegetable oils such as palm, soybean or spent coffee grounds, but they did not use the more complex microalgal oil as raw material for biodiesel production.

In this work, biodiesel (FAEEs) were produced using enzyme catalysis from lipids extracted with ethyl acetate from both wet and dry biomass from microalgae. For this purpose, two enzymes from different origins were assessed, namely lipase B from the fungus *Candida antarctica* (CalB) and lipase from the bacterium *Pseudomonas cepacia* (PC) which were supported on amino-functionalized SBA-15. The results were compared to those obtained with the commercial Novozym 435[®] catalyst. In addition, both enzymes were combined in different proportions to evaluate the synergistic activity and selectivity towards biodiesel production.

2. Results and Discussions

2.1. Characterization of the Synthesized Enzymatic Catalyst

This section contains the most relevant results of the synthesis of the mesoporous silica material SBA-15 as well as the modifications thereof when incorporating amino groups, glutaraldehyde and the enzymes.

The textural properties are shown in Table 1. The pore surface and diameter were reduced slightly after functionalization of the surface with amino groups and further glutaraldehyde linkage

as spacer-arm to favor the subsequent binding of the enzyme. Before functionalization, pore size of SBA-15 was 59.6 Å, and the surface area was the largest, 847.3 m²/g, and both parameters decreased to 56.3 Å and 841.8 m²/g, respectively, as chain length increased after amine group and glutaraldehyde introduction [29]. However, pore volume and surface area were drastically reduced by ~70% with respect to the original SBA-15 after the introduction of enzymes.

Table 1. Textural properties and enzyme fixed with synthesized mesoporous materials.

Material	S _{BET} (m ² /g) ¹	D _P (Å) ²	V _P (cm ³ /g) ³	Protein/Material (mg/g)
SBA-15	847.3	59.6	1.307	-
SBA-15-NH ₂	843.2	58.5	1.233	-
SBA-15-NH ₂ G	841.8	56.3	1.186	-
SBA-15-NH ₂ G-PC	275.4	55.0	0.378	35
SBA-15-NH ₂ G-CalB	262.3	56.2	0.393	33

¹ BET surface area, ² BJH pore diameter, ³ Pore volume at P/P₀ = 0.95.

The amount of enzyme loaded in each biocatalyst was determined by mass balance measuring the enzyme concentration in the supernatant solution using the Bradford's assay. The immobilization values were 35 and 33 mg/g for SBA-15-NH₂G-PC and SBA-15-NH₂G-CalB, respectively, similar to those obtained by Bautista et al. [4] (36.1 mg/g) and slightly lower than that obtained by Serra et al. [30] (44 mg/g), both using CalB as enzyme. The slightly higher incorporation of enzyme in the case of PC may be due to the fact that its size is somewhat smaller (3 × 3.2 × 6.6 nm) [31] and can enter into the pores more easily than CalB (3 × 4 × 6 nm) [32].

Regarding the structure, XRD (Figure 1) shows three reflection peaks at 2θ = 0.97°, 1.6° and 1.9° corresponding to *p6mm* mesoporous hexagonal symmetry (planes (100), (110) and (200), respectively) [33]. Based on the results obtained, the immobilization of enzymes covalently bound to the material did not modify its structure [10].

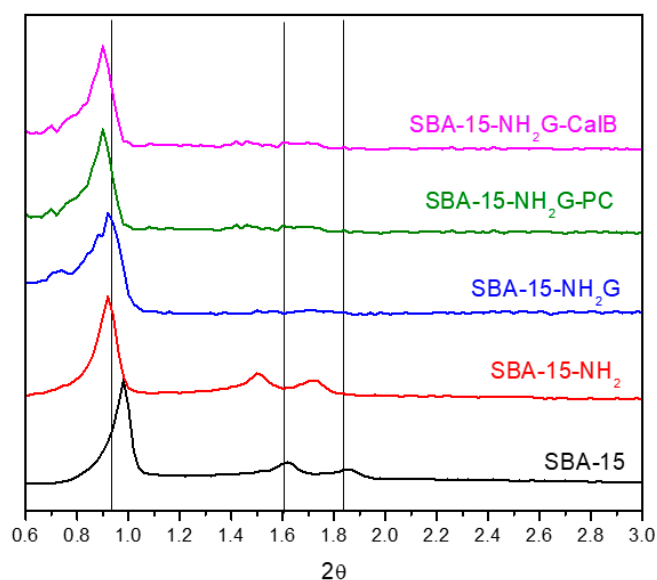


Figure 1. X-ray diffractograms of the mesoporous materials.

Figure 2 shows TEM images obtained from SBA-15 (a) and modified material with anchored lipases from PC (b) and CalB (c). It is clearly noted that both samples show the arrays of long-range mesopore channels, which are similar to the image of SBA-15 reported in the literature [19]. When the SBA-15 contains enzymes partially occupying the interior of its pores, a morphology showing the different degrees of sharpness of the channels, similar to that described by Mohammadi et al. [34], can be observed. However, as explained by Abdullah et al. [11], the fact that the modification of

the surface of the SBA-15 material was made by grafting a posteriori prevents major structural changes from taking place and only intervening in the formation of new links with Si-O-Si that are on the surface.

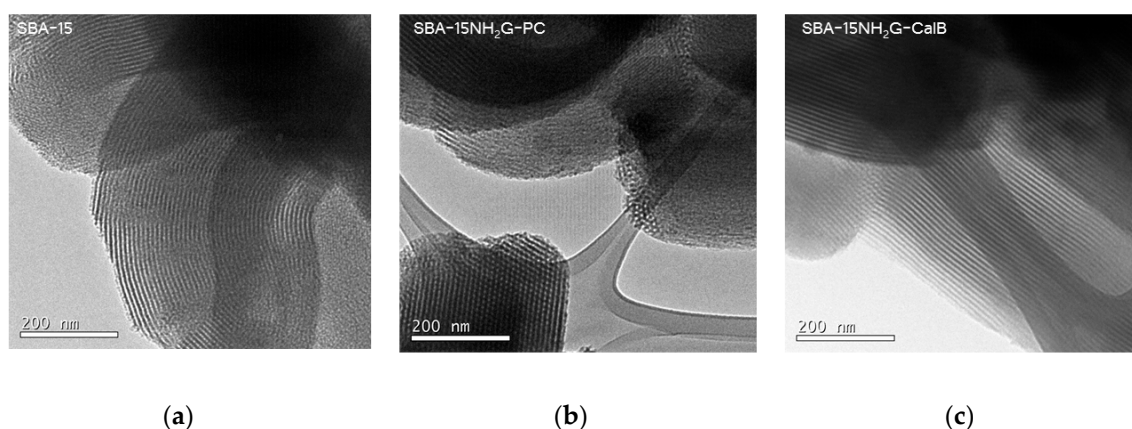


Figure 2. TEM micrographs: (a) SBA-15, (b) SBA-15-NH₂G-PC, (c) SBA-15-NH₂G-CalB.

The ²⁹Si MAS-NMR spectrum (Figure 3) of pure silica shows one wide signal within the range −90 to −110 ppm that correspond to the Si(OSi)₄ (Q⁴), HOSi(OSi)₃ (Q³) and (HO)₂Si(OSi)₂ (Q²) sites of the silica framework [35]. Around a displacement −60 to −78 ppm, a broad signal can be seen that corresponds to two signals created by the Si-C links corresponding to RSi(OSi)₃ (T³) and RSi(HO)(OSi)₂ (T²) [36]. These last two signals are due to the incorporation of the organic part (CH₃CH₂CH₂NH₂) by linking on the surface of the material. The peak areas were calculated after Gaussian deconvolution of the spectra considering the associated species and the respective intensities. The results of the integrations (Table 2) show that the greatest contribution to the areas is given by the peak corresponding to a Si atom linked to four Si-O structures, i.e., corresponding to the Q⁴ signal. Overlapped with this peak, two signals of smaller area corresponding to the formation of one or two Si-OH bonds, signals Q³ and Q² respectively. When the amino group was incorporated by grafting on the SBA-15 surface by using 3-aminopropyltriethoxysilane (APTES), the material SBA-15-NH₂ was formed, showing a significant increase in T signals (from 0% to ~10% for both T² and T³) and a concomitant decrease in Q signals, which evidenced the formation of Si-C bonds. However, the addition of glutaraldehyde (SBA-15-NH₂G) and CalB or PC lipases (SBA-15-NH₂G-CalB and SBA-15-NH₂G-PC, respectively) did not modified the peak contribution of T and Q signals significantly, proving that these incorporations are according to the immobilization scheme shown in Figure 4, where the silica framework was not affected thereafter. These results confirm that the structure is not modified when both organic modifiers and lipases are introduced [37].

Table 2. Relative peak area in the ²⁹Si NMR spectra.

Material	Q ⁴ (%)	Q ³ (%)	Q ² (%)	T ³ (%)	T ² (%)
SBA-15	83.8	13.4	2.77	-	-
SBA-15-NH ₂	66.0	10.2	3.42	10.32	10.23
SBA-15-NH ₂ G	67.3	9.27	3.21	11.26	9.87
SBA-15-NH ₂ G-PC	68.5	7.9	2.8	10.76	9.96
SBA-15-NH ₂ G-CalB	63.9	7.8	2.49	11.87	12.21

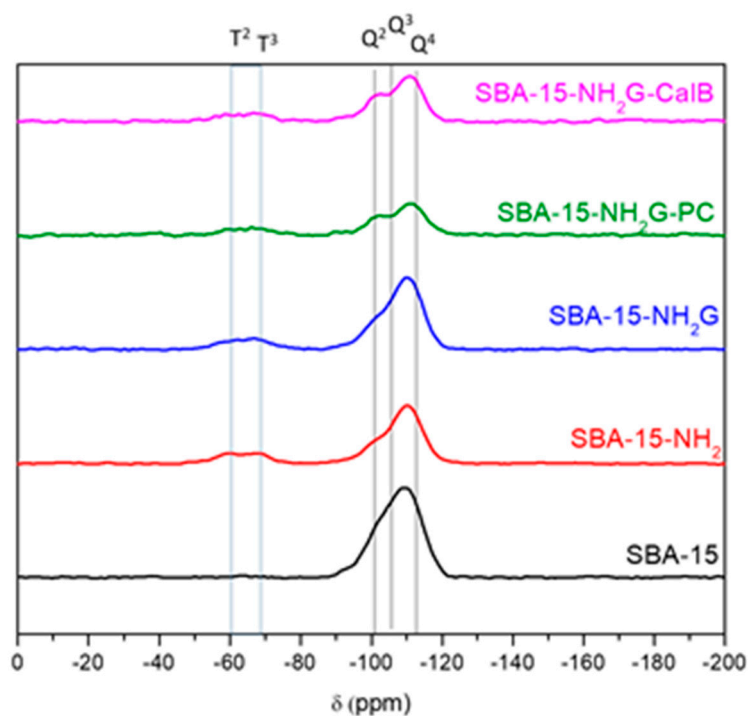


Figure 3. Solid state ^{29}Si MAS-NMR spectra of the different materials.

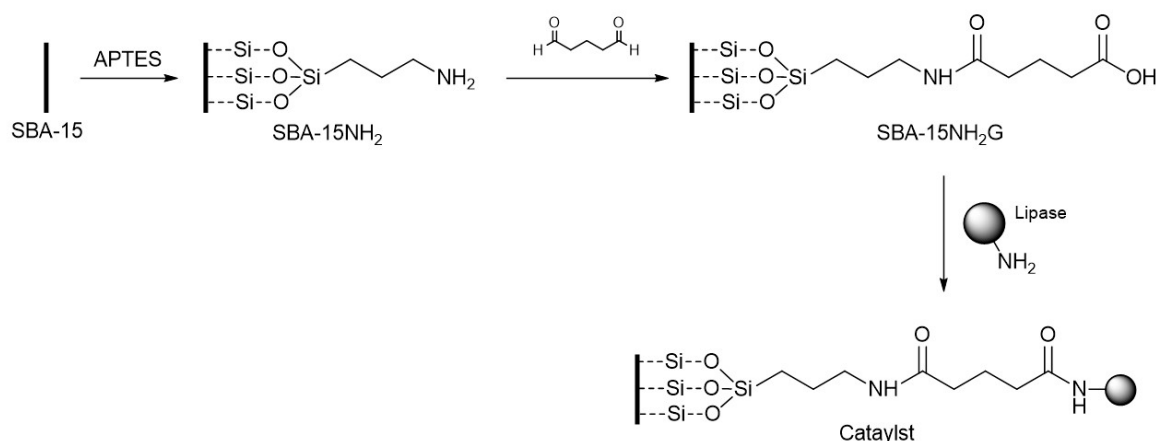


Figure 4. Synthesis of the enzymatic catalyst supported on SBA-15.

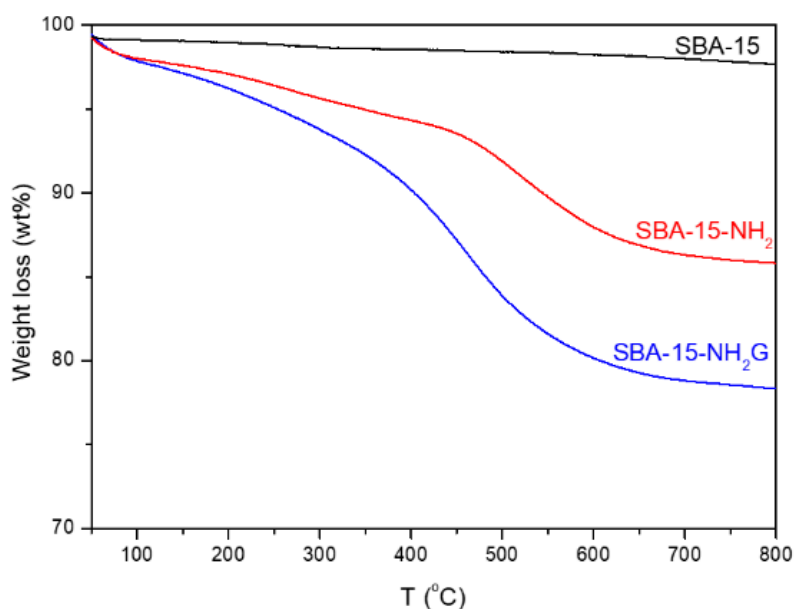
The elemental analysis shows how the composition of hydrogen, carbon, nitrogen and oxygen varies as new functional groups, amino and aldehyde are introduced into the structure, as well as with the incorporation of enzymes. The results shown in Table 3 are coherent since the initial mesoporous structure contains only silicon and hydrogen. The presence of nitrogen, carbon and oxygen are due to the incorporation of the different functional groups in the SBA-15. The introduction of the amino group from APTES increases the nitrogen concentration to ~2.8%, but the incorporation of glutaraldehyde decreases the nitrogen content to 2.2%, due to the addition of carbon, hydrogen and oxygen into the structure. Similar results were reported previously for different functionalization of SBA-15 materials [18]. Conversely, an increase in all the elements after lipase immobilization was observed in SBA-15NH₂G-PC and SBA-15NH₂G-CalB because the incorporation of the lipase molecules added N, C and H, as expected.

Table 3. Elemental analysis of synthesized mesoporous materials.

Material	N (%)	C (%)	H (%)	S (%)
SBA-15	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.01	n.d.
SBA-15NH ₂	2.77 ± 0.01	8.01 ± 0.04	1.97 ± 0.02	n.d.
SBA-15NH ₂ G	2.2 ± 0.1	15.1 ± 0.5	2.23 ± 0.09	n.d.
SBA-15NH ₂ G-PC	2.59 ± 0.02	16.8 ± 0.2	2.71 ± 0.04	n.d.
SBA-15NH ₂ G-CalB	2.49 ± 0.01	21.07 ± 0.02	3.28 ± 0.02	0.01 ± 0.01

n.d.: not detected (lower than the detection limit).

After water removal at $T < 100$ °C, thermogravimetric analysis shows a weight loss between 200 and 300 °C that corresponds to glutaraldehyde followed by the loss of the amino group at temperatures between 300 and 600 °C (Figure 5). The results of this analysis show an organic loss, corresponding to propylamine ($\text{CH}_3\text{CH}_2\text{CH}_2\text{NH}_2$), of 11.8 wt% in all the materials. The nitrogen content present in the material SBA-15-NH₂ according to this test (2.8 wt%) corroborates the results of elemental analysis (Table 3).

**Figure 5.** Thermogravimetric analysis of SBA-15 and functionalized materials.

A weight loss of 7.48% is observed in the material SBA-15-NH₂G, which implies an incorporation of glutaraldehyde of 37 wt% with respect to the amount of available amino groups. This indicates that part of amino groups incorporated into the material did not form bonds with glutaraldehyde. In addition, this test allowed to corroborate the results of nitrogen content (2.35 wt%) with that obtained by elemental analysis (2.23 wt%).

2.2. Production of FAEEs Using Single Lipase Biocatalysts

The activity of both synthesized biocatalysts (SBA-15-NH₂G-CalB and SBA-15-NH₂G-PC) towards FAEEs production from wet and dry extracted lipids from *I. galbana* was evaluated and compared with the commercial Novozym 435. The results were assessed based on the molar conversion of saponifiable lipids measured by ¹H NMR (Table 4). As it can be seen, the lipid extraction method had a large impact on lipase-catalyzed conversion to FAEEs. When the microalgal oil, used as feedstock, was extracted by the wet method, the results showed a reduction in FAEEs production from 97 to 85.5 mol% and from 91 to 87 mol% using CalB and PC lipase-based biocatalysts, respectively. The commercial Novozym 435 also showed a negative effect when lipids extracted by the wet method were used. Microalgal oils from both the wet and dry extraction methods did not contain water. Therefore, the decrease

in conversion may be caused by the extraction of water-soluble lipase-inhibitor compounds during the wet route that are not present in the oil extracted from dry biomass, because lipase activity was negatively affected by polar compounds causing inhibition or denaturation [38].

Table 4. Conversion to FAEEs using single lipases.

Lipid Extraction Method	Catalyst	Conversion (mol%)
Dry	CalB	97 ± 1
	PC	91 ± 2
	N435	75 ± 2
Wet	CalB	85.5 ± 0.8
	PC	87 ± 2
	N435	69.5 ± 0.6

The highest conversion to FAEEs (97 mol%) using dry extracted lipids with ethyl acetate from *I. galbana* microalgae was achieved using SBA-15-NH₂G-CalB as biocatalyst. For the wet extraction route, both CalB and PC lipase-based biocatalysts showed similar activity (85.5 and 87 mol%, respectively) so that SBA-15-NH₂G-CalB seems to be more sensitive to the presence of water or other polar compounds likely extracted during the wet process. However, in all cases, the synthesized biocatalysts SBA-15-NH₂G-CalB and SBA-15-NH₂G-PC were significantly more active towards FAEEs production than the commercial Novozym 435, proving the better performance of both biocatalysts synthesized in the present work.

2.3. Production of FAEEs Using Combi-Lipase Biocatalysts

In recent years, the search for new biocatalysts calls for the combination of different enzymes of different specificity to produce a higher production yield of FAEEs [26,27]. Consequently, a biocatalyst formed by the combination in different proportions of CalB and PC supported on SBA-15 modified with amino groups and glutaraldehyde is used in order to evaluate the FAEEs production in the same conditions used previously, using the lipids extracted by wet route with ethyl acetate as raw material.

Table 5 shows the synergistic effect of different combi-lipases on the conversion to FAEEs, along with those values achieves with the corresponding single-lipase biocatalysts. It is observed that the combi-lipase 25:75 produced the highest conversion to FAEEs (97.2 mol%) which is more than 10% higher than those produced by the biocatalysts formed by CalB or PC alone. This is a remarkable result because it demonstrates that it is possible to design a catalyst containing an optimized mixture of different lipases that maximizes the conversion to FAEEs.

Table 5. Conversion to FAEEs using combi-lipases.

Calb:PC Lipase Ratio	Conversion (mol%)
0:100	87 ± 2
25:75	97.2 ± 0.5
50:50	81 ± 2
75:25	89.7 ± 0.3
100:0	85.0 ± 0.8

The fatty acid profile of FAEEs produced by the optimized 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC is shown in Table 6. The major saturated fatty acids are myristic (C14:0) and palmitic (C16:0), while palmitoleic (C16:1) and oleic (C18:1) acids represented the main monounsaturated and linolenic (C18:3) the most abundant polyunsaturated fatty acid. It is important to highlight the value of linolenic acid (20.2%), a concentration about 10% higher than that regulated by EN 14,214 standard (Table 7). This would require further actions after biodiesel production to meet the required specifications,

such as mixing with biodiesel from other lipid sources whose linolenic acid content is lower than that of the microalgae used in this work.

Table 6. Fatty acid profile of FAEs obtained with combi-lipase 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC.

Fatty Acid		Composition (%)
Myristic	C14:0	9.0
Palmitic	C16:0	11.3
Palmitoleic	C16:1	3.6
Stearic	C18:0	0.6
Oleic	C18:1	14.5
Linoleic	C18:2	5.1
Linolenic	C18:3	20.2
Arachidic	C20:0	0.5
Behenic	C22:0	18.2
Erucic	C22:1	0.2
Lignoceric	C24:0	16.8
Saturated		56.3
Monounsaturated		18.3
Polyunsaturated		25.4

Table 7. Biodiesel properties obtained by the combi-lipase 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC.

Property	EN 14214	Combi-Lipase 25:75
Iodine value (g I ₂ /100 g)	<120	72
Group I metals (Na, K) (mg/kg)	<5	179.1
Group II metals (Ca, Mg) (mg/kg)	<5	205.1
Phosphorus (mg/kg)	<10.0	250.6
Sulfur (mg/kg)	<10	0
Monoglycerides (%)	<0.7	0.6
Diglycerides (%)	<0.2	<0.1
Triglycerides (%)	<0.2	<0.1
Linolenic esters (%)	<12	20.2

Other key properties of the biodiesel produced by the combi-lipase biocatalyst 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC using the lipids obtained after wet extraction with ethyl acetate are shown in Table 7. Iodine value as well as mono, di and triglyceride content and sulfur content fulfill the European standard EN 14214. However, metals and phosphorus content are higher than those regulated by the above standard, which would require a further purification stage.

2.4. Study of the Reuse of the Heterogeneous Enzymatic Catalyst

As previously mentioned, one of the key features of heterogeneous catalysts for their economic viability is their possible recovery and reuse in order to reduce the costs of the biodiesel production process [39]. Therefore, after each reaction, the catalyst was washed with 3 mL of ethanol and dried in order to remove possible impurities that could affect the conversion [40]. The combi-lipase 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC was reused for 10 consecutive cycles under the same operating conditions (Figure 6).

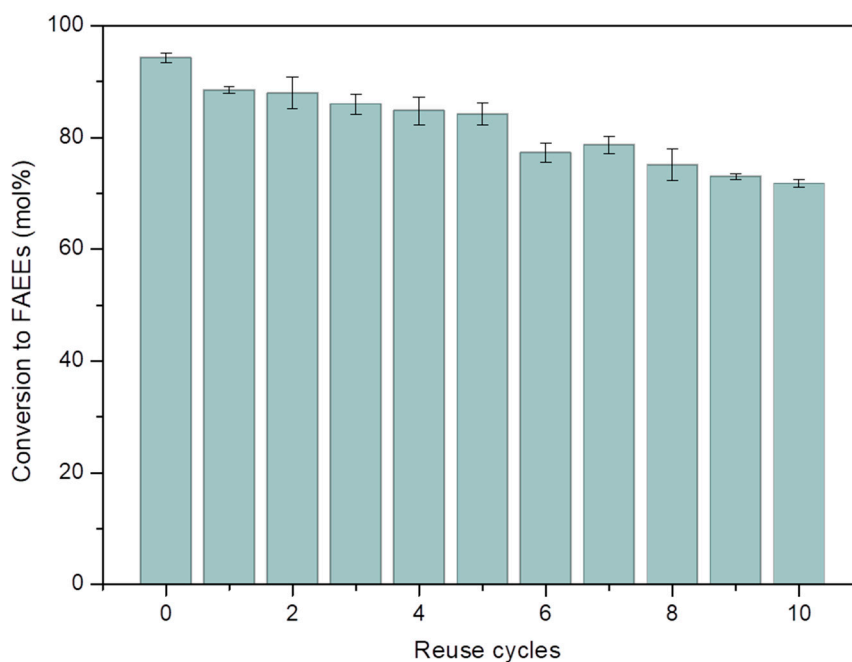


Figure 6. Reuse of combi-lipase 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC.

The results showed that the conversion was above 80 mol% during the first five cycles, decreasing by 10% with respect to the first reaction. Subsequently, the conversion gradually decreased, reaching a conversion of around 70 mol% after the tenth reuse. These results are comparable although better than others reported in the literature for the lipase from *P. cepacia* where after the first use, 10% of the activity was lost and in the fourth cycle the activity was reduced by 53% [41]. This loss of activity can be caused by a possible deterioration of the biocatalyst by enzyme poisoning [42].

3. Materials and Methods

3.1. Microalga

The microalga used in this work was *Isochrysis galbana* and it was supplied by AlgaEnergy S.A. (Madrid, Spain). This strain has been selected due to its high lipid content (33.5 wt%), which makes it favorable for the production of FAEs.

3.2. Lipid Extraction

The extraction of lipids from the microalga, in the dry biomass procedure, was carried out with a dry microalga:solvent ratio of 1:20, using ethyl acetate as solvent. The samples were stirred vigorously with the help of a vortex (Finepcr, Gunpo-si, South Korea) for 5 min at 11,000 rpm at room temperature to obtain the lipids contained in the microalgae. In the case of the extraction of lipids from wet biomass, the same procedure was carried out starting from a suspension of microalga in water with a concentration of 50 g/L. Lipid yields using ethyl acetate were 17.9 ± 0.7 wt% and 17.6 ± 0.1 wt% from dry and wet microalgal biomass, respectively.

3.3. Synthesis of Lipase-Based Biocatalysts

The production of FAEs was carried out using lipase B from *Candida antarctica* (CalB) and lipase from *Pseudomonas cepacia* (PC), both from Sigma-Aldrich (St. Louis, MO, USA), supported on SBA-15 modified with amino groups and glutaraldehyde following protocols previously described [43–45] (Figure 4).

In a typical synthesis for SBA-15, 8 g of block copolymer surfactant Pluronic 123 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved at room temperature under stirring in 250 mL of 1.9 M HCl

(Scharlab, Barcelona, Spain). The solution was heated up to 40 °C and 17.8 g of TEOS (tetraethyl orthosilicate, Sigma-Aldrich) were added to the solution. The resultant mixture was then stirred at that temperature for 20 h and hydrothermally aged at 110 °C for further 24 h. The template was removed by calcination at 550 °C for 5 h at a heating rate of 1.8 °C/min [44].

The protocol for covalent immobilization of lipases (Figure 4) was adapted from Wang's procedure [36]. Thus, 2 g of calcined mesoporous material SBA-15 were immersed into a solution of 1 g of APTES (3-aminopropyltriethoxysilane, Sigma-Aldrich) in 30 mL of anhydrous toluene (Sigma-Aldrich). The mixture was refluxed at 110 °C for 24 h under inert nitrogen atmosphere. Then, the suspension was filtered and washed three times with anhydrous toluene. The solid was placed in glass vials and vacuum dried for 24 h at 110 °C, yielding the amine-functionalized support named SBA-15-NH₂. Then, 1 g of SBA-15-NH₂ was blended with 1 mL of 25 vol% aqueous glutaraldehyde (Sigma-Aldrich) and 9 mL of 0.1 M phosphate buffer (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. The solid material (SBA-15-NH₂G) was filtered and washed with phosphate buffer.

The functionalized materials were used as carriers for lipase immobilization. 100 mg of amino-functionalized and glutaraldehyde linked mesoporous material was blended with 1 mL of a 2.5 mg/mL stock solution of lipase in 4 mL of 0.1 M phosphate buffer at pH = 7.0 and the mixture was shaken at 200 rpm and 25 °C for 3 h. Then, the material was filtered and washed three times with 5 mL phosphate buffer. The final biocatalysts produced were named as SBA-15-NH₂G-CalB and SBA-15-NH₂G-PC, containing immobilized CalB and PC lipase, respectively.

3.4. Characterization of Biocatalysts

The evaluation of the textural properties of SBA-15 mesoporous materials was performed by N₂ adsorption/desorption isotherms in a Tristar model 3000 equipment (Micromeritics, Norcross, GA, USA). Prior to the analysis, a degasification stage is carried out based on the controlled heating of the samples up to 250 °C and 120 °C for inorganic and organically functionalized samples, respectively, and the use of a nitrogen flow in a SmartPrep type degasser. The specific surface area was calculated by the Brunauer-Emmett-Teller (BET) method, the pore diameter was measured through the Barret-Joyner-Halenda technique (BJH) and pore volume was determined by using the Harkins-Jura technique.

X-ray diffraction experiments were performed in a X'Pert MPD (Philips, Amsterdam, The Netherlands) using monochromatic Cu K α radiation, with a wavelength of 1.54 Å. The step size was 0.02° with an accumulation time per step of 5 s for a sweep of angles 2 θ of 5–50°.

Transmission electron microscopy (TEM) analysis was performed with a Tecnai 20 microscope (Philips, Amsterdam, The Netherlands) with a resolution of 0.27 nm and $\pm 70^\circ$ inclination of the sample, equipped with an EDX detector.

²⁹Si NMR spectroscopy allow to determine the relationship between condensed and non-condensed silicon species, obtaining structural information about the mesoporous siliceous material [37]. Solid-state ²⁹Si MAS-NMR experiments were performed on a Bruker Avance III/HD 400 MHz spectrometer fitted with a 9.4 T magnetic field. These nuclei resonate at 79.41 MHz. An H/X 7.5 mm MAS probe and ZrO₂ rotors spinning at 6 kHz were used. In addition, to allow an accurate quantification of silicon groups, ²⁹Si MAS-NMR spectra using one pulse with spinal-64 ¹H decoupling were obtained. The ²⁹Si experiments were performed for 500 scans, $\pi/2$ pulse of 4.0 μ s, and 60 s of repetition time. ²⁹Si chemical shifts were externally referenced to tetramethylsilane.

The content of hydrogen, carbon, nitrogen, sulfur and oxygen was measured in a Vario EL III element analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) using sulphanic acid as standard.

To evaluate the thermal stability of the SBA-15 materials, thermogravimetric analysis (TGA) were carried out under inert atmosphere (nitrogen) in an 1100 TGA/DSC1 model (Mettler Toledo, Columbus, OH, USA). The thermal analysis ranged from 40 °C to 800 °C with a ramp of 10 °C/min.

Finally, the amount of enzyme immobilized was calculated by mass balance measuring the enzyme concentration in the supernatant solution using the Bradford's protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as standard [46]. All the analyses were performed in triplicate.

3.5. FAEEs Production

FAEE production experiments were carried out in 12 mL glass pressure tubes (Sigma-Aldrich). To carry out the reaction, 0.1 g of extracted lipids were added to the reaction tube together with 15 mg of the synthesized biocatalyst [4]. The reaction mixture was maintained at 50 °C and 300 rpm during 24 h. After the reaction time, the mixture was cooled to room temperature (25 °C) and vacuum filtered to recover the catalyst. The liquid phase containing FAEE was washed twice with 10 mL of water, then with 10 mL of hexane:diethylether (80:20) and, finally, with 10 mL of water again. Then, the solvent was removed in a rotary evaporator (Heidolph Instruments, Schwabach, Germany) and the purified FAEEs were obtained. To carry out a comparative study, the commercial biocatalysts Novozym 435® (N345) (Novozymes A/S, Bagsværd, Denmark) was used following the same protocol. Conversion of saponifiable lipids into FAEEs was measured by ¹H-NMR analyses (Figure 7) performed in a Varian Mercury Plus 400 unit (Varian, Palo Alto, CA, USA), following the procedure reported in the literature [47], according to Equation (1)

$$\text{FAEE conversion (mol\%)} = 100 \cdot \frac{A_{\delta 1} - A_{\delta 2}}{A_{\delta 3}} \quad (1)$$

where $A_{\delta 1}$ is the area of the peaks corresponding to glyceryl methylenic hydrogens of the oil together with the hydrogen of the ethoxy group of the ethyl ester appearing within the range 4.10–4.17 ppm, $A_{\delta 2}$ is the area of glyceryl methylenic hydrogens of oil integrated from 4.28 to 4.32 ppm and $A_{\delta 3}$ corresponds to the area of the α -acyl methylenic hydrogens in the oil, in the ethyl esters and in the free fatty acids, between 2.2 and 2.4 ppm.

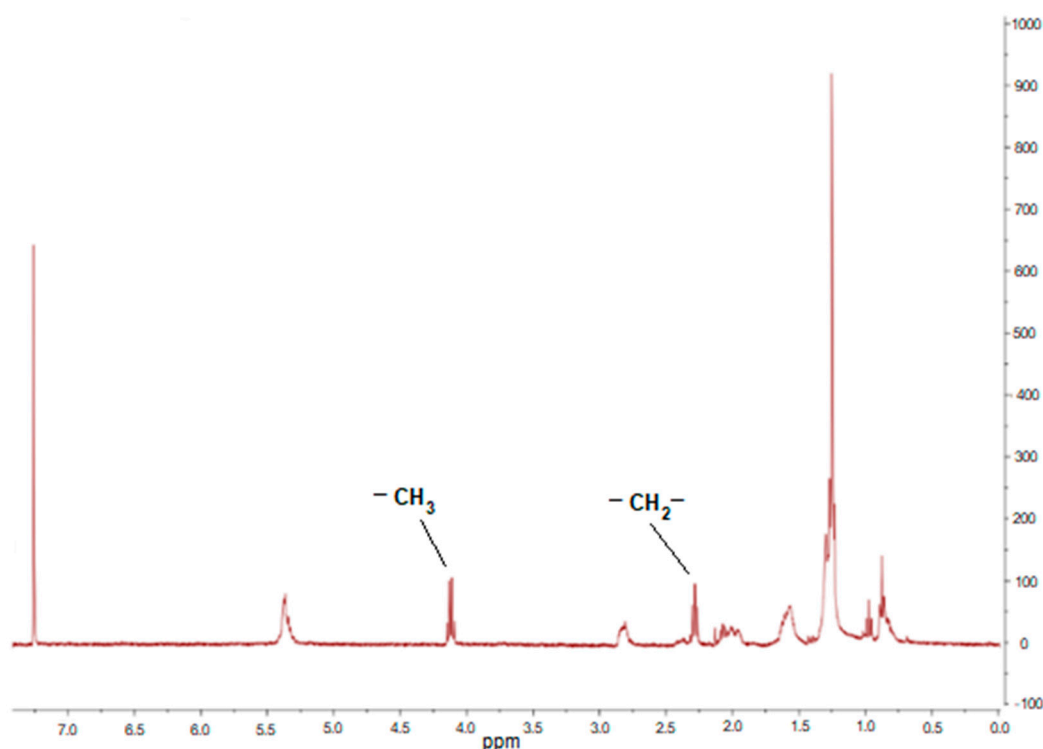


Figure 7. Typical ¹H-NMR spectrum of FAEE.

FAEE production reactions were also carried out using combi-lipase biocatalysts with the following SBA15-NH₂G-CalB:SBA15-NH₂G-PC ratios: 25:75, 50:50 and 75:25 wt%. Finally, successive reactions were carried out under the same conditions to assess the reuse capacity.

3.6. FAEE Characterization

The characteristics of the FAEE to be used as biodiesel must be according to EN 14,214 standard, in which the protocol for determining the iodine value of the biodiesel obtained is included. In addition, the content of mono-, di- and triglycerides was determined by thin layer chromatography (TLC) following the method described by Vicente et al. [2], where the sample was diluted with hexane. The TLC plate was developed, stained with iodine and digitized. The software Un-Scan-It Gel 6.1 (Silk Scientific Inc., Orem, UT, USA) was used for the quantification of each lipid fraction using the corresponding standards. The non-saponifiable extracted matter was determined by a gravimetric procedure described elsewhere [48].

Fatty acid profile of FAEE was performed by gas chromatography in a CP-3900 gas chromatograph (Varian) fitted with flame ionization detector and a TRB-FFAP capillary column (60 m length, 0.32 mm I.D., 0.25 µm film thickness. Teknokroma, Barcelona, Spain) following EN 14,103 standard.

The sulfur content was measured by elemental analysis in a Vario EL III elemental analyzer and metals were determined by inductively coupled plasma atomic emission spectroscopy in a Vista AX CCD simultaneous ICP-AES equipment coupled to a spectrophotometer (Varian. Palo Alto, CA, USA) following EN 14,538 (for Na, K, Ca and Mg) and EN 14,107 (for P) standards, respectively.

4. Conclusions

Lipids from *Isochrysis galbana* extracted with ethyl acetate using both dry and wet biomass were used to produce biodiesel (FAEEs) with heterogeneous lipase-based biocatalysts. Lipase B from *Candida antarctica* (CalB) and lipase from *Pseudomonas cepacia* (PC) were covalently immobilized on amino-functionalized SBA-15. Their catalytic activity was compared to that of the commercial catalyst Novozym 435. The conversion to FAEEs using the synthesized biocatalysts SBA-15-NH₂G-CalB (97 and 85.5 mol% from dry and wet extraction, respectively) and SBA-15-NH₂G-PC (91 and 87 mol%) resulted in higher conversions for both dry and wet ones than those obtained with the commercial catalyst Novozym 435 (75 and 69.5 mol%, respectively). Due to the heterogeneous nature of the lipid composition of the microalgae, mixtures with different proportions of CALB:PC biocatalysts were used to improve the conversion of wet extracted lipids. The results showed that a combi-lipase biocatalyst 25:75 SBA-15-NH₂G-CalB:SBA-15-NH₂G-PC produced a conversion of FAEE (97.2 mol%) significantly higher than those from wet extracted oil by biocatalysts containing a single lipase and similar to the maximum conversion using oils from wet extraction. Therefore, the combi-lipase presented can help to improve the viability of a microalgal biorefinery since the biomass-drying step for biodiesel production can be avoided.

Author Contributions: A.S.-B. performed the experimental work (synthesis of biocatalysts, catalytic experiments and sample characterization) and made a first draft of the manuscript. V.M. and R.R. analyzed and discussed the results of biocatalysts characterization and V.M. collaborated in the writing of the first draft of the manuscript. L.F.B. and G.V. devised the experimental work, analyzed the catalytic tests and wrote the final version of the manuscript. The listed authors have contributed substantially to this work.

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