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Article

Biodiesel production (FAEEs) by heterogeneous combi-lipase biocatalysis 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 PS (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 optimised 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

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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 microalga oil that is esterified or transesterified with methanol or ethanol into methyl or ethyl esters of fatty acids (FAMEs or FAEEs, respectively) in the presence of a catalyst. Due to its high content of free fatty acids, the most common way to carry out the transesterification and esterification reactions of the lipids extracted from microalgae 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. Immobilised 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)[10–14] or carbon nanotubes[15,16] 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[10,14,17–20].

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*[16,21], *P. fluorescens*[16,22], *Thermomyces lanuginosus*[16,23], *Candida rugosa*[16,24] and *C. antarctica*[11,16]. The last one is frequently used in the form of the commercial catalyst Novozym 435[6,16,25,26]. 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[5,21]. Therefore, the concept of combi-lipase biocatalysts have been recently developed to improve the biodiesel production processes[27–29]. However, the previous studies using combi-lipase systems used oils from vegetable crops such as palm, soybean or coffee, but they did not used the more complex microalgal oil as a source for biodiesel.

In this work, biodiesel (FAEEs) were produced using enzymatic catalysts from lipids extracted with ethyl acetate from both wet and dry biomass from microalgae. For this purpose, two enzymes from different origin were assessed: 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 are 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, 847.2 m^2/g, which decreased to 46.3 Å and 841.8 m^2/g, respectively, as chain length increased after amine group and glutaraldehyde introduction [30]. However, pore volume and surface area were drastically reduced with the introduction of enzymes, both properties decreasing by ~70% with respect to the original SBA-15.

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_2G	841.8	56.3	1.186	-
SBA-15-NH_2G-PC	275.4	55.0	0.378	35
SBA-15-NH_2G-CalB	262.3	56.2	0.393	33

[1] BET surface area, [2] BJH pore diameter, [3] Pore volume at $P/P_0 = 0.95$.

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-NH_2G-PC and SBA-15-NH_2G-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. (44 mg/g) [31], both using CalB as enzyme. The slighting higher incorporation enzyme in the case of PC may be due to the fact that its size is somewhat smaller (3 x 3.2 x 6.6 nm)[32] and can enter into the pores more easily than CalB (3 x 4 x 6 nm)[33].

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)[34]. Based on the results obtained, the immobilization of enzymes covalently bound to the material does not modify its structure[10].

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[20]. When the SBA-15 contains enzymes partially occupying the interior of its pores, it can appreciated with the different degrees of sharpness of the channels, a morphology similar to that observed by Mohammadi et al.[35]. However, as explained by Abdullah et al.[12], the fact that the modification of the surface of the

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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.

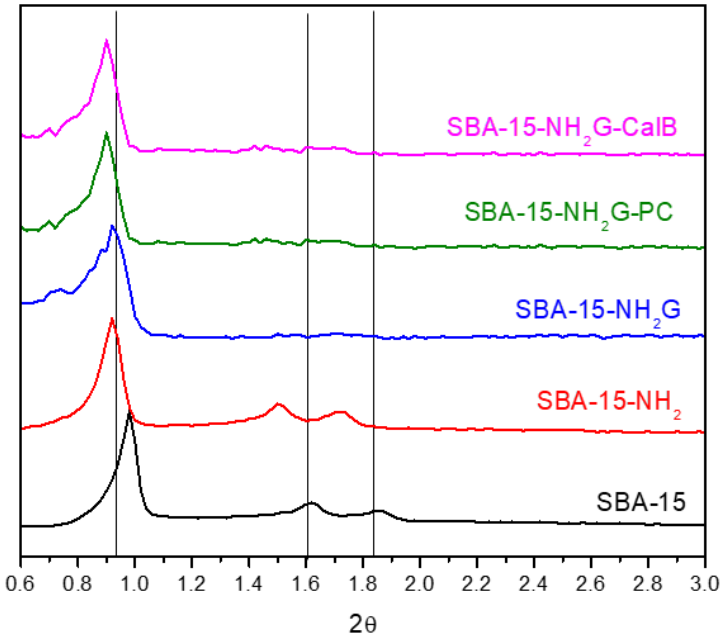
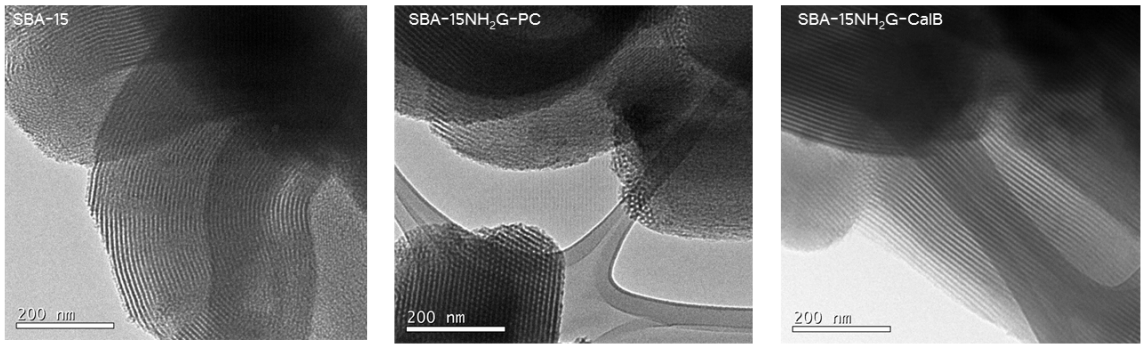


Figure 1. X-ray diffractograms of the mesoporous materials.



(**a**) (**b**) (**c**)

Figure 2. TEM micrographs: (a) SBA-15, (b) SBA-15-NH_2G-PC, (c) SBA-15-NH_2G-CalB.

The ^{29}Si NMR spectrum (Figure 3) of pure silica shows one wide signal within the range -90 to -110 ppm that correspond to the $Si(OSi)_4$ (Q^4), $HOSi(OSi)_3$ (Q^3) and $(HO)_2Si(OSi)_2$ (Q^2) sites of the silica framework[36]. 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 $RSi(OSi)_3$ (T^3) and $RSi(HO)(OSi)_2$ (T^2)[37]. These last two signals are due to the incorporation of the organic part ($CH_3CH_2CH_2NH_2$) 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 Q^4 signal. Overlapped with this peak, two signals of smaller area corresponding to the formation of one or two Si-OH bonds, signals Q^3 and Q^2 respectively. The T signals appear when the surface of the material is modified with the

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previously mentioned organic group and can be observed in the spectrum into overlapping signals of areas similar one each other. These results confirm that the structure is not modified when both organic modifiers and lipases are introduced[38].

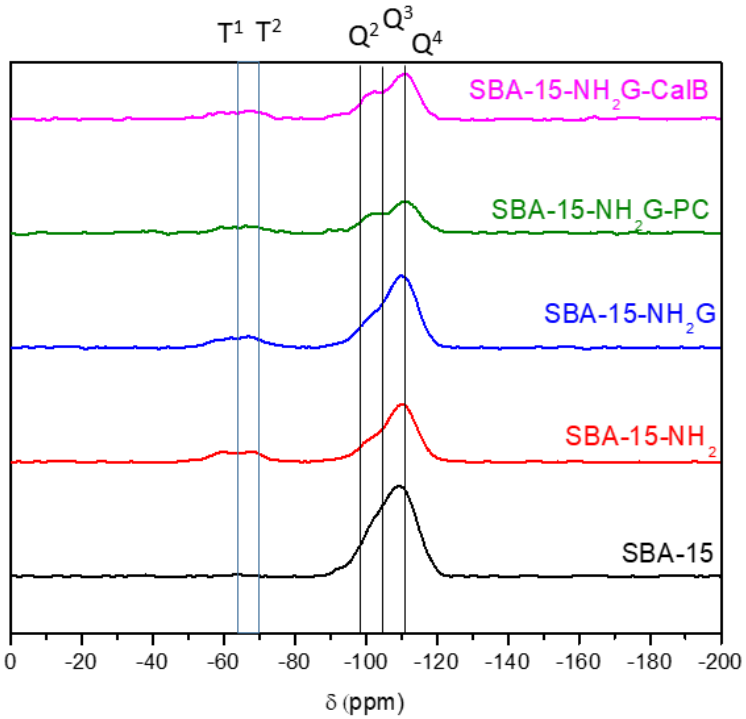

Figure 3. ^{29}Si NMR spectra of the different materials.

Table 2. Relative peak area in the NMR-^{29}Si 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	10.2	3.42	10.32	10.23
SBA-15-NH_2G	64.5	13.4	2.41	9.78	9.93
SBA-15-NH_2G-PC	68.5	7.9	2.8	10.76	9.96
SBA-15-NH_2G-CalB	63.9	7.8	2.49	11.87	12.21

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 presence of carbon, hydrogen and oxygen into the structure. Similar results are reported previously for different functionalization of SBA-15 materials[19]. Conversely, an increase of all the elements is observed after lipase immobilisation in SBA-15NH_2G-PC and SBA-15NH_2G-CalB because the incorporation of the lipase molecules added N, C and H, as expected.

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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_2G	2.2±0.1	15.1±0.5	2.23±0.09	n.d.
SBA-15NH_2G-PC	2.59±0.02	16.8±0.2	2.71±0.04	n.d.
SBA-15NH_2G-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 4). The results of this analysis show an organic loss, corresponding to propylamine ($CH_3CH_2CH_2NH_2$), of 11.8 wt% in all the materials. The nitrogen content present in the material SBA-15-NH_2 according to this test (2.8 wt%) corroborates the results of elemental analysis obtained (Table 3).

A weight loss of 7.48% is observed in the material SBA-15-NH_2G, which implies an incorporation of glutaraldehyde in 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 has allowed to corroborate the results of nitrogen content (2.35 wt%) with that obtained by elemental analysis (2.23 wt%).

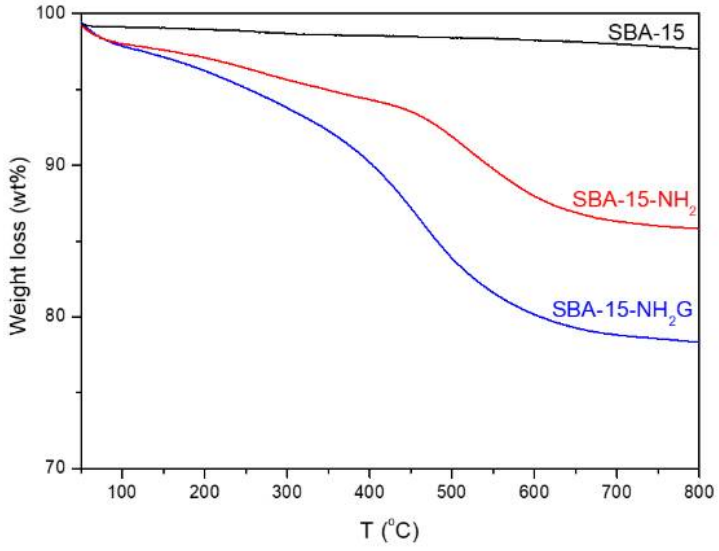

Figure 4. Thermogravimetric analysis of SBA-15 and functionalized materials.

2.2. *Production of FAEEs using single lipase biocatalysts*

The activity of both synthesized biocatalysts (SBA-15-NH_2G-CalB and SBA-15-NH_2G-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 ^{1}H NMR (Table 4). As it can be seen, the lipid extraction method had a large impact on lipase-catalysed conversion to FAEEs. When the microalgal oil, used as feedstock, was extracted by the wet method, the results showed a reduction in FAEEs production

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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 was 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 it is negatively affected by polar compounds that can cause inhibition or denaturation [39].

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-NH_2G-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-NH_2G-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 synthesised biocatalysts SBA-15-NH_2G-CalB and SBA-15-NH_2G-PC were significantly more active towards FAEEs production than the commercial Novozym 435, proving the better performance of both biocatalysts synthesised 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[27,40]. 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. 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

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

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combi-lipase 25:75 produced the highest conversion to FAEEs (97.2 mol%) which is more than 10% higher than biocatalysts formed by CalB or PC alone. This is a remarkable result because it demonstrates that it is possible to design a catalyst that contains an optimized mixture of different lipases that maximizes the conversion to FAEEs.

Table 6. Fatty acid profile of FAEEs obtained with combi-lipase 25:75 SBA-15-NH_2G-CalB:SBA-15-NH_2G-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

The fatty acid profile of FAEEs produced by the optimised 25:75 SBA-15-NH_2G-CalB:SBA-15-NH_2G-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 14214 standard (Table 7). This would require further actions after biodiesel production, such as mixing with biodiesel from other lipid sources whose linolenic acid content is lower than that of the microalgae used in this work, to meet the required specifications

Table 7. Biodiesel properties obtained by the combi-lipase 25:75 SBA-15-NH_2G-CalB:SBA-15-NH_2G-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
Phosphorous (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

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Other key properties of the biodiesel produced by the combi-lipase biocatalyst 25:75 SBA-15-NH_2G-CalB:SBA-15-NH_2G-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[41]. 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[42]. The combi-lipase 25:75 SBA-15-NH_2G-CalB:SBA-15-NH_2G-PC was reused for 10 consecutive cycles under the same operating conditions (Figure 6).

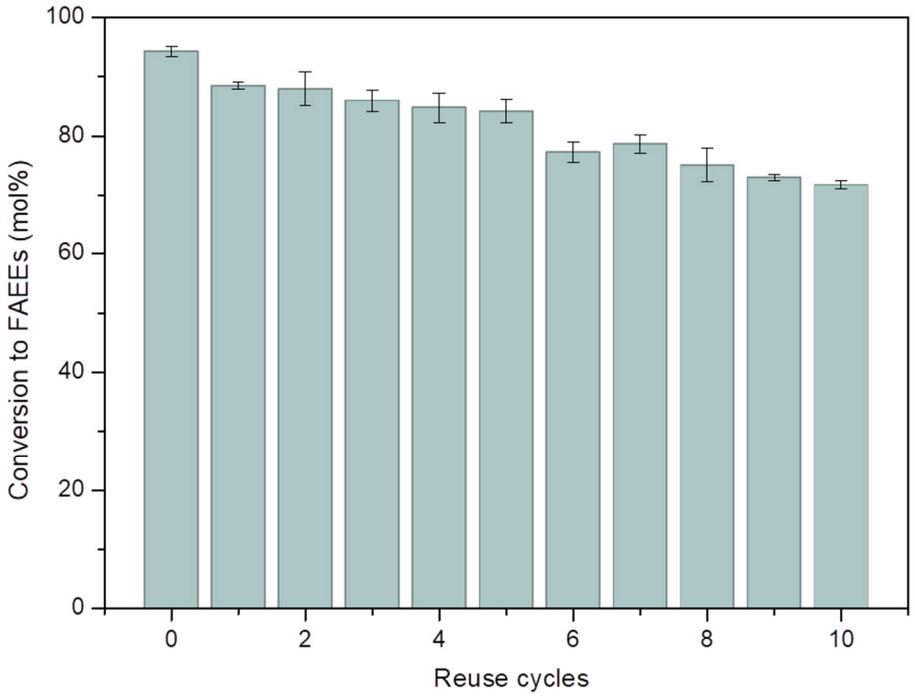

Figure 6. Reuse of combi-lipase 25:75 SBA-15-NH2G-CalB:SBA-15-NH2G-PC.

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%[43]. This loss of activity can be caused by a possible deterioration of the biocatalyst by enzyme poisoning[44].

3. Materials and methods

3.1. Microalga

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

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 stirrer vigorously with the help of a vortex (Finecorp, Korea) for 5 min at 11000 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.

3.3. Synthesis of lipase-based biocatalysts

The assays for the production of FAEEs were carried out using lipase B from *Candida antarctica* (CalB) and lipase from *Pseudomonas cepacia* (PC), both from Sigma-Aldrich (USA), supported on SBA-15 modified with amino groups and glutaraldehyde following protocols previously described[45–47] (Figure 7).

In a typical synthesis for SBA-15, 8 g of block copolymer surfactant Pluronic 123 (Sigma-Aldrich) were dissolved at room temperature under stirring in 250 mL of 1.9 M HCl (Scharlab. 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[46].

The protocol for covalent immobilization of lipases (Fig. 7) was adapted from Wang's procedure[37]. Thus, 2 g of calcined mesoporous material SBA-15 were immersed into a solution of 1 g of APTES (*n*-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 3 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-NH_2. Then, 1 g of SBA-15-NH_2 was blended with 1 mL of 25 vol% aqueous glutaraldehyde (Sigma-Aldrich) and 9 mL of 0.1 M phosphate buffer (Sigma-Aldrich) for 2 h. The solid material (SBA-15-NH_2G) 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 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-NH_2G-CalB and SBA-15-NH_2G-PC, containing immobilized CalB and PC lipase, respectively.

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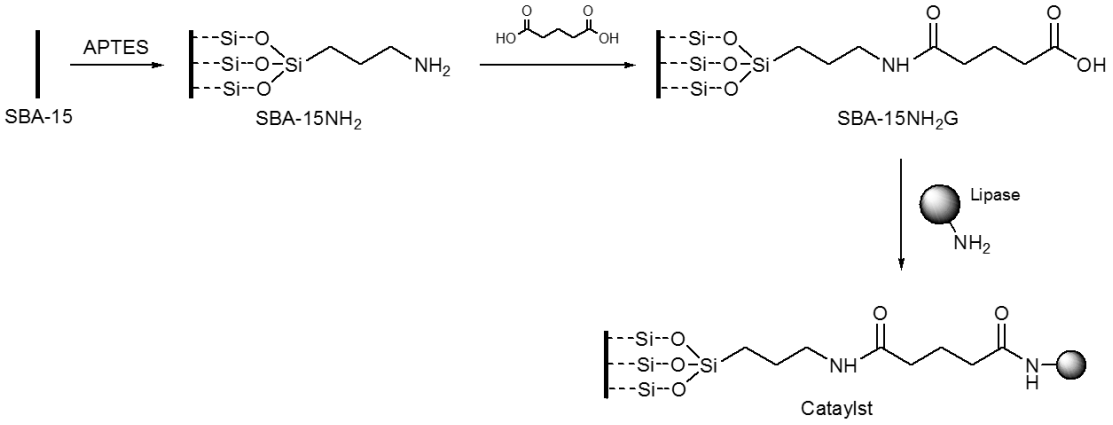

Figure 7. Synthesis of the enzymatic catalyst supported on SBA-15

3.4. Characterization of biocatalysts

The evaluation of the textural properties of SBA-15 mesoporous materials was performed by N_2 adsorption/desorption isotherms in a Tristar model 3000 equipment (Micromeritics. 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 current 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. The Netherlands) using monochromatic Cu Kα radiation, with a wavelength of 1.54 Å. The step size was 0.02° with an accumulation time per step of 5 seconds for a sweep of angles 2θ of 5–50°.

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

^{29}Si NMR spectroscopy allow to determine the relationship between condensed and non-condensed silicon species, obtaining structural information about the mesoporous siliceous material[38]. These experiments were performed in an Infinity AS400 NMR spectrometer (Varian. USA) operating at 9.4 Tesla.

The content of hydrogen, carbon, nitrogen, sulfur and oxygen was measured in a Vario EL III element analyzer (Elementar Analysensysteme GmbH. Germany) using sulphanilic 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. 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 assay with bovine serum albumin (BSA) as standard[48]. 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). 0.1 g of extracted lipids , 2 mL of ethanol as solvent and 15 mg of the synthesized biocatalyst were employed[11]. The reaction mixture was maintained at 50°C and 300 rpm for 24 h. After the reaction time, the mixture was cooled to room temperature generating two liquid phases. The upper phase containing FAEE was washed twice with 10 mL of water and then with 10 mL of hexane:diethylether mixture (80:20). To carry out a comparative study, the commercial biocatalysts Novozym 435® (N345) was used. Conversion of saponifiable lipids into FAEEs was measured by ^{1}H-NMR analyses performed in a Varian Mercury Plus 400 unit, following the procedure reported in the literature[49].

FAEE production reactions were also carried out using combi-lipase biocatalysts with the following SBA15-NH_2G-CalB:SBA15-NH_2G-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 have to be evaluated based on the standard EN 14214, 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. 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[50].

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 Inc. Germany) following EN 14538 (for Na, K, Ca and Mg) and EN 14107 (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 immobilised on amino-functionalised SBA-15. Their catalytic activity was compared with that of the commercial catalyst Novozym 435. The conversion to FAEEs using the synthesized biocatalysts SBA-15-NH_2G-CalB (97 and 85.5 mol% for dry and wet extraction, respectively) and SBA-15-NH_2G-PC (91 and 87 mol%) resulted in higher conversions for both dry and wet ones than those obtained with the commercial catalyst N435 (75 and 69.5 mol%). 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-NH_2G-CalB:SBA-15-NH_2G-PC produced a conversion of FAEE (97.2 mol%) significantly higher than those produced 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 can be avoided for biodiesel production.

Author Contributions: ASB performed the experimental work (synthesis of biocatalysts, catalytic experiments and sample characterization) and made a first draft of the manuscript. VM and RR analyzed and discussed the results of biocatalysts characterization and VM collaborated in the writing of the first draft of the manuscript. FB and GV 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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References

(1) Demirbas, A. Political, Economic and Environmental Impacts of Biofuels: A Review. *Appl. Energy* **2009**, *86*, S108–S117. https://doi.org/10.1016/j.apenergy.2009.04.036.

(2) Vicente, G.; Bautista, L. F.; Rodríguez, R.; Gutiérrez, F. J.; Sádaba, I.; Ruiz-Vázquez, R. M.; Torres-Martínez, S.; Garre, V. Biodiesel Production from Biomass of an Oleaginous Fungus. *Biochem. Eng. J.* **2009**, *48* (1), 22–27. https://doi.org/10.1016/j.bej.2009.07.014.

(3) Carrero, A.; Vicente, G.; Rodríguez, R.; Peso, G. L. del; Santos, C. Synthesis of Fatty Acids Methyl Esters (FAMEs) from *Nannochloropsis gaditana* Microalga Using Heterogeneous Acid Catalysts. *Biochem. Eng. J.* **2015**, *97*, 119–124. https://doi.org/10.1016/j.bej.2015.02.003.

(4) Bautista, L. F.; Vicente, G.; Mendoza, Á.; González, S.; Morales, V. Enzymatic Production of Biodiesel from *Nannochloropsis gaditana* Microalgae Using Immobilized Lipases in Mesoporous Materials. *Energy and Fuels* **2015**, *29* (8), 4981–4989. https://doi.org/10.1021/ef502838h.

(5) Guldhe, A.; Singh, B.; Mutanda, T.; Permaul, K.; Bux, F. Advances in Synthesis of Biodiesel via Enzyme Catalysis: Novel and Sustainable Approaches. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1447–1464. https://doi.org/10.1016/J.RSER.2014.09.035.

(6) Navarro López, E.; Robles Medina, A.; Esteban Cerdán, L.; González Moreno, P. A.; Macías Sánchez, M. D.; Molina Grima, E. Fatty Acid Methyl Ester Production from Wet Microalgal Biomass by Lipase-Catalyzed Direct Transesterification. *Biomass and Bioenergy* **2016**, *93*, 6–12. https://doi.org/10.1016/j.biombioe.2016.06.018.

(7) Park, J.-Y.; Park, M. S.; Lee, Y.-C. Advances in Direct Transesterification of Algal Oils from Wet Biomass. *Bioresour. Technol.* **2015**, *184*, 267–275. https://doi.org/10.1016/j.biortech.2014.10.089.

(8) Dalla-Vecchia, R.; Nascimento, M. D. G.; Soldi, V. Aplicações Sintéticas de Lipases Imobilizadas Em Polímeros. *Quim. Nova* **2004**, *27* (4), 623–630. https://doi.org/10.1590/S0100-40422004000400017.

(9) Hanefeld, U.; Gardossi, L.; Magner, E. Understanding Enzyme Immobilisation. *Chem. Soc. Rev.*; 2009; Vol. 38. https://doi.org/10.1039/b711564b.

(10) Rios, N. S.; Pinheiro, M. P.; dos Santos, J. C. S.; de S. Fonseca, T.; Lima, L. D.; de Mattos, M. C.; Freire, D. M. G.; da Silva, I. J.; Rodríguez-Aguado, E.; Gonçalves, L. R. B. Strategies of Covalent Immobilization of a Recombinant *Candida antarctica* Lipase B on Pore-Expanded SBA-15 and Its Application in the Kinetic Resolution of (R,S)-Phenylethyl Acetate. *J. Mol. Catal. B Enzym.* **2016**, *133*, 246–258. https://doi.org/10.1016/j.molcatb.2016.08.009.

(11) Bautista, L. F.; Vicente, G.; Mendoza, Á.; González, S.; Morales, V. Enzymatic Production of Biodiesel from *Nannochloropsis gaditana* Microalgae Using Immobilized Lipases in

Mesoporous Materials. *Energy and Fuels* **2015**, *29* (8), 4981–4989. https://doi.org/10.1021/ef502838h.

(12) Abdullah, A. Z. Z.; Sulaiman, N. S. S.; Kamaruddin, A. H. H. Biocatalytic Esterification of Citronellol with Lauric Acid by Immobilized Lipase on Aminopropyl-Grafted Mesoporous SBA-15. *Biochem. Eng. J.* **2009**, *44* (2–3), 263–270. https://doi.org/10.1016/j.bej.2009.01.007.

(13) Canilho, N.; Jacoby, J.; Pasc, A.; Carteret, C.; Dupire, F.; Stébé, M. J.; Blin, J. L. Isocyanate-Mediated Covalent Immobilization of *Mucor miehei* Lipase onto SBA-15 for Transesterification Reaction. *Colloids Surf. B. Biointerfaces* **2013**, *112*, 139–145. https://doi.org/10.1016/j.colsurfb.2013.07.024.

(14) Zhou, Z.; Piepenbreier, F.; Marthala, V. R. R.; Karbacher, K.; Hartmann, M. Immobilization of Lipase in Cage-Type Mesoporous Organosilicas via Covalent Bonding and Crosslinking. *Catal. Today* **2015**, *243* (C), 173–183. https://doi.org/10.1016/j.cattod.2014.07.047.

(15) Zniszczoł, A.; Herman, A. P.; Szymańska, K.; Mrowiec-Białoń, J.; Walczak, K. Z.; Jarzebski, A.; Boncel, S. Covalently Immobilized Lipase on Aminoalkyl-, Carboxy- and Hydroxy-Multi-Wall Carbon Nanotubes in the Enantioselective Synthesis of Solketal Esters. *Enzyme Microb. Technol.* **2016**, *87–88*, 61–69. https://doi.org/10.1016/j.enzmictec.2016.02.015.

(16) Dhawane, S. H.; Kumar, T.; Halder, G. Recent Advancement and Prospective of Heterogeneous Carbonaceous Catalysts in Chemical and Enzymatic Transformation of Biodiesel. *Energy Convers. Manag.* **2018**, *167*, 176–202. https://doi.org/10.1016/J.ENCONMAN.2018.04.073.

(17) Rios, N. S.; Pinheiro, M. P.; Lima, M. L. B.; Freire, D. M. G.; da Silva, I. J.; Rodríguez-Castellón, E.; de Sant'Ana, H. B.; Macedo, A. C.; Gonçalves, L. R. B. Pore-Expanded SBA-15 for the Immobilization of a Recombinant *Candida antarctica* Lipase B: Application in Esterification and Hydrolysis as Model Reactions. *Chem. Eng. Res. Des.* **2018**, *129*, 12–24. https://doi.org/10.1016/j.cherd.2017.10.032.

(18) Ashjari, M.; Mohammadi, M.; Badri, R. Selective Concentration of Eicosapentaenoic Acid and Docosahexaenoic Acid from Fish Oil with Immobilized/Stabilized Preparations of Rhizopus oryzae Lipase. *J. Mol. Catal. B Enzym.* **2015**, *122*, 147–155. https://doi.org/10.1016/j.molcatb.2015.08.017.

(19) Lee, J. S.; Yim, J. H.; Jeon, J. K.; Ko, Y. S. Polymerization of Olefins with Single-Site Catalyst Anchored on Amine-Functionalized Surface of SBA-15. *Catal. Today* **2012**, *185* (1), 175–182. https://doi.org/10.1016/j.cattod.2011.12.003.

(20) Yiu, H. H. P.; Wright, P. A.; Botting, N. P. Enzyme Immobilisation Using SBA-15 Mesoporous Molecular Sieves with Functionalised Surfaces. *J. Mol. Catal. Enzym.* **2001**, *15* (1–3), 81–92. https://doi.org/10.1016/S1381-1177(01)00011-X.

(21) Tongboriboon, K.; Cheirsilp, B.; H-Kittikun, A. Mixed Lipases for Efficient Enzymatic Synthesis of Biodiesel from Used Palm Oil and Ethanol in a Solvent-Free System. *J. Mol. Catal. B Enzym.* **2010**, *67* (1–2), 52–59. https://doi.org/10.1016/J.MOLCATB.2010.07.005.

(22) Guldhe, A.; Singh, B.; Rawat, I.; Permaul, K.; Bux, F. Biocatalytic Conversion of Lipids from Microalgae *Scenedesmus Obliquus* to Biodiesel Using *Pseudomonas fluorescens* Lipase. *Fuel* **2015**, *147*, 117–124. https://doi.org/10.1016/J.FUEL.2015.01.049.

(23) He, Y.; Wu, T.; Wang, X.; Chen, B.; Chen, F. Cost-Effective Biodiesel Production from Wet Microalgal Biomass by a Novel Two-Step Enzymatic Process. *Bioresour. Technol.* **2018**, *268*,

583–591. https://doi.org/10.1016/J.BIORTECH.2018.08.038.

(24) Guldhe, A.; Singh, P.; Renuka, N.; Bux, F. Biodiesel Synthesis from Wastewater Grown Microalgal Feedstock Using Enzymatic Conversion: A Greener Approach. *Fuel* **2019**, *237*, 1112–1118. https://doi.org/10.1016/J.FUEL.2018.10.033.

(25) Bauer, G.; Lima, S.; Chenevard, J.; Sugnaux, M.; Fischer, F. Biodiesel via in Situ Wet Microalgae Biotransformation: Zwitter-Type Ionic Liquid Supported Extraction and Transesterification. **2017**. https://doi.org/10.1021/acssuschemeng.6b02665.

(26) Castillo López, B.; Esteban Cerdán, L.; Robles Medina, A.; Navarro López, E.; Martín Valverde, L.; Hita Peña, E.; González Moreno, P. A.; Molina Grima, E. Production of Biodiesel from Vegetable Oil and Microalgae by Fatty Acid Extraction and Enzymatic Esterification. *J. Biosci. Bioeng.* **2015**, *119* (6), 706–711. https://doi.org/10.1016/J.JBIOSC.2014.11.002.

(27) Banerjee, A.; Singh, V.; Solanki, K.; Mukherjee, J.; Gupta, M. Combi-Protein Coated Microcrystals of Lipases for Production of Biodiesel from Oil from Spent Coffee Grounds. *Sustain. Chem. Process.* **2013**, *1* (1), 14. https://doi.org/10.1186/2043-7129-1-14.

(28) Alves, J. S.; Vieira, N. S.; Cunha, A. S.; Silva, A. M.; Záchia Ayub, M. A.; Fernandez-Lafuente, R.; Rodrigues, R. C. Combi-Lipase for Heterogeneous Substrates: A New Approach for Hydrolysis of Soybean Oil Using Mixtures of Biocatalysts. *RSC Adv.* **2014**, *4* (14), 6863–6868. https://doi.org/10.1039/C3RA45969A.

(29) Bajaj, A.; Lohan, P.; Jha, P. N.; Mehrotra, R. Biodiesel Production through Lipase Catalyzed Transesterification: An Overview. *J. Mol. Catal. B Enzym.* **2010**, *62* (1), 9–14. https://doi.org/10.1016/J.MOLCATB.2009.09.018.

(30) Lee, H. W.; Cho, H. J.; Yim, J. H.; Kim, J. M.; Jeon, J. K.; Sohn, J. M.; Yoo, K. S.; Kim, S. S.; Park, Y. K. Removal of Cu(II)-Ion over Amine-Functionalized Mesoporous Silica Materials. *J. Ind. Eng. Chem.* **2011**, *17* (3), 504–509. https://doi.org/10.1016/j.jiec.2010.09.022.

(31) Serra, E.; Mayoral, Á.; Sakamoto, Y.; Blanco, R. M.; Díaz, I. Immobilization of Lipase in Ordered Mesoporous Materials: Effect of Textural and Structural Parameters. *Microporous Mesoporous Mater.* **2008**, *114* (1–3), 201–213. https://doi.org/10.1016/j.micromeso.2008.01.005.

(32) Sun, Q.; Fu, C. W.; Aguila, B.; Perman, J.; Wang, S.; Huang, H. Y.; Xiao, F. S.; Ma, S. Pore Environment Control and Enhanced Performance of Enzymes Infiltrated in Covalent Organic Frameworks. *J. Am. Chem. Soc.* **2018**, *140* (3), 984–992. https://doi.org/10.1021/jacs.7b10642.

(33) Rabbani, G.; Ahmad, E.; Khan, M. V.; Ashraf, M. T.; Bhat, R.; Khan, R. H. Impact of Structural Stability of Cold Adapted *Candida antarctica* Lipase B (CaLB): In Relation to pH, Chemical and Thermal Denaturation. *RSC Adv.* **2015**, *5* (26), 20115–20131. https://doi.org/10.1039/C4RA17093H.

(34) Gómez-Orozco, S. Y.; Huirache-Acuña, R.; Pawelec, B.; Fierro, J. L. G.; Rivera-Muñoz, E. M.; Lara-Romero, J.; Alonso-Nuñez, G. Characterizations and HDS Performances of Sulfided NiMoW Catalysts Supported on Mesoporous Titania-Modified SBA-15. *Catal. Today* **2018**, *305*, 152–161. https://doi.org/10.1016/j.cattod.2017.08.009.

(35) Mohammadi M.; Habibi, Z.; Gandomkar, S.; Yousefi, M. A Novel Approach for Bioconjugation of *Rhizomucor Miehei* Lipase (RML) onto Amine-Functionalized Supports; Application for Enantioselective Resolution of Rac-Ibuprofen. *Int. J. Biol. Macromol.* **2018**. https://doi.org/10.1016/j.procbio.2018.02.010.

(36) Zhao, X. S.; Lu, G. Q.; Whittaker, A. K.; G. J. Millar, A.; Zhu, H. Y. Comprehensive Study of

Surface Chemistry of MCM-41 Using 29Si CP/MAS NMR, FTIR, Pyridine-TPD, and TGA. *J. Phys. Chem. B* **1997**, *33*, 6525–6531. https://doi.org/10.1021/JP971366+.

(37) Wang, C.; Zhou, G.; Li, Y.-J.; Lu, N.; Song, H.; Zhang, L. Biocatalytic Esterification of Caprylic Acid with Caprylic Alcohol by Immobilized Lipase on Amino-Functionalized Mesoporous Silica. *Colloids Surfaces A Physicochem. Eng. Asp.* **2012**, *406*, 75–83. https://doi.org/10.1016/J.COLSURFA.2012.04.053.

(38) Simonutti, R.; Comotti, A.; Bracco, S.; Sozzani, P. Surfactant Organization in MCM-41 Mesoporous Materials As Studied by 13C and 29Si Solid-State NMR. *Chem. Mater.* **2001**, *13* (3), 771–777. https://doi.org/10.1021/cm001088i.

(39) Guncheva, M.; Yancheva, D.; Ossowicz, P.; Janus, E. Structural Basis for the Inactivation of *Candida rugosa* Lipase in the Presence of Amino Acid Ionic Liquids. *Bulg. Chem. Commun.* **2017**, *49*, 132–136.

(40) Alves, J.; S. Vieira, N.; S. Cunha, A.; M. Silva, A.; Ayub, M.; Fernandez-Lafuente, R.; Rodrigues, R. Combi-Lipase for Heterogeneous Substrates: A New Approach for Hydrolysis of Soybean Oil Using Mixtures of Biocatalysts. *RSC Adv.* **2014**, *4*, 6863–6868. https://doi.org/10.1039/c3ra45969a.

(41) Amini, Z.; Ilham, Z.; Ong, H. C.; Mazaheri, H.; Chen, W.-H. State of the Art and Prospective of Lipase-Catalyzed Transesterification Reaction for Biodiesel Production. *Energy Convers. Manag.* **2017**, *141*, 339–353. https://doi.org/10.1016/j.enconman.2016.09.049.

(42) Galgali, A.; Gawas, S. D.; Rathod, V. K. Ultrasound Assisted Synthesis of Citronellol Laurate by Using Novozym 435. *Catal. Today* **2018**, *309*, 133–139. https://doi.org/10.1016/j.cattod.2017.08.052.

(43) Piligaev, A. V.; Sorokina, K. N.; Samoylova, Y. V.; Parmon, V. N. Lipid Production by Microalga Micractinium Sp. IC-76 in a Flat Panel Photobioreactor and Its Transesterification with Cross-Linked Enzyme Aggregates of *Burkholderia cepacia* Lipase. *Energy Convers. Manag.* **2018**, *156* (August 2017), 1–9. https://doi.org/10.1016/j.enconman.2017.10.086.

(44) Navarro López, E.; Robles Medina, A.; González Moreno, P. A.; Esteban Cerdán, L.; Martín Valverde, L.; Molina Grima, E. Biodiesel Production from *Nannochloropsis gaditana* Lipids through Transesterification Catalyzed by Rhizopus oryzae Lipase. *Bioresour. Technol.* **2016**, *203*, 236–244. https://doi.org/10.1016/j.biortech.2015.12.036.

(45) Lai, J. Q.; Hu, Z. L.; Wang, P. W.; Yang, Z. Enzymatic Production of Microalgal Biodiesel in Ionic Liquid [BMIm][PF 6]. *Fuel* **2012**, *95*, 329–333. https://doi.org/10.1016/j.fuel.2011.11.001.

(46) Zhao, D.; Feng, J.; Huo, Q.; Melosh, N.; Glenn, H.; Chmelka, B. F.; Stucky, G. D.; Zhao, D.; Feng, J.; Huo, Q.; et al. Triblock Copolymer Syntheses of Mesoporous Silica with Periodic 50 to 300 Angstrom. *Science (80-.).* **1998**, *279* (5350), 548–552.

(47) Salis, A.; Pinna, M.; Monduzzi, M.; Solinas, V. Biodiesel Production from Triolein and Short Chain Alcohols through Biocatalysis. *J. Biotechnol.* **2005**, *119* (3), 291–299. https://doi.org/10.1016/j.jbiotec.2005.04.009.

(48) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72* (1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

(49) Jaiswal, S. K.; Tejo Prakash, N.; Prakash, R. 1 H NMR Based Quantification of 1 H NMR Based Quantification of Ethyl Ester in Biodiesel: A Comparative Study of Product-Dependent

Derivations. *Anal. Chem. Lett.* **2016**, *6* (5), 518–525. https://doi.org/10.1080/22297928.2016.1246977.

(50) Panreac Quimica, S. . *Métodos Analíticos En Alimentaria*; 1999.