Journal of Cleaner Production 139 (2016) 495–503

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Towards sustainable biofuel production: Design of a new biocatalyst to biodiesel synthesis from waste oil and commercial ethanol

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ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
9 August 2016
Accepted 9 August 2016
Available online 18 August 2016

Keywords:
Biodiesel
Enzymatic immobilization
SBA-15
Soybean
Sunflower and friyng oils
Commercial ethanol
Biocatalysis
Alternative energies

ABSTRACT

The sustainable technologies to produce alternative energies as biofuel with focus on the harnessing of renewable sources and waste biomass is gradually gaining ground. Although the biodiesel is a very attractive biofuel its production from vegetable oils competes with the feed generation with the consequent socio-economic costs involved. Therefore, the reuse of waste oils appears as an alternative highly promising. However the high content of free fatty acid and water in this raw material difficult its use in the conventional processes employed in the actuality. In this work, we successfully develop hybrid catalysts based in *Pseudomonas fluorescens* lipase immobilized over Ca and Na modified mesoporous SBA-15 supports. The physic-chemical properties of the supports were determined by Small-angle X-ray Scattering (SAXS), Transmission Electron Microscopy (TEM), Scanning Electron Microscope (SEM), Infrared Spectroscopy (IR), Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP) and N_2 adsorption measurements. These hybrid catalysts were capable to process waste oils, and even, commercial ethanol (96%) obtained from a fermentative process An optimum activity, with around 90% of FAEE yield, was achieved with lipase immobilized on Ca modified SBA-15 using 4% of water respect to oil, 1:4 oil/ethanol ratio, 400 mg/g of enzyme immobilized, at 37 °C and 180 oscillations/min. This catalyst could contribute to development of a more environmentally and economically viable process to biodiesel production.

1. Introduction

The consciousness of sustainable technologies is gradually gaining ground. The need to replace the non-renewable fossil fuel by an alternative energy has been the objective of extensive research in recent years (Araújo, 2014). Biodiesel has been identified as one of the most notable options to replace or at least to complement conventional fuels consume (Aransiola et al., 2014). As biodiesel is derivate from vegetable oil or animal fats, it is renewable and biodegradable. Its use reduces the energy dependence on petroleum and most exhaust emissions (with the exception of nitrogen oxides, NO_x) resulting in an environmental benignity. Biodiesel presents a higher flash point, leading to safer handling and storage; therefore, it also can be stored and transported using diesel tanks and equipment. Since biodiesel molecule is oxygenated, it is a better lubricant than diesel fuel, increasing the life of engines, and is combusted more completely (Knothe et al., 2005; Vasudevan and Briggs, 2008).

Biodiesel can be produced by different kinds of raw materials (refined, crude, non edible or frying oils) and with different types of catalysts, such as basic compounds (sodium or potassium hydroxides), acids (sulfuric acid, ion exchange resins), heterogeneous solids (zeolites, CaO), enzymes (lipases) and supercritical fluids.

Currently, the homogeneous catalysis with sodium hydroxide and methanol is the most used for the biodiesel production (Vasudevan and Briggs, 2008). However, the alcohol must be substantially anhydrous and vegetable oil must have a low free fatty acid (FFA) content, because the presence of water lowers the activity of catalyst and FFA reacts with the catalyst to produce soaps. The formation of soaps reduces the biodiesel yield, and difficulty the product separation and purification (Freedman et al., 1984). In fact, the purification process represents between 60 and 80% of the

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http://dx.doi.org/10.1016/j.jclepro.2016.08.047
0959-6526/

biodiesel production cost (Atadashi et al., 2011; Wassell and Dittmer, 2006). Ineffective biodiesel separation and purification cause severe diesel engine problems such as plugging of filters, coking on injectors, more carbon deposits, excessive engine wear, oil ring sticking, engine knocking, and thickening and gelling of lubricating oil (Demirbas, 2009).

Then, the high consumption of energy and separation costs in the homogeneous process requires the development of heterogeneous catalysts for transesterification reaction, which can be easily separated from the reaction mixture and recycled. Several authors have used heterogeneous catalysts in order to eliminate the neutralization and washing steps needed in the homogeneous processes but they were faced with major problems such as higher temperature of transesterification reaction or catalyst deactivation (Atadashi et al., 2011; Chew and Bhatia, 2008; Dias et al., 2013).

On the basis of the above and in order to achieve a truly environmentally-friendly production of biodiesel, the enzyme immobilization appears as a promising technology to obtain a solid as biocatalyst. The use of enzyme-catalyzed transesterification reactions avoids drawbacks such as feedstock pretreatment, catalyst removal, continuous use, prevention of product contamination, reduction of effluent problems, material handling and high-energy requirement with respect to conventional homogeneous catalysts (Stoytcheva et al., 2011). The enzymes that present this activity are called lipases (EC 3.1.1.3 triacylglycerol acylhydrolase) and represent a group of water soluble enzymes that originally catalyze the hydrolysis of ester bonds in water insoluble lipid substrates. A disadvantage of use the free enzyme in non-aqueous media, is that these free enzymes tend to form aggregates that difficult the optimum subtract flux to inner of the same and therefore only the enzyme molecules present on the aggregate surface can work (Lawson et al., 2004). These lipases can be immobilized on several materials to improve enzyme stability and reusability obtaining biocatalysts with high selectivity, efficiency and yield into methyl/ethyl esters in milder reaction conditions (Lima et al., 2015; Liu et al., 2010; Salis et al., 2008). Moreover, the glycerol recovered via this enzymatic process has a higher grade of purity compared to that one obtained from the conventional alkaline process.

On the other hand, to achieve the maximum of active sites working and the full catalytic power, it is convenient to disperse the enzyme on high specific areas like those of mesoporous materials. These usually affect the enzymatic performance given its specific properties such as the pore size and the surface nature. To exploit all surface of the material and to avoid the enzyme leaching, the pore size of material has to be similar to the enzyme size. This allows preserving enzyme activity and specificity (Salis et al., 2010, 2009; Tran and Balkus, 2011). SBA-15 is one of the most popular supports to enzyme immobilization because they are highly ordered materials, with pore sizes in the mesopore range, high areas, and large pore volumes (Alam et al., 2010; Hudson et al., 2005). Moreover, the possibility of surface modification with different species confers new properties to the support that can affect the enzyme performance (Kim et al., 2006; Salis et al., 2009; Tran and Balkus, 2011), opening the gates to a very interesting area to explore.

The present work is aimed at evaluating the role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase. Enzyme immobilization on pure SBA-15 material and modified with calcium (Ca/SBA-15) was evaluated in the ethanolysis of sunflower, soybean and waste frying oil as a high industrially interested reaction to obtain biodiesel. Interestingly, the biocatalyst worked efficiently with commercial ethanol and the mentioned oil sources, which represent important economic and ecologic advantages over homogeneous catalysts.

2. Experimental section

2.1. Chemicals

The lipase from *Pseudomonas fluorescens* (PFL, ≥20,000 IU/g at 55 °C, pH 8.0) was acquired from Sigma-Aldrich Co. (St. Louis, USA). This is an enzyme with a high lipolytic activity produced by "Amano Labs". This enzyme was characterized by the Amano researchers and has a molecular weight of about 33 kD, an isoelectric point of pI = 4, a pH stability range 4 < pH < 10, and an optimum pH of activity in the range 8 < pH < 10 (Amano, 2008).

Sunflower oil used ("Vicentin" brand) was acquired in a local store; this oil is in agreement with the Argentinean specifications for food oil. The soybean oil used was graciously ceded by Bunge Argentina S.A. The waste frying oil was obtained from a voluntary collection of waste frying oil from different domestic sources. The reagents employed were KH_2PO_4, K_2HPO_4, (Anedra), ($Ca(NO_3)_2$) and $NaNO_3$ (Cicarelli), absolute ethanol 99.8% (analytical grade, Taurus), commercial bioethanol 96% v/v (Porta Hnos.), hydrochloric acid (HCl) (analytical grade, Cicarelli), n-Hexane (analytical grade, Merck), isopropanol (analytical standard, FLUKA), acetonitrile (analytical grade, Merck) and miliQ water. Syringe filters (polypropylene, 25 mm diameter and with 0.2 μm of pore size) were supplied by VWR.

2.2. Synthesis and modification of SBA-15

Pure SBA-15 was synthesized dissolving 4.0 g of Pluronic P123 in 30 g of water and 120 g of 2 M HCl solution with stirring at 40 °C. Then 8.50 g of TEOS were added into this solution with stirring at 40 °C for 20 h. The mixture was aged at 100 °C overnight without stirring. The solid product was filtered, washed, and air-dried at 60 °C overnight. Calcination was carried out by a rate of 1 °C/min to 500 °C and heating at 500 °C for 6 h. Modified material with metals was obtained using the wet impregnation method. Aqueous solutions of metal salt (($Ca(NO_3)_2$) and $NaNO_3$) with different concentrations were used to reach theoretical metal loadings between 2.5 and 15 wt%. The SBA-15 host (0.75 g) was dispersed in the precursor solution at room temperature and then, the solvent (water) was removed slowly by rotary evaporation at 50 °C for 30 min. The resulting powder was dried at 60 °C and calcined for 8 h at 500 °C to obtain the modified material (Elías et al., 2011). The samples were named as M/SBA-15(x) where "M" is the metal used and "x" is the theoretical loading in wt%.

2.3. Material characterization

SAXS analysis was carried out at the SAXS-1 beamline at the Brazilian Synchrotron Light Laboratory (LNLS) at Campinas, Brazil. The detector was a Pilatus 300 k from Dectris. Typical accumulation times were 1–10 s, the sample-detector distances were between 80 and 95 cm, and the wavelength was 1.55 Å. The empty Kapton cell was measured and subtracted from the signals after normalization. Data was radially integrated by using FIT2D V 12.077 from Andy Hammersley at ESRF. Powder X-ray diffraction patterns (XRD) of the samples were recorded in a PANalytical X'Pert Pro diffractometer with Cu Kα radiation (λ = 1.5418 Å) in the range of 2θ from 20° to 80°. SEM micrographs were obtained in a JEOL model JSM 6380 LV. Gold coverage was applied to make samples conductive. The acceleration voltage was 20 kV. TEM images were obtained in a JEOL Model JEM-1200 EXII System, working voltage: 120 kV. A small drop of the dispersion (sample in water-ethanol 50% solution) was deposited on the copper grid and then evaporated in air at room temperature. The calcium and sodium content in the samples was determined by Inductively Coupled Plasma Atomic Emission

Spectroscopy (ICP) using a spectrophotometer VISTA-MPX CCD Simultaneous ICP-OES-VARIAN. The specific surface was determined using a Micromeritics Pulse Chemisorb 2700. Samples were previously dried using a N_2 flux for 3 h at 350 °C. The specific surface was determined by the Brunauer-Emmett-Teller (BET) method. The organic species on the samples were detected by infrared spectroscopy using a JASCO-FT/IR-5300 at room temperature. Spectra were recorded between 400 and 4600 cm^{-1}.

2.4. Immobilization of Pseudomonas fluorescens lipase on support

Five solutions with different concentration (in the range of 5–10 mg/mL) of the commercial lipase were prepared. The same amount of support powder was suspended in each solution to obtain five different preparations with different loadings, namely: 400, 480, 600, 800 and 1000 $mg_{protein}/g_{support}$. In a typical procedure, pure or modified SBA-15 (125 mg) was suspended in 10 mL of enzyme solution in 25 mM phosphate buffer (pH 8). The suspension was kept under gentle stirring at room temperature by 24 h and then centrifuged and washed two times with 10 mL of 25 mM phosphate buffer (pH 8). Determination of protein content was carried out according to the Bradford assay [48]. The supernatant of the first centrifugation (10 μL) was mixed with a mixture of 200 μL of water and 50 μL Bradford reagent. After exactly 6 min, the absorbance at λ = 595 nm was determined by means of a Cary 50 spectrophotometer. Protein content was estimated by means of a calibration curve obtained using concentrations of 0.05, 0.1,0.25, 0.5 and 1 mg/mL of BSA (98%) as protein standard (Protein and Protocol, 1976). Then, hybrid materials obtained from an enzymatic immobilization of 400 $mg_{protein}/mg_{support}$ were named as L_{PF}/M/SBA-15(x), where "M" is the metal used and "x" is the theoretical loading in wt%. An abbreviation description table (Table 2) is shown in supplementary material.

In order to study the calcium effect on the enzymatic activity, lipase (50 mg) was pre incubated with solutions of $CaCl_2$ (5 and 8 mM) and NaCl (5 mM) in 25 mM Tris-HCL buffer pH = 8. Then 125 mg of pure SBA-15 was added and the enzyme immobilization procedure was carried out as it was indicated above.

2.5. Biocatalytic ethanolysis of oils

The reaction mixture was obtained by mixing oil and ethanol in an alcohol/oil molar ratio = 4/1. Water content in the reaction mixture varied from 0 to 5 wt% with respect to oil. The reactions were carried out at 37 °C and started by adding either L_{PF}/M/SBA-15(x) (87.5 or 175 mg/g oil) or free enzyme (50 mg/g oil) to the substrates mixture. Reaction vials were shaken at 180 oscillations/min through a horizontal shaker. Samples (20 μL) were collected at different times. The resulting mixtures were diluted to a volume of 1 mL with acetonitrile, filtered with a 0.45 μm pore size filter and analyzed by HPLC. All reactions were performed at least in duplicate and the results were expressed as mean values with relative percentage differences between them always less than 5% of the mean.

2.6. Chromatographic analysis (HPLC)

The analyses were conducted with a HPLC Perkin Elmer with UV–vis detector Series 200 equipped with a solvent delivery unit for binary gradient elution, a column Agilent Eclipse Plus 18 (C18 with a diameter of 4.6 mm × 25 cm length and a particle size of 5 μm) and the TotalChrom software for remote management and quantification.

The wavelength of the UV detector was set at 210 nm, the column temperature was maintained at 30 °C during the assays and the flow rate was 1 ml/min. For the quantification methyl-heptadecanoate was used as internal standard at a final concentration of 10 mg/ml in acetonitrile. For the chromatographic runs, we used a gradient chromatographic technique consisting of the following: 6 min of 30%/70% water/acetonitrile, 10 min of 100% acetonitrile, 15 min of 80%/20% acetonitrile/isopropanol-n-henane (5/4), 29 min in gradient up 30%/70% acetonitrile/isopropanol-n-henane (5/4).

2.7. Lipid extraction and profiling

The fatty acid composition of the oils were determined by gas–liquid chromatography of the fatty acid methyl esters (FAMEs). The FAMEs were obtained by transesterification with a cold methanolic solution of potassium hydroxide. Oil samples (0.1 g) were diluted in 2 ml hexane, to which 0.2 ml 2 N methanolic potassium hydroxide solution was added. After stratification, 1 μl from the upper layer, containing the fatty acid methyl esters, was injected into a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer Life and Analytical Sciences, Shelton, CT 06484, USA) fitted with a 25 m capillary column (CP-Wax 52 CB; Chrompack, Holland) with a 0.32 mm I.D., 0.25 μm film thickness and equipped with split injection and an FID detector. Injector and detector temperatures were set to 250 and 300 °C, respectively. Oven temperature was programmed at 180 °C for 5 min, increased from 180 to 240 °C at 4 °C min^{-1}, and then was set at 240 °C for 10 min. Individual fatty acids were determined by comparison with retention times of known standards (AOCS-1, Sigma–Aldrich, St. Louis, MO). Fatty acids are expressed as the relative percentage of each individual fatty acid of the total fatty acids present in the sample. All determinations were performed in triplicate.

3. Results and discussion

3.1. Support characterization

The structural and textural characterization of the mesoporous supports was obtained by small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and power XRD patterns in the high angle region. The scattering vector q is employed in SAXS analysis and it is related to the diffraction angle (θ) by using the Bragg equation, $\lambda = 2d\,\theta$, as followed: $q = 4\pi \sin\theta/\lambda$, where λ is 1.5418 Å. The corresponding spectra of pure SBA-15 and modified with a theoretical calcium content of 2,5% show the presence of well-defined peaks in the low angle range that can be indexed to the (100), (110), and (200) planes and are associated to the presence of a highly ordered porous structure with hexagonal pore array (Fig. 1a). Moreover, as it can be observed, the calcium impregnation on SBA-15 did not alter the highly ordered mesoporous structure. In Fig. 1b, the powder XRD pattern in the high angle region of the Ca/SBA-15(2,5) sample exhibited the typical reflections of cubic CaO. Thus, narrow peaks indicated the existence of oxide crystallites on the surface of the mesoporous support.

TEM images for SBA-15 and Ca/SBA-15(2.5) samples are presented in Fig. 2. In these images, well-ordered parallel nanotubular pores, could be clearly observed along the long axis. These images can be taken when the electron beam is perpendicular to the main axis of the cylindrical pores. However, when the electron beam is parallel to pore axis, their frontal views could be observed (see circle in Fig. 2) accounting for the good structure of the obtained solids. Thus, the regular hexagonal array of uniform channels in which each pore is surrounded by six neighbors could be clearly observed. These results demonstrated that the hexagonal array of the original mesostructured SBA-15 silica is preserved throughout

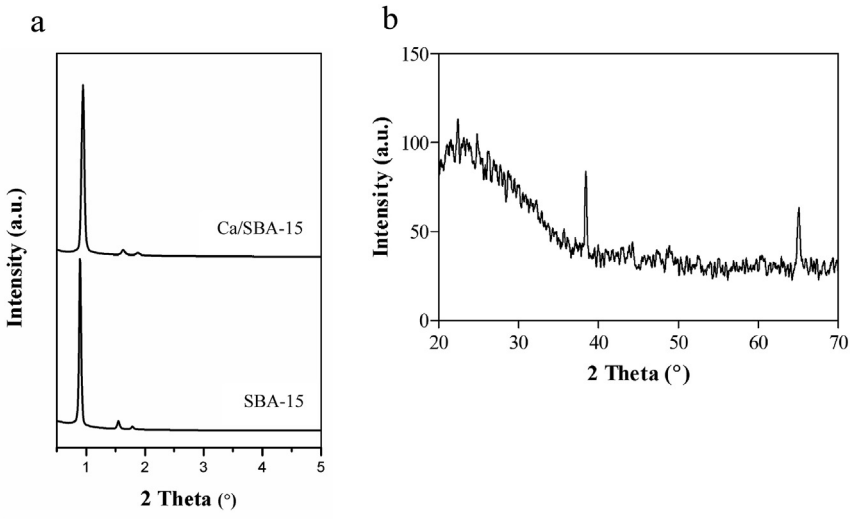

Fig. 1. Structural characterization of mesoporous supports: a) SAXS patterns for pure and Ca/SBA-15(2,5), b) XRD patterns at high angle of Ca/SBA-15(2.5).

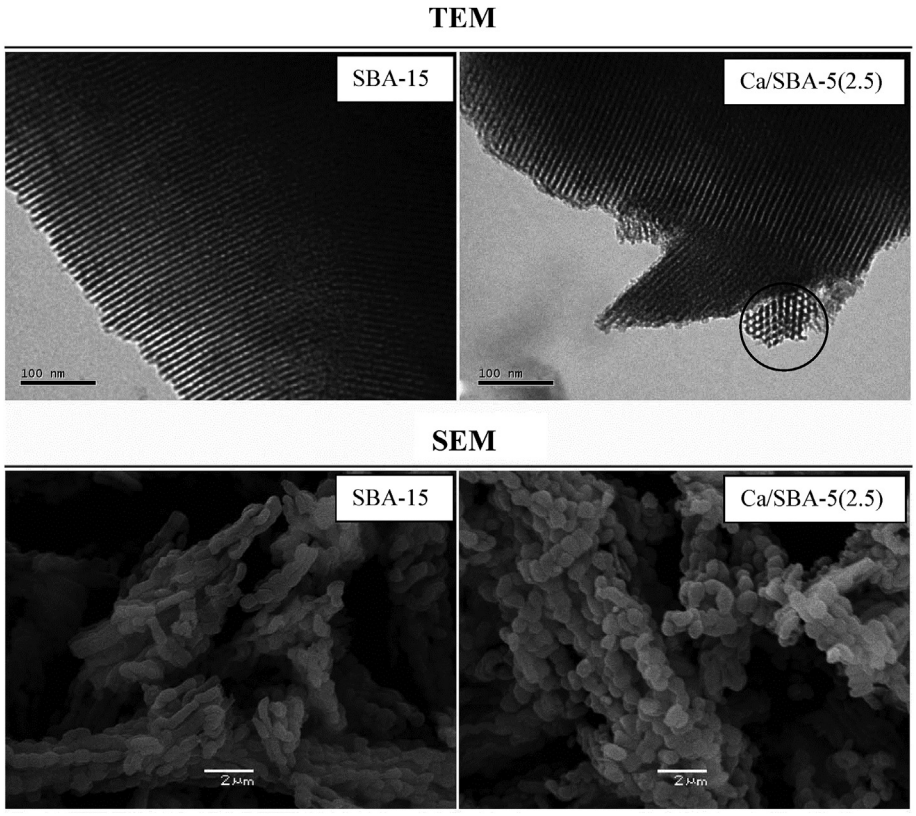

Fig. 2. TEM and SEM images of SBA-15 and Ca/SBA-15.

chemical modification of its surface with calcium. In the SEM images of SBA-15 and Ca/SBA-15(2.5), an irregular morphology formed by agglomerates was observed. This fact suggests the confluence and aggregation of primary small particles that form micron-sized aggregates as it has previously been proven by other researchers (Prasetyanto and Park, 2008).

The specific surface obtained from SBA-15 and the Ca/SBA-15(2.5) was 794 m^2/g and 481 m^2/g respectively. This decrease in the specific surface could be attributed to the presence of CaO crystallites blocking some mesopores in the Ca/SBA-15(2.5). However, the pore size was similar for both materials and around 7 nm. Therefore, good area values and a pore size that matches with the enzyme size can favor a suitable immobilization. It is important to highlight here that a host with pore sizes smaller or larger than enzymes presents disadvantages. In the first case, the enzyme is just adsorbed on the external surface and the interior surface is not accessible. In the second case, the enzyme can move freely in the pores and is susceptible to leaching in the process.

Finally, the calcium content in Ca/SBA-15 (2.5) determined by ICP was 1.96% wt. This metal increases the hydrophilic behavior

which could affect the enzyme immobilization and activity.

3.2. Immobilization of Pseudomonas fluorescens lipase

The behavior of the immobilized enzyme depends on several factors as: chemical composition of the support, hydrophilic or hydrophobic groups, pore size, specific surface and kind of immobilization (Tran and Balkus, 2011). In this work, the physical adsorption was used to immobilize the *Pseudomonas fluorescens* lipase according to Salis et al. (Salis et al., 2010).

Firstly, the performance of enzyme immobilized on the SBA-15 (L_{PF}/SBA-15) was determined with respect to the free enzyme. Taking account that the enzymes work at an initial rate that is approximately linear for a short period after the start of the reaction, in our conditions, the reaction was stopped after 2 h. It is to work in a linear condition to the enzymatic reaction and be able to compare the several catalysts activities.

Fig. 3 shows the FAEE yield in function of the amount of enzyme loaded on pure SBA-15, at ethanol/sunflower oil molar ratio = 4/1, 4 wt% of water with respect to oil, 87.5 mg of catalyst/g of oil and 37 °C. As it is shown, the FAEE yield increases when the enzyme quantity loaded increases. However, from 600 $mg_{protein}/g_{support}$ the protein amount determined on supernantante also increases, indicating that no all protein was bound on the material. Thus, the protein in the selected loading in this work was 400 $mg_{protein}/g_{support}$ (L_{PF}/SBA-15), because it leads to higher activity than a loading of 480 $mg_{protein}/g_{support}$ and protein was not detected in the supernantante.

3.3. Lipase activity in function of water content and catalyst concentration in the reaction mixture

Lipases cover their active sites by an amphiphilic peptidic loop (like a lid or flap) that makes it inaccessible to substrates in this closed conformation. However, the lipase architecture changes in the presence of an oil/water hydrophobic interface and adopt a lid opening conformation. This permits the subtract access to the active sites. Therefore, the water is necessary to lipase activity and its concentration in the reaction medium must be carefully considered (Salis et al., 2010; Schmid and Verger, 1998; Stoytcheva et al., 2011). For this reason, the effect of water concentration in the medium on the ethanolysis reaction was evaluated from 0 to 5 wt% with respect to oil mass, using sunflower oil, absolute ethanol and 87.5 mg of L_{PF}/SBA-15/g of oil. As it is shown in Fig. 4, the activity of

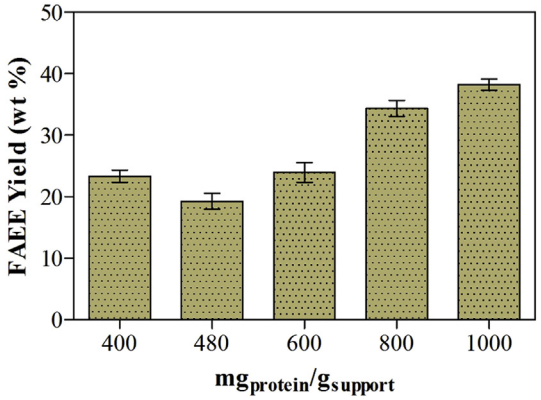

Fig. 3. Effect of enzyme loading over support on the transesterification activity. Reaction conditions: ethanol/oil molar ratio = 4/1; 4 wt% of water with respect to oil; 87.5 mg of catalyst/g of oil; sunflower oil and absolute ethanol as reactives; 37 °C, reaction time = 2 h and constant shaking (180 oscillations/min).

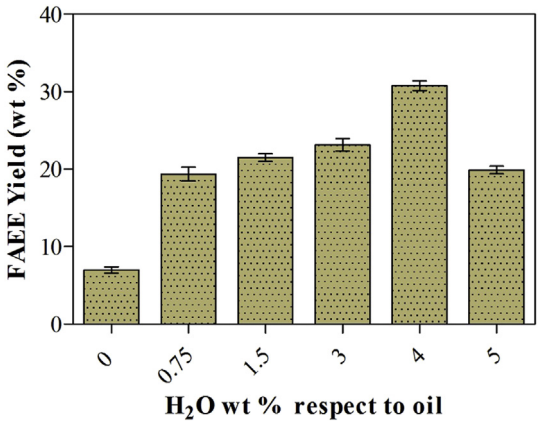

Fig. 4. Effect of water content on transesterification activity of L_{PS}/SBA-15 (400 $mg_{protein}/g_{support}$). Reaction conditions: ethanol/oil molar ratio = 4/1; 87.5 mg of catalyst/g of oil; sunflower oil and absolute ethanol as reactives; 37 °C, reaction time = 2 h and constant shaking (180 oscillations/min).

the enzyme immobilized on pure SBA-15 increased as the water concentration increased up to 4 wt%. Under such conditions, FAEE yield was 31 wt% compared with 7 wt% when the mixture does not contain water. When the optimum water concentration, 4 wt% respect to oil, is overcame the activity decreased as it was also observed by other authors (Salis et al., 2008).

On the other hand, the catalyst concentration in the reaction mixture is also a parameter to evaluate. Thus, when the L_{PS}/SBA-15 concentration was duplicate (175 mg of catalyst/g of oil), using the optimum water concentration, the FAEE yield increased from 31 wt % to 46 wt% in 2 h of reaction (datum shown in Fig. 5).

Finally the transesterification reaction conditions selected in this work were: ethanol/oil molar ratio = 4/1; 4 wt% of water with respect to oil; 175 mg of catalyst/g of oil (enzyme loading over support = 400 $mg_{protein}/g_{support}$), 37 °C and constant shaking (180 oscillations/min).

3.4. Modification of lipase activity by the support nature

As some authors mentioned, calcium ions can modify the lipase activity (Yu et al., 2007; Zheng et al., 2012). In order to evaluate this, *Pseudomonas Fluorescens* lipase was immobilized on SBA-15 modified with calcium (Ca/SBA-15(2.5)) and compared with enzyme immobilized on SBA-15. Using the selected reaction conditions and sunflower oil and absolute ethanol as reactives, the FAEE yield in function of time was evaluated (Fig. 5). As it can be

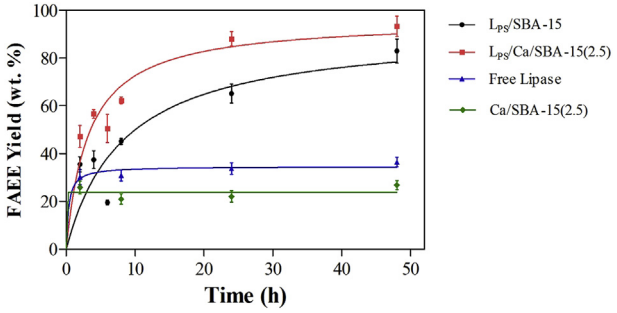

Fig. 5. FAEE yield comparison in function of time using different catalysts. Reaction conditions: ethanol/oil molar ratio = 4/1; 175 mg of catalyst/g of oil; 4 wt% of water with respect to oil; sunflower oil and absolute ethanol as reactives; 37 °C and constant shaking (180 oscillations/min).

seen, the enzyme immobilized on Ca/SBA-15 (L_{PS}/Ca/SBA-15(2.5)) present the major biodiesel yield at all reaction times. Thus, FAEE yield reached 88 wt% at 24 h and 93 wt% at 48 h of reaction. Meanwhile, when the L_{PS}/SBA-15 was employed, FAEE yield reached only a 65 wt% at 24 h and 83 wt% at 48 h. Similar behaviors have been reported in other systems (Salis et al., 2009; Shah and Gupta, 2007).

Moreover, as control, we have also determined the performance of the free enzyme and of the supports without absorbed enzyme (SBA-15 and Ca/SBA-15(2.5)). Free lipase forms aggregates during the reaction, which reduces the number of active sites exposed and as it is shown in Fig. 5 its activity also decreases. On the other hand, although SBA-15 didn't present transesterification activity (data not shown), Ca/SBA-15(2.5) showed some activity for the oil conversion, probably due to the basic character that the particles of CaO dispersed on the surface confer to the support (Albuquerque et al., 2008). In view of these results, a better catalytic performance of lipase immobilized on Ca/SBA-15(2.5) may be due to the calcium ions which active the enzyme (Zheng et al., 2012) or to the nature of the new species dispersed on the material.

3.5. Evaluation of calcium ions as lipase activators

The improvement of the catalytic performance of lipase adsorbed on Ca/SBA-15(2.5) with respect to lipase adsorbed on SBA-15 was evaluated by taking as hypothesis that metal ions of calcium can modify the lipase activity. To approach this, lipase was pre-incubated with two $CaCl_2$ solutions of 5 mM (suggested by bibliography (Yu et al., 2007; Zheng et al., 2012)) and 8 mM (in order to reproduce the Ca/L_{PF} molar ratio presented in the L_{PF}/Ca/SBA-15(2.5) catalyst) concentrations. Then, the pre-incubated lipase was loaded on SBA-15 and its activity, after 2 h of reaction, was compared with respect to L_{PF}/SBA-15 and L_{PF}/Ca/SBA-15(2.5) (Fig. 6). As it can be observed, none of the catalysts obtained from the enzyme pre-incubated with calcium achieved the L_{PF}/Ca/SBA-15(2.5) activity (33 wt%). Therefore, the behavior of these catalysts is similar to that of LPF/SBA-15, indicating that the calcium ions are not activating the enzyme and the presence of calcium species loaded on surface of support is necessary to increase the enzyme activity.

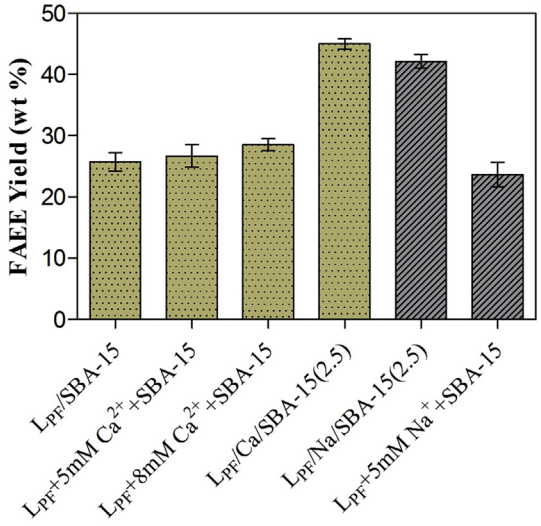

Fig. 6. Evaluation of Calcium and Sodium ions as activator of the lipase. Reaction conditions: ethanol/oil molar ratio = 4/1; 175 mg of catalyst/g of oil; 4 wt% of water with respect to oil; sunflower oil and absolute ethanol as reactives; 37 °C, reaction time = 2 h and constant shaking (180 oscillations/min).

On the other hand, Granados et al (Granados et al., 2010). describe as diglycerides and other organic components react on the CaO surface, forming several species that favor the catalyst activity. In fact, these organic species were detected by FT-IR on the Ca/SBA-15(2.5) catalyst used for 2 h in the oil ethanolysis under reaction conditions described in the 3.4 section (Fig. 7). Thus, this spectrum shows absorption peaks at: 3471.58 cm^{-1} due to longitudinal vibration of water, 2926.14 cm^{-1} and 2854.77 cm^{-1} assigned to (C–H) stretching of the saturated carbon-carbon bonds, 1746.62 cm^{-1} assigned to (C=O) stretching of the carbonyl functionalities and 1650.0 cm^{-1} assigned to (C=C) stretching of the carbon-carbon double bonds. Therefore, the presence of these species on the Ca/SBA-15(2.5) surface would account for an interaction between the calcium species on the SBA-15 and the tri, di or monoglycerides of the reaction mixture, which are the substrates of lipase.

As it is known, the transesterification reaction is the result of a nucleophilic attack on the carbonyl carbon atom from ester groups of the substrate by the lipases (Hasan et al., 2009). Thus, we suggest that the basic properties conferred by the calcium species to the surface would favor the interaction between the substrates and the surface and, consequently, the interaction of substrate with enzyme supported, increasing the FAEE yield from the L_{PF}/Ca/SBA-15(2.5) with respect to L_{PF}/SBA-15. This synergism, enzyme-support has not been described by other authors at the moment. For this reason we addressed our attention over this point.

3.6. Influence of support basicity on the enzyme activity

To strengthen the hypothesis of the necessity of a basic behavior on the material surface, mesoporous SBA-15 was also modified with sodium to obtain Na/SBA-15(2.5). Then the enzyme was immobilized and the oil conversion after 2 h was similar to the one obtained when Ca/SBA-15(2.5) was used as support (Fig. 6). It should be noted that, the possible activator effect of Sodium ions in the lipase was also tested here. Thus, a sodium-enzyme pre-incubation was carried out before the enzyme immobilization on SBA-15 and an increase in the biodiesel yield was not detected (Fig. 6).

In order to evaluate if a major basicity of the Ca/SBA-15 surface increases the catalyst activity, the enzyme was immobilized on material with different theoretical calcium loadings, from 2.5 wt% to 15 wt%. As it can be observed in Fig. 8, as the calcium content increases on the support, transesterification activity decreases. An explanation for this behavior is that the optimum pH for enzyme activity is pH = 8 (Amano, 2008); when this pH is overcame the

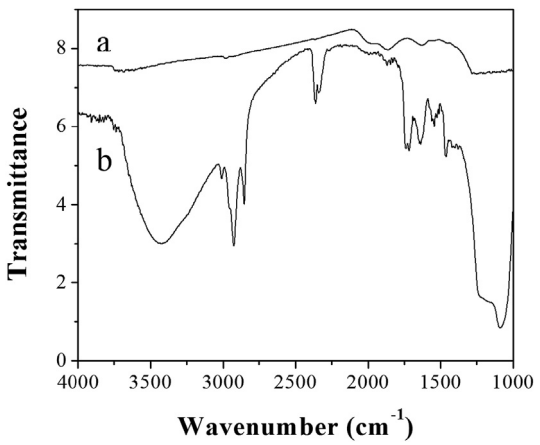

Fig. 7. IR spectra of: a) Ca/SBA-15(2.5) taken as reference, b) Ca/SBA-15(2.5) incubated with the reaction mixture in the described conditions.

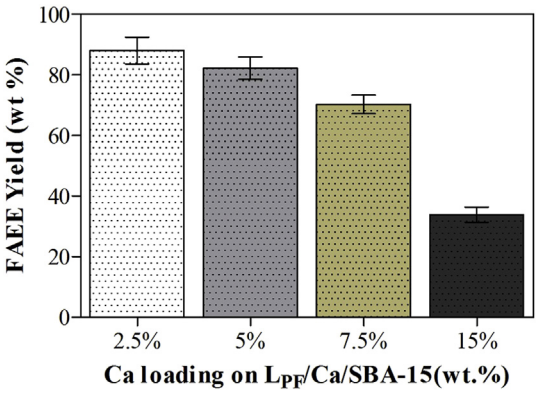

Fig. 8. Effect of calcium loading over the SBA-15 on the catalytic activity. Reaction conditions: ethanol/oil molar ratio = 4/1; 175 mg of catalyst/g of oil; 4 wt% of water with respect to oil; sunflower oil and absolute ethanol as reactives; 37 °C, reaction time = 24 h and constant shaking (180 oscillations/min).

activity markedly decreases. After the enzyme is immobilized on Ca/SBA-15(2.5) the supernatant pH is 8, which matches with the optimum pH for the activity of the enzyme. However, when theoretical calcium content increases until 15%, the pH of the supernatant increases until pH = 8.3 and this could be the cause of the decreased enzyme activity. That is, a major basicity could improve the biocatalyst activity and reagents interaction as it was mentioned in 3.5 section, however this increase in the basicity is detrimental to the lipase activity by modifying its pH optimum.

3.7. Effect of FFA and commercial ethanol on the biocatalyst activity

One the main inconveniences of the use of waste or non edible oil is the presence of FFA. When the FFAs level is >5%, the homogeneous catalyst is consumed decreasing the catalytic activity and promoting the soap production. This fact contributes to emulsion formation and hinders the glycerol-methyl esters separation (Atadashi et al., 2011; Knothe et al., 2005). An acid esterification has to be realized as pretreatment in order to reduce the FFA (usually at least of 1%), producing alcohol esters from the FFA before the basic transesterification (Sulistyo et al., 2015). This previous step can be removed when the enzymatic via is used because the enzyme has an esterification activity over FFA (Wu et al., 1996). To evaluate this activity, the reaction was carried out over L_{PS}/Ca/SBA-15(2.5%) adding palmitic acid, as FFA, in concentrations of 2.5, 5 and 10% respect to sunflower oil. Fig. 9 shows that the FFA didn't affect the transesterification reaction at any concentration. In fact, the FAEE yield was similar to that obtained without FFA addition (Fig. 5). This is, the catalyst prepared by us permitted to achieve an excellent activity using oil with high FFA contents without the necessity of a pretreatment. We propose that the ethyl esters obtained from the enzymatic esterification of FFA in presence of ethanol can act as emulsifiers. This fact would favor the mixture between the oil and alcohol, improving the interaction between the substrates and the catalyst (Shimada et al., 2002; Zhou et al., 2006).

Another problem in the biodiesel production is the water content in the raw material, since it produces soap formation in the traditional homogeneous process. The use of absolute ethanol in this process increases the costs, but it is necessary due to the water content in the commercial ethanol (96% v/v) (Knothe et al., 2005). Meanwhile, as methanol can be easily obtained anhydrous, it is chosen for the homogeneous process; however this cannot be obtained by fermentation from renewable sources as ethanol.

On the other hand, as it was previously mentioned in section 3.3, the water content is a critical factor for lipase activity. Here, the water amount necessary to achieve an optimum transesterification activity was determined and shown in Fig. 4. Nevertheless, to design an economical route for the production of biodiesel, in this work, the absolute ethanol was replaced by commercial bioethanol (4% V/V of water), maintaining the final content of water in 4 wt% with respect to the oil (Shah and Gupta, 2007). This replacement did not affect the sunflower oil conversion (Fig. 10).

Hence, under these conditions FAEEs can be produced using commercial bioethanol, without affecting the catalyst activity. This fact positively impacts over the economic considerations for developing a sustainable technology, since a cheaper ethanol produced from a fermentation process can be used with high FFA content oils, like waste o non comestible oils.

3.8. Performance of biocatalyst using alternative oil subtracts

The capacity of L_{PS}/Ca/SBA-15(2.5) catalyst to work with different subtracts is an important point of study. Herein, soybean oil and waste frying oil were also used as substrates. Soybean oil was selected because the Argentinean Republic is the first exporter of soybean oil and the third largest soybean producer in the world. Therefore, in the future, an increase in the soybean-based biodiesel production is expected. However, in order to meet the sustainability criteria of the biodiesel production in Argentina, the

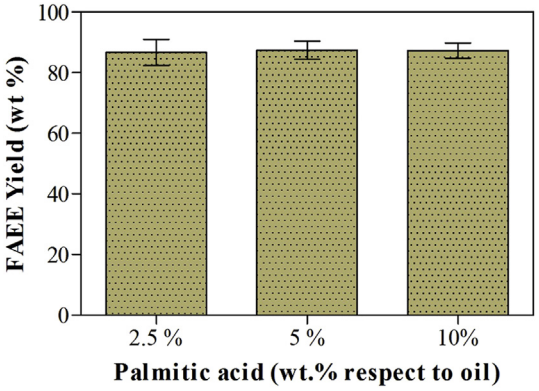

Fig. 9. Effect of FFA content in the reaction medium on the catalytic activity. Reaction conditions: ethanol/oil molar ratio = 4/1; 175 mg of catalyst/g of oil; 4 wt% of water with respect to oil; sunflower oil and absolute ethanol as reactives; 37 °C, reaction time = 24 h and constant shaking (180 oscillations/min).

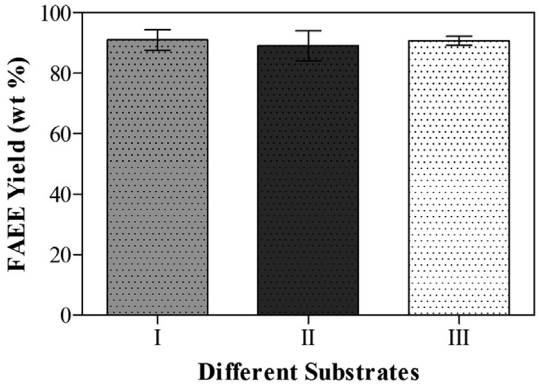

Fig. 10. Determination of transesterification activity of L_{PF}/Ca/SBA-15(2.5) using commercial ethanol with different substrates, I) sunflower oil, II) soybean oil, III) frying waste oil. Reaction conditions: ethanol/oil molar ratio = 4/1; 175 mg of catalyst/g of oil; 4 wt% of water with respect to oil; sunflower oil and bioethanol as reactives; 37 °C, reaction time = 24 h and constant shaking (180 oscillations/min).

Table 1
Lipid profile and properties of vegetal oils used.

Fatty acids	Sunflower (%)	Soybean (%)	Frying waste oil (%)
Saturated			
Palmitic	5.71	10.76	6.09
Stearic	3.13	4.7	3.46
Mono-unsaturated			
Palmitoleic	0.11	0.09	0.20
Oleic	29.95	21.28	37.65
Poly-unsaturated			
Linoleic	58.57	53.91	50.96
a-Linolenic	0.14	7.84	0.15
Acid value (**mg NaHO g^{-1}**)	0.32	0.31	0.51
Molecular weight (**g/mol**)	876.1	873.7	876

environmental performance of the traditional homogeneous process has to be evaluated (Panichelli et al., 2009).

According to literature data, the use of waste frying oil as raw material reduces the costs in up to 80% and results in an environment-friendly process, since waste oils are responsible for high environmental impact (Hasheminejad et al., 2011; Wassell and Dittmer, 2006). In addition, variations in the waste oil characteristics can affect product quality. A fresh vegetable oil and its waste differ significantly in water and FFA contents and, as it was mentioned, both affect the performance of homogeneous and heterogeneous inorganic catalysts (Knothe et al., 2005; Sulistyo et al., 2015).

However, these disadvantages were not a problem for the performance of catalyst developed in this work, because it could work with high activity in presence of 4% of water and even 10% of FFA respect to oil. Table 1 describes the fatty acid composition and the properties of the different vegetable oils used in this work. All oils contained a high percentage of unsaturated fatty acids. However, an interesting point is the decrease in the polyunsaturated fatty acids, molecules susceptible of oxidation, in the frying oil. The use of this oil to produce biodiesel, could lead to a reduction in the use of antioxidants which are necessary to improve the oxidation stability of biodiesel (Sulistyo et al., 2015). Nevertheless, frying waste oil has an acid value superior to that of soybean and sunflower oils, indicating a major FFA content. When soybean and frying waste oils were used as substrates, the FAEE yields employing absolute ethanol were higher than 91 wt% (data not shown). Then, to achieve an actually sustainable process, the transesterification reaction of both oils was also carried out using commercial bioethanol. Thus, Fig. 10 shows the FAEE yield from the different substrates described in Table 1. As it can be observed, the biodiesel yields were 89% from soybean oil and 90% from waste oil, similar to the ones obtained from sunflower oil (91%).

Finally, some conclusions can be reached from this experiment that contribute to the novelty and regional perspective of this work: the synthesized catalyst is active against different oil sources and commercial ethanol can also be used to produce high FAEE yield. Moreover, neither pre-treatments for waste oils nor reaction condition modification, are necessary. Therefore, the catalyst developed in this work allowed us to propose an economical and ecological process with important advantages compared to homogeneous process. This catalyst can work with renewable and economical substrates such as waste frying oils and commercial ethanol obtained from a fermentation process in Argentine. This could stimulate future technology transference from laboratory to the industry for biodiesel production.

4. Conclusion

Pseudomonas fluorescens lipase was successfully immobilized on mesoporous SBA-15 by physisorption in order to obtain active hybrid catalysts for the transesterification reaction of vegetables oils. Firstly, an optimum enzyme loading of 400 $mg_{protein}/g_{support}$ was determined. Meanwhile, the suitable water concentration to optimize the enzymatic activity was 4 wt% with respect to oil mass. In this work, we could demonstrate how the nature of support can improve the enzymatic activity and how the basic species formed on the surface can synergistically act with the enzyme. This synergistic effect favors the substrate-surface interaction and consequently the substrate availability for the immobilized enzyme. Thus, the L_{PF}/Ca/SBA-15(2.5) catalyst showed the highest activity (around 90% FAEE yield) in presence of different oils (sunflower, soybean and even oils with high free fatty acid content like waste frying oil) and using commercial bioethanol 96 V/V%. Therefore, such catalyst is capable of using renewable raw materials to produce biodiesel, increasing a 16% the yield in comparison to the lipase immobilized on pure SBA-15. Such increase, although apparently low at laboratory scale, can play a crucial role for the selection of a catalyst in the industry, providing even more sustainability to the process. Moreover, it represents an exceptional option for the biodiesel production in Argentina, because both vegetable oils and bioethanol are largely produced in the country.

Acknowledgements

G.O.F, H.J.R, C.E.A and G.A.E area members of CONICET. The authors are grateful to CONICET and UTN for the financial support. The authors acknowledge beam time to the Brazilian Synchrotron Light Laboratory (LNLS) at Campinas, Brazil, experimental stations SAXS-1 and SAXS-2 and Dr. R. Oliveira for helpful discussions. The authors are gratefully to Ph.D Vaschetto E.G. for the TEM and SEM images.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jclepro.2016.08.047.

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