Biodiesel production from alternative raw materials using a heterogeneous Low Ordered Biosilicified Enzyme as biocatalyst

Gabriel Orlando Ferrero*[a], Edgar Maximiliano Sánchez Faba [a] and Griselda Alejandra Eimer*[a].

[a] CITeQ-UTN-CONICET, Universidad Tecnológica Nacional, Fac. Regional Córdoba, Maestro López esq. Cruz Roja, Ciudad Universitaria, CP:5016, Córdoba Capital, Argentina.

* Corresponding authors

E-mail address: gferrero@frc.utn.edu.ar (G. Ferrero)

geimer@frc.utn.edu.ar (G. Eimer)

Abstract

Background: Nowadays, as an alternative to the production of fuels and chemicals from the fossil platform, renewable feedstocks are widely investigated. For biomass conversion, a new generation of catalysts with specific characteristics such as high activity and selectivity, easy recovery and reusability is necessary. The design of highly efficient and stable heterogeneous catalysts represents a challenge in this field, mainly to overcome current energy and environmental issues. The combination of enzymatic and heterogeneous inorganic catalysis generates an unprecedented platform that combines the advantages of both. Among the techniques for producing solid catalysts, enzymatic mineralization with an organic silicic precursor to obtain hybrid biocatalysts (biosilicification) is highlighted. This technique can provide exceptional stability to the biocatalyst in drastic conditions of use.

Results: Then, under these criteria, this work presents the one-step synthesis of a solid enzymatic catalyst, denominated Low Ordered Biosilicified Enzyme (LOBE) due to their structural properties. *Pseudomonas Fluorescens* lipase forms aggregates that are contained in the heart of a silicon-covered micelle, providing active sites with the ability to process different raw materials (commercial sunflower and soybean oil, *Jatropha excisa* oil, waste frying oil, residual soybeans, and pork fat) to produce first and second generation

biodiesel. Obtaining yields between 81 and 93% by weight depending on the used raw material.

Conclusions: Therefore, refined, non-edible and residual oils (with high water and free fatty acid contents) can be transformed into biodiesel through LOBE catalysts with commercial ethanol as co-substrate.

Keywords

Enzymatic biosilicification, Second generation biodiesel, Alternative oils, Mesoporous material, *Pseudomonas fluorescens* lipase.

Background

The processing of biomass for the production of fuels and chemicals is often not economical due to the high cost of materials, the number of purification steps and low selectivity. Thus, it is necessary to consider novel synthesis routes or novel process technologies for the valorization of biomass and to use more efficiently the raw materials, decreasing the processing time based on the principles and metrics of green chemistry and sustainable development [1,2]. Over the past decades, enzymes were established as a new class of catalysts in the field of modern synthetic chemistry, and they continue to gain importance through discovery of new applications and development of even more efficient systems. Consequently, applying in-vivo enzymatic transformations (biocatalysis) to industrial processes is an attractive strategy in order to circumvent the existing laborious design and synthesis of artificial catalysts [3–5]. The fast progression of these biological catalysts has numerous reasons, such as high catalytic efficiency and selectivity (stereo-selectivity, regio-selectivity and chemo-selectivity), mild operational conditions, no use of protecting groups, minimized side reactions, easier separation, and fewer environmental problems. However, such practical applications are hindered by enzymes fragile nature, such as low thermal stabilities, narrow optimum pH ranges, low tolerances to most organic solvents and many metal ions, etc. [6–

8]. To improve the use of enzymes in biocatalytic applications, immobilization of enzymes on mesoporous silica supports has been introduced as a novel method [9–12]. These mesoporous materials have unique advantages as immobilization support in the following aspects: structure, porosity and surface. Thus, these structures offer mechanical, thermal and chemical resistance, with a pore system in the order of 2 nm to up to 5 µm that makes possible discriminate molecules according to their size and allow the diffusion of substrates and products. The size of pore can be adjusted to match with that of the given enzyme; likewise the pore shape can be ordered in different arrangements, such as hexagonal or cubic [13,14], allowing the enzymes loading and also providing a protective environment where the enzymes can often tolerate higher temperature, extreme pH and more salt concentrations [15–17] . Mesoporous solids present high specific surfaces that can be modified to increase their hydrophobic or hydrophilic behavior, or with different catalytically active species. Such features can improve the enzyme stability and the enzymatic function (with a synergic effect enzyme-support), simplifying the biocatalyst recycling and enhancing the product recovery and yields [16,18,19].

Among the several techniques to immobilize enzymes, co-precipitation is found. By this methodology, recently reported by Luque et al., enzymes are immobilized on the support during the synthesis process. Thus a silicic acid

93 organic precursor is mineralized over the enzyme and this process, denominated
94 *biosilicification*, can provides an exceptional stability to the biocatalyst under
95 drastic conditions [20–22].

96 In this article, *Pseudomonas Fluorescens* lipase was biosilicified with
97 TEOS (Tetraethyl orthosilicate) as silicious source. In this way, a Low Ordered
98 Biosilicified Enzyme (LOBE) is obtained in only one step, with mesoporous
99 structure and ability to produce biodiesel from several oils and commercial
100 ethanol as raw materials. Herein, the synthesis conditions, characterization and
101 enzymatic performance are discussed in detail. Finally, we restated the
102 alternative of replace the homogeneous catalysis in the transesterification
103 reaction to produce biodiesel by heterogeneous enzymatic catalysis.

104

105 **Results and discussion**

106 **Synthesis and evaluation of biosilicified *Pseudomonas fluorescens* lipase**

107 Based on previous reports [20–22,25], the proposed one-pot encapsulation
108 technique was evaluated regarding enzyme content to obtain an optimum
109 transesterification activity. Table 1 summarizes the tested lipase content in the
110 synthesized material.

Table1. *Pseudomonas Fluorescens* lipase contents in the synthetized biocatalysts.

Biocatalyst	*P. F.* Lipase (mg)
Control	0
LOBE 1	1
LOBE 2	5
LOBE 3	10
LOBE 4	50
LOBE 5	100
LOBE 6	150

Biosilicification conditions: 6.36 mL Phosphate Buffer 50 mM, pH=7.5; 1.1 ml TEOS; 0.625 ml DDA; 6.36 mL ACN.

The activity of biocatalyst to produce biodiesel was studied using sunflower oil and commercial ethanol (96 v/v %) as raw materials. Commercial ethanol was chosen for three reasons: first, the enzyme needs the water present in ethanol to be active (4% v/v remaining), and moreover, this water act as lubricant allowing the active site lid to open to interact with the substrates[13,16,26]. Secondly, it is renewable since it is produced by

fermentation and finally, the ethanol has lower flammability and toxicity than methanol.

Transesterification activity was detected for all biocatalysts that contain the *Pseudomonas Fluorescens* lipase. The yield of different biocatalysts is showed in Figure 1. As it can be observed, the activity increases in conjunction with the amount of immobilized enzyme. However, only the enzyme contents higher than 50mg (LOBE4) present a better FAEE production. A normalized FAEE production was used to select the best biocatalyst. Thus converted oil mass per minute and per biosilicified enzyme mass was determined (see formula in 2.6 section). As showed in Table 2 and Figure 2, as the reaction time increases, the normalized FAEE production decreases because the triglycerides (the raw material) are consumed. On the other hand, at the same reaction time, when the biosilicified lipase mass increases, the production of FAEE decreases; it is because the biocatalyst is working below the optimum level, i.e. substrate/enzyme ratio lower than that it could process.

For these reasons, LOBE4 exhibits the best performance using the catalyst with the possible lower lipase content and with the major normalized FAEE production. Thus, it was select to follow the research.

Table 2. Normalized FAEE production for biocatalysts (mgFAEE/mg biosilicified enzyme per min).

Time (min)	LOBE4	LOBE5	LOBE6
120	8037.91	5185.77	3727.78
240	3988.04	2770.51	2448.33
480	2482.62	1493.72	1275.10
1440	856.96	552.39	503.90
2880	468.58	294.81	248.43

On the other hand, when the free lipase is used, it forms aggregates in the organic reaction mixture, which reduces the number of exposed active sites and, therefore, its activity also decreases (32 wt% FAEE yield after 48 h) [16].

As a control, a synthesis without enzyme was carried out and tested. No transesterification activity was detected when employing this non-enzimatic material.

3.2. Biocatalysts characterization

The structural and textural characteristics of the biosilicified enzymes were studied by X-ray diffraction (XRD), transmission electron microscopy

(TEM), N2 adsorption to obtain the specific areas by BET method and Fourier Transform Infrared Spectroscopy.

An ordering typical of mesoporous silicates was detected when the control and LOBE4 were analyzed by XRD. As it can be observed in Figure 3, the patterns exhibit two maxima peaks, assigned to (2 1 1) and (2 2 0) reflections. In addition, the ratio value d220/d2110 approximated to 0.87 is according with a cubic structure of MCM-48 [8,20–22]. This indicates a certain structural ordering of the biosilicified enzymes.

DDA is a molecule with polar head and hydrophobic chain, which can form micelles by acting as a surfactant [25,27]. Evidently, the DDA in solution gives rise to a liquid crystal micellar phase that can be employed as structure directing agent to form mesoporous structures, such as MCM-48, when TEOS is mineralized [28,29]. Likewise, the enzyme is able to insert itself in the formed cubic phase, not interfering with the formation of the micelle. Therefore, the type of three-way pore structure could be determined by a micellar cubic phase of the DDA that acts as a template while the enzyme could be acting as a swelling agent does (Figure 4).

TEM images show a nanotubular structure such as a nanofiber network with canals according to the reported by Garcia *et. al.* [20], indicating that the

siliceous mineralization has been realized over the enzyme (Figure 5). However, this biosilicification results permeable to the substrates, which can access to active sites to be transesterificated (Figure 1). To facilitate the observation of nanofiber network architecture, LOBE4 images are showed in original and inverted colors.

Infrared spectroscopy is a widely used technique in the study of substructure of peptides and proteins and it can be used to monitor the proteins presence on the supports [30,31]. In Figure 6a, different functional groups characteristic of the free *Pseudomonas Fluorescens* Lipase was evidenced by FT-IR. A broad and intense band between 3600 and 3200 cm^{-1} was assigned to the stretching vibrations of –OH groups, which would be also masking the –N–H stretching vibrations. Then, there is a band at 2930 cm^{-1} assigned to the stretching C–H vibrations of –CH2 and –CH3 groups. The typical stretching vibrations of carbonyl groups appear around 1700 cm^{-1} and stretching vibration of ≡C–O– is observed at 1180 cm^{-1}. The signal of the –OH deformation vibrations appears at 1400 cm^{-1} [32]. Finally, the signals for most characteristic functional groups of the pure enzymes, amide I and amide II, are seen at 1645 cm^{-1} and 1542 cm^{-1}, respectively [33,34].

After biosilicification, the LOBE4 FT-IR spectrum was analyzed (Figure 6b). The intensities and wavenumbers of amides bands decreases, indicating

that the immobilization of the lipase inside the silica matrix was successful [35]. In addition, the presence of the amide I and amide II bands indicates that the secondary structure and bioactivity of enzyme are conserved in the formed nanostructure, as it was evaluated in section 3.1 [36]. The presence of siliceous can be corroborated by the band of ≡Si–O bond vibration at 1070 cm^{-1} and the signals among 800 cm^{-1} and 460 cm^{-1} [37–39]. In addition, the bands at 2930 cm^{-1} and 2850 cm^{-1} assigned to (C–H) stretching of the saturated –CH2 and –CH3 groups increase their intensity respect to the free enzyme, due to the presence of the surfactant in the biocatalyst. However, these bands disappear when the biocatalyst is calcined at 773 K for 8 h, indicating that the organic material was removed (Figure 6c).

Likewise, the FT-IR spectrum of the calcined biocatalyst reveals bands characteristic of a siliceous matrix. The region of 3750 cm^{-1} is attributed to terminal silanol stretching vibrations ≡Si–OH, owing to the presence of surface OH groups with strong H-bending interactions [40]. This band may be poorly visible because of the influence of physically adsorbed water on the SiO2 surface that exhibits a band at 3440 cm^{-1} [41]. However, a shoulder at 960 cm^{-1} can be attributed to ≡Si−OH bending. The band at around 1100 cm^{-1} was assigned to ≡Si–O bond vibration [42]. Bands around 800 and 460 cm−1

correspond to ≡Si–O–Si≡ symmetric stretching and bending, respectively [37–39].

Therefore, the FT-IR spectrum analysis of the LOBE4 confirmed the presence of a number of functional groups typical of the enzyme and the siliceous material, indicating the effective lipase immobilization by biosilicification.

The specific surface of biocatalysts was determined on control and LOBE4, calcined and non-calcined (Table 3).

Table 3. Specific areas of Control and LOBE4 pre and post calcined

Sample	Specific areas m^2/g (C)	Specific areas m^2/g (NC)
Control	221.01	4.97
LOBE4	289.54	6.12

C=Calcined, NC= Non calcined

As expected, after calcination at 773 K the specific area should increase due to the elimination of the organic phase (surfactant and lipase). Nevertheless, when an XRD was realized an ordered structure was non detected (Figure 7), which indicate that the architecture of siliceous network is not stable at high temperatures.

From the obtained results, it could be suggested the formation of an orderly incomplete siliceous-organic hybrid structure, where silicification fails to cover the entire organic structure. Therefore, by removing the organic scaffolding, this nano architecture collapses. However, this incomplete mineralization leads to the biocatalyst some flexibility allowing the diffusion of substrates and products to the active sites of the enzymes. Due to these characteristics, these materials are denominated: Low Ordered Biosilicified Enzyme (LOBE).

3.3. Activity of LOBE4 with alternative raw materials

To verify the versatility of LOBE4, its activity to produce biodiesel was tested with the following feedstocks: Soybean oil, frying oil, *Jatropha Excisa* oil, residual soybean oil and pork fat (Figure 8a). The selection of these raw materials is justified as following. In 2018, Argentine was the world leading exporter of soybean oil and the world third largest exporter of sunflower oil [43]. Therefore, the use of soybean oils to produce biodiesel not present a detrimental effect at the local population because the sunflower oil is destined mainly to human consume. Even more, the industrialization and employment generation in agricultural regions can be promoted by this activity [44]. In

addition, during the process of soybean oil purification, an oily waste is generated, containing a large amount of free fatty acids (50-80% approx.), and in a minor proportion a mixture of phospholipids, tocopherols, sterols, degraded oxidized components, pigments, salts, color bodies, triglycerides, diglycerides and monoglycerides [45,46]. Alternatively, converting this residual oil into biodiesel could give it added value.

On the other hand, the frying used oils, which have low food value but high energy content, are a waste of the domestic and gastronomic industry. These are available in large quantities at a minimal cost and are generally discarded in the drain, causing obstructions in the sewer system and contamination of water resources. An alternative to avoid this inconvenience could be to reuse them for the biodiesel production, substantially reducing the price of the biofuel [47–49].

A substitute to avoid competing with the demand for food is the use of non-edible oilseeds. *Jatropha excisa* is an endemic specie from the semiarid and arid northwest of Argentina and its oil is presumably toxic; native people use it as purgative and emetic for medicinal purposes [50]. *J. excisa* is a non-conventional oilseed specie with an oil concentration approx. 34%. Then, it does not represent competition with crops of agricultural food and diversify

farmland. For these reasons, it also presents an economic potential as alternative oil to biofuels generation [51,52].

Table 4. Characterization of raw materials.

Feedstock	Density (g/cm^3)	Kinematic Viscosity (mm^2/s) [1]	Acid Value (mg_{KOH}/g_{oil})	FFA Content (wt %) [2]	Water Content (ppm)	Triglycerides content (wt %)
Soybean oil	0.93	18.38	0.13	0.07	626	97.70
Frying oil	0.94	20.48	0.21	0.11	671	95.73
Jatropha Excisa oil	0.92	15.75	1.55	0.78	980	94.60
Residual Soybean oil	0.96	10.94	153.72	76.91	5221	9.18
Pork Fat	0.90	21.85	0.67	0.33	590	97.64

[1] At 60 °C (reaction temperature). [2] Calculated from the acid value (EN 14104: 2003) and expressed as oleic acid [55].

Animal fats are interesting sources for biodiesel production due to the fact that their cost is considerably lower than that of the vegetable oils. These feedstocks are currently added to pet food and animal feed or used for soap

272 production in the industry. However, this raw material can be transform into
273 high-quality biodiesel that meets the ASTM specifications for biodiesel [53,54].

274 Excluding soybean oil, the other raw materials present a really interesting
275 source for the production of second-generation biofuels. However, the high
276 contain of FFA and water does not permit their use directly in the homogenous
277 process to obtain biodiesel (Table 4). Firstly, the homogeneous esterification of
278 free fatty acids with sulfuric acid and methanol must to be realized. Next, the
279 acid must be neutralized, and the product should be washed and dried. After this
280 pretreatment, the resulting raw material (a mixture of esters of free fatty acids
281 and triglycerides) can be used in the transesterification reaction with a basic
282 homogeneous catalyst. Finally, neutralization, washing and drying steps must
283 be performed again to use the product obtained as fuel [56,57]. This
284 methodology increases the cost of the process because the used strong acids and
285 bases cause the oxidation and corrosion of the reactor, decreasing its useful life
286 besides being aggressive with the environment. These stages can be avoided if
287 a biocatalyst is used.

288 As it can be observed in Figure 8b, the five oily raw materials were
289 converted into biodiesel in presence of commercial ethanol and LOBE4,
290 indicating that the biocatalyst, in addition to carrying out the acylglycerol

transesterification reaction, can also esterify the FFA without any previous treatment.

4. Conclusions

In this work, a heterogeneous enzymatic catalyst was developed in a single stage according to the following proposed mechanism: first, the enzymatic solution is mixed with the organic solvent (acetonitrile), forming aggregates that would leave its hydrophobic groups exposed to the solvent. By adding the surfactant, these enzymatic aggregates can be inserted into the DDA micelles but maintaining their acquired agglomerated structure due to hydrophobic heart of such micelles. That is, enzyme remain grouped without the need to perform a nonspecific cross linking to keep them together, which can affect their activity. Then, when this molecular macrostructure is stiffened by covering with an incomplete silicon network, a solid hybrid enzyme catalyst is synthetized. The concentration of active sites of the aggregated enzymes and the flexibility of an incomplete silicon network allows the diffusion of substrates and products through a Low Ordered Biosilicified Enzyme (LOBE). Such hybrid systems present the ability to produce biodiesel from a variety of edible, non-edible, residual oils or animal fats.

Methods

Materials

Pseudomonas Fluorescens lipase (PFL, ≥20,000 IU / g at 55 °C, pH 8.0) was purchased from Sigma-Aldrich Co. (St. Louis, USA). Sunflower and soybean oil (commercial, brand "Vicentin") and pork fat were purchased at a local store. Waste frying oil was obtained from different domestic sources and filtered before being used. Residual soybean oil was generously gifted by a local company. *Jatropha excisa* oil was kindly ceded by Dr. Fracchia. Other used reagents were: KH_2PO_4, K_2HPO_4 and KOH (Anedra), commercial bioethanol 96% v/v (Porta Hnos.), n-dodecylamine (Sigma-Aldrich), acetonitrile (Anedra), n-hexane (analytical grade, Merck), isopropyl alcohol (Fluka), acetonitrile (analytical grade, Merck), Tetraethyl orthosilicate (TEOS) (Aldrich) and milliQ water. Syringe filters (polypropylene, 25 mm diameter and 0.2 micron pore size) were supplied by VWR.

Acid value determination

The feedstocks acid value were determines by volumetric titration according to the standard EN ISO 14104 (2003). The required oil mass was mixed with 2-propanol in a conical flask (0.25 g sample/ml solvent), and titrated using an

KOH 0.1 M aqueous solution. Phenolphthalein was used as the final point indicator. Results are expressed in mg KOH/g sample.

Pseudomonas fluorescens lipase biosilicification

Different lipase amounts (0.00-150.00mg) were dissolved with 6.36 mL of 50 mM phosphate buffer (pH= 7.5). Then, 6.36 mL of acetonitrile (ACN) and 0.625 mL of n-dodecylamine (DDA) was added to the solution, with magnetic stirring. Finally, 1 mL of tetraethoxysilane (TEOS) was added. The solution containing a visible solid precipitate after a few minutes was stirred for another 3 h at 293 K. After this, the obtained solid was filtered, washed with water and dried at room temperature for 24 h. The liquid supernatant was utilized to determinate the protein content according to Bradford method [23]. The hybrid material obtained from the enzymatic immobilization was named as LOBE X, where X is related with the enzyme content.

Support characterization

The X-ray diffraction (XRD) patterns were recorded in air at room temperature on a PANalytical X-Pert Pro X-ray powder diffractometer, with a

Bragg-Brentano geometry. A CuKα lamp was used (40 kV, 40 mA) in 2Θ range between1.5-7◦. The interplanar distance (d(1 0 0)) was estimated using the position of the first X-ray diffraction line. BET method was employed to measure the specific surface of the materials using a Micrometrics Pulse ChemiSorb 2700 equipment. The samples were previously heated for 1 h at 300 ◦C under N2 flow. FT-IR analysis were performed on a Thermo Scientific Nicolet iS10 spectrometer, with Smart OMNI-Transmission accessory. The measure range was from 400 to 4000 cm−1 with a resolution of 8 cm−1 and 50 scans. Samples were prepared by the KBr technique.

Transesterification reaction

The reactions were carried out in a screw vials placed in an orbital shaker at 80 rpm, 37 °C and oil/ethanol molar ratio of 1/4. The reactions were started when the biocatalysts were added. Samples were taken at different times to be analyzed by high performance liquid chromatography (HPLC) [16].

Chromatographic analysis

The analyzes were performed with a Perkin Elmer HPLC with UV-vis detector of the 200 series equipped with a solvent delivery unit with gradient of elution, a KNAUER Vertex Plus column (250 mm × 4.6 mm, 5 μm) Eurospher II 100-5 C18 P and the software used was TotalChrom. The wavelength of the UV detector was set at 205 nm, the column temperature was maintained at 30 °C and the flowrate was 1 mL/min. For chromatographic runs, a stepwise method was used: 100% of methanol in 0 min, 50% of methanol and 50% of 5:4 2-propanol/n-hexane in 10 min maintained with isocratic elution for 10 min [24]. All reactions were performed at least in duplicate and the results were expressed as mean values that the percentage differences between them were always less than 5% of the mean.

The normalized FAEE production of biocatalysts was calculated according:

NFAEE: FAEE production normalized

mgFAEE: mg of FAEE produced

t: reaction time in minutes

mgLOBEX: mg of *Pseudomonas fluorescens* lipase biosilicified

$$\text{NFAEE} = \frac{\text{mgFAEE}}{t * mg\ biosilicified\ enzyme}$$

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with submission to Biotechnology for Biofuel.

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Competing interests

There are no competing interests associated with this manuscript.

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Author contributions

G.O.F. was involved in conceptualization, methodology, investigation, formal analysis, writing-original draft and writing-review & editing. E.M.S.F. was involved in experiments validation, formal analysis, conceptualization and writing-review & editing. G.A. E. was involved in resources, conceptualization, writing-review & editing, supervision and project administration.

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Figure legends

Figure 1: Reaction surface of FAEE yield versus enzyme loading in the LOBE catalyst and reaction time.

Figure 2: Normalized FAEE production of LOBE4, 5 and 6 respect to reaction time (mgFAEE/mg of biosilicified enzyme*min).

Figure 3: X-ray diffractions patterns of control and LOBE4.

Figure 4. Micelle formation with DDA: a) Molecular formula of DDA, b) mechanism of micelle-enzyme formation.

Figure 5: TEM images of LOBE4 catalyst at different amplifications.

Figure 6: FT-IR analysis of biocatalysts: a) free Pseudomonas Fluorescens Lipase, b) LOBE4 and c) calcined LOBE4.

Figure 7: X-ray diffractions of calcined control and LOBE 5.

Figure 8: Performance of LOBE4 catalyst. a) General aspect of the used feedstocks. b) Obtained FAEEs yields from different raw materials. Reaction conditions: ethanol/oil molar ratio = 4/1; 125 mg of LOBE4/g of oil; commercial ethanol as co-substrate, 37 °C, reaction time = 48 h and constant shaking (80 oscillations/min).

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