Utilization of discard bovine bone as a support for immobilization of recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris.*

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Abstract

In this study the possibility of using discard bovine bone as support for immobilization of *Rhizopus oryzae* lipase expressed in *Pichia pastoris* was analyzed. Discard bovine bone were milled and then subjected to a chemical treatment with acetone in order to remove lipids and blood traces. Two types of supports were evaluated: bovine bone and calcined bovine bone for 2 hours at 600°C. Supports were characterized by: ICP, SEM, XRD, FTIR, XPS and N_2 adsorption isotherms.

Calcined bovine bone presented appropriate characteristics for the lipase immobilization due to the removal of collagen: high porosity, large surface area and suitable porous structure. Biocatalysts were prepared with different initial enzyme load. For the equilibrium adsorption studies, the Langmuir isotherm was used to fit the data results. The immobilization occurs in monolayer to a value of 35 UA mg^{-1}.

The activities of biocatalysts were tested in transesterification reaction of olive oil. For the enzyme load used in the test, a final yield percentage of 49.6 was achieved after six methanol additions and 180 min of reaction, similar values were obtained using Relizyme as support. Therefore, the bovine bone discard is an economical and appropriate choice for use support immobilization of enzymes.

Keywords: biocatalysis, bovine bone, lipase immobilization, transesterification, biodiesel.

1. Introduction

Biodiesel production using enzymes as biocatalysts represents an environmentally more attractive option to the conventional process as basic catalysis, especially when oils have an important % of free fatty acids [1]. The application of enzymes allows mild reaction conditions and the use of oils from varied resources including waste oils with a high content of fatty acids and low cost, because they have the ability to transfer both triacylglycerols and free fatty acids into biodiesel[2].

Lipases (E.C. 3.1.1.3., triacylglycerol acylhydrolases) are widely employed in food and chemical industries because they possess advantageous properties such as high specificity and selectivity. Furthermore they have excellent catalytic activity and stability in non-aqueous media, which facilitate the transesterification reaction during biodiesel production [3,4].

The use of immobilized enzyme as biocatalyst is a real alternative to classical chemical catalysis in biodiesel production but one of the disadvantages for industrial application of lipase is the high cost of the biocatalyst. At this point, the use of recombinant enzymes and cheap supports in order to minimize economic costs of the biocatalyst are the solution [5]. Immobilization by adsorption involving weak forces (i.e. van der Waals and hydrophobic interactions) or by covalent binding between the enzyme and the solid carrier are the most classical approach.

Adsorption to a solid carrier is the most widely employed method for lipase immobilization because is relatively easy, of low cost and can be conducted under mild conditions without loss of activity [6]. Moreover, the support can easily be recovered for repeated immobilization.

An appropriate support for immobilization by adsorption would have a high porosity, large surface area, an adequate internal porous structure and high affinity for the lipase in use. Furthermore, it must be abundant and be inexpensive. Examples of solid carriers used in this type of immobilization are reported in the literature: acrylic resin, textile membrane, glass, polypropylene and celite [7-9].

In many countries, bovine bone is generated as a discard in large quantities. In Argentina 155.000 ton of bovine bone were discarded only in the first quarter of 2015. Bone is a porous, resistant and relatively cheap material, obtained as a by-product from slaughterhouse, where a large amount of this is used to produce bone meal for animal consumption, the rest is disposed in open dumps or landfills causing an environmental impact. The use and revalorization of discards has been a topic of great interest in the scientific community.

Mechanical and stiffness properties of bone are due to its structure that is composed by 65-70% hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$). The remaining 30-35% (dry basis) is attributed to organic compounds, mainly collagen. There are other organic compounds existing in minor concentrations such as chondroitin sulphate, keratin sulphate and lipids (e.g. phospholipids, triglycerides, fatty acids, cholesterol, etc.) [10].

The aim of this work was the revalorization of an industrial discards as bovine bone evaluating its use as a support material for the immobilization of the recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris* (rROL).This lipase has a high regiospecificity on the sn-1- and sn-3-position of triacylglycerols, and showed a 44-fold higher specific activity compared to a commercially available lipase obtained directly from native *R. oryzae* [1,11].

In previous research, bones from pigs, chickens and fish were used as supports for enzyme immobilization [12-14] but none of the works have used bovine bone as support of recombinant lipase for transesterification reaction in biodiesel production.

This work includes: physicochemical characterization of support, immobilization process analysis and optimization of the conditions of the transesterification reaction in biodiesel production (enzyme loading, and activity).

2. Materials and methods

2.1. Chemicals

Methanol (>99.9%), n-hexane (>99.9%) and heptane (>99.9%) were purchased from Panreac (Barcelona, Spain). Acetone (>99.9%) was supplied by Sintorgan (Buenos Aires, Argentina). Discard bovine bones were obtained from a local slaughterhouse and virgin olive oil from Carbonell (Córdoba, Spain).

2.2. Lipases

Recombinant *R. oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autónoma de Barcelona (UAB). This lipase was obtained by a mixed substrate fed-batch cultivation of a recombinant *P. pastoris* using methanol as the inductor [15]. The culture broth was centrifuged and micro-filtered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system (New York, USA) equipped with an Omega membrane with a 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris-HCl buffer pH 7.5 and thereafter lyophilized [16].

2.3. Support: Bovine bone

Discard bovine bone was milled to an appropriate particle size (20-40 mesh) and then subjected to a chemical treatment with acetone in order to remove lipids and blood traces. The treatment was developed in a stirred autoclave with a capacity of 500 cm^3, equipped with a controlled heating. The treatment was performed under inert atmosphere (N_2) at the following conditions: T = 50°C, P = 7 bar, stirring speed = 300 rpm, time = 3h. Finally the material was dried overnight at 50°C to remove residual solvent. The sample thus obtained was called BB. A portion of BB was calcined in a muffle at T = 600°C for 2 h in order to remove organic components that might still occupy the pore space after the chemical treatment, thereby generating void volume. The sample thus obtained was called BBC600.

2.4. Support characterization

The composition of the bovine bone was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, in a Perkin Elmer, Optima 2100 DV) after digestion in an acid solution of a mixture of concentrated nitric: perchloric acid of 1:5 (v/v). The content of Ca, P, As, Cd, Pb and the relation Ca/P were determined.
The nitrogen content in the bovine bone was determined by the Kjeldahl method indicated by Approved Method 56-10 (AACC, 2005). The sample was digested in a Technicon II digester for 4 h, and then was distilled. Crude protein was calculated as N × 6.25.
The lipid content was obtained by extraction with n-hexane (1:4 wt wt^{-1} ratio) in a Soxhlet extractor. The extraction was continued for 6 h until the extracted hexane was colorless. Hexane was then removed in a rotary vacuum evaporator.
The adsorbents were characterized by means of nitrogen adsorption at -194.6ºC. Before the adsorption, the samples of bone were degassed under vacuum at 200°C for 2 h. Adsorption isotherms were measured in an Automatic Micromeritics equipment. The specific area and pore size distribution were estimated by the BET (Brunauer, Emmett

and Teller) and the BJH (Barrett-Joyner-Halenda) methods, respectively [17]. Equiment error 1%.

The external surface of bovine bone was examined using a JEOL, JSM-35C scanning electron microscope (SEM) with magnification of 48 and 1000.

The X-ray photoelectron spectroscopy (XPS) measurements were carried out using a multitechnique system (SPECS) equipped with a dual Mg/Al X-ray source and a hemispherical PHOIBOS 150 analyzer operating in the fixed analyzer transmission (FAT) mode. The spectra were obtained with pass energy of 30 eV; an Mg/Al X-ray source was operated at 100 W. The working pressure in the analysing chamber was less than 2.10^{-8} mbar. The reference binding energy (BE) was C 1s peak at 284.6 eV.

X-ray diffraction (XRD) measurements of powdered samples were obtained using Shimadzu XD-D1instrument with a CuKα radiation (λ= 1.5405 Å) in the $20 < 2\theta < 55$ º at a scan speed of 2º min^{-1}.

Infrared spectra (FTIR) were obtained with a Shimadzu 8101 M spectrometer. Samples were prepared in the form of pressed wafers (ca. 1% sample in KBr). All spectra involved the accumulation of 80 scans at 4 cm^{-1} resolution.

2.5. Lipase Activity and Protein Concentration

Lipase activity assay was followed spectrophotometrically in a Cary Varian 300 (Varian, Palo Alto) spectrophotometer at 30°C in 200 mM Tris–HCl buffer at pH 7.25 using the Roche lipase colorimetric kit (*lipase colorimetric assay* Roche kit 11821792, Mannheim, Germany)) as previously described [18]. The measurement was done at 580 nm, and every analysis was carried out in triplicate.

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard [19]. All samples were analysed in duplicate.

For every experiment, the activity and protein content of the supernatant were analysed at the beginning and at the end of the assay.

2.6. Immobilization process

Different enzyme loadings of activity (4.000-110.000 UA) for rROL in 30 ml of 5 mM phosphate buffer at pH 7, were shaken under magnetic stirring for 30 min at 4°C. Then, the solutions were centrifuged for 20 min at 12.000 rpm in a Biofuge Fresco Heraeus centrifuge. Each supernatant obtained was mixed with approximate 700 mg of BB or BBC600 in a roller MoviROL (P-selecta, Abrera, Spain) for 7 h at 4°C. After this time, the support with the immobilized lipase was separated from the solution by vacuum filtration and washed with 200-300 ml of the same buffer used at the start of immobilization. In order to remove all moisture of the biocatalyst obtained, it was stored a desiccator until its weight reached a constant value (between 1 and 3 days). Finally, the support with the immobilized lipase was stored at 4°C. For each experiment a solution control, without support, was run under the same conditions (blank experiment).

The following parameters were studied in every immobilization assay:

$$\%\ Immobilization: \frac{(Blank\ activity - Final\ supernatant\ activity)}{Blank\ activity}\ x\ 100$$

$$\%\ Immobilized\ protein: \frac{(Initial\ supernant\ protein\ content - Final\ supernant\ content)}{Initial\ supernant\ protein\ content}\ x\ 100$$

$$Enzyme\ load: \frac{Blank\ activity - Final\ supernatant\ activity}{mg\ of\ support}\ (UA\ mg^{-1}\ of\ support)$$

2.7. Studies of adsorption on bovine bone

For the equilibrium adsorption studies, the Langmuir isotherm was used to fit the data results.

$$Qe = \frac{K_L * Ce}{1 + a_L Ce} \quad (1)$$

Where:
Qe= Lipase activity adsorbed on support in the equilibrium [UA mg support^{-1}];
Ce= Lipase activity in solution in the equilibrium [UA ml^{-1}];
K_L [ml mg support^{-1}] and a_L [ml UA^{-1}] are the Langmuir constants. K_L is related to the support maximum capacity and the energy of adsorption, a_L is related to the energy of adsorption and the affinity of the support for the lipase, and $K_L\ a_L^{-1}$ [UA mg support^{-1}] represents the monolayer capacity of the support.
The OriginPro 8 software was used to fit experimental equilibrium points to the Langmuir isotherm.

2.8. Transesterification reaction

Enzymatic transesterification were carried out with 1 ml of the dilution of 1 g of olive oil with 10 ml of hexane. The molar ratio methanol:oil was=3:1. The major problem for the enzymatic biodiesel production is the inactivation of the enzyme caused by the polar short chain alcohols such as methanol [20]. A strategy to solve this problem is the methanol stepwise addition, this solution is still the first choice as it can achieve a high yield using simple operating conditions [6]. The amount of methanol in each step (4 µL) was added to the reaction media to reach a maximum 14% of FAMEs yield (one step). The biocatalyst mass was adjusted according to the test performed. The temperature and stirring rate were kept constant at 30°C and 200 rpm respectively, using a shaker - incubator (IKA KS 400 i control, Satufen, Germany). Optimum parameters of temperature and stirring rate for enzyme rROL were reported by Guillen et al. [14].
The amounts of methyl esters were determined by gas chromatography in accordance with the method described by Canet et al.[1]. A blank assay using only the support as a catalyst (without immobilized lipase) was performed under the same reaction conditions (negative control).
All reactions were done in duplicate.

3. Results and discussion

3.1. Physicochemical characterization of supports.

The chemical analysis of BB and BBC600 are shown in Table 1. According to the chemical formula of the standard hydroxyapatite, the calcium to phosphorous molar ratio is 1.67. The results obtained by ICP for BB and BBC600 were 1.62 and 1.65, respectively; these values were lower than the theoretic value calculated from HAP molecular formula due to the presence of small amount of microelements such as arsenic, cadmium and plumb and other organic substances.

Initially the discard bone without acetone treatment contain a 22% of proteins and 26% of lipids, after treatment the lipid content is reduced to <0.5%, whereas protein content not varied. After calcination at 600ºC, proteins and lipids are not detected in BBC600. These results indicate that the chemical treatment performed with acetone may be effective in lipids removing, which found inside the pores of the bone, but it is not adequate to remove the collagen, the main protein component.

The amount of immobilized enzyme by adsorption is often limited by factors related to the support´s structure such as surface area, pore shape and size. The results of surface area, pore volume and mean pore diameter for both supports are shown in Table 2. The surface area and pore volume increased 13.2 and 16.9 times, respectively, when discard bovine bone are calcined. Calcination at 600°C is necessary to remove the organic component into the pores to generate an adequate porosity and to increase internal surface.

Figure 1 A and B show adsorption isotherms obtained for both supports. They can be classified as type IV, according to IUPAC nomenclature [21]. This type of isotherms is characteristic of mesoporous solids (pore diameters between 20-300 Å) [22]. When the two isotherms are compared it is clear that effectively the calcination treatment generates porosity.

Figure 1: Nitrogen adsorption isotherms. BB (A), BBC600 (B).

A good support material must have a suitable pore structure to allow the access of the enzymes to its internal surface without difficulty. Moreover, in reaction, it must allow the free access of the reactants to its internal surface. This latter topic is critical when large molecules (i.e., triglycerides) must diffuse with sizes similar to those of the pores of the supports. Figure 2 shows the pore size distribution of both supports, it also include the approximate length of the lipase and the triglycerides [23, 24]. As shown in this figure, BBC600 provides an appropriate porous structure so that both, lipase and triglyceride molecules, can diffuse inside of the pore network.

Figure 2: Pore size distributions of supports. BB (A), BBC600 (B).

According to the results obtained, BBC600 would be a suitable support for immobilization of rROL, therefore this support is chosen to be analysed by scanning electron micrograph (SEM). Figure 3 shows the images of BBC600. It can be seen an irregular surface and interconnected pores of different sizes. The SEM micrographs confirmed that the calcined bone is a highly porous material.

Figure 3: SEM micrographs showing different-scale views: (A) general overview. Magnification: 48, (B) high magnification for BBC600. Magnification: 1.000.

Figure 4 shows X-ray diffraction pattern of BB and BBC600 supports. The obtained XRD spectra are compared with JCPDS standard HAP card (Nº 9-432, 1996) [25]. The X-ray analysis showed that the main peaks corresponding to standard HAP are present in the spectrum of BBC600 (2θ = 32.32, 33.19 and 40.0º), which has a higher crystallinity (83.4%). For the BB sample not all the standard HAP peaks have been formed. It may be due to presence of fibrous collagen inside the pores which disperses the X-ray radiations [13].

Figure 4: X-ray diffraction pattern of samples. BBC600 (A) and BB (B).

To confirm that the removal of collagen was complete, FTIR and XPS analysis were performed using BBC600 as sample.
The FTIR spectra of BB and BBC600 are illustrated in Figure 5. FTIR analysis indicated the presence of PO_4^{-3} and OH^- ions in all the samples. The 1045 and 962 cm^{-1} bands were assigned to the stretching vibrations of PO_4^{-3} ions and 603 and 569 cm^{-1} bands were assigned to the deformation vibrations of PO_4^{-3} ions [26]. The bands at 3546 cm^{-1} and 640 cm^{-1}, were due to the vibration motion of the OH^- ions [27]. The FTIR spectra also indicated the absorption peak of carbonate ions around 1450 cm^{-1}. The collagen as a protein can be detected by FT-IR analysis in three main amide regions: amide I, II and III [28]. Amide I: the bands representing this amide are created at 1620, 1636 and 1685cm^{-1}. Amide II band has major peak at 1559cm^{-1} and there are minor bands at 1521, 1533 and 1543cm^{-1}. Amide III band having characteristic peaks at 1231, 1248, and 1281cm^{-1}. All these peaks are present only in this spectra of BB, therefore the results might confirm that the collagen and other organic compounds present in the bovine bone without thermal treatment were eliminated after calcination.

Figure 5: FTIR spectra of BB (A) and BBC600 (B).

XPS spectra of BBC600 sample is shown in Figure 6. It can be identified: carbon, oxygen, calcium, and phosphorus as the major constituents of the sample. In accordance with the results obtained by FTIR, the major C1s contribution is due to $CaCO_3$. Oxygen found on the surface of the sample can be attributed to compounds such as oxides, OH^- groups, PO_4^{-3} and H_2O. The calcium contribution is due to $CaCO_3$ molecules, while phosphorus is found in the PO_4^{-3} [29]. The value of binding energy for N1s is about 398.9 eV, the absence of this peak in the XPS spectra indicate a complete elimination of collagen after thermal treatment. This agrees with the results obtained by FTIR and Kjeldahl methods.

Figure 6: XPS spectra of BBC600 sample.

3.2. *Selection of immobilization supports*

Carpio et al.[30] immobilized b-galactosidase and amyloglucosidase on bone. They reported that enzyme adsorption was mainly due to interactions between the enzyme and the protein components of the matrix (essentially collagen). In order to analyze the incidence of collagen content on the adsorption of rROL, the immobilization was carried out employed BB and BBC600. The initial activity loaded was 4.000 UA. Concerning with the immobilization percentage obtained, BBC600 was the support that enabled the coupling of a higher amount of enzyme, 56.6%, while BB retained only 31%. The enzyme immobilization increases twice when the support used was BBC600. This result means that the collagen is not the major cause for enzyme adsorption. Therefore, the enzyme immobilization yields are strongly dependent on the properties of the support. BBC600 has possessed an adequate porous structure, pore size and large specific surface as a result of having removed most the organic material, however although BBC600 has a surface area 13 times higher than BB (Table 1), the percentage of immobilization increases only twice. Then, it can not rule out a promoter effect of collagen on enzyme adsorption.
In accordance with the results obtained, the support BBC600 was selected for further experiments.

3.3. Immobilization on BBC600

The effect of increasing the enzyme load in the initial immobilization solution (4.000 to 110.000 UA) was studied (Figure 7) employing approximate 700 mg of support. It can be seem that both, enzyme immobilization and the immobilized protein percentages obtained for the rROL, decreased when the initial enzyme load was increased. Conversely, the amount of enzyme adsorbed on the support (UA mg^{-1}) increases with the initial charge of enzymes to a value of near 50.000 UA and then remained constant, probably due to the saturation of the support.

Figure 7: Effect of the initial enzymes load on % immobilized protein (A), % immobilization (B) and enzyme loaded (UA mg^{-1})(C).

The adsorption isotherm was determined from the data of Figure 7 and the results are plotted in Figure 8. Generally, adsorption isotherms of globular proteins such as lipases can usually be fitted to the Langmuir equilibrium isotherm [31,32]. Some considerations can be made about the experimental isotherm:
i) It reflects a "favourable" equilibrium curve, in the range of load tested, for the lipase adsorption. The isotherm shape describes the support affinity for lipase [33], therefore in this study BBC600 displays affinity for rROL.
ii) It can be described by the Langmuir model which implied the formation of lipase monolayer on the support surface and an energetically homogeneous surface where all sites were identical.

Figure 8: Equilibrium isotherm of Lipase rROL immobilized on BBC600.

The Langmuir constant obtained by fitting the experimental points are present in Table 3. The monolayer capacity $K_L\ a_L^{-1}$ is close to 35 UA mg^{-1}. Because the limit of solubility of initial preparation is exceeded when high enzyme loads are employed. Under these conditions multilayer formation was not detected.
Blanco et al. [34] immobilized lipase from *Candida antarctica* B, and the support for enzyme was prepared by functionalization of mesoporous silica with octyltriethoxysilane. In comparison with BBC600 (Table 1 and 3), silica support has a greater BET surface area, 254 m^2g^{-1} and a less monolayer capacity 3.94 UA mg^{-1}. This means that despite to the surface area has an important role in lipases adsorption, not all surface sites are able to retain the enzyme. The reported value of $a_{L,}$ $4.77x10^{-3}$ ml UA^{-1}, is higher than the obtained when BBC600 was employed as support (Table 3), this would indicate a higher affinity of the enzyme for mesoporous octyl silica. This could be explained due to the lipases may become strongly adsorbed in the presence of hydrophobic support. In this case the octyl groups provide very hydrophobic properties to the silica, causing an increase in the affinity of the support by the enzyme.

3.4. Optimization of transesterification reaction conditions

3.4.1. Effect of enzyme load

The identification of the optimum enzyme loading has important economic and operational implications, in terms of the quantity of lipase purchased. In order to choice the optimum initial enzyme loading, the biocatalysts obtained previously were used in the transesterification reaction.
The reaction mixture contained 1ml of the dilution 1:10 oil:solvent solution, with 68 mg of biocatalyst and 4µl of methanol (stoichiometric relation in order to achieve a 14% yield). The time of the reaction was 1 hours. A blank assay using only BBC600 as a catalyst (without immobilized lipase) was performed under the same reaction conditions. No catalytic effect was observed. The values of methyl ester yield for different UA mg^{-1} of support are presented in Figure 9. The results suggest that biocatalyst is active in the reaction of transesterification, the interaction between enzyme and support, reduce any conformational change involved in enzyme inactivation. The maximum values of conversion are obtained for the range of 9.45 to 22.5 UA mg^{-1}. These results suggest that the activity of the system is presumably being controlled by the rate of diffusion of substrates through the pore structure of the particles.
For the purpose of avoiding saturation of the support and use the least amount of enzyme, a load of 9.45 UA mg^{-1} is chosen for further experiment.

Figure 9: The effect of rROL load in BBC600 on FAMEs yield. Enzyme load: 9.45 UA mg^{-1}.

3.4.2. Effect of support load on FAMEs yield

In order to setting the enzyme load to obtain the maximum conversion in one hour, the transesterification reaction was carried out using different amount of biocatalyst with an

enzyme surface load of 9.45 UA mg^{-1}. The maximum yield to be achieved in the reactions is 14%, according to the methanol added (4 µl). From figure 10 it can be observed that the maximum conversion of 14% is not achieved in any case. From 600 UA a constant conversion of 10% is obtained, despite to increasing enzyme loading. Therefore in the next experiment, 600 UA are employed in the reaction.

Figure 10: Effect of enzymatic load on FAMEs yield.

3.5. Stepwise methanolysis of olive oil

Stepwise addition of methanol was the most common strategy to avoid lipase inactivation caused by the polar short chain alcohols. In order to know evolution profile achieving a higher methylester (FAME) yield, a methanol stepwise addition reaction was carried out. The transesterification reaction was carried out with 1 µl of 1:10 dilution olive oil: hexane and 68 mg of immobilized biocatalyst. Methanol was added every 30 min, in a total of 6 additions. It is important to mention that because of the regiospecificity of rROL that acts in the locations 1 and 3 of triglyceride, the maximum yield achieved cannot be over of 66% of FAMEs [35]. Figure 11 shows the evolution of FAMEs yield as a function of reaction time. For the enzyme load used in the test, a final yield of 49.6±1.49% was achieved after the 6 methanol additions and 180 min of reaction. This value of conversion is lower to the theoretical value. Similar results were reported by Duarte et al. [35], employing rROL immobilized on Relizyme OD403 (polymethacrylate), olive oil as substrate and six methanol additions, a yield of 54.3 % is obtained after 250 min of reaction. The results suggest that rROL immobilized on BBC600 is very active in the reaction.

Figure 11: Time evolution of FAMEs yield.

4. Conclusions

The removal of lipids and subsequent calcination at 600ºC of the discard bovine bone, allows obtaining adequate support for the immobilization of rROL. The collagen is not the major cause for rROL adsorption but it may be important. The enzyme immobilization yields are strongly dependent on the properties of the support. BBC600 has the suitable characteristics of a good support: an appropriate porous structure, pore size and large specific surface. The immobilization was carried out by adsorption, and the monolayer enzyme loading on this support was 35 UA mg^{-1}. The biocatalyst was active in the reaction of transesterification without conformational change of the lipase or inactivation. A final yield of 49.6% was achieved after the 6 methanol additions and 180 min of reaction.

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Conflict of interest

The authors declare no conflict of interest

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Table 1: Chemical composition of supports.

Support	Component					Ca/P (mol/mol)	Proteins (%)	Lipids (%)
	Ca (%)	P (%)	As (%)	Cd (ppm)	Pb (ppm)			
BB	25.4±0.001	12.1±0.001	0.019±0.001	<0.04	<0.01	1.62	22±0.25	<0.5±0.01
BBC600	36.2±0.001	17.0±0.001	0.021±0.001	<0.04	<0.01	1.65	n.d.	n.d.

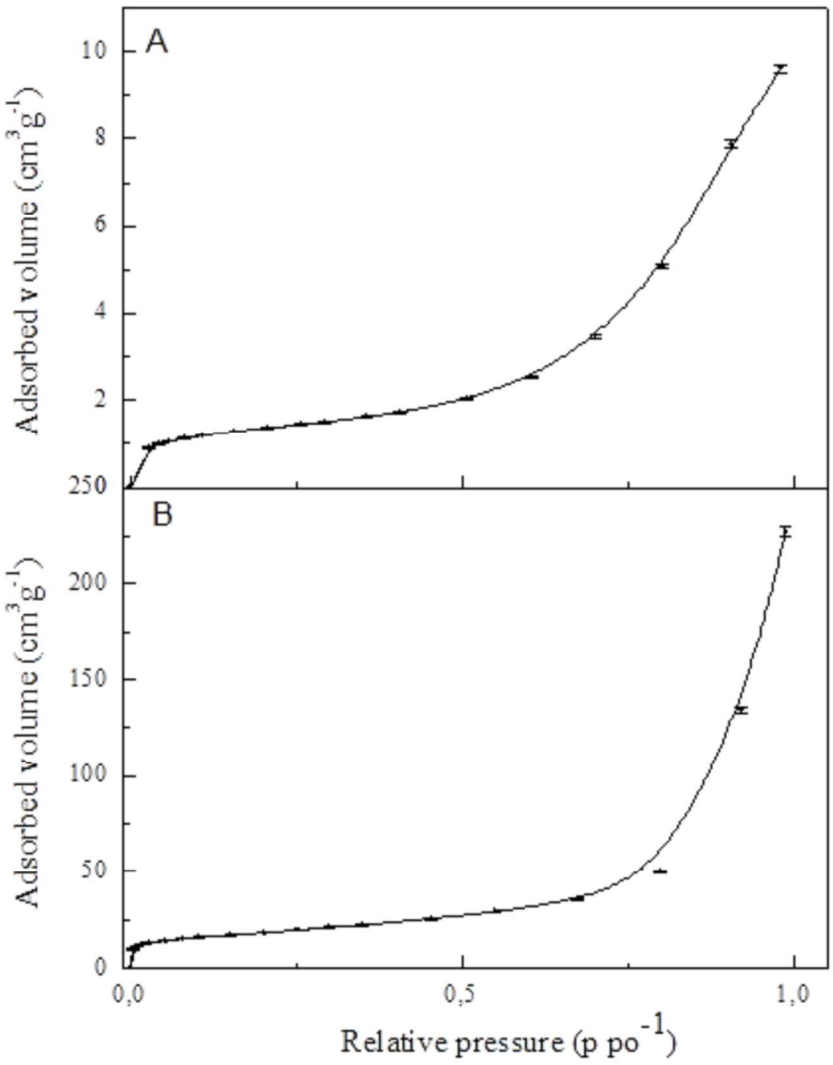
n.d.: not detected.

Table 2: Physical properties of supports. Surface area (Sg BET), pore volume (Vp) and mean pore diameter (Dp Wheeler) of supports.

Support	Dp wheeler (Å)	Sg BET (m^2g^{-1})	Vg ($cm^3\ g^{-1}$)
BB	106.7	4.9	0.013
BBC600	135.7	64.9	0.220

Table 3: Values of Langmuir constants for rROL lipase immobilized on BBC600.

Langmuir constants		
K_L (ml mg^{-1})	a_L (ml UA^{-1})	K_L/a_L (UA mg^{-1})
$3.68 \times 10^{-2} \pm 5.68 \times 10^{-3}$	$1.06 \times 10^{-3} \pm 5.51 \times 10^{-4}$	35



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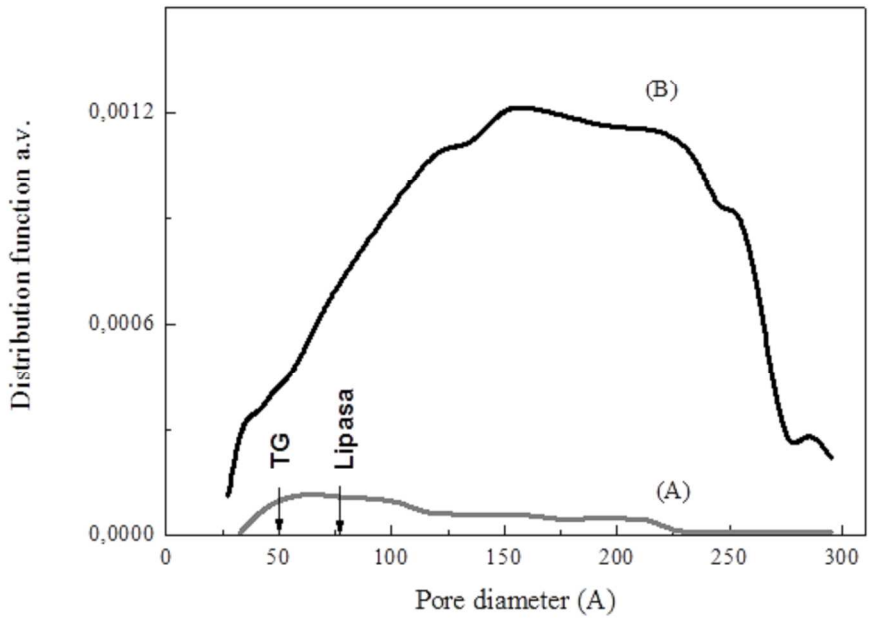

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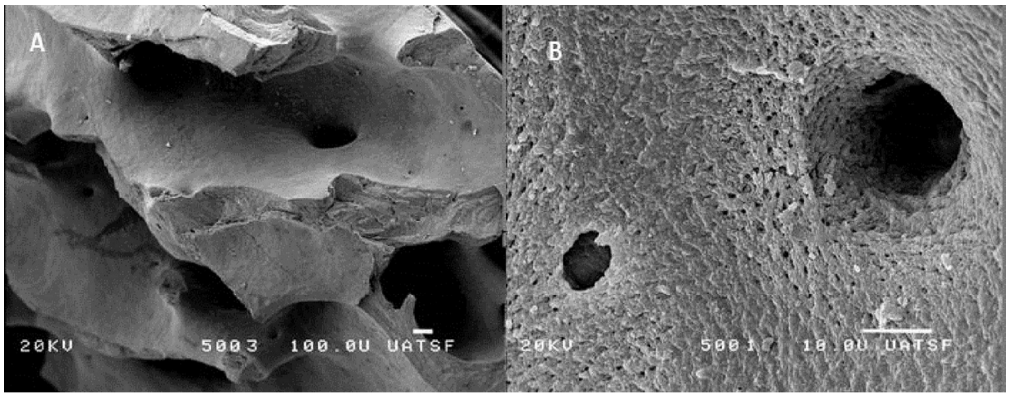

Figure 3: SEM micrographs showing different-scale views: (A) general overview. Magnification: 48, (B) high magnification for BBC600. Magnification: 1.000.
280x108mm (150 x 150 DPI)

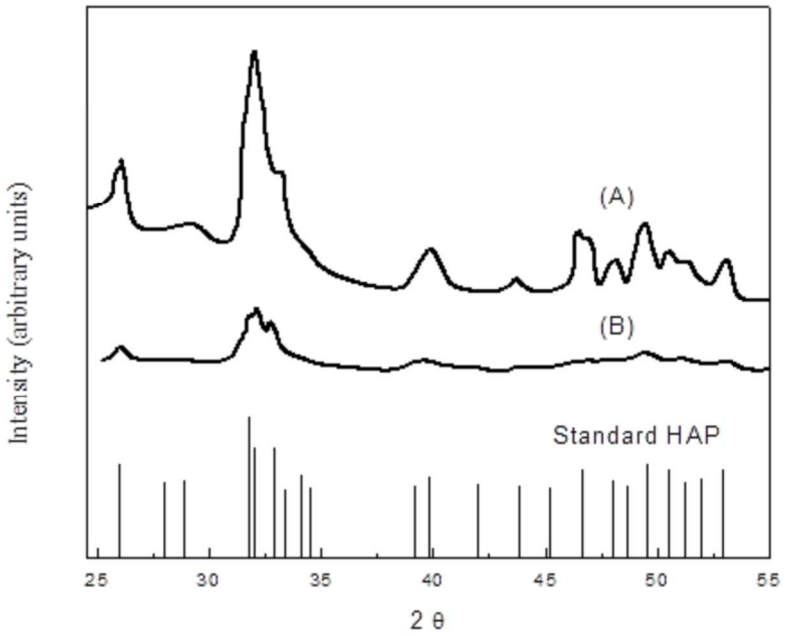

Figure 4: X-ray diffraction pattern of samples. BBC600 (A) and BB (B).
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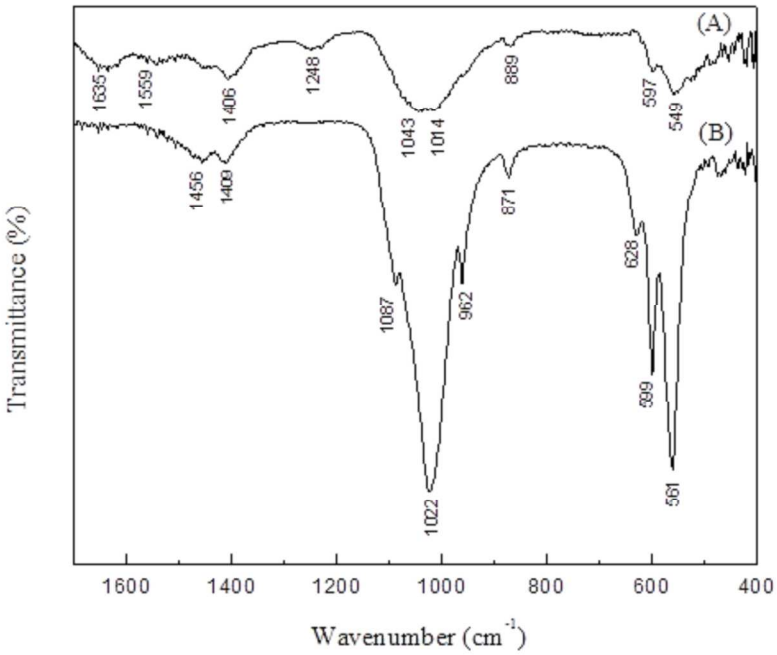

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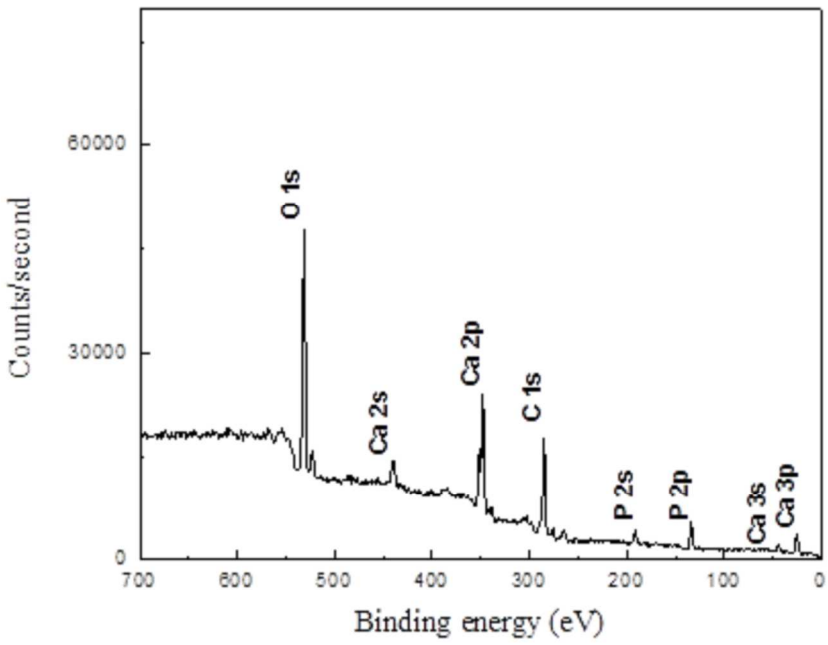

Figure 6: XPS spectra of BBC600 sample.
937x722mm (96 x 96 DPI)

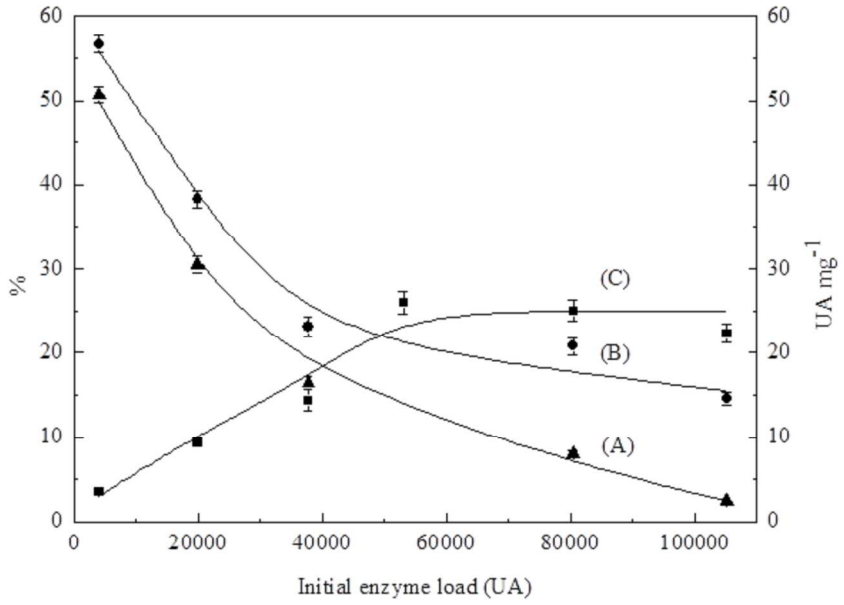

Figure 7: Effect of the initial enzymes load on % immobilized protein (A), % immobilization (B) and enzyme loaded (UA mg-1)(C).

937x660mm (96 x 96 DPI)

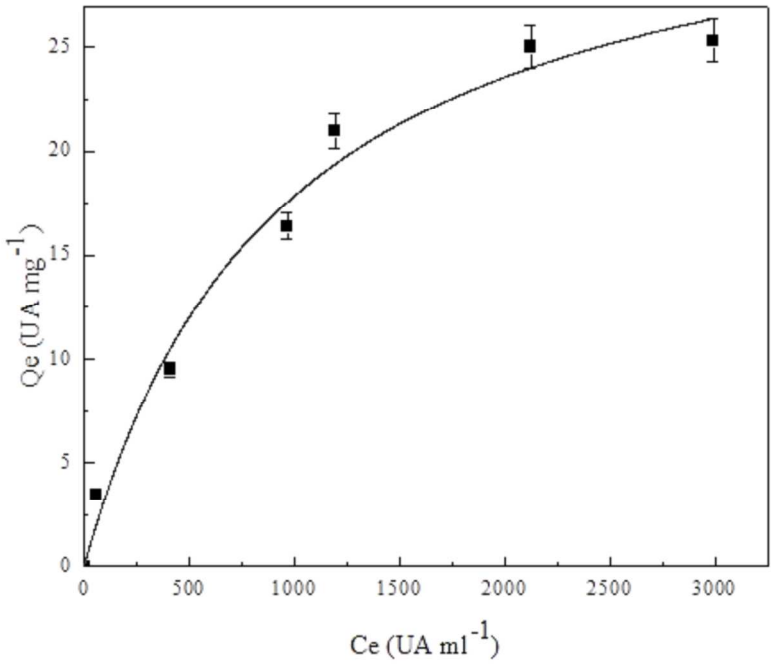

Figure 8: Equilibrium isotherm of Lipase rROL immobilized on BBC600.
937x731mm (96 x 96 DPI)

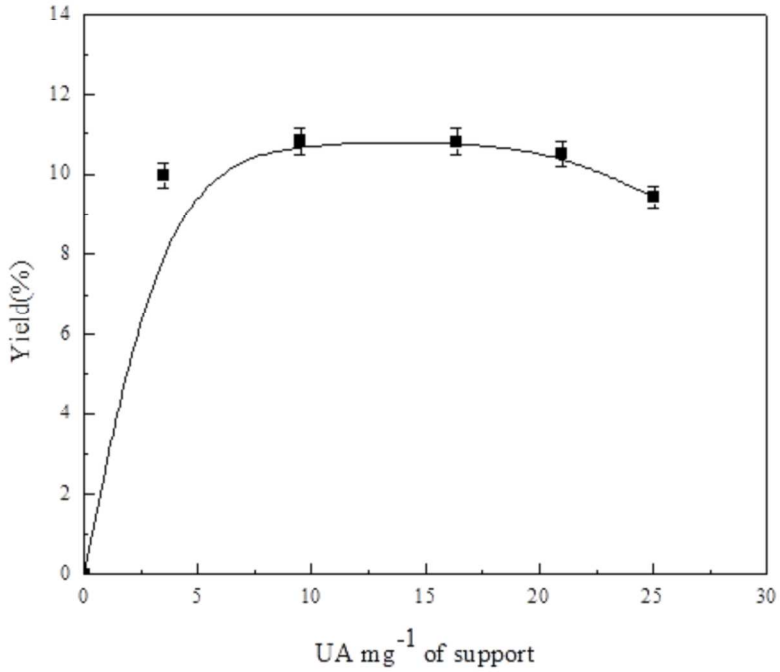

Figure 9: The effect of rROL load in BBC600 on FAMEs yield. Enzyme load: 9.45 UA mg-1.
937x731mm (96 x 96 DPI)

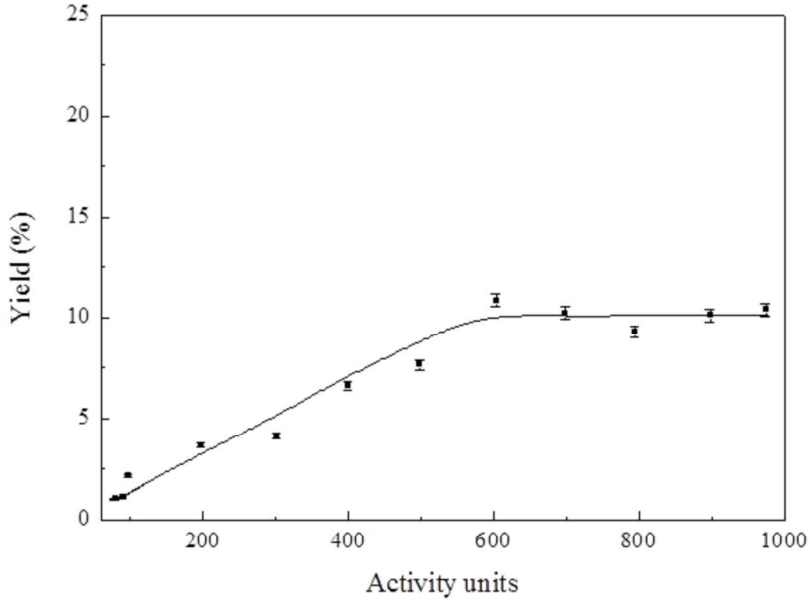

Figure 10: Effect of enzymatic load on FAMEs yield.
937x660mm (96 x 96 DPI)

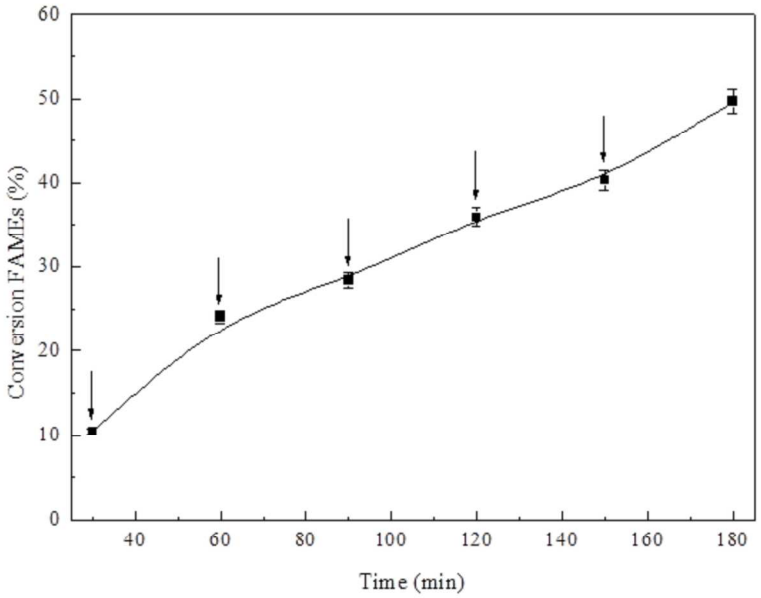

Figure 11: Time evolution of FAMEs yield.
937x660mm (96 x 96 DPI)