

# Utilization of Discard Bovine Bone as a Support for Immobilization of Recombinant *Rhizopus Oryzae* Lipase Expressed in *Pichia Pastoris*

**Adriana L. Clementz**

Institute for Research on Catalysis and Petrochemistry (INCAPE) FIQ-UNL-CONICET, Parque Tecnológico Litoral Centro, 3000, Santa Fe, Argentina

**Gonzalo Del Peso**

Dept. of Chemical, Biological and Environmental Engineering, Escola d'Enginyeria, Universitat Autònoma de Barcelona, Bellaterra 08193, Barcelona, España

**Albert Canet**

Dept. of Chemical, Biological and Environmental Engineering, Escola d'Enginyeria, Universitat Autònoma de Barcelona, Bellaterra 08193, Barcelona, España

**Juan C. Yori**

Institute for Research on Catalysis and Petrochemistry (INCAPE) FIQ-UNL-CONICET, Parque Tecnológico Litoral Centro, 3000, Santa Fe, Argentina

**Francisco Valero**

Dept. of Chemical, Biological and Environmental Engineering, Escola d'Enginyeria, Universitat Autònoma de Barcelona, Bellaterra 08193, Barcelona, España

DOI 10.1002/btpr.2321

Published online July 12, 2016 in Wiley Online Library (wileyonlinelibrary.com)

*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 h at 600°C. Supports were characterized by: ICP, SEM, XRD, FTIR, XPS, and N<sub>2</sub> 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<sup>-1</sup>. 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. © 2016 American Institute of Chemical Engineers Biotechnol. Prog., 32:1246–1253, 2016*

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

## 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.<sup>1</sup> 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.<sup>2</sup>

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.<sup>3,4</sup>

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

Correspondence concerning this article should be addressed to Dr. Francisco Valero at francisco.valero@uab.cat

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.<sup>5</sup> 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.<sup>6</sup> 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.<sup>7–9</sup>

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,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ). 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.)<sup>10</sup>

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*.<sup>1,11</sup>

In previous research, bones from pigs, chickens and fish were used as supports for enzyme immobilization<sup>12–14</sup> 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).

## Materials and Methods

### 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).

### Lipases

Recombinant *R. oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). This lipase was obtained by a mixed substrate fed-batch cultivation of a recombinant *P. pastoris* using methanol as the inductor.<sup>15</sup> The culture broth was centrifuged and micro-filtered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system (NY, 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.<sup>16</sup>

### 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<sup>3</sup>, equipped with a controlled heating. The treatment was performed under inert atmosphere (N<sub>2</sub>) at the following conditions:  $T = 50^\circ\text{C}$ ,  $P = 7$  bar, stirring speed = 300 rpm, time = 3 h. 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  $T = 600^\circ\text{C}$  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.

### 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  $\text{N} \times 6.25$ .

The lipid content was obtained by extraction with n-hexane (1:4 wt wt<sup>-1</sup> 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  $-194.6^\circ\text{C}$ . 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.<sup>17</sup> Equipment 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 analyzing 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-D1 instrument with a  $\text{CuK}\alpha$  radiation ( $\lambda = 1.5405 \text{ \AA}$ ) in the  $20 < 2\theta < 55^\circ$  at a scan speed of  $2^\circ \text{ min}^{-1}$ .

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  $4 \text{ cm}^{-1}$  resolution.

### Lipase activity and protein concentration

Lipase activity assay was followed spectrophotometrically in a Cary Varian 300 (Varian, Palo Alto) spectrophotometer at  $30^\circ\text{C}$  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.<sup>18</sup> 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.<sup>19</sup> All samples were analyzed in duplicate.

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

### 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  $4^\circ\text{C}$ . Then, the solutions were centrifuged for 20 min at 12,000 rpm in a Biofuge Fresco Heraeus centrifuge. Each supernatant obtained was mixed with  $\sim 700$  mg of BB or BBC600 in a roller MoviROL (P-selecta, Abrera, Spain) for 7 h at  $4^\circ\text{C}$ . 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 in a desiccator until its weight reached a constant value (between 1 and 3 days). Finally, the support with the immobilized lipase was stored at  $4^\circ\text{C}$ . 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 :

$$\frac{(\text{Blank activity} - \text{Final supernatant activity})}{\text{Blank activity}} \times 100$$

% Immobilized protein :

$$\frac{(\text{Initial supernatant protein content} - \text{Final supernatant content})}{\text{Initial supernatant protein content}} \times 100$$

Enzyme load (UA mg – 1 of support) :

$$\frac{\text{Blank activity} - \text{Final supernatant activity}}{\text{mg of support}}$$

### Studies of adsorption on bovine bone

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

$$Q_e = \frac{K_L C_e}{1 + a_L C_e} \quad (1)$$

Where:

$Q_e$  = Lipase activity adsorbed on support in the equilibrium [UA mg support<sup>-1</sup>];

$C_e$  = Lipase activity in solution in the equilibrium [UA ml<sup>-1</sup>];

$K_L$  [ml mg support<sup>-1</sup>] and  $a_L$  [ml UA<sup>-1</sup>] 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<sup>-1</sup>] represents the monolayer capacity of the support.

The OriginPro 8 software was used to fit experimental equilibrium points to the Langmuir isotherm.

### 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.<sup>20</sup> 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.<sup>6</sup> The amount of methanol in each step (4  $\mu\text{L}$ ) 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  $30^\circ\text{C}$  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.<sup>14</sup>

The amounts of methyl esters were determined by gas chromatography in accordance with the method described by Canet et al.<sup>1</sup> 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.

## Results and Discussion

### 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  $600^\circ\text{C}$ , proteins and lipids are not detected in BBC600. These results indicate that the chemical treatment performed with acetone may be effective

**Table 1. Chemical composition of supports**

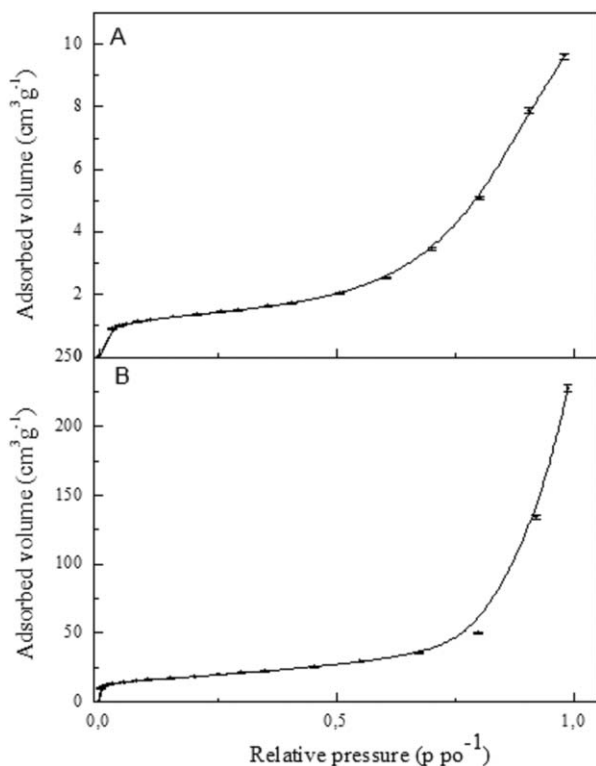
Support	Component					Ca/P (mol/mol)	Proteins (%)	Lipids (%)
	Ca (%)	P (%)	As (%)	Cd (ppm)	Pb (ppm)			
BB	25.4 ± 0.001	12.1 ± 0.001	0.019 ± 0.001	<0.04	<0.01	1.62	22 ± 0.25	<0.5 ± 0.01
BBC600	36.2 ± 0.001	17.0 ± 0.001	0.021 ± 0.001	<0.04	<0.01	1.65	n.d.	n.d.

n.d.: not detected.

**Table 2. Physical properties of supports**

Support	Dp wheeler (Å)	Sg BET (m <sup>2</sup> g <sup>-1</sup> )	Vg (cm <sup>3</sup> g <sup>-1</sup> )
BB	106.7	4.9	0.013
BBC600	135.7	64.9	0.220

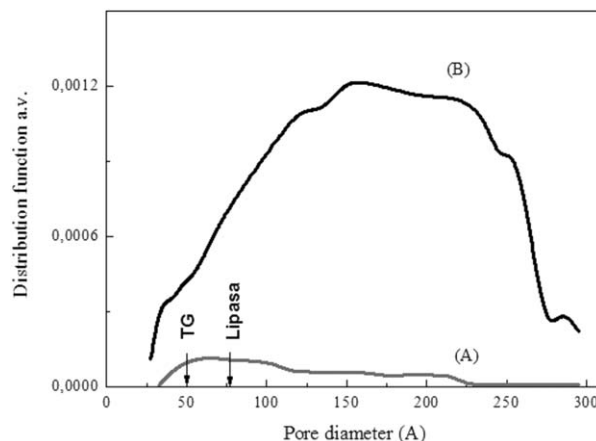
Surface area (Sg BET), pore volume (Vp) and mean pore diameter (Dp Wheeler) of supports.

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

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

**Figure 2. Pore size distributions of supports. 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.<sup>23,24</sup> 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.

According to the results obtained, BBC600 would be a suitable support for immobilization of rROL, therefore this support is chosen to be analyzed 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 4 shows X-ray diffraction pattern of BB and BBC600 supports. The obtained XRD spectra are compared with JCPDS standard HAP card (N° 9-432, 1996).<sup>25</sup> The X-ray analysis showed that the main peaks corresponding to standard HAP are present in the spectrum of BBC600 ( $2\theta = 32.32, 33.19$  and  $40.0^\circ$ ), 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.<sup>13</sup>

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  $\text{PO}_4^{3-}$  and  $\text{OH}^-$  ions in all the samples. The 1045 and  $962\text{ cm}^{-1}$  bands were assigned to the stretching vibrations of  $\text{PO}_4^{3-}$  ions and

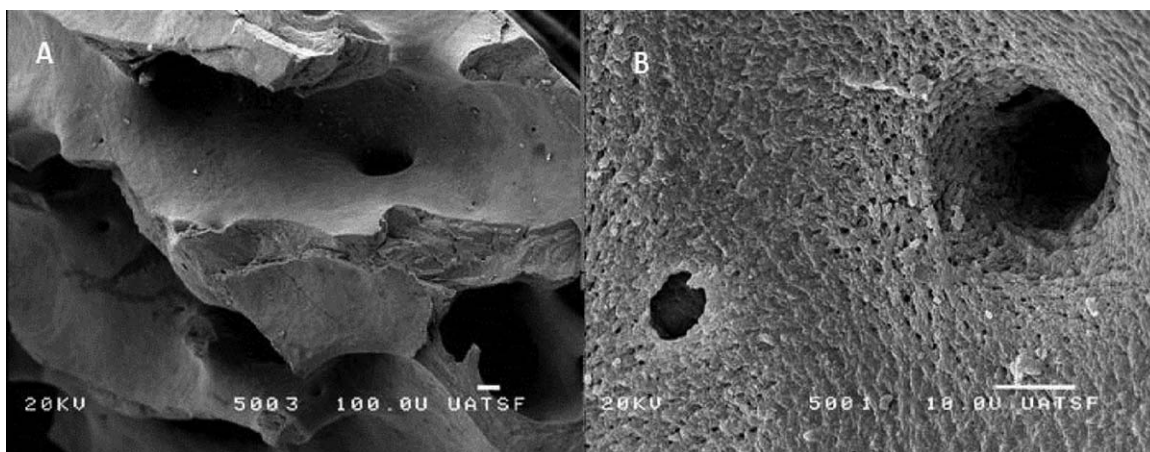


Figure 3. SEM micrographs showing different-scale views: (A) general overview. Magnification: 48, (B) high magnification for BBC600. Magnification: 1,000.

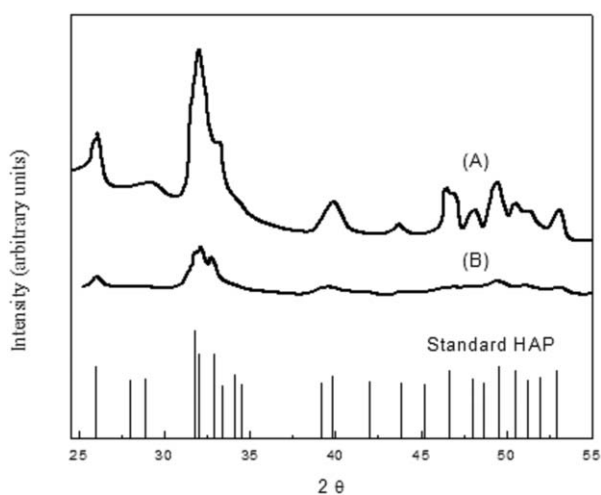


Figure 4. X-ray diffraction pattern of samples. BBC600 (A) and BB (B).

603 and  $569\text{ cm}^{-1}$  bands were assigned to the deformation vibrations of  $\text{PO}_4^{3-}$  ions.<sup>26</sup> The bands at  $3546\text{ cm}^{-1}$  and  $640\text{ cm}^{-1}$ , were due to the vibration motion of the  $\text{OH}^-$  ions.<sup>27</sup> The FTIR spectra also indicated the absorption peak of carbonate ions around  $1450\text{ cm}^{-1}$ . The collagen as a protein can be detected by FTIR analysis in three main amide regions: amide I, II, and III<sup>28</sup>. Amide I: the bands representing this amide are created at  $1620$ ,  $1636$ , and  $1685\text{ cm}^{-1}$ . Amide II band has major peak at  $1559\text{ cm}^{-1}$  and there are minor bands at  $1521$ ,  $1533$ , and  $1543\text{ cm}^{-1}$ . Amide III band having characteristic peaks at  $1231$ ,  $1248$ , and  $1281\text{ cm}^{-1}$ . 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.

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  $\text{CaCO}_3$ . Oxygen found on the surface of the sample can be attributed to compounds such as oxides,  $\text{OH}^-$  groups,  $\text{PO}_4^{3-}$  and  $\text{H}_2\text{O}$ . The calcium contribution is due to  $\text{CaCO}_3$  molecules, while phosphorus is found in the  $\text{PO}_4^{3-}$ .<sup>29</sup> The value of binding energy for N1s is about  $398.9\text{ eV}$ , the

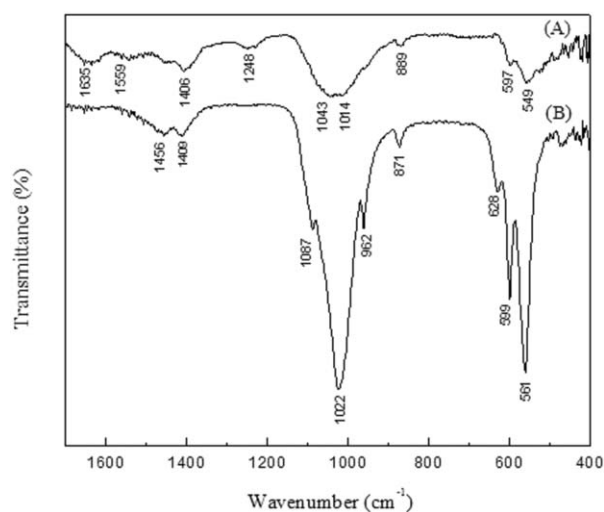


Figure 5. FTIR spectra of BB (A) and BBC600 (B).

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.

#### Selection of immobilization supports

Carpio et al.<sup>30</sup> immobilized  $\beta$ -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  $4.000\text{ UA}$ . 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

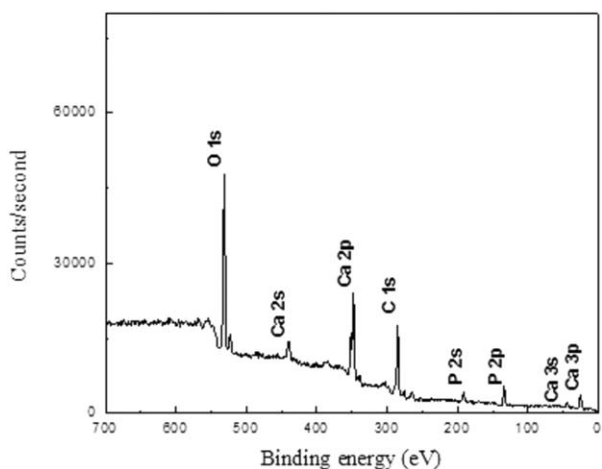


Figure 6. XPS spectra of BBC600 sample.

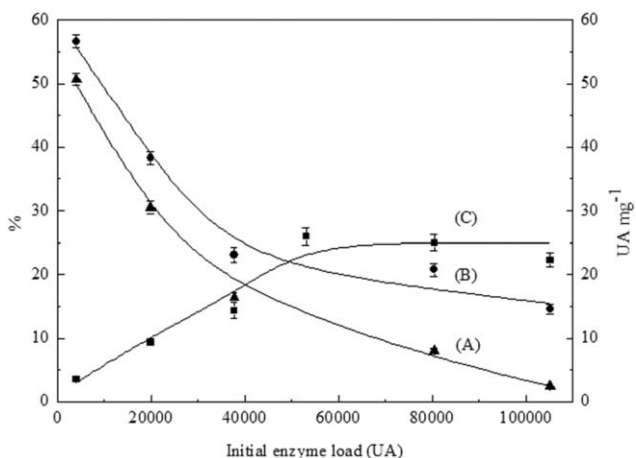


Figure 7. Effect of the initial enzymes load on % immobilized protein (A), % immobilization (B) and enzyme loaded (UA mg<sup>-1</sup>)(C).

increases only twice. Then, it cannot rule out a promoter effect of collagen on enzyme adsorption.

In accordance with the results obtained, the support BBC600 was selected for further experiments.

**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 seen 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<sup>-1</sup>) 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.

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.<sup>31,32</sup> Some considerations can be made about the experimental isotherm:

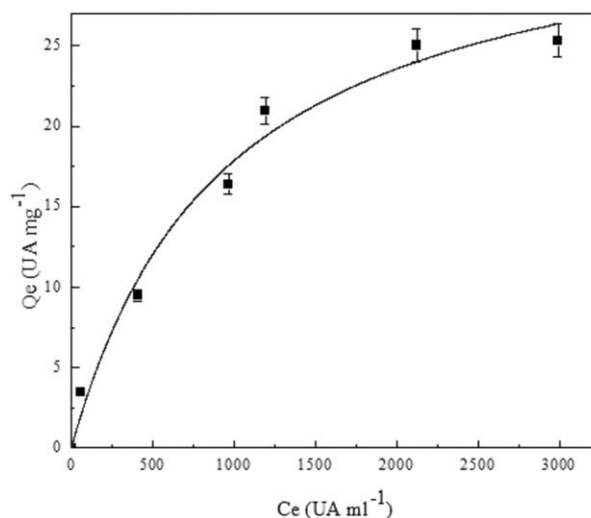


Figure 8. Equilibrium isotherm of Lipase rROL immobilized on BBC600.

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

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

- 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<sup>33</sup>; 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.

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<sup>-1</sup>. 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.<sup>34</sup> immobilized lipase from *Candida antarctica* B, and the support for enzyme was prepared by functionalization of mesoporous silica with octyltriethoxysilane. In comparison with BBC600 (Tables 1 and 3), silica support has a greater BET surface area, 254 m<sup>2</sup>g<sup>-1</sup> and a less monolayer capacity 3.94 UA mg<sup>-1</sup>. 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<sup>-3</sup>ml UA<sup>-1</sup>, 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.

**Optimization of transesterification reaction conditions**

*Effect of enzyme load.* The identification of the optimum enzyme loading has important economic and operational

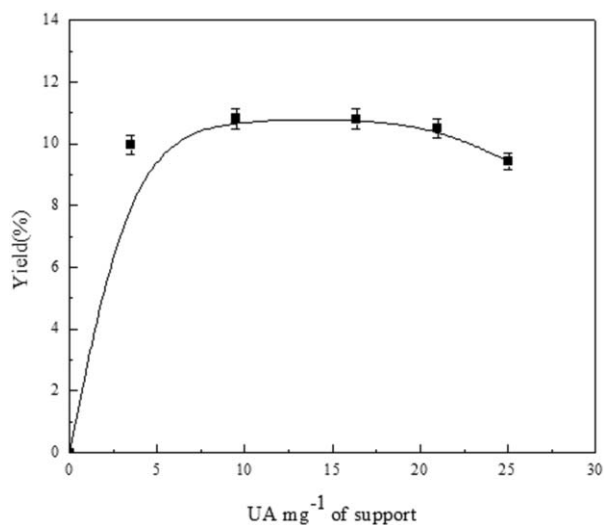


Figure 9. The effect of rROL load in BBC600 on FAMES yield. Enzyme load: 9.45 UA mg<sup>-1</sup>.

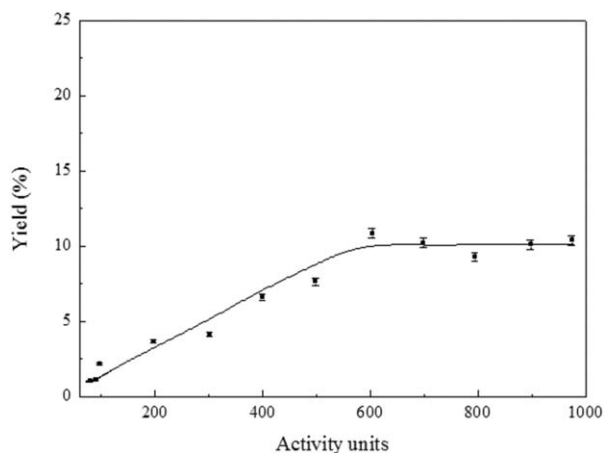


Figure 10. Effect of enzymatic load on FAMES yield.

implications, in terms of the quantity of lipase purchased. In order to choose the optimum initial enzyme loading, the biocatalysts obtained previously were used in the transesterification reaction.

The reaction mixture contained 1 ml of the dilution 1:10 oil:solvent solution, with 68 mg of biocatalyst and 4  $\mu$ l of methanol (stoichiometric relation in order to achieve a 14% yield). The time of the reaction was 1 h. 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<sup>-1</sup> 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 9.45–22.5 UA mg<sup>-1</sup>. 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<sup>-1</sup> is chosen for further experiment.

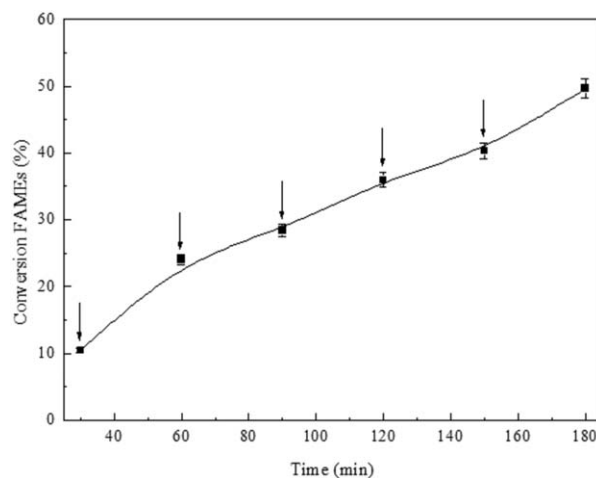


Figure 11. Time evolution of FAMES yield.

*Effect of support load on FAMES yield.* In order to setting the enzyme load to obtain the maximum conversion in 1 h, the transesterification reaction was carried out using different amount of biocatalyst with an enzyme surface load of 9.45 UA mg<sup>-1</sup>. The maximum yield to be achieved in the reactions is 14%, according to the methanol added (4  $\mu$ l). 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.

#### 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  $\mu$ l 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.<sup>35</sup> 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  $\pm$  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.,<sup>35</sup> 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.

#### Conclusions

The removal of lipids and subsequent calcination at 600°C 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<sup>-1</sup>. 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.

### Acknowledgements

The authors wish to thank Debora Manuale and María Fernanda Mori for their valuable cooperation. This work was carried out with the financial support of CONICET (Argentina) and the project CTQ2013-42391-R of the Spanish Ministry of Economy and Competitiveness. The group is member of 2014-SGR-452 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya).

### Literature Cited

- Canet A, Benaiges MD, Valero F. Biodiesel synthesis in a solvent-free system by recombinant *Rhizopus oryzae* lipase. Study of the catalytic reaction progress. *J Am Oil Chem Soc.* 2014;91:1499–1506. [TQ1]
- Yunjun Y, Xiang L, Guilong W, Gui X, Guanlin L, Feng S, Xiaofeng W, Tao L. Biotechnological preparation of biodiesel and its high-valued derivatives: A review. *Appl Energy* 2014; 113:1614–1631.
- Villeneuve P, Muderhwa JM, Graille J, Haas MJ. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *J Mol Catal B: Enzym* 2000;9: 113–148.
- Guillén M, Benaiges MD, Valero F. Immobilization and stability of a *Rhizopus oryzae* lipase expressed in *Pichia pastoris*: comparison between native and recombinant variants. *Biotechnol Prog.* 2011;27:1232–1241.
- Tecelão C, Guillén M, Valero F, Ferreira-Dias S. Immobilized heterologous *Rhizopus oryzae* lipase: a feasible biocatalyst for the production of human milk fat substitutes. *Biochem Eng J.* 2012;67:104–110.
- Tianwei T, Jike L, Kaili N, Li D, Fang W. Biodiesel production with immobilized lipase: a review. *Biotechnol Adv.* 2010;28: 628–634.
- Torres P, Batista-Viera F. Improved biocatalysts based on *Bacillus circulans*  $\beta$ -galactosidase immobilized onto epoxy-activated acrylic supports: applications in whey processing. *J Mol Catal B: Enzym.* 2012;83:57–64.
- Salis A, Sanjust E, Solinas V, Monduzzi M. Characterisation of accurel MP1004 polypropylene powder and its use as a support for lipase immobilization. *J Mol Catal B Enzym.* 2003;24–25: 75–82.
- Nakanishi K, Sakiyama T, Imamura K. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *J Biosci Bioeng.* 2001;91:233–244.
- Barakat NAM, Khil MS, Omran AM, Sheikh FA, Kim HY. Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. *J Mater Process Technol.* 2009;209:3408–3415.
- Guillen M, Benaiges MD, Valero F. Biosynthesis of ethyl butyrate by immobilized recombinant *Rhizopus oryzae* lipase expressed in *Pichia pastoris*. *Biochem Eng J.* 2012;65:1–9.
- Schafhauser DY, Storey KB. Immobilization of amyloglucosidase onto granular chicken bone. *Appl Biochem Biotechnol.* 1992;32:89–109.
- Mukatata S, Negishi S, Sato S, Takahashi J. Effect of substrate presoaking treatment of support materials on the activity of immobilized glucoamylase. *Enzyme Microb Technol.* 1993;15: 229–233.
- Negishl S, Sato S, Mukatata S, Takahashi J. Utilization of powdered pig bone as a support for immobilization of lipase. *J Ferment Bioeng.* 1989;67:350–355.
- Arnau C, Ramon R, Casas C, Valero F. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. *Enzyme Microb Technol.* 2010;46:494–500.
- Guillén M, Benaiges MD, Valero F. Comparison of the biochemical properties of a recombinant lipase extract from *Rhizopus oryzae* expressed in *Pichia pastoris* with a native extract. *Biochem Eng J.* 2011;54:117–123.
- Barrett EP, Joyner LG, Halenda PP. The determination of pore volume and area distributions in porous substances. I. Computations from nitrogen isotherms. *J Am Chem Soc.* 1951;73:373–380.
- Resina D, Serrano A, Valero F, Ferrer P. Expression of a *Rhizopus oryzae* lipase in *Pichia pastoris* under control of the nitrogen source-regulated formaldehyde dehydrogenase promoter. *J Biotechnol.* 2004;109:103–113.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem.* 1976;72:248–254.
- Shimada Y, Watanabe Y, Sugihara A, Tominaga Y. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *J Mol Catal B: Enzym.* 2002;17:133–142.
- Sing KSW, Everett DH, Haul RAW, Moscov L, Pierotti RA, Rouquérol J, Siemieniowska T. Reporting physisorption data for gas/solid systems with special reference to the determination of surface area and porosity. *Pure Appl Chem.* 1985;57:603–619.
- Sing KSW. Adsorption methods for the characterization of porous materials. *Adv Colloid Interface Sci.* 1998;76:3–11.
- Manuale D, Greco E, Clementz A, Torres GC, Vera CR, Yori JC. Biodiesel purification in one single stage using silica as adsorbent. *Chem Eng J.* 2014;256:372–379.
- Derewendat Z, Derewenda U. The crystal and molecular structure of the *Rhizomucor miehei* triacylglyceride lipase at 1.9 Å resolution. *J Mol Biol.* 1992;227:818–839.
- JCPDS File No. 9-432, 1980 International Centre for Diffraction Data, Pennsylvania, USA.
- Featherstone JD, Pearson S, Le Geros RZ. An infrared method for quantification of carbonate in carbonated apatites. *Caries Res.* 1984;18:63–66.
- Zhang S, Gonsalves KE. Preparation and characterization of thermally stable nanohydroxyapatite. *J Mater Sci Mater Med.* 1997;8:25–28.
- Changa MC, Tanaka J. XPS study for the microstructure development of hydroxyapatite–collagen nanocomposites cross-linked using glutaraldehyde. *Biomaterials* 2002;23:4811–4818.
- Kaciulis S, Mattogno G, Pandolfi L, Cavalli M, Gnappi G, Montenero A. XPS study of apatite-based coatings prepared by sol–gel technique. *Appl Surf Sci.* 1999;151:1–5.
- Carpio C, González P, Ruales J, Batista-Viera F. Bone-bound enzymes for food industry application. *Food Chem.* 2000;68: 403–409.
- Gitlesen T, Bauer M, Adlercreutz P. Adsorption of lipase on polypropylene powder. *Biochem Biophys Acta.* 1997;135:188–196.
- Kim DW, Jeong YK, Lee LK. Adsorption kinetics of exoglucanase in combination with endoglucanase from *Trichoderma viride* on microcrystalline cellulose and its influence on synergistic degradation. *Enzyme Microb Technol.* 1994;16:649–658.
- Al-Duri B, Yong YP. Lipase immobilization: an equilibrium study of lipases immobilized on hydrophobic and hydrophilic/hydrophobic supports. *Biochem Eng J.* 2000;4:207–215.
- Blanco RM, Terreros P, Fernández-Pérez M, Otero C, Díaz-González G. Functionalization of mesoporous silica for lipase immobilization. Characterization of the support and the catalysts. *J Mol Catal B Enzym.* 2004;30:83–93.
- Duarte SH, Del Peso Hernández G, Canet A, Benaige MD, Maugeri F, Valero F. Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant *Rhizopus oryzae* lipase. *Bioresour Technol.* 2015;183:175–180.