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Immobilization of the Lipase B from *Candida antarctica* on Urban Solid Waste

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Abstract: The adsorption of the lipase B from *Candida antarctica* (CALB) over polyethylene terephthalate (PET), polypropylene (PP), and derivatives, abundant components of urban solid waste (USW), was investigated. The characterization of the supports and biocatalysts synthesized by SEM-EDS and FTIR is presented. Two immobilization strategies were evaluated, conventional and total adsorption. The adsorbed protein was determined by Bradford and through high-resolution inductively coupled plasma atomic emission spectroscopy (ICP-AES). In this sense, the adsorption of CALB in all the proposed supports was evidenced, obtaining the highest protein loads in bis-(2-hydroxyethyl) terephthalate (BHET). Subsequently, the biocatalysts were applied to the esterification of *rac*-ibuprofen with ethanol. CALB immobilized in BHET showed remarkable activity, achieving conversions of 30%. In this context, immobilization on this support was optimized, studying the addition of sorbitol-glycerol. Thus, in the presence of 0.91 g of polyols, a catalyst with a protein load of 33.3 mg·g^{-1} was obtained, achieving productivity of 0.298 mmol min^{-1} mg^{-1}. Additionally, no differences were found when using BHET from USW bottles of various colors. This research shows the potential of materials derived from PET as enzymatic supports, unreported materials, that we can use as tools to achieve sustainable biotechnological applications.

Keywords: biocatalysis; enzymes; polyethylene terephthalate; polypropylene; bis-(2-hydroxyethyl) terephthalate; green chemistry; profens

Citation: Llerena Suster, C.R.; Fuentes, C.A.; Sambeth, J.E.; José, C. Immobilization of the Lipase B from *Candida antarctica* on Urban Solid Waste. *Catalysts* **2023**, *13*, 1324. https://doi.org/10.3390/catal13101324

Academic Editors: Ileana Daniela Lick and Paula Osorio-Vargas

Received: 26 June 2023
Revised: 22 July 2023
Accepted: 25 July 2023
Published: 26 September 2023

1. Introduction

Cells and enzymes are suitable and effective biocatalysts in the synthesis of pharmaceuticals, herbicides, insecticides, and alternative biofuels and are currently used in the textile and detergent industries, among others. In fact, biological biocatalysts are not only regio and stereoselective, but they are able to efficiently catalyze reactions under environmentally friendly conditions such as mild temperatures and pH [1].

Lipases [EC 3.1.1.3] are a family of enzymes that, in their natural environment, catalyze the hydrolysis of fats. These enzymes are well known for their versatility, having the ability to catalyze many reactions such as hydrolysis, alcoholysis, esterification, transesterification, acidolysis, aminolysis, and amidation, depending on environmental conditions. The versatility and wide range of substrate specificity make lipases very attractive from the perspective of industrial biocatalysis, and this is the reason for the variety of reported applications [2]. However, lipases, like other enzymes, have some limitations in their free form, such as less stability in non-conventional media, difficulty in separating the product, and difficult recovery and reuse of the catalyst. These factors determine the high costs of homogeneous enzymatic processes. The immobilization of enzymes on stable supports is a tool that allows us to overcome these restrictions. In recent years, various approaches for lipase immobilization have been demonstrated, such as adsorption, encapsulation, cross-linking, and covalent binding [3–6].

The adsorption of lipases on hydrophobic supports has been proposed as a simple and effective method for obtaining active biocatalysts. The hypothesis behind this immobilization strategy involves contacting the lipases with hydrophobic materials mimicking their natural substrates. In fact, the immobilization of the enzymes in an open conformation allows for increasing their catalytic activity through the effect known as interfacial activation [7,8]. In this context, there are numerous reports of the adsorption of various lipases on hydrophobic materials and their applications [9]. Palomo et al. reported the hyperactivation of lipases from *Mucor miehei, Candida rugosa*, and *Candida antarctica* due to their adsorption on strongly hydrophobic materials such as octadecyl–sepabeads [10]. In addition, the preparation of active catalysts in the synthesis of ethyl oleate by immobilized *Candida antarctica* lipase B and *Rhizopus oryzae* lipase (RoL) on polypropylene was also reported [11,12]. Pencreac'h et al. reported 100% conversions and improved selectivity (compared with the free enzyme) in the hydrolysis of p-nitrophenol acetate and p-nitrophenol palmitate in 2-propanol catalyzed by *Pseudomonas cepacia* lipase adsorbed on microporous polypropylene [13]. Bosley et al. studied the activity of lipases from *Humicola special, Rhizopus niveus*, and *Candida antarctica* B adsorbed on EP100 [14]. dos Santos et al. reported studies of covalent immobilization and adsorption of CALB on agarose beads functionalized with CNBr, divinylsulfones, and octyl groups [15]. These studies show that the differences observed in activity, stability, and specificity of the different enzyme preparations are due to different structures of the CALB molecules after immobilization. Zisis et al. reported the immobilization of CALB on hydrophobic surfaces of densely packed alkyl chains (methyl, propyl, octyl, or octadecyl functionalized glass beads) and the evaluation of substrate size on catalytic performance [16].

The well-known performance of the commercial biocatalyst Novozym®435, composed of CALB adsorbed on polymethylmethacrylate, is somehow evidence of the influence of hydrophobic polymers on lipase activity [17]. Previous investigations on some of us demonstrated that Novozym® 435 catalyzed the kinetic resolution of *rac*-ibuprofen towards a mixture of 70:30 molar ratio of the S- and R-enantiomers up to a 60% conversion [18]. However, the cost of the commercial catalyst as well as the reports that evidences the negative effects of short-chain alcohols on the stability of the biocatalyst, are serious drawbacks [19–22]. More recently, further investigations reported by some of us demonstrated that the biocatalysts based on CALB immobilized by adsorption on hydrophobic supports (acrylic resin, polymethylmethacrylate, polystyrene, epoxy resin, and polypropylene) possess higher conversion of ibuprofen and enantiomeric excess towards the S-enantiomer than CALB immobilized onto hydrophilic materials [23]. Those results were the driving force and motivation to further investigate the immobilization of CALB onto alternative materials, chemically stable, accessible, and low cost, considering polymers from urban solid waste as potential enzyme supports.

Even though the development and application of novel environmentally benign technologies are becoming more relevant every day, the associated cost somehow limits its expansion. In this sense, the recovery and reuse of wastes as enzymatic supports might become an incentive for the application of biocatalysis in the industry. In this context, it is interesting to analyze the composition of the urban solid waste (USW from now on) of a highly populated city such as Buenos Aires and also identify the recyclable fraction. The government survey regarding the composition of the USW indicates that the recyclable fraction is composed of 14.4% of paper and cardboard, 12.60% of plastics (mainly: polyethylene terephthalate PET, polypropylene PP, and low-density polyethylene LDPE), and 3.86% of glass. Additionally, 43.6% is food waste, 6% diapers and dressings, and the remaining 20% is composed of different wastes such as textile material, pruning, metals, construction material, pathogenic waste, hazardous waste, medicines, electronic material, and others, each of them in a content less than 4% [24].

This research is the first report regarding the use of PET and its derivatives as enzymatic supports. Specifically, the enzymatic immobilization of CALB by adsorption on PET and PP, major components of USW, is presented. Additionally, the synthesized catalysts

are applied to the esterification of *rac*-ibuprofen with ethanol, a reaction of interest in the pharmaceutical industry.

2. Results and Discussion

2.1. Characterization of Polyethylene Terephthalate, Its Derivatives, and Immobilized CALB

The images of commercial (PETc) and waste (PET) polyethylene terephthalate obtained through scanning electron microscopy are shown in Figure 1. This analysis shows morphological differences between both types of plastics. PETc possesses a rough surface with the presence of fibers, while the PET surface is homogeneous and smooth. These results correspond to the difference in intrinsic viscosity (η) and viscometric average molecular weight (Mη) reported by some of us for both PET samples. PETc corresponds to fiber grade, resulting in a lower intrinsic viscosity than bottle grade PET (10–40 mL/g vs. 70–85 mL/g) [25].

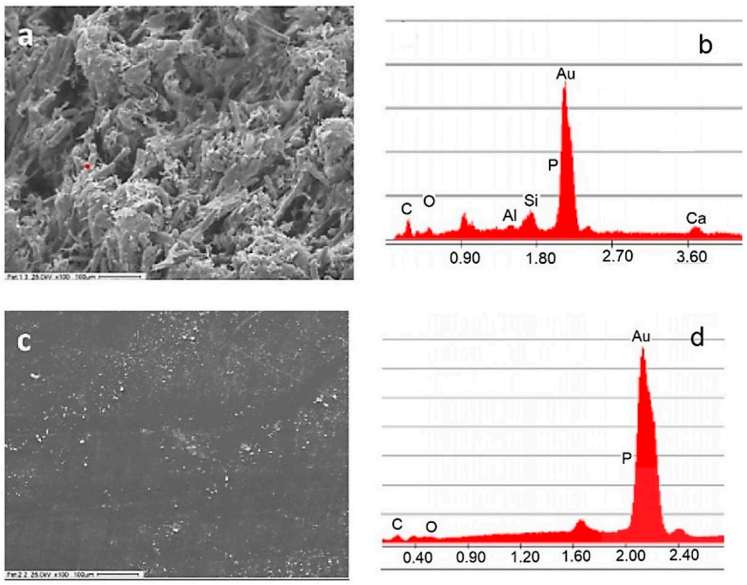


Figure 1. Morphological analysis (×100) and EDS. (**a**,**b**) Correspond to commercial PET (PETc); (**c**,**d**) correspond to PET waste (PET). The signal of Au belongs to the coating of the samples performed for analysis purposes.

In addition, the electron microanalysis evidenced the presence of Al, Si, P, and Ca in PETc, while in PET, only phosphorus was detected. The presence of Si in PETc agrees with the report of the commercial product, which details glass content as a reinforcer (Section 3.1). The presence of P can be attributed to the addition of P compounds as thermal stabilizers and flame retardants. Aluminum hydroxide is also reported as a stabilizing additive in polymers [26,27].

According to Jabłońska's report, the absence of Si, Al, and Ca in PET may be due to leaching in the intense wash of the starting material [28].

The infrared spectra of PETc and PET show signals corresponding to the methylene group $–CH_2–$, the carbonyl group C=O, and the aromatic hydrocarbon group, characteristic infrared signals of polyethyleneterephthalate. Table 1 summarizes the assignments of the infrared signals observed in the spectra of PETc, PET, and their derivatives. Similarly, the partial (PETpdp) and total (BHET) degradation derivatives of PET and the commercial monomer (BHETc) possess the infrared signals of the starting polymer along with the bands belonging to the stretching vibration of the –OH groups. Previously, Fuentes et al. reported a detailed characterization and identification of the glycolysis product used in this work as enzymatic support [29] and the initial PET [25]. The authors demonstrated that the product obtained by glycolysis of waste PET consists of high-purity bis-(2-hydroxyethyl) terephthalate.

Table 1. Summary of the functional groups and infrared signals detected in supports and biocatalysts derived from PET.

	ν –CH_2	ν C=O	ν –OH	ν C=C aromatic	Amide II (δ N–H; ν C–N)	Amide A (ν N–H)
PETc	2700–3000	1715–1750		1418, 1514		
PET	2700–3000	1715–1750		1419, 1510		
PETpdp	2963, 2908	1729	3457	1416, 1520		
BHETc	2887, 2967	1725	3456	1416, 1514		
BHET	2887, 2968	1725	3456	1416, 1514		
CALB		1652			1557	3357
C/BHET	2887, 2968	1652, 1729	3457	1514, 1416	1557	3357
C/PETpdp	2908, 2963	1652, 1729		1520, 1416		3332

PET, polyethylene terephthalate from waste bottles; PETc, commercial polyethylene terephthalate; PETpdp, partially digested purified PET; BHETc, commercial bis-(2-hydroxyethyl) terephthalate; BHET, obtained through glycolysis; CALB, lipase of *Candida antarctica*; C/BHET, C/PET, C/PETpdp, CALB immobilized on BHET, PET, and PETpdp.

Figure 2A,B shows the infrared spectra of the supports PETpdp, BHET, the free lipase (CALB), and the immobilized biocatalysts C/PETpdp and C/BHET. In addition, Table 1 presents the assignments of the infrared signals observed in those spectra.

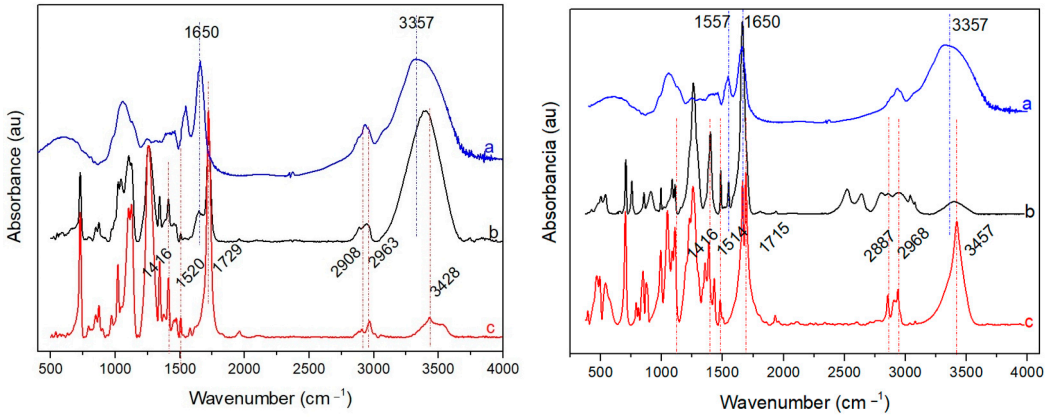


Figure 2. (**A**) Infrared spectra of lipase B de *Candida antarctica* (CALB) (a), immobilized lipase C/PETpdp (b), and bare PET partially digested and purified (PETpdp) (c). (**B**) Infrared spectra of lipase CALB (a), immobilized lipase C/BHET (b), and bis-(2-hydroxyethyl)terephthalate (BHET) (c).

The infrared spectra show the signals associated with both the support and the CALB lipase, providing clear evidence of the effectiveness of the immobilization of the lipase onto the supports. The infrared spectra of CALB possess an intense signal centered at 3357 cm^{-1} arising from the stretching vibration of the intramolecular hydrogen-bonded N–H species that is superimposed with the stretching vibrations of O–H species [30]. Additionally, the lipase possesses intense bands corresponding to the Amide I and Amide II signals centered at 1650 cm^{-1} and 1557 cm^{-1}, respectively [30,31]. The infrared signal, known as Amide I, arises from the stretching vibration of the carbonyl bond C=O of the backbone structure of the proteins. The infrared signal called Amide II is attributed to the out-of-plane combination of the in-plane bending mode of the N–H bond and the stretching vibration of the C–N bond of the protein.

Figure 3 shows SEM-EDS analysis of BHETc, BHET, and the lipase B of *Candida antarctica* immobilized on bis-(2-hydroxyethyl) terephthalate, called C/BHET. BHET possesses a laminar surface with splinter-shaped particles. BHETc also presents a laminar surface, along with fibers and chips that are irregular in size and thickness. The morphology of the biocatalyst C/BHET (see micrograph Figure 3e) goes from a laminar surface to a completely

rough one that might be ascribed to the presence of the lipase and/or other components of the crude extract.

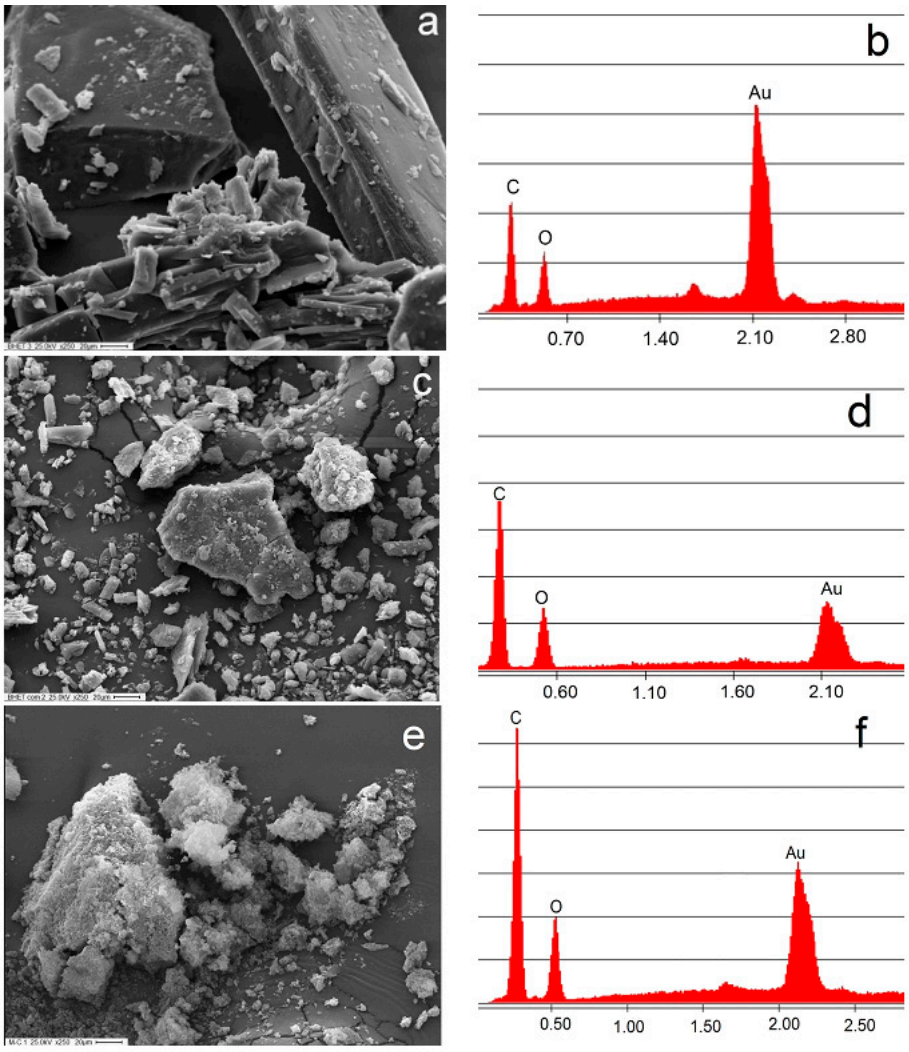


Figure 3. Morphological analysis (250×) and EDS. (**a**,**b**) Correspond to the BHET, (**c**,**d**) correspond to BHETc, and (**e**,**f**) correspond to the biocatalyst C/BHET. The signal of Au belongs to the coating of the samples performed for analysis purposes.

2.2. Characterization of Polypropylene, Derivatives, and Immobilized CALB

Figure 4 shows the micrographs of polypropylene (PP) before and after treatment with ethanol (PPetoh). "Cauliflower"-type granules gathered in large irregular particles are observed in both samples regardless of the treatment with the alcohol. Nevertheless, the morphology of PPetoh is more uniform than the not-treated sample, which is in agreement with the literature [32]. The elemental analysis shows only the presence of carbon in accordance with the chemical structure of the polymer and the absence of impurities.

Figure 5A shows the infrared spectra of polypropylene before and after treatment with ethanol, and the table presents a detailed assignment of the signal. The spectra of the bare PP and PPetoh show three groups of bands assigned to the tension movements of the C–H (2900 cm^{-1}), C–C bonds (1350–1450 cm^{-1}), and bending of CH_3 (1000–1200 cm^{-1}) that are fingerprints of the chemical structure of the polymer. Additionally, an important signal is observed in both cases at 3450 cm^{-1}, indicating the presence of O–H and/or N–H bonds. This signal can be attributed to the remains of nucleants, such as sorbitol, used in the industrial manufacture of the material [33].

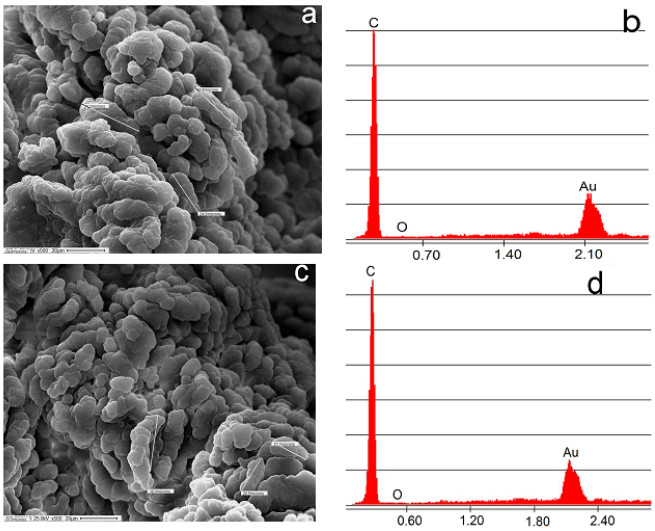


Figure 4. Morphological analysis (250×) and EDS. (**a**,**b**) Correspond to polypropylene (PP); (**c**,**d**) correspond to polypropylene post-treatment with ethanol (PPetoh).

Figure 5B shows the infrared spectra of CALB immobilized on PP and PPetoh and the pure lipase for comparison. The enzymatic adsorption is evidenced by the characteristic signals assigned to the proteins Amide I and Amide II. In these cases, the Amide A band does not provide information regarding enzymatic immobilization due to overlapping signals.

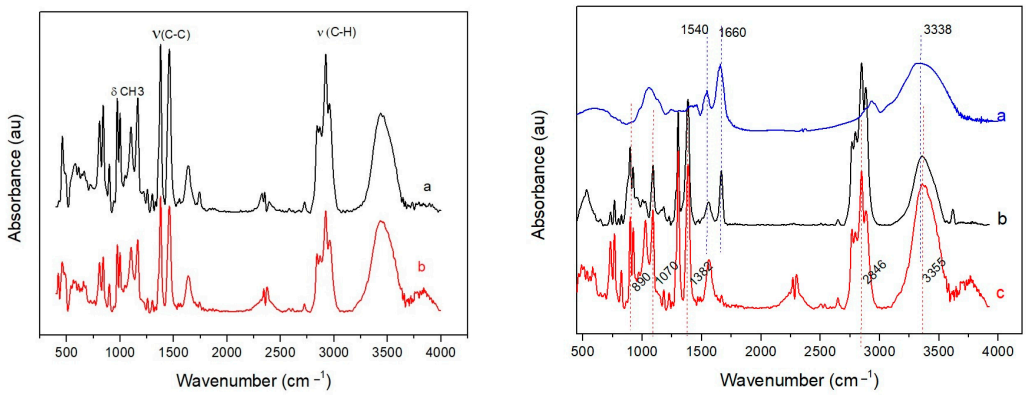


Figure 5. (**A**) (a) Infrared spectra of polypropylene (PP) and (b) polypropylene treated with ethanol (PPetoh). (**B**) (a) Infrared spectra of lipase B de *Candida antarctica* (CALB), (b) biocatalyst C/PPetoh, and (c) polypropylene treated with ethanol (PPetoh). Signal assignments are detailed in Table 2.

Table 2. Summary of the functional groups and infrared signals detected in supports and biocatalysts derived from PP.

	ν –CH– alkyl	**δ –CH_3**	**ν C–C**	**Amide I (ν C=O)**	**Amide II (δ N–H; ν C–N)**	**Amide A (ν N–H)**
PP	2900	1000, 1200	1350–1455	----	-----	3355
PP_{etOH}	2900	1000, 1200	1350–1455	----	----	3355
CALB	----	----	----	1652	1557	3357
C/PP	2925	1167, 996	1459	1652	1557	3434
C/PP_{etOH}	2846	890–1070	1382	1660	1557	3338

PP, polypropylene; PPetoh, polypropylene treated with ethanol; CALB, lipase B of *Candida antarctica*; C/PP and C/PPetoh, CALB immobilized on PP and Ppetoh, respectively.

2.3. Effect of the Immobilization Strategy on the Enzymatic Loading and Biocatalytic Activity

The immobilization of the lipase onto the investigated support was performed through the total and conventional adsorption methods. These methodologies and the effect on both the amount of protein adsorbed and catalytic activity are discussed in this section.

Table 3 shows the amount of the support, the volume of the solution of the crude extract used (containing 14.9 mg of protein per mL) in the immobilization, loading of protein immobilized onto the supports, *rac*-ibuprofen conversion, and specific activity.

Table 3. Amount of support, the volume of the solution of the crude extract used (containing 14.9 mg of protein per mL) in the total immobilization procedure, loading of protein immobilized onto the support, *rac*-ibuprofen conversion, and specific activity.

Biocalatyst	V_{CE} (mL)	Mass Support (mg)	Protein Loading ($mg \cdot g^{-1}$)	Ibuprofen Conversion (X%)	Specific Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
Free CALB	0.25	----	----	42.0	0.139
C/PPetoh	0.15	74.5	30.0	5.2	0.016
C/PET	0.30	149.8	29.8	0.8	0.005
C/PETpd	0.30	150.7	29.7	4.2	0.024
C/BHET	1.00	202.2	40.7 *2	3.4	0.008
C/BHET *1	1.00 *1	200.2	43.6 *2	5.4	0.012

*1 The "adapted" CE solution was used (13.4 mg of protein per mL). *2 Enzyme loading per gram of biocatalyst.

The biocatalyst called C/BHET* was prepared with an enzymatic solution containing less amount of non-protein substances (i.e., sorbitol and glycerol) than the crude extract. This strategy was tested due to some drawbacks observed in the other preparations. In this context, it is worth noticing that the high concentration of glycerol in the CE led to a sticky type of material and also negatively influenced the lyophilization of the biocatalysts because the polyols are not properly sublimated.

The total immobilization method assures that the whole amount of protein in the starting solution (along with the other components of the CE) is retained in the solid after lyophilization [34]. In this context, the protein loading reported in Table 3 (i.e., 30–40 $mg \cdot g^{-1}$) was calculated with the volume and the protein concentration of the CE used in the preparation. The observation that the conversion of ibuprofen was low (around 5%) regardless of the protein loading might indicate either a certain protein inactivation or agglomeration upon immobilization through this methodology.

In the particular case of conventional adsorption, the amount of protein adsorbed over time was determined. Figure 6 shows the kinetics of the adsorption of CALB on PP, PPetoh, PETpd, and PETpdp supports, and Table 4 presents the protein loading, the yield of the immobilization, conversion, and specific activity of the esterification of *rac*-ibuprofen with ethanol.

Figure 6 shows fast adsorption of the lipase in the first 10 min of exposure to the solution containing the enzyme. The adsorption on the polypropylene supports (PP and PPetoh) increases until 180 min. In the particular case of Ppetoh, certain desorption was observed at a longer time of exposure.

In the case of the PET-derived supports, the adsorption of lipase on the PETpd levels off after 30 min of exposure in contrast with the PETpdp support, which continues adsorbing the protein above 7 h of exposure.

The protein loading and immobilization yield values were calculated from the protein concentrations in the initial and final immobilization solutions. The polypropylene-based supports that PP and PPetoh possess a protein loading equal to 5.2 and 8.3 $mg \cdot g^{-1}$, respectively. These values are lower than the ones previously reported by Foresti and Ferreira for a similar type of polypropylene-based support [11]. In fact, this observation is attributed to the low specific surface (1 $m^2 \cdot g^{-1}$) of the material used in the present investigation. In addition, the low catalytic activity values obtained with C/PP and C/PPetoh are related

to the low enzyme loadings. The higher protein loading obtained with the PET-based supports than PP is somehow related to the adsorption sites, such as –OH groups of the polyethylene terephthalate partially digested, as described in Section 3.2. Nevertheless, no improvement in the catalytic activity was observed.

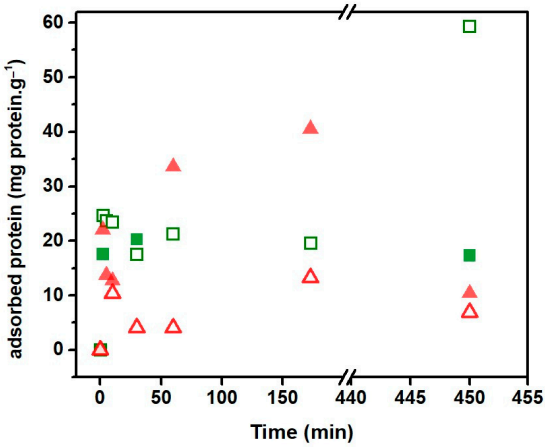


Figure 6. Protein adsorbed (in mg) per gram of PP (Δ), PPetoh (▲), PETpd (□), and PETpdp (■) as a function of time.

Table 4. Protein loading (expressed as the amount of protein in mg per gram of support), a yield of the immobilization, conversion, and specific activity of the esterification of *rac*-ibuprofen with ethanol.

Biocatalyst	Protein Loading (mg·g^{-1})	Immobilization Yield (%)	Ibuprofen Conversion (%)	Specific Activity (μmol·min^{-1}·mg^{-1})
C/PP	5.2	6.3	2.52	0.103
C/PPetoh	8.3	10.0	1.74	0.045
C/PETpdp	63.1	57.4	3.8	0.091
C/PETpd	17.5	15.9	nd	----
C/BHET	245.0 *1	nd	29.1	0.025

nd, not determined; *1 amount of protein per gram of biocatalyst.

It is worth noticing that, in the particular case of the BHET support, the supernatant in contact with this material during the adsorption process shows a certain turbidity attributed to species coming out of the support material. This observation made it difficult to follow the amount of protein adsorbed over time as performed with the other supports. Nevertheless, the protein loading was calculated as the amount of protein per weight of the biocatalyst (instead of support).

2.4. Effect of Preincubation of Biocatalyst in Stability and Activity

Figure 7 shows the catalytic activity of the biocatalysts named C/PPetoh, C/PET, C/PETpd, and C/PETpdp after being in contact with a solution mimicking the reaction media (without *rac*-ibuprofen), as described in Section 3.9. The biocatalysts synthesized through total adsorption showed an increase in the conversion of the esterification of *rac*-ibuprofen upon contact with the solution. In this sense, C/PPetoh doubles the value, C/PET six times, and C/PETpdp triples the conversion value (X_2%) with respect to the same system without preincubation in isooctane-ethanol (X_1%). This observation is somehow related to a rearrangement of the enzyme onto the support that is driven in the liquid media. In fact, the total adsorption methodology is forced non-specific adsorption through the simple mixing of the protein along with other components of the crude extract and the polymeric support. Thus, the preincubation allows both the enzyme molecules and other components of the crude extract to desorb and/or reorganize on the surface of the support, allowing an improved enzyme–substrate interaction and, therefore, an increased

conversion. In this sense, the UV–visible analysis of the obtained supernatants showed lixiviation of non-protein components with absorption at 240–245 nm (not shown).

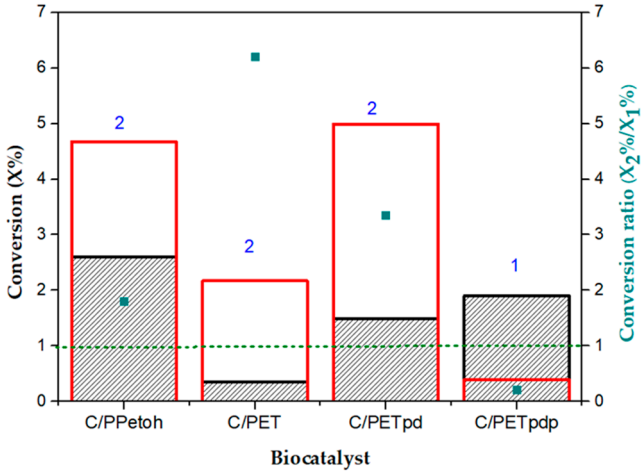


Figure 7. Ibuprofen conversion before (X_1%, black) and after (X_2%, red) incubation of the biocatalysts in the reaction medium. The numbers (1) and (2) indicate conventional and total adsorption methodologies, respectively. (■) Ratio between conversion values X_2/X_1. The dotted line indicates a ratio X_2/X_1 equal to one.

In contrast, the exposure of C/PETpdp, synthesized by simple adsorption, with the reaction mixture has a negative effect on the catalytic activity. The fact that no protein desorption was detected by UV–visible spectrophotometric analysis indicates that the decrease in the catalytic performance is attributed to an effect on the protein conformation.

2.5. Effect of the Enzyme Loading and Polyols in the Biocatalytic Activity

The promising catalytic activity of the *Candida antarctica* lipase B adsorbed on BHET (named C/BHET) driven further investigations on the influence of the protein loading and the presence of co-adsorbed polyols in order to improve the catalytic performance. It is worth noticing that the crude extract of CALB possesses a certain amount of both sorbitol and glycerol that were previously quantified by some of us [35]. The crude extract is composed of 11 mg·mL^{-1} of protein, 4% total organic solids, 25% glycerol, 25% sorbitol, 46% water, 0.2% sodium benzoate, and 0.1% potassium sorbate.

The biocatalysts obtained in the various assays listed in Table 5 were synthesized by conventional adsorption using various amounts of crude extract (that contains sorbitol already) and the addition of various volumes of a 50 % v/v solution of glycerol. Then, the weight of the polyol presented in Table 5 accounts for the whole amount of sorbitol (within the crude extract) and added glycerol. In addition, Table 5 shows the protein loading determined through ICP-AES methodology, the specific activity (UI/mg protein) in the esterification of *rac*-ibuprofen with ethanol, and the productivity of the biocatalysts (UI/mg biocatalyst).

The results of assays B and C show that the adsorption of CALB in BHET does not affect the enzymatic activity with respect to the free enzyme, observing similar specific activity values 0.116 and 0.136 μmol·min^{-1}·mg^{-1}, respectively.

In assays C, H, and I, immobilization was carried out in the presence of increasing amounts of glycerol. The results show a negative effect on the specific activity and the productivity of the catalyst with the increase of glycerol in the medium, which can be attributed to the simultaneous adsorption of protein and polyols, which then affects their activity by diffusional and/or steric limitation in enzyme–substrate interaction.

Although low amounts of added glycerol favor protein loading (see trials C and H), the effect on activity is negative. At higher additions of glycerol, the observed behavior

is attributed to the competition between the polyol and the protein with the consequent decrease in immobilized protein and to diffusion effects that directly affect the activity.

Table 5. Weight of added polyols (sorbitol from the crude extract and added glycerol), protein loading expressed as weight of protein in mg per weight of biocatalyst in grams, specific activity, and productivity in the esterification of *rac*-ibuprofen of CALB dispersed onto BHET.

Assays	Crude Extract (mL)	Polyols (g)	Protein Loading ($mg \cdot g^{-1}$)	Specific Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Productivity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
A	1 *	0.36	28.67	0.065	0.185
B	1	0.45	12.58	0.105	0.132
C	1	0.46	15.68	0.116	0.182
D	1 **	0.50	26.10	0.062	0.160
F	2	0.91	33.31	0.089	0.298
G	3	1.50	25.99	0.058	0.150
H	1	1.25	24.13	0.048	0.115
I	1	1.96	17.43	0.027	0.047

* Immobilization in saturated BHET solution. ** Semi-purified CALB or "adapted" CALB solution.

In assays C, F, and G, the volume of crude extract increases. Thus, the amounts of protein, sorbitol, and glycerol proportionally increase. Comparing the results of assays C and F, a proportional increase in the protein load is observed with the volume of crude extract used because in F, the volume of crude extract is doubled with respect to assay C, and the amount of immobilized protein is doubled. Otherwise, a decrease in the specific activity is observed, which can be explained by the previously mentioned diffusive effects. In this sense, it is observed that when tripling the volume of crude extract (assay G), a decrease in both the enzymatic load and specific activity is observed with respect to assay F.

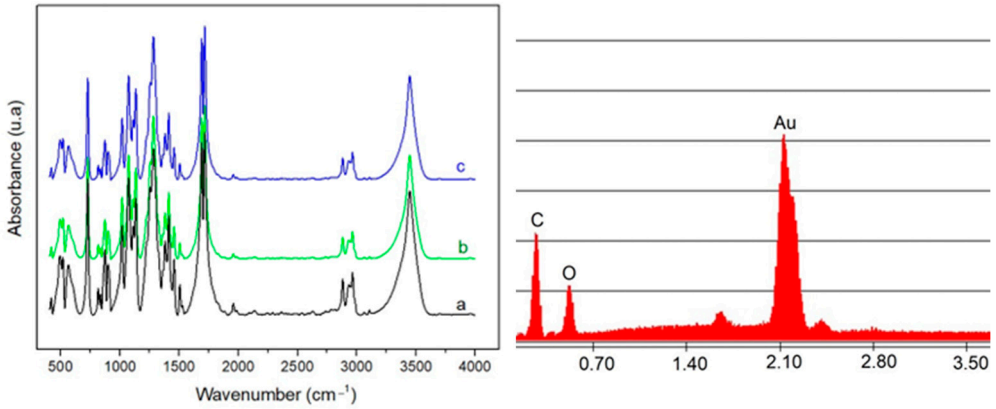
In assay D, the amount of CALB in the immobilization medium was doubled, maintaining the concentration of polyols with respect to assay C, observing an increase in the amount of adsorbed protein and a 50% decrease in the specific enzymatic activity. This result shows that the presence of polyols in the immobilization stage is essential to favor the adsorption of active enzymes. That is, in adequate amounts, a structural stabilization effect can be attributed to polyols and a better surface distribution of enzymes associated with co-adsorption. In the presence of a high concentration of polyols, negative effects on protein load and activity are evident, as previously indicated.

In this sense, assay F showed the best productivity value of the biocatalyst, 0.298 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, evidencing an optimal relationship between the amount of initial protein and polyols to be used in the immobilization stage, managing to optimize the relationship between protein load and activity.

In addition, considering the immobilization tests A–I, it is evident that an amount of polyols greater than 2.7% with respect to the adsorbed protein load is detrimental to the specific activity.

2.6. Investigation of BHET from Various Sources

This section discusses the use of BHET monomer as support obtained from colored PET bottles. Figure 8 shows the characterization studies of the products of glycolysis of colorless, green, and sky-blue PET bottles. The infrared spectra were equivalent for the three samples from waste and for the commercial monomer, evidencing that the glycolysis product is BHET of high purity regardless of the bottle color used, without evidence of pigment signals in the product obtained (see Figure 8A). The EDS elemental analysis shows that the obtained product only contains carbon and oxygen regardless of the starting bottle, according to the chemical structure of the monomer, with no evidence of metals or pigments used to color the bottles (see Figure 8B).



(**A**) (**B**)

Figure 8. (**A**) Infrared spectra of PET of the glycolysis product of colorless (a), green (b), and sky-blue (c) bottles. Signal assignments are detailed in Table 1. (**B**) Elemental analysis (EDS) corresponds to the BHET obtained from green bottle PET. The Au signal belongs to the coating of the samples made for analysis purposes.

Table 6 shows the protein loading and the yield of the adsorption process obtained when using BHET of various sources, including the commercial one. The conversion and specific activity of the esterification of *rac*-ibuprofen catalyzed with those materials are also presented.

Table 6. Protein loading expressed as the weight of protein in mg per weight of biocatalyst in grams, ibuprofen conversion, and catalytic performance of CALB immobilized on BHET obtained from different bottles.

BHET Source	Protein Loading ($mg \cdot g^{-1}$) [1]	*rac*-Ibuprofen Conversion (X%)	Specific Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Productivity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
Green Bottle	18.05	11.3	0.065	0.118
Sky-Blue Bottle	19.49	11.9	0.064	0.124
Commercial	15.68	17.5	0.116	0.182

[1] determined by ICP-S.

The results show that the protein load in commercial BHET is similar to that obtained in the colored bottles. The protein loading in BHET obtained from colorless bottles is substantially higher because, in this assay, immobilization was carried out for 7 h (see Section 3.5.1).

It is interesting to note that the characterization of the materials used as supports did not show differences between BHETs of different types of bottles (see Figure 8A,B) without being able to attribute the results to the presence of additives or pigments that could affect immobilization.

However, the specific activity of the materials tested is similar, indicating that the inherent activity of the adsorbed lipase is comparable between the different BHETs, regardless of the source. In general, the most relevant conclusion of this essay is that the residual PET bottles are useful sources for obtaining BHET suitable as enzyme support without requiring color classification in the selection of urban solid waste.

3. Materials and Methods

3.1. Materials

The *Candida antarctica* B lipase (CALB L, batches LCN02102 and LCN02103) was supplied by NovozymesLatin America LTDA (Paraná, Brazil), and pure *Candida antarctica* B lipase (35,500 g/mol) was purchased from Sigma Aldrich, Saint Louis, MO, USA). The commercial biocatalyst Novozym® 435 (batch LC200217) was obtained as a gift from

Novozymes Latin America LTDA (Paraná, Brazil). Polypropylene (PP) powder was obtained via Ziegler Natta (Petroken, La Plata, Buenos Aires, Argentina). Commercial polyethylene terephthalate (PETc, η = 42.45 mL g^{-1}, Mη = 22.23 kg mol^{-1} melting point of 250–255 °C, contains 30% glass particles as reinforcing filler) and bis-(2-hydroxyethyl) terephthalate (BHETc) from Sigma Aldrich were also used in this investigation [25]. Additionally, waste bottles of various colors (green, light blue, and transparent) were used as a source of PET (η = 84.04 mL g^{-1}, Mη = 60.91 kg mol^{-1}) and BHET [25].

Other reagents used in this study were *rac*-ibuprofen (99.23% Parafarm, Buenos Aires, Argentina), ethanol (99.8% Carlo Erba, Cornaredo, Italy), isooctane (Merck pro-analysis, Buenos Aires, Argentina), methanol (99.8% Carlo Erba), glacial acetic acid, trimethylamine, potassium hydroxide in ethanol 1M (Riedel-de Haen), phenolphthalein (99.0% Ennox), Coomassie Brillant Blue R250 (ultra-pure, USB), and p-nitrophenyl dodecanoate (Sigma Aldrich, Saint Louis, MO, USA).

3.2. Preparation of Support Materials Derived from PET

Polyethylene terephthalate (PET) pellets from mineral water bottles were used. The bottles were crushed, washed with distilled water, and dried at 50 °C.

Bis-(2-hydroxyethyl) terephthalate is the monomer of PET and was also evaluated as an enzyme support. The BHET was obtained by glycolysis (tertiary recycling) of PET bottles. This reaction involves the insertion of the diol (ethylene glycol EG) through the transesterification between the diol and the ester groups of PET catalyzed with ZnO [29]. The reaction was carried out with 2 g of PET and a PET: catalyst and PET: EG molar ratios of 100:1 and 1:8, respectively. The glycolysis was performed in a three-neck glass reactor operating at 196 °C, 300 rpm under reflux at atmospheric pressure for 2 h [29]. The glycolysis of PET produces oligomers of various molecular weights as byproducts. The fraction extracted in the first filtration of the monomer production process is called partially digested PET (PETpd). Further treatment with alcohol was performed in order to partially remove the low molecular weight oligomers. The material obtained is called partially digested purified PET (PETpdp).

3.3. Treatment of Commercial PP

Commercial PP was exposed to ethanol for 10 min at 800 rpm, followed by 50 min at 200 rpm to decrease the hydrophobicity of the microenvironment. The solid (called PPetoh) was separated and dried in a desiccator.

3.4. Characterization of the Supports and the Biocatalysts

3.4.1. Scanning Electron Microscopy Analysis

The surface morphology of the samples was analyzed through scanning electron microscopy (SEM) in a Philips SEM 505 microscope coupled with an electron microprobe EDAX DX PRIME 10 (Philips, Amsterdan, The Netherlands) The topographic analysis and morphology evaluation of the supports and the biocatalysts were performed at various magnifications (100×, 250×, 500×, 1000×). Additionally, a qualitative composition analysis was performed using the microprobe.

3.4.2. Infrared Spectroscopy

FTIR spectra were obtained in a Bruker Vertex 60 (Bruker, Billerica, MA, USA) infrared spectrometer with KBr optics and DTGS detector in the 400–4000 cm^{-1} range. The spectra were obtained with 200 scans collected at 4 cm^{-1}.

3.4.3. Specific Surface Area and Pore Size

The specific surface area and pore diameter of the supports were determined through the physical adsorption of nitrogen at −195.8 °C using the Brunauer Emmett–Teller (BET) method. The assay was performed using the equipment Micromeritics ASAP 2020 (Micromeritics Instrument Corporation, Norcross, GA, USA).

3.4.4. Stability of BHET under Enzyme Immobilization and Esterification Conditions

In a glass bottle, 451.5 mg of BHET were weighed, and 15 mL of deionized distilled water was added. The mixture was left at 30 °C under magnetic stirring. Aliquots of 1 mL were taken at 5, 10, 30, 60, and 180 min and centrifuged at 10,000 rpm for 5 min. The stirring was stopped after 6 h, and the system was centrifuged at 9000 rpm for 10 min. The pellets obtained both in the aliquots taken at different times and at the final time were dried and weighed. The amount of solubilized monomer was determined through the mass difference between the various samples.

The stability of BHET under esterification conditions was determined by contacting 20 mg of BHET with 10 mL of the reaction mixture (0.12 M ethanol in isooctane). The system was stirred for 24 h at 45 °C and centrifuged afterward. The supernatant was evaluated by UV–visible spectroscopy.

3.5. Immobilization Procedure

3.5.1. Immobilization through Simple Adsorption

A total of 100 mg of support was added to 10 mL of enzyme solution in the case of PP, Ppetoh, and BHET and 20 mL for PETpd and PETpdp supports. The enzymatic solution was obtained by diluting 1/20 the commercial extract Lipozyme® CalB L (LCN02102) with deionized distilled water. The mixture was kept for 7 h at 30 °C under magnetic stirring. The course of immobilization was followed by withdrawing 0.2 mL aliquots at various times, and the solids were separated from the supernatants. The catalysts were washed with distilled water, lyophilized, and stored at 4 °C. The supernatants were stored at 4 °C for protein quantification through the Bradford method. Additionally, the amount of protein was quantified on the starting solution (time zero T0 of the immobilization).

Subsequently, considering the results of the immobilization kinetics and experimental observations, new adsorption tests on BHET were performed. The enzyme solution used was obtained by diluting the commercial extract of CALB (EC) 1:11 in deionized water. A volume of 10.0 mL of this solution was mixed with 300.0 mg of ground BHET in a glass bottle. It was left under magnetic stirring at 30 °C for 1 h and centrifuged at 9000 rpm for 30 min at 10 °C. The solids were lyophilized for 48 h, weighed, and stored at 4 °C. Supernatants were stored at 4 °C for Bradford analysis. Several tests showed a significant turbidity in the supernatants after a few seconds of separation; therefore, they were re-centrifuged in order to separate the solids and the supernatants that were kept at 4 °C for further analysis.

Additionally, blank tests were performed by replacing the volume of diluted EC with deionized water.

In addition, the lipase immobilization was performed under the following conditions: (1) BHETc with different heat treatment were used as support; (2) BHET from blue and green bottles; (3) the addition of 50% (*v*/*v*) glycerol replacing 1.5 and 3 mL of deionized water; (4) various EC dilutions of 2:11 and 3:11; (5) saturated solution of BHET instead of deionized water and (6) "adapted" CALB solution as described in Section 3.6.

3.5.2. Immobilization by Total Adsorption

This immobilization strategy was used to synthesize biocatalysts on PPetoh, PET, PETpd, and BHET as supports according to the procedure published by Morcelle et al. (2009) [34]. In this sense, 150 mg of support was contacted with 300 µL of crude extract (~5.04 mg of protein) at room temperature and vigorously vortexed for 5 min. The samples were lyophilized and stored at 4 °C. Additionally, this synthesis was performed with a pretreated EC (called "adapted EC") that possesses a lower content of glycerol than the original crude extract. The treatment is described in the next section.

3.6. Semi-Purified CALB or "Adapted" CALB Solution

Deionized water (6 mL) was added to 1.5 mL of EC and transferred to a concentrator tube (10K, 50 mL falcon). It was centrifuged twice at 6000 rpm for 15 min until a concen-

trated solution of around 1.5 mL was obtained. The initial and final solutions, and the washing ones, were analyzed through Bradford and esterase activity containing 13.4 mg of protein per mL.

3.7. Characterization of the Biocatalysts: Determination of Enzyme Loading

The protein concentration in the biocatalyst was determined through the conventional Bradford method and measuring the sulfur content through high-resolution ICP-AES (Shimadzu ICPE 9000, Kyoto, Japan) [36]. The Bradford method was used for protein quantification of the enzyme solutions [37]. In each assay, 10 µL of the sample was mixed with 200 µL of Bradford's reagent. Absorbance readings were made at 595 nm using a TECAN infinite 200 pro plate reader (TECAN, Männedorf, Switzerland) after 10 min of contact. The samples (including blank experiments) were assayed in sextuplicate. The calibration curve was performed with purified CALB [35].

The sulfur content was determined by ICP in samples obtained by acid digestion of the biocatalysts. Because the sulfur exclusively comes from the immobilized protein, the enzyme load was calculated considering that a molecule of CALB (molecular weight equals 33 kDa) possesses ten amino acids with sulfur in the structure [38].

3.8. Kinetic Resolution of rac-Ibuprofen

The esterification of *rac*-ibuprofen with ethanol was studied by adding 40 mg of catalyst to 10 mL of a solution of 0.12 M ethanol and 0.12 M ibuprofen in isooctane. The system was kept at 45 °C for 48 h in an orbital shaker at 200 rpm. Blank tests (without the addition of the biocatalysts) were also performed.

The quantification of ibuprofen was performed through acid-base titration with a basic solution of potassium hydroxide in ethanol. Phenolphthalein was used as the end-point indicator. The performance of each biocatalyst is reported in terms of the percentage of conversion (X%) of ibuprofen, which was determined by the relative reduction in the acidity of the samples. Additionally, the analysis of ibuprofen was performed by high-performance liquid chromatography (HPLC) with a C18 column (VP-ODS, Shim-pack Shimadzu Kyoto, Japan) under a flow of 1.3 mL·min^{-1} of a mixture of methanol (Carlo Erba 99.8%, Conaredo, Italy) and tetraethylammonium acetate buffer TEAA 0.1% v/v (Fluka) in an 80:20 ratio at pH = 4, with a UV detector at 230 nm (elution time of ibuprofen: 3.4 min; elution time of ethyl ester: 7.8 min). The conversion (X%) and specific activity of the enzyme (µmol/min mg protein) are calculated.

3.9. Stability of the Biocatalysts

The stability of the biocatalyst upon contact with the reaction media was investigated by treating 20 mg of biocatalyst with 10 mL of a 0.12 M ethanol mixture in isooctane for 24 h at 45 °C in an orbital shaker. The supernatant and the solid biocatalyst were separated afterward. Then, both phases were allowed to react either by adding *rac*-ibuprofen to the supernatant or fresh reaction mixture to the recovered solid. For both samples, the activity was determined according to the procedure described in the previous section. Analysis of the supernatant through the Bradford method did not show the presence of protein.

4. Conclusions

This investigation presents a methodology of valorization of various polymers (and their derivatives) that compose many products of everyday use, such as polypropylene PP and polyethylene phthalate PET. It is worth noticing that PP- (typically plastic bags) and PET- (typically plastic bottles) based materials are neither biodegradable nor renewable and constitutes an environmental hazard.

The adsorption of the lipase B of *Candida antarctica* through contacting the support with the protein in a liquid environment proved to provide a more active biocatalyst rather than just mixing and lyophilization of the protein and the support. This observation is

attributed to the plugging of the active sites due to a non-adequate arrangement of the lipase onto the support.

The oligomers derived from the glycolysis of PET and the bis-(2-hydroxyethyl) terephthalate BHET are suitable as protein supports, providing biocatalysts with a protein loading of an order of magnitude higher than PP (typically, 5–8 $mg \cdot g^{-1}$ for bare PP and treated with ethanol vs. 17–245 $mg \cdot g^{-1}$ for oligomers and BHET from PET). That combination of a higher protein loading of the PET derived supports than PP and the addition of polyols (as activity promoters) are key factors for improving the specific activity in the esterification of *rac*-ibuprofen with ethanol. In fact, an increase of an order of magnitude (40%) of the productivity (specific activity of biocatalyst) was observed when a biocatalyst containing 33 $mg \cdot g^{-1}$ of protein loading with 0.9 g of polyols dispersed on BHET was assayed.

Finally, the adsorption of CALB onto BHET obtained from the glycolysis of plastic bottles of PET of various colors (typically waste plastic bottles) demonstrated that they are suitable sources of support without finding differences with respect to the color of the bottles used.

Thus, this research presents innovative and economic supports for lipases that allow obtaining active biocatalysts in reactions of industrial interest.

Author Contributions: Methodology, C.R.L.S., C.A.F., J.E.S. and C.J.; investigation, C.R.L.S. and C.J.; data curation, C.R.L.S. and C.J.; writing—original draft, C.R.L.S. and C.J.; writing—review and editing C.J. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge the financial support provided by the Consejo Nacional de Investigaciones Científicas y Técnicas CONICET of Argentina (project PIP 11220200102016 CO), Agencia Nacional de Promoción Científica y Tecnológica of Argentina (PICT2020-01118), and Universidad Nacional de La Plata (project 11X-898).

Data Availability Statement: Not applicable.

Conflicts of Interest: We declare that we have no financial or personal relationships with other people or organizations that could influence the present contribution.

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