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Evaluation of Designed Immobilized Catalytic Systems: Activity Enhancement of Lipase B from *Candida antarctica*

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Abstract: Immobilized enzymatic catalysts are widely used in the chemical and pharmaceutical industries. As *Candida antarctica* lipase B (CALB) is one of the more commonly used biocatalysts, we attempted to design an optimal lipase-catalytic system. In order to do that, we investigated the enantioselectivity and lipolytic activity of CALB immobilized on 12 different supports. Immobilization of lipase on IB-D152 allowed us to achieve hyperactivation (178%) in lipolytic activity tests. Moreover, the conversion in enantioselective esterification increased 43-fold, when proceeding with lipase-immobilized on IB-S861. The immobilized form exhibited a constant high catalytic activity in the temperature range of 25 to 55 °C. Additionally, the lipase immobilized on IB-D152 exhibited a higher lipolytic activity in the pH range of 6 to 9 compared with the native form. Interestingly, our investigations showed that IB-S500 and IB-S60S offered a possibility of application in catalysis in both organic and aqueous solvents. A significant link between the reaction media, the substrates, the supports and the lipase was confirmed. In our enzymatic investigations, high-performance liquid chromatography (HPLC) and the titrimetric method, as well as the Bradford method were employed.

Keywords: supports; immobilization; *Candida antarctica* lipase B; reaction medium; lipolytic activity; enantioselectivity; Immobeads; biocatalysis

1. Introduction

Over the past decade, there has been a rapid increase in the synthesis of chiral compounds and lipolysis reactions using enzymes immobilized on different supports. This enzyme technology is a promising solution that offers more environmentally-friendly and cost-effective methods to improve catalytic activity [1,2]. Lipases are among the most common biocatalysts used in the pharmaceutical and chemical industries. These proteins play an extremely important role in the chiral synthesis and enzymatic kinetic resolution of racemic compounds and in the hydrolysis and transesterification of oils. *Candida antarctica* lipase B (CALB) is a widely used catalyst from the catalyst class of lipases [3–6]. The structure of CALB includes a very short oligopeptide helix that may serve as a lid, but does not fully cover the active site of the enzyme [7]. CALB has a propensity to be adsorbed on hydrophobic

surfaces (hydrophobic supports and oil drops) by the hydrophobic region close to the active site and the hydrophobic part of the internal side of the lid. It is because of this small lid that CALB does not tend to form lipase–lipase dimers [8]. By modifying the conformational structure of CALB through its immobilization on different supports, it is often possible to modulate the enzyme's catalytic (lipolytic, enantioselective, etc.) activity. However, despite the numerous reports in the literature that describe interfacial activation of CALB, there is still insufficient scientific evidence confirming interfacial activation of this lipase [9–11].

Immobilization of enzymes on supports (carriers) is a technique that is aimed to increase the activity (hyperactivation by the stabilized open conformation of an enzyme), selectivity, and stability of biocatalysts (influence of e.g., temperature and pH). Additionally, the use of solid supports can greatly facilitate the recovery of an enzyme from the reaction medium [12–16]. The binding of an enzyme onto a support can be achieved by means of physical (hydrophobic, van der Waals, etc.) interactions, and by means of ionic or covalent bonds. Physical binding (adsorption), due to its nature, is not strong enough to maintain the enzyme on the carrier during repeated use. The second type of immobilization, ionic bonding, is usually more stable than physical adsorption, whereas the covalent bond is the most durable type of linkage, being characterized by low enzyme leaching [17–19]. Notably, the covalent bond may result in a higher rigidity of protein molecules, which may in turn decrease the catalytic activity [20]. On the other hand, the technique of immobilization using physical adsorption or ionic bonding (electrostatic interaction: repulsive or attractive) or combinations thereof is ordinarily uncomplicated and, due to the lack of chemical modification, offers a possibility to achieve high activity retention of the enzyme [21,22]. Lipase adsorption on hydrophobic supports is an extremely popular strategy because the hydrophobic material is, to some extent, similar to a natural hydrophobic substrate, allowing the binding to occur by interfacial activation. The hydrophobic bonds formed in this way, according to some authors, result in greater stabilization of the enzyme (open form) than a multipoint covalent attachment [23,24].

The activity of the immobilized lipase largely depends on the enzyme molecules, the immobilization protocol, the reaction medium, and the type of support. It has been demonstrated that the intensity of the enzyme–support, enzyme–substrate (in the reaction medium) and support–substrate interactions affect the stability and specificity of the biocatalysts [25,26]. It has also been shown that the reaction medium can affect the enzyme–substrate molecular recognition process [27]. Interestingly, a suitable material for enzyme immobilization can capture substrates through hydrophobic or electrostatic linking, and thus contribute to a positive partition effect of the substrate in the reaction medium [28]. In recent years, materials for CALB immobilization (via the physical, ionic, and covalent bonds) that are based, among others, on acrylic resin, silica, polystyrene, polypropylene, agarose, chitosan, and activated carbon, have been widely studied [29–39].

Due to the growing need for enzymatic catalysts for the purpose of preparing drug enantiomers, we used (*R*,*S*)-flurbiprofen as the model compound in our investigations of CALB enantioselectivity. This drug belongs to the pharmacological class of nonsteroidal anti-inflammatory drugs which are widely used in the treatment of pain and inflammation. It is a derivative of 2-arylpropionic acid, with a chiral center. It exists in two enantiomeric forms: (*R*)- and (*S*)-flurbiprofen. (*S*)-flurbiprofen is responsible for anti-inflammatory effects, while (*R*)-enantiomer has antinociceptive and antiproliferative properties, and in various animal models (in vitro), it stops the progression of colon and prostate cancer [40]. Another extremely important aspect of enzymatic activity studies is the determination of lipolytic activity. This activity, manifested by the hydrolysis of triglycerides, is indisputably one of the most significant features of lipases. It is notable that these enzymes have some applications in the formation of free fatty acids [41]. Therefore, extensive studies were performed on the lipolytic activity of CALB in free and immobilized form. As a model substrate for evaluating the lipolytic activity, we chose olive oil, which is one of the most popular vegetable oils, rich in ω-9 PUFAs (polyunsaturated fatty acids) [42].

CALB is a very active enzyme towards the resolution of alcohols and amines, and at the same time displays moderate enantioselectivity towards carboxylic acids [43]. Given the great current interest in the preparation of chiral carboxylic acids, the search for solutions aimed at increasing the catalytic activity of lipase towards these compounds is a significant challenge for researchers. To overcome these disadvantages tests of substrates, the reaction medium, as well as implement enzyme engineering including lipase immobilization on supports should be performed [44].

Unfortunately, using lipase immobilization techniques on carriers is associated with serious problems. One of them is enzyme leaching, which occurs when lipase is immobilized by interfacial activation on hydrophobic support after treatment with high temperature, organic co-solvents, or detergent-like compounds. Another problem is carrier solubility in organic media, e.g., ethanol and other alcohols, and next polymers incorporating into the products. Further, serious problems are posed by the fragility of support and the retention of hydrophilic compounds (water and glycerin) by lipases. Certain measures are taken to contain these inconveniences, such as cross-linking of the enzyme to prevent leaching, coating of the biocatalyst to avoid breakage, application of molecular sieves to minimalize adsorption of water or glycerin by the lipase, and modification of the matrix of the support [41].

In this paper, we present the results of comprehensive investigations of enantioselectivity and lipolytic activity of CALB immobilized on 12 commercially available supports that differed in physical and chemical properties. Enantioselectivity was investigated by enantioselective esterification of (*R*,*S*)-flurbiprofen, while lipolytic activity was assessed by the hydrolysis reaction of an olive oil emulsion. We also present and discuss the screening of the supports, the types of lipase–carrier binding, and the effects of the reaction medium (aqueous and organic) and acyl acceptors on the catalytic activity. Analyses of enantioselectivity were performed by high-performance liquid chromatography (HPLC) with chiral stationary phases. The immobilized lipase was quantified with the Bradford method and the lipolytic activity was calculated from the titration results. We also investigated the effects of temperature and pH on the lipolytic activity of the resulting catalytic systems.

2. Results and Discussion

2.1. Enantioselectivity

Screening of Enzyme Carriers, Types of Binding, Reaction Media, and Acyl Acceptors

The enantioselectivity of CALB immobilized on 12 commercially available carriers was assessed. The reactions were carried out in three different solvents, due to the solubility of (*R*,*S*)-flurbiprofen: dichloromethane (DCM), 1,2-dichloroethane (DCE), and 1,2-dichloropropane (DCP) were chosen (Figure S1 and Table S1). The monitoring of catalytic parameters (conversion and enantiomeric excess) during the reaction enabled us to determine the optimal reaction time (94 h for the reactions in DCM, and 46 h for the reactions in DCE and DCP). The carriers differed in terms of features and properties of the matrix, the type of bond with the lipase, the functional groups on the surface of the support, and the size (Table 1).

For the purpose of the experiment, the carriers were divided into three groups depending on the type of bond offered to the lipase: covalent bond (IB-150A and IB-150P)-Group 1; ionic (cationic/anionic) bond (IB-D152, IB-C435, IB-A161, IB-A171, and IB-A369)-Group 2; and non-ionic bond (IB-EC1, IB-S861, IB-S500, IB-S60S, and IB-S60P)-Group 3. The best results in terms of enantioselectivity (enantiomeric excess: ee_s and ee_p) and conversion (*C*) were obtained with Group 1 and 3 carriers (covalent and non-ionic bonds), while the lowest catalytic activity was observed during tests of Group 2 supports (ionic type) (Table 2, Figure 1, and Scheme 1). It is notable that a different quantity of lipase was immobilized on each carrier.

Table 1. Properties of the carriers used (data received from the supplier, ChiralVision).

Support	Type of Bond	Matrix	Functional Group	Particle Size (μm)
IB-150P	covalent	polyacrylic	epoxide, polar	150–300
IB-150A	covalent	polyacrylic	epoxide, apolar	150–300
IB-D152	cationic	polyacrylic	carboxylic acid	350–700
IB-C435	cationic	polyacrylic	carboxylic acid	350–700
IB-A161	anionic, strong	polystyrene	quaternary ammonium type	350–700
IB-A171	anionic, strong	polystyrene	quaternary ammonium type	350–700
IB-A369	anionic, weak	polystyrene	quaternary ammonium type	350–700
IB-EC1	non-ionic	polyacrylic	carboxylic ester	350–700
IB-S861	non-ionic	polystyrene	aromatic	350–700
IB-S500	non-ionic	polypropylene	alkyl	150–1500
IB-S60P	non-ionic	silica, porous	hydroxyl	60–200
IB-S60S	non-ionic	silica, super porous	hydroxyl	60–200

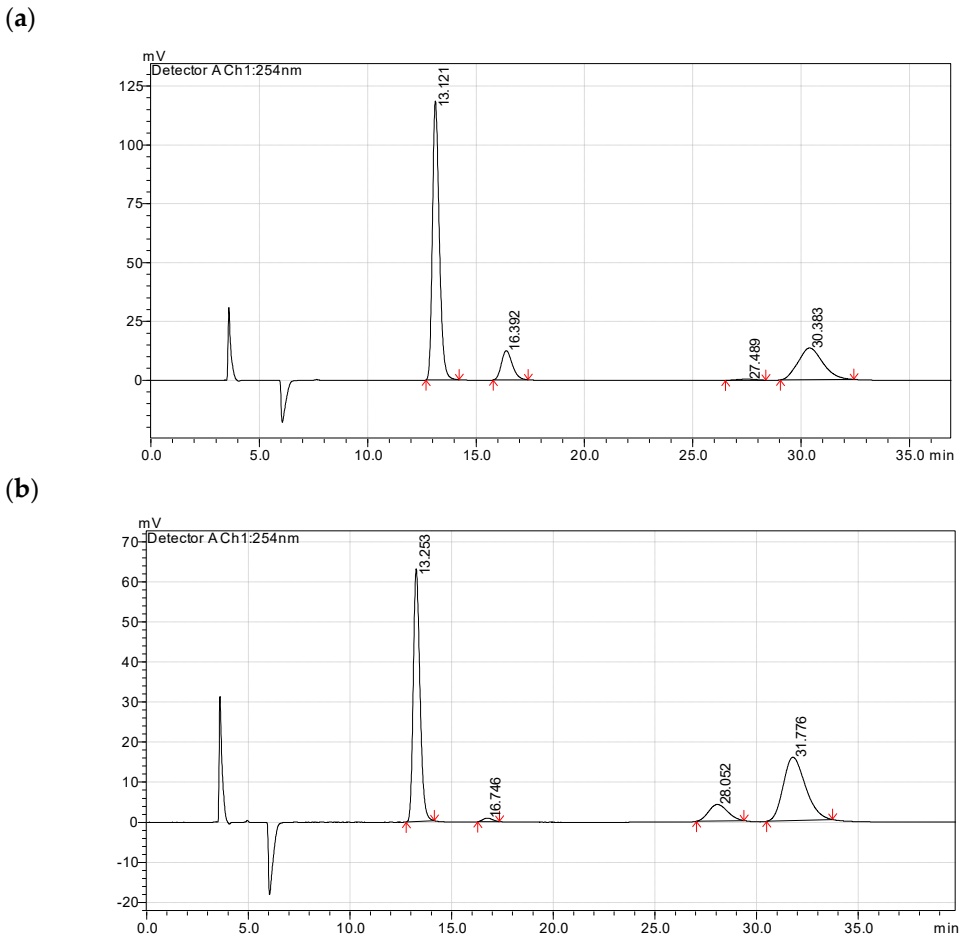


Figure 1. High-performance liquid chromatography (HPLC) chromatograms of (*R*,*S*)-flurbiprofen and its methyl esters. (**a**) (*R*,*S*)-flurbiprofen and its methyl ester, reaction time 46 h, IB-150A, DCE; t_R 13.121 (*R*)-enantiomer of methyl ester, t_R 16.392 (*S*)-enantiomer of methyl ester, t_R 27.489 (*R*)-flurbiprofen, t_R 30.383 (*S*)-flurbiprofen; (**b**) (*R*,*S*)-flurbiprofen and its methyl ester, reaction time 96 h, IB-S861, DCM; t_R 13.253 (*R*)-enantiomer of methyl ester, t_R 16.746 (*S*)-enantiomer of methyl ester, t_R 28.052 (*R*)-flurbiprofen, t_R 31.176 (*S*)-flurbiprofen; Chromatographic conditions: Lux Cellulose-3 (4.6 mm × 250 mm × 5 μm) column; mobile phase: *n*-heptane/2-propanol/trifluoroacetic acid (97.6/2.4/0.2 *v*/*v*/*v*), flow rate = 1 mL/min, t = 15 °C, UV = 254 nm.

Table 2. The effects of supports and reaction media on the parameters of kinetic resolution of (*R*,*S*)-flurbiprofen, after 46 h (1,2-dichloroethane (DCE) and 1,2-dichloropropane (DCP)) and 94 h (dichloromethane (DCM)) of incubation.

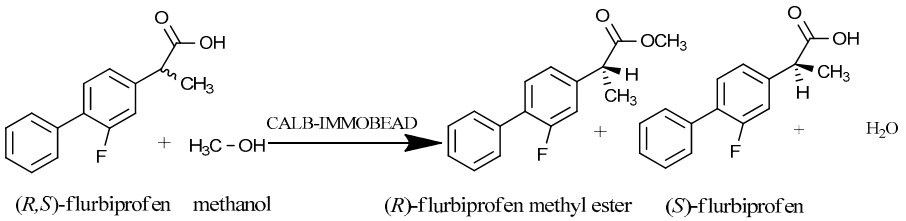
Support	Lipase Loading (mg/50 mg Support)	Reaction Medium	Stereopreference	ee_s (%)	ee_p (%)	*C* (%)
IB-150A	7.37	DCM	(*R*)	60	91	39
		DCE		95	71	57
		DCP		91	40	69
IB-150P	6.87	DCM	(*R*)	26	96	21
		DCE		62	90	41
		DCP		91	83	52
IB-D152	1.26	DCM	(*R*)	-	-	-
		DCE		-	-	-
		DCP		-	-	-
IB-C435	1.06	DCM	(*R*)	2	96	2
		DCE		2	93	2
		DCP		2	89	2
IB-A161	1.89	DCM	(*R*)	*	*	*
		DCE		*	*	*
		DCP		26	89	23
IB-A171	1.78	DCM	(*R*)	*	*	*
		DCE		*	*	*
		DCP		15	89	14
IB-A369	1.39	DCM	(*R*)	-	-	-
		DCE		*	*	*
		DCP		26	88	22
IB-EC1	6.69	DCM	(*R*)	87	89	49
		DCE		70	60	54
		DCP		91	39	70
IB-S861	4.15	DCM	(*R*)	61	95	39
		DCE		83	87	49
		DCP		88	79	53
IB-S500	3.73	DCM	(*R*)	50	92	35
		DCE		93	80	54
		DCP		48	22	69
IB-S60S	6.49	DCM	(*R*)	15	96	14
		DCE		26	92	22
		DCP		40	82	33
IB-S60P	5.04	DCM	(*R*)	6	97	6
		DCE		16	92	15
		DCP		21	84	20

Reaction conditions: racemic flurbiprofen (4.8 mg, 0.02 mM), methanol (2.44 μL, 0.06 mM), immobilized CALB (50 mg of support), medium (DCM, DCE, or DCP) (700 μL), molecular sieve 4 Å, reaction temp 37 °C, shaking at 600 rpm; *C*—conversion, ee_s—enantiomeric excess of the substrate, ee_p—enantiomeric excess of the product, (−) no reaction, (*)—impossible to calculate.

The Group 1 carriers (covalent bond) are made of a polyacrylic matrix with epoxide functional groups. With the application of IB-150A (which is apolar), (*S*)-flurbiprofen of high enantiopurity (ee_s of 95%) was obtained at the conversion rate of 57% in DCE, whereas, the (*R*)-flurbiprofen methyl ester was obtained with an ee_p of 96%, when using IB-150P (which is polar) in DCM at a conversion rate of 21%. In Group 2 (cationic bond), no or low activity of the enzyme immobilized on IB-D152 and IB-C435 was observed, respectively. These findings may suggest an interaction between the supports (polyacrylic matrix with functional groups: carboxylic acid) and substrates in organic solvents. These data may be

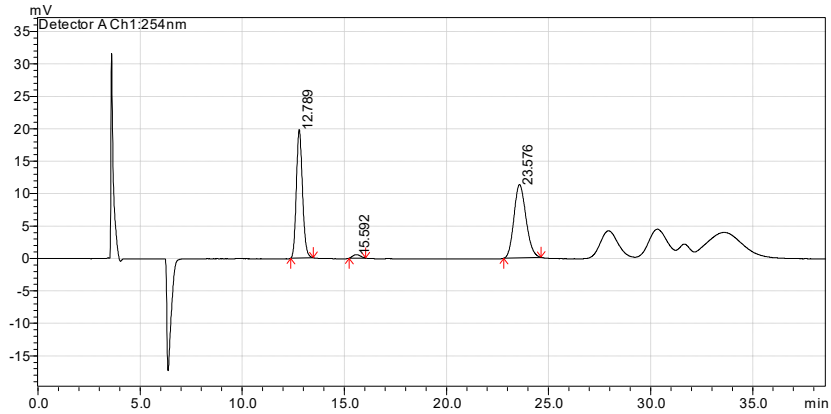
linked to the inhibitory effect of the carriers used and the cationic bond on the enzymatic activity of the catalytic system formed in the organic reaction medium. It is assumed that in the case of lipase immobilized on these supports, structural changes occur, which might influence the enzyme's catalytic activity in organic solvents.

Noteworthy are the results obtained when carriers offering an anionic bond (IB-A161, IB-A171, IB-A369) and a polystyrene matrix with the quaternary ammonium functional group were studied. Changes in the chromatograms in the area of retention time for (*R*,*S*)-flurbiprofen peaks were noticed when carriers with a strong anionic bond (IB-A161 and IB-A171) in DCM and DCE were used (Figure 2).



Scheme 1. Enantioselective esterification of (*R*,*S*)-flurbiprofen catalyzed by *Candida antarctica* lipase B (CALB) immobilized on polymeric supports, Immobead, with the use of methanol.

(**a**)



(**b**)

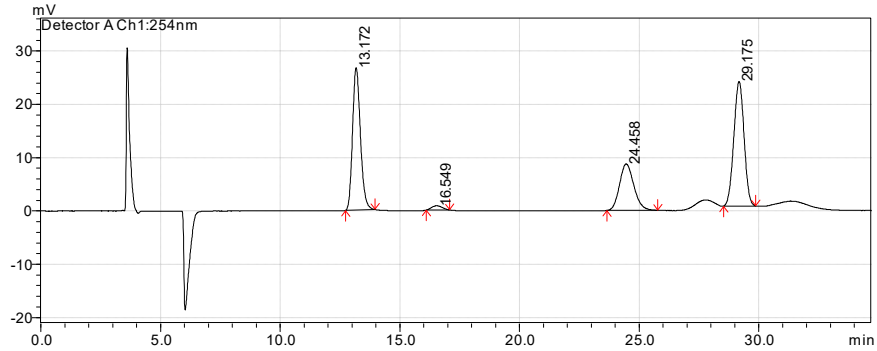


Figure 2. HPLC chromatograms of (*R*,*S*)-flurbiprofen methyl esters. (**a**) (*R*,*S*)-flurbiprofen methyl ester, reaction time 96 h, IB-A171, DCM; t_R 12.789 (*R*)-enantiomer of methyl ester, t_R 15.592 (*S*)-enantiomer of methyl ester; (**b**) (*R*,*S*)-flurbiprofen methyl ester, reaction time 46 h, IB-A171, DCE; t_R 13.172 (*R*)-enantiomer of methyl ester, t_R 16.549 (*S*)-enantiomer of methyl ester; Chromatographic conditions: Lux Cellulose-3 (4.6 mm × 250 mm × 5 µm) column; mobile phase: *n*-heptane/2-propanol/trifluoroacetic acid (97.6/2.4/0.2 *v*/*v*/*v*), flow rate = 1 mL/min, t = 15 °C, UV = 254 nm.

We assume that this is caused by the effect of the carriers used (offering a strong anionic bond) in the reaction system because these changes are less pronounced for IB-A369, which is characterized by a weak anionic bond. We observed this only for these three carriers (out of the 12), which could suggest an interaction between the matrix (a type of bond), reaction medium (DCM and DCE), and substrates. The peaks detected in the chromatograms require further investigation to determine the types of chemical reactions that occurred in the mixture. It was impossible to calculate ee_s, ee_p, and *C*, therefore they are marked with an asterisk in the table (Table 2). Characteristic changes in the chromatograms were not observed or were observed to a negligible extent in reactions performed in DCP, also implying a link between the intensity of the changes and the type of solvent.

Good catalytic activity was also achieved by immobilizing CALB on IB-S861 (Group 3, non-ionic, polystyrene, and aromatic functional groups)—ee_p 95% at 39% conversion, as well as on IB-S500 (non-ionic, polypropylene, and alkyl functional group)—ee_s 93%, at 54% conversion. Kahar et al. [45] suggested that because of the structural changes in the immobilized enzyme, significant changes in enzyme product specificity (or selectivity) have taken place. It is assumed that, among others, the hydrophobic and hydrophilic properties of the support affect the activity of enzymes and their biochemical behavior. In our experiments, in the group of carriers studied, the enantiopreference of the lipase was found to remain the same but the enzyme activity significantly depended on the type of carrier being used.

The investigation of the silica supports resulted in higher conversion values when applying IB-S60S (super porous, 6.49 mg CALB/50 mg carrier) compared to IB-S60P (porous, 5.04 mg CALB/50 mg carrier). It is believed that the presence of pores in the material may be conducive to the achievement of higher values of conversion because more lipase can be loaded on the support. Increasing the enzyme amount will increase the reaction yield, up to the moment when further addition of the biocatalyst will stop its positive effect on the conversion [46–48]. It was noticed that the enantioselectivity of lipases on these two supports was characterized by a similar profile.

The carriers we tested were made of the various matrices: polyacrylic, polystyrene, polypropylene, and silica. High enzymatic activity with the application of polyacrylic supports with a covalent bond and epoxide functional groups (Group 1) was observed. In contrast, lipase immobilized on polyacrylic supports with a cationic bond and carboxylic acid functional groups did not show any catalytic activity. It is suggested that both the bond type and the functional groups on the surface of polyacrylic carriers in association with their chemical character to have a significant effect on the enzyme activity. The use of IB-S861 with a polystyrene matrix (non-ionic type and aromatic functional groups) enabled us to obtain good lipase activity. The use of polystyrene carriers with an anionic bond and quaternary ammonium functional groups was of interest when characteristic changes in the chromatogram were observed (as mentioned earlier). These findings also indicate that type of bond and functional groups on the carrier are important for lipase activity. When assessing the impact of the carriers from Group 3 (the non-ionic type), the best results for enzyme activity were obtained with the application of the polystyrene matrix with the aromatic groups, then polypropylene matrix with alkyl groups, polyacrylic matrix with carboxylic ester groups, and the lowest on silica matrix with hydroxyl groups. Based on these results, it can be concluded that the three of the carriers tested (IB-150A, IB-150P, and IB-S861) had a significant positive effect on CALB activity under the reaction conditions tested (organic solvents). In the investigated group of 12 carriers, the best enantioselectivity (expressed as enantiomeric excess) was achieved when using carriers with polyacrylic matrix, epoxide groups (covalent bond, apolar), and polystyrene matrix, aromatic groups (non-ionic type). Therefore, IB-150A (high ee_s value) and IB-S861 (high ee_p value) were selected for further tests (Figures S2–S4).

A comparison of free and immobilized lipase activity parameters is presented in Table 3. The investigations of free and immobilized lipase activity were carried out under the same reaction conditions and with the same amount of enzyme, both free and immobilized, as was determined on the appropriate support. The free lipase was used as the control. Lipase immobilized on IB-150A demonstrated over 11-fold higher enzymatic activity, while in the case of IB-S861, the lipase catalytic

activity was over 43-fold higher compared to the free form. Water generated as a by-product was adsorbed on molecular sieves shifting the equilibrium towards esterification. However, it should be noted that the reactions with the free form were performed without the addition of molecular sieves due to a lack of conversion in their presence.

Table 3. The Comparison of catalytic activity of immobilized and free form of CALB.

Medium, Time	Form of CALB	ee_s (%)	ee_p (%)	*C* (%)
DCE, 46 h	free form (7.37 mg)	5	96	5
DCE, 46 h	immobilized on IB-150A (7.37 mg of lipase)	95	71	57
DCM, 94 h	free form (4.15 mg)	0.8	91	0.9
DCM, 94 h	immobilized on IB-S861 (4.15mg of lipase)	61	95	39

Reaction conditions: racemic flurbiprofen (4.8, 0.02 mM), methanol (2.44 µL, 0.06 mM), immobilized CALB (50 mg of support), medium (DCM and DCE) (700 µL), reaction temp 37 °C, shaking at 600 rpm; *C*—conversion, ee_s—enantiomeric excess of the substrate, ee_p—enantiomeric excess of the product.

The further goal of the study was to determine the impact of the log P value of the reaction media tested on the lipase catalytic activity. According to the literature [48,49], biocatalysts in hydrophobic solvents with a high log P value offer higher catalytic activity compared to the activity of the enzyme in solvents with a lower log P value. Additionally, hydrophilic solvents are more likely to strip bound water (essential for catalytic activity) from the enzyme molecules. In these papers, there are also indications that log P is not optimal to correlate with enzyme activity. In our experiments, a link was found between log P of the solvent and the enzymatic activity. In all the reactions, the increasing values of log P of the medium have resulted in increasing values of the reaction conversion. The log P values of solvents used in the investigations are 1.25, 1.48, and 1.98–2.28, for DCM, DCE, and DCP, respectively (data received from the suppliers). In the next step of the project, the effect of acyl acceptors (methanol, ethanol, *n*-propanol, and *n*-butanol) on the enantioselectivity of lipase immobilized on IB-150A and IB-S861 was determined (Tables 4 and 5). The investigations carried out on IB-150A showed that the highest conversion values were obtained when methanol and ethanol were used, while the ee_s values reached the highest levels when methanol and *n*-butanol were used. Therefore, methanol and *n*-butanol were concluded to be the best alcohols for this catalytic system. Analyzing the results for IB-S861, the highest ee_p values were obtained using methanol and ethanol. Using *n*-propanol allowed us to achieve the highest value of conversion but at the same time the lowest value of ee_p was recorded. Based on the values of conversion and ee_p, the best alcohols for this catalytic system were demonstrated to be methanol and ethanol. It was described in the literature that CALB was more active in the presence of low molecular weight alcohols, which may positively affect the efficiency of catalysis [50].

Table 4. Effect of alcohol on the parameters of kinetic resolution of (*R*,*S*)-flurbiprofen. CALB immobilized onto IB-150A after 20 h (in DCE) of incubation.

Alcohol	ee_s (%)	ee_p (%)	*C* (%)
methanol	82	87	49
ethanol	74	83	47
n-propanol	59	85	41
n-butanol	76	92	45

Reaction conditions: racemic flurbiprofen (4.8 mg, 0.02 mM), methanol (2.44 µL, 0.06 mM), ethanol (3.52 µL, 0.06 mM), *n*-propanol (4.51 µL, 0.06 mM), *n*-butanol (5.52 µL, 0.06 mM), immobilized CALB (50 mg of support), medium (DCE for IB-150A) (700 µL), molecular sieve 4 Å, reaction temp 37 °C, shaking at 600 rpm; *C*—conversion, ee_s—enantiomeric excess of the substrate, ee_p—enantiomeric excess of the product.

Table 5. Effect of alcohol on the parameters of kinetic resolution of (*R*,*S*)-flurbiprofen. CALB immobilized on IB-S861 after 94 h (in DCM) of incubation.

Alcohol	ee_s (%)	ee_p (%)	*C* (%)
methanol	61	95	39
ethanol	65	94	41
n-propanol	77	90	46
—*n*-butanol	70	92	43

Reaction conditions: racemic flurbiprofen (4.8 mg, 0.02 mM), methanol (2.44 µL, 0.06 mM), ethanol (3.52 µL, 0.06 mM), *n*-propanol (4.51 µL, 0.06 mM), *n*-butanol (5.52 µL, 0.06 mM), immobilized CALB (50 mg of support), medium (DCM for IB-S861) (700 µL), molecular sieve 4 Å, reaction temp 37 °C, shaking at 600 rpm; *C*—conversion, ee_s—enantiomeric excess of the substrate, ee_p—enantiomeric excess of the product.

The use of CALB in the kinetic resolution of (*R*,*S*)-flurbiprofen by enantioselective esterification with different primary alcohols serving as an acyl acceptor has been described in the literature [51–53]. The catalytic activity values presented in this paper are similar to those described in our previous publication in which Novozym 435 was used in the kinetic resolution of (*R*,*S*)-flurbiprofen (*C* = 35.7%, ee_p = 96.3%, and ee_s = 53.6%) [40]. We created a new catalytic system based on a polystyrene matrix with the aromatic functional group enabling us to achieve results for the enantioselectivity of the kinetic resolution of (*R*,*S*)-flurbiprofen that were as good as those when using commercial biocatalysts, namely Novozym 435 (poly(methacrylic acid) cross-linked with divinylbenzene). The new system can be further optimized and tested for the preparation of drug enantiomers.

There are few publications in the scientific literature describing the use of Immobeads manufactured by ChiralVision in biotransformation, especially in the kinetic separation of chiral drugs, so the results described in this paper are an important contribution to the state of art in the field of catalysis. Selected results from using Immobeads are discussed below (Table S2). In a paper by Mitsuya et al. [54], the high activity recoveries of immobilized laminarinase ULam111 with the use of IB-S60P (19.4%), IB-S60S (15.6%), IB-150P (11.9%), and IB-C435 (7.1%) are described. On the other hand, no activity was observed when IB-A161, IB-A171, IB-A369, IB-EC1, IB-S861, and IB-S500 were applied. Rodríguez-Alonso et al. [55] immobilized four enzymes necessary for L-amino acid formation on, among others, IB-150, IB-C435, IB-161, IB-A171, IB-A369, IB-S861, IB-S60S, and IB-S60P. Each enzyme demonstrated a preference for binding on one or several carriers. The best results with the use of IB-161, IB-A171, and IB-A369 were only achieved for one enzyme, among the four of the tested ones, namely *N*-carbamoyl-amino acid racemase. Soriano-Maldonado et al. [56] performed bio-enzymatic production of enantiopure L-α-amino acids using IB-A161 with good results: the enantiomeric excess of the amino acid was >99.5%. De Lima et al. [57] performed the synthesis of short-chain carboxylic acid esters by esterification catalyzed, among others, by lipase *Thermomyces lanuginosus* immobilized on IB-150, achieving significant results of conversion (>80%) after 24 h of reaction. Becker et al. [58] immobilized the laccase on Immobead IB-EC-1 to apply in the investigation of the degradation of endocrine-disrupting compounds. They concluded that the immobilized enzymes displayed better performance in comparison to the free enzyme. De Souza et al. [25] applied IB-150P in the glycerol carbonate formation by transesterification reaction reaching a selectivity of >99%.

The literature broadly describes the application of Novozym 435 (microporous acrylic polymer resin) in the production of optically pure products by hydrolysis, synthesis, transesterification, or a combination thereof. The use of Novozym 435 makes it possible to obtain enantiomers with high enantiopurity. Its use is described in the production of such compounds as (*S*)-β-halohydrin (ee of 99%), (*R*)-ester of flurbiprofen (ee_p 98.9%), benzoxazole derivatives. Furthermore, this biocatalyst was used, among others, in the 2-phenylpropionic acid esterification, resolution of *rac*-1-adamantylethanol, resolution of (±)-1-methyl-3-phenylpropylamine, and the resolution of racemic-2-pentanol [41]. It is also worth mentioning that Barbosa et al. [5] obtained high ee (96%) performing acylation of racemic atenolol in toluene with the application of CALB immobilized on epoxy groups from commercial

Eupergit C. Whereas, Rios et al. [36] using modified pore-expanded mesoporous silica (SBA-15) for CALB immobilization carried out the kinetic resolution of (*R*,*S*)-phenylethyl acetate with ee_p > 99%.

2.2. Lipolytic Activity

The lipolytic activity of CALB immobilized on 12 porous polymer carriers was determined by the hydrolysis of olive oil. The amount of protein immobilized on the support (the total amount of lipase) was measured with the use of the Bradford method. The carriers were also divided into three groups, depending on the type of binding of the lipase onto support: Group 1 (covalent bond), Group 2 (ionic bond), and Group 3 (non-ionic bond). The highest loading of the lipase was found on the carriers from Group 1, IB-150A, and IB-150P (147.4 mg/g support and 137.4 mg/g support, respectively) and on supports from Group 3, IB-EC1 (133.8 mg/g support) and IB-S60S (129.8 mg/g support) (Table 6). On the other hand, the lowest amount of enzyme was demonstrated on the supports from Group 2 with a cationic/anionic bond (amounts in the range from 21 to 38 mg/g support). In the case of silica carriers (IB-S60S and IB-S60P), more lipase was immobilized on IB-S60S than on IB-S60P, which may be associated with the porous matrix of IB-S60S (super porous—data received from the supplier). A larger amount of bonded protein on the carriers indicates a stronger affinity of the lipase for the supports caused by the type of bond (covalent and non-ionic), the chemical nature of the carriers, and/or their porosity. A low loading of protein from Group 2 of the supports tested may be due to ionic interactions (cationic and anionic bond) of the protein with the surface of the carriers. There are reports [45,46,59] that the support's porosity can be advantageous by offering a high degree of enzyme retention. It has also been pointed out that small deep pores in some Immobeads may reduce the interaction between substrates and enzyme because of low substrate penetration, and thus influence the decrease of catalytic activity. Likewise, the large molecules of protein located inside the pores of supports can decrease the intra-particle diffusion rates of substrates and products.

Table 6. The results of the CALB immobilization procedure.

Catalyst	Lipase Loading (mg/g Carrier)	Activity Retention (%)	Activity [U] (μmol/mL/min)	Specific Activity (U/mg Lipase)	Activity (U/g Carrier)
Free CALB	n/a	n/a	2.69 *	1.010 *	n/a
IB-150A	147.4	41.2	1.17	0.16	23.4
IB-150P	137.4	17.6	0.5	0.07	10.0
IB-D152	25.2	178.0	4.17	3.31	83.4
IB-C435	21.2	80.0	2.0	1.89	40.0
IB-A161	37.8	21.4	0.5	0.26	10.0
IB-A171	35.6	28.6	0.67	0.37	13.4
IB-A369	27.8	42.9	1.0	0.72	20.0
IB-EC1	133.8	17.6	0.5	0.07	10.0
IB-S861	83.0	14.3	0.42	0.1	8.4
IB-S500	74.6	85.7	2.0	0.54	40.0
IB-S60S	129.8	80.0	2.67	0.41	53.4
IB-S60P	100.8	71.4	2.5	0.5	50.0

Reaction conditions: immobilized CALB onto polymer supports (50 mg), or free lipase, phosphate buffer (100 mM, pH 7.4), the emulsion of gum arabic and olive oil, temperature 37 °C, incubation 30 min. n/a—not applicable; * The average value with the use of the quantity of free lipase in the range of 1.06 to 7.37 mg (the same range as was determined for the carriers).

When analyzing the results of activity retention and lipase activity [U], it is noticeable that, despite the small amount of lipase immobilized onto carriers of the second group (cationic type), a hyperactivation (activity retention of 178%) was observed for the IB-D152 support. The kinetic parameters for the lipase immobilized on this carrier (IB-D152) were also determined. The Michaelis constant value (K_m) was 556.87 mg/mL and the turnover numbers (k_{cat}) was 0.077 s^{-1}. We consider that such high activity retention may be the result of electrostatic effects of the carboxylic acid-functional

polyacrylic support with fatty acid triglycerides (the carbonyl group is polarized) in the aqueous medium (slightly alkaline). Therefore, the concentration of fatty acid triglycerides should be higher near the active site of the enzyme. This may be an indication of a positive partition effect by the enhanced capture of the substrate. Moreover, the small amount of lipase on this carrier may influence the improvement of the substrate's availability for the lipase. Additionally, protein aggregation and steric hindrance are probably limited. It is expected that the strong electrostatic interactions of the carrier and long-chain substrates (olive oil) may create a friendly environment for the catalytic activity of lipase in the aqueous medium. According to the literature [60,61], the values over 100% of enzyme activity retention may come up from the fact, that free form is in the catalytically less active conformation, whereas the immobilized biocatalyst shows more open conformation. The hyperactivation of the enzyme using the procedure with Immobead 150 has been described in the cited papers. In other papers, it was also pointed out that changes in the enzyme conformation caused by the immobilization of the enzyme via different orientation may modify the final conformation of the lipase [8]. Additionally, immobilization of lipase in hydrophobic carriers at low ionic strength creates the open form of the lipase and hyperactivation of the enzyme upon immobilization on octyl support [19]. It should be noted that in our experiment the lipase immobilized on the IB-D152 carrier did not show activity in enantioselectivity assays, which may be related, as previously mentioned, too weak carrier interactions in the organic medium with the model substrate (flurbiprofen) (Table 2). Summarizing, the results showed that, due to electrostatic interactions, the application of a polyacrylic carrier and cationic bond type increases the affinity between the hydrophobic substrate and support in the aqueous medium and thus increases the lipolytic activity of the immobilized enzyme. However, it should be emphasized that is difficult to clearly state what mainly influenced the hyperactivation of the lipase. It is worth noting that for a better comparison of causes, the enzyme loading on the carriers should be the same.

Results of moderate activity retention of lipase immobilized onto the IB-S500 and IB-S60S carriers were demonstrated. The IB-S500 is built from a polypropylene matrix with alkyl function groups on the surface. This carrier has a good affinity for the hydrophobic substrate (triglyceride of fatty acid) in an aqueous medium and what should be noticed, the catalytic activity of lipase on this support in an organic medium is also high. The lipase immobilized onto the IB-S60S silica carrier showed activity retention of 80%. The functional hydroxyl groups on this silica matrix can form hydrogen bonds with triglycerides, promoting interaction with the substrate. What is important, during the enantioselectivity study in an organic medium, a good activity of the CALB was observed. Chemical properties of these supports allow the use of both organic and aqueous reaction media (Figure 3).

Despite a large amount of lipase absorbed onto the polyacrylic matrix of IB-EC1 with carboxylic ester functional groups (non-ionic bond), low lipolytic activity was detected. It can be assumed that the weak interaction of the support surface with the triglycerides in aqueous medium occurred and/or enzyme overloading has taken place. What is interesting, good results were obtained when the IB-EC1 support in the lipase enantioselectivity study was tested. These results can be linked with the chemical properties of the matrix, functional groups of the carrier, and type of bond. It is worth noticing that activity retention of lipase immobilized onto the polystyrene carrier (IB-S861) was 14.3%, which is probably related to the construction of the carrier and weak interaction in an aqueous medium with the long-chain substrate. However, because of the presence of aromatic functional groups on the surface, this carrier has shown high catalytic activity in the organic medium. Supports from the second "anionic type" group (IB-A161, IB-A171, and IB-A369) characterized by quaternary ammonium type functional groups, exhibited low lipolytic activity, probably due to the negative influence of the carrier (functional group) used in the aqueous buffer medium on the hydrolysis of the olive oil. Important is that the lipase enantioselectivity could not be determined with the use of these supports because of an unknown supports-solvent phenomenon (Table 2).

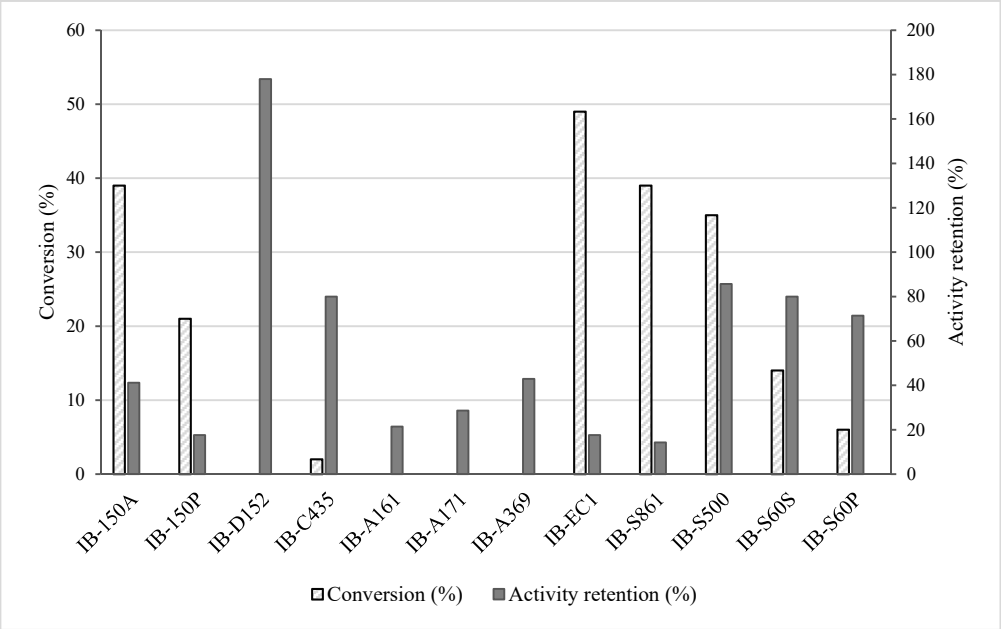


Figure 3. The catalytic activity of immobilized CALB. Activity retention (%) in buffer medium-hydrolysis of olive oil; conversion (%) in DCM–esterification of (*R*,*S*)-flurbiprofen with methanol; values of conversion and activity retention from Tables 2 and 6, respectively.

It should be emphasized that even though the largest loading of lipase was on the carriers from the first group, the lipolytic activity of the enzyme was low. This may be connected with a weak influence of the carrier in the buffer medium on triglycerides of fatty acids and worse penetration of substrates into the pores of the support. It could also be associated with an enzyme overload on these supports and steric hindrance by short epoxide spacer arms, which in consequence may reduce the availability of the active site of the enzyme for long-chain substrates. On the polar support IB-150P, activity retention was lower than on the apolar IB-150A. This might also be caused by an interaction between the polar support and molecule of lipase, presumably affecting the enzyme conformation and its active site. According to Kahar et al. [45], hydrophobic-hydrophilic interactions between the surface of the support and enzyme might result in changes in the substrate-binding pocket near to the active site. On the other hand, the enantioselectivity of that lipase-catalytic system was high, which can be related to a strong interaction of the epoxide functional group-carrier in the organic reaction medium with the substrates (flurbiprofen and alcohol).

Based on the achieved results of enantioselectivity and lipolytic activity studies, the supports were divided into three groups, depending on target reaction media of biocatalysis: the first group of carriers was dedicated for use in an organic medium; the second—for use in an aqueous medium, and the third—carriers with properties that allow application both in an organic and water medium. The division included the type of matrix, functional groups, and type of binding to the enzyme. The carriers belonging to the first group were characterized by a polyacrylic matrix with epoxy functional groups and a type of covalent bond (IB-150A and IB-150P); a polyacrylic matrix with carboxylic ester functional groups and non-ionic lipase binding (IB-EC1); a polystyrene matrix with aromatic functional groups and a non-ionic bond (IB-S861). The second group of carriers was characterized by a polyacrylic matrix with carboxylic acid functional groups and cationic bond (IB-D152 and IB-C435). The last group of carriers has a matrix made of polypropylene with alkyl functional groups and a type of non-ionic bond (IB-S500); silica (super porous) with hydroxyl functional groups and binding enzyme with the non-ionic type (IB-S60S). It should be emphasized that the proposed assignment to groups requires further investigation covering other substrates (e.g., differing in the amount of carbon atom) and the wider range of applied reaction media.

Due to the values of activity retention and activity [U], CALB immobilized onto the IB-D152 carrier was chosen for further studies of the influence of pH and temperature.

2.2.1. Effect of Temperature

The lipolytic activity of the CALB in native and IB-D152-immobilized form was tested at a temperature range of 25–65 °C (Figure 4). The results showed that the immobilized form exhibited close to the maximum activity in the broader range of temperatures, while native one was characterized by the narrower range of temperatures in which was highly active. Above 55 °C, the activity of the studied lipase forms has been reduced, although the reduction was less intensive for the immobilized form of the enzyme. It can be assumed that above this temperature, inactivation of the catalytic protein has taken place, manifested by a decrease in activity [50]. Analyzing the data for the native lipase, the activity increased with increasing temperature, reaching maximum activity at 55 °C, and after exceeding this point, the activity decreased. In contrast, the immobilized form exhibited a constant high catalytic activity of 25 °C to 55 °C. The optimum temperature range for the immobilized form is 25–55 °C, while for a native one 55 °C. Interestingly, Borges et al. [59] described lower thermal stability of immobilized enzyme onto the Immobead IB-D152, in comparison with the native form. In addition, they stated that immobilization by adsorption (such as ionic bonds) does not usually cause any significant enzyme stabilization, because of the nature of the binding of lipase with the matrix. The studies on the influence of temperature on lipase B from *Candida antractica* were described previously [62,63].

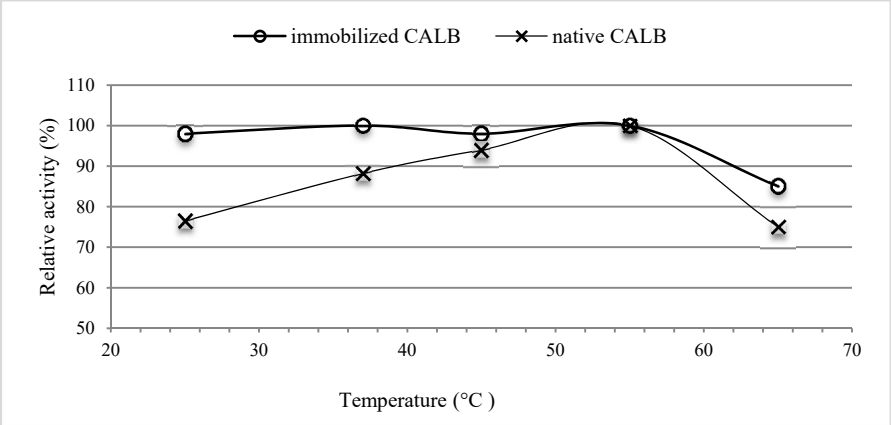


Figure 4. Effect of temperature on CALB activity. Reaction conditions: native lipase (1.26 mg) and immobilized CALB onto IB-D152 (50 mg of support), phosphate buffer (100 mM and pH 7.4), the emulsion of gum arabic and olive oil, the temperature in the range of 25–65 °C, and incubation 30 min.

2.2.2. Effect of pH

The catalytic activity of native and immobilized lipase onto the IB-D152 polyacrylic support was tested at a reaction medium pH range of 5 to 9 (Figure 5). Algöz et al. [61], reported that the pH has a meaningful impact on the enzyme protonation and in consequence changes in molecule activity. The data showed an increase in enzymatic activity of CALB with the increase of pH value up to 8, while above this value the activity for both forms had dropped. Therefore, the optimum pH for immobilized and native forms was pH 8. Between pH 6–9, the profiles of pH-dependent enzymatic activity of the tested enzyme forms were similar, but the differences in values of activity for CALB in the immobilized form were more noticeable.

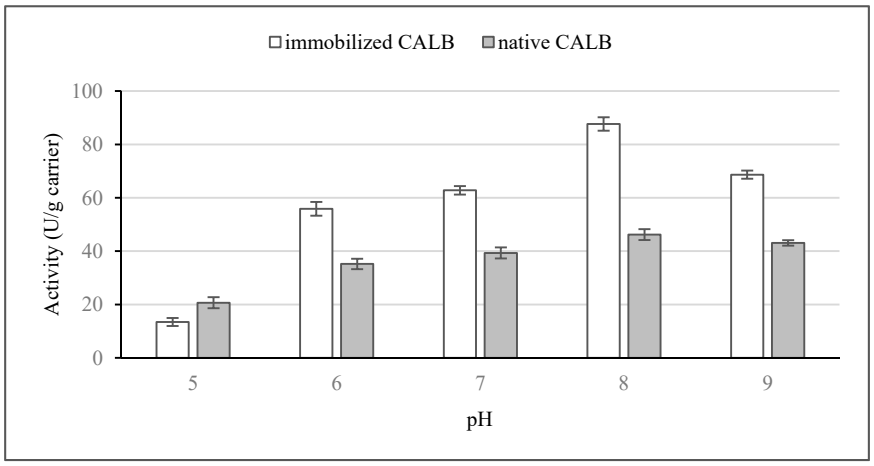


Figure 5. Effect of pH on CALB activity (U/g carrier). Reaction conditions: native lipase (1.26 mg) and immobilized CALB onto IB-D152 (50 mg of support), one of the buffer solutions: citrate buffer (100 mM and pH 5 and 6), phosphate buffer (100 mM and pH 7–8), tris-base buffer (100 mM and pH 9), the emulsion of gum arabic and olive oil, temperature 37 °C, and incubation 30 min. Data are presented as a means ± standard deviations of three analyses (n = 3). The error bars represent the standard deviations of the mean. To make the results more comparable, the data of enzymatic activity of native form were multiplied 20 times.

At pH 5, the immobilized form exhibited lower activity in comparison with the native form, which can be a result of the negative effect of the carrier on the protein in an acidic buffer medium. This phenomenon may probably be linked with the change of the dielectric constant of the microenvironment, as was mentioned in the above-mentioned paper [61]. It should be emphasized that the immobilized form in the pH range of 6 to 9 is characterized by higher activity than the native one. As based on the results obtained, there was no protective effect of immobilization on enzyme activity at pH 9 observed. Similar conclusions were reported more recently [45,47], where the authors described that enzymes immobilized onto Immobeads had almost the same profile of properties as the native form. In our study, the optimum values of pH for the native and immobilized forms and profiles of catalytic activity did not differ significantly. The effect of pH on the catalytic activity of lipase B from *Candida antarctica* was presented in very recent papers [64,65].

3. Materials and Methods

3.1. Materials

(*R*,*S*)-Flurbiprofen, (*R*)-flurbiprofen, *n*-heptane, 2-propanol (IPA), Bradford reagent, olive oil, Trizma Base, 1,2-dichloropropane (DCP), and trifluoroacetic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Lipase B from *Candida antarctica* (CALB, produced in yeast), Immobeads: IB-150A, IB-150P, IB-D152, IB-C435, IB-A161, IB-A171, IB-A369, IB-EC1, IB-S861, IB-S500, IB-S60S, and IB-S60P were acquired from ChiralVision (Leiden, the Netherlands). Dichloromethane (DCM), 1,2-dichloroethane (DCE), methanol, ethanol, *n*-propanol, *n*-butanol, and molecular sieves 4 Å, gum arabic, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate dihydrate, sodium hydroxide solution (0.1 mol /L), acetone, citric acid, *o*-phosphoric acid, hydrochloric acid, and phenolphthalein were procured from POCH (Gliwice, Poland). The (*R*)- and (*S*)-esters of flurbiprofen were achieved as products of an esterification reaction of racemic flurbiprofen and (*R*)-flurbiprofen with acyl acceptors (methanol, ethanol, *n*-propanol, and *n*-butanol) with the use of sulfuric acid [3]. The water used in the study was prepared with a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All incubations of enantioselectivity were conducted at a temperature of 37 °C in Thermomixer comfort (Eppendorf, Hamburg, Germany). Lipolytic activity studies were performed in incubator Unimax 1010 (Heidolph, Germany). Spectrophotometer UV-Vis U-1800

(Hitachi, Tokyo, Japan) and pH-meter SevenMulti (Mettler Toledo, Schwerzenbach, Switzerland) were also used in experiments.

3.2. Immobilization of CALB onto Immobeads

The procedure was performed according to the described methodology by ChiralVison, with few modifications. In brief, 50 mg of each of the tested supports (Immobeads) were placed into centrifuge tubes (Eppendorf). Next, 10 mg lipase B from *Candida antarctica* was inserted into the next centrifuge tubes and 1 mL of 0.1 M phosphate buffer at pH 7 was added. To remove air from the pores in the carriers, the vials with supports were rinsed with 2-propanol (0.3 mL) and put away for 15 min. In the next step, the supports were transferred to a filter and rinsed with distilled water (1.5 mL). Followed by, the supports were added into tubes containing the CALB suspension and mixed with a spoon in an ice bath (5 min) and then incubated overnight in a fridge (4 °C). After incubation, lipase concentration in the supernatant was determined with the use of the Bradford method. Immobilization procedures were performed in triplicate.

3.3. Determination of the Amount of Immobilized CALB

The amount of immobilized lipase adsorbed onto the Immobeads was calculated by measuring the initial concentration of CALB and its final concentration in the supernatant after immobilization with the application of the Bradford protein assay method [66]. A calibration curve of CALB solution of known concentration (1.0–10 mg/mL) was used in the determination of protein concentration in the initial solution and supernatant. All data were average of triplicate of tests.

3.4. Enantioselectivity

The enantioselectivity of the immobilized CALB was determined by enantioselective esterification of (*R*,*S*)-flurbiprofen. The reaction mixture consisted of solvent (DCM, DCE, or DCP) (700 µL) and (*R*,*S*)-flurbiprofen (4.8 mg, 0.02 mM) and the acyl acceptor: methanol (2.44 µL, 0.06 mM), ethanol (3.52 µL, 0.06 mM), *n*-propanol (4.51 µL, 0.06 mM), or *n*-butanol (5.52 µL, 0.06 mM), and molecular sieves 4 Å (reactions with free form were carried out without using of molecular sieves). The reaction was started by adding to the mixture the appropriate amount of free lipase or 50 mg of support with immobilized CALB. The suspensions were incubated and shaken at a temperature of 37 °C. The samples (50 µL) were collected and dried at room temperature and then dissolved (2-propanol and 0.9 mL), and after filtration (0.45 µm), injected (5 µL) on the HPLC column. Analyses were performed in triplicate.

3.5. Lipolytic Activity

The lipolytic activity of free and immobilized CALB was evaluated by titration of free fatty acids, which came from the hydrolysis of olive oil [67–69]. An emulsion of olive oil was made by mixing olive oil (50 mL) and gum arabic suspension (50 mL, 7%, *w*/*v*). The mixture composed of emulsion (5 mL), phosphate buffer (2 mL, pH 7.4, and 100 mM,) and free lipase (the same amount as was immobilized onto appropriate support, 1 mL) or immobilized CALB (50 mg support in 1 mL buffer). Oil hydrolysis was performed in a shaking water bath (37 °C, 30 min, and 150 rpm). The reaction was stopped by the addition of ethanol–acetone solution (1:1 and 10 mL). The liberated fatty acids were calculated based on the results of titration with NaOH solution (50 mM) with the application of phenolphthalein indicator. The control was carried out without lipase (as a blank). One unit of CALB activity (U) was determined as the amount of lipase that hydrolyzed olive oil liberating 1 µmol fatty acid per minute under the assay condition. Activity retention (%) remaining after immobilization was the ratio between the activity of immobilized CALB and the activity of the same amount of free protein in the solution that had been immobilized onto the support. Analyses were performed in triplicate.

3.6. Effect of pH and Temperature on CALB Activity

The pH effect on the free (the same amount as was immobilized onto appropriate support, 1 mL) and immobilized lipase (50 mg of the support) activity were investigated by enzyme incubation in the mixture of emulsion (gum arabic and olive oil) and one of the buffer solutions: citrate buffer (100 mM and pH 5–6), phosphate buffer (100 mM and pH 7–8), tris-base buffer (100 mM and pH 9). The studies with the use of the standard activity assay procedure (mentioned above) were performed and lipase activity (U/g support) was evaluated. The tests of the effect of the temperature on the activity of free (the same amount as was immobilized onto appropriate support, 1 mL) and immobilized lipase (50 mg of immobeads) were carried out in phosphate buffer (100 mM and pH 7.4), in the temperature range of 25–65 °C, in 30 min, with the use of an emulsion of gum arabic and olive oil (standard activity assay). Titration was performed at room temperature. The values of relative activity (%) as the ratio between the activity of every sample and the maximum activity of the sample were calculated. Analyses were performed in triplicate.

3.7. Chromatographic Conditions

The optimal mobile phase was *n*-heptane/2-propanol/trifluoroacetic acid (97.6/2.4/0.2 *v*/*v*/*v*) at a flow rate of 1 mL/min. The Lux Cellulose-3 HPLC column was selected for the separation of (*R*,*S*)-flurbiprofen and its esters, as it was described in a previous paper [40]. The temperature of the chromatographic process was 15 °C and the wavelength was 254 nm. The enantiomeric excesses of the substrate (ee_s) and the product (ee_p) as well as the conversion (*C*) were calculated with the application of the equations presented by Ghanem and Aboul-Enein [70].

The ee_s and ee_p values were expressed as:

$$(\%)ee_s = \frac{|R_s - S_s|}{R_s + S_s} \times 100$$
$$(\%)ee_p = \frac{|R_p - S_p|}{R_p + S_p} \times 100$$

R_s, S_s—enantiomers of the substrate (*R*,*S*-flurbiprofen); represent the peak areas of the *R*- and *S*-enantiomers, respectively.

R_p, S_p—enantiomers of the product (ester of (*R*,*S*)-flurbiprofen); represent the peak areas of the *R*- and *S*-enantiomers, respectively.

The conversion (*C*):

$$(\%)C = \frac{ee_s}{ee_s + ee_p} \times 100$$

The HPLC instrumentation and chromatograms of (*R*,*S*)-flurbiprofen and its esters were demonstrated and described in detail in a previous publication [40].

3.8. Kinetic Parameters

Kinetic parameters for immobilized CALB on IB-D152 were determined based on the reaction described in Section 3.5. The kinetics of the activity was investigated using olive oil in various concentration (100–800 mg/mL). The value of the Michaelis constant (K_m) and the turnover numbers (k_{cat}) were calculated from the Lineweaver-Burk plot using the following equations:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

where V, V_{max}, K_m, and $[S]$ are the initial velocity, the maximum velocity, the value of the Michaelis constant, and the initial substrate concentration, respectively [68].

$$k_{cat} = \frac{V_{max}}{[E_\tau]}$$

where V_{max} and $[E_\tau]$ are the maximum velocity and the total amount of the enzyme in the reaction mixture, respectively [61].

4. Conclusions

In this paper, investigations of the enantioselectivity and lipolytic activity of CALB immobilized on polymeric carriers were described. The interactions between the carriers, lipase, substrates, and reaction media were evaluated. The results indicate that with a well-designed catalytic system, it is possible to achieve a significantly enhanced lipase activity compared to the native form of the enzyme. A 43-fold increase in catalytic activity (expressed as a conversion value) was achieved in the reaction of enantioselective esterification of (*R*,*S*)-flurbiprofen in an organic medium with the application of lipase immobilized on IB-S861 in comparison to the native form. Moreover, hyperactivation (activity retention of 178%) of CALB immobilized on IB-D152 in the investigations of the lipolytic activity in the buffer medium was documented. The screening that was carried out demonstrated what an extremely difficult, often impossible, task it was to create a universal catalytic system dedicated to a wide substrate spectrum. The carriers with the potential for application both in organic and aqueous reaction media were IB-S500 and IB-S60S. The design of a catalytic system (carrier–enzyme-bond) should be considered individually, including substrates and the reaction medium. It should be emphasized that the phenomena of strong dependencies between the reaction medium (aqueous and organic), substrates ((*R*,*S*)-flurbiprofen, olive oil), carriers, and the immobilized lipase were demonstrated. These interactions can cause enhanced or decreased enzyme activity. Meaningful differences in the activity of the tested immobilized CALB were observed in reactions in which the variable parameters were only the reaction medium and the substrates. The immobilized form exhibited a constant high catalytic activity in the temperature range of 25 to 55 °C. Furthermore, a higher catalytic activity of the immobilized form in the reaction media pH range of 6–9 was found in comparison with the native form. The data allow us to conclude that at the stage of designing the catalytic system when it is desirable to achieve an enhanced catalytic activity of the enzyme, the interaction of lipase with the carrier, carrier with substrate, and carrier with the reaction medium should be taken into account.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/8/876/s1, Figure S1. Reactors with CALB immobilized on IB-S861: (a) in DCM (b) in DCE (c) in DCP. Figure S2. Effect of time on the conversion. Figure S3. Effect of time on the enantiomeric excess of product. Figure S4. Effect of time on the enantiomeric excess of substrate. Table S1. The solubility of (*R*,*S*)-flurbiprofen. Table S2. The state of the art in enzyme immobilization on Immobeads.

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