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Synthesis, Characterization and Synthetic Applications of Nanobioconjugates of Lipase B from *Candida antarctica*

PhD Thesis Abstract

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1. Introduction

Biocatalysis represents a major research area in both industry and academia. Biocatalysis, defined as the chemical synthesis mediated by a biocatalyst, brings a clean and friendly alternative to the environment for carrying chemical processes [1,2]. Biocatalysis posses many advantages such as high selectivities, mild reaction conditions, lower cost, all these making the chemical transformations catalyzed by biocatalysts intensively used as compared to the conventional chemical processes. Over the last decades, the research regarding the immobilization of the enzymes or even the whole cell, presented very few limitations when those preparates are used as catalysts in organic synthesis [3-5].

Enzymes are protein compounds with a catalytic function, synthesized by living organisms. Similar to conventional catalysts, they increase the reaction rate by lowering the activation energy. In addition to classical catalysts, they make possible the biotransformatios of substances in conditions where the traditional catalyst can not do it. These transformations are due to specificity, selectivity (chemo-, regio- and stereoselectivity), but above all, to the high enzymatic activity. All these are important factors in the successful applications of the enzymes, but besides these ones, the stability and reusability are also critical factors in bioprocesses development [3,6].

Pure enantiomeric compounds present a major interest in the pharmaceutical industry, since the two enantiomers of a chiral compound can have different physiological effects, the utilization of enzymes in this field, lead to optically pure compounds, through selective and biocompatible reactions. A major concern regarding the engineering process is represented by the capability to establish an easy method to separate and reuse the biocatalysts [7].

Originally, it was thought that enzymes function exclusively in aqueous media, that they are incompatible with organic solvents, hypothesis that was later shown to be false. The enzymes need a layer of water to ensure conformational flexibility, water acting like a lubricant for the moves of the polypeptide chain. In the absence of this layer, the enzymes become rigid, which does not necessarily mean its denaturation, but a decrease of its activity was generally observed [5,8].

There are many reasons to the use of organic solvents in enzymatic reactions such as: the reduced solubility of some substrates in water, the avoidance of secondary processes like hydrolysis or water addition reactions or the avoidance of substrate or product inhibition. Additionaly, when using water as a solvent microbial contamination, leading to more complicated separation steps and reduced conversions, occurs. Volatile organic solvents bring the advantage of being easily removed from the reaction system, allowing the recovery of both, product and used enzyme [9].

Increasing the stability of enzymes in organic solvents can be achieved by immobilization, process of passing enzymes in a solid phase. By immobilizing the enzymes, makes possible their recyclability, and switching to a continuous process operational system, that presents advantages in terms of productibility and the possibility of automatization. Although immobilization has many advantages, it has also some downfalls, the major of them being an induced rigidity by the support on which is carried out the enzyme, leading to a decrease on its activity [10,11].

The main objective of this work is to obtain new bioconjugates of lipase B from *Candida antarctica*, their characterization and investigation in the enzymatic kinetic resolution of different heteroaryl secondary alcohols.

In the first part of original contribution (Chapter 4) are described new stable enzymatic preparates obtained through the covalent immobilization of lipase B from *Candida antarctica*

(CaL-B) onto chitosan-coated magnetic nanoparticles using different arm-spacers. The newly enzymatic preparates were used in the stereoselective synthesis of optically pure heteroaryl secondary alcohols in batch system [12,13-18,19].

The second part of Chapter 4 presents the immobilization of the same enzyme through entrapment technique into polyvinyl alcohol and chitosan nanofibers. The obtained bioconjugate was further tested in the *O*-transestericafion of two series of phenothiazinyl ethanols (*N*-alkyl-phenothiazin-2-yl-ethanols and *N*-alkyl-phenothiazin-3-yl-ethanols) [20-23].

The third and final part of original contributions is represented by the immobilization of CaL-B onto (adsoption technique) and into polyvinyl alcohol and polylactic acid nanofibers. The bioconjugates obtained were used in the enzymatic kinetic resolution of 1-benzo[b]thiophen-2-yl-ethanol in batch and continuous systems, providing a promising background for the process development [10,22,24].

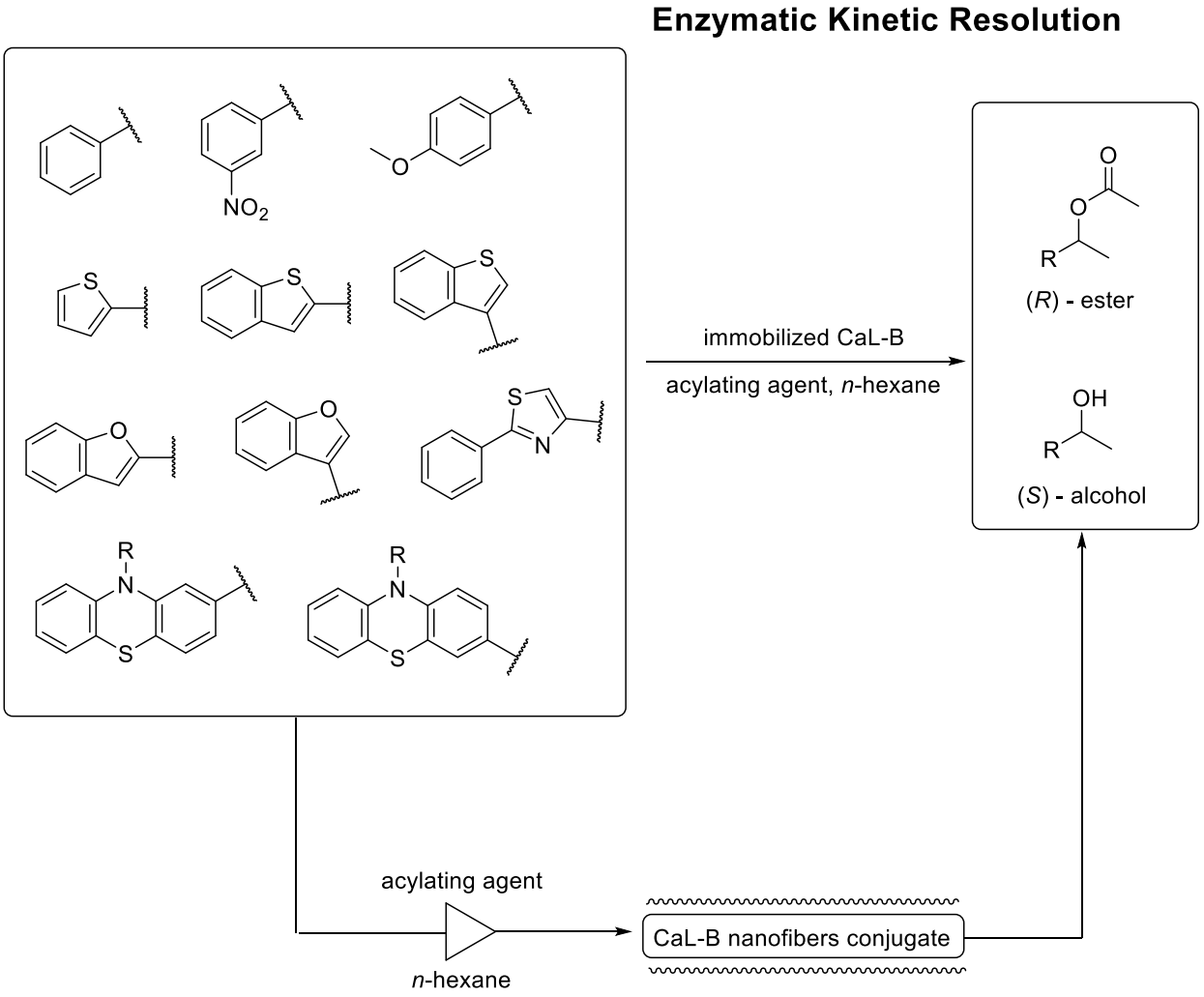
Lipase B from *Candida antarctica*, also known as CaL-B, is considered to be one of the most promising enzymes for industrial processes, due to its specificity and activity in transesterification reactions when the substrates are secondary alcohols [25-27].

Even this lipase has great properties, it also has some disadvantages when is used in its free form, making it difficult to recover the enzyme from the reaction mixture in order to reuse it, and presents a lower stability compared to its immobilized form, on different supports such as: chitosan, silica, magnetic nanoparticles, or nanofibers [10,28,29,30].

2. Literature Data

3. Aim of the Thesis

The objective of the current work was to develop stable and active bioconjugates of lipase B from *Candida antarctica* using nanosupports such as chitosan-coated magnetic nanoparticles (MNP-CS) or polymeric nanofibers of polyvinyl alcohol (PVA) or polylactic acid (PLA). The obtained biocatalysts were tested in the EKR of (hetero)aromatic secondary ethanols through *O*-transesterification, in batch and continuous systems, in order to obtain optically pure compounds (**Scheme 1**).



Scheme 1. EKR of (hetero)aromatic secondary ethanols through *O*-transesterification, in batch and continuous systems.

(Hetero)aromatic secondary alcohol were chosen as substrates due to their importance in the pharmaceutical field as building blocks for different drugs. Phenothiazine, benzofuran, benzo[*b*]thiophene or 2-phenylthiazol are known cores for drugs used as anti-cancer, anti-viral, anti-bacterial, anti-fungal, asthma or antipsychotic agents [70-75].

4. Personal Contributions

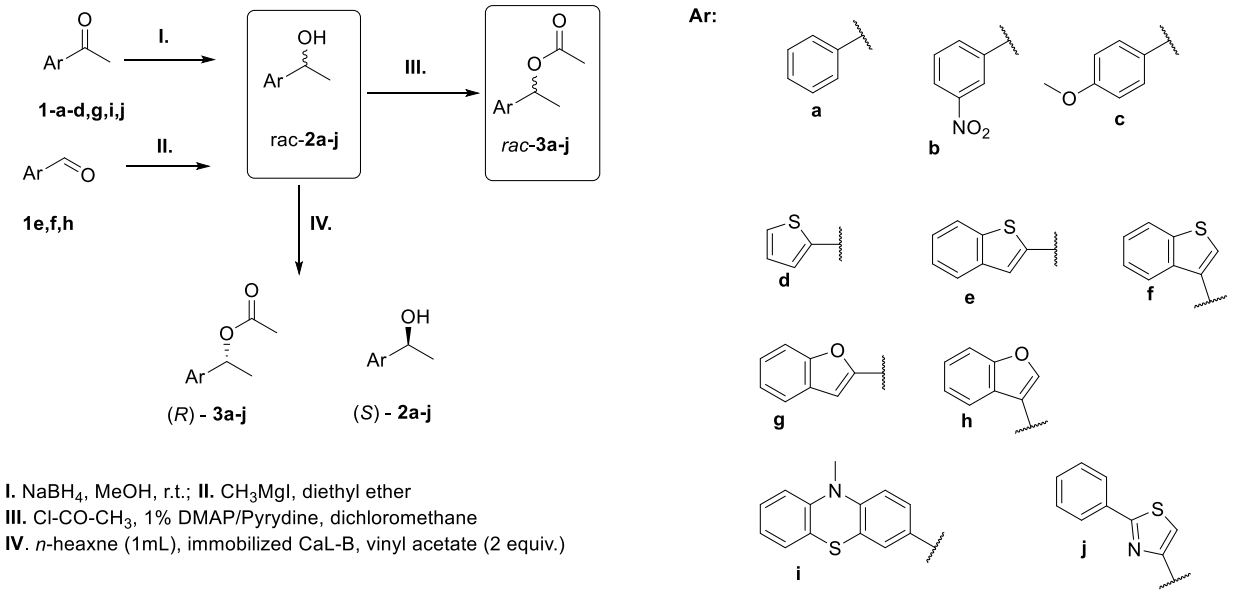
4.1. Chitosan-Coated Magnetic Nanoparticles Bioconjugates of CaL-B Obtained Through Covalent Immobilization

4.1.1. Introduction

4.1.2. Results and Discussion

4.1.2.1. Chemical Synthesis of Racemic Heteroaryl Ethanols (rac-2a-j) and Their Corresponding Acetates (rac-3a-j)

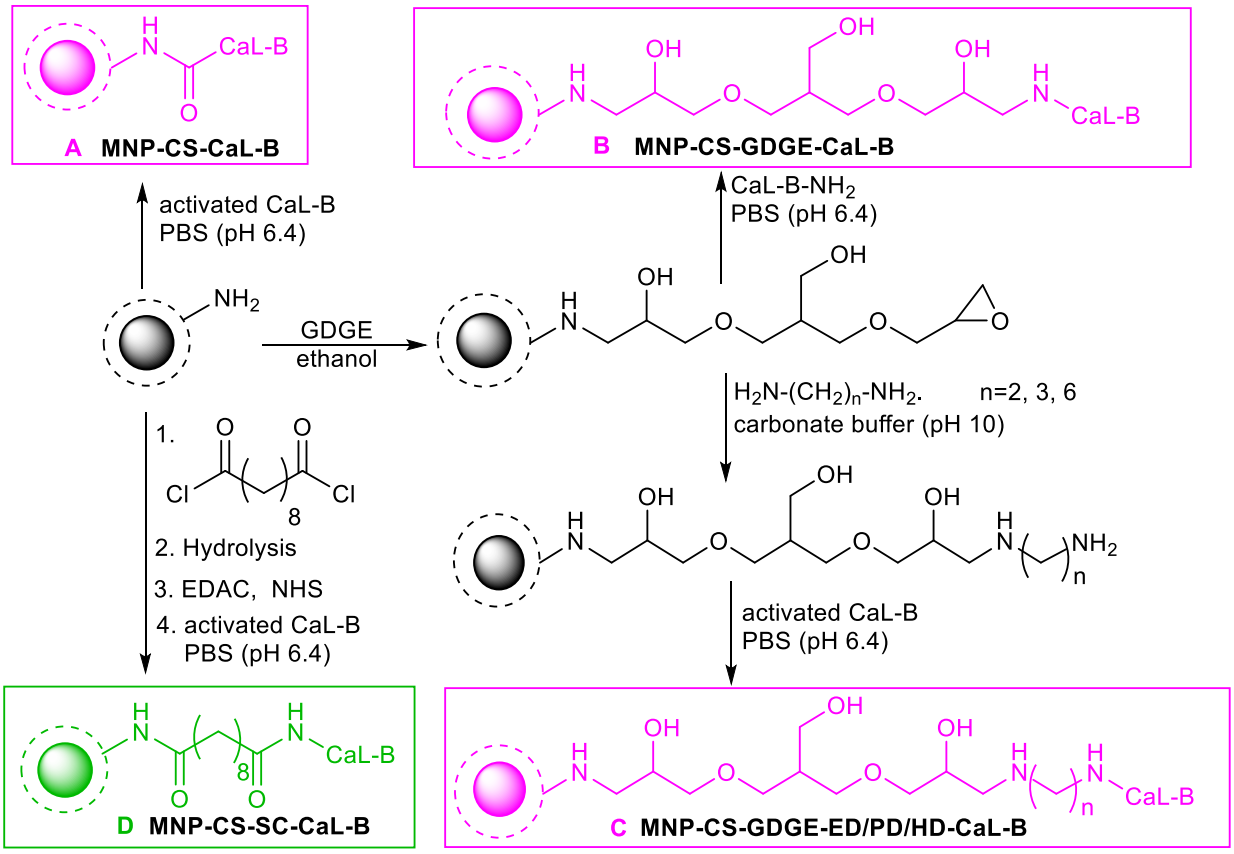
The racemic heteroaromatic ethanols were chemically synthesized using known methods [97-100]. Racemic heteroarylethanols *rac*-**2a–d,g,i,j** were obtain through chemical reduction with sodium borohydride of the corresponding prochiral heretoaryl-methyl-ketones **1a–d,g,i,j**, while the heretoarylethanols *rac*-**2e,f,h** were prepared through Grignard reaction from the corresponding aldehydes **1e,f,h (Scheme 2).** The racemic ethanols were used as substrates in the *O*-transesterification reaction (**Scheme 2**), and as starting materials for the chemical synthesis of their corresponding esters, in order to set-up the chromatographic separation methods.



Scheme 2. Chemical synthesis of racemic ethanols *rac*-**2a–j** and their acetates *rac*-**3a–j** and *O*-transesterification mediated by immobilized CaL-B of racemic ethanols *rac*-**2a–j**.

4.1.2.2. Covalent Immobilization of CaL-B on Chitosan-Coated Magnetic Nanoparticle (MNP-CS) Supports

Having the aim of developing efficient bioconjugates of CaL-B, four methods for were used the covalent immobilization of lipase B onto chitosan-coated magnetic nanoparticles (MNP-CS) involving different interactions between support and enzyme: A) the direct covalent binding of the activated enzyme to the amino groups of chitosan; B) the covalent binding on the residual epoxy groups of glycerol-diglycidyl ether (GDGE) after its covalent attachment to amino groups of chitosan; C) the covalent binding through an alkyl-diamine linker (ethyl diamine —ED, propyl diamine —PD and hexyl diamine —HD) previously linked to the GDGE activated particles and D) the covalent binding on the sebacoyl chloride (SC)-derivatized MNP-CS *via* the activated ester method (**Scheme 3**).



Scheme 3. Covalent immobilization of CaL-B on MNP-CS based supports.

4.1.2.3. Synthetic Activity of the Biocatalysts

Since the catalytic activity of lipases in organic solvents is not reflected by their hydrolytic activity determined in aqueous solution, a sensitive colorimetric method has been developed, based on the amount of acetaldehyde released when vinyl esters are used in the transesterification reactions, named *synthetic activity* [101]. Alongside the obtained bioconjugates, the synthetic activity of commercially immobilized CaL-B (Novozym 435) as reference was determined. The obtained results are presented in **Tabel 1**.

Table 1. Synthetic Activity of the newly obtained enzymatic preparates.

Entry	Preparate	Synthetic Activity[a] (mmol/min*$g_{preparate}$)
1.	lyophilized CaL-B	0.44
2.	Novozym 435	1.96
3.	MNP-CS-CaL-B	0.13
4.	MNP-CS-GDGE-CaL-B	0.13
5.	MNP-CS-GDGE-ED-CaL-B	0.21
6.	MNP-CS-GDGE-PD-CaL-B	0.20
7.	MNP-CS-GDGE-HD-CaL-B	0.09
8.	MNP-CS-SC-CaL-B	1.95

[a] according to Lambert-Beer law, where ε = 14,300 [101].

For the synthetic activity determination, the same amount of immobilized enzyme was used. As observed from **Table 1**, the chitosan-coated magnetic nanoparticles bearing the sebacoyl moiety (Entry 8) had the same synthetic activity as the commercially immobilized CaL-B (Entry 2), which has the drawbacks to be unstable, under stirring may become fragile, leading to

enzyme leakage, consequently in a decrease of the biocatalyst's activity [102,103]. Comparing the most active bioconjugate (Entry 8) with the lyophilized CaL-B (Entry 1), the bioconjugate presents a much higher synthetic activity, probably because of the hydrophobic residue that blocks the enzyme-enzyme interactions at the support surface.

4.1.2.4. Enzymatic Kinetic Resolution Optimization

To corroborate the results obtained for the synthetic activity, regarding the efficiency of the bioconjugates, they were tested in the *O*-transesterification reaction of racemic 1-phenyl-1-ethanol *rac*-**2a** chosen as model substrate. The obtained results were in concordance with the ones obtained in the activity study, proven the most efficient enzymatic preparate to be the one bearing the sebacoyl moiety (**Figure 1**). This new conjugate allows a maximum conversion (50%) in the shortest reaction time (13 hours) and high enantiomeric excesses (ee_S and ee_P >99.9%). For this study and the next two the used substrate-enzyme weight *ratio* was 5:1, with the corresponding amount of preparate containing 1 mg of enzyme, in 1 mL of *n*-hexane as solvent and at 45 °C and 1000 rpm.

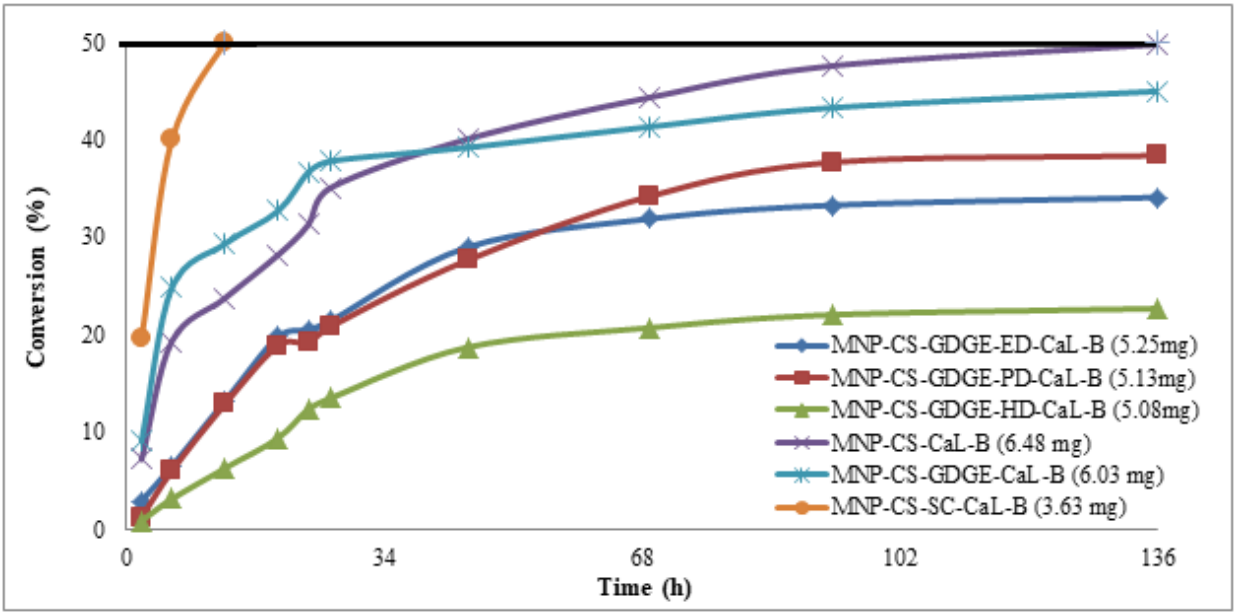


Figure 1. Biocatalyst screening in the EKR of model substrate racemic1-phenyl-1-ethanol (*rac*-**2a**).

4.1.2.5. Optimal Parameters for EKR

4.1.2.5.1. Influence of Temperature on the Biocatalyst's Activity

Since it is known that temperature plays an important role in the reaction rate of enzyme-catalyzed reactions, the next study focused on determining the optimal temperature for the most efficient bioconjugate of CaL-B: MNP-CS-SC-CaL-B. The reactions were performed in 1 mL of *n*-hexane, with vinyl acetate as acylating agent (2 equiv.) and a substrate-enzyme weight *ratio* of 5:1. The temperature range was between 30 °C and 60 °C, gradually increased with 5 °C. The samples were taken after 2 hours and analyzed on HPLC. After analyzing the experimental data, the optimal temperature was determined as 45 °C (**Figure 2**), thus all further experiments will be performed at this value.

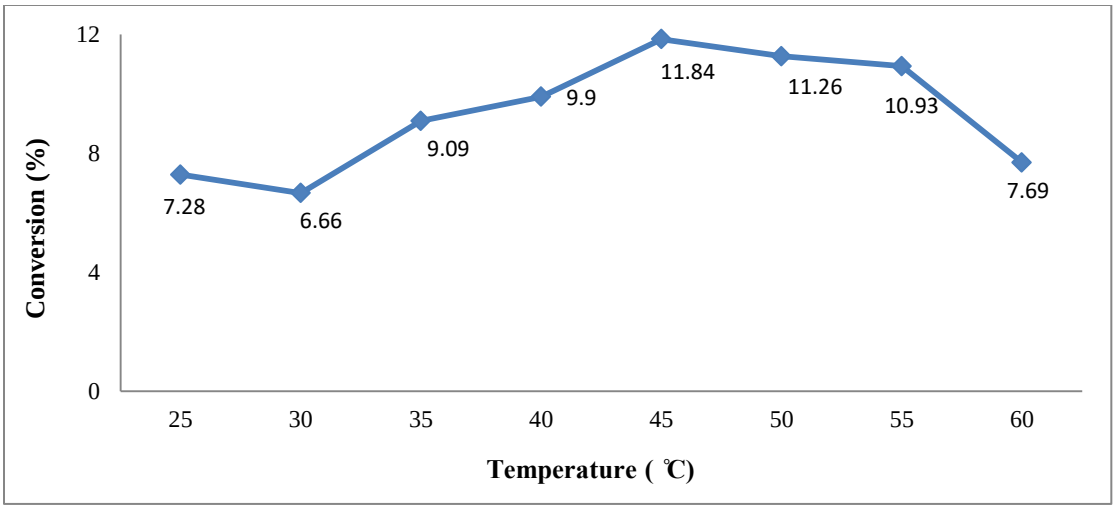


Figure 2. Temperature effect on enzymatic activity of MNP-CS-SC-CaL-B in the EKR of racemic 1-phenyl-1-ethanol (conversion after 2 hours).

4.1.2.5.2. Determination of the Optimal Reaction Medium

The enzyme conformation strongly depends on the reaction media; thus, its efficiency depends on the solvent nature. For the screening of the solvent, five nonpolar and polar aprotic solvents were used (*n*-hexane, toluene, tetrahydrofuran (THF), tert-butyl-methyl-ether (MTBE) and dichloromethane (DCM)), under similar conditions as the previous preliminary studies: vinyl acetate (2 equiv.), substrate-enzyme weight *ratio* 5:1, temperature 45 °C. The obtained experimental data showed *n*-hexane as optimal reaction medium (obtaining a conversion of 42% after 6 hours) as compared to toluene, where the conversion was only 12%. In the case of the other three solvents (THF, MTBE and DCM), the obtained conversion was less than 2%. Samples were taken after 6 hours and analyzed on HPLC.

4.1.2.5.3. Influence of Substrate: Enzyme Weight *Ratio* over the EKR

Next, to increase the productivity of the EKR, we studied the optimal substrate-enzyme weight *ratio*, using 5 mg of the model substrate *rac*-**2a**, 2 equiv. of vinyl acetate, *n*-hexane as solvent (1 mL), at 45 °C and 1000 rpm. In order to study the substrate-enzyme weight *ratio*, the amount of substrate was maintained for all the experiments, modifying the quantity of enzyme and enzymatic preparate respectively. Samples were taken after 6 hours and analyzed on HPLC. After analyzing the obtained results, a substrate-enzyme weight *ratio* 5:1 was chosen as optimal, resulting in a conversion of 45%, compared with the higher *ratio* (15:1) where the obtained conversion was 12% or with the lower *ratio* (2.5:1) where the conversion was 30%.

4.1.2.5.4. Influence of the Acylation Agent

It is a known fact that the nature of the acylating agent plays a significant role in the selectivity of the enantiomer selective acylation. Three vinyl esters (2 equiv.): acetate, butanoate and decanoate were tested in the *O*-transesterification of the model compound *rac*-**2a**, in the previously established conditions: 1 mL of *n*-hexane, substrate-enzyme weight *ratio* 5:1 at 45 °C and 1000 rpm. Samples were taken every 2 hours until the completion of reaction. As observed in **Table 2**, the best acylating agent has proven to be vinyl acetate, allowing the maximum conversion (50%) in the shortest reaction time (13 hours) (Entry 1), and with higher enantiomeric excesses (ee_S and ee_P > 99.9%).

Table 2. Determination of the optimal acylation agent

Entry	Acylation agent	Time (h)	ee_S[a] (%)	ee_P[a] (%)	c[b] (%)
1	Vinyl acetate	13	>99.9	>99.9	50
2	Vinyl butyrate	79	>99.9	>99.9	50
3	Vinyl decanoate	24	77.6	85	43.7

[a] determined from peak areas of HPLC chromatograms; [b] calculated with the formula $c = [ee_S/(ee_S+ee_P)]$

4.1.2.5.5. Substrate-Vinyl Acetate *Ratio* Effect

Another parameter that can influence both the reaction rate and the synthetic activity is represented by the substrate-vinyl acetate *ratio*. The experiments were performed with the previously determined conditions (1 mL of *n*-hexane, substrate-enzyme weight *ratio* 5:1, vinyl acetate as acylating agent, at 45 °C and 1000 rpm), on the substrate model *rac*-**2a**. The *ratios* between the substrate and vinyl acetate (equiv.) used were: 1:0.75, 1:1, 1:2 and 1:4. When 2 and 4 equiv. of acylating agent were used, the maximum conversion was reached (50%), the biocatalyst having similar synthetic activity (**Figure 3**). Due to economical and environmental reasons, the *ratio* between substrate and vinyl acetate used in the further experiments was 1:2.

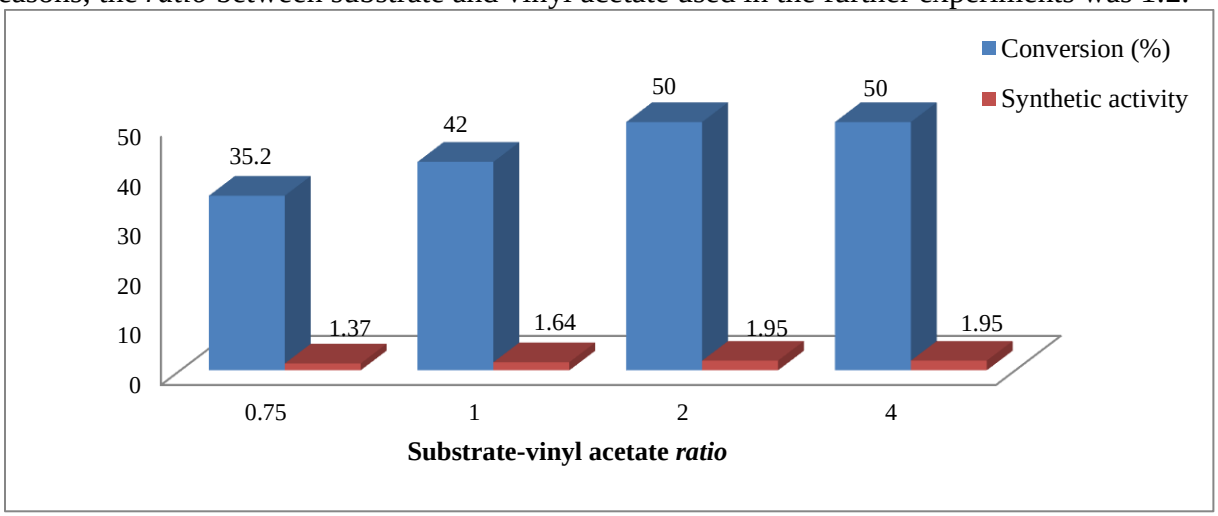
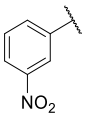
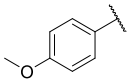
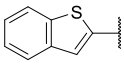
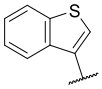
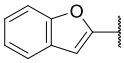
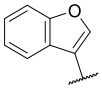
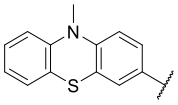
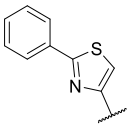


Figure 3. The influence of substrate: vinyl acetate *ratio* on the conversion and synthetic activity in the EKR of racemic 1-phenyl-1-ethanol (*rac*-**2a**).

4.1.2.6. Analytical-Scale Lipase-Mediated O-Transesterification Reactions of Racemic Heteroarylethanols rac-2a–j

Having the optimal parameters previously determined for the enzymatic kinetic resolution, next the substrate domain for the new bioconjugate of lipase B from *Candida antarctica* (MNP-CS-SC-CaL-B) was established. Samples were taken periodically from the reaction mixture, diluted with *n*-hexane and analyzed on HPLC using the appropriate chiral columns (**Table 3**, **Table 4** from section 5.1.). The obtained results showed a very good conversions (c>49%), high optical purities (ee_P> 99.9%, ee_S>96%, E>200), for all investigated heteroarylethanols. The bioconjugate presented an excellent catalytic property and a wide substrate domain composed of aromatic and heteroaromatic substrates bearing phenyl, thiophene, benzothiophene, furan, benzofuran, phenothiazine and thiazole moieties.

Table 3. EKR of heteroarylethanols *rac*-**2a-j**

Entry	Substrate		Reaction time (h)	ee_S [a] (%)	c [b,c] (%)
1	*rac*-**2a**		13	>99.9	50
2	*rac*-**2b**		5	>99.9	50
3	*rac*-**2c**		5	98.9	49.7
4	*rac*-**2d**		4.5	>99.9	50
5	*rac*-**2e**		4	97.6	49.4
6	*rac*-**2f**		16	96.9	49.2
7	*rac*-**2g**		8	96.1	49
8	*rac*-**2h**		12	98.2	49.6
9	*rac*-**2i**		10	96.8	49.2
10	*rac*-**2j**		3	>99.9	50

[a] determined from peak areas of HPLC chromatograms; [b] calculated with the formula $c = [ee_S/(ee_S+ee_P)]$; [c] ee_P >99.9% in all cases, E »200.

4.1.2.7. Recycling Experiments

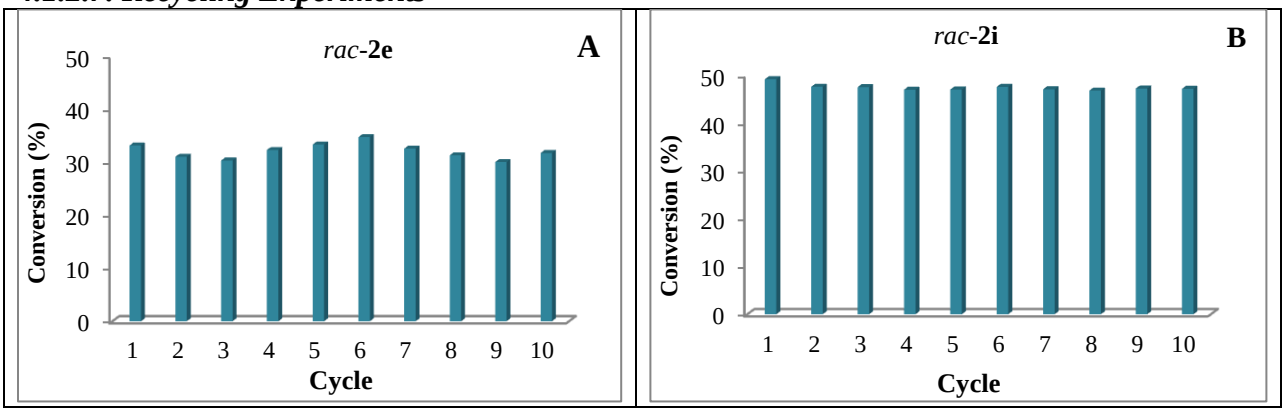


Figure 4. A. The reusability of the optimal biocatalyst MNP-CS-SC-CaL-B in the EKR of racemic benzothiophene-2-ethanol (*rac*-**2e** - 1 hour reaction time) and **B.** of racemic *N*-ethyl-phenothiazinyl-3-ethanol (*rac*-**2i** -10 hours reaction time)

Since the reusability and stability are important factors for every biocatalyst and a crucial requirement in any applications, allowing the development of sustainable processes, the most

efficient bioconjugate (MNP-CS-SC-CaL-B) was tested in the EKR of *rac*-**2e** (reaction time 1 hour) and *rac*-**2i** (reaction time 10 hours) (**Figure 4A, 4B**) perfected in the previously determined conditions. The reaction was performed 10 consecutive times, the immobilized enzyme being washed with *n*-hexane (3×0.5 mL) after each cycle and immediately used in the next one. The biocatalyst presented a high stability in *n*-hexane, especially showed in the EKR of *rac*-**2i**, the compound bearing a phenothiazine moiety. The conversion decreased with less than 3% after a total of 100 h in the organic solvent. In the case of *rac*-**2e**, compound with a benzothiophene moiety, the biocatalyst maintained its activity through all 10 reaction cycles, the conversion decreasing with less than 3%, after an overall reaction time of 10 h reaction time.

4.1.3. Conclusions

The optimization of enzymatic kinetic resolution of heteroarylethanols mediated by lipase B from *Candida antarctica* covalently immobilized onto chitosan-coated magnetic particles was performed. As demonstrated, the covalent immobilization method offers the possibility of developing efficient biocatalysts for different applications by varying the spacer-arm positioned between the enzyme and support. Finding the optimal parameters that influence the reaction rate, activity, and selectivity (temperature, substrate-enzyme *ratio*, solvent, acylating agent and substrate-vinyl acetate *ratio*), the enzymatic preparate bearing the sebacoyl moiety both as activating agent and spacer-arm, provided high efficiency for each tested substrate. The newly obtained bioconjugate has proved to be highly stable in organic solvents, its activity remains high in both recyclability cases even after 10 cycles, decreasing with less than 5%.

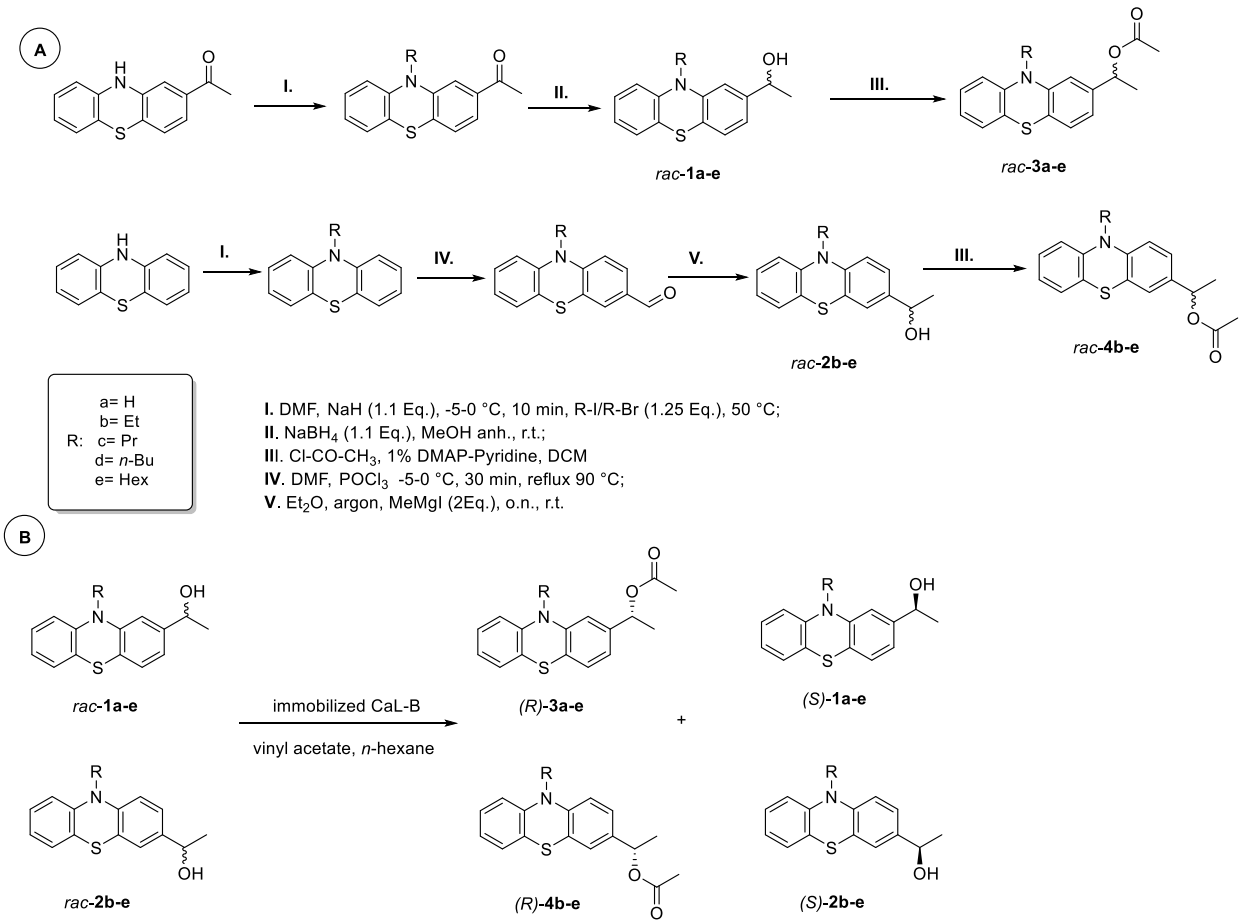
4.2. Nanobiocatalyst Based on PVA-CS Nanofibers for Phenothiazinyl-Ethanols Resolution

4.2.1. Introduction

4.2.2. Results and Discussion

4.2.2.1. Chemical Synthesis of Phenothiazinyl-Ethanols (rac-1a-e and rac-2b-e)

The racemic ethanols containing a phenothiazine moiety were synthesized using known methods [97-99]. The racemic heteroarylethanols *rac*-**1a–e** were obtained through chemical reduction with sodium borohydride of the corresponding prochiral heteroaryl-methyl-ketones, while the heteroarylethanols *rac*-**2b–e** were obtained by Grignard reaction from the corresponding aldehydes **(Scheme 4A).** The obtained racemic ethanols were used as substrates in the *O*-transesterification reactions (**Scheme 4B**), and also as starting materials for the chemical synthesis of their corresponding acetates (*rac*-**3a–e** and *rac*-**4b–e**), in order to set-up the chromatographic separation methods (**Table 5** in section 5.1).



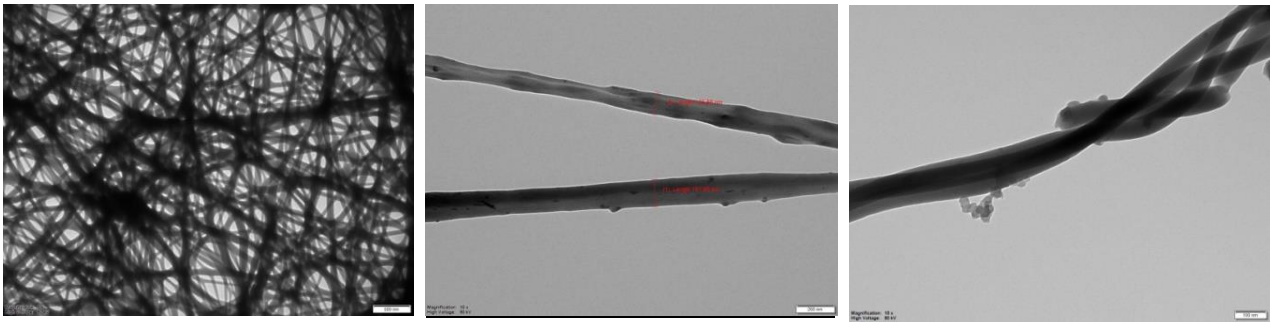
Scheme 4. A. Chemical synthesis of racemic ethanols *rac*-**1a–e** and *rac*-**2b–e** their acetates *rac*-**3a–e** and *rac*-**4b–e**; **B.** Enantiomer selective *O*-transesterification of racemic ethanols mediated by immobilized CaL-B.

4.2.2.2. Electrospinning Process

Even if the bioconjugate of CaL-B was obtained through electrospinning technique, we can still consider that the new biocatalyst was obtained by using the entrapment method. The optimal enzyme loading through entrapment is the lowest amount of the enzyme assuring the highest biocatalytic activity [135]. According to the literature, the most used protocol for enzyme entrapment in polymeric nanofibers based on PVA and chitosan, uses a mixture of 8% PVA and 1.35% CS solutions, with the volumetric *ratio* of PVA: chitosan=8:2 (*v*/*v*) [19,21,22].

4.2.2.2.1. Biocatalyst Morphology Characterization

The nanofibers were characterized through electronic transmission microscopy (TEM). The images of PVA-CS nanofibers prepared as reference (**Figure 5a,b**) indicated a good homogeneity and an uniform dimensional distribution, while in the presence of lipase (**Figure 5c**), the enzyme molecules are visible mostly into nanofibers, indicating the enzyme immobilization preponderant through entrapment and less by adsorption onto the polymeric support surface.

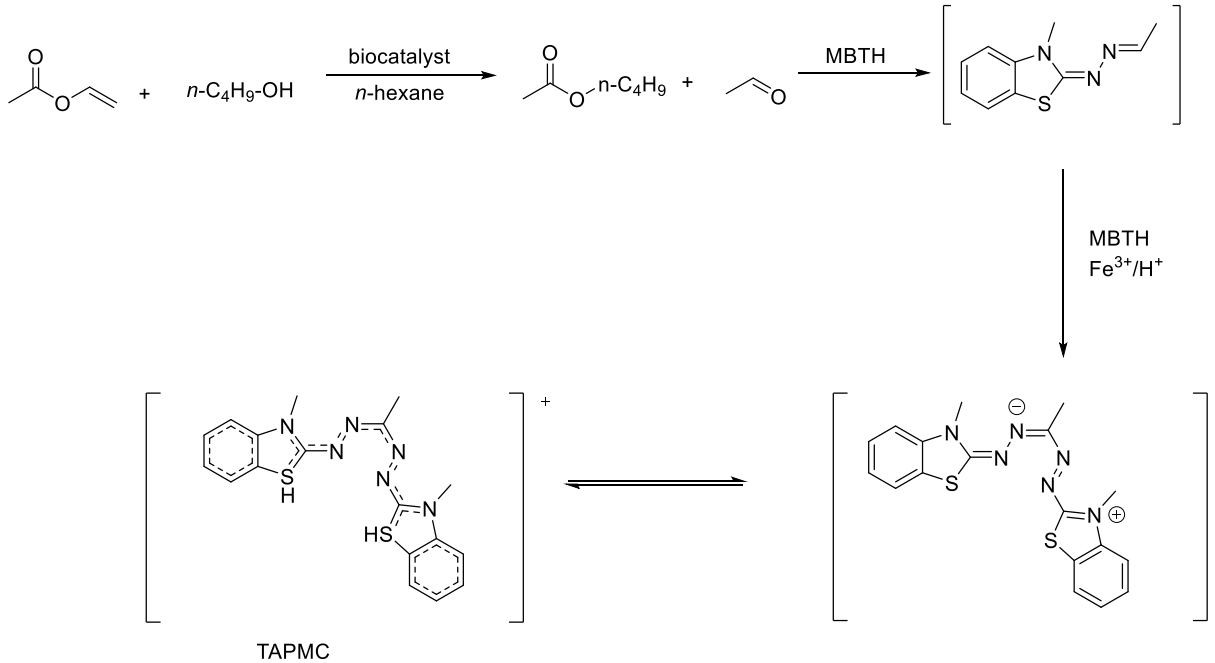


a **b** **c**

Figure 5. TEM images of the new biocatalyst (PVA-CS-CaL-B). **a.** PVA-Chitosan nanofibers homogeneity and uniform distribution, with a magnification of 5× (500 nm) and high voltage (80kV); **b.** PVA-Chitosan fibers length determined between 120-152 nm, using a magnification of 10× (200 nm) and high voltage (80kV). **c.** PVA-Chitosan nanofibers containing CaL-B at an amplification allowing to observe the enzyme molecules, using a magnification of 15× (100 nm) and high voltage (80kV).

4.2.2.2.2. Synthetic Activity of the Enzymatic Preparate

The first evaluation of the new biocatalyst was determining its synthetic activity, based on the previous mentioned method [101]. The synthetic activity assay is based on the reaction of *n*-butanol with vinyl acetate, the released acetaldehyde being quantified after derivatization with 3-methyl-2-benzothalininone hydrazone (MBTH); the aldazine intermediate reacts further with another MBTH molecule by oxidative coupling in the presence of ammonium iron (III) sulfate ($NH_4Fe(SO_4)_2 \times 12\ H_2O$) forming a blue tetraazapentamethylene-cyanine (TAPMC) with a maximum absorption at 598 nm (**Scheme 5**) [101].



Scheme 5. Reactions involved in the synthetic activity protocol.

Using this assay, a high value for the synthetic activity of the nanobioconjugate (PVA-CS-CaL-B) 6.1 $mmol \times min^{-1} \times g_{biocatalyst}^{-1}$ was obtained as compared with those of Novozym 435 (1.96 $mmol \times min^{-1} \times g_{biocatalyst}^{-1}$) or with previously reported CaL-B covalently immobilized onto magnetic nanoparticles coated with chitosan (MNP-CS-SC-CaL-B, 1.95 $mmol \times min^{-1} \times g_{biocatalyst}^{-1}$), with the mention that each biocatalyst contained the same amount of enzyme (1 mg of CaL-B), resulting in 5 mg PVA-CS-CaL-B, 10 mg Novozym 435 and 3.63 mg CaL-B immobilized

onto magnetic nanoparticles. The approximately 3 fold increased synthetic activity of the new developed biocatalyst is related to the used immobilization technique (enzyme entrapment in polymeric nanofibers); the enzyme molecules are dispersed into a nanofibrous system with a large surface area and as result the biocatalyst's molecules are much more flexible (as compared with those covalently bonded on MNP-CS-SC-CaL-B or those physically adsorbed in the case of Novozym 435, the last one being known for the problems related to the enzyme desorption [100]). Moreover, the polymeric matrix proved to be suitable for binding a larger lipase quantity, resulting in a higher immobilization capacity, which can be important for synthetic applications requiring high-loaded biocatalysts.

4.2.2.3. Lipase Mediated EKR Studies Through O-Transesterification

4.2.2.3.1. Determination of the Optimal Reaction Medium

Since the solvent plays a crucial role in the stability of both support and enzyme and can influence the enzyme's catalytic properties [132], we determined the optimal solvent, based on the nanofiber's support stability. Four nonpolar and polar aprotic solvents were tested: dichloromethane (DCM), tetrahydrofuran (THF), chloroform and *n*-hexane. The polar solvents influenced through stereoregularity the properties of PVA, which was already influenced by the dipole-dipole interactions or hydrogen bonds with other polar groups [133]. Based on this experimental facts, *n*-hexane was chosen as solvent, since after keeping under stirring at 50 °C and 1000 rpm for 24 hours, only a loss of 0.46% was recorded, as compared with chloroform (of 1.47%), THF (2.77%) or DCM (8.1 %), demonstrating the impressive biocatalyst stability in organic media.

4.2.2.3.2. Determination of the Substrate Domain and the Substrate: Enzyme Weight *Ratio* for Each Compound

In order to determine the substrate domain of the new biocatalyst, the EKR of nine substrates were performed in *n*-hexane (based on nanofibers stability) using 10 mg of substrate (*rac*-1**a–e**, *rac*-2**b–e**, **Figures 6A,B**), 2 equiv. of vinyl acetate, 5 mg of enzyme preparate (containing 1 mg of CaL-B) and 1 mL of *n*-hexane, at 50 °C and 1000 rpm. Samples were taken periodically and analyzed on HPLC.

In the phenothiazine-2-yl-1-ethanols series (**Figure 6A**), the maximum conversion was obtained for the *N*-ethyl- (*rac*-**1b**) and *N*-butyl (*rac*-**1d**) derivatives in 10–12 hours, while the *N*-propyl- (*rac*-**1c**) and the unsubstituted phenothiazine-2-yl-1-ethanol (*rac*-**1a**) were transformed much slower. For *rac*-**1e**, bearing a voluminous hexyl group at the nitrogen atom, the conversion remains low even after 12 hours (approx. 2.8%).

The *N*-ethyl and *N*-butyl derivatives react faster and the conversion of 50% was reached after 10, respectively 12 hours. Based on these results it was concluded that the new bioconjugate presents a good activity in the EKR of large substrates, but for molecules with an alkyl group longer then butyl the interaction of the substrate with the enzyme is weaker. In all EKR processes studied the immobilized enzyme presented high enantiomer selectivity (enantiomeric excesses close to 100% at 50% conversions).

In the phenothiazine-3-yl-1-ethanols series the obtained results (**Figure 6B**) were moderate to modest 6.7–40.23%, even after 48 hours, our data supporting the dependence of the conversion on the ethanol's position on the phenothiazine nucleus and the substrate-enzyme interaction strength.

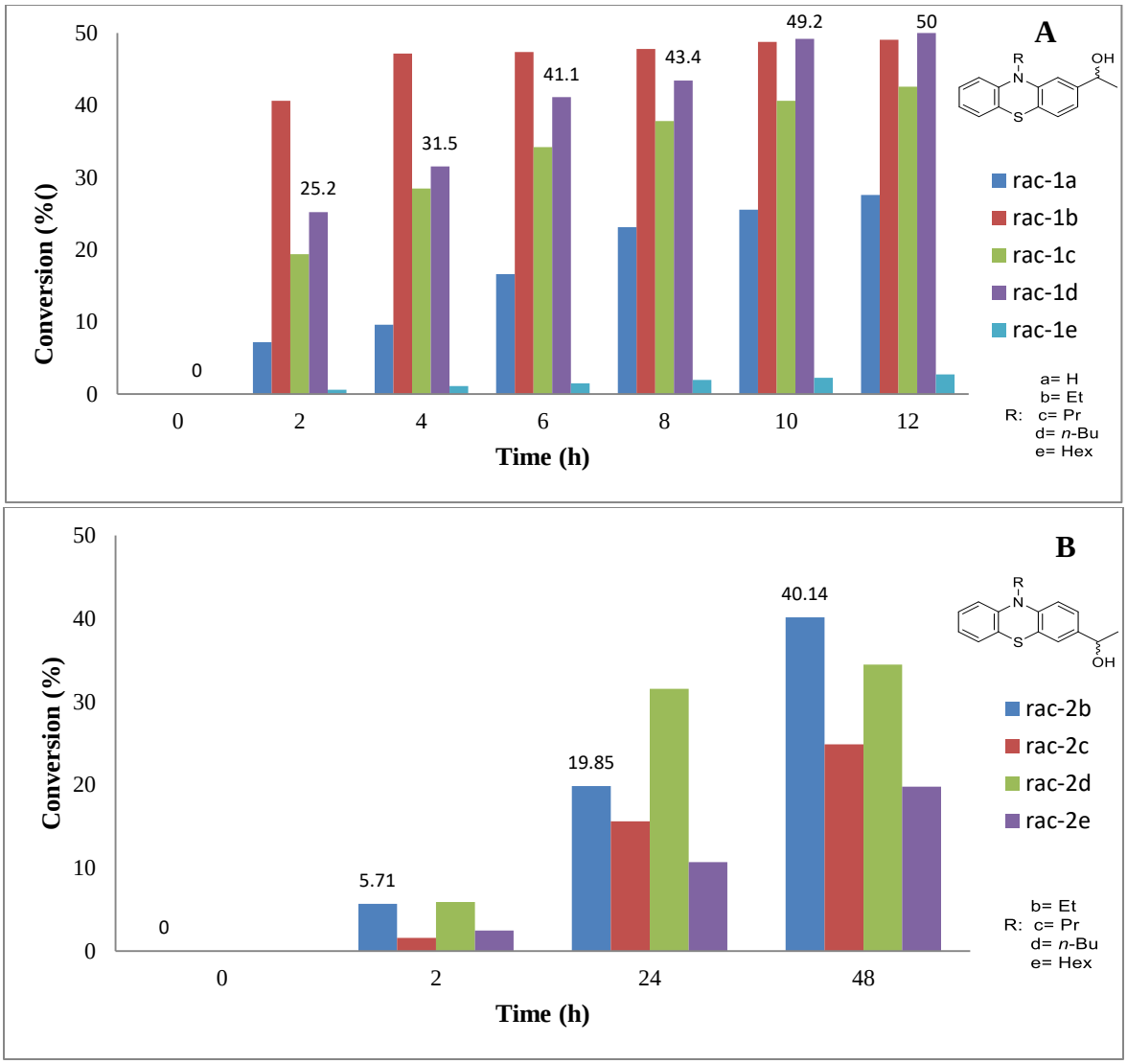


Figure 6. The enzymatic kinetic resolution (EKR) of the racemic *N*-alkyl-phenothiazine-2-yl-1-ethanols (**A**) and *N*-alkyl-phenothiazine-3-yl-1-ethanols (**B**).

Since in the EKR of *N*-substituted phenothiazine-3-yl-1-ethanols (*rac*-**2b–e**) only moderate to modest conversions were recorded with vinyl acetate as acylating agent even after 48 hours, vinyl butanoate was next tested (**Figure 7**). Some improvements (conversions higher with approximately 5–10%) were observed for all four derivatives. The maximum 50% conversion was reached after 48 hours only in the case of 1-(10-ethyl-10*H*-phenothiazine-3-yl)ethan-1-ol (*rac*-**2b**). It is important to note now the behaviour of the *N*-propyl- and *N*-hexyl-phenotiazine-3-yl-1-ethanols *rac*-**2c,e**, reacting with similar rates when the asymmetric carbon of the ethanol moiety is fixed at de carbon 3 of the phenothiazine structure.

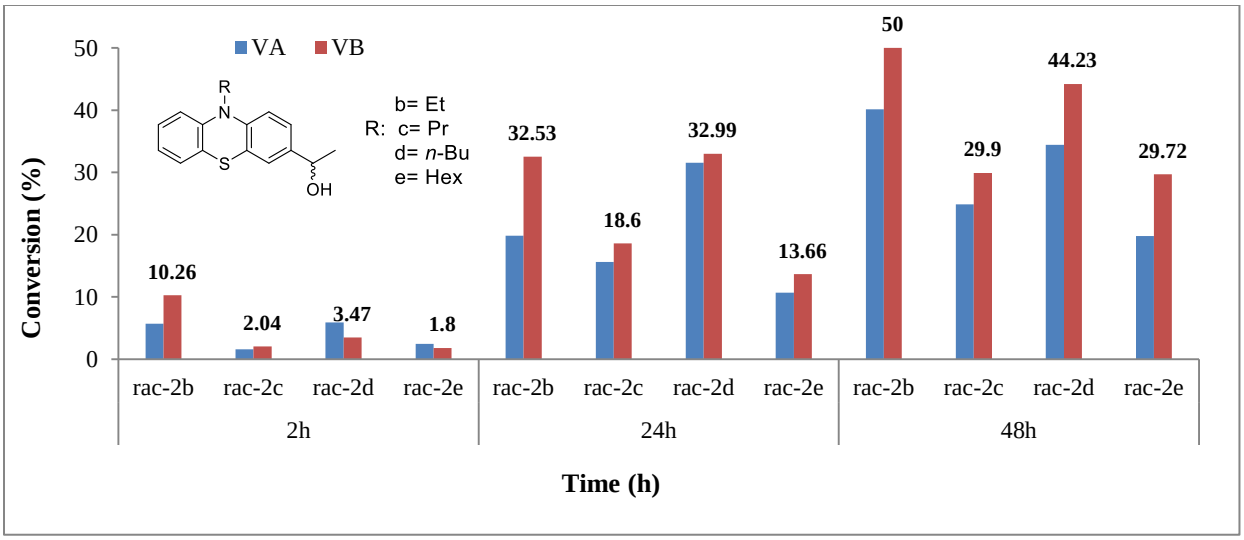


Figure 7. The EKR of *N*-alkyl-phenothiazine-3-yl-1-ethanols (*rac*-**2b–e**) with acylating agents (VA- vinyl acetate and VB- vinyl butanoate).

In order to improve the productivity, the influence of substrate-enzyme weight *ratio* over the reaction rate and conversion for each substrate was next studied (**Figures 8–12**). For the *N*-alkyl-phenothiazine-2-yl-1-ethanols eight different *ratios* between 10:1 and 100:1 were tested, while for *N*-alkyl-phenothiazine-3-yl-1-ethanols only three different *ratios* were investigated (10:1, 50:1 and 100:1) (**Figure 13**). It was already noticed that the conversion decreases as the length of the alkyl chain increases and with the increase of substrate concentration. At lower substrate concentrations, when *rac*-**1b** was used as substrate, good conversions were obtained. Samples were taken every two hours, until 12 hours (EKR of *rac*-**1d** was complete) and analyzed on HPLC.

The biocatalyst presented high selectivity and activity for all the substrates at the lowest substrate:ezyme *ratio* (10:1). For the unsubstituted *N*-alkyl-phenothiazine-2-yl-1-ethanol, the highest conversion was obtained when a *ratio* of 50:1 was used (*c*= 37.9 %) (**Figure 8**) with the enantiomeric excess of product >99.9.

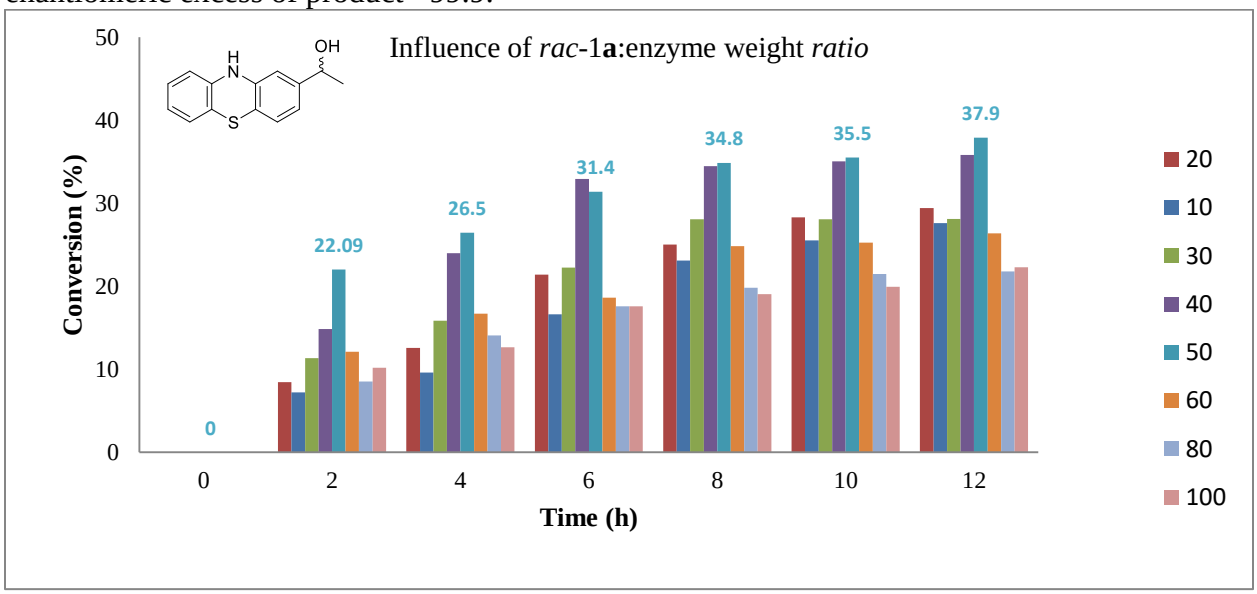


Figure 8. Influence of enzyme weight *ratio* on the conversion of *rac*-**1a**

In the case of *N*-ethyl-phenothiazine-2-yl-1-ethanol *rac*-**1b**, the highest conversion was obtained at a substrate:enzyme weight *ratio* of 30:1 (*c*= 49.8%). As we expected, the conversion increased as compared to the one at the lowest *ratio* used (10:1) (**Figure 9**).

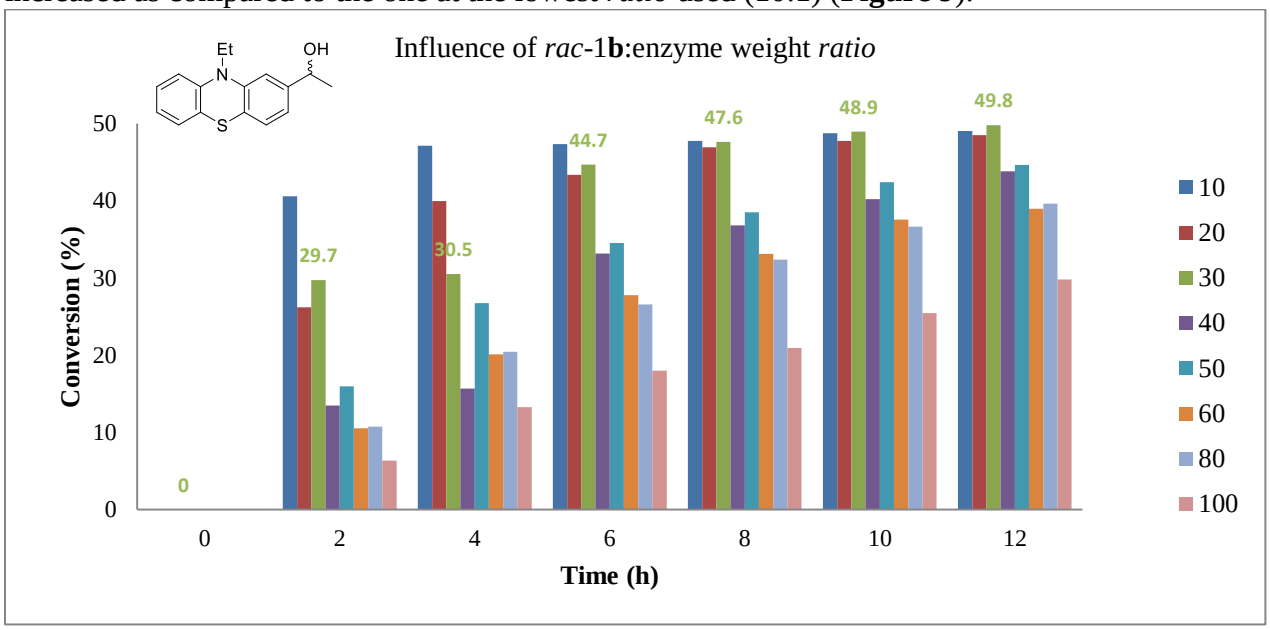


Figure 9. Influence of enzyme weight *ratio* on the conversion of *rac*-**1b**.

For the *N*-propyl-phenothiazine-2-yl-1-ethanol, the conversion for the *ratios* 10:1 and 40:1 is comparable, 42.57%, respectively 42.2 % (**Figure 10**).

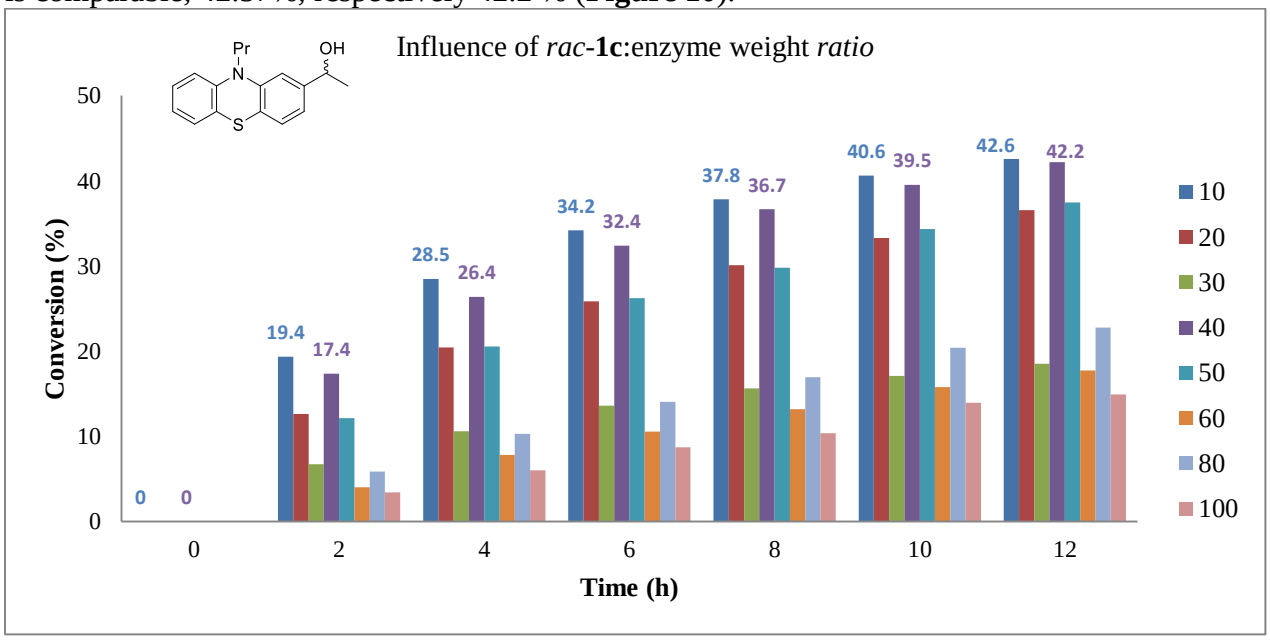


Figure 10. Influence of enzyme weight *ratio* on the conversion of *rac*-**1c**.

The exception in this study was the *N*-butyl derivative (*rac*-**1d**), when the approximate maximum conversion was reached, regardless of the substrate-enzyme weight *ratio* used (**Figure 11**).

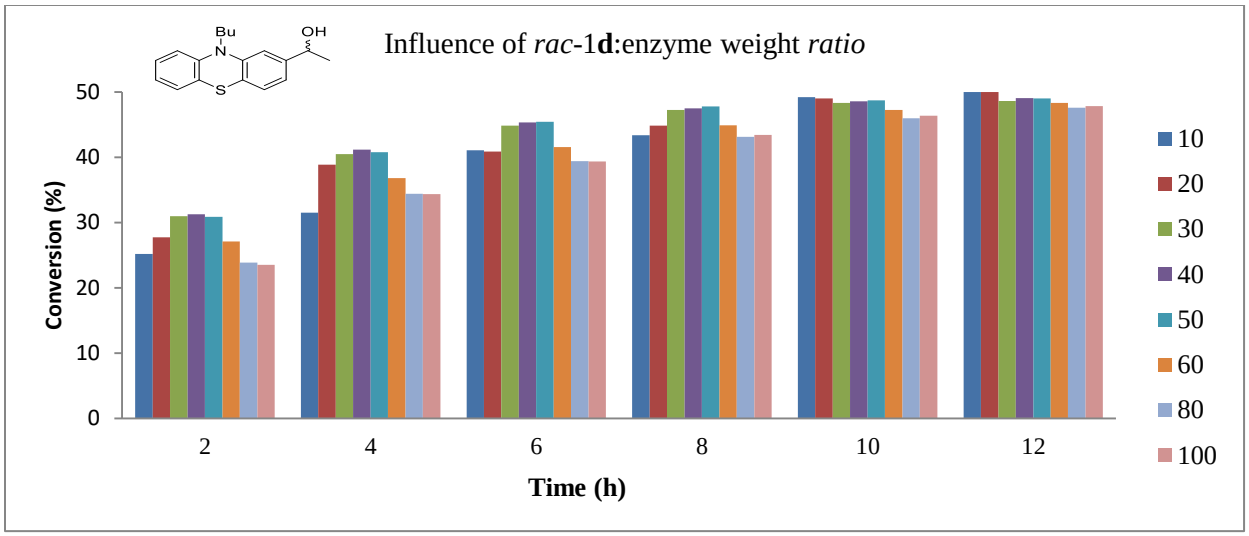


Figure 11. Influence of enzyme weight *ratio* on the conversion of *rac*-**1d**.

In the case of the *N*-hexyl-derivative, *rac*-**1e**, the substrate was transformed in insignificant quantities even at the lowest concentration (2.7%. conversion) (**Figure 12**).

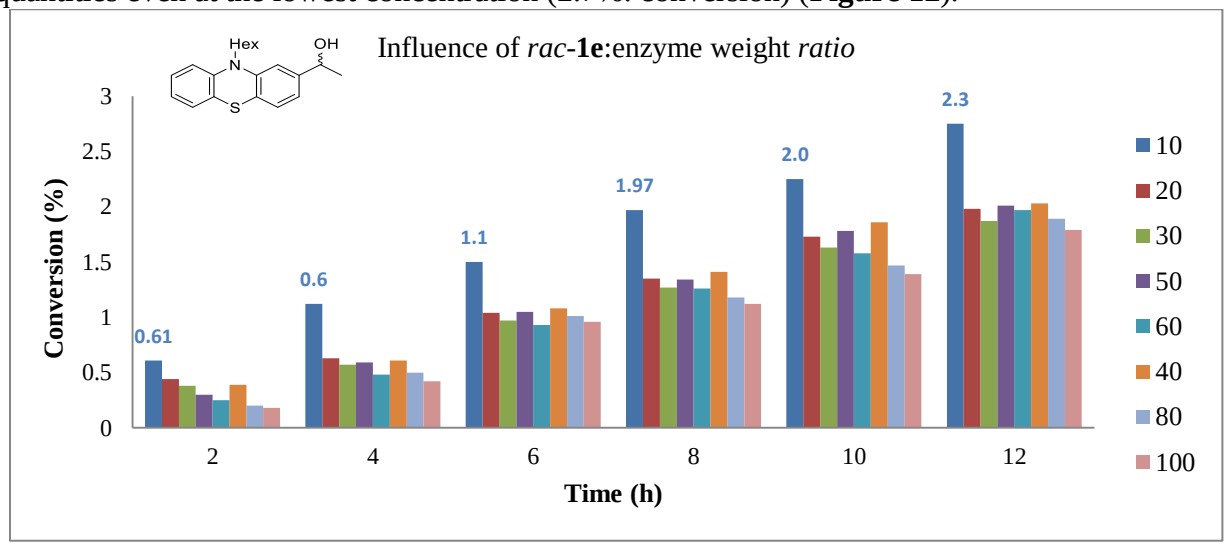


Figure 12. Influence of enzyme weight *ratio* on the conversion of *rac*-**1e**.

In the phenothiazine-2-yl-1-ethanols series, the maximum 50% conversion was obtained for *N*-ethyl- (*rac*-**1b**, substrate:enzyme *ratio*=10:1) and *c*>47.5% was obtained for *N*-butyl (*rac*-**1d**, regardless of the substrate:enzyme *ratio* used) derivatives in 10–12 hours, while the *N*-propyl- (*rac*-**1c**) and the unsubstituted phenothiazine-2-yl-1-ethanol (*rac*-**1a**) were transformed much slower. For *rac*-**1e**, bearing a voluminous hexyl group at the nitrogen atom, the conversion remains low even after 12 h (approx. 2.8%). In order to improve the activity of the biocatalyst, it was also tested in the EKR of *rac*-**1d** using as acylating agent vinyl butanoate, but even after 72 h the reaction did not reached the maximum conversion, only 47%.

Based on these results, it was concluded that the new bioconjugate presents a good activity in the EKR of large substrates, but for molecules with an alkyl group longer than butyl the interaction of the substrate with the enzyme is weaker.

Next, the new bioconjugate was tested in the EKR of a series of phenothiazine-3-yl-1-ethanols (*rac*-**2b-e**). When using vinyl acetate as acylating agent, the obtained conversions were moderate to modest after 48 h. Having these conversion values, vinyl butanoate was also tested

as acylating agent for the phenothiazine-3-yl-1-ethanols (*rac*-**2b-e**). There were observed improvements for all substrates, the obtained conversions were higher with 5-10% than the ones obtained when vinyl acetate was used as acylating agent. The maximum conversion was obtained in the case of *rac*-**2b** (ethyl derivative) after 48 h.

Vinyl acetate was used as acylating agent in all experiments due to the price of vinyl butanoate. As expected, at lower concentrations of substrate, good conversions have been obtained for *rac*-**2b** (approximatively 40%, after 48 hours). The conversion follows the same pattern as described above, decreasing with the increase of alkyl chain, with the exception of 1-(10-butyl-10*H*-phenothiazine-3-yl)-ethan-1-ol (*rac*-**2d**) (**Figure 13**).

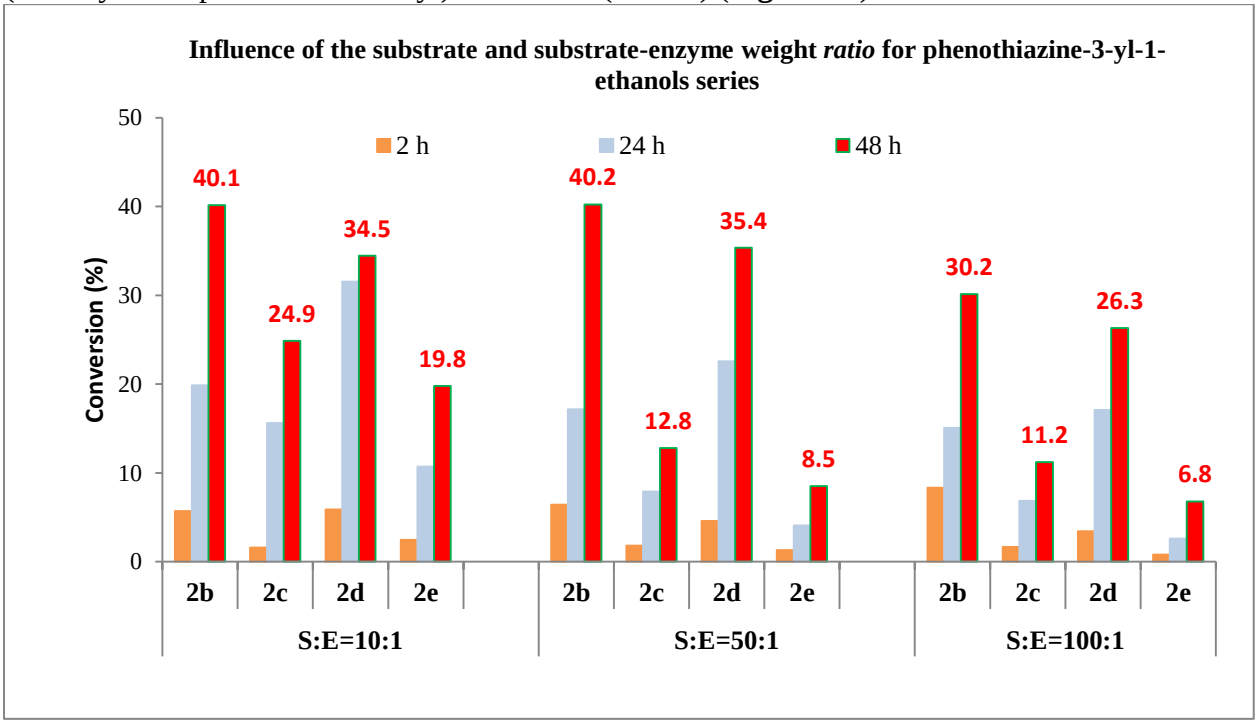


Figure 13. Influence of the substrate and substrate-enzyme weight *ratio* for phenothiazine-3-yl-1-ethanols series

4.2.2.4. Recyclability Experiments

The recyclability and stability of every new enzymatic preparate is an important requirement in any process, allowing to develop its sustainability. The reusability of this new bioconjugate of CaL-B was studied in the enantioselective acylation of *rac*-**1d** with vinyl acetate as acylating agent, and a substrate: enzyme weight *ratio* of 10:1. As presented in **Figure 14**, its activity remains high even after 10 cycles, decreasing with less than 3%. As consequence, the operational and long-term stability makes this biocatalyst promising in the continuous-flow system, allowing a higher productivity. Samples were taken after 30 min and analyzed on HPLC.

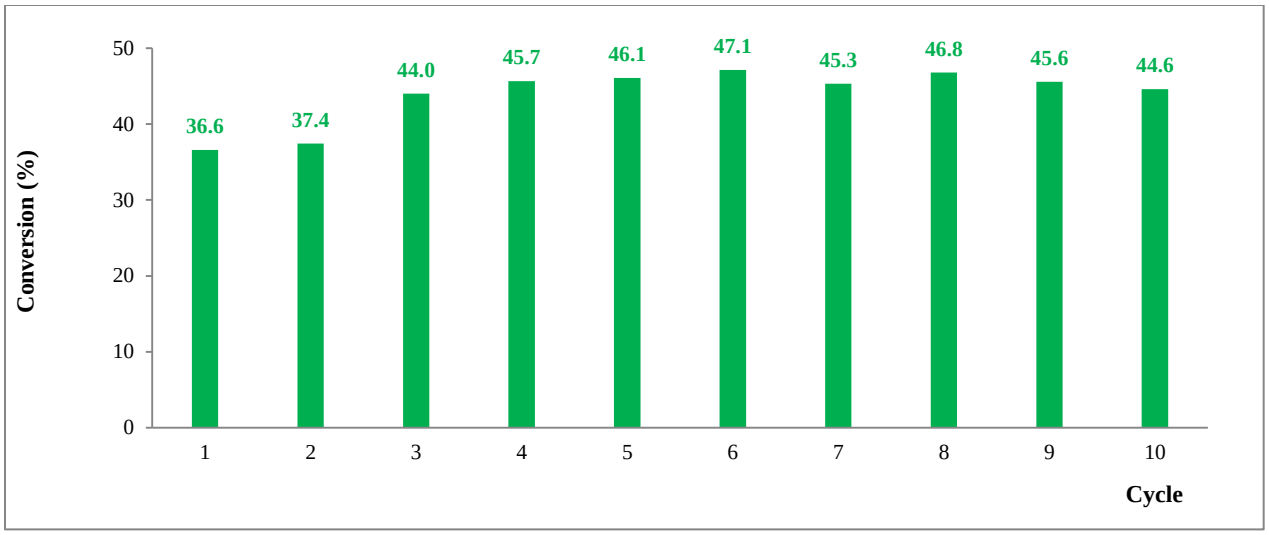


Figure 14. The reusability of PVA-CS-CaL-B in the EKR of *rac*-**1d** (30 min reaction time).

4.2.3. Conclusions

The immobilization of lipase B from *Candida antarctica* through entrapment into PVA-CS electrospun nanofibers represents a promising alternative for the preparation of high-surface-area, active and stable membrane containing enzyme, efficient in the EKR of bulky racemic phenothiazine-yl-ethanols, as compared with previously reported results (19–24 hours reaction time and 1:2 substrate:biocatalyst weight *ratio*) [99,100]. As consequence of the enzyme fine dispersion in the polymer matrix and large surface area of the resulted nanofibers, a significant increase in the lipase synthetic activity was noticed as compared with other preparates (lipase adsorbed on polyacrylamide beads- Novozyme 435 or covalently bonded on activated chitosan coated MNP). Moreover, this study demonstrates for the first time that PVA-CS lipase entrapment is suitable to form membrane biocatalysts with high activity, selectivity and excellent stability for synthetic application, obtaining high conversion values for bulky substrates containing phenothiazine skeleton with a substrate:enzyme weight *ratio* of at least 10:1 and relatively short reactions time (aproximately 12 hours). At the same time, the enzyme preserved more that 95% of its activity even after 10 reaction cycles, making it a promising candidate for continuous flow applications.

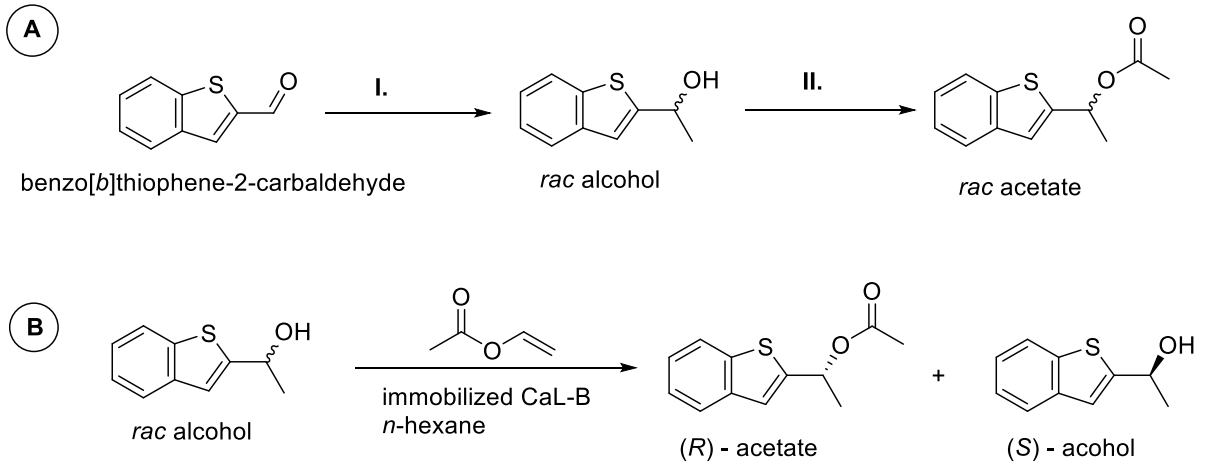
4.3. Nanocomposites of CaL-B Based on Biopolymeric Nanofibers of PLA/PVA

4.3.1. Introduction

4.3.2. Results and Discussion

4.3.2.1. Chemical Synthesis of racemic 1-Benzo[b]Thiophen-2-yl-Ethanol

The racemic 1-benzo[*b*]thiophen-2-yl-ethanol was synthesized using known methods [94-97]. Racemic 1-benzo[*b*]thiophen-2-yl-ethanol was obtained by Grignard reaction from the corresponding aldehyde (**Scheme 6A**). The obtained racemic ethanol was used as substrate in the *O*-transesterification reaction (**Scheme 6B**) and as starting material for the chemical synthesis of the corresponding acetate, in order to set-up the chromatographic separation method.



I. Et_2O, argon, MeMgI (2Eq.), o.n., r.t.
II. Cl-CO-CH_3, 1% DMAP-Pyridine, DCM

Scheme 6. A. Chemical synthesis of *rac* 1-benzo[*b*]thiophen-2-yl-ethanol and its acetate; **B.** Enantiomer selective transesterification of *rac* 1-benzo[*b*]thiophen-2-yl-ethanol mediated by immobilized CaL-B.

4.3.2.2. Cal-B Immobilization by Adsorption and Entrapment in PLA and PVA Nanofibers

The most used immobilization methods in industry are physical adsorption, inclusion, cross-linking and covalent bonding, the first two being preferred due to the price-cost efficiency *ratio*. [79]. Cross-linking involves both inclusion and covalent bonding using specific chemical agents, most often glutaraldehyde vapors. After binding by physical adsorption, enzyme molecules maintain their initial conformation and usually the resulting biocatalyst presents high activity.

The enzyme entrapment method protects the biocatalyst, allows the transport of low molecular weight compounds, can be used in a continuous regime, is easily separated from the reaction medium, and allows the controlled release of the product. However, this type of immobilization presents mass transfer limitations and low enzyme loading. Moreover, enzyme molecules can create a compact structure changing the nanofibers skeleton, making them impossible to use as catalyst in many cases [66].

4.3.2.3. The Nanofibers Morphology Characterization

The obtained nanofibers were analyzed by electronic transmission microscopy (TEM). It confirmed the nanodimensions of the fibers having a diameter between 126 and 439 nm (**Figure 15A**). In the images recorded before (**Figure 15B**) and after (**Figure 15C**) the CaL-B immobilization onto PLA nanofibers by adsorption, the presence of enzyme molecules at the surface of the nanofibers were confirmed and some structural changes were noticed as irregular conglomerates onto the nanofibers surface.

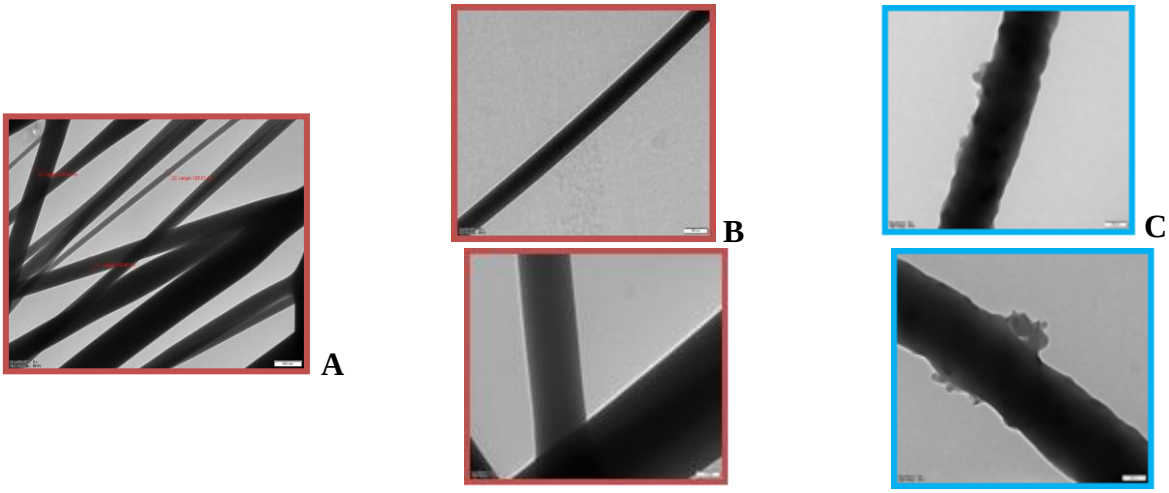


Figure 15. A. PLA fibers length in the range 126–439 nm, using a magnification of 6× (500 nm) and high voltage (80kV); **B.** PLA nanofibers homogenity and uniform distribution, with a magnification of 20× (100 nm) and high voltage (80kV); **C.** TEM images of the new biocatalyst PLA nanofibers with CaL-B adsorbed on their surface, with a magnification of 20× (100 nm) and high voltage (80kV).

The entrapment of CaL-B into PLA nanofibers (**Figure 16A**) using a procedure described in the literature [68], led in our experiments to a compact structure, determined through scanning electron microscopy (SEM), (**Figure 16B**), which probably do not allow the crossing of organic molecules, in order to interact with the entrapped enzyme.

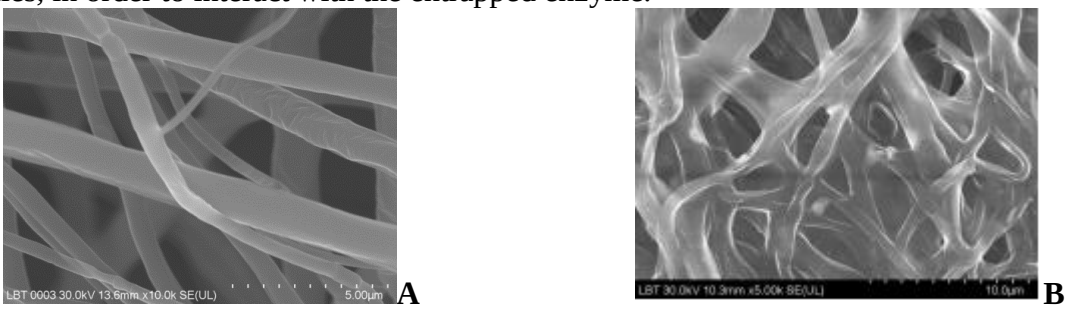


Figure 16. A. PLA nanofibers without enzyme; **B.** PLA nanofibers with entrapped CaL-B molecules.

Six enzyme preparates were obtained: four based on PVA (8 and 10% *w*/*v*) nanofibers, one was through enzyme entrapment and three through adsorption and two based on PLA (8% *w*/*v*) nanofibers:one through adsorption and one through entrapment. All six enzymatic biocatalysts were tested in the EKR of *rac* -benzo[*b*]thiophen-2-yl-ethanol.

4.2.3.4. EKR of Racemic 1-Benzo[b]Thiophen-2-yl-Ethanol in Discontinuous System

4.2.3.4.1. PVA Nanofibers Based Biocatalysts

The activity and selectivity of the four PVA nanofibers-based biocatalysts were tested in the *O*-acylation of racemic 1-benzo[*b*]thiophen-2-yl-ethanol by transesterification with vinyl acetate. In order to improve the productivity of the process, two different substrate:enzyme weight *ratios* (8:1 and 10:1) were used in *n*-hexane as solvent, at 30 °C and 4 equiv. of vinyl acetate (**Figure 17**). Samples were taken every two hours, until the reaction reached the maximum conversion (c=50%) with high enantiomeric excesses (ee_S and ee_P >99.9%).

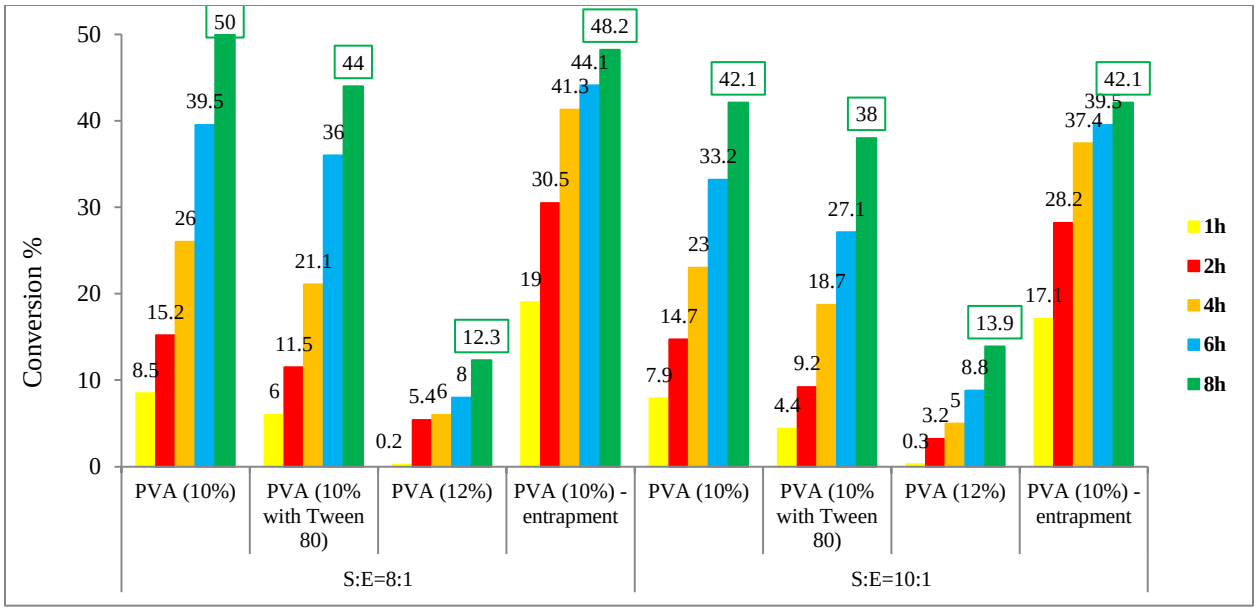


Figure 17. The influence of substrate:enzyme weight *ratio* on the transesterification of *rac* 1-benzo[*b*]-thiophen-2-yl-ethanol with vinyl acetate (4 equiv.) in *n*-hexane, mediated by PVA based biocatalysts.

According to the experimental data, the best preparate was the one based on enzyme immobilized through adsorption on PVA nanofibers obtained from a 10% polymer solution. In the case of adsorbed CaL-B onto nanofibers prepared from PVA 12% solution, the conversions were much lower. This outcome might be explained by the elevated polymer concentration and a limited mass transfer, making the substrate access to the enzyme's catalytic site problematic, or by a higher enzyme loading, leading to conglomerates of enzyme's molecules, decreasing its activity.

In order to find the optimal parameters, the influence of substrate:vinyl acetate *ratio* was also studied in the EKR mediated by the best PVA preparate. The reactions were monitored by taking samples every two hours until the maximum conversion was reached. As already determined, the best biocatalyst reached the 50% conversion after eight hours when 4 equiv. of vinyl acetate were used. With 2 equiv. the reaction reached only a 36.8% conversion after eight hours.

4.2.3.4.2. PLA Nanofibers Based Biocatalysts

The preparates based on PLA nanofibers were also tested in the *O*-tranesterification of *rac* 1-benzo[*b*]-thiophen-2-yl-ethanol with vinyl acetate as acylating agent and the previous determined substrate:enzyme weight *ratio* of 8:1. The samples were taken periodically, until the reaction reached the maximum conversion (50%) (**Figure 18**), resulting in products high enantiomeric excesses (ee_S and ee_P >99.9%).

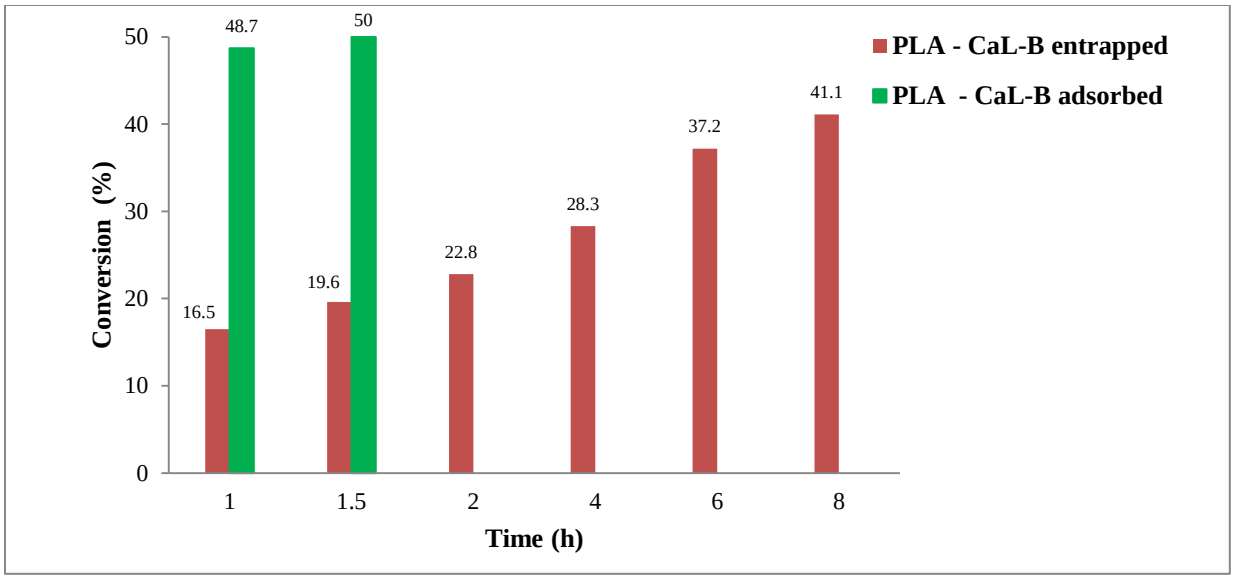


Figure 18. The influence of the immobilization method on the O-transesterification of *rac* 1-benzo[b]thiophen-2-yl-ethanol with vinyl acetate (2 equiv.) in *n*-hexane, mediated by PLA based biocatalysts.

Based on experimental data, the optimal PLA nanofibers preparate was obtained by lipase adsorption onto the polymeric nanofibers. The maximum conversion was reached in a relatively short period of time (1.5 hours), as compared to the preparate obtained by enzyme entrapment into the nanofibers, when 41% conversion was reached in 8 hours. A possible explanation might be the enzymatic conglomerates observed in SEM images (see **Figure 16B**), leading to a decrease of enzyme's mobility that has been trapped into the polymeric network, leading also into a decrease of enzyme's activity. Besides, the substrate molecules need to traverse the polymeric hydrophobic membrane to access the catalytic site of enzyme, and during this crossover the polymer molecules can interact with the substrate.

Next, in the optimal conditions previously determined: *n*-hexane as solvent, vinyl acetate as acylating agent, substrate:enzyme *ratio (w/w)* of 8:1 and 30 °C, the influence of substrate:vinyl acetate *ratio* was studied using 2 and 4 equiv. of acylating agent. Thus, when the PLA nanofibers biocatalyst was used, the optimal *ratio* was 2 equiv. of vinyl acetate, the maximum conversion was reached in 1.5 hours, as compared to 6 hours when 4 equiv. were used. Since the enzymatic preparate having PLA nanofibers as skeleton was the most active of the six biocatalysts, reaching the maximum conversion in the EKR of *rac* 1-benzo[*b*]thiophen-2-yl-ethanol in the shortest period of time requiring only 2 equiv. of vinyl acetate, this biocatalyst was chosen for the recycling experiments.

4.2.3.4.3. Recycling Experiments

The enantioselective acylation of racemic 1-benzo[*b*]thiophen-2-yl-ethanol with vinyl acetate was further used for testing the biocatalyst reusability (reaction time 1 hour). The reaction was performed 10 consecutive times, separated immobilized enzyme being washed with *n*-hexane (3×0.5 mL) after each cycle and immediately used in the next one. As presented in **Figure 19**, its activity remains high even after 10 cycles, decreasing by less than 12%. As conclusion, the operational and long-term stability makes this active biocatalyst promising in the continuous-flow system, allowing a higher productivity.

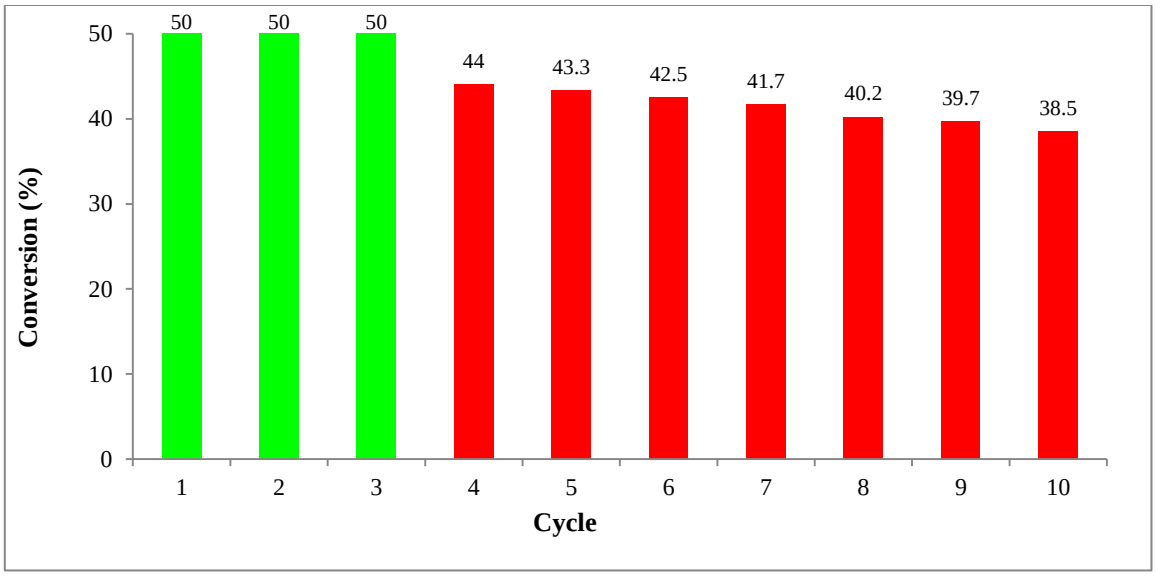
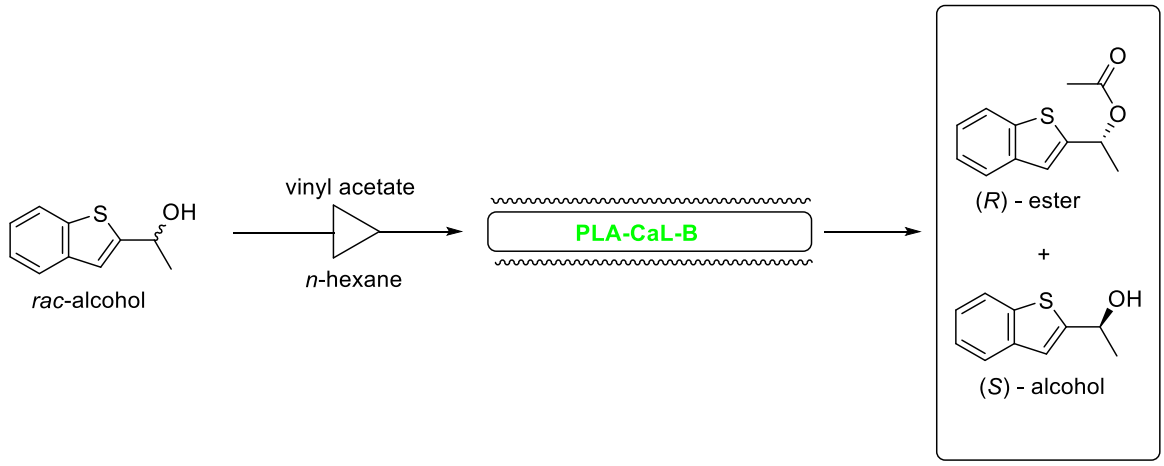


Figure 19. The reusability of PLA-CaL-B prepared by adsorption in the EKR of *rac* 1-benzo[b]thiophen-2-yl-ethanol (after 1 h reaction time) with vinyl acetate (2 equiv.) in *n*-hexane at 30 °C.

4.2.3.5. Continuous-Flow PLA-CaL-B-mediated O-acylation of rac 1-Benzo[b]Thiophen-2-yl-Ethanol with Vinyl Acetate

With the aim to increase the efficiency of the biocatalytic process, the best CaL-B bioconjugate based on polymeric nanofibers (PLA-CaL-B) was tested in continuous-flow experiments, using packed-bed reactor (30×4.6 mm) in the *O*-acylation of *rac* 1-benzo[*b*]thiophen-2-yl-ethanol with vinyl acetate (2 equiv.) in *n*-hexane, using a substrate concentration of 8 mg/mL (**Scheme 7**). Two of the most important parameters that influence the productivity in continuous-flow system, temperature, and flow rate, were investigated.



Scheme 7. Lipase-mediated *O*-acylation of racemic 1-benzo[*b*]thiophen-2-yl-ethanol in continuous-flow packed-bed reactor.

The process was investigated at different flow-rates in the range of 0.2–0.5 mL/min and different temperatures in the range of 30–50 °C, using a substrate concentration of 8 mg/mL and 2 equiv. of vinyl acetate. In all four cases, the conversion increased with the temperature, the maximum conversion being reached at 50 °C at flow-rates of 0.2 and 0.3 mL/min. As observed in **Figure 20**, the influence of temperature is relatively insignificant at constant flow of 0.2 mL/min. At 0.3 mL/min flow-rate, there is a slight increase from 46% to the maximum conversion of 50%. In the cases of higher flow-rates, 0.4 and 0.5 respectively, a slight increase

was also noticed, from 38% to 43% at 0.4 mL/min and 35% to 41% for 0.5 mL/min flow-rate. In conclusion, when using lower flow-rates, an increased temperature is not justified, it will only increase the overall cost of the process. As expected, the conversion increased with the temperature, but decreased with the increasing of the flow-rate.

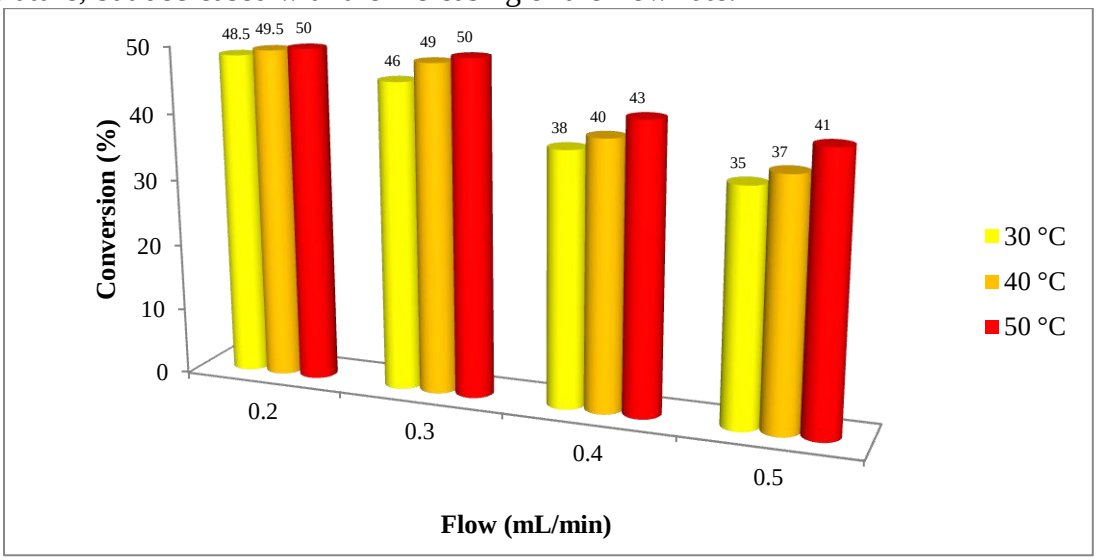


Figure 20. Influence of temperature at constant flow-rates on the continuous-flow EKR of *rac* 1-benzo[b]thiophen-2-yl-ethanol with vinyl acetate (2 equiv.) in *n*-hexane.

4.3.3. Conclusions

This study demonstrated that the CaL-B-nanofiber composites prepared through adsorption allow the development of stable and active biocatalyst for the enzymatic kinetic resolution of heteroarylethanols having a bulky skeleton. The PLA nanofibers-based biocatalyst has proven to be the most efficient, reaching the maximum conversion (50%) in the shortest period of time (1.5 hours). The operational stability of this preparate was studied in reusability experiments, conserving 77% from its initial activity, making it a valuable candidate for continuous-flow investigations. Two parameters that can influence the reaction rate were investigated: temperature (30–50 °C) and flow-rate (0.2–0.5 mL/min), maintaining constant the substrate concentration (8 mg/mL). The obtained experimental data confirmed the expected trend regarding the reaction rate: the conversion increased with the temperature, reaching its maximum at 50 °C, when lower flow-rates were used (0.2–0.3 mL/min). At the same time, the influence of flow-rate over the reaction rate was also confirmed, the last one decreasing by 18–28% with the increase of the flow-rate.

5. Experimental Part

6. Conclusions

The studies performed in the current thesis present the optimization of lipase-mediated kinetic resolution processes using different heteroaryl secondary alcohols regarding the stability, activity, and productivity of the enzymatic preparates used as catalysts.

Six enzymatic preparates of CaL-B were obtained through covalent immobilization onto chitosan-coated magnetic nanoparticles (MNP-CS) using different linkers and spacer-arms. The most active preparate was the one obtained with sebacoyl chloride as spacer-arm, presenting a synthetic activity similar to the commercially available form of CaL-B (Novozym 435). After setting the optimal parameters (45 °C, *n*-hexane as solvent, vinyl acetate as acylating agent,

substrate:enzyme optimal weight *ratio* (5:1) and the optimal substrate:vinyl acetate *ratio* (1:2), the preparate was tested on a series of heretoaromatic secondary alcohols, obtaining high values of conversion (*c*>49%) and maximum product enantiomeric excess (ee_P>99.9%) in relatively short reaction time (3–16 h). At the same time, it was demonstrated that the nature of the acylating agent influences the activity of the immobilized enzyme. Based on the promising conversions in relatively short reactions time, the reusability of this preparate was also tested in the transesterification reactions of *rac*-**2e** (1-benzo[*b*]thiophen-2-yl-ethanol) and *rac*-**2i** (*N*-ethyl-phenothiazinyl-3-ethanol). The newly developed biocatalyst presented very good stability and activity (after an overall reaction time of 10 h and 100 h respectively), its activity decreasing with less than 5% after 10 cycles, making it a promising candidate for EKR processes in continuous system.

An enzymatic preparate obtained through entrapment by electrospinning was synthesized, using chitosan-PVA nanofibers as immobilization support. After finding the optimal *ratio* between the two polymers, the obtained fibers were analyzed and characterised and the synthetic activity of the bioconjugate was determined, obtaining a value of 6.1 $mmol \times min^{-1} \times g_{biocatalyst}^{-1}$ (three times higher than Novozym 435 or MNP-CS-SC-CaL-B). The stability of the preparate in organic solvents was studied and *n*-hexane was found to be optimal. This new bioconjugate of CaL-B was studied in the EKR of two series of phenothiazinyl chiral secondary ethanols: *N*-alkyl-phenothiazin-2-yl-1-ethanols and *N*-alkyl-phenothiazin-3-yl-1-ethanols. All substrates were synthesized according to the literature, alongside the new *N*-hexyl derivatives for each series, that have not been previously reported. The PVA-CS-CaL-B bioconjugate presented a high activity in the *N*-alkyl-phenothiazin-2-yl-ethanols serie, obtaining good conversions for ethyl-, propyl- and butyl-derivatives in a relatively short time (10-12 h). As for the *N*-alkyl-phenothiazin-3-yl-ethanols series it has been observed that vinyl acetate was not the optimal acylating agent, the conversions were lower than 40%, even a higher reaction time (48 h), vinyl butanoate was further tested and increased conversions were obtained (5–10% in the case of all derivatives after 48 h).

The recyclability of this preparate was tested in the EKR of *rac*-**1d** (*N*-butyl-phenothiazin-2-yl-1-ethanol). The biocatalyst maintained its activity even after 10 cycles (30 min of reaction time for each cycle), with an activity loss less than 3%, making it a promising candidate for the continuous flow studies.

CaL-B was also immobilized through entrapment and adsorption methods into/onto PLA and PVA nanofibers, obtained through electrospinning technique.

The obtained biocatalysts were tested in the EKR of *rac*-1-benzo[*b*]-thiophen-2-yl-ethanol with vinyl acetate as acylating agent and *n*-hexane as solvent at 30 °C. Based on the obtained results, the best performing biocatalyst based on PLA nanofibers was further tested in recyclability experiments using 2 equiv. of vinyl acetate and a substrate:enzyme weight *ratio* of 8:1. Based on the promising results obtained in the batch system, this preparate was further used in the continuous flow process. Two process parameters were studied: temperature (30–50 °C) and flow rate (0.2–0.5 mL/min). Based on the obtained results, the conversion increases with the temperature, reaching its maximum at 50 °C, when lower flow-rates were used (0.2–0.3 mL/min). The influence of flow-rate over reaction rate was also confirmed, the last one decreasing by 18–28% with the increase of the flow-rate (0.4–0.5 mL/min).

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