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PSEUDOMONAS FLUORESCENS LIPASE AS BIOCATALYST IN THE ENZYMATIC KINETIC RESOLUTION OF CHIRAL PHENOTHIAZIN ETHANOLS

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ABSTRACT. The synthesis of both enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol by enzymatic kinetic resolution of the racemic alcohol or his corresponding acetate was performed using lipase from *Pseudomonas fluorescens* (PFL) as biocatalyst. PFL was next immobilized by sol-gel encapsulation. The stability, activity and reusability of the obtained enzyme preparations were also determined.

Keywords: *enzymatic kinetic resolution, lipase from Pseudomonas fluorescens, sol-gel encapsulation, chiral phenothiazine derivatives*

INTRODUCTION

Phenothiazines and related compounds have shown diverse biological activities.[1] They bind to physiological targets or receptors, producing many possible mechanisms of actions. Due to the chiral nature of pharmacological receptors, their interaction with the individual enantiomer of the same compound could be significantly different and the synthesis of each of them is an important target in the modern medicinal chemistry.

Enantiopure secondary alcohols are key intermediates in the synthesis of a large number of pharmaceutical products. In particular, non-racemic alcohols and their derived amines containing heterocyclic fragments are known for their biological activity.[2] The lipase mediated kinetic resolution of some heteroaryl-1-ethanols have been already successfully performed.[3,5,8]

Kinetic resolution (KR) of racemates is by far the most common transformation catalyzed by lipases in which the enzyme discriminate between the two enantiomers of racemic mixture, so that one enantiomer is transformed to the product faster than the other one. In the ideal case, at 50% conversion, a final mixture of 50% reactant and 50% product, both in optically pure form,

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can be obtained. For example, starting from the racemic ethyl 3-hydroxy-3-(10-alkyl-10*H*-phenothiazin-3-yl)propanoates as substrates, a multienzymatic procedure was developed for the efficient synthesis of the corresponding highly enantiomerically enriched (*R*)- and (*S*)-3-heteroaryl-3-hydroxypropanoic acids.[4] Lipase A and B from *Candida antarctica* were found as efficient catalyst not only for KR and dynamic kinetic resolution (DKR)[5] of the enantiomer selective resolution of various heteroaryl cyanohydrins, including *N*-alkylated phenothiazin-3-yl-cyanohydrins,[6] but also for phenothiazine based 3-hydroxy-propanoic acids,[7] methanols and ethanols.[8,10]

The synthesis of 2-substituted *N*-acylphenothiazines derivatives and their antibacterial and antifungal activities was reported.[9] The chiral heteroaryl-ethanol obtained by carbonyl reduction represent an interesting compound in the field of medicinal chemistry, especially if the individual enantiomers are used as optically active intermediates.

To obtain highly enantiomerically enriched or optically pure heterocyclic ethanols,[10] the synthesis of both enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol by enzymatic kinetic resolution of the racemic alcohol or his corresponding acetate was our object of enquiry. We note that the enzymatic kinetic resolution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol and their acetate with the lipase A and B from *Candida antarctica* was reported by our group recently.[11] However, to apply these results in industrial processes, the use of an efficient, cheap and stable catalyst is necessarily.

Thus, the most used method for improvement of biocatalysts performance is their immobilization. Many effective immobilization methods have been developed, including binding to a carrier, cross-linking and encapsulation in an organic or inorganic polymeric matrix.

Lipases from *Pseudomonas fluorescens* (PFL) belongs to commonly used commercial lipases. Their immobilization using adsorption,[12] cross-linking,[13] sol-gel encapsulation,[14] combination of the methods[15] or other techniques[16] has been under extensive studies.

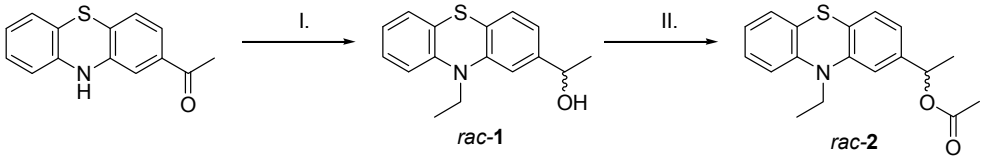
In the present work, PFL encapsulated in sol-gels was prepared and studied for the enzymatic kinetic resolution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol and their acetate, for obtaining both enantiomers of the heterocyclic ethanol, a valuable intermediate for different chiral active compounds. The stability, activity and reusability of enzyme preparations were determined.

RESULTS AND DISCUSSION

1. Chemical synthesis

The 1-(*N*-ethyl-phenothiazin-2-yl)ethanol *rac*-**1** was chosen as model compound for this study, due to its availability by simple reaction starting from the commercial ketone. The racemic substrate was prepared using a modified previously described method,[17] as shown in Scheme 1. By alkylation of

1-(10*H*-phenothiazin-2-yl)ethanone with ethyl iodide in DMF in presence of sodium hydride at room temperature, followed by chemical reduction of the carbonyl group with sodium borohydride in methanol at reflux or by reduction followed by *N*-alkylation in the same conditions, the racemic ethanol can be obtained in high yield. Chemical acetylation of the alcohol with acetyl chloride in presence of triethylamine and catalytic amount of DMAP gave the corresponding racemic acetate *rac*-**2** (Scheme 1).



I. 1. NaH, EtI, DMF; 2. $NaBH_4$, NaOH/methanol or 1. $NaBH_4$/methanol; 2. NaH, EtI, DMF;
II. AcCl, NEt_3, DMPA, CH_2Cl_2

Scheme 1. *Chemical synthesis of the racemic substrates*

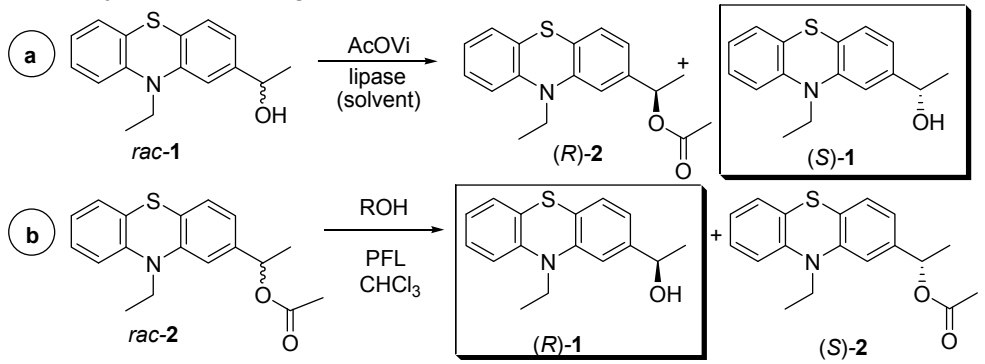
To investigate the stereoselectivity of the subsequent enzymatic reactions, the chromatographic separation of the enantiomers of the racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)-1-ethanol (*rac*-**1**) and his acetate (*rac*-**2**), was first established. The base-line separation of enantiomers was performed by HPLC using a Chiralpack IB column.

2. Biotransformations with lipases

2.1. Analytical scale biotransformations

2.1.1. Analytical scale enzymatic acetylation of rac-1

First, the analytical scale enantiomer selective lipase catalyzed acetylation of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol (*rac*-**1**) was studied in neat vinyl acetate using commercial available lipases (Scheme 2a).



Scheme 2. *Lipase mediated enzymatic kinetic resolution processes*

Most of them exhibited good enantiomer selectivities and activities. The best results were obtained with immobilized lipases B from *Candida antarctica* (CaL-B), commercialized as Novozym 435 and with lipase from *Pseudomonas fluorescens* (PFL) (Table 1, entry 1-2, *E* >>200, at 50% conversion). Lipase A from *Candida antarctica* (CaL-A) was also selective and active (*E* = 198 at 48% conversion), while lipases from *Candida rugosa* (CrL), *Candida cilindracea* (CcL) and from *Thermomyces lanuginosa* (TLIM) were selective but less active (only 15-34 % conversion after 24 h). Other commercial lipases such as that from *Mucor javanicus* and pancreas pig lipase were only moderate active and selective (*E*= 30-70, at 10-25% conversion, data not presented in Table 1).

Table 1. Lipase catalyzed acetylations of *rac*-**1** in neat vinyl acetate in the presence of some lipases after 24 h

Entry	Lipase	$ee_{(R)-2}$ (%)	$ee_{(s)-1}$ (%)	c (%)	*E*
1	CaL-B	>99	>99	50	>>200
2	PFL	>99	>99	50	>>200
3	CaL-A	89	97	48	198
5	CcL	>99	18	15	>200
6	CrL	>99	31	25	>200
7	TLIM	>99	52	34	>200

It is known that the nature of the solvent could significantly influence the selectivity of the enantiomer selective acylation. Thus the acylation of *rac*-**1** with vinyl acetate in presence of PFL as selected catalyst was tested in several organic solvents (Table 2). Chloroform proved to be the most appropriate solvent for the acetylation (*E*> 200, *c* = 50% after 24 hours, Table 2, entry 1).

Table 2. Lipase catalyzed acetylations of *rac*-**1** (5 mg) with vinyl acetate (10 µL) and PFL (5 mg) in different solvents (200 µL) after 24 h

Entry	Solvent	$ee_{(R)-2}$ (%)	$ee_{(s)-1}$ (%)	c (%)	*E*
1	Chloroform	>99	96.3	50	>200
2	*n*-Hexane	>99	77.1	56	44
3	Toluene	>99	72.7	58	36
5	Tetrahydrofuran	>99	87.1	53	85
6	Dichlorometane	>99	45.9	68	14
7	Acetonitrile	95.2	94	50	122

2.1.2. Analytical scale enzymatic alcoholysis of rac-2

It is known that lipases usually retain their enantiomer preference in hydrolysis or alcoholysis.[18] Consequently, such reactions should result in the opposite enantiomeric forms of the reaction counterparts (Scheme 2b). The degree tested using four alcohols (methanol, ethanol, propan-1-ol and butan-1-ol) of enantioselectivity in alcoholysis of the racemic acetate *rac*-**2** was

with PFL as catalyst. The highest selectivity was achieved for ethanolysis (Table 3, entry 2), yielding the highly enantiomerically enriched opposite forms: (*R*)- **1** and (*S*)-**2**.

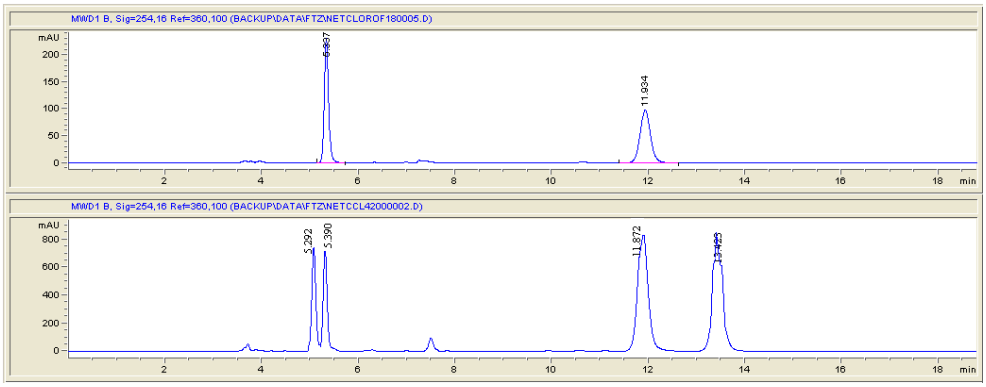


Figure 1. The chromatogram of the mixture obtained after the EKR of ***rac*-1** with PFL in chloroform (up) in comparison with the chromatogram of a mixture of ***rac*-1** and ***rac*-2**.

Table 3. Lipase catalyzed alcoholysis of *rac*-**2** with PFL in chloroform after 24 h

Entry	Alcohol	$ee_{(S)\text{-}2}$ (%)	$ee_{(R)\text{-}1}$ (%)	c (%)	*E*
1	Methanol	95	79	45	94
2	Ethanol	99	99	50	>>200
3	*n*-Propanol	91	85	48	58
4	*n*-Butanol	85	75	47	28

2.2. The use of immobilized biocatalysts

2.2.1. Preparation of immobilized PFL catalysts

Using a combination of methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS), a hydrophobic matrix, suitable to enhance the activity of the entrapped lipase, was prepared by using an optimized[19] method of Reetz.[20] This method consists of the base-catalyzed *in situ* polymerization of the silanes. The previously reported optimal conditions (TMOS/MTMS=1/5, molar *ratio*; water/gel= 9/1 molar *ratio* and fluoride ion-catalyzed hydrolysis of silane precursors[19]) were used. The influence of celite and sucrose as additives on the performance and stability of the biocatalyst in the studied EKR processes was also investigated.

2.2.2. Acylation of rac-1 and ethanolysis of rac-2 with sol-gel immobilized PFL

PFL sol-gel catalysts were tested for the acylation of *rac*-**1** (0.1 M) with vinyl acetate and ethanolysis of *rac*-**2** (0.1 M) in chloroform. The results were excellent with respect to the applicability of our modified catalyst,

obtained in the presence of both celite and sucrose, in the studied enzymatic resolution processes (*E*>> 200 with all enzyme preparations).

2.2.3. Reuse of immobilized enzymes

One of the most important features of immobilized enzymes in synthetic applications is their reusability. For testing the recycling capacity, the acylation of *rac*-**1** (0.1 M) with vinyl acetate (0.2 M) in chloroform was repeated with the same enzyme preparation up to 4 times. Every reaction was allowed to proceed to approx. 50% conversion before the catalyst was subjected to the next cycle. Between the cycles, the catalyst was washed with dry chloroform and reused without drying. The results (as enantiomeric excesses of the robtained compounds and enantiomeric *ratio E* of each reaction) are presented in Table 4.

Table 4. Reuse of the sol-gel PFL enzyme preparations (1.5 mg protein/mL) for the *O*-acylation of *rac*-**1** (0.1 M) with vinyl acetate (0.2 M) in chloroform (1 mL) at room temperature

	PFL cycle				
	powder	1	2	3	4
Time (h)	24	20	20	22	22
c (%)	50	49	48	50	49
$ee_{(S)\text{-}\mathbf{1}}$	96	97	97	96	94
$ee_{(R)\text{-}\mathbf{2}}$	99	98	96	95	93
E	>>200	>200	>200	183	115

The enzymatic activity decreased slowly (5%) after 3 cycles and significantly (10%) after 4 times, respectively. The reuse had no significant effect on enzymatic enantioselectivity, allowing the preparation of (*S*)-**1** and (*R*)-**2** from *rac*-**1** in highly enantiomerically enriched forms (ee>95%) still on the 4th reuse cycle.

3. Preparative scale synthesis of both (R)- and (S)-1

The resulted optimum issued from the analytical scale studies was successfully applied at preparative scale synthesis of both highly enantiomerically enriched stereoisomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol and his acetate. To demonstrate the usefulness of these enzymatic procedures, 500 or 600 mg of the racemic substrate were used for each EKR as acetylation and alcoholysis. Whereas the enzymatic acetylation afforded the acetate (*R*)-**2** and the alcohol (*S*)-**1a** in high enantiomeric excess, alcoholysis of the racemic acetate *rac*-**2**, yielded the opposite enantiomeric forms (*R*)-**1** and (*S*)-**2**.

The absolute configuration of these enantiopure products was assigned using the earlier published data for optically pure enantiomers of **1**.[11,17a]

EXPERIMENTAL PART

1. Analytical methods

High Performance Liquid Chromatography (HPLC) analyses were conducted with a HP 1200 instrument using Chiralpak IB column (4.6 x 250 mm) and a mixture of *n*-hexan: 2-propanol, 90:10 (v/v) as eluent for enantiomeric separation of *rac*-**1,2** at 1 mL/min flow rate. Retention times for (*R*)- and (*S*)-**1,2** are: 5.29 and 5.47 min for **2** and 11.87 and 13.42 min for **1**.

Thin Layer Chromatography (TLC) was carried out using Merck Kieselgel 60F_{254} sheets. Spots were visualized by treatment with 5% ethanolic phosphomolybdic acid solution and heating. Preparative chromatographic separations were performed using column chromatography on Merck Kieselgel 60 (63-200 μm). Melting points were determined by hot plate method and are uncorrected. The enantiomeric excess of the resolution products were calculated using the peak area from the chiral HPLC analysis.

The determination of enantiomeric ratio (*E*) is based on the equation $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$ with $c=ee_S/(ee_S+ee_P)$[21]

2. Materials

Lipozyme TLIM and CaL-B (immobilized and commercialized as Novozym 435) were products of Novozymes, Denmark. Lipases from *Candida rugosa* (CrL) was purchased from Fluka, whereas PPL, lipase from *Candida cylindracae* (CcL) and *Mucor miehei* were obtained from Sigma. Lipase from *P. fluorescens* (PFL) was purchased from Amano Pharmaceuticals Co., Ltd. (Nagoya, Japan) and used as received. All organic, inorganic reagents and solvents were products of Sigma-Aldrich or Fluka. All solvents were purified and dried by standard methods as required.

A. Chemical synthesis

*1. Synthesis of racemic 1-(10-ethyl-10H-phenothiazin-2-yl)ethanol rac-***1**

Into a stirred solution of 1-(10*H*-phenothiazin-2-yl)ethanone (241 mg, 1 mmol) in anhydrous DMF (20 mL) NaH (26.5 mg. 1.1 mmol) was added in small portion maintaining the mixture temperature at 0-5 °C. After 10 min., ethyl iodide (125 μL, 1.25 mmol) was added and the resulted mixture was stirred at 50 °C. After completion of the reaction (TLC monitoring), the solvent was removed under reduced pressure to dryness. The solid resulting residue was dissolved in CH_2Cl_2 (20 mL), filtered and concentrated *in vacuum*. The crude 1-(10-ethyl-10*H*-phenothiazin-2-yl)-ethanone was then crystallized from ethanol to provide a yellow semisolid material which was used as such in the next step without further purification.

To a stirred solution of the 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanone (215 mg , 0.8 mmol) in dry methanol (5 mL) $NaBH_4$ (38 mg, 1 mmol,) was added in small portion at room temperature with stirring. After the reduction was complete (TLC monitoring), the reaction was quenched by dropwise addition of 2N HCl solution (1 mL), then evaporated to a final volume of about 1 mL. To this mixture water (3 mL) and CH_2Cl_2 (6 mL) were added. After separating the two layers, the aqueous one was extracted with CH_2Cl_2 (6 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and the solvent was removed. The residue was purified by column chromatography on silica gel using CH_2Cl_2 as eluent to yield the desired product as colorless semisolid.

*2. Chemical acetylation of the racemic 1-(10-ethyl-10H-phenothiazin-2-yl)ethanol rac-***1**

Into a solution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol (*rac*-**1**, 1.5 g, 5.61 mmol) in dry CH_2Cl_2 (15 mL), Et_3N (6.16 mmol, 624 mg, 860 µL), acetyl chloride (6.17 mmol, 484.3 mg, 438.7 µL) and DMAP (0.16 mmol, 20 mg) were dropwise added in this order. The mixture was stirred at room temperature overnight and quenched with water (15 mL). After separation, the organic layer was dried over anhydrous sodium sulfate then evaporated to dryness under reduced pressure.The crude product was purified by vacuum-chromatography on neutral aluminum oxide (Brockmann IV) using CH_2Cl_2 as eluent to give *rac*-**2** as semisolid.

The structure of racemic **1,2** was confirmed by spectral analysis. All data are in good accordance with those previously reported in literature.[11]

B. Biotransformations with lipases

1. Analytical scale biotransformations

*1.1. Analytical scale enzymatic acetylation of rac-***1**

Into a solution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol *rac*-**1** (27.1 mg, 0.1 mmol) in vinyl acetate (1 mL), the tested lipase (50 mg) was added. The reaction mixture was shaken at 300 rpm at room temperature. For HPLC analysis, samples were taken from the reaction mixture (10 µL), diluted to 500 µL with 2-propanol and filtered before injection. Data on conversion and enantiomeric composition of the products for the tested lipases are presented in Table 1.

*1.2. Enzymatic kinetic resolution of racemic 1-(10-ethyl-10H-phenothiazin-2-yl)ethanol rac-**1** with vinyl acetate in different solvents with PFL*

Into a solution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol *rac*-**1** (27.1 mg, 0.1 mmol) in different solvents (1 mL), vinyl acetate (0.4 mmol, 34.4 mg, 36.8 µL) and PFL (50 mg) were added. The reaction mixtures were shaken at 300 rpm at room temperature. Samples were analyzed by

HPLC as described. Data on conversion and enantiomeric composition of the products for the tested solvents are presented in Table 2.

*1.3. Analytical scale enzymatic alcoholysis of rac-**2***

Into the mixture of *rac*-**2** (0.1 mmol, 31.3 mg) in chloroform (1 mL), PFL (50 mg) and the tested alcohol (1 mmol) were added. The reaction mixture was shaken at 300 rpm at room temperature. Samples were analyzed by HPLC. Data on conversion and enantiomeric composition of the products are presented in Table 3.

2. The use of immobilized L-AK

2.1. Preparation of immobilized L-AK catalysts

PFL powder (150 mg) was dissolved in Tris/HCl-buffer (0.1 M, pH 8, 5 mL). When additives (Celite 50 mg or/and sucrose, 100 mg) were used, they were included in the PFL solution. Into this solution, an aqueous sodium fluoride (50 µL of a 1 M solution) was added under vigorous shaking until the mixture turned to homogeneous. After the addition of the gel precursors (MTMS and TMOS, TMOS/MTMS= 1/5, water/gel *ratio*= 9/1) in one portion, the mixture was vigorous shaken for 10-15 s. The gelation was completed under gentle shaking. For maturation, the sol-gel preparations were kept overnight in a cooled (4 ^{o}C) desiccator. The next day, the sol-gel preparations were gently crushed, sequentially washed with 2-propanol, water, 2-propanol and *n*-hexane and finally freeze-dried (12 h, 0.2 mbar).

*2.2. Acylation of rac-**1** with sol-gel immobilized PFL*

The experiment was carried out in the presence of 1.5 mg/mL of protein. The PFL preparation was added into the solution of the substrate *rac*-**1** in chloroform (1 mL) before vinyl acetate (2 eq.) was added to start the reaction. The reaction mixture was shaken at 160-180 rpm at room temperature (23-24 ^{o}C).

2.3. Reuse of immobilized enzymes

The immobilized PFL preparation (1.5 mg protein/ mL) was added into the solution of *rac*-**1** (0.1 M) in chloroform (1 mL) before the addition of vinyl acetate (2 eq.) as described above. Progress and enantioselectivity of reaction were followed by sample analyses with HPLC. The reactions were stopped at 50% by decantation of the liquid phase. The solid lipase preparations were washed with dry chloroform before subjected to a new reaction with a fresh portion of reagents. Reuse was repeated 5 times under identical conditions.

3. Preparative scale synthesis of both (R)- and (S)-1

The optimum conditions determined at analyticale scale reactions were succesfully applied for the preparative scale synthesis of the highly enantiomerically enriched (*R*)- and (*S*)-1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol and their acetate ((*R*)- and (*S*)-**1** and **2**) using in each case 500 mg of the substrate.

Into a solution of racemic 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol (*rac*-**1**, 500 mg) in chloroform (20 mL), vinyl acetate (175 μL) and PFL (500 mg) were added. The reaction mixtures were shaken at 300 rpm at room temperature until the conversion reach 50% (approx. 24 h). The enzyme was filtered off and washed with acetone (2×5 mL). Solvents were distilled off from the combined filtrates and the residue was purified by column chromatography (silica gel, CH_2Cl_2) resulting in the optically active alcohol [(*S*)-**1**] and acetate [(*R*)-**2**] fractions as white semisolids. IR, NMR and MS spectra of the optically active alcohol and acetate were indistinguishable from that of their racemates. Data on yield and enantiomeric excess of the products [(*S*)-**1** and (*R*)-**2**] are shown in Table 5 (entry 1).

Into the mixture of 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethyl acetate *rac*-**2** (600 mg) in chloroform (20 mL), PFL (500 mg) and ethanol (1 mL) were added. The reaction mixture was shaken at 300 rpm at room temperature until the conversion reach 50% (approx. 24 h). Further purifications were performed as described above. Data on yield and enantiomeric excess of the products [(*S*)-**2** and (*R*)-**1**] are shown in Table 5 (entry 2).

Table 5. Preparative scale enzymatic resolution of *rac*-**1,2** with PFL in chloroform

Entry	Substrate	ee (%)		η^* (%)	*E*	ee (%)		η^* (%)	*E*
		(*R*)-**2**	(*S*)-**1**			(*R*)-**1**	(*S*)-**2**		
1	*rac*-**1**	99	96	47	>>200				
2	*rac*-**2**					99	99	48	>>200

*based on the *rac*- **1** or *rac*- **2** as starting compund

CONCLUSIONS

The synthesis of both enantiomers of 1-(10-ethyl-10*H*-phenothiazin-2-yl)ethanol **1** and his acetate **2** has been achieved by enzymatic kinetic resolution with PFL as efficient biocatalyst.

Whereas the enzymatic acetylation of the racemic ethanol **1** afforded the (*R*) enantiomer of acetate [(*R*)-**2**] and the (*S*) enantiomer of the alcohol [(*S*)-**1**] in high enantiomeric excess, the ethanolysis of the racemic acetate *rac*-**2** yielded the opposite enantiomeric form [(*R*)-**1** and (*S*)-**2**].

The stability, activity and reusability of the sol-gel encapsulated PFL were determined. The immobilized biocatalyst provide to be efficient in both studied resolution processes.

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