Babeş-Bolyai University

Cluj-Napoca

Faculty of Chemistry and Chemical Engineering

Tailored lipases for bioprocess development

PhD Thesis Abstract

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Key words: biocatalysis, enzymatic kinetic resolution, enzymatic transesterification, chiral heteroaromatic ethanols, lipase, sol-gel immobilization, batch reactor, continuous-flow reactor, kinetic resolution products separation, click reaction.

Chapter 1. General introduction

Biocatalysis is an active area of research in both academia and industry. The steady need to develop technologies with increased efficacy, productivity and selectivity, but reduced costs and minimal environmental impact has driven researchers' and engineers' interest and effort into the field of biocatalysis. The studies and work carried out in the past decades demonstrated that there are only a few limitations to the application of enzymes or whole cells as catalysts in organic synthesis.

Besides high catalytic activity and selectivity (chemo-, regio- and stereoselectivity), decisive factors for the successful application of enzymes are stability and recyclability. Moreover, the economic viability of a certain process strongly depends on the productivity of the biocatalyst. Increased stability in non-natural media (for instance in organic solvents) and the possibility to recover and reuse the biocatalyst can be achieved by enzyme immobilization. In addition to this, continuous-flow operation of immobilized enzymes offers means of upgrading the productivity.

Due to the fact that the two enantiomers of a chiral compound can have different physiological effects the use of enzymes as catalysts is particularly appealing for pharmaceutical industry where they can offer biocompatible and selective routes for the synthesis of optically pure compounds. It is worth mentioning that the potential of a compound does not depend only on structural complexity or optical purity but also on the ability to obtain it through an up-scalable technology. Furthermore, an important aspect regarding the process engineering is represented by the possibility to ensure a facile downstream procedure enabling the high-yield recovery of the desired product(s).

The present thesis addresses several aspects regarding the application of lipases in kinetic resolution processes in organic solvents for obtaining highly useful enantiomerically enriched secondary alcohols.

The first part of Chapter 4 (Personal contribution), **Tailor-made sol-gel immobilized lipases for the enzymatic kinetic resolution of heteroarylethanols in batch and continuous-**

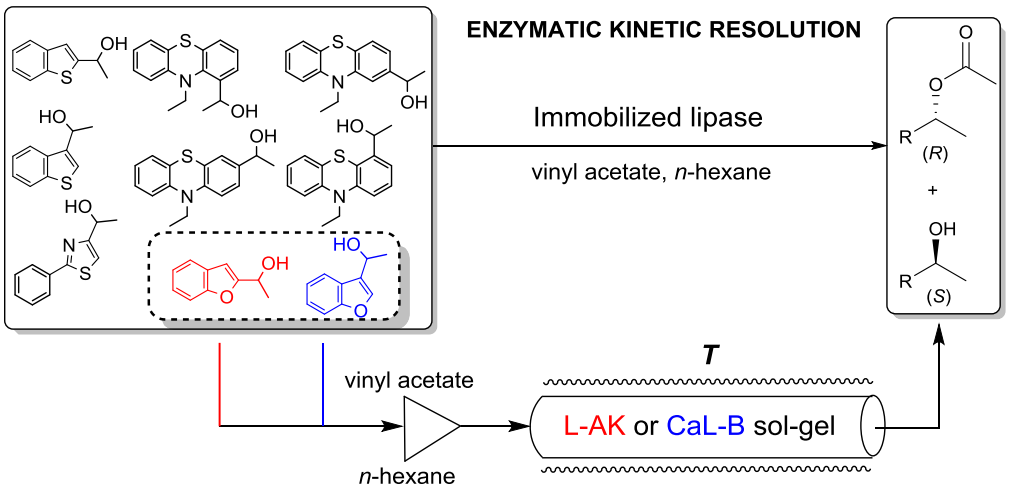
flow systems, describes the application of various highly stable lipase preparates immobilized through sol-gel entrapment for the efficient stereoselective synthesis of optically pure heteroaryl secondary alcohols in batch and continuous-flow reactors, affording a good background for additional process development.

The second part of Chapter 4, **Click reaction-based downstream strategy applied in the enzymatic kinetic resolution of (hetero)aromatic secondary alcohols**, referring to the challenges of separating the post-kinetic resolution mixtures at industrial level, presents the development of a new, facile downstream procedure for enzymatic kinetic resolution processes, allowing the recovery of both enantiomers of (hetero)aromatic ethanols in high yields conserving the excellent enantiomeric purities obtained through the EKR processes.

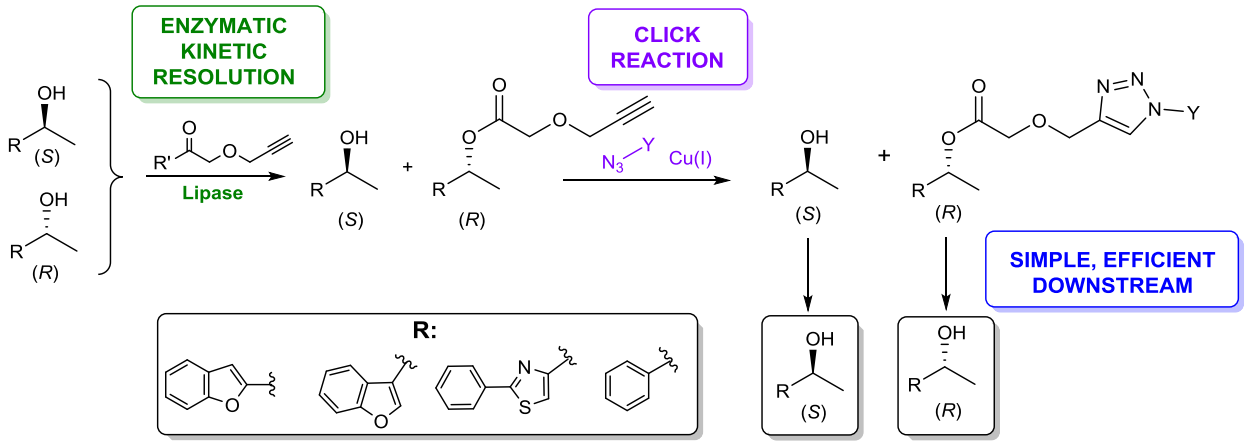
Chapter 2. Literature overview (literature data)

Chapter 3. Aim of the thesis

➢ to develop efficient enzymatic kinetic resolution processes for the synthesis of valuable enantiomerically enriched heteroaromatic ethanols using tunable sol-gel entrapped lipases from *Pseudomonas fluorescens* and *Candida antarctica* B in batch and continuous-flow reactors;



➢ to develop a simple, cost-effective and up-scalable strategy for the separation of kinetic resolution products with the aid of mild and selective click reactions. The study focused on the separation of alcohol-ester mixtures obtained after lipase-mediated enantioselective transesterifications of racemic (hetero)aromatic secondary alcohols.



Chapter 3. Aim of the thesis

(Hetero)aromatic ethanols were chosen as compounds of interest due to their undisputed importance as versatile chiral synthons in pharmaceutical and fine chemicals industries. Heteroaromatic structures like benzofuran, benzo[*b*]thiophen, phenothiazine and 2-phenylthiazol occurring in natural products and drugs, are associated with anti-bacterial, anti-viral, anti-fungal, anti-cancer, anti-malarial, anti-inflammatory, analgesic or anticoagulant activities.[1-4]

Chapter 4. Personal contribution

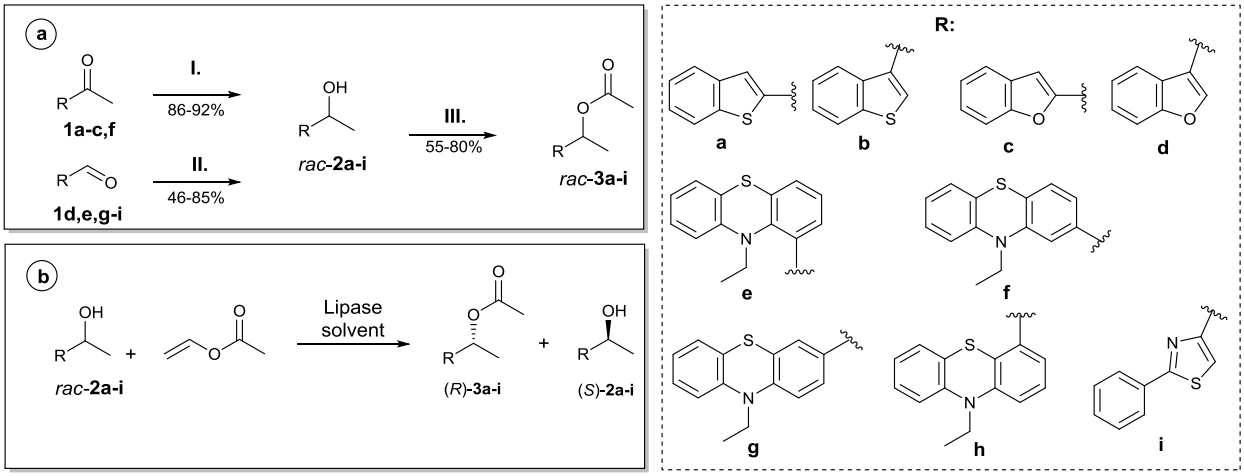
4.1. Tailor-made sol-gel immobilized lipases for the enzymatic kinetic resolution of heteroarylethanols in batch and continuous-flow systems

4.1.1. Introduction (literature data)

4.1.2. Results and discussion

4.1.2.1. Chemical synthesis of racemic heteroaromatic ethanols *rac*-2a-i and their corresponding acetates *rac*-3a-i

Firstly the chemical synthesis of racemic substrates and products was performed using known methods (**Scheme 1a**).**Error! Bookmark not defined.-Error! Bookmark not defined.** Racemic ols *rac*-**2a-i** were further used as substrates in lipase-mediated *O*-acylation reactions (**Scheme 1b**).



Scheme 1. a) Chemical synthesis of racemic ethanols and corresponding acetates; **b)** Lipase-mediated EKR; **Reagents and reaction conditions**: **I.** $NaBH_4$, MeOH, rt; **II.** CH_3MgI, dry Et_2O; **III.** Cl-CO-CH_3, 1% DMAP/Pyridine, dry CH_2Cl_2

4.1.2.2. Optimization of EKR of racemic ethanols *rac*-2a-i through *O*-acylation with vinyl acetate and immobilized lipase preparates in organic solvents

The immobilization of lipases was accomplished by adsorption on Celite 545, sol-gel entrapment in silica materials obtained from binary (BS) or ternary (TS) silane precursors mixtures, or the combination of these two methods, as presented in **Table 1**. The sol-gel entrapment of enzymes was performed by two different methods: **Method 1** using NaF as catalyst for simultaneous hydrolysis and polycondensation and **Method 2** using a sol prepolymer obtained with HCl.

Table 1. Immobilized lipase preparates description

Preparate code[a]	**Immobilization method**	**Silane precursors**[b] **(molar *ratio*)**
CaL-B 1 BS	Method 1	VTMOS:TMOS (4:1)
CaL-B 1 BSC	Method 1 + adsorption on Celite 545	VTMOS:TMOS (4:1)
CaL-B 1 TS	Method 1	PhTMOS:VTMOS:TMOS (1.6:0.4:1)
CaL-B 1 TSC	Method 1 + adsorption on Celite 545	PhTMOS:VTMOS:TMOS (1.6:0.4:1)
CaL-B 1 TS*	Method 1	PhTMOS:MeTMOS:TMOS (1.6:0.4:1)
CaL-B 2 TS	Method 2	PhTMOS:MeTMOS:TMOS (1.6:0.4:1)
CaL-B 2 TSC	Method 2 + adsorption on Celite 545	PhTMOS:MeTMOS:TMOS (1.6:0.4:1)
CaL-B Celite	Method 3 (adsorption on Celite 545)	-
AK 1 BS	Method 1	OcTMOS:TMOS (1:1)
AK 1 BSC	Method 1 + adsorption on Celite 545	OcTMOS:TMOS (1:1)
AK 1 TS	Method 1	PhTMOS:VTMOS:TMOS (1.6:0.4:1)
AK 1 TSC	Method 1 + adsorption on Celite 545	PhTMOS:VTMOS:TMOS (1.6:0.4:1)
AK 2 TSC	Method 2 + adsorption on Celite 545	PhTMOS:MeTMOS:TMOS (1.6:0.4:1)

[a] Lipases source: CaL-B- lipase B from *Candida antarctica*, AK- lipase from *Pseudomonas fluorescens*; [b] VTMOS (vinyltrimethoxysilane), PhTMOS (phenyltrimethoxysilane), MeTMOS (methyltrimethoxysilane), OcTMOS (octyltrimethoxysilane) and TMOS (tetramethoxysilane).

With the aim of finding possible applications of the investigated lipase preparates in the enzymatic synthesis of enantiomerically enriched heteroaromatic compounds, biocatalyst and solvent screenings were performed. Accordingly, the transesterification of the selected substrates *rac*-**2a-i** (40 mM) was first accomplished in neat vinyl acetate in the presence of immobilized lipases. Since the nature of the solvent could have a significant influence upon the selectivity and activity of the biocatalyst, the enzymatic acylation of racemic ethanols (10 mM) with vinyl acetate (3 equiv.) in presence of the previously selected optimal biocatalysts was tested in several dry organic solvents with different polarities, such as *n*-

hexane, toluene, diisopropyl ether (DIPE), *tert*-butyl methyl ether (MTBE), acetonitrile (ACN) and dichloromethane. The biocatalytic performance was assessed in each case using three parameters: conversion *c*, enantiomeric excesses (of the untransformed substrate ee_S and of the formed product ee_P), and enantiomeric *ratio E*.

Some preliminary general observations regarding the screenings were made. It was noticed from the biocatalyst screening results that all lipase preparates were highly enantioselective towards the racemic heteroaromatic ethanols in neat vinyl acetate, except for two L-AK preparates, AK 1 TSC and AK 2 TSC in the acylation reaction of *rac*-**2b** (*E*= 25-58). The conversion, on the other hand, was strongly influenced by the structure of the substrate and the immobilization method. Moreover, when performing the solvent screening, it was generally observed that while the selectivities remained high, the reaction times decreased significantly as compared to experiments carried out in neat vinyl acetate.

The best results obtained in the optimized EKRs are summatively presented for each substrate in **Table 2**.

Table 2. Lipase-catalysed *O*-acylation of *rac*-**2a-i** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C[a,b]

Entry	Substrate *rac*-**2a-i**	Biocatalyst	Reaction time (h)	ee_S[c] (%)	*c*[d] (%)
1	*rac*-**2a**	CaL-B 1 BSC	1	>99	50
		CaL-B Celite		>99	50
2	*rac*-**2b**	CaL-B 1 TS*	5	92 (± 1.6)	48 (± 0.5)
		CaL-B Celite		>99	50
3	*rac*-**2c**	AK 1 BS	21	86 (± 1.2)	46 (± 0.5)
4	*rac*-**2d**	CaL-B 1 TS*	19	94 (± 2.1)	49 (± 0.5)
		CaL-B Celite	2	92 (± 2.5)	48 (± 0.8)
5	*rac*-**2f**	AK 1 BS	8	>99	50
6	*rac*-**2g**	AK 1 BS	9	>99	50
7	*rac*-**2h**	CaL-B Celite	27	99 (± 0.5)	50
8	*rac*-**2i**	CaL-B 2 TSC	28	95 (± 2.2)	49 (± 0.8)

[a] ee_P>99%, *E*»200; [b] experiments were performed in triplicate and standard deviations from average values are given in brackets; [c] determined from peak areas of GC or HPLC chromatograms; [d] calculated with the formula $c = [ee_S/(ee_S+ee_P)]$

4.1.2.3. Operational stability of the immobilized lipase preparates in batch processes

One of the essential requirements for an industrially relevant biocatalyst is long-term catalytic efficiency. In this regard, the reusability of the best performing lipase immobilizates was studied in the enantioselective transesterifications of *rac*-**2a,c,d,g,i** with vinyl acetate, in *n*-hexane (**Figures 1-4**). Each reaction was repeated with the same lipase preparate up to 10 times and was allowed to proceed to ~50% conversion, or as long as the preparate maintained its activity and enantioselectivity, before subjecting the biocatalyst to the next cycle. Between reaction cycles the catalyst was washed three times with anhydrous *n*-hexane and afterwards immediately reused.

As **Figure 1A-B** indicates, the selected immobilized lipases were efficient catalysts for the acylation of the benzothiophenic ethanol *rac*-**2a** in ten consecutive reaction cycles. The adsorbed lipase (CaL-B on Celite) showed even higher reuse efficiency than the enzyme entrapped in a binary sol-gel matrix containing vinyl pending groups (CaL-B 1 BSC).

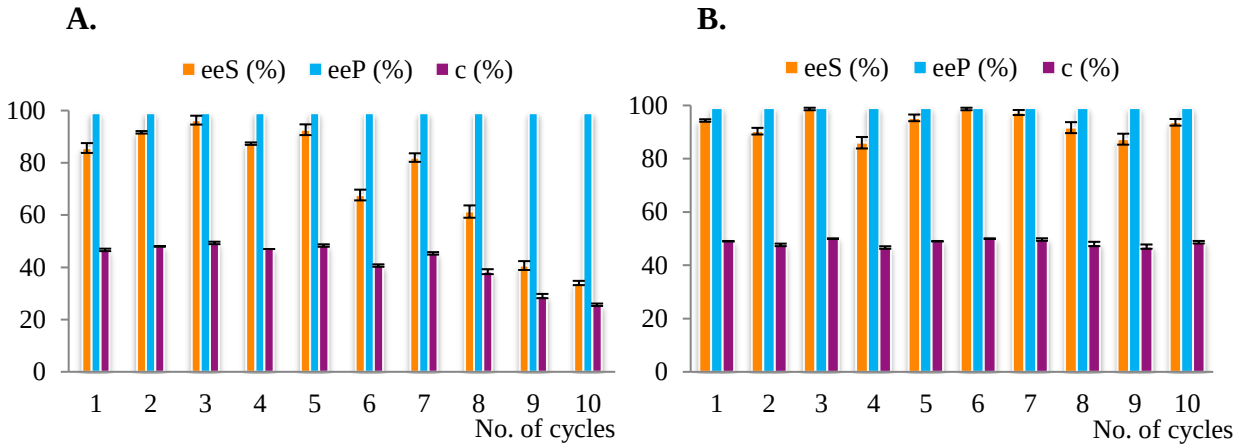


Figure 1. Recycling capacity of the selected biocatalysts for the EKR of *rac*-1-(benzo[*b*]thiophen-2-yl)ethan-1-ol *rac*-**2a** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C using: **A**) CaL-B 1 BSC and **B**) CaL-B on Celite 545 (after 40 min reaction time). Error bars represent standard deviations from average.

The multiple use results for AK 1 BS in the transesterification of *rac*-**2c** (**Figure 2A**) show a significant conversion decrease from 42 to 23% *c* in just three acylation cycles; the enantiopurity of the product however remained maximum. Additionally, the same biocatalyst, AK 1 BS, was

subjected to recyclability tests in the acetylation of *rac*-**2g** and in this case it was observed that the conversion started to decline only after five reaction cycles, from 50 to 35% *c* (**Figure 2B**). These results indicate that the reusability of a biocatalyst depends on the substrate structure.

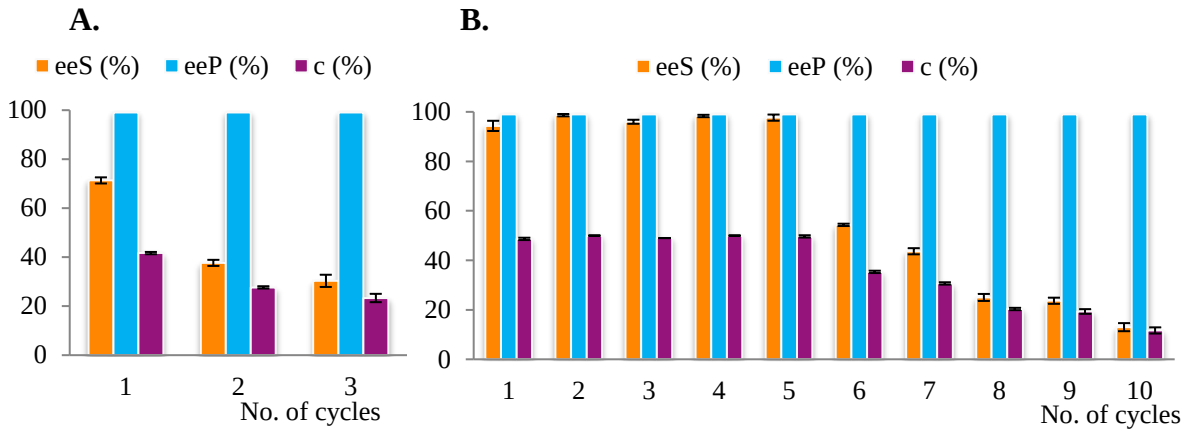


Figure 2. Recycling capacity of AK 1 BS in the EKR of: **A**) *rac*-1-(benzofuran-2-yl)ethan-1-ol *rac*-**2c** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C (after 20 hours reaction time) and **B**) *rac*-1-(10-ethyl-10*H*-phenothiazin-3-yl)ethan-1-ol *rac*-**2g** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C (after 8 hours reaction time). Error bars represent standard deviations from average.

Benzofuranic ethanol *rac*-**2d,** although being a positional isomer of *rac*-**2c**, was observed to have a different behaviour in the lipase-mediated EKR process. While for *rac*-**2c** lipase AK was the most efficient biocatalyst, for *rac*-**2d** CaL-B was more appropriate, the best immobilized preparate (CaL-B 1 TS*) demonstrating high catalytic efficiency throughout repeated use (**Figure 3**).

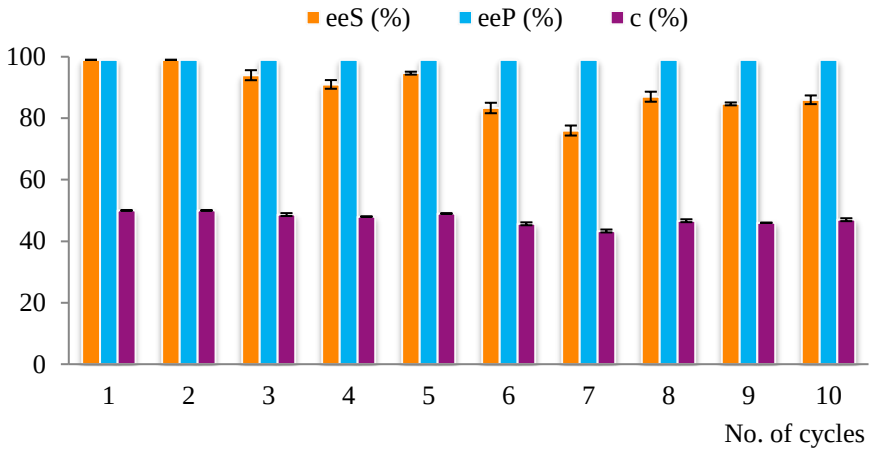


Figure 3. Recycling capacity of CaL-B 1 TS* in the EKR of *rac*-1-(benzofuran-3-yl)ethan-1-ol *rac*-**2d** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C (after 20 hours reaction time). Error bars represent standard deviations from average.

Furthermore the operational stability of a lipase preparate obtained through **Method 2** of sol-gel immobilization (CaL-B 2 TSC), was studied in the EKR of the phenyl-thiazolyl secondary alcohol *rac*-**2i.** The results presented in **Figure 4** indicate that this immobilized CaL-B preparate is not an appropriate candidate for multiple use, although it showed (in the screening study) comparable catalytic power and enantioselectivity with the best performing immobilizates obtained with **Method 1**. A rapid decrease in catalytic performance was observed, as the conversion fell from 49 to 17% *c* in the second cycle.

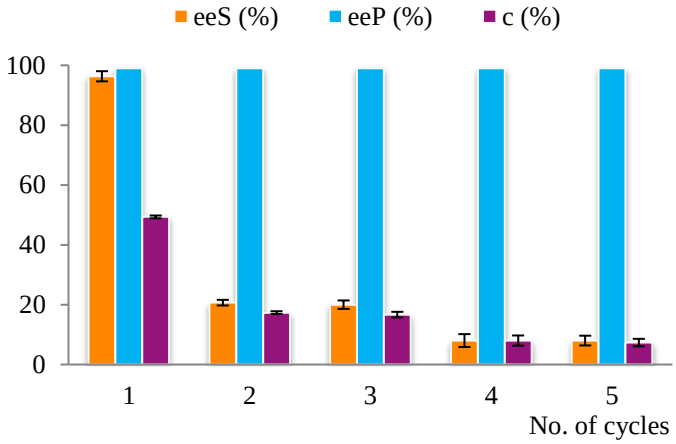


Figure 4. Recycling capacity of CaL-B 2 TSC in the EKR of *rac*-1-(2-phenyl-thiazol-4-yl)-ethanol *rac*-**2i** (10 mM) with vinyl acetate (3 equiv.) in *n*-hexane at 25 °C (after 28 hours reaction time). Error bars represent standard deviations from average.

4.1.2.4. Continuous-flow lipase-mediated EKR of racemic heteroarylethanols using packed-bed reactors

In an effort to upgrade the efficiency of the investigated biotransformation processes, another aim of the present study was to test the best performing lipase immobilizates in continuous-flow experiments using packed-bed reactors (50 × 2.1 mm). In this regard, two from the previously selected efficient biocatalysts, one from each lipase source - CaL-B 1 TS* and AK 1 BS - were chosen and investigated, while benzofuranyl-ethanols *rac*-**2c,d** were elected as substrates (**Figure 5)**. The most important parameters influencing the productivity of a continuous system process – temperature, flow rate and substrate concentration - were tested.

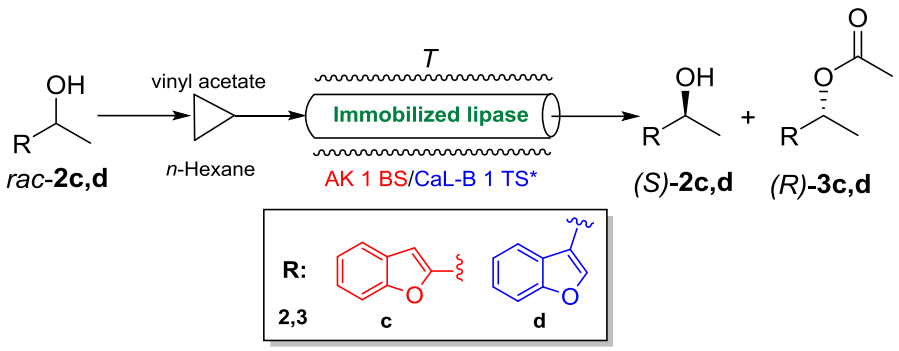


Figure 5. Lipase-mediated *O*-acylation of racemic benzofuranyl-ethanols *rac*-**2c,d** in continuous-flow packed-bed reactors

In the first set of experiments the continuous-flow EKRs were investigated at different temperatures in the range of 25 – 65 °C using a 10 mM substrate solution at 0.5 mL min^{-1} flow rate (**Figure 6A**). In both studied cases the conversion increased with the temperature; the maximum value was reached at 65 °C. Noteworthy, the continuous-flow EKRs proceeded enantioselectively (*E* » 200) throughout the investigated temperature domain (25 – 65 °C).

Next, for enhancing the conversion of the studied EKR processes involving racemic ethanols *rac*-**2c,d**, the flow rate was gradually decreased from 0.5 to 0.1 mL min^{-1}, while the temperature was set at 65 °C. As **Figure 6B** shows, the racemic substrates were almost completely resolved (48% and 50% conversion).

Moreover, in an attempt to increase the productivity of the continuous-flow packed-bed reactor, substrate concentrations in the range of 10 – 100 mM were investigated at 65 °C at 0.1 (**Figure 6C**) and 0.5 mL min^{-1} flow rate. Regarding CaL-B 1 TS*-mediated *O*-acylation of *rac*-**2d**, no significant drop in reaction conversion was observed over the studied substrate concentration domain at 0.1 mL min^{-1} flow rate. In the case of AK 1 BS preparate, the conversion of the acylation of *rac*-**2c** decreased with increased substrate concentration, at 100 mM the conversion being half of the initial value. Importantly, the enantiomeric excesses of the products were maximum (>99%) even at higher substrate concentrations.

The maximum productivity achieved using lipase AK 1 BS in the EKR of *rac*-**2c** was 37.4 µmol of product min^{-1} g^{-1} at 65 °C, 0.5 mL min^{-1} flow rate and 100 mM substrate concentration,

while the maximum productivity of CaL-B 1 TS* in the EKR of *rac*-**2d** was 157.5 μmol of product min^{-1} g^{-1} at 65 °C, 0.5 mL min^{-1} flow rate and 50 mM substrate concentration.

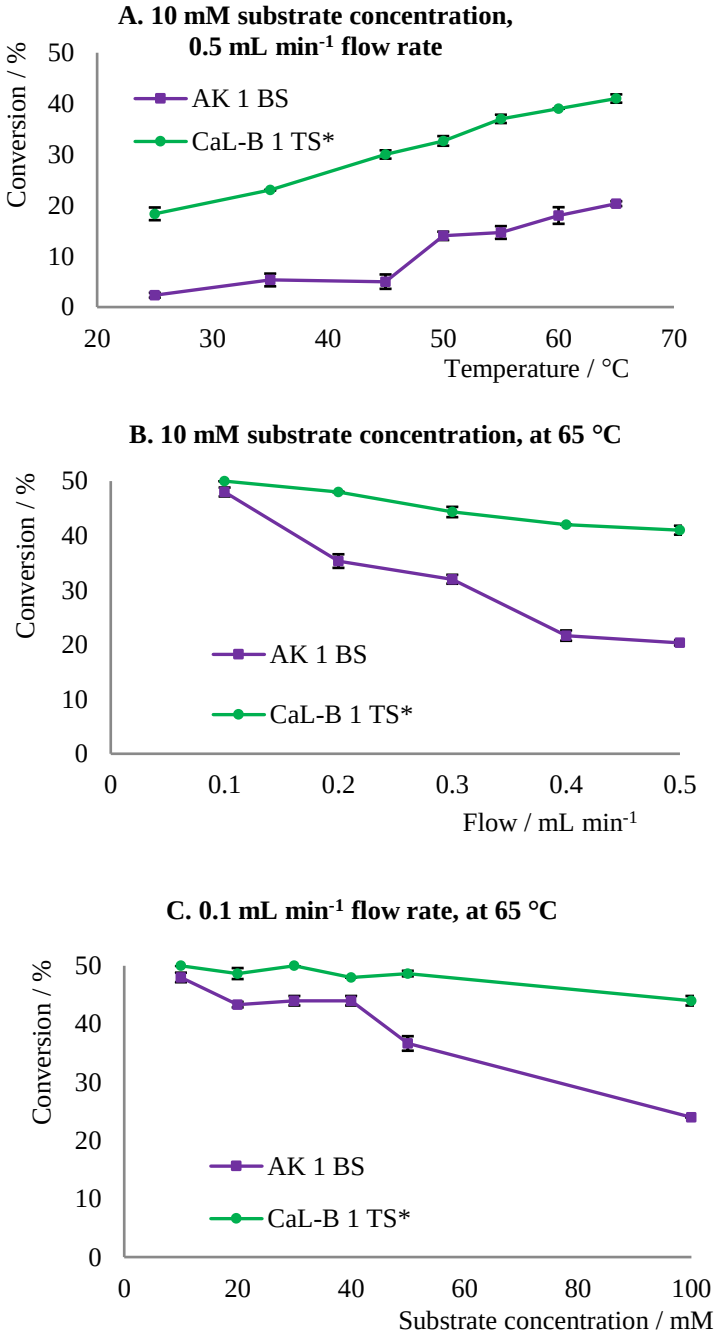


Figure 6. The effect of temperature (**A**), flow rate (**B**) and substrate concentration (**C**) on the continuous-flow EKRs of *rac*-**2c** (–□–) and *rac*-**2d** (–○–) with vinyl acetate (0.75 and 3 equiv., respectively) in *n*-hexane mediated by lipases AK 1 BS (–□–) and CaL-B 1 TS* (–○–), respectively. Error bars represent standard deviations from average.

Another aim was the study of the long-term operational stability of the two selected biocatalysts in continuous-flow mode, using a 10 mM substrate concentration and 0.1 mL min^{-1} flow rate. The results indicated that the immobilized lipase AK 1 BS preserved almost 95% of its initial activity at 55 °C after 100 h of continuous operation, while CaL-B 1 TS*, proved even higher stability, the results showing practically no decline of activity after 100 h at 65 °C (**Figure 7**). In this case, once again, the enantioselectivities of both lipase-mediated continuous-flow EKRs were not altered during continuous long-term operation of the biocatalysts.

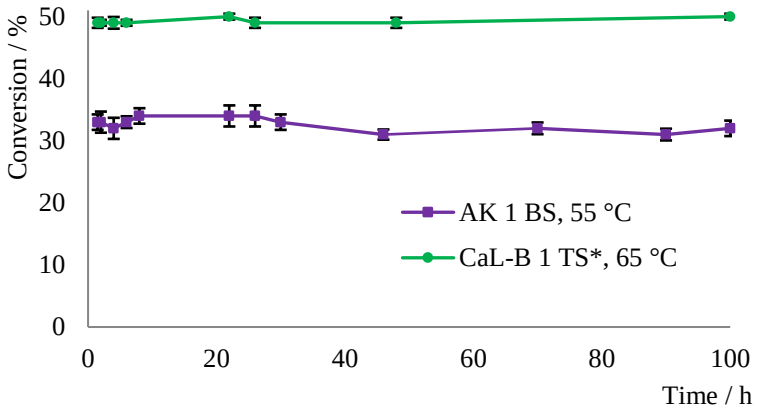


Figure 7. Long-term operational stability of immobilized lipase preparates AK 1 BS and CaL-B 1 TS* in the continuous-flow EKRs of *rac*-**2c,d** (10 mM) with vinyl acetate (0.75 and 3 equiv., respectively) in *n*-hexane at 55 °C and 65 °C, respectively and 0.1 mL min^{-1} flow rate. Error bars represent standard deviations from average.

Additionally, the productivities of the investigated continuous-flow systems and those obtained in batch processes were compared by means of specific reaction rates values calculated with Equations **5** and **6.** [70]

$$r_{batch} = \frac{n_P}{t \times m_e}\left[\frac{\mu mol}{\min \times g}\right] \qquad \text{(Equation 5)}$$

$$r_{flow} = \frac{[P] \times f}{m_e}\left[\frac{\mu mol}{\min \times g}\right] \qquad \text{(Equation 6)}$$

A major difference in specific reaction rates values was observed for AK 1 BS-mediated batch and continuous-flow acylation of *rac*-**2c**, the biocatalyst affording under continuous-flow operation a >4 times higher productivity (r_{batch} = 1.1 µmol min^{-1} g^{-1}, r_{flow} = 4.5 µmol min^{-1} g^{-1}, at

48% conversion, 65 °C, 10 mM substrate concentration, 0.75 equiv. of vinyl acetate and 0.1 mL min^{-1} flow rate). On the other hand, for CaL-B 1 TS*-catalysed acylation of *rac*-**2d** higher productivities were obtained in both systems; however, the specific reaction rate calculated for continuous-flow mode was nearly 1.5 times higher than the one correspoding to batch mode (r_{batch}= 6.7 µmol min^{-1} g^{-1}, r_{flow}= 9.8 µmol min^{-1} g^{-1}, at 50% conversion, 65 °C, 10 mM substrate concentration, 3 equiv. of vinyl acetate and 0.1 mL min^{-1} flow rate).

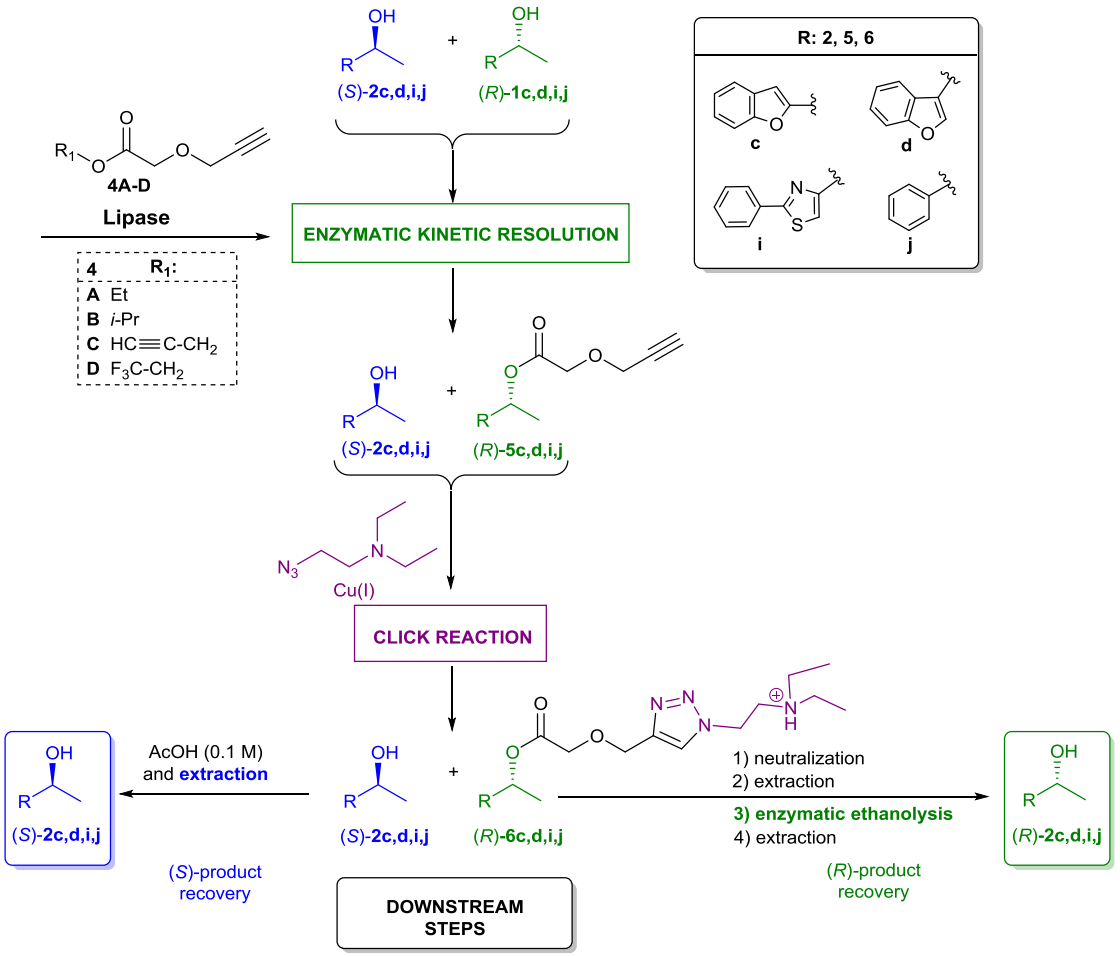
4.2. Click reaction-based downstream strategy applied in the enzymatic kinetic resolution of (hetero)aromatic secondary alcohols

4.2.1. Introduction (literature data)

4.2.2. Results and discussion

In order to apply the click reaction-based products separation in the EKR of (hetero)aromatic ethanols one of the mixture components, the formed enantiopure ester, should bear either an alkynyl or an azido group. Therefore, in order to set-up an efficient click reaction-based processing procedure for the EKR process, an appropriate reagent which plays two roles should be selected: **1)** it has to be an efficient acylating agent in the EKR step and **2)** it can form an ester product reactive in CuAAC. The most accessible approach uses 2-(prop-2-yn-1-yloxy)acetates as acylating agents, forming enantiomerically enriched clickable esters.

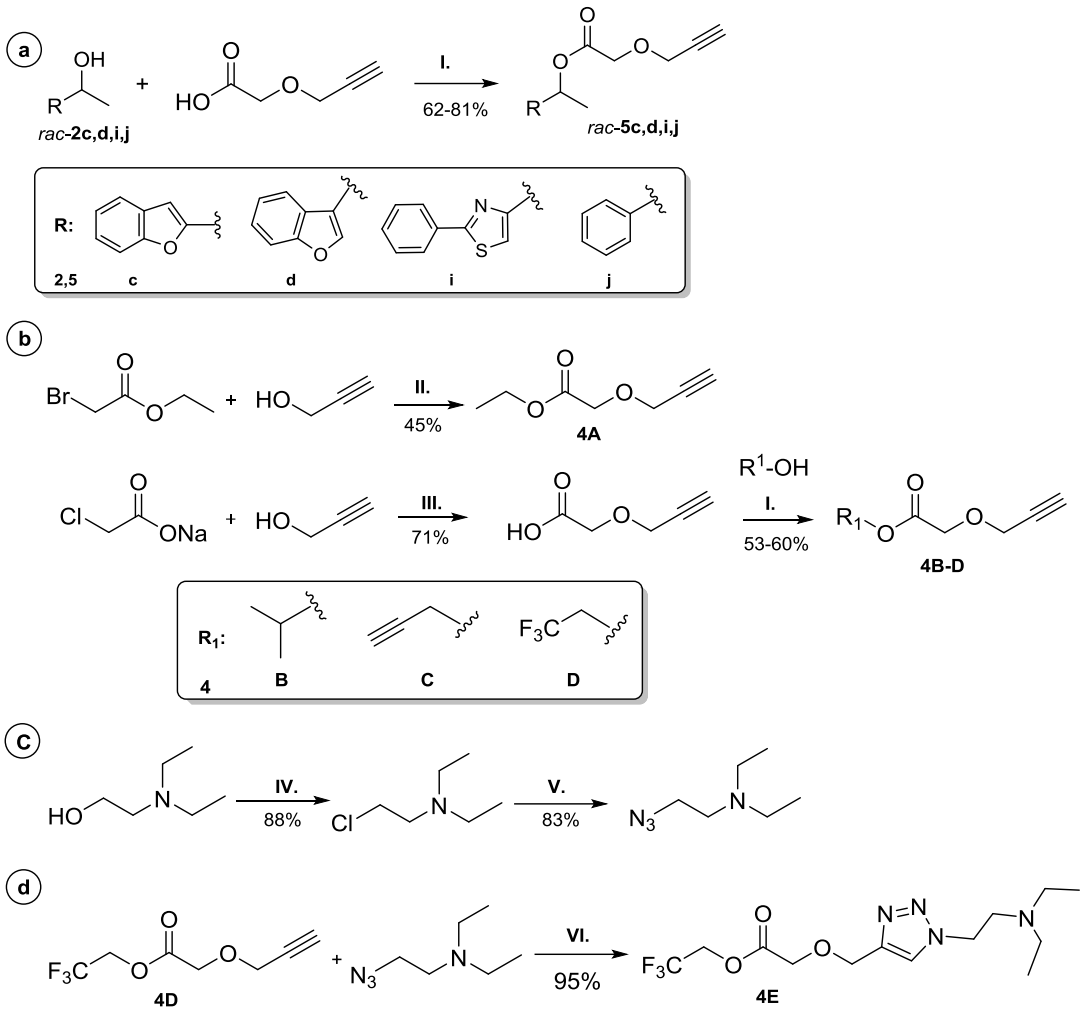
According to this click-chemistry-aided EKR products separation methodology various 2-(prop-2-yn-1-yloxy)acetates **4A-D** were tested as acylating agents for the enatioselective lipase-mediated KRs of racemic (hetero)arylethanols *rac*-**2c,d,i,j**. As shown in **Scheme 2**, the reaction mixture, containing the untransformed alcohol enantiomer [(*S*)-**2c,d,i,j**] and the formed ester bearing an alkynyl group [(*R*)-**5c,d,i,j**] was reacted with an azido-functionalized tertiary amine, the ester being quantitatively transformed into ionisable aminoalkyl-triazoles [(*R*)-**6c,d,i,j**]. Through pH adjustment-extraction steps the facile separation and isolation of enantiomerically enriched ethanols (*S*)-**2c,d,i,j** and triazoles (*R*)-**6c,d,i,j** was achieved without affecting their enantiopurity. The last step consisting in a mild enzymatic deprotection of the click product (*R*)-**6c,d,i,j** allowed the recovery of the reactive enantiomer of the substrate (*R*)-**2c,d,i,j** with unaltered enantiopurity.



Scheme 2. Click reaction-aided separation of EKR products

4.2.2.1. Chemical synthesis of racemic 2-(prop-2-yn-1-yloxy)acetates *rac*-5c,d,i,j, clickable acylating agents 4A-E and reactants

The chemical synthesis of the racemic aromatic ethanols *rac*-**2c,d,i,j** was performed as already presented; the corresponding racemic 2-(prop-2-yn-1-yloxy)acetates *rac*-**5c,d,i,j**[102], 2-(prop-2-yn-1-yloxy)acetic acid and its ethyl[103], isopropyl, propargyl and 2,2,2-trifluoroethyl esters[Error! Bookmark not defined.] **4A-D,** 2-azido-*N*,*N*-diethylethan-1-amine and the triazolic ester **4E** were synthesized according to **Scheme 3.** All compounds were characterized through ^{1}H and ^{13}C-NMR spectroscopy and mass spectrometry.

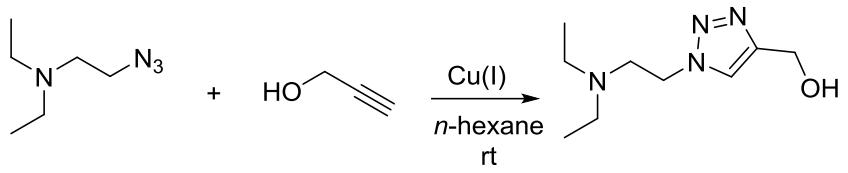


Scheme 3. Chemical synthesis of **a**) racemic clickable esters *rac*-**5c,d,i,j**, **b**) acylating agents **4A-D**, **c**) azido-functionalized tertiary amine and **d**) triazolic acylating agent **4E**. **Reagents and reaction conditions: I.** DCC, DMAP, dry CH_2Cl_2, rt, 3 h; **II.** NaH, dry THF, Ar, rt, 12 h; **III.** MTBE, NaOH, TBAB, rt; **IV.** $SOCl_2$, $CHCl_3$, 0 °C - reflux, 4 h; **V.** NaN_3, DI H_2O, 80 °C; **VI**. CuI, *n*-hexane, rt, 12 h.

4.2.2.2. Copper(I) source screening for the click reaction

With the aim to find the optimal reaction conditions for the click reactions of the investigated compounds a preliminary click reaction test and the optimization of Cu(I) source were next performed on the model click reaction between propargyl alcohol and 2-azido-*N*,*N*-

diethylethan-1-amine in apolar solvents (**Scheme 4**). From the tested catalysts [CuI, CuI(PPh_3) and $Cu_2SO_4 \times 5H_2O$ + $N_2H_4 \times H_2O$], CuI was selected as most efficient copper(I) source.



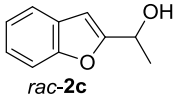
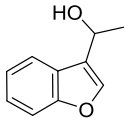
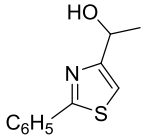
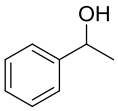
Scheme 4. Optimization study on preliminary model click reaction

4.2.2.3. Optimization of lipase-catalysed *O*-acylation of racemic (hetero)arylethanols *rac*-2c,d,i,j with 2-(prop-2-yn-1-yloxy)acetates 4A-E

In order to obtain enantiomerically enriched compounds with high conversions, the lipase-catalysed enantioselective transesterifications of *rac*-**2c,d,i,j** with 2-(prop-2-yn-1-yloxy)acetates **4A-D** were investigated in various organic solvents at analytical scale. Lipase B from *Candida antarctica* in the form of the commercially available preparate Novozyme 435 (CaL-B adsorbed on macroporous acrylic resin), previously found as efficient in the enantioselective *O*-acylation of racemic (hetero)arylethanols *rac*-**2d,i,j** with vinyl esters,[Error! Bookmark not defined.,Error! Bookmark not defined.] was chosen as biocatalyst for the EKR/click reaction-based separation process of these substrates as well. Similarly, lipase from *Pseudomonas fluorescens* (L-AK) found optimal for the enzymatic transesterification of racemic 1-(benzofuran-2-yl)ethan-1-ol, *rac*-**2c**, with vinyl acetate[Error! Bookmark not defined.] was selected as biocatalyst for the present study. Various organic solvents frequently used in lipase-mediated reactions, solubilizing both the substrates and the products [*n*-hexane, dichloromethane, acetonitrile (ACN), tetrahydrofuran (THF), *tert*-butyl methyl ether (MTBE), diisopropyl ether (DIPE), cyclohexane and toluene] were tested.

Initially, analytical scale enzymatic transesterifications of *rac*-**2c,d,i,j** using ethyl 2-(prop-2-yn-1-yloxy)acetate **4A** as acyl donor were tested in the selected organic solvents to determine the most appropriate reaction medium for each substrate (**Table 3**).

Table 3. Solvent screening for the enzymatic acylations of *rac*-**2c,d,i,j** (10 mM) with ethyl 2-(prop-2-yn-1-yloxy)acetate **4A** (1 equiv.) at 30 °C and substrate:biocatalyst *ratio* 1:1, w/w[a]

Compound	 *rac*-**2c**	 *rac*-**2d**	 *rac*-**2i**	 *rac*-**2j**				
Solvent	$c_{rac\text{-}2c}$[b,c] **(%)**	ee_{5c}[b,c] **(%)**	$c_{rac\text{-}3d}$[d,e] **(%)**	ee_{5d}[d,e] **(%)**	$c_{rac\text{-}2i}$[e,f] **(%)**	ee_{5i}[e,f] **(%)**	$c_{rac\text{-}2j}$[e,g] **(%)**	ee_{5j}[e,g] **(%)**
n-Hexane	17 (± 1.4)	>99	10 (± 1.6)	>99	n.d.	<5	17 (± 0.8)	>99
CH_2Cl_2	n.d.	n.d.	11 (± 1.4)	>99	15 (± 0.5)	>99	n.d.	n.d.
ACN	n.d.	n.d.	18 (± 1.4)	>99	n.d.	n.d.	n.d.	n.d.
THF	n.d.	n.d.	15 (± 0.5)	>99	n.d.	n.d.	n.d.	n.d.
MTBE	n.d.	n.d.	18 (± 0.9)	>99	n.d.	<5	n.d.	n.d.
DIPE	n.d.	n.d.	21 (± 0.9)	>99	4 (± 0.5)	30 (± 1.2)	n.d.	n.d.
Cyclohexane	n.d.	n.d.	30 (± 1.2)	>99	25 (± 1.4)	39 (± 1.2)	16 (± 0.5)	>99
Toluene	n.d.	n.d.	26 (± 0.8)	>99	18 (± 2.1)	86 (± 2.1)	n.d.	n.d.

[a] experiments were performed in triplicate and standard deviations from average values are given in brackets; [b] after 120 h; [c] Lipase from *Pseudomonas fluorescens* (L-AK); [d] after 15 h; [e] Lipase B from *Candida antarctica* (Novozyme 435); [f] after 130 h; [g] after 4 h; n.d. not detected.

It is notable that both Novozyme 435 and L-AK were highly enantioselective in all EKRs involving *rac*-**2c,d,j** (ee_P > 99%) providing detectable conversion, while for *rac*-**2i** high enantiomeric purity of the product was achieved only in CH_2Cl_2. However, using the ethyl ester **4A** as acyl donor could only lead to unsatisfactory conversions, the maximum value, 30% *c*, being reached after 15 hours in the case of *rac*-**2d**. Unfortunately, allowing the reactions to proceed for longer reaction times and investing the effect of several parameters such as temperature, amount of acyl donor and biocatalyst did not significantly enhance the conversions, which can be explained by ethanol liberation reversing the transesterification reaction.

Consequently three other esters **4B-D** [the isopropyl, propargyl and 2,2,2-trifluoroethyl esters of 2-(prop-2-yn-1-yloxy)acetic acid] were tested as acyl donors in EKRs of racemic ethanols using the selected most suitable solvents (*n*-hexane for *rac*-**2c,j**, cyclohexane for *rac*-**2d** and CH_2Cl_2 for *rac*-**2i**). By performing the enzymatic acylations of *rac*-**2c,d,i,j** with esters **4B-D** (**Figure 8**), sterically hindered or weakly nucleophilic alcohols with decreased reactivity were liberated[Error! Bookmark not defined.], thus shifting the equilibrium of the transesterification reactions towards product formation. The 2,2,2- trifluoroethyl ester **4D** proved high efficiency as acyl

donor for all investigated substrates *rac*-**2c,d,i,j**, affording maximum or close to maximum conversions (*c* = 48–50% after 7-90 h) without decrease in enantiomer selectivity (ee_P > 99% in all cases).

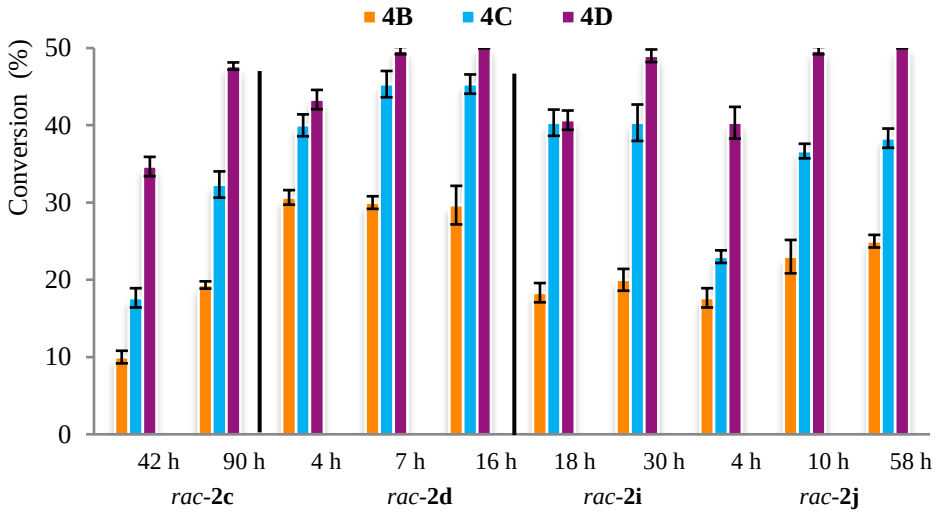


Figure 81. Acyl donors **4B-D** (1 equiv.) screening in the kinetic resolutions of *rac*-**2c,d,i,j** (10 mM) mediated by Novozyme 435 (for *rac*-**2d,i,j**) or L-AK (for *rac*-**2c**) in the optimal solvents at 30 °C and substrate:biocatalyst *ratio* 1:1, w/w; ee_P > 99% in all cases. Error bars represent standard deviations from average.

The ester 2,2,2-trifluoroethyl 2-((1-(2-(diethylamino)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)acetate **4E** (**Scheme 3d**), was also tested as potential acyl donor in the enzymatic acylation of the model substrate *rac*-**2j** but proved to be unsuccessful.

4.2.2.4. Set-up of click reaction-based protocol for the separation of EKR products

In order to establish the click reaction-based protocol for the separation of enzymatic kinetic resolution products, mixtures of racemic compounds *rac*-**2c,d,i,j** and *rac*-**5c,d,i,j**, mimicking the post-EKR reaction composition, were used, following the methodology described in **Scheme 2**. In this way, ethanols *rac*-**2c,d,i,j** and triazoles *rac*-**6c,d,i,j** were isolated using extractive metods with >94% recovery for each component.

4.2.2.5. Isolation of enantiomerically enriched (*S*)- and (*R*)-(hetero)arylethanols 2c,d,i,j by enzymatic *O*-acylation/click reaction-aided separation/enzymatic alcoholysis tandem process

For isolation of both enantiomers of chiral (hetero)aromatic ethanols **2c,d,i,j** with high enantiopurity the whole procedure was repeated at preparative scale maintaining the optimal reaction conditions found at analytical scale tests but applying 30-fold higher substrate concentration. The recovery of the reacting ethanol enantiomers (*R*)-**2c,d,i,j** was achieved through lipase-catalysed alcoholysis of enantiomerically enriched triazoles (*R*)-**6c,d,i,j**. The highly stable and thermotolerant form of lipase B from *C. antarctica*, obtained through covalent immobilization on single-walled carbon nanotubes (CaL-B-SWCNT)[105] proved to be an efficient biocatalyst for the alcoholysis of (*R*)-**6c,d,i,j**, allowing the recovery of the reacting alcohols (*R*)-**2c,d,i,j** from the racemates in good overall yields (87-91%) with maximum enantiomeric excesses (**Table 4**).

Table 4. Isolated products and intermediates in the lipase-mediated KR/click reaction-based separation/enzymatic alcoholysis tandem process

Non-reacting enantiomer	Yield[a] (%)	*ee* (%)	Click-product	Yield[a] (%)	*ee* (%)	$[\alpha]_D^{27}$[b]	Reacting enantiomer	Yield[a] (%)	*ee* (%)
(*S*)-**2c**	96	93	(*R*)-**6c**	94	>99	+76.3	(*R*)-**2c**	88	>99
(*S*)-**2d**	97	>99	(*R*)-**6d**	95	>99	+13.9	(*R*)-**2d**	90	>99
(*S*)-**2i**	96	97	(*R*)-**6i**	94	>99	+63.2	(*R*)-**2i**	87	>99
(*S*)-**2j**	97	>99	(*R*)-**6j**	96	>99	+48	(*R*)-**2j**	91	>99

[a] determined after separation of EKR products; 100% theoretical yield for one enantiomer corresponds to 50% of the racemate;
[b] c =1 mg mL^{-1} in acetonitrile

To prove the feasability of this new methodology, the whole procedure was repeated at gram-scale for racemic 1-phenylethan-1-ol *rac*-**2j** (1.5 g) under the same conditions. It was noticed that the activity and selectivity of the employed biocatalysts remained similar to those previously observed, but the isolated yields for (*S*)-**2j**, (*R*)-**6j** and (*R*)-**2j** slightly increased (>99%, for each).

4.2.2.6. Continuous-flow CaL-B-SWCNT-mediated *O*-acylation of *rac*-1-phenylethan-1-ol *rac*-2j with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yloxy)acetate 4D

With the purpose of increasing the efficiency of large-scale production of (hetero)arylethanols through the novel developed methodology, next, the new and efficient acylating agent **4D** was investigated in continuous-flow enzymatic *O*-acylation of the model compound, the racemic 1-phenyl-1-ethan-1-ol *rac*-**2j**, using a packed-bed reactor (30 × 4.6 mm, **Figure 9**). The remarkably stable, already described lipase preparate CaL-B-SWCNT was selected as biocatalyst as it previously demonstrated high operational stability.**Error! Bookmark not defined.**

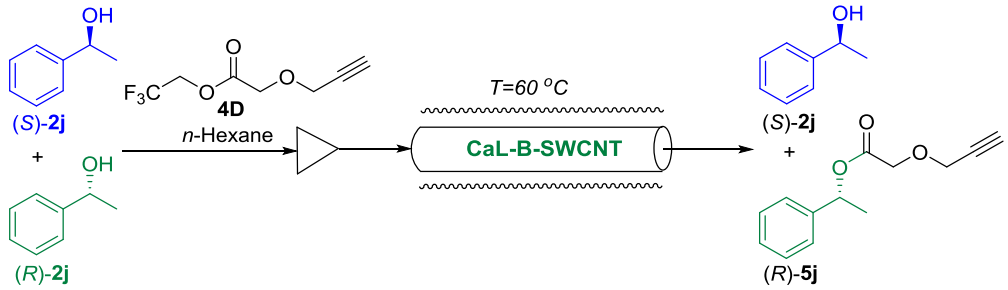


Figure 92. Continuous-flow transesterification reaction of *rac*-1-phenylethan-1-ol *rac*-**2j** with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **4D** in *n*-hexane at 60 °C in a packed-bed reactor

Initially, the continuous-flow acylation reaction was investigated using a 10 mM substrate concentration and 0.75 equiv. of acylating agent **4D** at 60 °C, at flow rates in the range of 0.1 – 0.5 mL min^{-1}. It was observed that the maximum conversion reached within this experiment was 42% at 0.1 mL min^{-1}. Consequently, with the aim to increase the conversion of the reaction, identical experiments were carried out using higher amounts of the acyl donor **4D** (2, 3 and 4 equiv.).

The results are represented in **Figure 10**. It can be noticed that the molar *ratio* of the acyl donor influences the conversion of the continuous-flow enzymatic reaction. Since no significant modifications of conversion were seen from 0.5 to 0.1 mL min^{-1} when 4 equiv. of acyl donor were used (48-50%), this amount of acyl donor was further used as optimal.

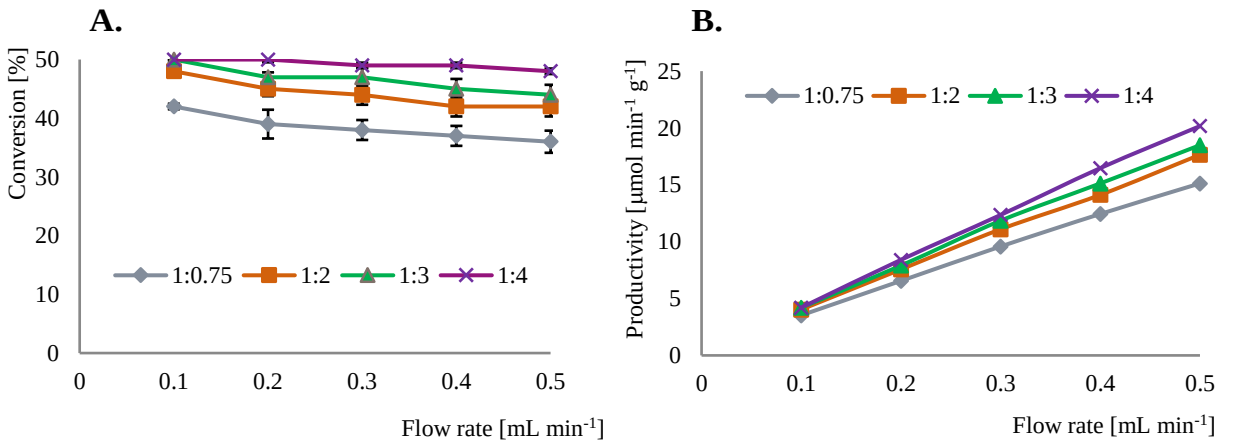


Figure 30. Influence of substrate:acyl donor *ratio* on the conversion (**A**) and productivity (**B**) of CaL-B-SWCNT-catalysed continuous-flow kinetic resolution of *rac*-**2j** (10 mM) with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **4D** in *n*-hexane at 60 °C. Error bars represent standard deviations from average. The productivity was calculated using the average value of conversion.

Next, in an effort to increase the productivity of the studied biocatalytic continuous-flow system, different substrate concentrations were tested at 60 °C using 4 equiv. of acyl donor **4D** at 0.1 and 0.5 mL min^{-1} (**Figure 11**). As expected, the productivity increased with the concentration of the substrate, the maximum value being obtained at 100 mM substrate concentration and 0.5 mL min^{-1} flow rate (88.2 µmol min^{-1} g^{-1}). Noteworthy, even at the highest studied substrate concentration (100 mM) the system afforded a very good conversion of *rac*-**2j** (45% at 0.1 mL min^{-1}).

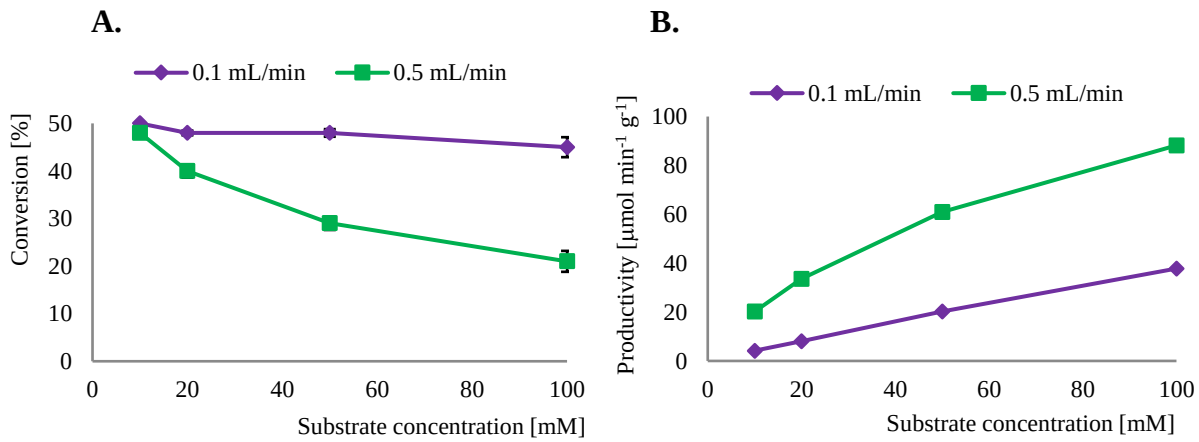


Figure 41. Influence of substrate concentration on the conversion (**A**) and productivity (**B**) of CaL-B-SWCNT-mediated continuous-flow kinetic resolution of *rac*-**2j** with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **4D** (4 equiv.) in *n*-hexane at 60 °C. Error bars represent standard deviations from average. The productivity was calculated using the average value of conversion.

Another goal of the study was to compare the productivities obtained in continuous-flow and batch systems for the acylation reaction of *rac*-**2j** with **4D** mediated by CaL-B-SWCNT. The immobilized preparate CaL-B-SWCNT proved remarkable productivity in continuous-flow mode as compared to the one obtained in batch mode (r_{batch} = 1.0 μmol min^{-1} g^{-1}, r_{flow} = 17.6 μmol min^{-1} g^{-1}, at 42% conversion, 60 °C, 10 mM substrate concentration, 2 equiv. of acyl donor **4D** and 0.5 mL min^{-1} flow rate), demonstrating the superiority of continuous-flow systems over batch processes.

Chapter 5. Experimental part (experimental data)

Chapter 6. General conclusion

The studies performed as part of this thesis present the optimization of lipase-mediated kinetic resolution processes involving (hetero)aromatic secondary alcohols on aspects of stability and productivity of the biocatalyst and separation of post-kinetic resolution mixtures.

Tailored sol-gel entrapped lipases from *Candida antarctica* B and *Pseudomonas fluorescens* were successfully applied in EKRs of various heteroaromatic ethanols. The stability and productivity of the biocatalysts were investigated in batch as well as in continuous-flow processes. Under continuous-flow operating conditions the tested biocatalysts (AK 1 BS and CaL-B 1 TS*) demonstrated improved productivity in the EKRs of racemic benzofuranyl-ethanols as compared to the batch processes. Moreover, both biocatalysts were highly stable under prolonged continuous operation (100 h) at high temperature (55 or 65 °C), as more than 95% of the initial activity was maintained, although at repeated use in batch processes only CaL-B 1 TS* showed remarkable stability (more than 10 recycles in the EKR of *rac*-**2d**), AK 1 BS manifesting modest recyclability (approx. 55% enzymatic activity after three reaction cycles in the EKR of *rac*-**2c**). These results clearly demonstrate the superior efficiency of continuous-flow operating systems.

Additionally, a novel procedure combining an efficient lipase-catalysed kinetic resolution of (hetero)arylethanols with a mild and selective click reaction-based downstream was developed, allowing the easy separation of post-KR mixtures and recovery of both enantiomers of secondary alcohols in good yields with excellent enantiomeric purities, avoiding the need for laborious, expensive chromatographic separation. The proposed methodology employs 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate as best performing acylating agent in EKRs of secondary alcohols *rac*-**2c,d,i,j**, resulting in enantiopure (hetero)arylethyl 2-(prop-2-yn-1-yloxy)acetates (*R*)-**5c,d,i,j**

which are further reacted with an azido-functionalized tertiary amine in presence of CuI as catalyst affording ionisable triazole derivatives (*R*)-**6c,d,i,j**. Enantiomerically enriched ethanols (*S*)-**2c,d,i,j** are isolated from the ionisable triazoles by simple extraction. Mild lipase-catalyzed ethanolysis, using a highly stable and active lipase preparate (CaL-B immobilized on single-walled carbon nanotubes) finally provided the enantiomerically enriched ethanols (*R*)-**2c,d,i,j**.

The up-scalability of this new methodology was demonstrated for the model compound *rac*-1-phenylethan-1-ol *rac*-**2j** in a batch process at gram-scale and also under continuous-flow operation using the highly active and stable CaL-B-SWCNT in a packed-bed reactor for the EKR of *rac*-**2j** with 2,2,2-trifluoroethyl 2-(prop-2-yn-1-yl-oxy)acetate **4D**, demonstrating >17 times higher productivity in comparison to the batch system.

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