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DEVELOPMENT OF IMMOBILIZED BIOCATALYSTS FOR SELECTIVE BIOTRANSFORMATIONS

PhD Thesisbook

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1. INTRODUCTION

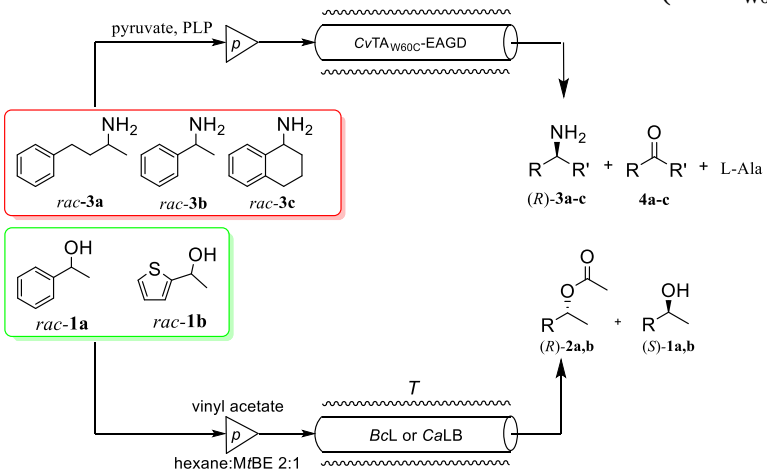
Recently, biotechnology has attracted growing attention both in industrial and academic fields as it facilitates environmentally-friendly processes.[1] Within the frames of white biotechnology, enzyme or whole-cell catalyzed processes are frequently used in the production of chiral pharmaceuticals, flavor and fragrance industry. However, biocatalytic processes may suffer from several drawbacks: reduced stability of enzymes under nonconventional circumstances (pH above 9, higher temperature), inhibition by non-natural substrates and/or products. Fortunately, methods for enzyme stabilization and enzyme engineering are available. Rise of gene technology enabled the design and fermentation of new, enhanced biocatalysts with extended lifetime and higher stability. Most recently, researchers focus on finding of enzymes among extremophiles (organisms are adapted to extreme condition, e.g. high temperature, pH or salt concentrations).[2] These enzymes can be directly employed in extreme reactions without enzyme engineering.

Nevertheless, biotechnology offers a powerful tool to improve the catalytic performance of already existing enzymes: the immobilization of enzymes or whole-cells. Usually, the development of an immobilized biocatalyst can be divided into three major steps:

- Selection of active and stable enzymes / whole-cells
- Development of immobilization techniques for enzyme stabilization
- Evaluation and characterization of immobilized biocatalysts in batch and continuous-flow reactors.

The goal of my PhD thesis is to highlight different topics of enzyme immobilization and present some possibilities to improve the enzymatic performance. The evaluation of various enzyme and whole-cell immobilization methods could broaden the applicability of enzymes in the continuous-flow kinetic resolutions. Scheme 1 illustrates the performed KRs with the developed enzyme preparations.

Scheme 1. Kinetic resolutions of *rac*-**1a,b** with immobilized *Burkholderia cepacia* lipase (*Bc*L) or *Candida antarctica* lipase B (*Ca*LB) in continuous-flow reactors; Kinetic resolution of *rac*-**3a-c** with immobilized *Chromobacterium violaceum* ω-transaminase (*Cv*TA$_{W60C}$)



1. S. Heux, I. Meynial-Salles, M.J. O'Donohue, C. Dumon, *Biotechnol. Advances*, **2015**, *33*, 1653–1670.
2. *Biotechnology of Extremophiles: Advances and Challenges*, P.H. Rampelotto, Ed. Springer, **2016.**

2. BACKGROUND

2.1. Biocatalyst immobilization techniques

Majority of biotechnological research projects is focusing on enzyme and/or whole-cell immobilization techniques. Designing the proper immobilization process and retaining the active conformation of the enzyme enables obtaining of active, stable and selective biocatalyst. Immobilization can be widely used in industry because of its low cost, high enzyme specifity and activity. However, the enzyme activity may be altered after immobilization. Enzyme immobilization methods can be classified according to several aspects, but the most important division is: physical methods (reversible) and chemical methods (irreversible). Immobilization of enzymes means attachment of enzymes to non-soluble support or creating enzyme aggregates and so obtaining heterogeneous biocatalyst. Proteins can be linked with crosslinking agents to supports or to each other. Fig.1. illustrates above mentioned immobilization methods (A, C and E represent physical methods, while B and D illustrate chemical methods for immobilization). Success of enzyme immobilization depends on immobilization method, nature and type of support and enzyme structure.[3,4]

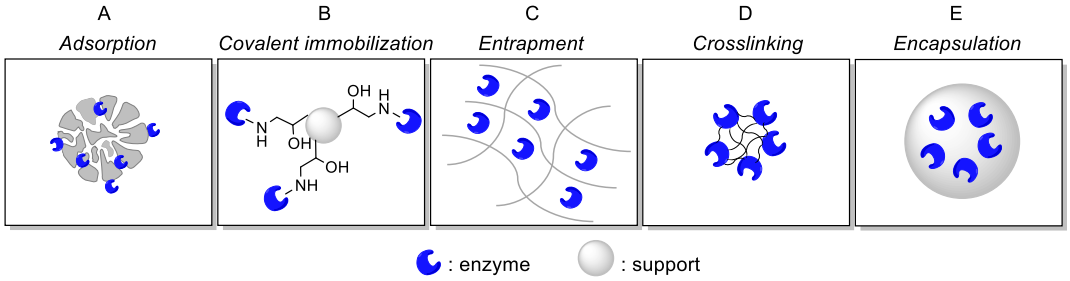


Fig.1. Examples of several immobilized biocatalysts

Physical methods include adsorption and dispersion of enzyme molecules on various solid supports or encapsulation of enzymes in matrices, while chemical methods modify the side chains of amino acids in enzymes. Adsorption is a mild immobilization process which results from intermolecular interactions (van der Waals forces, hydrogen bonding, hydrophobic interactions) between the enzyme and the support surface.[5] Immobilization conditions strongly influence the enzyme performance: hydrophobic group density, pH, ionic strength, temperature, solvent polarity. Taking into account that enzyme immobilization is reversible, support can be recovered after the reaction. Encapsulation is a great choice for enzyme immobilization when the enzyme can be easily deactivated. Consequently, to create a stable, active and selective biocomposite, several aspects of immobilization have to be taken into account. Chemical and physical nature of the support will strongly determine the properties of immobilized biocatalyst, but methods of activation can alter the performance as well.[6] Rational selection of carriers with proper physical nature (shape, mechanical and chemical stability, particle size, pore size, porosity, and mode of pore distribution) not only influences the performance of enzymes, but meets the requirements for some types of enzyme reactors. The surface of the carrier can be divided to external and internal surface. For bio-immobilization, high internal surface with accessible pores is desirable. High surface area is a

3. A.B. Salleh, *New lipases and proteases*, Nova Science Publishers, Inc., New York, **2006.**
4. G. Bayramoğlu, B. Kaya, M.Y. Arica, *Food Chemistry*, **2005**, *92*, 261–268.
5. Enzyme Stabilization and Immobilization, ed. Shelley D. Minteer, **2011**, Humana Press
6. N.R. Mohamad, N.H.C. Marzuki, N.A. Buang, F. Huyop, R.A. Wahab, *Biotech. & Biotechnol. Equip.*, **2015**, *29*, 205–220.

crucial point for high enzyme loading leading to higher enzyme activity. Pores have to be 3-9 times larger than the diameter of enzyme molecules (>30 nm) thus the enzymes can freely diffuse within the pores. Furthermore, the carrier must be mechanically stable in order to reduce the particle breakage resulting from high shearing forces and back-pressures.

2.2. Covalent enzyme immobilization

Biocatalysis offers an efficient route to obtain enantiopure chemicals, however leaching of biocatalyst from different supports could lead to serious product contamination issues. One method to overcome this issue is immobilization of enzymes with irreversible covalent linkages onto different supports: establish the crosslinking between the enzyme molecules and the carrier surface. Enzymes have various functional groups on their side chains (e.g. amino, thiol, carboxyl or hydroxyl groups), which are the targets of crosslinking. The thiol group of cysteine is the most potent nucleophile, but the presence of amino groups is more likely. Some lysine residues may be located near to the active site and are essential for the enzyme activity. If these amino groups are involved in crosslinking, it could lead potentially to the full loss of biocatalytic activity. Until recently, glutaraldehyde has been used for covalent immobilization of proteins on amino-activated carriers or as crosslinking agent creating protein-protein aggregates (Fig.2.).[7,8] In ideal case, aldehyde groups of glutaraldehyde are highly reactive and form Schiff bases with lysine residues on enzyme surfaces.

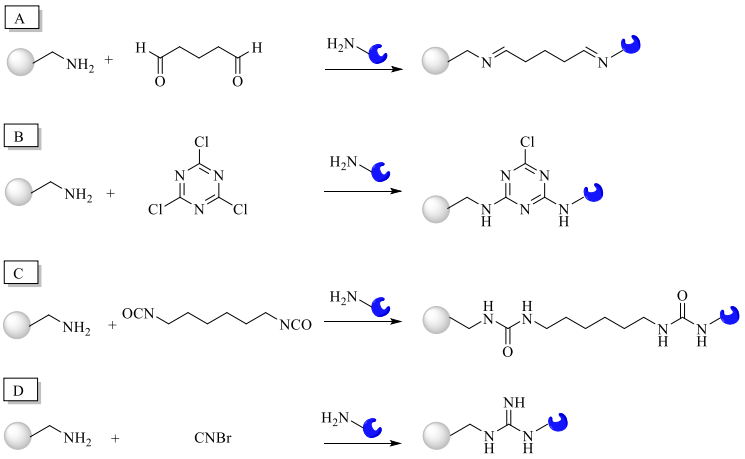


Fig.2. Some examples of covalent enzyme immobilizations on amine-functionalized carriers

Although the preparation of glutaraldehyde activated carriers is an easy process, the short-term stability of activated carriers is a major drawback. Many studies have shown that activated supports were freshly prepared prior enzyme immobilization.[9,10] Other bifunctional agents used for covalent enzyme immobilizations included cyanuric chloride, hexamethylene diisocyanate and cyanogen bromide (Fig.2B, 2C and 2D). However, industry needs a protocol and method resulting in stable and robust enzyme carrier. Epoxy activated supports are

7. F. Lopez-Gallego, L. Betancor, C. Mateo, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J.M. Guisan, R. Fernandez-Lafuente, *J. Biotechnol.*, **2005**, *119*, 70–75.
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9. H. Zaak, L. Fernandez-Lopez, C. Otero, M. Sassi, R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, **2017**, *106*, 67–74.
10. O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, *Process Biochem.*, **2012**, *47*, 1220–1227.

excellent options for immobilized biocatalyst preparation due to the stability of epoxy groups. Additionally, epoxy groups can react with other types of functional groups besides amine groups (thiol, aromatic hydroxyl group) and the excess of epoxy groups can be easily blocked by other types of nucleophiles (e.g. amino acids).[11] The presence of hydrophobic groups on surface is essential and promotes enzyme immobilization. Controlling the hydrophobic and epoxy group ratio, the carriers can be excellently fine-tuned for the immobilization of particular proteins.

3. EXPERIMENTAL SECTION

3.1. Immobilization of lipases (BcL or CaLB)

3.1.1. Immobilization of BcL by adsorption on mesoporous silica gel with mixed grafting

To a solution of *Bc*L (30.0 mg) in Tris buffer (14.3 mL, 100 mM, pH = 7.5, ionic strength controlled with NaCl) were added surface functionalized silica gel (300.0 mg), additive (30.0 mg) and 2-propanol (750 µL) as co-solvent. The mixture was shaken at 400 rpm at 4 °C for 24 h. The adsorbed *Bc*L biocatalyst was filtered off on a glass filter (G4), washed with distilled water (10 mL), phosphate buffer (10 mL, 20 mM, pH 7.2), 2-propanol (2 × 10 mL), *n*-hexane (10 mL), dried at room temperature (2 h) and stored at 4 °C.

3.1.2. Immobilization of CaLB on bisepoxide-activated aminoalkyl resins

*Ca*LB (40.0 mg, lyophilized powder) was dissolved in phosphate buffer (10.0 mL, 100 mM, pH 7.5), and then the support (200.0 mg) was added to the solution. The enzyme-support suspension was shaken at 400 rpm for 24 h at 25 °C. The immobilized *Ca*LB preparation was filtered off on a glass filter (G3), washed with isopropanol (2 × 15 mL) and *n*-hexane (15 mL), dried for 4 h at room temperature and stored at 4 °C. Protein concentration of the *Ca*LB solution before immobilization and in supernatant after immobilization were determined according to Bradford's method.[12] Immobilization yield (IY) was calculated according to equation IY (%) = $P/P_0 \times 100$ (where P_0 [mg mL^{-1}] is the initial protein concentration before immobilization, and P [mg mL^{-1}] is the protein concentration in supernatant after immobilization).

3.2. Immobilization of ω-transaminase on bisepoxide-activated aminoalkyl resins

In 1.5 mL Eppendorf tube purified transaminase (210 µL, 4.8 mg mL^{-1}) was diluted with HEPES buffer (790 µL, 50 mM, pH 7.0), and then the support (10.0 mg) was added to the solution. The enzyme:support ratio was kept constant at 1:10. Suspension was shaken at 900 rpm for 24 h at 25 °C in Eppendorf shaker. The immobilized transaminase preparation was centrifuged, washed with HEPES buffer (2 × 1.0 mL). Protein concentration in supernatant

11. M. Mihailović, M. Stojanović, K. Banjanac, M. Carević, N. Prlainović, N. Milosavić, D. Bezbradica, *Process Biochem.*, **2014**, *49*, 637–646.
12. M.M. Bradford, *Anal. Biochem.*, **1976**, *72*, 248–254.

was measured before and after immobilization on Nanodrop 2000. Immobilization yield (IY) was calculated according to equation IY (%) = $P/P_0 \times 100$ (where P_0 [mg mL^{-1}] is the initial protein concentration before immobilization, and P [mg mL^{-1}] is the protein concentration in supernatant after immobilization). After immobilization transaminase preparations were immediately used in kinetic resolutions.

3.3. Immobilization of whole-cells in templated sol-gel matrices

In the first step, the silica sol was prepared: 0.72 mL TEOS was added to a solution containing 65 µL 0.1 M HNO_3 and 0.25 mL distilled water, and the mixture was then sonicated for 5 min and cooled at 4 °C for 24 h. Then, 150 mg of MAT540 additive was added to the mixture of 3 mL cell paste solution (0.5 g of centrifuged cell paste –*E. coli* or *Le*KRED cells- dissolved in phosphate buffer, 3 mL, 100 mM, pH 7.5), and the suspension was hardly shaken until homogeneous. The prepared silica sol was mixed with the solution and was shaken for 5 min. The gelation occurred within 30 min at RT and the gel was aged at 4 °C for 48 h. Then the crude products were washed with phosphate buffer (2 × 15 mL, 100 mM, pH 7.5), centrifuged and dried at room temperature (24 h), and stored at 4 °C.

4. RESULTS AND DISCUSSION

4.1. Systematic study of the effect of additives enhancing catalytic properties of lipase from Burkholderia cepacia immobilized on mixed-function-grafted mesoporous silica gel

Adsorption and covalent binding of *Burkholderia cepacia* lipase (*Bc*L) was carried out in the presence of more than 40 additives as bioimprinting agents, stabilizers or surface coating polymers. The biocatalytic properties of immobilized *Bc*Ls were investigated in the KRs of racemic secondary alcohols 1-phenylethanol (*rac*-**1a**) and 1-(thiophen-2-yl)ethan-1-ol (*rac*-**1b**) in batch mode and in continuous-flow bioreactors (Scheme 1).

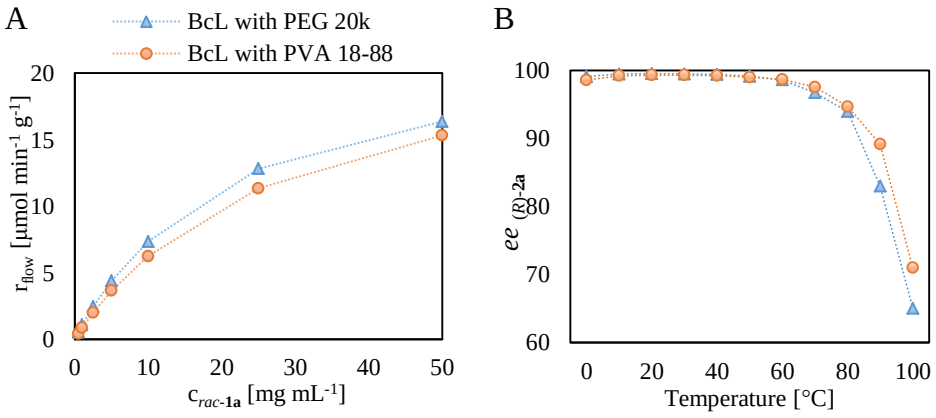
When direct adsorption of *Bc*L (without additive) was applied, conversion reached only 2.3% after 4 h of KR of *rac*-**1a**. Major impact on the activity had addition of polyvinyl alcohols (PVAs) and polyethylene glycols (PEGs). PVAs applied in the adsorption represented different molecular weight and hydrolysis degree. Addition of PVA 4-88 resulted in the most significant 11-fold activity enhancement. The most active adsorbed *Bc*L/PEG 20k biocatalyst had 13-fold higher enzyme activity in KR of *rac*-**1a** than *Bc*L adsorbed without any additives. The enantiomer selectivity of *Bc*L increased in each case. After performing the tests of adsorbed *Bc*Ls, covalent immobilization was also carried out on glycerol diglycidyl ether (GD)-activated silica gels.

Table 1. Biocatalytic properties of immobilized *Bc*Ls on mixed function-grafted mesoporous silica gel in the kinetic resolution of *rac*-**1a** in batch mode[a]

Additive	*c* (%)	$ee_{(R)\text{-}2a}$ (%)	*E*	U_b (μmol g^{-1} min^{-1})
-	2.3	98.1	>100	1.6
Tween 80	9.9	99.2	>200	6.7
PVA 4-88	26.1	99.5	»200	17.9
PVA 18-88	22.5	99.4	>200	15.4
PVA 13-23-88	25.8	99.3	>200	17.6
PVA 72-98	14.8	98.9	>200	10.1
PEG 8k	24.6	99.5	»200	16.8
PEG 20k	30.0	99.0	>200	20.5
Lauric acid	13.8	99.3	>200	9.4
Oleic acid	23.3	99.3	>200	15.9
Trilaurin	14.2	99.4	>200	9.7

[a] Reactions were performed at 30 °C for 4 h.

Two *Bc*Ls with PVA 18-88 and PEG 20k were selected to study the effect of substrate concentration and temperature on KR of *rac*-**1a** in continuous-flow PBRs (Fig.3A). The productivity of the two *Bc*L biocatalysts (r_{flow}, μmol g^{-1} min^{-1}) was investigated as a function of substrate concentration ($c_{rac\text{-}1a}$, mg mL^{-1}). Productivity – temperature profiles of the adsorbed *Bc*L / PEG 20k and *Bc*L / PVA 18-88 were different (Fig.3C). *Bc*L / PEG 20k had its optimum working temperature at 30 °C, so it started to lose its activity over 40 °C. On the other hand, *Bc*L / PVA 18-88 was thermostable up to 80 °C and deactivated only at higher temperatures. Both adsorbed *Bc*Ls showed maxima of *E* at certain temperature (Fig.3D). *Bc*L/PEG 20k exhibited higher enantiomer selectivity in the lower temperature range (0–50 °C) with a maximum at around 20 °C but selectivity decreased drastically over the breakdown temperature of this form (~30 °C). Enantiomer selectivity of *Bc*L/PVA 18-88 had a maximum at higher temperature (~30 °C) with a much slower monotonic decrease up to 100 °C. This phenomenon may be explained by assuming different solubility of the two additives. In case of PEG 20k, the additive has good solubility in the solvent (2/1 mixture of hexane/*tert*-butyl methyl ether) and thus dissolved out continuously from the biocatalyst resulting in rapid loss of its positive effects. On the other hand, the PVA 18-88 additive is nearly insoluble in non-aqueous media due intermolecular hydrogen bondings.[13]



13. P. Chetri, N.N. Dass, N.S. Sarma, *Chin. J. Polym. Sci.*, **2008**, *26*, 399–404.

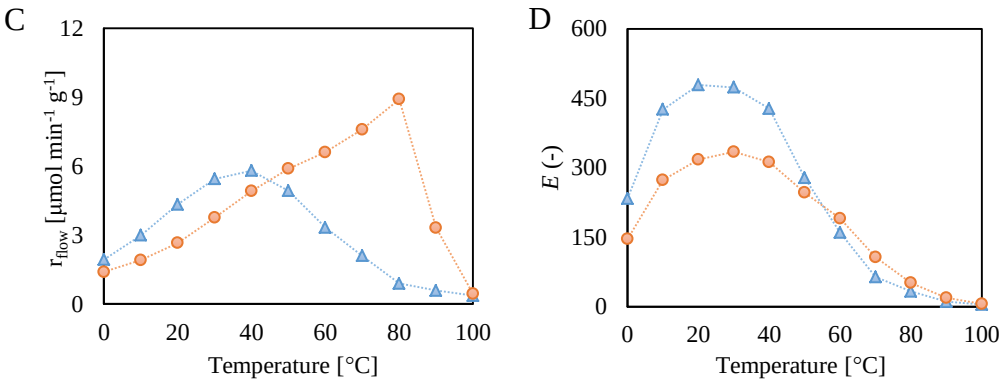


Fig.3. Kinetic resolutions of *rac*-**1a** continuous-flow PBR. Effect of the substrate concentration on specific reaction rate, r_{flow} (A) at 30 °C; temperature on enantiomeric excess, $ee_{(R)\text{-}\mathbf{2a}}$ (B), specific reaction rate, r_{flow} (C) and temperature on enantiomeric ratio, E (D) at concentration of 5 mg mL^{-1}.

4.2. Bisepoxide-activated aminoalkyl resins designed for covalent immobilization of Candida antarctica lipase B

Our aim was to activate the aminoalkyl supports with different bisepoxides for protein immobilizations. As already proved the efficacy of glycerol diglycidyl ether (GD) in enzyme immobilization, several bisepoxides bearing different length of spacer arms were selected for this study (Fig.4). After pre-activation, immobilization of *Ca*LB was carried out in phosphate buffer (pH 7.5) at RT for 24 h. To characterize the biocatalytic properties of the biocatalysts, kinetic resolution of *rac*-**1a** was selected as the test reaction as shown in Scheme 1. According to the results of batch reactions, use of bisepoxides in immobilization significantly altered the activity of covalently immobilized *Ca*LBs compared to *Ca*LBs immobilized by only adsorptive interactions (on unmodified resins). *Ca*LB immobilized on 1,4-cyclohexanedimethanol diglycidyl ether (CH)-, 1,6-hexanediol diglycidyl ether (HD)- and glycerol diglycidyl ether (GD)-activated resins displayed the highest activity. We have tested also the operational stability of these preparations in successive KRs. Although every biocatalyst used in this study lost its initial activity cycle-by-cycle, HAHD *Ca*LB retained 90% of its initial activity after 13 cycles. This demonstrated that spacer arms with proper length and hydrophobicity between enzyme molecule and support could contribute not only to the initial activity but also to the long-term stability of the immobilized enzyme.

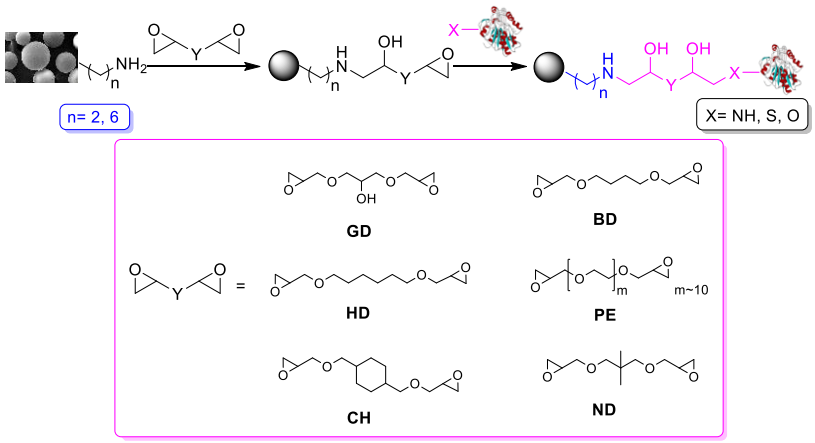


Fig. 4. Covalent immobilization of *Ca*LB on bisepoxide-activated resins

(Abbreviations of bisepoxides: GD: glycerol diglycidyl ether, HD: 1,6-hexanediol diglycidyl ether, CH: 1,4-cyclohexanedimethanol diglycidyl ether, BD: 1,4-butanediol diglycidyl ether, PE: poly(ethylene glycol)diglycidyl ether, ND: neopentyl glycol diglycidyl ether)

The thermal stability of *Ca*LBs linked to polymer resins was investigated in continuous-flow KR of *rac*-**1a** in bioreactors varying the temperature in the range of 0–110 °C in 10 °C steps. Productivity (r_{flow}) of the *Ca*LB derivatives as a function of temperature was compared to commercial CV *Ca*LB containing the lipase immobilized on epoxy-functionalized acrylic resin (Fig.5B). The r_{flow} of CV *Ca*LB increased only up to 70 °C, then a significant drop of enzyme activity was observed. The productivity of *Ca*LB immobilized on EA-resin activated by CH and GD bisepoxide increased almost linearly with increasing temperature up to 110 °C. The productivity of *Ca*LB immobilized *via* longer and more flexible linkers (the HD-activated EA-resin and the CH- or GD-activated HA-resins) increased up to 110 °C, although with a slowdown in the increase starting at around 70 °C. The *ee*–temperature (Fig.5C) and *E*–temperature (Fig.5D) curves exhibited maxima at various temperatures between 50 and 80 °C. Characteristic differences of enantiomer selectivity optima were found between *Ca*LB on bisepoxide activated EA-resins (~80 °C) compared to *Ca*LB on bisepoxide activated HA-resins (~60 °C) indicating that increased flexibility of the linker allows a lower degree of selectivity.

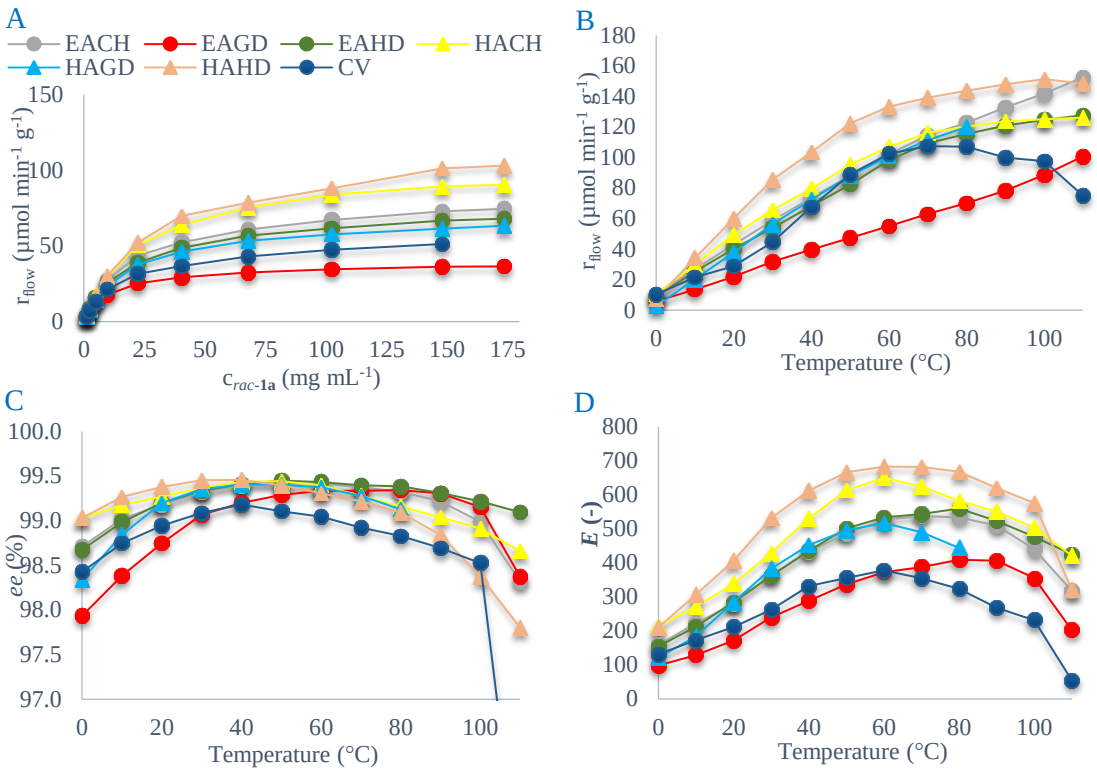


Fig.5. Continuous-flow kinetic resolution of *rac*-**1a** catalyzed by *Ca*LB immobilized on bisepoxide-activated resins. Biocatalytic properties (r_{flow}, *ee*, *E*) of immobilized *Ca*LB biocatalysts on ethylamine or hexylamine resin activated by bisepoxides (CH, GD or HD) compared to (**A**) Effect of the substrate concentration on specific reaction rate at 30 °C (r_{flow}); (**B**) Effect of temperature on specific reaction rate (r_{flow}); (**C**) Effect of temperature on enantiomeric excess (*ee*); (**D**) Effect of temperature on enantiomeric ratio (*E*) at 40 mg mL^{-1} substrate concentration.

4.3. Covalently immobilized Trp60Cys mutant of ω-transaminase from Chromobacterium violaceum for kinetic resolution of racemic amines in batch and continuous-flow modes

Bisepoxide modification of polymer resins is a cheap and easy-to-perform technique for fine tuning of the surface of enzyme carriers, therefore our aim was to prove, that the same carriers are perfect for the immobilization for a different type of enzyme (e.g. transaminase). Three different bisepoxides as activating agents were selected for the study: HD, CH having relatively long linkers of hydrophobic character and GD with a short and hydrophilic linker. *Cv*TA mutant W60C was chosen due to its improved activity, enantioselectivity and PLP-tolerance compared to the wild-type. Because transaminases operate mainly in aqueous environment, it is extremely important to prevent enzyme leaching from the support. The preparations were applied as biocatalysts in the KR of various racemic amines (Scheme 1).

As shown in Table 2, without surface modification, EA and HA could fix *Cv*TA$_{W60C}$ only by adsorption. *Cv*TA$_{W60C}$ attached to the commercial epoxy resin (EP) resulted in low conversion of *rac*-**3a**. The absence of spacer arms between the enzyme and the surface of EP carrier resulted in limited flexibility during catalysis by assuming multipoint attachment to the surface. The difference of c_{app} from c_{theor} could be explained by assuming selective adsorption of the product **4a** onto the surface of the resin. Surface modification of the aminoalkyl polymers with the hydrophilic GD resulted the most efficient carriers for *Cv*TA$_{W60C}$ ensuring high enzyme activity. Transaminase immobilized on the GD-activated resins (*Cv*TA$_{W60C}$-EAGD and *Cv*TA$_{W60C}$-HAGD, respectively) applied as biocatalysts in the KR of *rac*-**3a** resulted in high conversions (49%) and excellent enantiomeric excess ($ee_{(R)\text{-}\mathbf{3a}}$ >99%) after 4 h. When EAHD and HAHD were applied for binding *Cv*TA$_{W60C}$, the activity decreased but the enzyme molecule could maintain its flexibility. When further cyclohexyl rings were attached to the surface of the aminoalkyl resins (HACH), the even more hydrophobic nature of the linker could explain the further decreased activity of the enzyme (only c_{app} = 20%).

Table 2. Kinetic resolution of *rac*-**3a** catalyzed by differently immobilized *Cv*TA$_{W60C}$ preparations[a]

Support	c_{app} (%)[a]	c_{theor} (%)[b]	$ee_{(R)\text{-}\mathbf{3a}}$ (%)
EP	9	21	26
EA	13	15	17
EAGD	49	50	>99
EAHD	48	50	>99
EACH	41	44	77
HA	14	17	20
HAGD	49	50	>99
HAHD	30	32	48
HACH	20	22	29

[a] Reactions were performed at 30 °C for 4 h. [b] Deviation of the apparent and theoretical conversion are due to selective adsorption of ketones on polymeric supports.

After proper optimization of reaction parameters and investigation of co-solvent tolerance of immobilized *Cv*TA preparations, recyclability study was performed. *Cv*TA$_{W60C}$-EAGD and *Cv*TA$_{W60C}$-HAGD were successfully used in 19 consecutive cycles in KR of *rac*-**3a**. To perform also continuous-flow KRs, the sufficient amount of immobilized enzyme was

prepared by flow-through approach (Fig.6.). Bioreactors were filled with supports and enzyme solution was recirculated. The immobilization efficiency was monitored. Then the kinetic resolution of *rac*-**3a-c** was performed at preparative scale.

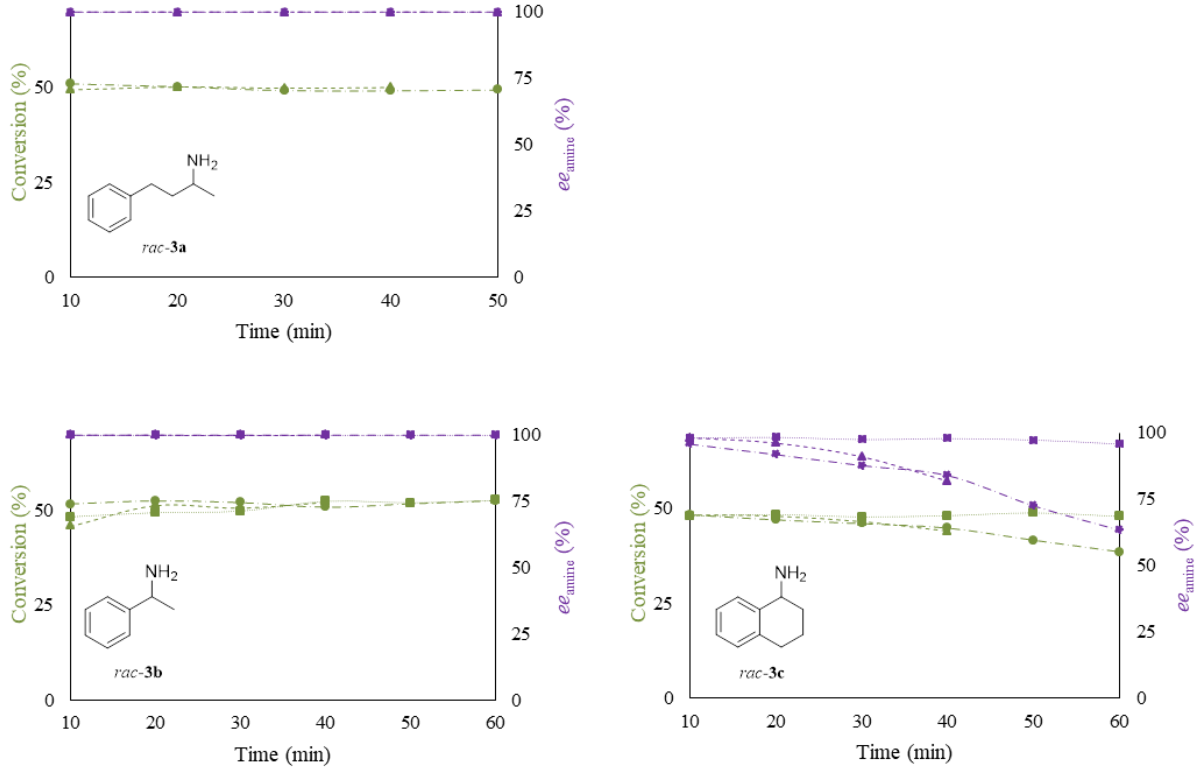
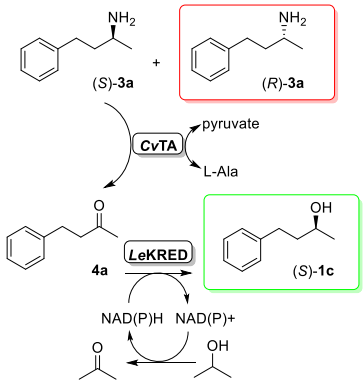


Fig.6. Continuous-flow kinetic resolutions of *rac*-**3a-c** at different flow rates in *Cv*TA$_{W60C}$-EAGD-filled packed-bed reactors

4.4. Immobilized E. coli cells containing transaminase and ketoreductase whole-cells for continuous-flow synthesis of enantiopure amines and alcohols

After developing immobilization processes for enzymes, our aim was to improve a sol-gel technique for whole-cell immobilization and continuous-flow application. As a first step, we determined the proper immobilization conditions for *E. coli* containing transaminase and *Le*KRED cells. In the presence of hollow silica gel and cells, acid-catalyzed hydrolysis of tetraethyl orthosilicate (TEOS) was followed by polycondensation. The activity of cells was evaluated in the kinetic resolution of racemic 4-phenylbutan-2-amine (*rac*-**3a**). In the first reaction catalyzed by *Cv*TA, (*S*)-**3a** was converted to ketone **4a**, the residual enantiomer (*R*)-**3a** remained in enantiopure form. In the second step catalyzed by *Le*KRED, **4a** was converted stereoselectively to (*S*)-**1c**.



Scheme 2. Cascade reaction employing immobilized transaminase and ketoreductase whole-cells

After composing an efficient system of immobilized whole-cells, KR followed by asymmetric reduction was carried out in continuous-flow mode. Two columns filled separately with *Cv*TA and *Le*KRED were connected in line and substrate solution (*rac*-**3a**) was pumped through the columns at 30 °C and at appropriate flow rate (50 µl min^{-1}). Cofactor (PLP) for *Cv*TA and isopropyl alcohol as hydrogen source for NADH regeneration was added to the solution. Reactions were performed at 30 °C for 24 h and conversion was determined based on alcohol formation. After determining the appropriate reaction conditions (flow rate 50 µl min^{-1}), flow synthesis of (*R*)-**3a** and (*S*)-**1c** was continued for 1 day. Results are shown in Fig.7. The first 3 h of biotransformation was fast without significant loss of activity. However, after 22 h reaction accumulation of ketone by-product led to lowering of conversion ($c_{(S)\text{-}\mathbf{1c}}$=40%). Stereoselectivity of transaminase ($ee_{(R)\text{-}\mathbf{3a}}$>99%) cells remained high.

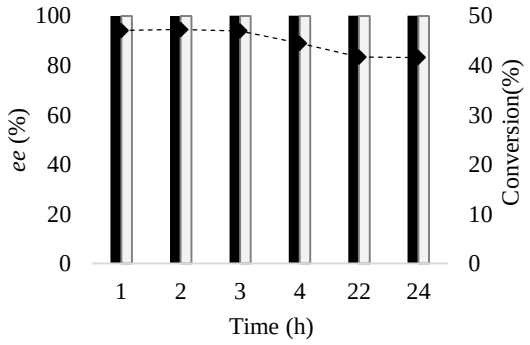


Fig.7. Operational stability of immobilized *Cv*TA and *Le*KRED whole-cells under continuous-flow conditions at 30 °C ($ee_{(R)\text{-}\mathbf{3a}}$ ■, $ee_{(S)\text{-}\mathbf{1c}}$ ■, conversion: ◆ $c_{(S)\text{-}\mathbf{1c}}$)

Additionally, we performed the KR with *E. coli* cells under the same reaction conditions without the addition of PLP cofactor (flow rate 50 µl min^{-1} at 30 °C). After 22 hours, the conversion and enantiomeric excess of amine dropped. During the flow synthesis, the cofactor continuously washed out from biocatalyst preparation and after one day of operation almost no cofactor was left in the system. Without the cofactor, the enzyme may dissociate to inactive monomer which can lead to loss of activity. We have investigated the reactivation of already "exhausted" biocatalyst. For the next two hours, the biocatalyst was washed with PLP cofactor at 30 °C and the KR of *rac*-**3a** was performed again in the presence of PLP. After the re-activation of *Cv*TA, the biocatalyst regained its catalytic activity and performed well in the KR of *rac*-**3a.**

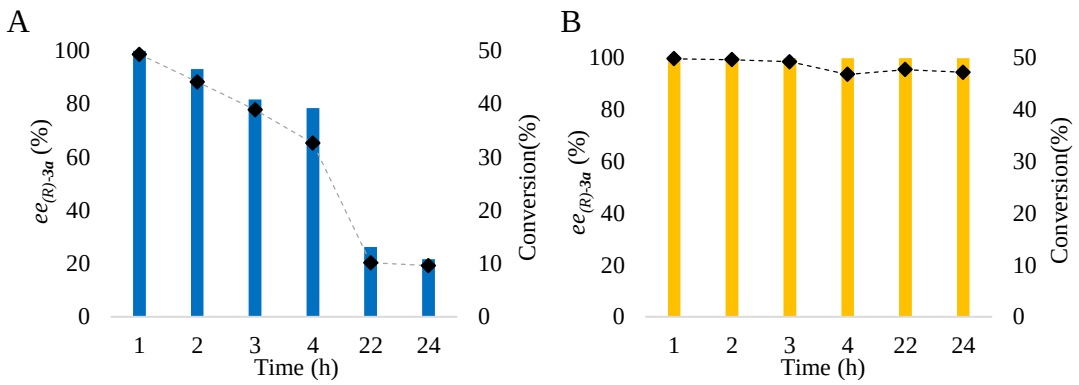


Fig.8. Kinetic resolution of *rac*-**3a** catalyzed by immobilized *E. coli* cells containing *Cv*TA without addition of PLP cofactor (A: $ee_{(R)\text{-}3a}$; ◆ c_{4a}) and after the reactivation with PLP (B: $ee_{(R)\text{-}3a}$; ◆ c_{4a})

5. THESIS POINTS

I. Adsorption and covalent immobilization of *Burkholderia cepacia* lipase was successfully carried out onto mixed-function-grafted mesoporous silica gels (phenyl-aminopropyl) in the presence of more than 40 additives. Thermotolerance of the *Bc*L / poly(vinyl alcohol) and *Bc*L / polyethylene glycol biocatalysts was examined in batch and continuous-flow kinetic resolution of racemic 1-phenylethanol as well. I have found a remarkable 50 °C increase of optimum temperature of lipase activity for *Bc*L co-immobilized with poly(vinyl alcohol) compared to *Bc*L co-immobilized with polyethylene glycol. [1]

II. We designed the covalent immobilization of *Candida antarctica* lipase B on aminoalkyl (ethylamine and hexylamine) polymer resins activated with different bisepoxides with various length of carbon chain and hydrophobicity. I have found that activation with 1,4-cyclohexanedimethanol diglycidyl ether with hydrophobic character significantly improved the stability of immobilized *Ca*LB. Continuous-flow kinetic resolution of racemic 1-phenylethanol revealed that *Ca*LBs immobilized on activated ethylamine resins had higher thermostability, while *Ca*LBs immobilized on activated hexylamine resins displayed higher specific activity and selectivity. [2]

III. We carried out the covalent immobilization of an improved ω-transaminase mutant of *Chromobacterium violaceum* on bisepoxide-activated aminoalkyl (ethylamine and hexylamine) resins. I proved that the transaminase immobilized on glycerol diglycidyl ether-activated resin with hydrophilic character retained its activity during several consecutive kinetic resolutions of racemic 4-phenylbutan-2-amine. The immobilized enzyme had remarkable organic solvent tolerance and it was active in media containing up to 50% v/v DMSO. [3]

IV. We have successfully carried out the immobilization of *E. coli* whole-cells containing *Chromobacterium violaceum* transaminase in sol-gel matrices templated with silica microspheres. I proved that *Cv*TA containing whole-cell biocatalyst is compatible with *Lodderomyces elongisporus* whole-cells with ketoreductase activity. The immobilized whole-cell biocatalysts were successfully applied in continuous-flow cascade reaction of 4-phenylbutan-2-amine for the preparation of enantiomerically pure amine and alcohol. [4]

6. POTENTIAL APPLICATIONS

Lipases as efficient biocatalysts in the preparation of enantiomerically pure esters already strengthened their position in industry. Therefore, we have investigated the covalent immobilization of lipases from bacterial (*Burkholderia cepacia*) and yeast origin (*Candida antarctica* lipase B). Bisepoxides containing different hydrophobic and hydrophilic moieties could serve as novel "2 in 1" agents for fine tuning and activating the surface of resins for enzyme immobilization. The prepared biocatalyst showed high stability and selectivity in the KR of racemic alcohols. In addition, we have proved that the bisepoxide-activated aminoalkyl resins can be used for the immobilization of different type of enzyme (e.g. *Chromobacterium violaceum* transaminase). At industrial scale, whole-cells may be also used in addition to purified enzymes. We proved that for immobilization of whole-cells templated silica matrix may be used. Cells can be directly immobilized after fermentation without the need for lyophilization which can significantly shorten the time between fermentation process and application of cells.

7. PUBLICATIONS

Scientific achievements of the Thesis are based on following articles:

[1] Emese Abaházi, Zoltán Boros, László Poppe, Additives Enhancing the Catalytic Properties of Lipase from *Burkholderia cepacia* Immobilized on Mixed-Function-Grafted Mesoporous Silica Gel, *Molecules*, **2014**, *19*(7), 9818–9837. IF: 2.416, contribution by the author: 70%.

[2] Emese Abaházi, Dávid Lestál, Zoltán Boros, László Poppe, Tailoring the Spacer Arm for Covalent Immobilization of *Candida antarctica* Lipase B—Thermal Stabilization by Bisepoxide-Activated Aminoalkyl Resins in Continuous-Flow Reactors, *Molecules*, **2016**, *21*(6), 767. IF: 2.861, contribution by the author: 60%.

[3] Emese Abaházi, Péter Sátorhelyi, Balázs Erdélyi, Beáta G. Vértessy, Henrik Land, Csaba Paizs, Per Berglund, László Poppe, Covalently immobilized Trp60Cys mutant of ω-transaminase from *Chromobacterium violaceum* for kinetic resolution of racemic amines in batch and continuous-flow modes, *Biochemical Engineering Journal*, **2018**, *132*, 270–278. IF: 2.892, contribution by the author: 100%.

[4] László Nagy-Győr, Emese Abaházi, Viktória Bódai, Péter Sátorhelyi, Balázs Erdélyi, Diána Balogh-Weiser, Csaba Paizs, Gábor Hornyánszky, László Poppe, Co-immobilized whole-cells with ω-transaminase and ketoreductase activity for continuous-flow cascade reaction, *ChemBioChem*, **2018**, doi:10.1002/cbic.201800286, IF: 2.847, contribution by the author: 49%.

Articles not included in the Thesis:

[5] Z. Boros, E. Abaháziová, D. Weiser, P. Kovács, A. Varga, Z. Valentin, L. Poppe, *Studia Universitas Babes-Bolyai Chemia*, **2014**, *59*, 33–38.

[6] Z. Boros, D. Weiser, M. Márkus, E. Abaháziová, Á. Magyar, A. Tomin, B. Koczka, P. Kovács, L. Poppe, *Process Biochem.*, **2013**, *48*, 1039–1047. IF: 2.524.

[7] Z. Boros, E. Abaháziová, M. Oláh, P. Sátorhelyi, B. Erdélyi, L. Poppe, *Chimica Oggi-Chemistry Today*, **2012**, *30*, 26–29. IF: 0.539.

[8] E. Farkas, E. Abaházi, L. Nagy-Győr, Z. Szakács, J. Kóti, P. Sátorhelyi, J. Éles, L. Poppe, G. Hornyánszky, XL. Kémiai Előadói Napok, Conference Book (ISBN: 978-963-9970-83-0), **2017.**

Lectures:

1. Z. Boros, E. Abaházi, L. Nagy-Győr, M. Oláh, P. Sátorhelyi, B. Erdélyi, Poppe L., XVIII. Nemzetközi Vegyészkonferencia, 22-25 November **2012**, Félixfürdő, Romania

2. Z. Boros, E. Abaházi, M. Oláh, L. Nagy-Győr, P. Falus, V. Bódai, P. Sátorhelyi, B. Erdélyi, Gy. Szakács, L. Poppe, MKE Vegyészkonferencia, 26-28 June **2013**, Hajdúszoboszló, Hungary

3. Z. Boros, M. Oláh, E. Abaházi, L. Nagy-Győr, P. Falus, V. Bódai, P. Sátorhelyi, B. Erdélyi, K. Kovács, D. Weiser, B. G. Vértessy, L. Poppe, COST CM1303 „SysBiocat" Kick-off Workshop, 10-11 April **2014**, Madrid, Spain

4. E. Abaházi, L. Poppe, Spring Wind Conference, 15-16 April **2016**, Budapest, Hungary

5. E. Farkas, E. Abaházi, L. Nagy-Győr, Z. Szakács, J. Kóti, P. Sátorhelyi, J. Éles, L. Poppe, G. Hornyánszky, XL. Kémiai Előadói Napok, 16-18 October **2017**, Szeged, Hungary

Posters:

1. Z. Boros, P. Falus, D. Weiser, K. Kovács, G. Hellner, M. Márkus, E. Abaházi, M. Oláh, B. G. Vértessy, L. Poppe, biocat2012 – 6th International Congress on Biocatalysis, 2-6 September **2012**, Hamburg, Germany

2. E. Abaházi, Z. Boros, L. Poppe, MKE Vegyészkonferencia, 26-28 June **2013**, Hajdúszoboszló, Hungary

3. Z. Boros, E. Abaházi, M. Oláh, L. Nagy-Győr, P. Falus, V. Bódai, P. Sátorhelyi, B. Erdélyi, L. Poppe, Biotrans, 11th International Symposium on Biocatalysis, 21 - 25 July **2013**, Manchester, United Kingdom

4. E.Abaházi, Z. Boros, L. Poppe, COST SysBiocat Training School, 28 May - 1 June **2014**, Certosa di Pontignano, Italy

5. Z. Boros, P. Falus, D. Weiser, M. Oláh, E. Abaházi, L. Nagy-Győr, V. Bódai, P. Sátorhelyi, B. Erdélyi, L. Poppe, COST SysBiocat Training School, 28 May - 1 June **2014**, Certosa di Pontignano, Italy

6. E. Abaházi, D. Lestál, Z. Boros, L. Poppe, XIIIth Conference of George Oláh PhD School, 11 February **2016**, Budapest, Hungary

7. L. Nagy-Győr, E. Abaházi, V. Bódai, P. Sátorhelyi, B. Erdélyi, G. Hornyánszky, L. Poppe, BioTrans, 13th International Symposium on Biocatalysis and Biotransformations, 9 - 13 July **2017**, Budapest, Hungary

8. E. Farkas, E. Abaházi, L. Poppe, BioTrans, 13th International Symposium on Biocatalysis and Biotransformations, 9 - 13 July, **2017**, Budapest, Hungary

9. E. Farkas, E. Abaházi, L. Nagy-Győr, D.J. Incze, Z. Szakács, J. Kóti, P. Sátorhelyi, J. Éles, L. Poppe, G. Hornyánszky, XVth Conference of George Oláh PhD School, 01 February **2018**, Budapest, Hungary