DEVELOPMENT OF IMMOBILIZED BIOCATALYSTS FOR SELECTIVE BIOTRANSFORMATIONS

PhD THESIS

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2018
DECLARATION

I, Emese Abaházi hereby declare, that this PhD thesis is my own work and I have used in this thesis only references listed in the list. Any parts that I have taken literally or in the same content but reworded from other sources are clearly indicated.

Budapest, 02 September 2018.

………………………………

NYILATKOZAT

Alulírott Abaházi Emese kijelentem, hogy ezt a doktori értekezést magam készíttem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltém.


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First, I would like to express my huge gratitude to my supervisor Prof. Dr. László Poppe, who has been helping me in the past seven years. Thank you for your motivation, guidance and endless discussions on paper writing and laboratory work planning.

Besides my professor, I would like to thank my former consultant Zoltán Boros for his precious advise, enormous help during my PhD years. And of course, thank you for your friendship and the holidays we spent together.

I would like to thank for financial support of Gedeon Richter’s Talentum Foundation, Pro Progressio Foundation and Varga József Foundation.

I thank to Fermentia company for the large scale fermentations and help in paper writing.

I would like to say a special thanks for Márk Oláh and László Nagy-Győr. Guys, without you the years of PhD would have not been the same. Thank you for this precious time of my life. Thanks for my fellow labmates at Bioorganic Research Group: Flóra Nagy, Emese Farkas, Bianka Szokol, Zsófia Bata, Gergő Bánóczy, Pál Csuka, Evelin Bell, Dr. Diána Balogh, Edit Tóth-Németh, Rita Molnárné Bernáth, and to my former colleagues in Déli II. laboratory: Attila Kalocsai, Ádám Czerman, Eszter Kókai, Péter Falus, Balázs Komjáti.

I thank to my students, Dávid Lestál, Renáta Horváth, Péter Kun, László Harsányi, Judit Lévai, who significantly contributed with their conscientious work to my PhD research. Thank you for your continuous curiosity which helped me to became a more mature person.

I thank my friends: Enikő Sipkó, Eszter Szaszák, Zsófia Marsi and Tímea Törekes for being my support during these years. Especially Enikő, thank you for listening me and sharing the room and flat with me. Girls, thank you for the time we spent together, it meant a lot to me.

És végül, de egyáltalán nem utolsó sorban, köszönöm Szüleinnek és bátyánnak, Krisztiánnak a támogatásukat és szeretetüket, mellyel elhalmoztak. Köszönöm, hogy bármikor számíthattam rájuk és minden gondot igyekeztek megoldani.
ABBREVIATIONS

ADH       alcohol dehydrogenase
AlaDH     alanine dehydrogenase
ApTMS    (3-aminopropyl) trimethoxysilane
BD        1,4-butanediol diglycidyl ether
BcL       *Burkholderia cepacia* lipase
c        conversion (%)
CaLB      *Candida antarctica* lipase B
CH        1,4-cyclohexanediol diglycidyl ether
CIP       Cahn-Ingold-Prelog
CV        *CaLB* immobilized on Chiral Vision carrier
CvTA      *Chromobacterium violaceum* transaminase
CvTAw60C  *Chromobacterium violaceum* transaminase mutant
Cys       cysteine
DMSO      dimethyl sulfoxide
E         enantiomeric ratio (−)
E          enzyme
E. coli   *Escherichia coli*
EA        ReliZyme™ EA403 (EA) ethylamine carrier
ee        enantiomeric excess
EP        ReliZyme™ EP403/S epoxy carrier
ES        enzyme-substrate complex
FAD       flavin adenine dinucleotide
GD        glycerol diglycidyl ether
GA        glutaraldehyde
GC        gas chromatography
HD        1,6-hexanediol diglycidyl ether
HA        ReliZyme™ HA403 (HA) hexylamine carrier
HEPES     4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IUBMB     International Union of Biochemistry and Molecular Biology
KR        kinetic resolution
KRED      ketoreductase
LeKRED    *Lodderomyces elongisporus* ketoreductase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MAT540</td>
<td>hollow silica microsphere with 3-aminopropyl and vinyl functions</td>
</tr>
<tr>
<td>ND</td>
<td>neopentyl glycol diglycidyl ether</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NME</td>
<td>New Molecular Entity</td>
</tr>
<tr>
<td>P</td>
<td>product</td>
</tr>
<tr>
<td>PBR</td>
<td>packed bed reactor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PDC</td>
<td>pyruvate decarboxylase</td>
</tr>
<tr>
<td>PE</td>
<td>poly(ethylene glycol)diglycidyl ether</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PMP</td>
<td>pyridoxamine phosphate</td>
</tr>
<tr>
<td>PTMS</td>
<td>phenyltrimethoxysilane</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>( r_{flow} )</td>
<td>specific reaction rate (continuous-flow) ((\mu\text{mol min}^{-1} \text{g}^{-1}))</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature (°C)</td>
</tr>
<tr>
<td>S</td>
<td>substrate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>T</td>
<td>temperature (°C)</td>
</tr>
<tr>
<td>TA</td>
<td>transaminase</td>
</tr>
<tr>
<td>TEOS</td>
<td>tetraethyl orthosilicate</td>
</tr>
<tr>
<td>TMOS</td>
<td>tetramethyl orthosilicate</td>
</tr>
<tr>
<td>( t_R )</td>
<td>residence time (min)</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>( \omega)-TA</td>
<td>(\omega)-transaminase</td>
</tr>
<tr>
<td>( U_b )</td>
<td>specific enzyme activity ((\mu\text{mol min}^{-1} \text{g}^{-1}))</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>( Y_s )</td>
<td>space time yield (g L^{-1} h^{-1})</td>
</tr>
</tbody>
</table>
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 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.
2. LITERATURE OVERVIEW

2.1. Significance of chirality in pharmaceutical industry

Discovery of molecular chirality dates back to 1848, when a French chemist and biologist Louis Pasteur separated enantiomers of sodium ammonium tartrate.³ It took a century for researchers to find out that chirality plays an extremely important role in drug-receptor interactions.⁴ Chirality is a geometric property of molecules. If three different substituents link to the central carbon atom, two mirror images of molecules can be distinguished. These two non-superimposable mirror-image forms of chiral molecules are called enantiomers.⁴ According to Cahn-Ingold-Prelog (CIP) convention, these four substituents bear different priorities. They can be counted based on these priorities clockwise [(R)-enantiomer] or anticlockwise [(S)-enantiomer]. An equimolar mixture of enantiomers is called racemate (racemic mixture).

![Diagram of Thalidomide enantiomers](image)

**Fig.2.-1.** Enantiomers of Thalidomide (Contergan). (R)-Enantiomer is teratogenic; while (S)-enantiomer is a sedative.

In the late 1950s and early 1960s, a chiral drug Thalidomide (Contergan) was prescribed for pregnant women as a slight sedative.⁵ However, Contergan was marketed as a racemic mixture of (R)- and (S)-enantiomers. When it was found that (S)-enantiomer has teratogenic side effects and caused birth defects, stricter regulations were introduced in pharmaceutical industry.⁶ Worldwide approvals of New Molecular Entities (NME) changed in the past ten years as shown in Fig.2.-2. In 2001, ~30% of approved drugs were marketed as achiral or racemates while in 2010 racemates almost diminished from drug market.⁷
Nowadays leading chiral NMEs are single enantiomers which can be prepared on an industrial scale utilizing different methods: resolution of racemates, chiral pool approach or enantioselective catalysis.  

Resolution of racemates can be carried out by classical crystallization of diastereomeric adducts or by kinetic resolution of racemic mixture. Although classical diastereomeric salt crystallization is a still predominant method in the separation of enantiomers, significant yield loss and high costs are associated with this technique. Chemical methods for the drug production are often inadequate: contamination of product with metals may occur, or the synthetized organocatalyst may have low enantioselectivity. Kinetic resolution can be achieved with chiral catalyst: enzyme or organocatalyst. Enzyme catalyzed kinetic resolution or enantioselective syntheses are now practical alternatives to traditional organic synthesis. Hydrolases or redox biocatalysts are frequently used on industrial scale. Enantiomerically pure alcohols or amines can be produced in the kinetic resolution of racemic mixture followed by enantiomer separation via physical processes.

Drugs containing chiral alcohol or amine moieties are presented in Fig.2.-3. In recent years, enantioselective biocatalytic route (ω-transaminase catalyzed synthesis) has been developed to obtain Sitagliptin (an antidiabetic drug). Synthetic pathway involved asymmetric hydrogenation of an enamine at high pressure using a rhodium-based chiral catalyst.
Biocatalysis has already demonstrated its enormous potential in the synthesis of enantiopure chiral compounds. This continuously evolving field can eliminate its drawbacks with utilization of enzyme engineering methods and meet the pharmaceutical regulation and legislation.⁹
2.2. Enzymes – catalytic biopolymers

Enzyme is a device of nature: catalytic polypeptide chain in a three-dimensional structure. During their evolutionary process, they adapted to their natural surroundings. These biopolymers are able to catalyze cata-anabolic processes of the living cells under neutral pH (pH 5-8) and mild temperature (20-40°C). Enzymes do not alter the position of equilibrium between substrates and products but accelerate the rate of reaction by lowering the activation energy of a reaction compared to the non-catalyzed reaction. Enzyme-catalyzed processes can be faster by $10^8$-$10^{10}$ times than corresponding non-catalyzed reactions. Biocatalyst are only needed in a small amount and remain unchanged in the end of the reaction.

![Energy diagram of non-catalyzed and enzyme-catalyzed reactions](image)

**Fig. 2.4.** Energy diagram of non-catalyzed and enzyme-catalyzed reactions

2.3. Enzyme structure and classification

Majority of enzymes are proteins (also catalytic RNAs are known), macromolecules built from amino acids linked by peptide bonds. Primary structure of proteins is determined by its amino acid sequence. Polar groups of amino acids (–OH, –NH$_3^+$, –COO$^-$, –SH…) take place on the surface of macromolecule and are hydrated in aqueous environment. Nonpolar alkyl chains are facing inside the globular structure of cytosolic protein forming the hydrophobic core.

Linear polymer of amino acids is folded into three-dimensional structure containing local segments: right handed α-helices and β-pleated sheets. These local segments are called secondary structure of protein and they are linked with loops to each other. The structure of these local motifs is stabilized via secondary interactions (hydrophobic, hydrogen bonding, disulfide bonds) and fold up further in three-dimensional structure, so called tertiary structure of protein. In many cases, proteins may contain smaller subunits from individual polypeptide chains forming the quaternary structure of protein. When these subunits are built up from the same polypeptide chains, protein is a homomultimer, distinct polypeptide chains form heteromultimer.
The International Union of Biochemistry and Molecular Biology (IUBMB) has established the nomenclature of enzymes.\(^{17}\) In general, name of the enzyme is derived from its chemical reaction it catalyzes with the word ending in \(\text{-ase}\).

For identification, enzymes have a four-digit number in general form: EC A.B.C.D. EC stands for Enzyme Commission, A represents the major class of enzyme, B codes the subtype, C indicates the nature of the co-substrate and D is an individual number for the enzyme.\(^{13}\) The major classes of enzymes are displayed in Table 2.-1.

### Table 2.-1. Classification of enzymes\(^{13}\)

<table>
<thead>
<tr>
<th>Enzyme class</th>
<th>Examples</th>
<th>Reaction catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidoreductases</strong></td>
<td>dehydrogenase</td>
<td>oxidation or reduction</td>
</tr>
<tr>
<td><strong>Hydrolases</strong></td>
<td>lipase, phosphatase</td>
<td>hydrolysis reaction in water, or formation of ester bonds in organic solvents</td>
</tr>
<tr>
<td><strong>Transferases</strong></td>
<td>transaminase</td>
<td>transfer of a functional group between substrate molecules</td>
</tr>
<tr>
<td><strong>Lyases</strong></td>
<td>phenylalanine ammonia lyase</td>
<td>addition or elimination of small molecules on C=C, C=N, C=O bonds</td>
</tr>
<tr>
<td><strong>Isomerases</strong></td>
<td>racemase</td>
<td>intramolecular rearrangement</td>
</tr>
<tr>
<td><strong>Ligases</strong></td>
<td>carboxylase</td>
<td>formation or cleavage of C-O, C-S, C-N, C-C bonds</td>
</tr>
</tbody>
</table>

Some enzymes need cofactors or coenzymes for their catalytic activity. In most cases, cofactors and enzyme molecules have only non-covalent interactions; however, there are some examples within transaminases binding pyridoxal 5’-phosphate cofactor covalently.

When utilizing enzymes in chemical reactions, their most important advantage is selectivity.\(^{18}\) Selectivity may be substrate-selectivity (distinction between different substrates in a mixture) or product-selectivity (differentiation between constitutionally identical groups or stereoheterotopic faces). Enzymes can utilize different types of selectivities:

- **chemoselectivity**: if substrate contains different functional groups of similar chemical reactivity; enzyme can selectively convert one of them.
- **regioselectivity**: substrate molecule may contain identical functional groups at constitutionally different positions (e.g. sugars with hydroxyl groups). Enzyme can distinguish between them and can regioselectively convert one of them.\(^{19}\)
- **diastereomer or diastereotopic selectivity**: enzymes can prefer one diastereomer from a stereoisomeric mixture or can distinguish between diastereotopic groups or faces.
- enantiomer or enantiotopic selectivity: enzymes are chiral biocatalyst, thus can prefer one enantiomer from racemic mixture (i.e. kinetic resolution) or one of the two enantiotopic groups or faces (i.e. asymmetric synthesis).

Relative use of enzymes depends on several factors: significance of product produced by enzyme, accessibility and stability of enzyme. Certain enzymes displaying poor stability can be improved before the industrial application. Scientists are constantly expanding the area of recombinant DNA techniques and biocatalyst engineering.

Industrial application of enzymes is illustrated in Fig.2.-5. Nearly two third of industrial enzymes are hydrolases used mainly in detergents, biodiesel production and food industry. Lipases significantly contribute to the enhancing of food flavor and fragrance. As detergent additives also proteases, lipases, oxidases, amylases and peroxidases are used; which are active at thermophilic temperatures and alkaline pH. One quarter of industrial enzymes represent oxidoreductases, other enzymes (transferases, lyases, ligases and isomerases) display only a small amount of industrially relevant enzymes.

![Distribution of enzyme classes according to industrial use (2006)](image)

**Fig.2.-5.** Distribution of enzyme classes according to industrial use (2006)

### 2.4. Mechanism of enzyme catalyzed reactions

In the past few decades, numerous studies and theories were elaborated regarding the mechanism of enzymatic reactions. The best-known models of enzymatic catalysis are the “Lock-and-key” model and the Induced-fit model.

The first model of enzymatic mechanisms was developed by E. Fischer in 1894. In the lock-and-key mechanism, enzyme has a rigid structure which can only accept substrates suitable for its active site. (Fig.2.-6.) According to this mechanism, binding occurs when the substrate is electrostatically complementary to the active site and no repulsive forces arise. However, this
model was not able to explain why enzymes could convert larger substrates, or even non-natural engineered substrates with bulky substituents.

![Fig.2.-6. Lock-and-key mechanism (the active site of enzyme is rigid structure)](image)

In the 1960’s, Koshland Jr. developed the induced-fit model which takes into account that enzymes are soft structures and can alter their conformation upon binding substrate molecule (Fig.2.-7).\(^{13}\) It also explains why the presence of some additional functional groups (hydrogen bonding, hydrophobic interactions etc.) is essential to bind the substrates.

![Fig.2.-7. Induced-fit model (the active site of enzyme can accommodate different substrate structures)](image)

### 2.5. Enzyme kinetics

Biocatalysis can be explained in a simple way: when substrate molecule binds to the active site of protein, they form an enzyme-substrate complex (ES\(^*\)). At the end of the reaction, this complex can further dissociate to product (P) and enzyme (E) or to substrate (S) and enzyme without reaction. In 1902, Brown investigated enzyme-catalyzed reactions and provided qualitative explanation of substrate dependence of reactions.\(^ {23} \)

\[
E + S \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \quad ES^* \quad \underset{k_2}{\rightarrow} \quad P + E
\]

The reaction velocity is proportional to enzyme-substrate complex and can be described as follows:

\[
v = k_2[ES^*] \quad \text{Eq. 1.}
\]

When mixing substrate solution with enzyme molecules and measuring product formation and substrate depletion as a function of time, we can observe progression curves shown in Fig.2.-8. At the beginning of reaction, substrate concentration changes rapidly with time giving first order
kinetics. For a short period of time at an initial rate, product formation is linear but after a certain time interval shows saturation.

Michaelis and Menten characterized enzymatic catalysis from mathematical perspective. Michaelis-Menten kinetics is one of the best-known models in enzyme catalysis. This approach supposes rapid equilibrium setting between the reactants (E and S) and ES complex:

\[
E + S \overset{K_S}{\rightleftharpoons} ES^* \overset{k_{cat}}{\rightarrow} P + E
\]

Seventeen enzymatic assays are available for the initial rate measurement of enzyme-catalyzed reactions at different substrate concentration. Fig.2.-9 shows initial velocity of enzyme catalyzed reactions at increasing substrate concentrations. In the beginning, bimolecular reaction occurs between active site of enzyme and substrate molecules. Velocity of reaction increases linearly with increasing substrate concentration. After a certain time interval, active sites of enzyme molecules become saturated with substrate molecules reaching a maximal velocity of reaction (\(V_{max}\)).
$V_{\text{max}}$ can be mathematically expressed as the multiplication of enzyme concentration with turnover number ($k_{\text{cat}}$). It defines the number of substrate molecules converted by the enzyme in a given period of time. Unit of $k_{\text{cat}}$ is reciprocal time (min$^{-1}$).

$$V_{\text{max}} = k_{\text{cat}}[E] \quad \text{Eq. 2.}$$

Michaelis-Menten equation describes how the rate of enzymatic reaction depends from substrate concentration:

$$v = V_{\text{max}} \frac{[S]}{K_M + [S]} \quad \text{Eq. 3.}$$

$K_M$ is the substrate concentration at which reaction rate is half of $V_{\text{max}}$. At low substrate concentration ($[S] < K_M$), $[S]$ is negligible from denominator, so reaction rate varies linearly with substrate concentration (first order kinetics). At relatively high substrate concentration $K_M$ is negligible, so reaction rate is independent on substrate concentration (zero order kinetics). Ratio of kinetic constants ($k_{\text{cat}}/K_M$) is used for comparison of catalytic efficiency of different enzymes towards substrate or for different substrates for the same enzyme.

Linearization of Michaelis-Menten kinetics helps visualization of kinetic data. This double reciprocal plot is a good graphical method for determination of $K_M$ and $V_{\text{max}}$. If reciprocal initial velocities are represented as a function of reciprocal substrate concentration, we obtain a linear plot. Slope of the line determines $K_M/V_{\text{max}}$ and intercept of the x axis and y axis determines $-1/K_M$ and $1/V_{\text{max}}$.\(^\text{14}\)

![Fig.2-10. Lineweaver-Burk linearization method for determination of $K_M$ and $V_{\text{max}}$](image)
2.6. Enzyme-catalyzed kinetic resolutions

Since enzymes are chiral biocatalysts composed from L-amino acids, they can distinguish between the two enantiomers of racemic substrate (enantioselectivity). One of the enantiomers fits better in the active site of the enzyme and it is converted to the product at higher rate (see Fig.2.-11., \(k_R > k_S\)).\textsuperscript{13} This method is called kinetic resolution (KR). One drawback of this method is that the product can be obtained only in 50% yield. In most cases, enzyme converts one enantiomer rapidly and the other one more slowly. The ratio of reaction rates of enantiomers is measurable. During the reaction constantly varies the ratio of substrate enantiomers, thus the optical purity of the substrate and product is a function of conversion.\textsuperscript{13}

![Kinetic Resolution Diagram]

**Fig.2.-11.** Kinetic resolution of racemic substrate (enantiomers are converted to products at different reaction rates)\textsuperscript{34}

One of the major goals in enzyme-catalyzed reactions is the synthesis of enantiomerically pure compounds. Enantiopurity of unreacted substrate (\(ee_S\), Eq.4.) and synthetized product (\(ee_P\), Eq.5.) can be calculated from concentrations of enantiomers:\textsuperscript{25}

\[
ee_S[{\%}] = \frac{[S_R] - [S_S]}{[S_R] + [S_S]} \times 100 \quad \text{Eq. 4.}
\]

\[
ee_P[{\%}] = \frac{[P_S] - [P_R]}{[P_S] + [P_R]} \times 100 \quad \text{Eq. 5.}
\]

Enantiomeric excess (\(ee\)) values of substrates and products are directly related to the conversion of the reaction according to Eq.6.:

\[
c = \frac{ee_S}{ee_S + ee_P} \quad \text{Eq. 6.}
\]

Dimensionless enantiomeric ratio (\(E\)) is a measure of enantioselectivity which can be used to compare different enzymes. If the \(E\) value of enzyme is low, the enzyme displays no selectivity towards the two enantiomers of substrate.\textsuperscript{13,26} For irreversible reactions (hydrolytic reaction catalyzed by lipases) \(E\) can be calculated from measurement of:
o conversion (c) and enantiomeric excess of product (eeₚ):

$$E_{c,eeₚ} = \frac{\ln[1-c(1+eeₚ)]}{\ln[1-c(1-eeₚ)]} \quad Eq. 7.$$ 

o conversion (c) and enantiomeric excess of substrate (eeₛ):²⁵

$$E_{c,eeₛ} = \frac{\ln[(1-c)(1-eeₛ)]}{\ln[(1-c)(1+eeₛ)]} \quad Eq. 8.$$ 

o enantiomeric excess of product (eeₚ) and enantiomeric excess of substrate (eeₛ):²⁷

$$E_{c,eeₚ} = \frac{\ln[(1-eeₛ)/(1 + eeₛ/eeₚ)]}{\ln[(1 + eeₛ)/(1 + eeₛ/eeₚ)]} \quad Eq. 9.$$ 

For very high and very low conversion Eq.7. and Eq.8. give not reliable results due to the measurement errors arising from sample manipulation. Little change in the enantiomeric excess causes large change is E value because of the logarithmic function of E.

If reactions are reversible, discrimination of the two enantiomers by enzyme are defined by values of E and K (equilibrium constant). For determination of E in reversible reaction, Eq.10. can be applied (eeₛ of substrate or eeₚ of product can be used):

$$E = \frac{\ln[1-(1+K)c(1+eeₚ)]}{\ln[1-(1+K)c(1-eeₚ)]} = \frac{\ln[1-(1+K)(c+eeₛ(1-c))]}{\ln[1-(1+K)(c-eeₛ(1-c))]} \quad Eq. 10.$$ 

Prognosis of kinetic resolutions carried out by enzymes with different enantioselectivities can be determined by different softwares (Fig.2.-12). In Fig.2.-12A and 2.-12B are shown enzymes with low enantioselectivities (A: E=5; B: E=20). In this cases, kinetic resolutions with low conversions (c<50%) give substrates and enzymes with highest enantiopurities. In Fig.2.-12C and 2.-12D enantioselectivities are higher (C: E=20; D: E=200). Around 50% conversion enantiopurity of products significantly increases and reaches a plateau at eeₚ>99%. When E=200, enantiopurity of substrate decreases rapidly with increasing conversion (c>50%). In 50% conversion enantiomers of substrate and product can be obtained in high enantiopurity.
In addition to above mentioned enantiomeric excess and enantioselectivity, we can define specific enzyme activity to precisely evaluate the native or immobilized biocatalysts in batch mode. It can be used to compare different preparations. The specific enzyme activity ($U_b$) is given in Eq.11.: 

$$U_b = \frac{n_P}{t \times m_B} \quad \text{Eq. 11.}$$

where $n_P$ is the mole of product formed ($\mu$mol), $t$ is the reaction time (min) and $m_B$ is the amount of applied biocatalyst (mg). 

---

**Fig.2.-12.** Conversion-dependence of optical purities of substrates and products in the kinetic resolution (Blue line: enantiomeric excess of substrate, red line: enantiomeric excess of product)$^{28,29}$
2.7. **Lipases- structure and mechanism of catalysis**

Lipases (E.C. 3.1.1.3) are ubiquitous enzymes in nature with a huge industrial potential. Lipases originate from plants, animals or microorganisms (bacteria, fungi). According to protein sequence alignments, they form three groups: mammalian lipases (porcine pancreatic lipase), fungal lipases (*Candida* and *Rhizomucor* families) and bacterial lipases (*Pseudomonas* and *Staphylococcus* families). They are widely used in laundry detergents, food industry (flavor and aroma constituents), paper industry, crude oil refining and for biodiesel production.

Lipases belong to the family of hydrolases that catalyze preferentially the hydrolysis of water insoluble triacylglycerides. Catalysis takes place at the water-lipid interface, resulting glycerol, free fatty acid and partially hydrolyzed monoacylglyceride and diacylglyceride. Under appropriate conditions (organic phase with low water activity) lipases catalyze enantio- and regioselective ester synthesis, alcoholysis or transesterification. The controlled water content of reaction media is essential for reversible ester synthesis.

Molecular mass of lipases ranges from 20 to 60 kDa. Optimum operating temperature range is between 30 °C and 60 °C. Most of the lipases have a helical segment (lid or flap) covering their active site in the aqueous environment. So called closed conformation of lipases can be altered to open conformation by lipid-induced interfacial activation. Open conformation of lipase permits the substrate to enter the active site and catalytic activity of lipase increases dramatically. Primary sequence of lipases is quite diverse, but they have a common motif, so called α/β hydrolase fold. Eight mostly parallel β-sheets are surrounded by α-helices. The active site of lipases is built up from a catalytic triad: aspartate/glutamate, histidine and serine. These amino acid residues in the active site are specially arranged and stabilized by hydrogen bonding.

Catalytic circuit of lipase catalyzed ester hydrolysis is presented in Fig. 2.-13. Hydroxyl group of serine performs nucleophilic attack on the carbonyl group of ester substrate. Thus first tetrahedral intermediate is created stabilized by hydrogen bonding. After rearrangement, alcohol is released from the active site leaving acyl-enzyme intermediate. Water acts as a nucleophile on acyl-enzyme complex first forming the second tetrahedral intermediate, then regenerating the free enzyme and releasing the second product, free carboxylic acid.
Lipases have different binding sites for alcohol (similar in all lipases) and acid portion of esters. Acid binding site shows great variability among lipases. Alcohol binding site contains two different channels: large hydrophobic pocket and a small pocket. The most common substrates of lipases are racemic secondary alcohols and its derivatives. Scientists elaborated a rule to predict which enantiomer of alcohol reacts faster in reactions. According to Kazlauskas’s rule, size of the substituents plays a crucial role in distinguishing between enantiomers of secondary alcohols.

As model enzymes in this PhD thesis, two different lipases were selected in order to investigate the effect of surface modification on enzymatic performance. Lipase from *Burkholderia cepacia* (*BcL*) is an extracellular lipase catalyzing the biodegradation of environmental pollutants, biological control of plant diseases (PDB code: 1YS1). *BcL* is often applied as biocatalyst in various biotransformations performed in non-aqueous media because of its thermostolerance. Fig.2.-14. illustrates the crystal structures of two investigated lipases (*Burkholderia cepacia* lipase as well as *Candida antarctica* lipase B).
The second lipase, *Candida antarctica* lipase B (PDB code: 1LBT) is a recombinant protein produced in *Aspergillus* fungus with a huge industrial potential.\textsuperscript{33,43} Structure of *CaLB* was resolved by Uppenberg in 1994.\textsuperscript{44} Molecular weight of lipase is 33 kDa and consists of 317 amino acids.\textsuperscript{45} *CaLB* has an isoelectric point of 6.0 and a small active site lid.\textsuperscript{46} Unlike other lipases, *CaLB* does not show interfacial activation, however it is greatly adsorbed on hydrophobic surfaces.\textsuperscript{33,47}

<table>
<thead>
<tr>
<th></th>
<th><em>Burkholderia cepacia</em> lipase (BcL)</th>
<th><em>Candida antarctica</em> lipase B (CaLB)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origin</strong></td>
<td>bacterial</td>
<td>yeast</td>
</tr>
<tr>
<td><strong>PDB code</strong></td>
<td>1YS1</td>
<td>1LBT</td>
</tr>
<tr>
<td><strong>Molecular weight (kDa)</strong></td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td><strong>Isoelectric point</strong></td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Optimum pH</strong></td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>Interfacial activation</strong></td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><strong>Number of composing amino acids</strong></td>
<td>320</td>
<td>317</td>
</tr>
</tbody>
</table>
2.8. \(\omega\)-Transaminases - valuable biocatalysts for chiral amine synthesis

Transaminases (often referred as amino transferases, EC 2.6.1.X.) catalyze the redox-neutral amino group transfer from amine donor to carbonyl group.\(^{13,48}\) They are cofactor dependent enzymes (pyridoxal 5'-phosphate - PLP, derivative of vitamin B\(_6\), where PLP serves as a molecular shuttle for ammonia and electrons.\(^{49}\) In living microorganisms, \(\alpha\)-transaminases catalyze the amino acid synthesis from corresponding keto acids. They are able to accept only \(\alpha\)-amino acids and \(\alpha\)-keto acids as substrates. Transaminases converting aliphatic amines and ketones are called \(\omega\)-transaminases.\(^{50}\) Having two substrate-binding pockets of different sizes, transaminases display remarkable enantioselectivity.\(^{49}\)

Transaminases (TA) are able to catalyze kinetic resolution of racemic amines (Fig.2.-15A), asymmetric synthesis of chiral amines starting from prochiral ketones (Fig.2.-15B) and deracemization of racemic amines (Fig.2.-15C).\(^{50,51}\) They were discovered about 50 years ago, but the use of transaminases was hampered by several drawbacks: majority of transaminases was only active on their natural substrates (in absence of bulky substituents) and transaminases were inhibited at higher substrate concentrations. Another major obstacle is that asymmetric synthesis is hardly feasible since equilibrium lies on the side of substrates.\(^{52}\)

**Fig.2.-15.** Reaction types catalyzed by transaminases. A: Kinetic resolution of racemic amines, B: asymmetric synthesis starting from ketones, C: one-pot deracemization of racemic amines catalyzed by two enantiocomplementary transaminases.\(^{53}\)

(Abbreviations: TA: transaminase, PLP: pyridoxal 5'-phosphate, PMP: pyridoxamine phosphate, AADH: amino acid dehydrogenase.)
In the kinetic resolution of racemic amines, one enantiomer is converted to ketone leaving one enantiomer of amine in optically pure form. Although maximum yield of enantiopure amine in the reaction is only 50%, this type of the reaction can be easily carried out. Which enantiomer is not converted, depends on the transaminase used: (S)-selective transaminases convert (S)-amines, leaving (R)-amines unreacted, while (R)-selective transaminases lead to enantiopure (S)-amines.

Although asymmetric synthesis of enantiopure amines can reach theoretically 100% yield, this type of reaction is difficult to implement. Equilibrium lies on the side of substrates (ketone and amino acid), thus researchers developed some tricks for equilibrium displacement shown in Fig. 2.16. If amine donor is isopropyl amine, formed acetone can be removed at reduced pressure or with nitrogen sparge or by the reduction to isopropyl alcohol catalyzed by alcohol dehydrogenase (ADH). Another common method is using amino acids as amine donors. In this case, formed pyruvate can be removed from equilibrium by lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC), alanine dehydrogenase (AlaDH) or by additional transaminase (TA).

![Diagram](image)

**Fig.2.16.** Methods for shifting the equilibrium in asymmetric synthesis of amines

(Abbreviations: TA: transaminase, PLP: pyridoxal 5'-phosphate, PMP: pyridoxamine phosphate, ADH: alcohol dehydrogenase, LDH: lactate dehydrogenase, PDC: pyruvate decarboxylase, AlaDH: alanine dehydrogenase.)

In the past few years non-conventional amine/diamine donors were developed for equilibrium displacement or for fast screening of transaminase activity. They are referred sometimes as
“smart” substrates as they undergo irreversible reactions (tautomerization or polymerization). After reaction, diamines (e.g. ortho-xylylenediamine) go through spontaneous polymerization and precipitation which provides efficient synthesis of optically pure amines. However, this method cannot be implemented in flow-through approaches, since precipitation in solution can lead to reactor clogging.

Deracemization of racemic amines requires two transaminases with opposite selectivities (Fig.2.-15C). Enantiopure amine is produced in a two-step catalytic cascade: kinetic resolution followed by a reductive amination step. Depending on the order of enzymes in cascade reaction, (R)- or (S)-enantiomers can be obtained, as well. Thermal treatment after first kinetic resolution is often required to ensure inactivation of firstly applied transaminase. While some racemic amines are more readily available than ketones, the limitation of kinetic resolution can be eliminated with this one-pot, two-step process.

Transaminase from Chromobacterium violaceum (CvTA) used in our study was discovered by Ward et al. in 2007. CvTA shows 38% sequence identity with Vibrio fluvialis and exhibits (S)-selectivity. The protein was described in details regarding the substrate spectrum, kinetic experiments and enzymatic properties. CvTA is widely used enzyme in chiral amine synthesis, however its low operational stability needs to be improved. In addition, CvTA is catalytically active as homodimer, dissociation of the dimer to monomers leads to protein inactivation. Fig.2.-17. illustrates the monomer and dimer of CvTA. Berglund et al. found that several additives (organic solvents, surfactants, substrates) can significantly alter the storage stability of this enzyme. Besides additives, protein engineering and immobilization of enzyme can be excellent methods for protein stabilization. Berglund et al. performed single point mutation in the active site of CvTA changing tryptophan to cysteine (W60C).

Table 2.-3. Properties of Chromobacterium violaceum ω-transaminase

<table>
<thead>
<tr>
<th>Chromobacterium violaceum transaminase (CvTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
</tr>
<tr>
<td>PDB code</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
</tr>
<tr>
<td>Optimum pH</td>
</tr>
<tr>
<td>Number of composing amino acids</td>
</tr>
<tr>
<td>Cofactor</td>
</tr>
</tbody>
</table>
Transaminase catalysis occurs in a catalytic cycle: pyridoxal 5’-phosphate is recycled to its initial form thus it is no need for additional cofactor regeneration. Fig. 2.-18. illustrates the catalytic cycle of transamination reaction (amino donor substrate is 1-phenylethylamine and amino acceptor is pyruvate). The reaction can be divided into two half reactions: in the first half reaction, PLP forms Schiff base with lysine residue in the active center of transaminase, an internal aldimine. In the next step, amino donor enters active site and forms a Schiff base with PLP. After electron and proton rearrangements hydrolysis occurs which yields the first product (acetophenone) and pyridoxamine phosphate (PMP) is formed. PMP is bound to the active center only by secondary interactions thus it can be competitively eliminated by phosphate salts. Then, the amino acceptor enters the active site and reverse reaction steps of the first half cycle will take place.
2.9. Oxidoreductases

Another major group of enzymes represent alcohol dehydrogenases (ADHs) and ketoreductases (KREDs) which belong to the class of oxidoreductases (EC 1.X.X.X.) and can be of bacterial or yeast origin. Ketoreductases are commercially available, but many of them can be isolated from human or soil samples. Reduction of prochiral ketones to enantiomerically pure alcohols occurs in the presence of cofactor as it is shown in Fig.2.-19. (nicotinamide adenine dinucleotide – NAD, nicotinamide adenine dinucleotide phosphate – NADP or flavin adenine dinucleotide– FAD) which transfers the electrons from one molecule to another. When using enzymes, effective cofactor regeneration is a key point of the process. Usually, glucose or isopropyl alcohol is used to maintain the excess of the hydrogen source to push the equilibrium towards alcohol formation.
Enzymes are particularly sensitive to high substrate concentration, co-solvent addition or any non-natural conditions, so instead of enzymes, whole-cells can be used for ketone reduction. Whole-cells provide suitable microenvironment for intracellular enzymes and their internal metabolism is able to recycle the cofactor and maintain the catalytic activity.\textsuperscript{98,99} Several ketones containing aromatic moieties were successfully reduced to the corresponding secondary chiral alcohols using lyophilized whole-cells of \textit{Wickerhamomyces subpelliculosus}, \textit{Debaryomyces hansenii} and \textit{Zygosaccharomyces rouxii}.\textsuperscript{100,101} Industrially relevant whole-cells e.g. \textit{Saccharomyces cerevisiae}, \textit{Rhodotorula pilimanae}, \textit{Geotrichum candidum} was efficiently utilized in the stereoselective reduction of the carbonyl groups.\textsuperscript{98}

\textbf{2.10. Cascade reactions involving transaminases and ketoreductases}

Based on the multistep cascades in the nature – the concentrations of reactants are low, intermediates are not separated and it is no by-product formation – artificial enzyme cascades have been developed by scientists.\textsuperscript{102} They enable the multistep synthesis of complex chiral compounds in contemporary pharmaceutical and agrochemical research. Cascade reactions reduce costs, shorten reaction times, avoid toxic intermediates, prevent the use of protecting groups and increase the atom efficiency.\textsuperscript{103-105}

Enzymatic cascade reactions are sometimes defined as tandem or domino reactions.\textsuperscript{106} Several reactions proceeding simultaneously can be divided into four groups: linear, parallel, orthogonal and cyclic cascades.\textsuperscript{107} In linear cascade reactions the enzyme converts the product of previous enzymatic reaction, thus accumulation of intermediates is prevented (Fig.2.-20A). Parallel cascades (Fig.2.-20C) can be found in the field of redox reactions, where cofactor is regenerated simultaneously with product formation (e.g. recycling of NAD(P)H). Orthogonal cascades can be employed by transaminases, which may be strongly inhibited by substrates and/or products (Fig.2.-20D). Applying a second enzyme in parallel cascade reaction can minimize enzyme inhibition and shift equilibrium toward product formation. In cyclic cascades, one product is converted back to starting material (Fig.2.-20B).

\textbf{Fig.2.-19.} Scheme of ketone reduction catalyzed by ketoreductases with isopropanol as co-substrate
Fig.2. Classification of cascade reactions: A: linear cascade, B: cyclic cascade, C: parallel cascade, D: orthogonal cascade

Either purified enzymes or whole-cell systems can operate under the same reaction conditions in one-pot cascade reactions. However, purified enzyme cascade systems are more difficult to establish. Many studies have shown cascade reactions employing isolated enzymes with low operational stability, cofactor addition and no possibility of biocatalyst recycling. The mentioned disadvantages can be eliminated with the use of proper recombinant whole-cell systems, or with the combination of two whole-cell systems which was investigated during our study as well.

ω-Transaminases due to their excellent compatibility with other class of enzymes: ketoreductases, transketolases, imine reductases and monoamine oxidases, represent a constantly expanding research area in recent years. Depending on the substrates, researchers have already shown that alcohol dehydrogenases and transaminases can be excellently combined: racemic alcohols were transformed into enantiopure amine (Fig.2.-21B) and in addition, in the kinetic resolution of racemic amines they can produce alcohols and amines simultaneously. By coupling the KR with stereoselective ketone reduction, product and substrate inhibitions can be overcome (Fig.2.-21A).
2.11. Biocatalyst immobilization techniques

Different enzyme formulations or immobilizations can be used in the same reaction both at laboratory scale or large scale. Table 2.-4. illustrates and compares the common biocatalysts and their formulations in terms of activity, specificity, stability and cost associated with its production. These properties need to be taken in account when choosing a proper biocatalyst. Although whole-cell production is cheap, the cells may contain another class of enzymes causing unwanted side-reactions. Crude cell extracts are also inexpensive (especially in the case of extracellular enzymes), but its purity also depends on additional enzymes and protein expression. Obtaining purified enzymes requires additional work-up associated with higher costs and the long-term stability of the preparation is really low.\textsuperscript{116} Lyophilized enzyme offers long storage time and high specificity, however the biocatalyst cannot be reused in consecutive reaction cycles.

<table>
<thead>
<tr>
<th></th>
<th>Whole-cell biocatalyst</th>
<th>Crude cell extract</th>
<th>Native enzyme</th>
<th>Lyophilized enzyme</th>
<th>Immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Medium</td>
<td>Medium/Low</td>
<td>High</td>
<td>High/Medium</td>
<td>High/Medium</td>
</tr>
<tr>
<td>Activity</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Stability</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
<td>Medium</td>
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</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
</tbody>
</table>

The advantages of biocatalyst application are highlighted in green, while disadvantages in red.
Majority of biotechnological research projects is focusing now 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. In addition, when enzymes are built up from different subunits or have low stability, one option for the enzyme structure stabilization could be immobilization. Further advantages of immobilized biocatalysts are reusability of enzymes several times in synthesis, minimization of pH and substrate inhibition effects and minimization of product contamination. 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. Fig. 2.22. illustrates 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.

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. 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.

Fig. 2.22. Examples of several immobilized biocatalysts
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.\textsuperscript{121} 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 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.

\subsubsection{Covalent enzyme immobilization}

In pharmaceutical industry, purity of chemicals and drugs is strictly regulated by competent authorities. 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.

After finding the suitable carrier with the desired properties, the next task is to establish the crosslinking between the enzyme molecules and the carrier surface. Most of the enzyme crosslinking methods belong to the $S_N2$-type nucleophilic substitution reactions.\textsuperscript{119} Enzymes have various functional groups on their side chains (e.g. amino, thiol, carboxylic 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.-23A).\textsuperscript{122,123} In ideal case, aldehyde groups of glutaraldehyde are highly reactive and form Schiff bases with lysine residues on enzyme surfaces. Conversely, glutaraldehyde molecules can react as intramolecular crosslinking agents within the protein, or can bind two adjacent biomolecules. In this case, intramolecular crosslinking may cause enzyme inactivation.
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.\textsuperscript{124,125} Other bifunctional agents used for covalent enzyme immobilizations included cyanuric chloride, hexamethylene diisocyanate and cyanogen bromide (Fig.2.-23B, 2-23C and 2-23D).

However, industry needs a protocol and method resulting in stable and robust enzyme carrier. Epoxy activated supports are excellent options for immobilized biocatalyst preparation due to the stability of epoxy groups (Fig.2.-24A). They can be directly used for enzyme immobilization. Diamines and glutaraldehyde modification can alter the spacer arms between the enzyme and support surface (Fig.2.-24B). Additionally, epoxy groups can react with other types of functional groups besides amine groups (thiol, aromatic hydroxyl group, carboxyl group, Fig.2.-24C) and the excess of epoxy groups can be easily blocked by other types of nucleophiles (e.g. amino acids).\textsuperscript{126} Based on previous studies, we can assume that the immobilization is a two-step process. The first step of enzyme immobilization is the hydrophobic adsorption of proteins on carrier surface followed by the covalent linkage formations between the molecules and surface in the second step. Thus, presence of hydrophobic groups on surface is essential and promotes enzyme immobilization. Controlling the hydrophobic and epoxy group ratio, the carriers can be excellently fined-tuned for the immobilization of particular proteins.
Sol-gel entrapment is an easy-to-perform process, which offers an effective way for the entrapment of sensitive cells or enzymes. In the first step, silane precursor (e.g. tetraethyl orthosilicate - TEOS, or substituted trialkoxysilanes – RSi(OMe)₃ where R is a non-hydrolyzable lipophilic alkyl group) undergoes hydrolysis which is followed by condensation of molecules leading to SiO₂ matrix. Acid- or base- catalyzed hydrolysis of silane precursors results in sol formation (liquid phase with solid particles, diameter: 1-1000 nm). After the interaction of these colloidal particles with large surface area and active silanol groups, liquid sol phase changes to gel phase. Commonly used catalyst is NaF which enables larger pore formation within the silica matrix. If the condensation occurs in the presence of whole-cells and possibly additives, a semipermeable porous membrane will encompass the cells. These nanoporous materials are inert, highly thermostable and nontoxic. In addition, different types of alkoxysilane precursors provide different hydrophobicity of silane matrix and other additives (e.g. celite, sucrose, PVA) are perfect for fine-tuning of this structure as well. Additives applied during the cell entrapment may include hollow silica spheres, which result in the formation of templated silica matrices (see Fig.2.-25.). Biocatalyst gain increased mechanical stability and activity.
2.11.3. Molecular imprinting

Enzyme immobilization may be used in combination with other types of techniques e.g. molecular imprinting. Molecular imprinting\textsuperscript{133} – which is called bioimprinting when enzymes are treated at their active site by substrates or their analogues – proved to be one of the most successful strategies for enhancing enzyme activity in organic solvent (Fig.2.-26.).\textsuperscript{134,135} According to recent studies, when the active site of the lipase is tuned with substrate analogues, surfactants or other additives, lipase activity may be improved in non-aqueous medium.\textsuperscript{136-139} During the bioimprinting process conformational changes occur and lead to opening the lid over the active site. After the immobilization in aqueous media, the ligand is washed away, so the enzyme is trapped in this conformation because it has adopted a rigid structure due to the strong intramolecular electrostatic interactions that occur in a solvent with a low dielectric constant.\textsuperscript{135,140} Biodegradable polymers such as polyvinyl alcohol (PVA) or chitosan were applied as additives in enzyme immobilizations.\textsuperscript{141} Further eco-friendly polymers such as gum arabic and chitosan were also useful for stabilization of enzymes by microencapsulation.\textsuperscript{142} In addition, PVAs were applied in preparation of sol-gel catalysts as lipase stabilizing additives. The beneficial effects of further additives such as crown ethers, β-cyclodextrin derivatives, surfactants and sugars were also studied in sol-gel encapsulations.\textsuperscript{143}

Fig.2.-26. Illustration of enzyme bioimprinting with substrate analogue
2.11.4. Immobilization of lipases

Immobilization of the biocatalysts onto solid supports has become a widely accepted and used industrial technique, which can improve enzyme activity, temperature stability and selectivity of enzymes.\textsuperscript{144} In addition, application of immobilized enzymes can reduce the costs of reactions and facilitate the downstream processing. Numerous methods have been already developed for enzyme immobilization,\textsuperscript{145-148} including adsorption, covalent attachment to solid supports and entrapment of enzymes within polymer matrices. Besides these enzyme immobilizations, several commercial immobilized CaLB preparations are available and presented in Table 2.5.

Physical adsorption process of the chosen lipase onto proper carrier is a convenient, one-step immobilization technique. Since the lipases display interfacial activation, use of hydrophobic supports can significantly alter the biocatalytic performance of adsorbed lipases.\textsuperscript{149} Mesoporous silica gels (MPSs)\textsuperscript{150} and polymer resins proved to be useful carriers for enzyme immobilization due to their functionalizable large surface area, tunable porosity, low cytotoxicity and favorable mechanical properties. Surface modification of silica gels and polymers with different functions can broaden their applicability as carriers for proteins and enzymes.\textsuperscript{150,151} Hydrophobic silica gels, such as butyl\textsuperscript{152} or octyl\textsuperscript{153} silica gels proved to be suitable carriers for adsorptive immobilization of lipases. Furthermore, modified silica gels were useful for differential adsorption of lipase A and lipase B from Candida antarctica. Mixed-function-grafted silica supports with amine groups allowed the immobilization of enzymes by adsorption as well as by covalent immobilization.\textsuperscript{154} Lipases after adsorptive immobilization can retain their activity in organic media but under aqueous conditions they can release the enzyme causing decrease of activity and product contamination. To avoid the problem of enzyme leaching, covalent immobilization offered a solution. For example, activated natural polymers (epichlorohydrin- or glutaraldehyde-activated chitosan or agarose gels) proved to be suitable carriers for multipoint covalent immobilization of proteins\textsuperscript{155} or silica gel grafted with glyoxyl groups enabled covalent enzyme immobilization.\textsuperscript{156}
Table 2.5. Examples of commercially available immobilized *Candida antarctica* lipase B preparations

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Mode of immobilization</th>
<th>Carrier</th>
<th>Particle size (µm)</th>
<th>Activity (PLU/g)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novozyme 435</td>
<td></td>
<td>Lewatit VP OC 1600</td>
<td>300 – 800</td>
<td>10000</td>
<td>Novozymes</td>
</tr>
<tr>
<td>CalB immo 1090</td>
<td>adsorption</td>
<td>Purolite ECR1030M</td>
<td>300 – 710</td>
<td>&gt; 9000</td>
<td>Purolite</td>
</tr>
<tr>
<td>CalB immo 8806</td>
<td></td>
<td>Purolite ECR1090M</td>
<td>300 – 710</td>
<td>8000</td>
<td>Purolite</td>
</tr>
<tr>
<td>CalB immo 8806</td>
<td></td>
<td>Purolite ECR8806M</td>
<td>300 – 710</td>
<td>10000</td>
<td>c-Lecta/Purolite</td>
</tr>
<tr>
<td>IMMACALB-T1-350</td>
<td>acrylic bead</td>
<td>styrene/methacrylate</td>
<td>NA</td>
<td>11500</td>
<td>Chiral Vision</td>
</tr>
<tr>
<td>NATE-1897</td>
<td></td>
<td>divinylbenzene cross-linked polymer based on methacrylic esters</td>
<td>300 – 1000</td>
<td>≥ 9000 U/g</td>
<td>SPRIN technologies</td>
</tr>
<tr>
<td>SPRIN liposorb</td>
<td></td>
<td>Purolite ECR8285</td>
<td>250 – 1000</td>
<td>5000</td>
<td>c-Lecta/Purolite</td>
</tr>
<tr>
<td>CALB</td>
<td>covalent</td>
<td>IB-150A</td>
<td>150 – 250</td>
<td>5000</td>
<td>Chiral Vision</td>
</tr>
</tbody>
</table>

* Unit definition: 1 PLU unit = 1 µmol propyl laurate formed per minute / g immobilized enzyme

2.11.5. Immobilization of ω-transaminases

For enzyme stabilization, immobilization is one of the easiest methods. Transaminases from different species (*Arthrobacter citreus, Vibrio fluvialis, Gibberella zeae*, *Neosartorya fischeri*, *Halomonas elongata*) were already immobilized either at whole-cell level or as isolated proteins. Immobilization may simplify the application of enzymes as biocatalysts due to enabled reuse, enhanced solvent stability of the enzyme and easier downstream processing. Transaminases as cell-free proteins were immobilized by several techniques, such as adsorption onto polymeric resins, covalent attachment onto chitosan beads or epoxy carrier, entrapment in sol-gel matrices, or calcium alginate beads. In addition, His-tagged transaminases were immobilized by binding to support-bound metals.

Whole-cell immobilization methods include entrapment of cells in inorganic (silica) or organic (alginate gel) matrices or attachment of cells to various supports. They are surrounded by a semipermeable membrane which permits the flow of the substrates and products but protects the cells. Biopolymers applicable as matrix are extremely diverse, but alginate gel is a widely used one. Alginate is a polysaccharide that can be found in brown algae species. In the presence of
divalent metal cations (such as Ca$^{2+}$) sodium alginate forms beads which can incorporate cells containing e.g. transaminase.$^{168}$ Transaminases as whole-cell biocatalysts were already immobilized by various methods: in calcium alginate beads,$^{169,170}$ in κ-carrageenan gel,$^{171}$ in poly(vinyl alcohol) hydrogel$^{172}$ or adsorptive immobilization of the whole-cells onto polymethacrylate resin can be carried out as well.$^{173}$ The overview of immobilized transaminases is presented in Table 2.-6.

Table 2.-6. Examples of immobilized ω-transaminases or whole-cells containing ω-transaminases

<table>
<thead>
<tr>
<th>ω-Transaminase</th>
<th>Selectivity</th>
<th>Mode of immobilization</th>
<th>Matrix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Native enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberella zeae</td>
<td>R</td>
<td>covalent</td>
<td>chitosan</td>
<td>158</td>
</tr>
<tr>
<td>Neosartorya fischeri</td>
<td>R</td>
<td>covalent</td>
<td>chitosan beads</td>
<td></td>
</tr>
<tr>
<td>Vibrio fluvialis</td>
<td>S</td>
<td>covalent</td>
<td>TentaGel S COOSu</td>
<td>162</td>
</tr>
<tr>
<td>Vibrio fluvialis JS17</td>
<td>S</td>
<td>covalent</td>
<td>Eupergit®</td>
<td></td>
</tr>
<tr>
<td>Halomonas elongata</td>
<td>S</td>
<td>covalent</td>
<td>metal-derivatised epoxy resin</td>
<td>163</td>
</tr>
<tr>
<td><strong>Whole-cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobacter citreus</td>
<td>S</td>
<td>adsorption</td>
<td>titanium oxide</td>
<td>170</td>
</tr>
<tr>
<td>(in E.coli)</td>
<td></td>
<td></td>
<td>calcium alginate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>carrageenan bead</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>polyacrylamide gel</td>
<td></td>
</tr>
<tr>
<td>Arthrobacter citreus</td>
<td>S</td>
<td>entrapment</td>
<td>calcium alginate</td>
<td>169</td>
</tr>
<tr>
<td>(in E.coli)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.coli EP8-10</td>
<td></td>
<td>entrapment</td>
<td>gelatin</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>κ-carrageenan bead</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>calcium alginate</td>
<td></td>
</tr>
<tr>
<td>TA-D44 (in E.coli)</td>
<td></td>
<td>entrapment</td>
<td>LentiKats®</td>
<td>172</td>
</tr>
</tbody>
</table>
2.12. Continuous-flow biocatalysis

Nowadays, flow chemistry represents a high potential in research and development of pharmaceutical drugs. Synthesis of new molecules whether on analytical or preparative scale requires efficient mixing, precise temperature control, minimization of side product formation and development of safe processes. Compared to batch reactions, flow systems enable fast synthesis of pure chemicals with minimum work-up.\textsuperscript{174}

Further advantages of reactor technology, such as increased product throughput, high enzyme or whole-cell stability, high reproducibility and automation contributed to the development of biocatalysis in continuous-flow regime.\textsuperscript{173,175,176}

Applicability of immobilized enzymes to continuous-flow biotransformations can significantly reduce product and/or substrate inhibitions. Particularly in flow-through approach substrate is continuously pumped through the column filled with biocatalyst and product is continuously taken away. Some of the main types of flow reactors are summarized in Fig.2.-27.: continuous stirred-tank reactor (CSTR), packed-bed reactor (PBR), fluidized-bed reactor (FBR) or continuously operated membrane reactor (MBR).\textsuperscript{177}

\textbf{Fig.2.-27.} Continuous-flow reactor types: A: continuously stirred tank reactor (CSTR), B: packed-bed reactor (PBR), C: fluidized-bed reactor (FBR), D: continuously operated membrane reactor (MBR).\textsuperscript{177}

CSTR consists of a tank and agitator.\textsuperscript{178} Reaction proceeds and upon complete mixing, concentration is evenly distributed in the reactor. Products are continuously withdrawn from the system. On the other hand, in PBR the conversion of the reaction depends on the length of the column and concentration gradients are normal to flow direction. Voidage is only 34% for PBRs
with immobilized catalyst, and 80–90% for CSTR. In stirred tank reactor, immobilized enzyme is subjected to higher shearing forces compared to packed bed reactor which can lead to damage of biocatalyst.30

When designing a continuous-flow process, one should consider kinetic and operational features of different reactor types. Ideal reactors have high space time yield to increase the efficiency of the biotransformation. When product inhibition is pronounced, a continuous-flow PBR has to be selected to achieve higher catalyst specific productivity.

So far, lipases as not cofactor-dependent enzymes were the most frequently used enzymes in packed-bed reactors,143,154,177,179 but systems with PLP-dependent transaminases were recently reported as well.163 The dimensions of the immobilized biocatalyst should be carefully selected; small particles may lead to high pressure drop or even to blocking. Selection of the proper enzyme carrier depends both on the reactor type and enzyme properties.

Similarly to batch biocatalysis, specific enzyme activity \( r_{\text{flow}} \) can be given for continuous-flow reactions:

\[
r_{\text{flow}} = \frac{[P]}{m_B} \quad \text{Eq. 12.}
\]

where \([P]\) is the product concentration (µmol mL\(^{-1}\)), \(v\) is the flow rate (mL min\(^{-1}\)) and \(m_B\) is the amount of applied biocatalyst (mg).30

If we divide the reactor volume \((V_R, \text{mL})\) by flow rate \((v, \text{mL min}^{-1})\), we can calculate the residence time \((t_R)\), which shows how much time spend the reactants inside the reactor:

\[
t_R = \frac{V_R}{v} \quad \text{Eq. 13.}
\]

Reactors of different sizes can be compared to each other, if the performance of the reactor is given related to the catalyst mass or volume. This quantity is known as the space–time yield \(Y_s\) (g L\(^{-1}\) h\(^{-1}\))180:

\[
\text{STY} = \frac{\text{desired product quantity}}{\text{catalyst volume} \times \text{time}} \quad \text{Eq. 14.}
\]
3. Materials and Methods

3.1. Materials

All chemicals and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA), Molar Chemicals Kft. (Budapest, Hungary), Merck KGaA (Darmstadt, Germany), or Fluka (Milwaukee, WI, USA) and used as received. Bisepoxides [1,6-hexanediol diglycidyl ether (HD), neopentylglycol diglycidyl ether (ND), 1,4-cyclohexanediol diglycidyl ether (CH), 1,4-butaneediol diglycidyl ether (BD), poly(ethylene glycol) diglycidyl ether (PE)] were products of Ipox Chemicals Ltd. (Budapest, Hungary). Glycerol diglycidyl ether (GD) was purchased from Sigma-Aldrich.

3.1.1. Additives

Polyethylene glycols: PEG 400 (M$_{w}$ = 380-420 g mol$^{-1}$), PEG 1k (M$_{w}$ = 950-1050 g mol$^{-1}$), PEG 4k (M$_{w}$ = 3600-4400 g mol$^{-1}$), PEG 8k (M$_{w}$ = 7000-9000 g mol$^{-1}$), PEG 20k (M$_{w}$ = 16000-24000 g mol$^{-1}$); polysaccharides: chitosan, gum arabic, sodium alginate, α-cyclodextrin, β-cyclodextrin; carboxylic and fatty acids: lauric acid, decanoic acid; glycerides: trilaurin, tristearin; 1-phenylethanol were purchased from Fluka (Milwaukee, WI, USA). Detergents: Brij 30, Triton X-100, Tween 80; poly(vinyl alcohols): PVA 4–88 (M$_{w}$=31 kDa, hydrolysis: 88%), PVA 18–88 (M$_{w}$=130 kDa, hydrolysis: 88%), PVA 13–23–88 (M$_{w}$=13–23 kDa, hydrolysis: 88%), PVA 60–98 (M$_{w}$=60 kDa, hydrolysis: 98%), PVA 72–98 (M$_{w}$=72 kDa, hydrolysis: 98%); xylane, carrageenan, starch, hexanoic acid, triolein, Hymono 9004, 2-octanol, saccharides: rhamnose, glucose, fructose, xylitol, xylose, sorbitol, mannitol, sucrose, maltose were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

3.1.2. Enzymes and whole-cells

Lipase from Burkholderia cepacia (BcL) was purchased from Aldrich (Milwaukee, WI, USA). Lipase B from Candida antarctica (CaLB, lyophilized) was purchased from c-LEcita GmbH (Leipzig, Germany). The plasmid encoding the W60C mutant of ω-transaminase from Chromobacterium violaceum (CvTA$_{W60C}$) was a kind gift from Prof. Per Berglund. The transaminase was overexpressed in E. coli BL21 (DE3). Fermentation was carried out at 10 L scale in Fermentia Microbiological Ltd. according to the protocol published by Cassimjee et al.$^{93}$
3.1.3. Supports

Ethylamine (EA)- and hexylamine (HA)-functionalized methacrylic polymer resins were purchased from Resindion S.r.l. (Rome, Italy). The MAT540 silica gel was purchased from Materium Innovations (Granby, Canada) and mesoporous silica gel Davisil®250 was purchased from Grace (Columbia, Maryland, USA). Davisil®250 silica gel was surface modified with (3-aminopropyl)trimethoxysilane (APTMS) and phenyltrimethoxysilane (PTMS) at 1:3 molar ratio and the surface modified silica gel is the product of SynBiocat Ltd. (Budapest, Hungary).

Table 3.1. Properties of supports used for enzyme immobilizations

<table>
<thead>
<tr>
<th>Support name (Abbreviation)</th>
<th>Davisol®250</th>
<th>MAT540®</th>
<th>ReliZyme™ EP403/S</th>
<th>ReliZyme™ EA403 (EA)</th>
<th>ReliZyme™ HA403 (HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support type</td>
<td>silica gel</td>
<td>silica gel</td>
<td>polymethyl methacrylate resin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional group</td>
<td>silanol</td>
<td>vinyl, amine</td>
<td>epoxy</td>
<td>ethylamine</td>
<td>hexylamine</td>
</tr>
<tr>
<td>Pore size (nm)</td>
<td>25</td>
<td>15-30</td>
<td>40-60</td>
<td>40-60</td>
<td>40-60</td>
</tr>
<tr>
<td>Particle size (µm)</td>
<td>40-63</td>
<td>10</td>
<td>100-300</td>
<td>100-300</td>
<td>100-300</td>
</tr>
<tr>
<td>Ion exchange capacity, Wet (µmol g⁻¹)</td>
<td>-</td>
<td>?</td>
<td>-</td>
<td>500</td>
<td>600</td>
</tr>
<tr>
<td>Water retention (%)</td>
<td>-</td>
<td>-</td>
<td>65-75</td>
<td>60-70</td>
<td>60-70</td>
</tr>
</tbody>
</table>
3.2. **Analytical methods**

3.2.1. **Scanning electron microscope analysis**

The surface morphology of the samples was investigated with a JEOL JSM-5500LV scanning electron microscope (SEM). The samples of immobilized CaLB were coated with gold prior to analysis. Electron beam energy of 25 kV was used for the investigations.

3.2.2. **Gas chromatography analysis**

GC analysis of lipase-catalyzed KRs were carried out on Agilent 4890 instrument equipped with FID detector using H₂ carrier gas (injector: 250 °C, FID: 250 °C, head pressure: 12 psi, split ratio: 50:1) on a Hydrodex β-6TBDM column (25 m × 0.25 mm × 0.25 µm film with heptakis-(2,3-di-O-methyl-6-O-t-butyldimethylsilyl)-β-cyclodextrine; Macherey & Nagel GmbH). Conversion (c), enantiomeric excess (ee) and enantiomeric ratio (E) were determined by GC measurements with base-line separations of the enantiomers using precise integration methods. Enantiomeric ratio (E) was calculated from the conversion (c) and enantiomeric excess of the product (eeₚ) using the equation Eq.7. Due to sensitivity to experimental errors, E values calculated in the 100–200 range are given as >100, in the 200–500 range as >200 and above 500 as >>200.

GC analysis of transaminase-catalyzed KRs were carried out on Agilent 5890 instrument equipped with FID detector using H₂ carrier gas (injector: 250 °C, FID: 250 °C, head pressure: 12 psi, split ratio: 50:1) on a Hydrodex β-6TBDAc column (25 m × 0.25 mm × 0.25 µm film with heptakis-(2,3-di-O-acetyl-6-O-t-butyldimethylsilyl)-β-cyclodextrin; Macherey & Nagel GmbH). Conversion (c) and enantiomeric excess (ee) were determined by GC measurements with base-line separations of the enantiomers of 3a-d and 4a-d using precise integration methods. The applied temperature programs are shown in Table 3.-2. For the kinetic resolution followed by asymmetric reduction of rac-3a, GC programs are shown in the Supporting Information of Article 4 in Annex.

Theoretical conversion of a selective and irreversible kinetic resolution (cₜheor) can be defined and calculated from Eq.15.:  

\[ c_{\text{theor}} = \frac{ee_p}{1 + ee_p} \quad \text{Eq. 15.} \]
Table 3.2. Temperature programs used for determination of conversions of lipase- and transaminase catalyzed kinetic resolutions

<table>
<thead>
<tr>
<th>KRs</th>
<th>Temperature program</th>
<th>Compounds of KRs</th>
<th>Retention time (min)</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>rac-1a</td>
<td>120 °C, 8 min</td>
<td>(S)-1a</td>
<td>5.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(R)-1a</td>
<td>5.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-2a</td>
<td>3.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(R)-2a</td>
<td>4.08</td>
<td>Hydrodex β-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(R)-1b</td>
<td>3.87</td>
<td>6TBDM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-1b</td>
<td>4.06</td>
<td></td>
</tr>
<tr>
<td>rac-1b</td>
<td>100-160 °C, 10 °C min⁻¹</td>
<td>(S)-2b</td>
<td>4.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(R)-2b</td>
<td>4.77</td>
<td></td>
</tr>
<tr>
<td>rac-3a</td>
<td>140-170 °C, 1 °C min⁻¹</td>
<td>(R)-3a</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-3a</td>
<td>22.7</td>
<td></td>
</tr>
<tr>
<td>rac-3b</td>
<td>100 °C, 15 min; 100-180 °C, 5 °C min⁻¹</td>
<td>(R)-3b</td>
<td>28.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-3b</td>
<td>28.4</td>
<td>Hydrodex β-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rac-3c</td>
<td>120 °C, 10 min; 120-180 °C, 6 °C min⁻¹; 180 °C, 10 min</td>
<td>(R)-3c</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(S)-3c</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>rac-3d</td>
<td>50 °C, 10 min; 50-60 °C, 2 °C min⁻¹; 60 °C, 10 min; 60-80 °C, 2 °C min⁻¹; 80-180 °C, 10 °C min⁻¹</td>
<td>4d</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rac-3d</td>
<td>39.8</td>
<td></td>
</tr>
</tbody>
</table>

3.3. *Surface activation of aminoalkyl polymer resins with bisepoxides or glutaraldehyde*

Dry polymer support (EA- or HA-resin, 500 mg) was added to a solution of bisepoxide (2.5 mmol, GD, HD, ND, CH, BD or PE) or glutaraldehyde (2.5 mmol, GA) in propan-1-ol (15 mL). The suspension of polymer support in activating agent solution was shaken at 400 rpm for 24 h at 25 °C. The activated support was filtered off on a glass filter (G3), washed with Patosolv® (3 × 10 mL),
dried at room temperature (4 h) and stored at 4 °C (in case of the GA-activated support, under argon).

### 3.4. Immobilization of lipases (BcL or CaLB)

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

To a solution of BcL (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 BcL 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.

#### 3.4.2. BcL immobilization by adsorption followed by cross-linking on mesoporous silica gel with mixed grafting

To a solution of additive (30.0 mg) in phosphate buffer (4 mL, 20 mM, pH 7.2) and ethanol (12 mL) was added the previously adsorbed BcL preparation (200.0 mg) and glycerol diglycidyl ether (320.0 µL). The mixture was incubated at 400 rpm at 25 °C for 24 h. The adsorbed and cross-linked BcL biocatalyst was filtered off on a glass filter (G4), washed with ethanol (3 × 10 mL), distilled water (10 mL), ethanol (3 × 10 mL), dried at room temperature (2 h) and stored at 4 °C.

#### 3.4.3. Immobilization of CaLB on bisepoxide-activated aminoalkyl resins

CaLB (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 CaLB 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 CaLB solution before immobilization and in supernatant after immobilization were determined according to Bradford’s method.\textsuperscript{181} As a standard, BSA was used. Immobilization yield (IY) was calculated according to equation IY (%) = \( \frac{P}{P_0} \times 100 \) (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).
3.5. Immobilization of \( \omega \)-transaminase on bisepoxide-activated aminoalkyl resins

In 1.5 mL Eppendorf tube purified *Chromobacterium violaceum* \( \omega \)-transaminase (210 \( \mu \)L, 4.8 mg mL\(^{-1} \)) was diluted with HEPES buffer (790 \( \mu \)L, 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 \(^\circ\)C in Eppendorf shaker. The immobilized transaminase preparation was centrifuged, washed with HEPES buffer (2 \( \times \) 1.0 mL). Protein concentration in supernatant was measured before and after immobilization on Nanodrop 2000. Immobilization yield (IY) was calculated according to equation \( \text{IY} (%) = \frac{P}{P_0} \times 100 \) (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.

3.6. Immobilization of *E. coli* and LeKRED 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 \( \mu \)L 0.1 M HNO\(_3\) and 0.25 mL distilled water, and the mixture was then sonicated for 5 min and cooled at 4 \(^\circ\)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* containing transaminase or LeKRED cells – dissolved in 3 mL 100 mM phosphate buffer pH 7.5), and the suspension was hardly shaken until homogeneous. Finally, the prepared silica sol was mixed with the solution, and was shaken for 5 min. The gelation occurred within 30 min at room temperature and the gel was aged at 4 \(^\circ\)C for 48 h. Then the crude products were washed with phosphate buffer (2 \( \times \) 15 mL, 100 mM, pH 7.5), centrifuged and dried at room temperature (24 h), and stored at 4 \(^\circ\)C.

The number of whole-cells prior to and after immobilization were determined in two ways: (i) the number of viable cells by dilution plating and viable plate counts and (ii) the total number of cells by Helber counting chamber. (i): Dilution plating was performed on agar plates containing the fermentation media for LeKRED (*L. elongisporus*) and CvTA (in *E. coli*) cells. (ii): Helber counting chamber (MOM Helber, Budapest, Hungary); chamber parameters: 0.01 mm, 1/400 mm\(^2\), 1/25 mm\(^2\).
3.7. Enantiomer selective acetylation of rac-1a and rac-1b

Immobilized BcL biocatalyst (25.0 mg; adsorbed, or adsorbed and cross-linked; with additive) or immobilized CaLB biocatalyst (25.0 mg) was added to a solution of the racemic alcohol (rac-1a: 50.0 mg; 0.409 mmol; rac-1b: 50.0 mg; 0.390 mmol) in n-hexane/tert-butyl methyl ether/vinyl acetate 6/3/1 (2.0 mL) in glass vial and the resulting mixture was shaken (1000 rpm) at 30 °C for 4 h (adsorbed BcLs or immobilized CaLBs) or 24 h (adsorbed and cross-linked BcLs). Samples (50 µl) were taken after 1, 2, 4 and 24 h and diluted with ethanol (1 mL). The samples were then analyzed by chiral stationary phase GC as described in Section 3.2.2.

3.8. Kinetic resolution of racemic amines catalyzed by immobilized transaminase preparations in batch mode

Racemic amine substrates rac-3a–d (20 mM) and pyridoxal 5’-phosphate monohydrate - PLP (0.2 mM) in HEPES buffer (1 mM, 50 mM, pH 7.0) containing DMSO as a co-solvent (50% v/v in case of rac-3c) was added to a 1.5 mL Eppendorf tube, containing the immobilized ω-transaminase catalyst (10 mg). Suspensions were incubated at 30°C for 15 minutes. Than sodium pyruvate (0.5 equiv., 10 mM) was added to the mixture. Reactions were shaken in Eppendorf shaker (950 rpm) at 30°C. Samples were taken after 2, 4 and 24 h and aqueous NaOH (1 M, 100 µL) was added followed by the extraction of the amine and ketone into ethyl acetate (600 µL). The organic phase was dried with Na2SO4, and was derivatized with acetic anhydride (1 µL, 60°C, 3 h) to determine the conversion and enantiomeric excess of (R)-3a-d by GC as described in Section 3.2.2.
3.9. Continuous-flow mode biotransformations

The continuous-flow KRs were performed in a laboratory flow reactor comprising an isocratic HPLC pump (K-120, Knauer, Berlin, Germany) attached to CatCart™ columns (ThalesNano Budapest, Hungary; stainless steel, inner diameter: 4 mm; total length: 70 mm; packed length: 65 mm; inner volume: 0.816 mL) filled with the immobilized BcL, CaLB or CvTA biocatalysts in an in–house made thermostated aluminum metal block column holder with precise temperature control. Before use, the BcL-, CaLB- or CvTA-filled columns were washed with a 2:1 mixture of hexane and tert-butyl methyl ether (0.5 mL min\(^{-1}\) or 1 mL min\(^{-1}\), 20 min). The adsorbed BcL biocatalysts (on mesoporous silica gel with mixed grafting in the presence of PVA 18–88 or PEG 20k) were packed into stainless steel CatCart™ columns according to the filling process of ThalesNano. Before packing, the BcL biocatalyst-filled columns were washed with distilled water, ethanol, n-hexane and acetone in an ultrasonic cleaner. For the continuous-flow enzymatic applications, the columns were sealed by silver metal filter membranes [Sterlitech Silver Membrane Filter from Sigma–Aldrich, Z623237, pore size 0.45 μm; pure metallic silver, 99.97% with no extractable or detectable contaminants] due to the known benefits of Ag (bacteriostatic). The sealings were made of PTFE [Whatman® Sigma–Aldrich, Milwaukee, WI, USA, WHA10411311, pore size 0.45 μm] filter membranes.

3.9.1. Kinetic resolutions of rac-1a with BcL- or CaLB-filled continuous-flow bioreactors

Two CatCart™ columns per enzyme were packed for the study (separate columns for temperature and substrate concentration test). Filling weights for BcL preparations were: BcL with PVA 18–88: 237.1 mg and 237.7 mg; BcL with PEG 20k: 233.6 mg and 245.9 mg). Filling weights for CaLB preparations were: CV CaLB, 250 ± 10 mg; HAGD CaLB, 230 ± 13 mg; HAHD CaLB, 220 ± 2 mg; HACH CaLB, 230 ± 15 mg; EAGD CaLB, 229 ± 5 mg; EAHD CaLB, 212 ± 9 mg; EACH CaLB, 214 ± 14 mg.

To study the effect of the substrate concentration, solutions with racemic 1-phenylethanol (rac-1a) at different concentrations (0.5, 1.0, 2.5, 5.0, 10, 25, 50 mg mL\(^{-1}\) for BcL preparations; 1, 2.5, 5, 9.5, 22, 40, 68, 103, 148, 174 mg mL\(^{-1}\) for CaLB preparations) in 6/3/1 mixture of hexane/tert-butyl methyl ether/vinyl acetate were pumped through the adsorbed BcL or CaLB biocatalyst-filled columns (adsorbed BcL with PVA 18–88 or PEG 20k) thermostated to 30 °C at a flow rate of 0.2 mL min\(^{-1}\). At each concentration, samples were analyzed by GC every 10 min up to 40 min after the beginning of the experiment. Samples were collected during stationary operation (30 min after...
changing the parameters), diluted with EtOH to 2 mg mL\(^{-1}\) and analyzed as described in Section 3.2.2.

To study the effect of the temperature, a solution of \(\text{rac-1a}\) (5.0 mg mL\(^{-1}\) for \(\text{BcL}\) preparations or 40 mg mL\(^{-1}\) for \(\text{CaLB}\) preparations) in 6/3/1 mixture of \(n\)-hexane/\(t\)-butyl methyl ether/vinyl acetate was pumped through the biocatalyst-filled columns thermostated to various temperatures (0 °C–100 °C for \(\text{BcLs}\) or 0 °C–110 °C for \(\text{CaLBs}\)) at a flow rate of 0.2 mL min\(^{-1}\). Samples were collected during stationary operation (30 min after changing the parameters) and analyzed as described above. The experiments were performed at 10 °C steps in the temperature range between 0 °C–100 °C or 0 °C–110 °C. After performing the various tests, the columns were washed with a 2/1 mixture of \(n\)-hexane/\(t\)-butyl methyl ether (0.5 mL min\(^{-1}\), 20 min) and stored at 4 °C. The \(\text{CaLB}\) samples were removed from columns and then the samples were analyzed by scanning electron microscope.

3.9.2. Continuous-flow immobilization of \(\text{CvTA}_{W60C}\) and kinetic resolution of racemic amines

Immobilization of \(\text{CvTA}_{W60C}\) was carried out at room temperature in triplicate. Enzyme solution (2 mg mL\(^{-1}\), in a volume corresponding to enzyme:support ratio 1:10) was recirculated in stainless-steel CatCart™ columns filled with EAGD support (support weights: 211.4±16.1 mg) at a flow rate of 0.5 mL min\(^{-1}\). Protein concentrations of the \(\text{CvTA}_{W60C}\) solution before immobilization and at several time points during immobilization were determined by NanoDrop 2000 spectrophotometer.

For KRs of the amines \(\text{rac-3a-c}\), a solution containing the amine \(\text{rac-3a-c}\) (20 mM), sodium pyruvate (14 mM) and PLP (0.2 mM) in HEPES buffer (50 mM, pH 7.0, for \(\text{rac-3a,b}\); containing DMSO as cosolvent (50% v/v) for \(\text{rac-3c}\)) was pumped through \(\text{CvTA}_{W60C}\)-EAGD-filled column thermostated to 30 °C at different flow rates (0.1, 0.3 or 0.5 mL min\(^{-1}\)). Samples were taken and analyzed (after extraction and derivatization as described in Section 3.8.) by GC every 10 min up to 60 min.

The products were isolated from the effluents collected during stationary phase of the operation (20-60 min after changing the parameters). The pH of the collected effluents was adjusted to 1 by aqueous HCl (5 M), and the remaining ketone was removed by extraction with ethyl acetate (3 x 10 mL). After removal of the ketone, pH of the aqueous phase was adjusted to 12 by addition of ammonium hydroxide (25%) and the residual amine was extracted with ethyl acetate (3 x 10 mL). From the combined extracts the solvent was evaporated resulting in the \((R)\)-amines in high yield [(\(R\))-3a: 49% yield (28 mg, \(ee_{(R)-3a}>99\%\)), from run at 0.5 mL min\(^{-1}\) flow rate; \((R)\)-3b: 48% yield
(27 mg, $ee_{(R)-3b} > 99\%$), from run at 0.5 mL min$^{-1}$ flow rate; $(R)$-3c: 48% yield (13 mg, $ee_{(R)-3c} = 96\%$), from run at 0.1 mL min$^{-1}$ flow rate].
4. **GOALS OF THE THESIS**

Aim of my PhD thesis was to develop efficient and stable immobilized biocatalysts with different activities (lipase, transaminase and ketoreductase) and apply these biocatalysts in the synthesis of enantiomerically pure products.

In the first part of my PhD thesis, comprehensive study of immobilization methods was carried out with two industrially relevant lipases (*Burkholderia cepacia* lipase and *Candida antarctica* lipase B). In case of *Burkholderia cepacia* lipase, our aim was to combine the bioimprinting effect and enzyme immobilization on surface modified mesoporous silica gel. As a support, phenylaminopropyl modified silica gel was selected, as it proved to be an efficient carrier for lipase in our previous study. Different additives could significantly alter the biocatalytic activity of enzymes by acting as substrate analogues (bioimprinting) or by embedding the enzyme in a polymer matrix in addition to enzyme immobilization. For the evaluation of immobilized biocatalysts, kinetic resolution of racemic secondary alcohols can be applied in batch as well as in continuous-flow reactors (see Scheme 4.-1.). After the promising results of bisepoxide application as novel crosslinking agent in the BcL immobilization, our goal was to extensively study the covalent immobilization of *Candida antarctica* lipase B with crosslinking agents of different length of spacer arm and hydrophobicity. Bisepoxides bearing different moieties can significantly alter the performance of enzymes in biotransformations. As a support, methacrylate polymer resin may be used due to its functionalizable surface, mechanical stability and proper pore size distribution.

**Scheme 4.-1.:** Kinetic resolution of secondary alcohols catalyzed by BcL and CaLB. Kinetic resolution of racemic amines catalyzed by immobilized CvTA<sub>W60C</sub>^a

(*Abbreviations: BcL: *Burkholderia cepacia* lipase, CaLB: *Candida antarctica* lipase B, CvTA<sub>W60C</sub>: *Chromobacterium violaceum* transaminase)
The second part of my PhD thesis is dedicated to the improved mutant of $\omega$-transaminase from *Chromobacterium violaceum*. The plasmid encoding $\text{CvTA}_{\text{W69C}}$ mutant was a kind gift from Prof. Per Berglund (KTH, Sweden). $\text{CvTA}$ as native enzyme, shows poor stability under the conditions of use, thus immobilization of enzyme may be very important. The quaternary structure of this homodimeric enzyme can be excellently stabilized by covalent linkages to porous supports. Our aim was to study the covalent immobilization and stabilization of this transaminase on bisepoxide-activated aminoalkyl resins and perform continuous-flow kinetic resolutions of racemic amines (see Scheme 4.1).

Another possibility to solve the enzyme stability issue is to use a whole-cell biocatalyst instead of native enzyme. After investigating the transaminase stability, our next goal was to apply the *E. coli* cells containing transaminase in biocatalysis and to study the co-application of cells with cells of different origin. *Lodderomyces elongisporus* whole-cells of yeast origin were selected for this study. The whole-cells with different catalytic activity are able to perform one-pot cascade reactions without the need for intermediate isolation and produce enantiomerically pure products.
5. **RESULTS AND DISCUSSION**

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

For industrial application of enzymes, immobilization may play a crucial role to enhance their selectivity, activity and more importantly, stability. According to our preliminary studies, mixed-function-grafted mesoporous silica gels proved to be suitable carriers for lipase (CaLB) immobilization (adsorptive as well as covalent attachment onto solid support). In addition, fine tuning of lipase biocatalysts in sol-gel process could significantly enhance the productivity of biocatalysts in continuous-flow processes.

Aim of our study was to investigate and combine immobilization method of *Burkholderia cepacia* (*BcL*) lipase with imprinting effect of additives. When enzymes are treated at their active site by substrates or their analogues during immobilization in aqueous environment, then placed to organic solvent, enzyme activity may be enhanced (see Fig.2.-26.). Adsorption and adsorption followed by covalent binding of *BcL* was carried out in the presence of more than 40 additives as bioimprinting agents, stabilizers or surface coating polymers. The selected additives were mono- (rhamnose, glucose, fructose, xylitol, xylose, sorbitol, mannitol), di- (sucrose, maltose) and polysaccharides (xylene, gum arabic, carrageenan, sodium alginate, chitosan, α- and β-cyclodextrins, starch); polyethylene glycols (tetaethylene glycol, PEG 400, PEG 1k, PEG 4k, PEG 8k, PEG 20k, PEG 1k dimethyl ether); polyvinyl alcohols (PVA 4–88, PVA 18–88, PVA 13–23–88, PVA 60–98, PVA 72–98), detergents (Brij 30, Triton X-100, Tween 80), carboxylic and fatty acids (hexanoic, octanoic, decanoic, lauric, palmitic, oleic acid); glycerol and glycerides (glycerol, trilaurin, triolein, tristearin, Hymono 9004—a mixture of unsaturated mono- and diglycerides, FAME—fatty acids methyl esters), and unnatural racemic lipase substrates (1-phenylethanol, 1-(thiophen-2-yl)ethan-1-ol, 2-octanol), which are capable of molecular imprinting.

Mesoporous silica gel with mixed grafting – (3-aminopropyl)trimethoxysilane (ApTMS) and phenyltrimethoxysilane (PTMS) in mole ratio of 1:3 – allowed efficient immobilization of lipase B from *Candida antarctica* by adsorption as well as by covalent immobilization in our previous study. Thus, hydrophobic mixed-grafted silica gels with amino groups on the surface were applied in the adsorption and covalent immobilization of *BcL* in the presence of the various additives. Our recent results showed that glycerol diglycidyl ether (GD) was an efficient crosslinking agent for the preparation of cross-linked enzyme aggregates. GD was used for surface activation of mesoporous silica gels instead of the previously used glutardialdehyde. Effects of the various additives on the biocatalytic properties of immobilized *BcLs* were investigated in the
kinetic resolutions of racemic secondary alcohols 1-phenylethanol (rac-1a) and 1-(thiophen-2-yl)ethan-1-ol (rac-1b) in batch mode (Table 5.-1.) and in continuous-flow bioreactors as well (Scheme 5.-1.).

**Scheme 5.-1.** Kinetic resolutions of rac-1a and rac-1b with immobilized BcL biocatalysts in batch and continuous-flow reactors

[Diagram of Scheme 5.-1]

*Abbreviations: rac-1a: racemic 1-phenylethanol, rac-1b: racemic 1-(thiophen-2-yl)ethan-1-ol, BcL: Burkholderia cepacia lipase, PEG: polyethylene glycol, PVA: poly(vinyl alcohol)*

Results from KR of rac-1a are summarized in Table 5.-1. Several additives significantly enhanced the catalytic properties of the adsorbed BcL. When direct adsorption of BcL (without additive) was applied, conversion reached only 2.3% ($U_b=1.6 \mu\text{mol g}^{-1}\text{min}^{-1}$; $ee_{(R)-2a}=98.1\%$) after 4 h of KR of rac-1a. Sugars and monosaccharides had no significant impact on activity of BcL (data not shown), the surfactant Tween 80 enhanced slightly the activity and selectivity of BcL ($U_b=6.7 \mu\text{mol g}^{-1}\text{min}^{-1}$; $ee_{(R)-2a}=99.2\%$). As expected, the natural substrates of lipases such as trilaurin and triolein resulted in greater enhancement of activity (e.g. triolein increased the specific activity by 10-fold). 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 ($U_b=17.9 \mu\text{mol g}^{-1}\text{min}^{-1}$; $ee_{(R)-2a}=99.4\%$). The most active adsorbed BcL / PEG 20k biocatalyst had 13-fold higher enzyme activity in kinetic resolution of rac-1a ($U_b=20.5 \mu\text{mol g}^{-1}\text{min}^{-1}$; $ee_{(R)-2a}=99.0\%$) than BcL adsorbed without any additives. Although racemic 1-phenylethanol rac-1a is neither an ester nor carboxylic acid-type substrate, the 2.5-fold activity enhancement of the rac-1a-treated BcL compared to the non-treated BcL preparation in KR of rac-1a indicated significant bioimprinting effect. Remarkably, the enantiomer selectivity of BcL increased in each case as well.
Table 5.1. Biocatalytic properties of BcLs adsorbed onto amino-phenyl function-grafted silica supports in the kinetic resolution (KR) of rac-1a and rac-1b in batch mode.

<table>
<thead>
<tr>
<th>Additive</th>
<th>KR of rac-1a</th>
<th>KR of rac-1b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c (%)</td>
<td>ee(1R,2R) (%)</td>
</tr>
<tr>
<td>Brij 30</td>
<td>2.3</td>
<td>98.1</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5.1</td>
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<tr>
<td>PVA 4-88</td>
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<td>99.2</td>
</tr>
<tr>
<td>PVA 18-88</td>
<td>26.1</td>
<td>99.5</td>
</tr>
<tr>
<td>PVA 13-23-88</td>
<td>22.5</td>
<td>99.4</td>
</tr>
<tr>
<td>PVA 72-98</td>
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<td>99.3</td>
</tr>
<tr>
<td>PEG 8k</td>
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</tr>
<tr>
<td>Hymono 9004</td>
<td>23.3</td>
<td>99.3</td>
</tr>
<tr>
<td>Trilaurin</td>
<td>22.5</td>
<td>99.5</td>
</tr>
<tr>
<td>Tritoine</td>
<td>14.2</td>
<td>99.4</td>
</tr>
<tr>
<td>rac-1a</td>
<td>10.8</td>
<td>99.0</td>
</tr>
<tr>
<td>rac-1b</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a The conversion (c) and enantiomeric excess of ester (ee(1R,2S)) was determined by chiral GC and enantiomeric ratio (E) was calculated from c and ee(1R,2a). The results of KR with immobilized BcLs are shown only for biocatalysts with U₃ > 5.0 µmol g⁻¹ min⁻¹. Reactions were performed at 30 °C for 4 h. The conversion (c) and enantiomeric excess of ester (ee(1R,2b)) was determined by chiral GC and enantiomeric ratio (E) was calculated from c and ee(1R,2b). The results of KR with immobilized BcLs are shown only for biocatalysts with U₃ > 3.0 µmol g⁻¹ min⁻¹. Reactions were performed at 30 °C for 2 h.

The results of KRs of rac-1b with the adsorbed BcL biocatalysts templated with the additives are summarized in Table 5.1. Since BcL has towards rac-1b moderate selectivity, effect of the additives in adsorptive immobilization of BcL can be investigated more precisely. KR of rac-1b catalyzed with the non-templated BcL biocatalyst resulted in only 2.1% conversion after 2 h (U₃ = 2.7 µmol g⁻¹ min⁻¹; ee(1R,2b) = 76.6%). Addition of additives significantly enhanced the productivity of BcL in many cases, but enantiomeric excesses of the forming esters were marginally decreased. Similarly to the KRs of rac-1a, surfactants enhanced the specific enzyme activity in KRs of rac-1b by 2- and 4-fold (U₃ = 5.5 and 11.5 µmol g⁻¹ min⁻¹; with Brij 30 and Tween 80, respectively). The natural substrates (triolein, trilaurin, oleic acid and lauric acid) as additive showed higher imprinting effects with the adsorbed BcL (2.8–7.1-fold increase of U₃). In the same way as in the KRs of rac-1a, the most active BcL biocatalysts in KRs of rac-1b were template with
RESULTS AND DISCUSSION

PVAs and PEGs as additives (13-fold enhancement, $U_b=35.9 \ \mu\text{mol} \ \text{g}^{-1} \ \text{min}^{-1}$ and 17-fold activity enhancement, $U_b=45.1 \ \mu\text{mol} \ \text{g}^{-1} \ \text{min}^{-1}$; with PVA 4-88 and PEG 20k, respectively). Expectedly, in the KR of rac-1b the rac-1b-treated BcL showed more significant bioimprinting effect (2.4-fold increase in $U_b$ compared to the non-treated BcL preparation) than the rac-1a-treated one (less than 2-fold enhancement of $U_b$).

5.1.1. Effect of additives on the biocatalytic properties of covalently immobilized BcL

Combining the benefits of bioimprinting and covalent binding of BcL onto mixed-function grafted silica gel can further improve the stability of the covalently-linked BcLs. It was already demonstrated that heterofunctional supports could be advantageous for enzyme immobilization from multiple points of view.\textsuperscript{184} Phenyl-modified silica gel was beneficial for hydrophobic adsorption and lipase activation\textsuperscript{185,186} and covalent immobilization of CaLB on amino-grafted-silica gel enhanced the thermal stability of the enzyme.\textsuperscript{154} Based on our previous studies, it was assumed that adsorption of BcL on an amino-phenyl mixed-function-grafted silica gel support followed by cross-linking may provide a more stable biocatalyst. Thus, the mixed-function-grafted mesoporous silica gel was selected as a carrier which offers the possibility of covalent enzyme attachment. The adsorption and covalent immobilization of BcL was achieved in the presence of seven well performing additives (PEG 4k, Tween 80, PVA 18–88, gum arabic, triolein, lauric acid and oleic acid). Bisepoxide GD was applied as cross-linking agent instead of the previously used glutaraldehyde. As it was already studied, GD may be particularly useful crosslinking agent due to its ability to form stable bonds under mild conditions not only with the amine groups of Lys residues but with sulfur and oxygen containing residues of Cys, Tyr, Glu or Asp as well.\textsuperscript{183} In addition, GD is an inexpensive, partially water soluble bisepoxide compound being less toxic than glutaraldehyde (GA). The discouraging results of GD cross-linking after adsorption without additives may be rationalized by taking into account the spatial distribution of the surface exposed Lys residues in the open conformation of BcL. The crystal structure of BcL (Fig.5.-1.) clearly indicates that the majority of the surface exposed Lys residues (four out of seven) are close to the lid domains and modulate the active site accessibility. Thus attaching the enzyme to the support by these residues may indicate—at least partially—the closure of the entrance to the active site thus resulting in inactive forms of the immobilized BcL.
Fortunately, all the eight selected additives (PEG 4k, PEG 20k, Tween 80, PVA 18-88, gum arabic, lauric acid, oleic acid, triolein) contributed to the enhancement of the biocatalytic properties of the cross-linked BcL preparations (Table 5.-2.). Addition of natural carboxylic acid substrates (e.g. lauric acid or oleic acid) during immobilization preserved the activity of BcL (almost tenfold increase in activity). Larger ester-type additives (e.g. triolein or Tween 80) further enhanced the conversion ($c = 7.3$ and $8.7\%$, respectively). The best results were obtained with large molecular weight polymeric additives such as PEG 20k ($c = 11.8\%$), gum arabic ($c = 13.5\%$) and PVA 18–88 ($c = 14.0\%$). The beneficial effect may be explained by the lower hydrophobicity of the polymeric additives forcing opposite orientation at the surface of the carrier and rigidification of the enzyme in proper conformation during the cross-linking process. In aqueous environment, treating the BcL with additives may alter the conformation of enzyme and this more active form is frozen in the second step of immobilization process.
**Table 5.-2.** Biocatalytic properties of covalently immobilized BcLs on mixed function-grafted mesoporous silica gel in the kinetic resolution of *rac*-1a and *rac*-1b in batch mode

<table>
<thead>
<tr>
<th>Additive</th>
<th>KR of <em>rac</em>-1a</th>
<th>KR of <em>rac</em>-1b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>e</em> (%)</td>
<td>ee(R)-2 (%)</td>
</tr>
<tr>
<td>-</td>
<td>0.3</td>
<td>89.8</td>
</tr>
<tr>
<td>PEG 4k</td>
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<td>97.9</td>
</tr>
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<td>99.5</td>
</tr>
<tr>
<td>Tween 80</td>
<td>7.3</td>
<td>99.6</td>
</tr>
<tr>
<td>PVA 18-88</td>
<td>14.0</td>
<td>99.8</td>
</tr>
<tr>
<td>Gum arabic</td>
<td>13.5</td>
<td>99.8</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>4.0</td>
<td>99.4</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>3.4</td>
<td>99.4</td>
</tr>
<tr>
<td>Triolein</td>
<td>8.7</td>
<td>99.7</td>
</tr>
</tbody>
</table>

* Reactions were performed at 30 °C for 24 h.

Evaluation of the biocatalytic properties of the BcL preparations cross-linked in the presence of the eight selected additives was also performed in the KR of *rac*-1b (Table 5.-2.). As seen in the KR tests with *rac*-1a, all the eight covalently bound BcLs prepared in the presence of additives showed enhanced biocatalytic features in the KRs with *rac*-1b as well. PEG 4k enhanced 2.6-fold the conversion, the bioimprinting additives caused even more pronounced effect (17- to 26-fold increase of the conversion with lauric acid, Tween 80, oleic acid and triolein). The most promising additives (large molecular weight polymeric additives PEG 20k, gum arabic and PVA 18-88) enhanced conversions in the KRs with *rac*-1b by 45- to 53-fold with covalently immobilized BcL. Unfortunately, the specific activity (*U*_b) of the cross-linked BcL preparations remained only 5-15% that of the adsorbed BcL biocatalysts. Due to the simplicity of the adsorption process and to the 10-fold higher specific activity of the adsorbed BcLs, only the adsorbed lipase biocatalysts were investigated further.

**5.1.2. Thermal stability of BcLs adsorbed onto mixed-function-grafted mesoporous silica gel**

Based on our previous results of KRs, five immobilized adsorbed BcLs in the presence of additives were selected for the further study on thermal stability. During the thermostability tests, the BcL samples adsorbed in the presence of the five selected additives were incubated in toluene at various temperatures (30, 50, 70, 90 and 110 °C) for 1 h and were tested in KR of *rac*-1a after cooling to 30 °C. Fig.5.-2. shows the specific activity (*U*_b) of the selected BcLs incubated at given temperatures in the KRs at 30 °C after 2 h.
In our previous KR tests with rac-1a at 30 °C, the BcL / PEG 20k turned out to be the most active preparation ($U_b = 18.7 \mu$mol g$^{-1}$ min$^{-1}$). However, after the incubation the BcL / PEG 20k form proved to be the least thermostable (retained only 18% of its initial activity after incubating at 70 °C for 1 h). The second most active form of adsorbed BcL at 30 °C was the one prepared with PVA 18-88 as additive ($U_b = 14.0 \mu$mol g$^{-1}$ min$^{-1}$). The BcL / PVA 18-88 form was one of the most thermostable preparations (87% of the initial activity was retained after incubating at 70 °C for 1 h). While the applied PVAs as additives had the same hydrolysis degree (88% hydrolyzed), their stabilizing effect can be explained based on their molecular weight. The higher molecular weight PVA 18-88 (130 kDa) or PVA 4-88 (31 kDa, as additive had almost the same relative stabilizing effect for BcL up to 70 °C, the lower molecular weight PVA 13-23-88 (13–23 kDa) resulted in lower degree of thermal stabilization (only up to 50 °C). Oleic acid imprinted BcL showed even less thermal stabilization. The most thermostable forms of BcL with PVA 4-88 and PVA 18-88 retained 44 and 49% of their initial activity after 1 h incubation at 90 °C, but all BcL forms lost their activity after incubating at 110 °C. The thermal behavior of adsorbed BcL with PEG 20k can be rationalized by assuming that enhanced thermostability is related to embedding the lipase molecule within a thin film of the high molecular weight additive PEG 20k which is diminished when the additive melts (Mp ~60 °C).

Finally, when we consider the results of PVAs, the more hydrophilic, almost fully (98%) hydrolyzed PVAs were much less active ($U_b = 10.1 \mu$mol g$^{-1}$ min$^{-1}$ with PVA 72-98: 72 kDa; and $U_b < 5 \mu$mol g$^{-1}$ min$^{-1}$ with PVA 60-98: 60 kDa; see Table 5.-1.) than PVAs with 88% hydrolysis ($U_b = 15.4–17.9 \mu$mol g$^{-1}$ min$^{-1}$ with PVA 18-88: 130 kDa, PVA 4-88: 31 kDa and PVA 13-23-88: 13–23 kDa; see Table 5.-1.), it can be assumed that partial hydrophobicity of less hydrolyzed PVAs forming a thin film embedding the lipase molecules contributed to the activation of BcL.
5.1.3. Reuse of adsorbed BcL biocatalysts in successive kinetic resolutions

For industrial applications of immobilized lipases, stability and reusability are of foremost importance. The operational stability and recycling of the BcLs adsorbed onto mixed-function-grafted mesoporous silica gel with additives were compared by repeated KRs of rac-1a. The five adsorbed BcL biocatalysts with the highest specific biocatalyst activity (\(U_b\)) were applied in successive KRs of rac-1a. Each biocatalyst was reused seven times and their \(U_b\) (initial values are listed in Table 5.-1.) was recorded after 1 h of KRs. Fig.5.-3. shows the retained relative specific activities of adsorbed BcL biocatalysts related to their initial specific biocatalyst activity defined as 100%.

![Graph](image)

**Fig.5.-3.** Recycling of the adsorbed BcL biocatalysts in kinetic resolutions of rac-1-phenylethanol (rac-1a)

Recycling of the adsorbed BcL biocatalysts in eight cycles of KR of rac-1a showed different effect of the additives on the operational stability of the adsorbed BcL in organic solvent. Among five investigated additives four (oleic acid and three PVAs) resulted in quite stable adsorbed BcL biocatalysts retaining their initial specific biocatalyst activity even after eight runs. In contrast, the BcL / PEG 20k lost gradually its activity and retained only 49% of its initial \(U_b\) after eight runs. Worth mentioning, that the apparent increase of the initial \(U_b\) may be due to the equilibration / partial loss of water content of the enzyme during repeated runs. The good mechanical properties of carrier resulted in good retaining of the biocatalyst during the recyclability test (the mass loss of biocatalysts was below 5% in each cycle).

5.1.4. Continuous-flow kinetic resolutions of rac-1a with BcL / PVA 18-88 and BcL / PEG 20k

Development of biocatalyst immobilization techniques – in particular for enzymes, especially for lipases significantly contributed to the improvement of continuous-flow enzymatic reactions. When a biotransformation is performed in a continuous-flow packed-bed
bioreactor, the immobilized biocatalyst is continuously retained thus no enzyme recovery is needed. Additionally, packed-bed flow reactors with immobilized catalyst have a clear advantage in that voidage is low: 34% compared to over 80–90% being typical for a stirred tank reactor.191

Since it was already demonstrated, that temperature30 or the mode of lipase immobilization154,192 can significantly influence the lipase-catalyzed KR processes in continuous-flow reactors, two well-working adsorbed BcL biocatalysts (with PVA 18-88 and PEG 20k as additives) were selected to study the effect of substrate concentration and temperature on KR of rac-1a in continuous-flow packed-bed bioreactors (Fig.5.-4A).

First, the productivity of the two BcL biocatalysts ($r_{\text{flow}}$, µmol g⁻¹ min⁻¹) was investigated as a function of substrate concentration ($c_{\text{rac-1a}}$, mg mL⁻¹). Because the quasi-linear range of $r_{\text{flow}}$ as a function of $c_{\text{rac-1a}}$ ended at 5 mg mL⁻¹ by both BcLs (0.041 mmol mL⁻¹, Fig.5.-4B), the further temperature effect studies in the range of 0–100 °C were performed at this substrate concentration (Fig.5.-4C-E). The reactor operated at the flow rate of 0.2 mL min⁻¹ (residence time: 4.1 min) with conversion ($c = 16.3\%$ for PEG 20k, $c = 11.3\%$ for PVA 18-88) resulting in space time yield of 11 and 16 g L⁻¹ h⁻¹.

Next, temperature effects on enantiomeric excess ($ee_{(R)2a}$, Fig.5.-4C), productivity ($r_{\text{flow}}$, Fig.5.-4D) and selectivity ($E$, Fig.5.-4E) of two BcL biocatalysts in continuous-flow KR of rac-1a were investigated between 0–100 °C. In accordance with the thermal stability tests conducted in batch mode, productivity – temperature profiles of the adsorbed BcL / PEG 20k and BcL / PVA 18-88 were quite different (Fig.5.-4D). BcL / PEG 20k had its optimum working temperature at 30 °C, so it started to lose its activity over 40 °C. On the other hand, BcL / PVA 18-88 was thermostable up to 80 °C and deactivated only at higher temperatures.

Analysis of the temperature-dependency of enantiomer selectivity with the two BcL biocatalysts in continuous-flow KR of rac-1a revealed similar trends as published previously for lipase-catalyzed KR of secondary alcohols and amines in continuous-flow bioreactors.30,154,192 Both adsorbed BcLs showed maxima of $E$ at certain temperature (Fig.5.-4E). BcL / 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 BcL / PVA 18-88 had a maximum at higher temperature (~30 °C) with a much slower monotonic decrease up to 100 °C. The two BcL biocatalysts treated with PEG 20k and PVA 18-88 displayed remarkably different behavior in the continuous-flow KR of rac-1a. 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
results and discussion

Effects. On the other hand, the PVA 18-88 additive is nearly insoluble in non-aqueous media due to intermolecular hydrogen bondings. These assumptions can rationalize the enormous, about 50 °C difference in the optimum temperature of lipase activity for BcL co-immobilized with PEG 20k and PVA 18-88. These results highlight also the significance of embedding the enzyme molecules in a thin and permeable matrix which can lead to enhanced stabilization of immobilized form.

![Diagram](image)

**Fig.5-4.** Kinetic resolutions of rac-1-phenylethanol (rac-1a) in continuous-flow PBR (A). Effect of the substrate concentration on specific reaction rate, $r_{\text{flow}}$ (B) at 30 °C; temperature on enantiomeric excess, $ee(R)_{2a}$ (C); temperature on specific reaction rate, $r_{\text{flow}}$ (D) and temperature on enantiomeric ratio, $E$ (E) at 5 mg mL$^{-1}$. 
5.2. **Bisepoxide-activated aminoalkyl resins designed for covalent immobilization of Candida antarctica lipase B—Enhanced thermal stabilization of the enzyme in continuous-flow reactors**

Based on our successful and detailed examination of proper additives in immobilization of Burkholderia cepacia lipase, our next aim was to extend the study on covalent immobilization of Candida antarctica lipase B. Both lipases belong to the class of hydrolases, which represent a significant proportion of industrially relevant enzymes. The lipase from yeast Candida antarctica (CaLB) is one of the most frequently used lipase in biotechnology due to its excellent biochemical properties e.g. resistance to organic solvents, high stereospecificity and enantioselectivity.

Selection of the supports (mesoporous polymer resins with amine functional groups) was a crucial point for this study on surface fine-tuning exploring variations created by different bisepoxide spacer arms. Because the final goal was to apply the immobilized CaLB derivatives in continuous-flow reactors, pore size and particle size were important parameters influencing the pressure drop and mass transfer properties. Worth mentioning that the different lengths of ethylamine and hexylamine functional groups on the two starting carriers can contribute to the different properties of the final spacer arms between the surface of carriers and the target protein.

5.2.1. **Covalent immobilization of CaLB on bisepoxide-activated supports and kinetic resolution of rac-1a**

Aminoalkyl supports can be activated in different ways as already presented in the Literature Overview (Chapter 2). Because only some type of bisepoxides (e.g. glycerol diglycidyl ether, poly(ethylene glycol) diglycidyl ether) was used in enzyme immobilizations so far, our aim was to extend the use of bisepoxides in protein immobilizations. They display different hydrophobicities and length of spacer arms contributing to the flexibility of the immobilized enzyme. In addition, bisepoxides are cheap and non-toxic agents widely used in adhesives. As a reference, glutaraldehyde (GA) activation of the two carriers was also performed. After pre-activation, immobilization of CaLB was carried out in phosphate buffer (pH 7.5) at room temperature for 24 h.
Because the epoxide groups on resin surface may form covalent bonds not only via the surface exposed amine functions of Lys residues but also via the S- and O-atoms of Cys, Glu, Tyr, Asp, covalent bond formation possibility may be higher with the bisepoxide-activated supports than with the GA-activated ones, especially in the cases where proteins have only a few surface exposed lysine residues. These differences may explain the higher activity of the immobilized CaLB achieved with several bisepoxide-activated carriers compared to the GA-activated ones. Moreover, the C–X bonds (X = NH, S, O) forming by immobilization with the bisepoxide-activated carriers, unlike the C=N bonds forming in GA-activated ones, are not susceptible to hydrolysis.

In addition, GA-activated resins are not suitable for long-term storage and they should be used for immobilization shortly after activation. On the other hand, the bisepoxide-activated supports—similarly to the usual epoxy carriers—can be stored for a long time enabling real separation of the carrier activation and enzyme immobilization steps in time.

To characterize the biocatalytic properties of the biocatalysts, kinetic resolution of 1-phenylethanol (rac-1a) was selected as the test reaction in batch mode (Scheme 5.-2). This test enabled gaining information on the activity and enantiomer selectivity of the CaLB variants. The activity of the preparations was tested before and after washing with Triton X-100 non-ionic detergent solution. This method was used to distinguish between enzyme fractions which were retained on the surface only by hydrophobic physical adsorption.

**Fig. 5.** Bisepoxide activation of aminoalkyl supports for covalent immobilization of CaLB (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)
Scheme 5.-2. Kinetic resolution of rac-1a catalyzed by covalently immobilized CaLB preparations in batch mode and in continuous-flow reactor

Table 5.-3. shows the wash resistance representing the covalent ratio of binding onto activated EA- and HA-resins and the biocatalytic performance of the covalently bound CaLB preparations. For a comparison, a commercial preparation (ChiralVision CaLB, CV) was selected in which CaLB was covalently attached to macroporous acrylic beads of 150–300 µm particle size by the aid of epoxy functions. In the case of CV CaLB, washing the preparation with Triton X-100 solution resulted in 125% activation possibly due to a slight bioimprinting effect. Wash resistance of EA CaLB slightly reached 7% and confirmed the fact that the non-activated resin did not contain groups for covalent attachment and only ionic interactions could retain the enzyme molecules causing residual activity. HA CaLB preparation with more hydrophobic surface compared to EA-resin (alkyl chain of hexylamine function contributed to the surface more hydrophobic) and ionic interactions could contribute the slightly higher wash resistance (11%).

When poly(ethylene glycol) diglycidyl ether (PE) was used as crosslinking agent, the wash resistance remained quite similar as observed by the unmodified resins (7% for EAPE CaLB and 17% for HAPE CaLB). These results can be explained with good adsorptive ability of the supports combined with only occasional covalent bond formation. During the activation both epoxy functions of the bisepoxide with a long spacer arm of high flexibility could react with the surface amine functions.

On the other hand, high wash resistance was obtained with CaLB immobilized on 1,4-cyclohexanediol diglycidyl ether (EACH)-, 1,6-hexanediol diglycidyl ether (EAHD)- and glycerol diglycidyl ether (EAGD)-activated resins (81%, 58% and 53%, respectively). This result can be attributed to the hydrophobic character of CH activating agent, since cyclohexyl rings on the surface increased the hydrophobicity of support. Barbosa et al. proved that proteins become immobilized on epoxy supports via a two-step mechanism: first, physical adsorption occurs and then covalent linkages are formed between the nucleophilic residues on the enzyme molecule and epoxy functions of the support. Thus, enhanced hydrophobicity of the spacer arm with cyclohexyl ring could result in stronger hydrophobic activation and consequently lead to CaLB
forms of higher activity. When compared bisepoxide-activated alkylamine resins to the widely applied glutaraldehyde activation, it can be clearly seen the value of the activation by a properly selected bisepoxide: approximately four-fold activity increase of the CaLB attached to CH-activated supports (\( U_b = 39.6 \, \mu{\text{mol min}}^{-1} \, g^{-1} \) for EACH CaLB and \( U_b = 47.6 \, \mu{\text{mol min}}^{-1} \, g^{-1} \) for HACH CaLB) compared to the activity of the corresponding CaLB form immobilized by the aid of the widely used glutaraldehyde-activation (\( U_b = 10.2 \, \mu{\text{mol min}}^{-1} \, g^{-1} \) for EAGA CaLB and \( U_b = 11.9 \, \mu{\text{mol min}}^{-1} \, g^{-1} \) for HAGA CaLB). The length of spacer arms significantly influenced the activity of prepared CaLB biocatalysts. Within both series (EA-resin-based or HA-resin-based), the highest degree of covalent immobilization (wash resistance) was achieved after CH activation (92% with HA-resin and 81% with EA-resin). HD- and GD- activation resulted in a somewhat lower degree of covalent binding (82% and 64% with HA-resin and 58% and 53% with EA-resin, respectively). However, only a modest degree of covalent binding was observed after BD-activation (51% with HA-resin and 22% with EA-resin). Residual activity of CaLB attached to the modified HA-resins after activation with any bisepoxide or glutaraldehyde was higher than that attached to the corresponding pre-activated EA-resin. Because the trends within the two series were the same, optimization of the bisepoxide-activation conditions for CaLB immobilization on either alkylamino resins was performed only with the three best bisepoxides (CH, HD and GD).

**Table 5.3.** Kinetic resolution of rac-1a catalyzed by CaLB on bisepoxide-activated polymer resins (molar amount of bisepoxide 5 mmol g\(^{-1}\) carrier). Results with the various forms of CaLB after washing with Triton X-100 are shown.

<table>
<thead>
<tr>
<th>Binding Function</th>
<th>Wash Resistance (%)</th>
<th>( U_b ) (( \mu{\text{mol min}}^{-1} , g^{-1} ))</th>
<th>( c ) (%)</th>
<th>ee(%(a))</th>
<th>( E )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV (^c)</td>
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<td>0.3</td>
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<td></td>
</tr>
<tr>
<td>EAGA</td>
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<td>7.4</td>
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<td>&gt;200</td>
</tr>
<tr>
<td>EAGD</td>
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<td>&gt;200</td>
</tr>
<tr>
<td>EABD</td>
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<td>6.2</td>
<td>98.2</td>
<td>&gt;200</td>
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<tr>
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<td>&gt;200</td>
</tr>
<tr>
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<td>99.5</td>
<td>&gt;200</td>
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<td>0.2</td>
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<td></td>
</tr>
<tr>
<td>HAGA</td>
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<td>98.9</td>
<td>&gt;200</td>
</tr>
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<td>17.4</td>
<td>99.6</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HABD</td>
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<td>24.5</td>
<td>17.9</td>
<td>99.2</td>
<td>&gt;200</td>
</tr>
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<td>14.7</td>
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<td>&gt;200</td>
</tr>
</tbody>
</table>

\(^{a}\)Wash resistance (%) = activity of samples washed with Triton X-100/activity of samples after immobilizationx100, in KR of rac-1a; \(^b\)Enantiomeric ratio; \(^c\)CV CaLB: CaLB immobilized onto epoxy-functionalized acrylic beads; \(^d\)Activity of CV CaLB washed with Triton X-100/activity of CV CaLBx100=125%; \(^e\)ee and \(E\) values are not given for reactions with \(c < 5\%\) due to their high uncertainty.
5.2.2. Optimization of the bisepoxide activation for covalent immobilization of CaLB

The microenvironment of enzymes after immobilization can be tuned by proper optimization of the process parameters. Our preliminary results proved that three bisepoxides (GD, HD, CH) applied in 5 mmol g\(^{-1}\) of carrier amounts (Table 5.3) significantly enhanced the catalytic features of CaLB. In this series of experiments, the amount of bisepoxides applied for surface modification of the two aminoalkyl resins was varied in order to investigate the effect of final epoxy group density on enzyme activity and immobilization yield (Fig.5.-6). As a reference, CV CaLB is shown on Fig.5.-6. Reduction of the amount of bisepoxides to 2.5 mmol g\(^{-1}\) carrier caused dramatic decrease in specific activity of EAHD CaLB and EAGD CaLB. With increasing molar amount of bisepoxides (up to 10 mmol g\(^{-1}\) carrier) increased the specific activity of the immobilized CaLB preparations. The specific enzyme activity only slightly decreased in case of EACH CaLB. Surface grafted with cyclohexyl rings could enhance or keep the activity of CaLB preparations even at lower density of epoxy groups. A further increase of the amount of bisepoxides to 20 mmol g\(^{-1}\) carrier resulted in negligible increase of activity of the immobilized CaLB compared to that of achievable by CaLB on the carriers treated by 10 mmol bisepoxide g\(^{-1}\) carrier. For both aminoalkyl resins, the optimal amount of bisepoxide for surface modification was defined as 10 mmol g\(^{-1}\) carrier.

![Graph A](image1.png) ![Graph B](image2.png)

**Fig.5.-6.** Optimization of the bisepoxide activation of EA and HA resins for CaLB immobilization. Effect of molar amount of bisepoxide to mass resin ratio (mmol g\(^{-1}\)) of CH, HD and GD on CaLB activity. Commercial CV CaLB is presented as a reference preparation.

Immobilization yields for 12 variants of bisepoxide-activated supports (EA- and HA-resins modified with 2.5 and 10 mmol g\(^{-1}\) bisepoxides) were determined by protein concentration measurements in the supernatant before and after immobilization of CaLB according to Bradford’s method.\(^{181}\) The results are shown in Fig.5.-7. Our results indicated that immobilization yields with the other two bisepoxides (CH, HD) were also significantly higher after bisepoxide-activation with
the optimal amount of bisepoxides (10 mmol g⁻¹ carrier) as compared to the resins modified with only 2.5 mmol bisepoxide g⁻¹ carrier. Supports modified with CH showed highest immobilization yields (EACH CaLB: 95% and HACH CaLB: 92%) which also confirmed the role of proper hydrophobicity of the linker which is beneficial for the first lipase adsorption step during the covalent immobilization on epoxy-activated supports. When comparing the high immobilization yields and specific activity of biocatalysts showed in Fig.5.-6., higher activity could be expected. However, results of activity assays are displayed after washing the preparations with detergent solution. The enzyme immobilized only by adsorptive interactions (which is calculated into immobilization yield) is removed from the surface of carriers and thus this enzyme proportion doesn’t contribute to the activity.

![Figure 5-7](image1)

**Fig.5.-7.** Effect of molar amount of bisepoxides to mass resin ratio on immobilization yields. Supports modified with 2.5 mmol g⁻¹ bisepoxide (dark grey bars); supports modified with 10 mmol g⁻¹ bisepoxide (light blue bars).

### 5.2.3. **Operational stability of the CaLB biocatalysts**

One of the great advantages of immobilized enzyme preparations is the possibility of re-use in repeated batch reactions, thus making the process more cost-effective.¹⁹⁸ Five CaLB preparations (EAGD CaLB, EACH CaLB, HAGD CaLB, HACH CaLB, HAHD CaLB) were selected for studying their stability during recycling. To test their operational stability, the CaLB biocatalysts were tested by repetitive cycles of KRs of rac-1a at 30 °C for 1 h in a shaken test tube (1000 rpm, in n-hexane/t-butyl methyl ether/vinyl acetate 6/3/1). Between each cycle, the CaLB biocatalysts were washed with n-hexane in order to remove substrates and products from pores. The relative residual activities of repeated batch experiments compared to the first experiment as 100% are presented in Fig.5.-8. Although every biocatalyst used in this study lost its initial activity cycle-by-cycle, HAHD CaLB 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. The EAGD CaLB in which the enzyme was attached by the shortest carbon chain retained only 70% of its initial activity after 13 cycles. Furthermore, CaLB immobilized on EA- or HA-resin activated with the cyclohexyl ring containing CH preserved 82% of their initial activity regardless of which resin was used.

**Fig. 5-8.** Operational stability of CaLB immobilized on ethylamine or hexylamine resins activated by bisepoxides. Relative activity(%) = activity in given cycle/activity in first cycle×100 was determined in kinetic resolutions of rac-1a.

5.2.4. *Long-term storage stability of the CaLB preparations*

In order to investigate the long-term stability, the biocatalysts were stored in screw capped vials at 4 °C for 12 months. After one year, the residual activity of the stored CaLB preparations was compared to the activity of the freshly prepared biocatalysts (see Table 5-4). After the 12-month storage, HACH CaLB and HAHD CaLB preserved almost entirely their initial activity (95% and 90%, respectively). Presence of the cyclohexyl ring in the linker was beneficial to preserve enzyme activity in case of CaLB attached to modified EA-resin (88% residual activity with EACH CaLB), while EAGD CaLB preserved only 67% of its initial activity. The enantiomeric excess of product (R)-2a remained high (ee(R)-2a = 99.4–99.6%).

**Table 5-4.** Long-term storage stability of the CaLB biocatalysts.

<table>
<thead>
<tr>
<th>Polymer Resin</th>
<th>Residual activitya (%)</th>
<th>ee(R)-2a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAGD</td>
<td>67</td>
<td>99.6</td>
</tr>
<tr>
<td>EAHD</td>
<td>81</td>
<td>99.4</td>
</tr>
<tr>
<td>EACH</td>
<td>88</td>
<td>99.6</td>
</tr>
<tr>
<td>HAGD</td>
<td>67</td>
<td>99.5</td>
</tr>
<tr>
<td>HAHD</td>
<td>90</td>
<td>99.6</td>
</tr>
<tr>
<td>HACH</td>
<td>95</td>
<td>99.6</td>
</tr>
</tbody>
</table>

*aThe residual activity of the biocatalysts after 12-month storage were compared to the activity of freshly prepared biocatalysts.
5.2.5. **Continuous-flow kinetic resolution of rac-1a catalyzed by CaLB preparations on bisepoxide-activated resins—Substrate concentration and temperature effects**

Flourish of continuous-flow processes became significant after the improvement of enzyme immobilization techniques. As seen previously, the mode of immobilization and operation temperature could significantly influence KR processes in continuous-flow bioreactors. Therefore, we have selected six different CaLB preparations from this study to investigate the effect of substrate concentration and temperature on KR of rac-1a catalyzed by the covalently immobilized enzymes. As a reference, this study was extended with the commercially available CV CaLB which contained CaLB covalently immobilized onto epoxy-functionalized acrylic resin.

During the substrate concentration dependency investigations, stainless-steel columns were filled with the actual immobilized CaLB preparation and then thermostated at 30 °C. The solution containing vinyl acetate and the substrate at various concentrations was pumped through the column at a constant flow rate. The productivity of the various CaLB preparations ($r_{\text{flow}}$, µmol g$^{-1}$ min$^{-1}$) was investigated as a function of substrate concentration (Fig. 5.-9A). The 2.5-fold higher activity of the most active HAHD CaLB than that of the commercially available CV CaLB indicated the significance of fine-tuning the bisepoxide activation for covalent immobilization. Similar to the batch-mode KR of rac-1a, CaLB on EA- and HA-resins activated with GD showed the lowest activity (EAGD CaLB 29.2 µmol g$^{-1}$ min$^{-1}$; HAGD CaLB 46.1 µmol g$^{-1}$ min$^{-1}$) among tested derivatives. Shorter spacer arms link enzyme molecules closer to the resin surface preventing greater flexibility. The behavior of CaLB on resin-surfaces grafted with epoxy function via a linker containing cyclohexyl ring (EACH CaLB and HACH CaLB derivatives) demonstrated that this lipase interacting with hydrophobic groups became more active in flow systems as well. The productivity–substrate concentration curve showed the typical saturation after an initial quasi-linear range with all seven CaLB preparations (Fig. 5.-9A). The rapidly increasing range of $r_{\text{flow}}$-[rac-1a] curves ended at around 40 mg mL$^{-1}$; therefore, the temperature effect studies were performed at this substrate concentration. At this substrate concentration with a flow rate of 0.2 mL min$^{-1}$ the space time yields were calculated as shown in Table 5.5. The EAGD preparation showed the lowest space time yield of 79 g L$^{-1}$ h$^{-1}$. The most active preparation HAHD resulted in almost 2-fold higher space time yield ($Y_s = 185$ g L$^{-1}$ h$^{-1}$), than commercial CaLB preparation CV CaLB ($Y_s = 111$ g L$^{-1}$ h$^{-1}$).
Table 5.5. Space time yield of covalently immobilized CaLB preparations used in kinetic resolutions of \textit{rac}-1a

<table>
<thead>
<tr>
<th>Polymer Resin</th>
<th>Space time yield * (g L (^{-1}) h (^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAGD</td>
<td>79</td>
</tr>
<tr>
<td>EAHD</td>
<td>129</td>
</tr>
<tr>
<td>EACH</td>
<td>142</td>
</tr>
<tr>
<td>HAGD</td>
<td>134</td>
</tr>
<tr>
<td>HAHD</td>
<td>185</td>
</tr>
<tr>
<td>HACH</td>
<td>164</td>
</tr>
<tr>
<td>CV</td>
<td>111</td>
</tr>
</tbody>
</table>

The major goal of this study was to investigate the thermal stability of CaLB linked to polymer resins by spacer arms with different length and hydrophobicity. Thus, KR of \textit{rac}-1a was performed in bioreactors filled with the selected CaLB preparations similarly as in the substrate-concentration tests but varying the temperature in the range of 0–110 °C in 10 °C steps (Fig.5.-9B–D).

**Fig.5.-9.** Continuous-flow kinetic resolution of \textit{rac}-1a catalyzed by CaLB immobilized on bisepoxide-activated resins. Biocatalytic properties (\(r_{flow}\), \(ee\), \(E\)) of immobilized CaLB 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.
Productivity ($r_{\text{flow}}$) of the six selected CaLB derivatives on bisepoxide-activated resins as a function of temperature was compared to commercial CV CaLB containing the lipase immobilized on epoxy-functionalized acrylic resin (Fig.5.-9B). The thermal behavior of CaLB attached to bisepoxide activated resins differed significantly from CV CaLB (linking the enzyme to the resin by a quite short linker). The $r_{\text{flow}}$ of CV CaLB increased only up to 70 °C, then a significant drop of enzyme activity was observed.

In contrast, productivity of CaLB immobilized on EA-resin activated by CH and GD bisepoxide increased almost linearly with increasing temperature up to 110 °C indicating the importance of properly selected linkers to achieve significant improvement of thermal stability. The productivity of CaLB immobilized via longer and more flexible linkers (the HD-activated EA-resin and the CH- or GD-activated HA-resins) increased also uninterruptedly up to 110 °C, although with a slowdown in the increase starting at around 70 °C. All CaLB preparations (immobilized on activated HA resin) remained highly active even at high temperatures.

Besides the productivity of the immobilized enzyme, dependence of enantiomer selectivity of the KR rac-1a on temperature is also an important issue. Thus, enantiomer selectivity of the process was characterized as a function of temperature by the enantiomeric excess of product (R)-2a ($ee$) and by the enantiomeric ratio ($E$) (Fig.5.-9C and 9D, respectively). The $ee$–temperature (Fig.5.-9C) and $E$–temperature (Fig.5.-9D) curves exhibited maxima at various temperatures between 50 and 80 °C. This behavior was in agreement with previously found maxima of $E$–temperature curves in KRs of secondary alcohols$^{200-202}$, although different resins displayed these maxima at different temperatures (Fig.5.-9D). Characteristic differences of enantiomer selectivity optima were found between CaLB on bisepoxide activated EA-resins (~80 °C) compared to CaLB on bisepoxide activated HA-resins (~60 °C) indicating that increased flexibility of the linker allows a lower degree of selectivity. CaLB immobilized on HA-resin activated by HD showed the highest enantiomer selectivity among all tested preparations at 60 °C, ($E \approx 700$ with $ee_{(R)-2a} = 99.3\%$), while the HAGD CaLB with shorter and less hydrophobic spacer arm showed a lower degree of enantiomer selectivity ($E \approx 490$ with $ee_{(R)-2a} = 99.2\%$). On the other hand, CaLB immobilized on EA-resins activated by the three bisepoxides (HD, CH and GD) showed maxima in enantioselectivity at around 80 °C ($E \approx 550$, 530 and 380, respectively). This is a remarkable shift compared to the enantiomer selectivity maxima of CaLB immobilized on HA-resins at a 20 °C lower temperature. Worth mentioning is that enantiomer selectivity maxima of both bisepoxide-activated series exceeded the 50 °C optimum found for CV CaLB without a long spacer between the resin surface and the enzyme.

In addition to their biocatalytic properties, mechanical stability of the immobilized biocatalysts is also an important issue. Thus, morphology of two selected CaLB forms (on the GD-activated EA-
resin and on the HD-activated HA-resin) representing both aminoalkyl-resin based series was investigated by scanning electron microscopy (SEM) before their use, after the recycling study and after the temperature dependence study (Fig.5.-10).

**Fig.5.-10.** Scanning electron microscopy (SEM) analysis of EAGD CaLB (a, b, c) and HAHD CaLB (d, e, f): (a) before kinetic resolution of rac-1a; (b) after 13 cycles of kinetic resolutions in batch; (c) after the temperature-dependence study in continuous-flow reactor up to 110 °C; (d) before kinetic resolution of rac-1a; (e) after 13 cycles of kinetic resolutions in batch; (f) after the temperature-dependence study in continuous-flow reactor up to 110 °C.

SEM investigations of EAGD CaLB and HAHD CaLB before their use in biotransformations showed intact morphology of spherical beads with diameters 150-300 μm (Fig.5.-10a and 5.-10d, respectively), indicating no mechanical damage during bisepoxide activation and enzyme immobilization steps. Remarkably, both CaLB preparations proved to be mechanically quite durable and remained intact even after 13 cycles of KR experiments in batch mode (Fig.5.-10b and 5.-10e). However, the SEM pictures after the temperature dependency study of KRs, performed in packed-bed reactors using continuous-flow mode in the 0–110 °C temperature range, indicated limitations in the temperature stability of these CaLB forms on polymer beads (Fig.5.-10c and 5.-10f). Although data sheets of the basic EA- or HA-resins declared them to be stable up to 60 °C, we tested CaLB on their bisepoxide-activated derivatives up to 110 °C in continuous-flow reactors. Thus the result indicating fracture of EAGD polymer beads at this elevated temperature was not surprising (Fig.5.-10c). Remarkably, HAHD beads remained apparently unaltered even after the temperature-dependency study ended at 110 °C.
5.3. Covalently immobilized Trp60Cys mutant of ω-transaminase from Chromobacterium violaceum for kinetic resolution of racemic amines in batch and continuous-flow modes

Enantiomerically pure amines are essential building blocks in the majority of active pharmaceutical ingredients. Pharmaceutical industry is facing a challenge when producing amine enantiomers as the synthesis often requires harsh reaction conditions and toxic transition metal catalysts. Discovering of transaminases significantly enhanced the development of amine synthesis thus biocatalysis offers now an alternative route to obtain amines of high enantiopurity.

As pointed out in our previous study, bisepoxide modification of polymer resins is an extremely cheap and easy-to-perform technique for fine tuning of the surface of enzyme carriers. Our aim was to prove, that the same carriers are perfect for the immobilization for the completely different type of enzyme. Amino-functionalized polymer resins were selected for the immobilization of CvTA_W60C as a model ω-transaminase. This variant of CvTA 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.

5.3.1. Bisepoxide activation of polymer resins for immobilization of CvTA_W60C and batch-mode kinetic resolution of racemic amines

For our study, a standard epoxy-functionalized resin (EP) and two aminoalkyl-functionalized carriers with ethylamine- and hexylamine- functions on their surface (EA, and HA) were selected as basic carriers (Fig.5.-11.). To evaluate several microenvironments for ω-transaminase (CvTA_W60C) during biocatalysis, three different bisepoxides as activating agents were selected for the present study: 1,6-hexanediol diglycidyl ether (HD) and 1,4-cyclohexanediethanol diglycidyl ether (CH) having relatively long linkers of hydrophobic character, and glycerol diglycidyl ether (GD) with a short and hydrophilic linker. As shown in Fig.5.-11., after activation of the aminoalkyl-methacrylate resins with the selected bisepoxides, immobilization of CvTA_W60C was achieved at room temperature.
Fig. 5.11. Immobilization of engineered o-transaminase CvTA\textsubscript{W60C} on bisepoxide-activated aminoalkyl resins (Bisepoxide abbreviations: GD: glycerol diglycidyl ether, CH: 1,4-cyclohexanediol methanol diglycidyl ether, HD: 1,6-hexanediol diglycidyl ether)

The immobilized CvTA\textsubscript{W60C} preparations were applied as biocatalysts in the KR of various racemic amines (Scheme 5-3). In our first experiments, 4-phenyl-2-butamide (\textit{rac}-3\textsubscript{a}) and sodium pyruvate (0.5 equiv.) as amine acceptor were used due to the synthetic significance of the residual enantiomer [(\textit{R})-3\textsubscript{a}] as a building block in the synthesis of Dilevalol.\textsuperscript{205} Since CvTA showed increased activity in HEPES buffer (50 mM, pH 7.0), these conditions were applied in our reactions as well.\textsuperscript{93}

**Scheme 5-3.** Kinetic resolution of racemic amines catalyzed by immobilized CvTA\textsubscript{W60C} mutant
Data of our first KR experiments are summarized in Table 5.-6. Influence of the spacer arms in the various CvTA<sub>W60C</sub> biocatalysts on KR of rac-3a was compared to CvTA<sub>W60C</sub> attached to a commercially available epoxy resin (EP) as a standard.

Table 5.-6. Kinetic resolution of rac-3a catalyzed by differently immobilized CvTA<sub>W60C</sub> preparations<sup>a</sup>

<table>
<thead>
<tr>
<th>Support</th>
<th>c&lt;sub&gt;app&lt;/sub&gt; (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>c&lt;sub&gt;theor&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ee&lt;sub&gt;(R)-3a&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP</td>
<td>9</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>EA</td>
<td>13</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>EAGD</td>
<td>49</td>
<td>50</td>
<td>&gt;99</td>
</tr>
<tr>
<td>EAHD</td>
<td>48</td>
<td>50</td>
<td>&gt;99</td>
</tr>
<tr>
<td>EACH</td>
<td>41</td>
<td>44</td>
<td>77</td>
</tr>
<tr>
<td>HA</td>
<td>14</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>HAGD</td>
<td>49</td>
<td>50</td>
<td>&gt;99</td>
</tr>
<tr>
<td>HAHD</td>
<td>30</td>
<td>32</td>
<td>48</td>
</tr>
<tr>
<td>HACH</td>
<td>20</td>
<td>22</td>
<td>29</td>
</tr>
</tbody>
</table>

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

Even the basic aminoalkyl-resins (EA and HA without surface modification), the standard commercial epoxy-resin (EP) and all of the bisepoxide activated aminoalkyl-resins (EAGD, EAHD, EACH, HAGD, HAHD and HACH) could bind CvTA<sub>W60C</sub> by adsorptive interactions. However, only the resins containing epoxy groups (EP, EAGD, EAHD, EACH, HAGD, HAHD and HACH) could form covalent bonds with the enzyme on their surface. Without surface modification, the basic aminoalkyl-resins (EA and HA) could fix CvTA<sub>W60C</sub> only by adsorption. While aminoalkyl-functionalized polymer resins were already claimed as proper ready-to-use enzyme carriers for two other transaminases,\textsuperscript{161} the basic aminoalkyl-resins (EA and HA) were not ideal carriers for immobilization of CvTA<sub>W60C</sub>. CvTA<sub>W60C</sub> on EA and HA resins resulted in low conversion of rac-3a to the corresponding ketone (4a) and poor enantiomeric excess of the unreacted amine (13% and 14% conversion for EA and HA respectively with ee<sub>amine</sub>&lt;20%).

CvTA<sub>W60C</sub> attached to the standard commercial epoxy-functionalized resin (EP) resulted in low conversion of rac-3a. Presumably, this carrier is capable of covalent binding, but the microenvironment of the enzyme immobilized in EP was not optimal for the catalysis. This can be explained with the absence of spacer arms between the enzyme and the surface of EP carrier resulting in limited flexibility during catalysis by assuming multipoint attachment to the surface. Furthermore, evaluation of the conversion and the corresponding enantiomeric excess of the unreacted amine fraction indicated significantly lower apparent conversion (c<sub>app</sub>) as could be calculated from the enantiomeric excess of the residual amine in a KR with exclusive enantiomer selectivity (c<sub>theor</sub>, determined according to Eq. 15., see Section 3.2.2.). The difference of c<sub>app</sub> from
The most significant difference between \( c_{\text{app}} \) and \( c_{\text{theor}} \) (12%) was found with EPM-\( CV_{\text{TA} W60C} \). Fortunately, when \( CV_{\text{TA} W60C} \) was immobilized onto bisepoxide-activated resins (EAGD, EAHD, EACH, HAGD, HAHD and HACH) the negligible differences between \( c_{\text{app}} \) and \( c_{\text{theor}} \) (<3%) indicated almost no product adsorption.

As expected, surface modification of the aminoalkyl polymers (EA and HA) with the hydrophilic glycerol diglycidyl ether (GD) resulted in the most efficient carriers for \( CV_{\text{TA} W60C} \) ensuring high enzyme activity. Transaminase immobilized on the glycerol diglycidyl ether-activated resins (\( CV_{\text{TA} W60C} \)-EAGD and \( CV_{\text{TA} W60C} \)-HAGD, respectively) applied as biocatalysts in the KR of \( \text{rac-3a} \) resulted in high conversions (49%) and excellent enantiomeric excess (\( ee_{(R)\text{-3a}} > 99\% \)) after 4 h. This could be rationalized with the flexible but hydrophilic spacer arms between the enzyme and the surface of the resins. When 1,6-hexanediol diglycidyl ether-activated resins (EAHD and HAHD) were applied for binding \( CV_{\text{TA} W60C} \), the activity decreased but the enzyme molecule could maintain its flexibility. Presumably, the length and hydrophobicity of the linker offered an acceptable microenvironment for the transaminase (\( c_{\text{app}} = 48\% \)), while the more hydrophobic surface on hexylamine-based resin (HAHD) could account for the slightly decreased conversion (\( c_{\text{app}} = 30\% \)). 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 \( c_{\text{app}} = 20\% \)).

After the comprehensive testing of carriers suitable for immobilization of \( CV_{\text{TA} W60C} \) in KR of \( \text{rac-3a} \), catalytic performance of the most efficient biocatalysts (\( CV_{\text{TA} W60C} \)-EAGD and \( CV_{\text{TA} W60C} \)-HAGD) was confirmed in KR of further racemic amines (\( \text{rac-3b-d} \)) bearing aliphatic and aromatic moieties and being known substrates of \( CV_{\text{TA}} \) (Table 5.-7.). In the KR of each racemic amine (\( \text{rac-3b-d} \)) \( CV_{\text{TA} W60C} \)-EAGD resulted in higher conversion than \( CV_{\text{TA} W60C} \)-HAGD. This indicated, that the more hydrophilic microenvironment provided by the shorter linker in EAGD was the best for immobilization of \( CV_{\text{TA} W60C} \).
Table 5.-7. Kinetic resolution of rac-3b-d catalyzed by CvTA_W60C-EAGD and CvTA_W60C-HAGDa

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Support</th>
<th>c_{app} b (%)</th>
<th>ee(R)-amine c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3b</td>
<td>EAGD</td>
<td>41</td>
<td>72</td>
</tr>
<tr>
<td>3b</td>
<td>HAGD</td>
<td>38</td>
<td>70</td>
</tr>
<tr>
<td>3c</td>
<td>EAGD</td>
<td>49</td>
<td>97</td>
</tr>
<tr>
<td>3c</td>
<td>HAGD</td>
<td>49</td>
<td>96</td>
</tr>
<tr>
<td>3d</td>
<td>EAGD</td>
<td>46</td>
<td>97</td>
</tr>
<tr>
<td>3d</td>
<td>HAGD</td>
<td>44</td>
<td>75</td>
</tr>
</tbody>
</table>

a Reactions were performed at 30 °C for 2 h with 0.5 equivalent of pyruvate. b Reaction was performed in DMSO cosolvent (50% v/v).

In the non-perfect KRs, the enantiomeric excess of the residual amine fractions was low due to the low conversions (ee(R)-3a-d <95%), reaction conditions were further optimized. By assuming that the applied 0.5 equivalent of sodium pyruvate as amine acceptor in the initial screening tests was not sufficient for the full conversion (50% in KR) the amount of the amine acceptor was increased. When comparing Table 5.-7. to Table 5.-8., the KRs of rac-3a-c (Table 5.-8.) catalyzed by CvTA_W60C-EAGD using 0.6 and 0.7 equivalents of sodium pyruvate resulted in higher conversions (c ~50%) leaving almost perfectly the unreacted amine in high enantiomeric excess (ee(R)-3a-c ≥98-99%). Nevertheless, the amount of added pyruvate had the most significant impact on the kinetic resolution of rac-3b. Because substrate 1,2,3,4-tetrahydro-1-naphthalenamine (rac-3c) is almost insoluble in water, DMSO (50% v/v) was used as co-solvent in the KR to enhance the solubility of the substrate. Fortunately, CvTA_W60C-EAGD proved to be sufficiently active under these conditions and resulted in good conversion (49%) and high enantiomeric excess of the residual amine (ee(R)-3c= 98%). Presumably, multipoint attachment of CvTA_W60C to the EAGD resin during immobilization stabilized the active structure of enzyme which could be preserved even at as high as 50% v/v concentration of DMSO.

Table 5.-8. Evaluation of pyruvate equivalent in KR of rac-3a-c catalyzed by CvTA_W60C-EAGD

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Equivalent of pyruvate</th>
<th>c_{app} d (%)</th>
<th>ee(R)-amine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>0.6</td>
<td>49</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3a</td>
<td>0.7</td>
<td>48</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3b</td>
<td>0.6</td>
<td>46</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3b</td>
<td>0.7</td>
<td>46</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3c b</td>
<td>0.6</td>
<td>49</td>
<td>98</td>
</tr>
<tr>
<td>3c b</td>
<td>0.7</td>
<td>49</td>
<td>98</td>
</tr>
</tbody>
</table>

a Deviation from c_{theor}= 50% might be due to selective adsorption of the product onto the carrier. b Reaction was performed in DMSO cosolvent (50% v/v).
5.3.2. Recycling of the CvTA<sub>W60C</sub> immobilized on EAGD resin

Besides high activity and selectivity of the target enzyme, another major goal of enzyme immobilization is to obtain a stable, heterogeneous form of the biocatalyst which can be easily recovered from the reaction and can be reused in successive cycles.

Having found suitable bisepoxide-activated resins for CvTA<sub>W60C</sub> immobilization (EAGD and HAGD) and reaction conditions optimization, recyclability of the CvTA<sub>W60C</sub>-EAGD and CvTA<sub>W60C</sub>-HAGD biocatalysts was tested in KR of rac-3a (Fig. 5.12). Between each cycle the CvTA<sub>W60C</sub> preparations were washed with HEPES buffer and centrifuged. Before starting a new cycle, PLP was added first to enzyme preparations to allow formation of PLP-transaminase complex. The recycling study of CvTA<sub>W60C</sub>-EAGD and CvTA<sub>W60C</sub>-HAGD was performed during 19 consecutive reaction cycles (each cycle lasted for 1 h). The results indicated that CvTA<sub>W60C</sub> attached onto proper bisepoxide-activated carriers could maintain its catalytic activity during at least 19 cycles (representing 19 h of operation) with excellent activity and selectivity (c ~50%, ee<sub>(R)-3a</sub> >99%).

![Relative activity (%) vs Cycle](image)

Fig.5.12. Successive kinetic resolutions of rac-3a catalyzed by immobilized CvTA<sub>W60C</sub>-EAGD (■) and CvTA<sub>W60C</sub>-HAGD (■)

5.3.3. Co-solvent stability of immobilized CvTA<sub>W60C</sub>-EAGD

Next, co-solvent stability of the most active biocatalyst (CvTA<sub>W60C</sub>-EAGD) was examined in media containing various DMSO concentrations. A detailed study on co-solvent tolerance of wild-type CvTA after storage in media containing different concentrations of DMSO and measuring the residual activity of enzyme at various DMSO concentrations indicated beneficial stabilization of wt-CvTA up to 50% v/v but significant deactivation was observed above 30% v/v. Thus, the CvTA<sub>W60C</sub>-EAGD biocatalyst was tested in KR of rac-3a in HEPES buffer media containing sufficient amount of PLP, sodium pyruvate and various concentrations of DMSO in the range of 0–
RESULTS AND DISCUSSION

50% v/v (Fig. 5.-13.). Fortunately, the covalently immobilized $CVTA_{W60C}$-EAGD performed well at all the tested DMSO concentrations up to 50% v/v and its enantiomer selectivity remained excellent. The beneficial enhancement of the co-solvent tolerance of $CVTA_{W60C}$-EAGD could be rationalized by assuming significant stabilization of the tertiary structure of $CVTA_{W60C}$ upon immobilization due to multipoint covalent fixation.

Fig.5.-13. Activity of immobilized $CVTA_{W60C}$-EAGD in buffer containing different concentrations of DMSO

5.3.4. Continuous-flow-mode kinetic resolution of racemic amines by immobilized $CVTA_{W60C}$-EAGD

A further way to enhance the robustness of applications of immobilized transaminases is to perform the $CVTA$-catalyzed process in packed-bed reactors operated in continuous-flow mode. Since transaminases are cofactor dependent enzymes, continuously operated reactions should be designed with sufficient PLP cofactor in the system. PLP plays an important role not only as cofactor but also contributes to the stabilization of the dimeric structure of $CVTA$.^85

When performing scaled-up immobilization of $CVTA$ in shake flasks to obtain sufficient amount of immobilized enzyme for studies in continuous-flow mode, precipitation of enzyme was observed. During the shaking process, enzyme is subjected to higher shearing forces as compared to the immobilization by flow-through approach. Therefore, to reduce immobilization time and to overcome enzyme precipitation, immobilization of $CVTA_{W60C}$ was performed onto the most efficient bisepoxide-activated resin (EAGD) filled in packed-bed column. In shake flask mode, immobilization of $CVTA_{W60C}$ was carried out at room temperature for 24 h in HEPES buffer (50 mM, pH 7.0). In the flow-through experiments – while keeping 10:1 as support to enzyme ratio similar to batch experiments – $CVTA_{W60C}$ solution was recirculated at 0.5 mL min$^{-1}$ at room temperature through an EAGD resin-filled stainless steel column and the transaminase content of the enzyme solution was checked regularly (Fig.5.-14.). It was demonstrated, that flow-through mode could shorten the immobilization time significantly (~100% immobilization yield could be
achieved within 2 h of constant recirculation. The shortened immobilization time is highly important since non-immobilized enzyme can be inactivated over the course of immobilization.

![Figure 5.14](Image)

**Fig. 5.14.** Immobilization of CvTA<sub>W60C</sub> on EAGD resin in flow-through mode. The residual concentration of CvTA<sub>W60C</sub> (●) and immobilization yield (■) were monitored over 120 min.

Activity and selectivity of CvTA<sub>W60C</sub>-EAGD in continuous-flow mode was evaluated in KRIs of racemic amines *rac*-3a-c at 30 °C (Fig.5.-15.). Before the KRIs, the CvTA<sub>W60C</sub>-EAGD-filled columns from flow-through immobilization were washed with PLP (0.2 mM) in HEPES buffer to further stabilize the dimeric structure of enzyme. To evaluate the effect of flow rate on kinetic resolutions, three different flow rates were applied: 0.5 mL min<sup>-1</sup>, 0.3 mL min<sup>-1</sup> and 0.1 mL min<sup>-1</sup> (residence times: 1.6 min, 2.7 min and 8.2 min respectively).

At first, KR of 4-phenyl-2-butamine (*rac*-3a) was investigated in continuous-flow mode. At 0.1 and 0.3 mL min<sup>-1</sup> flow rates perfect conversion (50%) was achieved leading to (*R*)-3a in excellent enantiomeric excess (*ee*<sub>(*R*)-3a</sub> >99%). After further increasing the flow rate to 0.5 mL min<sup>-1</sup>, the CvTA<sub>W60C</sub>-EAGD-filled reactor still provided excellent conversion (50%) and enantiomeric excess (*ee*<sub>(*R*)-3a</sub> >99%). The effluent was collected during stationary phase of the KR and enantiopure (*R*)-3a (*ee*<sub>(*R*)-3a</sub> >99%) was isolated in high yield (49%).

Next, 1-phenylethylamine (*rac*-3b), a widely used model substrate for transaminase catalyzed reactions, was examined in continuous-flow KR. Due to the excellent conversion (~50%) at every flow rate (0.1, 0.3 and 0.5 mL min<sup>-1</sup>), enantiopure (*R*)-3b (*ee*<sub>(*R*)-3b</sub> >99%) could be obtained in all cases. From the effluent collected at the stationary phase of this KR enantiopure (*R*)-3b (*ee*<sub>(*R*)-3b</sub> >99%) was isolated in high yield (48%). Overall, in these two series, the CvTA<sub>W60C</sub>-EAGD-filled column operated continuously over 2 × 3 h without significant loss of activity.

Finally, KR of 1,2,3,4-tetrahydro-1-naphthalenamine (*rac*-3c) in HEPES buffer containing DMSO as co-solvent (50% v/v) was investigated under continuous-flow conditions. At a flow rates of 0.3
mL min\(^{-1}\), non-perfect conversion was observed (39%) after 1 h of operation with \(ee_{(R)-3c} = 63\%\). When the residence time was increased by decreasing the flow rate to 0.1 mL min\(^{-1}\), the conversion increased to 48% providing the unreacted substrate \((R)-3c\) in good enantiomeric excess \((ee_{(R)-3c} = 96\%)\). Isolation of the product from the effluent of this reaction could be performed in high yield (48%). Conversion and enantiomeric excess of amines from effluents were determined by chiral gas chromatography.

**Fig.5.** Continuous-flow kinetic resolution of rac-3a-c at different flow rates in CvTA\(_{Whic}\)-EAGD-filled packed-bed reactor at 30 °C [conversion at 0.5 mL min\(^{-1}\)(▲), 0.3 mL min\(^{-1}\) (●) and 0.1 mL min\(^{-1}\) (◆); enantiomeric excess of \((R)-3a-c\) at 0.5 mL min\(^{-1}\)(▲), 0.3 mL min\(^{-1}\) (●) and 0.1 mL min\(^{-1}\)(◆)].
5.4. Immobilized E. coli cells containing transaminase and ketoreductase whole-cells for continuous-flow synthesis of enantiopure amines and alcohols

Multienzymatic synthesis of enantiopure chiral compounds is an ever-evolving field in chemistry and technology. Many studies have already shown cascade reactions employing isolated enzymes with low operational stability and no possibility of biocatalyst recycling. Use of whole-cells in chemical synthesis—instead of purified enzymes—simplifies these complex reactions as they have an internal cofactor regeneration system. In addition, reduced stability of cells under harsh conditions or inhibition by non-natural substrates and/or products can be significantly improved by applying immobilization techniques.

Combination of transaminases with other class of enzymes (e.g. oxidoreductases, transketolases, hydrolases, etc.) facilitates cascade reactions without the need for intermediate isolation which can lower the overall product yield. In the past few years, our research group has been working in close collaboration with Fermentia Microbiological Ltd. on isolating new types of microorganisms with ketoreductase activity. Whole-cell ketoreductases can selectively catalyze the reduction of ketones to optically pure secondary alcohols. They are widely used building blocks in complex drug structures.

As a part of my PhD thesis, we envisioned a miniaturized packed bed reactor system with immobilized whole-cell biocatalysts from Chromobacterium violaceum (CvTA) and Lodderomyces elongisporus (LeKRED).\textsuperscript{207} The aim of our study was to develop a combined cell system consisting of two different biocatalysts and to prove the compatibility with CvTA with other class of enzymes.

Experimental work related to this topic was carried out in collaboration with László Nagy-Győr. Scaled-up fermentations of transaminase and ketoreductase whole-cells were performed in Fermentia Microbiological Ltd.

5.4.1. Sol-gel immobilization process for E. coli cells containing transaminase

In the previous chapter, immobilization process for purified transaminase was shown. Based on the study performed by Kato et al.,\textsuperscript{208} our aim was to improve their sol-gel technique and develop a templated matrix suitable for whole-cell containing transaminase immobilization and prove its compatibility with other cells of different origin. As a first step, we determined the proper immobilization conditions for E.coli containing \( \omega \)-transaminase and LeKRED whole-cells. In the presence of hollow silica gel and whole-cells, acid-catalyzed hydrolysis of tetraethyl orthosilicate
RESULTS AND DISCUSSION

(TEOS) is followed by polycondensation. After maturation of gel, biocatalysts were gently crushed and washed with buffer.

The biocatalytic activity of cells was evaluated in the KR of racemic 4-phenylbutan-2-amine (rac-3a) as shown in Scheme 5.4. Amine 3a is a common substrate for ω-transaminase from Chromobacterium violaceum (CvTA) as previously shown.204

**Scheme 5.4.** Cascade reaction employing immobilized *E. coli* cells containing transaminase and ketoreductase whole-cells

To evaluate the compatibility of whole-cells, in batch reactions separately immobilized transaminase and ketoreductase cells were added together to the reaction mixture. After 24 h, samples were taken and analyzed by chiral gas chromatography. Results are shown in Table 5-9, which indicated that immobilization conditions were suitable for both whole-cells. Application of *E. coli* whole-cells in KR of rac-3a led to full conversion (ee(R)-3a>99%). The combination of *E. coli* and LeKRED cells resulted in slightly lower conversion, but we found that the co-application of whole-cells is suitable for cascade reaction.

**Table 5-9.** Evaluation of biocatalyst activity in batch-mode

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>( c^b ) (%)</th>
<th>( c_{\text{theor}}^c ) (%)</th>
<th>( ee_{(R)-3a} ) (%)</th>
<th>( ee_{(S)-1c} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CvTA</td>
<td>50</td>
<td>50</td>
<td>&gt;99</td>
<td>-</td>
</tr>
<tr>
<td>LeKRED</td>
<td>82</td>
<td>100</td>
<td>-</td>
<td>&gt;99</td>
</tr>
<tr>
<td>CvTA+LeKRED</td>
<td>40</td>
<td>50</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

\(^a\) Results are not published. \(^b\) conversion is based on ketone or alcohol formation. \(^c\) maximum theoretical conversion
5.4.2. Cascade reaction in continuous-flow reactor employing whole-cells containing transaminase

Kinetic resolution followed by asymmetric reduction was carried out in continuous-flow mode. Two columns filled separately with *E. coli* (CvTA) and LeKRED 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 CvTA was added to the solution. Reactions were performed at 30 °C for 24 h and conversion was determined based on alcohol formation. Results are shown in Fig.5.-16. Tuning the reaction parameters and maintaining high conversion, enabled the synthesis of enantiomerically pure amine. The first 3 h of biotransformation was fast without significant loss of activity. Stereoselectivity of transaminase cells (*ee*(*R*)-3a>99%) remained high, thus transaminase cells were compatible with cells of different origin.

![Operational stability of immobilized E. coli cells and LeKRED whole-cells under continuous-flow conditions](image)

**Fig.5.-16.** Operational stability of immobilized *E. coli* cells and LeKRED whole-cells under continuous-flow conditions (*ee*(*R*)-3a ■, *ee*(*S*)-1c ◆, conversion: ◆ *c*(*S*)-1c) at 30 °C

5.4.3. Cofactor-induced reactivation of *E. coli* cells containing transaminase under flow conditions

During the flow experiments, sufficient cofactors were present in the system. Additionally, we performed the kinetic resolution with *E. coli* cells containing transaminase under the same reaction conditions without the addition of PLP cofactor (flow rate 50 µl min⁻¹ at 30 °C). We monitored the reaction for the first 4 hours then took samples the next day. The activity of cells continuously
decreased which resulted in the drop in enantiomeric excess as well. After 22 hours, the conversion and enantiomeric excess of amine dropped (c_{4a}=10\% with ee_{(R)}-3a=26\%). 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 (Fig. 5.-17A). The presence of cofactor is important to keep the catalytically active dimer form of transaminase. Without the cofactor, the enzyme may dissociate to inactive monomer which can lead to loss of activity.\cite{85}

As already mentioned, an advantage of immobilization methods is the reuse of biocatalyst in the same reaction. In this case, 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.

As shown in the Fig. 5.-17B, after the re-activation of CvTA, the biocatalyst regained its catalytic activity and performed well in the KR of rac-3a (c_{4a}=47\% with ee_{(R)}-3a>99\%).

In conclusion, we have demonstrated that the cost-effective entrapment of CvTA whole-cells in templated sol-gel matrix is suitable for continuous-flow mode biotransformations. The compatibility of CvTA cells with ketoreductase cells was well characterized.
6. **CONCLUSION**

In the past few years as a member of the Bioorganic Research Group, I was involved in the development of novel immobilization methods for both native enzymes and whole-cells. Aim of my PhD thesis was to investigate the immobilization methods (adsorption, covalent immobilization as well as sol-gel entrapment) and the applicability of different enzyme preparations in the continuous-flow kinetic resolutions.

Lipases as efficient biocatalysts in the preparation of enantiomerically pure esters already strengthened their position in industry. Therefore, the first part of my thesis deals with the immobilization of lipases from different origins. Bacterial lipase from *Burkholderia cepacia* with lysine residues on its outer surface may lose its catalytic activity after covalent immobilization. However, bioimprinting effect (treating lipases with their substrate analogues at their active site during immobilization) is a great tool to facilitate activity enhancement of lipase in organic media. We have investigated the effect of more than 40 additives on the immobilization process of *Bc*L and the resulting biocatalysts were tested in the kinetic resolution of racemic alcohols. We have demonstrated that combination of immobilization with bioimprinting significantly altered the activity of lipases. Additionally, the stereoselectivity of *Bc*L may be improved with the additives. Continuous-flow kinetic resolutions showed, that polyethylene glycol– and polyvinyl alcohol–treated *Bc*L preparations had remarkably different thermal stability.

*Candida antarctica* lipase B (*CaLB*) – an industrially relevant enzyme – was our next target enzyme in the study of different activating agents in the covalent enzyme immobilization. Bisepoxides containing different hydrophobic and hydrophilic moieties could serve as “2 in 1” agents for fine tuning the surface of aminoalkyl resins. Macroporous carriers with ethylamine and hexylamine functions after bisepoxide activation provided a suitable microenvironment for *CaLB* during the re-use and long-term stability. Continuous-flow kinetic resolution of racemic 1-phenylethanol revealed, that spacer arms of various lengths, hydrophobicity and rigidity remarkably altered the biocatalytic properties of *CaLB* attached to polymer resins.

To demonstrate the broad applicability of developed bisepoxide-activated resins, an advanced mutant W60C of the ω-transaminase from *Chromobacterium violaceum* was immobilized on macroporous resins. Homodimeric transaminase is extremely sensitive and may lose its catalytic activity at higher temperatures or in the presence of organic solvents. After enzyme purification and covalent immobilization on bisepoxide-activated resins (glycerol diglycidyl ether, 1,4-cyclohexanediethanol diglycidyl ether and 1,6-hexanediol diglycidyl ether) the biocatalyst was successfully used in the continuous-flow kinetic resolution of racemic amines. The synthesis of enantiomerically pure amines was carried out on preparative scale (several milligrams) as well.
Nowadays, cascade reactions are gaining more interest as they can prevent the accumulation of unwanted intermediates. After the development of enzyme immobilization methods, our next aim was to investigate the application of whole-cells containing transaminase in combination with ketoreductase whole-cells. *Lodderomyces elongisporus* (*LeKRED*) and *Chromobacterium violaceum* were immobilized in sol-gel matrices templated with spherical silica gel and successfully employed in kinetic resolution of racemic 4-phenylbutan-2-amine followed by enantiotope selective reduction of product benzylacetone. The developed biocatalysts were applied in continuous-flow reactors as well. In this way, a universal immobilization method was developed for the immobilization of whole-cells of different origin.
7. **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 BcL / poly(vinyl alcohol) and BcL / 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 BcL co-immobilized with poly(vinyl alcohol) compared to BcL 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-cyclohexanedicarboxyl diglycidyl ether with hydrophobic character significantly improved the stability of immobilized CaLB. Continuous-flow kinetic resolution of racemic 1-phenylethanol revealed that CaLBs immobilized on activated ethylamine resins had higher thermostability, while CaLBs 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 CvTA 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]
Scientific achievements of the Thesis are based on following articles:


Articles not included in the Thesis:


Lectures:


Posters:


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


8. TÉZISPONTOK

I. Megvalósítottuk a *Burkholderia cepacia* lipáz adsorpciós és kovalens rögzítését kevert (fenil-aminpropil) felületmódosítású mezopórusos szilikagékre több, mint 40 különböző additív jelenléteben. A poli(vinil-alkohol) és polietilén-glikol additívvek jelenléteben rögzített biokatalizátorok hőtűrését szakaszos és folytonos üzemű kinetikus rezolválásokban is megvizsgáltuk. Megállapítottam, hogy a racém 1-feniletanol kinetikus rezolválása során a polietilén-glikol jelenléteben immobilizált *BcL* hőmérsékleti optimuma 50 °C-al eltért a poli(vinil-alkohol) jelenléteben immobilizált *BcL*-hez képest. [1]

II. Megvalósítottuk a *Candida antarctica* B lipáz kovalens rögzítését eltérő lánchosszúságú és hidrofób karakterű biszepoxidokkal aktivált aminalkil (etil-amin és hexil-amin) polimer hordozókon. Megállapítottam, hogy a hidrofób karakterű 1,4-ciklohexándimetanol-diglicidiléter (CH) biszepoxiddal felületmódosított hordozó jelentősen javította az enzim stabilitását. A racém 1-feniletanol folytonos üzemű kinetikus rezolválásával kimutattam, hogy a biszepoxiddal aktivált etil-amin hordozókon immobilizált enzimnek magasabb hőmérsékleti optimuma volt, míg az aktivált hexil-amin hordozókon immobilizált enzim aktívabb és szelektívebb volt. [2]

III. Elsőként valósítottuk meg a *Chromobacterium violaceum* ból származó tisztított ω-transzamináz kovalens rögzítését biszepoxiddal aktivált polímer alapú hordozókon (etil-amin és hexil-amin). Bebizonyítottam, hogy a hidrofil karakterű glicerin-diglicidiléterrel aktivált hordozón rögzített transzamináz stabilitását több konszekutív kiszerkezetű kinetikus rezolválásban (racém 4-fenilbután-2-amin) is jelentős mértékben megőrizte. A kovalensen rögzített enzim szerves oldószer toleranciája jelentősen megnőtt, aktív maradt 50% v/v DMSO jelenléteben is. [3]

IV. Sikeresen rögzítettük a *Chromobacterium violaceum* transzamináz kifejező *E. coli* egész sejteket továbbfejlesztett szilikat alapú polimer mátrixba. Bebizonyítottam, hogy az enzimkészítmény kompatibilis ketoreduktáz aktivitását egész sejtes *Lodderomyces elongisporus* élesztő törzzsel. A rögzített egész sejtes készítmények aktivitását a racém 4-fenilbután-2-amin folytonos üzemű kaszkád reakciójában vizsgáltuk, mely során enantiomertiszta alkoholt és amint állítottunk elő. [4]
9. References

29. http://biocatalysis.uni-graz.at/enantio/cgi-bin/enantio.pl


