## 1. Introduction

Process chemists have long accepted the need to consider both environmental and economic metrics when developing processes that ultimately will find application in chemicals manufacture. Beyond stoichiometric reagent use, catalysis offers important benefits, and today, growing emphasis is being placed on biologically mediated chemical reactions, more generally termed bioconversion. The scope of bioconversion is wide-ranging.[1] Developing sustainable bio-based processes for production of fuels, chemicals, and materials is increasingly attractive due to increased concern for the environment. The chemical synthesis processes are generally high-yield, however they are regularly environmentally-unfriendly and are associated with the production of unwanted products, thus dropping efficiency and rising the downstream costs. In comparison with chemical catalysis, the biocatalysis offer some unique advantages and provides an efficient and environmentally-friendly alternative to old-style chemical synthesis for production of bulk and fine chemicals[2]. In the past several decades, many biocatalytic processes have been implemented to produce a wide variety of products in various industries[3]. A modern, green technique for product optically active compounds that could be highly valuable building blocks of numerous drugs is the continuous-flow biotransformation. The importance of biotransformation is showed by the industrial application of enzymes. For example, more than 3000 tons/year of chiral amines are produced by BASF in lipase mediated continuous-flow biotransformation[4].

The goals of my thesis was to develop biocatalytic system for production of optically active compounds. Our aim was to find yeast strains with multifaceted biocatalytic activity for synthesis of various alcohol enantiomers and immobilize their whole cells preserving two different kinds of biocatalytic activities. Furthermore our aim was to prove the applicability of the immobilized whole-cell preparations as “on demand switchable” biocatalyst for different types of biotransformations (bioreduction or acyloin condensation), even in a consecutive manner where the performance in the actual reaction is independent from the reaction performed in the previous cycle. Therefore, the primary aim of the present work is to demonstrate that proper immobilization of yeast whole-cells can result in a multipurpose biocatalyst, which is storable and recyclable from at least two different kinds of biotransformation without „remembering of the previous reaction”. Moreover we focused to develop a biocatalytic cascade system comprising of co-immobilized cells with two different biocatalytic activity. Thus, a rational strategy was developed to (co)immobilize various whole-cells with different biocatalytic activities directly after harvesting fermentation(s) providing easy recovery, long term storability and good mechanical stability of the produced biocatalyst.

1 J.M. Woodley, M. Breuer, D. Mink, *Chem Eng Res Des*, **2013**, *91*, 2029− 2036.
2 J. Wachtmeister, D. Rother, *Curr Opin Biotechnol*. **2016**; *42*, 169–77.
3 K.A. Curran, H.S. Alper, *Metab Eng*. **2012**, *14*, 289–97.
4 T.C. Nugent, „*Chiral Amine Synthesis: Methods, Developments and Applications*”, Wiley-VCH, Weinheim, **2010**.

## 2. Background

The single enzymes are versatile biocatalysts catalyzing a given type of biotransformation in a selective and environmentally benign way. Microbial cells as rich natural source of several enzymes, are acting as redoubtable biocatalysts, enabling various biotransformations of a wide range of natural but also synthetic substrates. Compared to preparative usage of cell-free isolated enzymes which are specific catalysts for a certain biotransformation, whole-cell biocatalysts offer a number of advantages such as easy handling, no need of cell lysis and enzyme purification, increased stability and presence of natural cofactors[5].

The most used whole-cell biocatalyst is baker's yeast (BY, *Saccharomyces cerevisiae*) as a non-pathogenic, inexpensive and simple to handle whole-cell system applicable for biotransformations with various types of chemo-, regio- and stereoselectivities.[6] BY as a versatile but also active biocatalyst is able to mediate various types of biotransformations, such as C=O and C=C bond reduction, oxidation and racemization reactions, hydrolytic reactions, and C-C bond formation. The benefits of dried or lyophilized yeast cells – the easy usage, storability and transportability – were earlier recognized. However, the rehydrated yeast cells cannot be recycled from aqueous reactions in their original form, therefore they are limited for a single use in batch mode for only a certain biotransformation (one type of reaction).

In recent years, ω-transaminases (ω-TAs) have been in the spotlight of biotechnological research[7]. They catalyze asymmetric reductive amination of prochiral ketones or thermodynamically more favored kinetic resolution of racemic amines. ω-TAs are pyridoxal-5'-phosphate (PLP) cofactor dependent enzymes which serves as molecular shuttle for ammonia and electrons between substrates and products. To explore the advantages of continuous-flow methodology (e.g. increased production, shorter reaction times, easier process control), The biotransformations with ω-TAs in packed-bed reactors under flow conditions were also performed. Due to the excellent compatibility of ω-TAs with other class of enzymes (e.g. ketoreductases, alcohol dehydrogenases, transketolases, imine reductases and monoamine oxidases) their application is a constantly expanding research area.

Many studies indicated that cascade reactions employing isolated enzymes proceeded with low operational stability and without possibility of biocatalyst recycling[8]. Application of whole-cell methods applying lyophilization and entrapment of whole-cells in polymer matrices for cell storage and stabilization could improve the applicability of multienzyme biotransformations. Co-immobilization and simultaneous operation of yeast and bacteria was described earlier in cider fermentation and biosensor construction.

Immobilization of yeast cells provided an advantageous solution to turn whole cells into recyclable biocatalysts. Entrapment of whole cells is a well-established method to prepare immobilized biocatalysts. Although many of the previous methods resulted in storable biocatalysts applicable for continuous-flow biotransformations, they suffered from several drawbacks. For example, dried alginate beads entrapping whole cells are swelling and rapidly disintegrate in the presence of low salt content solutions.[9] While various immobilization methods were developed for creating recyclable biocatalysts for a certain biotransformation, the preservation and sequential repeated utilization of the multifaceted bioactivities within immobilized wild-type microbial whole-cell biocatalysts still represent unexplored area.

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5 B. Pscheidt, A. Glieder, *Microbiol. Cell Fact.* **2008**, *7*, 1–36.
6 B. Erdélyi, Á. Szabó, G. Seres, L. Birincsik, J. Ivanics, G. Szatzker, L. Poppe, *Tetrahedron: Asymmetry* **2006**, *17*, 268–274.
7 M. L. Contente, F. Paradisi, *Nature Catal.* **2018**, *1*, 452–459.
8 L.Päiviö, T. Kanerva, *Process Biochem*. **2013**, *48*, 1488–1494.
9 V. De Vitis, F. Dall'Oglio, A. Pinto, C. De Micheli, F. Molinari, P. Conti, D. Romano, L. Tamborini, *Chem. Open* **2017**, *6*, 668–673.

Lipases are useful biocatalysts which can provide regio- and enantioselectivity in a wide range of reactions. They are one of the most extensively utilized bio- catalysts in organic synthesis. Lipases (EC 3.1.1.3) are essential in the digestion, transport and processing of lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. Immobilization can enhance activity, thermal and operational stability, and also reusability of enzymes which are essential advantages in industrial applications.[10] Among the many available immobilization methods, including adsorption, covalent attachment to solid supports and entrapment within polymers, hydrophobic adsorption onto suitable carriers was found to be an efficient way not only for immobilization but also for the separation of lipases. Lipases immobilized by various procedures proved to be useful both in batch mode and in continuous-flow biotransformations.

## 3. Experimental section

### *3.1. Immobilization/co-immobilization of the whole cells in templated sol-gel matrices.*

Silica sol was prepared by addition of tetraethoxysilane (TEOS, 0.72 mL) to a solution of distilled water (0.25 mL) containing 0.1 M $HNO_3$ (65 μL) followed by sonication of the resulted mixture for 5 min at RT and cooling at 4 °C for 24 h. Supported cell suspension was prepared by addition of MAT540 support (150 mg) to whole cell suspension [obtained by resuspending centrifuged whole cell paste (0.5 g) or mixture 0.33 g of centrifuged *Lodderomycess elongisporus* cell paste and 0.16 g of centrifuged *Escherichia coli* (expressing ω-transaminase *Chromobacterium violaceum*) cell paste (2:1 w/w) - for co-immobilized biocatalyst - in phosphate buffer (3 mL, 0.1 M, pH 7.5)] followed by shaking until it became homogeneous (~4-6 min at RT). Finally, silica sol was mixed with the supported cell suspension and the resulting mixture was shaken at RT for 5 min. After gelation at RT (occurred within 30 min), the resulting gel was aged at 4 °C for 48 h. The crude biocatalyst was washed with phosphate buffer (2×15 mL, 100 mM, pH 7.5), centrifuged and dried at room temperature (24 h), and stored at 4 °C.

### *3.2. On demand switchable biotransformations under continuous-flow conditions.*

The biotransformations in continuous-flow mode were performed in a laboratory scale flow reactor built from a Knauer Azura P4.1S isocratic HPLC pump attached to SynBioCart columns filled with the immobilized whole-cell biocatalyst (stainless steel, inner diameter: 4 mm; total length: 70 mm; packed length: 65 mm; inner volume: 0.86 mL) in an in-house made aluminum metal block column holder with precise temperature control.

The consecutive sequence involving three Runs of different biotransformations under continuous-flow conditions was performed by using a single SynBioCart column filled with the corresponding immobilized whole-cell biocatalyst (*L. elongisporus* or *P. carsonii*). For the three Runs, the following inlet streams were applied: Run 1 – phenylacetone (**1a**, 17 mM) in phosphate buffer (100 mL, 100 mM, pH 7.5) containing NADH (0.1 mM) and 2-propanol (2 v/v%); Run 2 – benzaldehyde (**3**, 20 mM) in citrate buffer (100 mL, 100 mM, pH 7.5) containing sodium pyruvate (3 equiv.), thiamine pyrophosphate (0.2 mM), $MgCl_2$ (1 mM) and 2-propanol (2v/v%); Run 3 – phenylacetone (**1a**, 17 mM) in recovered phosphate buffer (100 mL, 100 mM, pH 7.5); temperature: 30 °C; flow rate: 50 μL $min^{-1}$; run time: 24 h, each. During each Run, samples were taken at different time intervals, extracted with ethyl acetate (700 μL) dried over $Na_2SO_4$ and analyzed by GC. After Run 1 (24 h), the column was washed by citrate buffer (100 mM, pH 5.0, 100 μL $min^{-1}$ for 60 min). After Run 2 (24 h), the column was washed by phosphate buffer (100 mM, pH 7.5, 100 μL $min^{-1}$ for 60 min). For Run 3, the

10 C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, *Enzyme Microb. Technol*., **2007**, *40*, 1451–1463.

NADH-containing buffer recovered from Run 1 was applied as reaction medium. The products of Runs 1-3 were isolated from the effluents and analyzed by GC

### *3.3. Continuous-flow cascade with two column co-immobilized whole-cells*

Kinetic resolution/bioreduction cascade in continuous-flow mode was performed by a SynBioCart column filled with the co-immobilized *Cv*TA - *Le*KRED whole-cells (filling weight in Column A: 402±13 mg, in Column B: 415±6 mg). Racemic amine (±)-**8b** or (±)-**8f** (7.5 mM) was dissolved in phosphate buffer (100 mL, 100 mM, pH 7.5) containing sodium pyruvate (30 mM), pyridoxal-5'-phosphate monohydrate (0.2 mM), NADH (0.1 mM) and 2-propanol (2% v/v). The co-immobilized *Cv*TA - *Le*KRED whole-cells-filled column was pre-washed by pumping through it a solution of phosphate buffer (100 μL $min^{-1}$ for 100 min) followed by pumping the substrate solution through the column (50 μL $min^{-1}$ for 24 h) at 30 °C. Samples were taken and analyzed (after extraction and derivatization) by GC.

## 4. Results

### *4.1. Yeast mediated biotransformations*

In the preliminary experiments, more than 300 different bacterium and yeast strains of Witaria Strain Collection (WY) were isolated and screened for biocatalytic activity by Fermentia Ltd. After the preliminary activity tests 9 candidates were selected for more detailed investigations. For selectivity screenings two different types of biotransformations – the enantiotope selective reduction of prochiral ketones (phenylacetone **1a**, 2-heptanone **1b**) and the acyloin condensation of benzaldehyde **3** – were selected (Figure 1.).

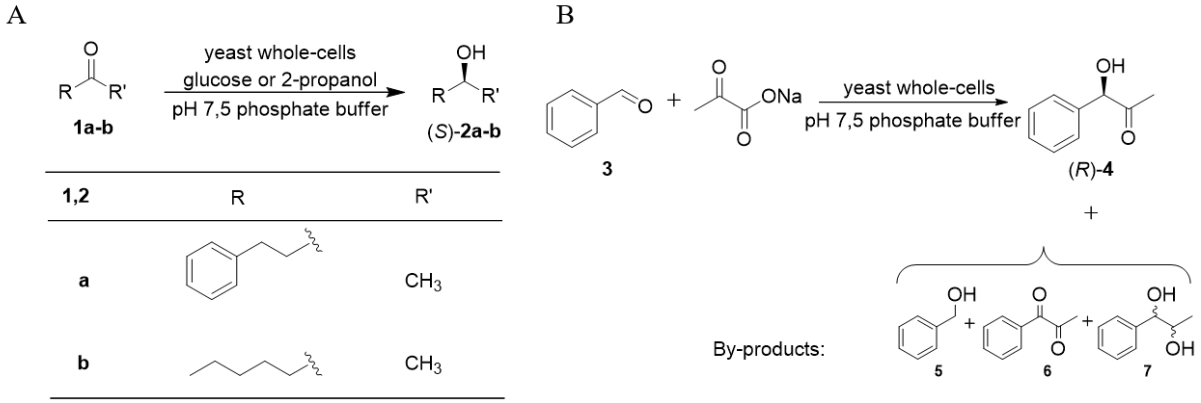


**Figure 1**. Yeast mediated bioreductions (Panel A) of prochiral ketones **1a-b** and acyloin condensation (Panel B) of benzaldehyde **3**.

Initially, the lyophilized cells of several yeast strains were investigated in the bioreduction of ketones **1a-b** in the presence of 2-propanol or glucose as cosubstrate. The preliminary tests [ketone (30 mM) with lyophilized cells, in phosphate buffer (100 mM, pH 7.5) in presence of 2-propanol (4 v/v%) or glucose (6 w/v%) at 30 °C for 24 h] revealed appropriate efficiency and enantiotopic selectivity in the bioreduction of **1a-b** by *L. elongisporus*, *P. carsonii*, *C. norvegica*, *D. fabryi, W. subpelliculosus WY3* and *W. subpelliculosus WY13* strains as presented in Table 1. The other investigated strains showed low or moderate activity in the bioreduction and were not investigated further.

**Table 1**. Comparison of lyophilized yeast cells in the bioreduction of **1a-b** in presence of 2-propanol (4 v/v%) or glucose (6w/v%) after 24 h.

| | 2-propanol as cosubstrate | | | | glucose as cosubstrate | | | |
|---|---|---|---|---|---|---|---|---|
| Strain name | $c_{\mathbf{1a}}$(%) | $ee_{(S)\text{-}\mathbf{2a}}$(%) | $c_{\mathbf{1b}}$(%) | $ee_{(S)\text{-}\mathbf{2b}}$(%) | $c_{\mathbf{1a}}$(%) | $ee_{(S)\text{-}\mathbf{2a}}$(%) | $c_{\mathbf{1b}}$ (%) | $ee_{(S)\text{-}\mathbf{2b}}$(%) |
| *Candida norvegica* | 97 | 97 | 91 | >99 | 6 | 63 | 15 | 50 |
| *Pichia carsonii* | 97 | >99 | 89 | >99 | 37 | 81 | 17 | 0 |
| *Lodderomyces elongisporus* | 96 | >99 | 90 | >99 | <1 | - | <1 | - |
| *Debaryomyces fabryi* | 92 | >99 | 78 | >99 | 2 | 89 | 5 | 71 |
| *W. subpelliculosus WY3* | 56 | >99 | 19 | >99 | 96 | >99 | 63 | >99 |
| *W. subpelliculosus WY13* | 40 | 70 | 13 | >99 | 99 | >99 | 50 | >99 |

To our best knowledge the two different strains from *Wickerhamomyces subpelliculosus* has never been mentioned as biocatalyst in any stereoselective bioreduction. Thus we described the application of two strains of *Wickerhamomyces subpelliculosus* in bioreductions in wide range. Both 2-propanol and glucose were investigated as cosubstrate in the bioreductions. As expected, glucose was tolerated at higher concentration (up to 16 w/v%) than 2-propanol (up to 4 v/v%). The excellent results achieved in bioreduction of phenylacetone **1a** even at high concentration of glucose (8 w/v %) as cosubstrate [$c$= 99.8%, $ee_{(S)\text{-}\mathbf{2a}}$= 99.6% with WY3; and $c$= 99.9%, $ee_{(S)\text{-}\mathbf{2a}}$= 99.8%] indicated the usefulness of these novel whole-cell biocatalysts. Both strains showed moderate tolerance of temperature (up to 40 °C) stability in wide pH range (3–10) and variable tolerance of a few organic solvents up to 5 w/v%. The *W. subpelliculosus* strains were used first for screening the bioreduction on analytical scale and later on preparative scale as well.

Next, the yield and selectivity of the acyloin condensation reaction from benzaldehyde **3** was investigated with the selected yeast strains suspended in aqueous buffer (Table 2.). The lyophilized form of *L. elongisporus* and *P. carsonii* cells proved to be efficient and highly selective biocatalyst for acyloin condensation ($x_{(R)\text{-}\mathbf{4}}$= 59% and $ee_{(R)\text{-}\mathbf{4}}$= 96% with *L. elongisporus* and $x_{(R)\text{-}\mathbf{4}}$= 53% and $ee_{(R)\text{-}\mathbf{4}}$= 96% with *P. carsonii*.

**Table 2.** Screening lyophilized yeast strains as biocatalysts for acyloin condensation of benzaldehyde **3**

| Strain name | $c_{\mathbf{3}}$ (%)[a)] | $ee_{(R)\text{-}\mathbf{4}}$ (%) | $x_{\mathbf{4}}$ (%)[b)] |
|---|---|---|---|
| *Lodderomyces elongisporus* | 100 | 96 | 59 |
| *Pichia carsonii* | 98 | 96 | 53 |
| *Candida norvegica* | 17 | 89 | 17 |
| *Debaryomyces fabryi* | 100 | 95 | 31 |
| *W. subpelliculosus WY3* | 29 | 93 | 24 |
| *W. subpelliculosus WY13* | 11 | 59 | 5 |

These yeast strains have been applied scarcely for biotransformations earlier. *Lodderomyces elongisporus* was identified as a strain capable of enantiomer selective oxidation of 1,3-butenediol and aldoketoreductases of them were characterized[11]. While redox reactions by *Pichia carsonii* were mentioned marginally[12], no data were found on use of *Candida norvegica* or *Debaryomyces fabryi* in reactions leading to enantioenriched alcohols.

[11] C. Ning, E. Su, D. Wei, *Arch. Biochem. Biophys*. **2014**, *564*, 219–228.
[12] M. Chartrain, R. Greasham, J. Moore, P. Reider, D. Robinson, B. Buckland, *J. Mol. Catal. B: Enzym.* **2001**, *11*, 503–512.

### *4.2. On-demand catalysis of ketone bioreduction or acyloin condensation*

The freshly harvested or lyophilized form of cells, were also not suitable for effective reuse in a next bioreduction or acyloin condensation. Thus, the four yeast strains from the previous screening (*L. elongisporus*, *P. carsonii*, *C. norvegica* and *D. fabryi*) were selected for immobilization by using sol-gel entrapment of the silica-supported whole cells and for further investigations. The sol-gel entrapment protocol required sol formation by acid-catalyzed hydrolysis of tetraethyl orthosilicate (TEOS) [13]. Suspension of whole-cells and hollow silica particles in phosphate buffer were added to silica sol in order to entrap the cells adsorbed onto the supporting silica particles during gelation. To optimize the immobilization process for the desired whole-cell biocatalysts, the effect of silica content on gelation process and biocatalyst performance in the bioreduction of **1a** was investigated. Different amounts of silica microspheres were mixed with the whole cells, and the immobilization process was examined without silica support as well. The 30% w/w silica microsphere content provided the highest specific activity of the immobilized whole-cell biocatalyst, thus this support content was applied in the further part of the study.

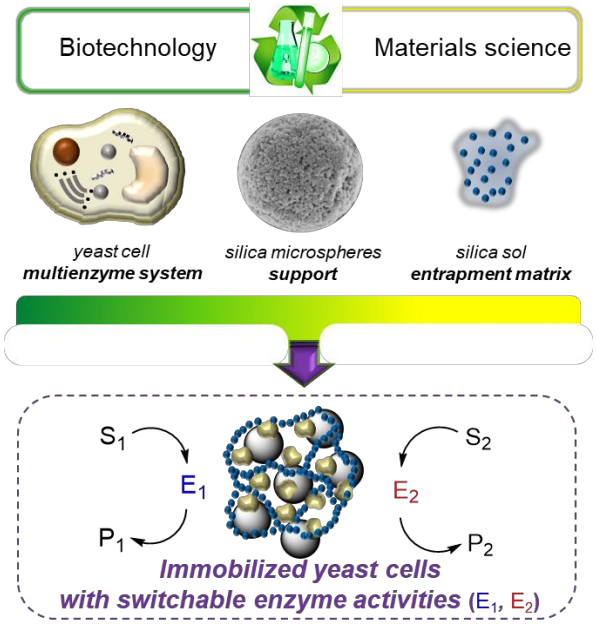


**Figure 2**. Immobilization process for yeast cells preserving two kinds of biocatalytic activities.

With the four selected immobilized yeast whole-cell biocatalysts in our hands, the bioreduction of **1a-b** and acyloin condensation of benzaldehyde **3** were investigated. The immobilized *L. elongisporus* and *P. carsonii* biocatalysts could mediate the enantiotope selective continuous-flow bioreduction of **1a** with high conversion and excellent selectivity ($c_{\mathbf{1a}}$= 95% for *L. elongisporus* and $c_{\mathbf{1a}}$= 92% for *P. carsonii*, both with $ee_{(S)\text{-}\mathbf{2a}}$ >99%). The *C. norvegica* and *D. fabryi* biocatalysts mediated bioreductions from ketones **1a** and **1b** showed lower conversions but high selectivity ($c_{\mathbf{1a}}$= 41%, $ee_{(S)\text{-}\mathbf{2a}}$ >99% and $c_{\mathbf{1b}}$= 49%, $ee_{(S)\text{-}\mathbf{2b}}$ >99% with *C. norvegica*; and $c_{\mathbf{1a}}$= 25%, $ee_{(S)\text{-}\mathbf{2a}}$ >99% and $c_{\mathbf{1b}}$= 22%, $ee_{(S)\text{-}\mathbf{2b}}$>99% with *D. fabryi*).

We have designed recovery process for used NADH cofactor (similar to that applied in multi-enzyme conversion of amines into alcohols)[9], thereafter the continuous-flow bioreductions were performed with the immobilized yeast cells using recovered NADH

[13] K. Kato, H. Nakamura, K. Nakanishi, *Appl. Surf. Sci*. **2014**, *293*, 312–317.

cofactor solution. Bioreduction of ketone **1a** in the presence of recovered NADH resulted in enantiopure alcohol (*S*)-**2a** ($ee_{(S)\text{-}\mathbf{2a}}$ >99%) with conversions ($c_{\mathbf{1a}}$= 92% with *L. elongisporus*; and $c_{\mathbf{1a}}$= 90% with *P. carsonii*; after 24 h) comparable to the reductions in presence of fresh NADH solution.

Fortunately, the performance of immobilized yeast cell biocatalysts in acyloin condensation from **3** improved when the reactions were performed in continuous-flow mode. The amount of formed (*R*)-**4** increased from 44% to 51% with the *L. elongisporus*-based biocatalyst and from 5% to 19% with the *P. carsonii*-based biocatalyst in the continuous-flow system. Moreover, the continuous-flow systems could be operated for 24 h The immobilized whole cells of the two different yeast strains (*P. carsonii* and *L. elongisporus*) which could mediate both enantiotope selective bioreduction and acyloin condensation effectively were investigated as multipurpose biocatalysts under continuous-flow conditions as well (Figure 3.).

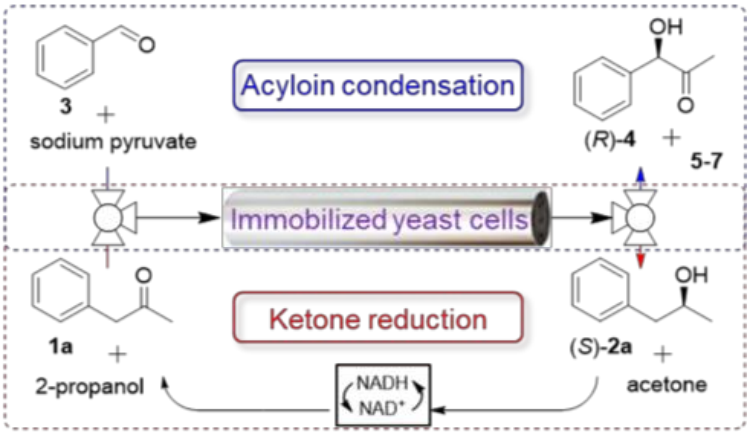


**Figure 3**. Application of the immobilized biocatalyst with switchable activity for continuous-flow ketone reduction and acyloin condensation.

Prior to continuous-flow experiments, the switchability of these biocatalyst was successfully tested in batch mode [immobilized *L. elongisporus*-mediated acyloin condensation from **3** as the first reaction ($x_{(R)\text{-}\mathbf{4}}$= 44%, $ee_{(R)\text{-}\mathbf{4}}$= 92%), followed by reduction of **1a** as the consecutive second reaction ($c_{\mathbf{1a}}$= 44%, $ee_{(S)\text{-}\mathbf{2a}}$ >99%); and immobilized *P. carsonii*-mediated acyloin condensation from **3** as the first reaction ($x_{(R)\text{-}\mathbf{4}}$= 5%, $ee_{(R)\text{-}\mathbf{4}}$= 92%), followed by reduction of **1a** as the consecutive second reaction ($c_{\mathbf{1a}}$= 52%, $ee_{(S)\text{-}\mathbf{2a}}$ >99%)]. Based on these results, the "on demand switchable" biotransformations were performed in consecutive continuous-flow runs.

To demonstrate the ketoreductase-acyloin condensation switchability of both selected immobilized biocatalysts in continuous-flow mode, a sequence involving three Runs (24 h continuous operation, 25 °C and 30 °C) was performed.

**Table 3**. Switching between biotransformations from ketone **1a** or from aldehyde **3** in microreactors filled with immobilized whole-cell biocatalyst in continuous-flow mode

| Biocatalyst | Run | Substrate | *Y* (%) | *ee* (%) |
|---|---|---|---|---|
| *L. elongisporus* | 1 | **1a** | 90 | >99 |
| *L. elongisporus* | 2 | **3** | 47 | 97 |
| *L. elongisporus* | 3 | **1a** | 91 | >99 |
| *P. carsonii* | 1 | **1a** | 86 | >99 |
| *P. carsonii* | 2 | **3** | 15 | 90 |
| *P. carsonii* | 3 | **1a** | 87 | >99 |

In Run 1, the enantiotope selective bioreduction of ketone **1a** with both selected immobilized cells yielded (*S*)-**2a** with excellent conversion and enantiopurity (for *L. elongisporus*; $c_{\mathbf{1a}}$= 95%; $ee_{(S)\text{-}\mathbf{2a}}$ >99%). After washing the biocatalysts with citrate buffer (pH 5.0, 100 mM), the second 24 h long Run 2 provided solely highly enantiopure acyloin (*R*)-**4** with acceptable conversion (for *L. elongisporus;* $x_{(R)\text{-}\mathbf{4}}$= 51±1.9%; $ee_{(R)\text{-}\mathbf{4}}$= 97±0.2%). In Run 3, after re-buffering with phosphate buffer (pH 7.5, 100 mM), bioreduction of **1a** was performed again providing conversion to (*S*)-**2a** comparable to the one in Run 1 with excellent enantiopurity (for *L. elongisporus*, $c_{\mathbf{1a}}$= 93±1.0%, $ee_{(S)\text{-}\mathbf{2a}}$ >99%). Products of each run were isolated from the effluents and purified successfully (Table 3.).

This system enabling consecutive syntheses of virtually enantiopure compounds [(*S*)-**2a**, in Runs 1 and 3; and (*R*)-**4**, in Run 2] by different types of biotransformations demonstrated the usefulness of these immobilized whole cells as multipurpose biocatalysts under continuous-flow conditions as well. Similar results with somewhat lower degrees of conversion in each Run were observed with the *P. carsonii*-based biocatalyst.

The three Runs revealed that the operational stability of both the immobilized *L. elongisporus* and *P. carsonii* cell-based biocatalyst is quite good during several days-long processes. Unfortunately, the second acyloin condensation of **3** attempted in a fourth run yielded product **4** with a significant decrease of the biocatalytic activity (Figure 4.). After 1 h, less than 5% (*R*)-**4** was produced, with further deactivation in time of both biocatalysts, presumably due to either loss of carboligase activity required for (*R*)-**4** production or due to degradation of the yeast cells.

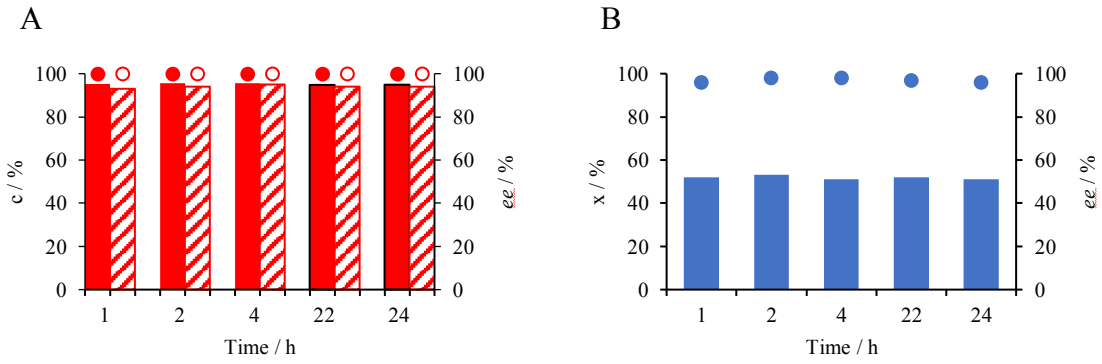


**Figure 4**. Operational stability of the immobilized *L. elongisporus* cell-based biocatalyst in a sequence of three Runs involving two types of continuous-flow biotransformations. Panel A) shows data for bioreduction of **1a** in Run 1 ($c_{\mathbf{1a}}$: ■, $ee_{(S)\text{-}\mathbf{2a}}$: ●) and in Run 3 ($c_{\mathbf{1a}}$: □, $ee_{(S)\text{-}\mathbf{2a}}$: ○); panel B) depicts data for acyloin condensation of **3** in Run 2 (amount of (*R*)-**4**: ■; $ee_{(R)\text{-}\mathbf{4}}$: ●).

### *4.3. Co-immobilized whole-cells with ω-transaminase and ketoreductase activity for continuous-flow cascade reaction*

To test the capabilities of the target two-cell co-immobilization system, the kinetic resolution of racemic 4-phenylbutan-2-amine [(±)-**8f**] coupled to enantiotope selective reduction of the forming ketone was selected as a model biotransformation (Scheme 1). 4-Phenylbutan-2-amine [(±)-**8f**] was selected as substrate and the products therefrom have real synthetic importance. The residual (*R*)-amine (*R*)-**8f** is a constituent of antihypertensive drug Dilevalol[14] and the forming (*S*)-alcohol (*S*)-**2f** is a flavoring ingredient and used also as a precursor to anti-hypertensive agents and spasmolytics (anti-epileptic agents) [15]. Amine [(±)-**8f**] is a known substrate of ω-transaminase from *Chromobacterium violaceum* (*Cv*TA) and the corresponding ketone **1f** was also found to be a good substrate of ketoreductase in *Lodderomyces elongisporus* (*Le*KRED). Thus, *Cv*TA catalyzed the conversion of (*S*)-**8f** in the first reaction to prochiral ketone **1f**, while the less reactive enantiomer (*R*)-**8f** remained in the solution. In the second step catalyzed by *Le*KRED, ketone **1f** was converted stereoselectively to (*S*)-**2f**.

Because the whole-cells of *E. coli* expressing W60C mutant of *Chromobacterium violaceum* ω-transaminase (*Cv*TA) and the whole-cells of yeast *Lodderomyces elongisporus* with ketoreductase activity (*Le*KRED) display different properties, optimization of their immobilization conditions should be performed. After maturation of the gel, the sol-gel biocatalysts were gently crushed and washed with buffer. Different amounts of silica particles were mixed with the whole-cells. For both bacterial and yeast whole-cells the 30% w/w silica content was found as the best condition for immobilization. Next, effect of *Cv*TA- to *Le*KRED-containing cell ratio on activity of biocatalyst was investigated on the model cascade system from [(±)-**8f**] in batch mode. The 1:1 weight ratio of cells resulted in low amount of enantiomerically pure alcohol (*S*)-**2f** ($c_{(S)\text{-}\mathbf{2f}}$= 19%). When the ratio of *Le*KRED:*Cv*TA was set to 2:1, the biocatalyst exhibited excellent behavior ($c_{(S)\text{-}\mathbf{2f}}$= 46% with $ee_{(R)\text{-}\mathbf{8f}}$ >99% and $ee_{(S)\text{-}\mathbf{2f}}$ >99%). Further increase in cell ratio to 4:1 resulted in a drop of cascade conversion of the forming alcohol.

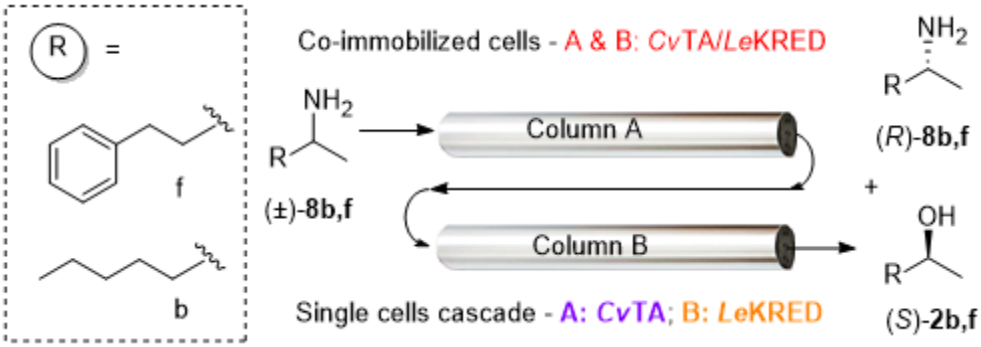


**Figure 5.** Continuous-flow conversion of a racemic amine [(±)-**8b,f**] to enantiopure amine (R)-**8b,f** and alcohol (S)-**3b,f** performed by a two column-single cell serial cascade or a co-immobilized whole-cells system.

Having established an efficient system to co-immobilize whole-cells of various activities, kinetic resolution followed by asymmetric reduction was carried out with the separately immobilized and with the co-immobilized whole-cell biocatalyst with *Cv*TA and *Le*KRED activities under continuous-flow conditions. To demonstrate the advantages of co-immobilization of whole-cells of different microbial origin with diverse enzyme activities, the operational stability of system utilizing co-immobilized *Le*KRED/*Cv*TA was compared to that of the single-cell *Cv*TA and *Le*KRED cascade (Figure 5.). This investigation showed that the co-immobilized whole-cells had better activity up to 24 h of continuous-flow mode operation

[14] J.E. Clifton, I. Collins, P. Hallett, D. Hartley, L.H.C. Lunts, P.D. Wicks, *J. Med. Chem*. **1982**, *25*, 670-679.
[15] A. Liese, K. Seelbach, C. Wandry, *Industrial Biotransforamtion*, Wiley-VCH, New-York, **2000**, 423 pp

with higher final conversion (46.7%) than that of the single-cell column system (41.6%). The average and SD values for the first three values of the long term runs (from values at 1 h, 2 h, 3 h: $x_{(S)\text{-}\mathbf{2f}} = 47.0\pm0.1\%$ for single-cell cascade and $x_{(S)\text{-}\mathbf{2f}} = 47.0\pm0.4\%$ for the mixed-cells system) compared to the last three values from the same runs (from values at 22 h, 23 h, 24 h: $x_{(S)\text{-}\mathbf{2f}} = 41.6\pm0.1\%$ for single-cell cascade and $x_{(S)\text{-}\mathbf{2f}} = 45.5\pm0.2\%$ for the mixed-cells system) showed significantly better long term conversion in the mixed-cells TA-KRED system as for the TA single-cell and KRED single-cell cascade system. This is due to the beneficial effect of KRED being present besides TA which enhanced the conversion of the TA reaction by removing the formed ketone from the equilibrium (Figure 6.).

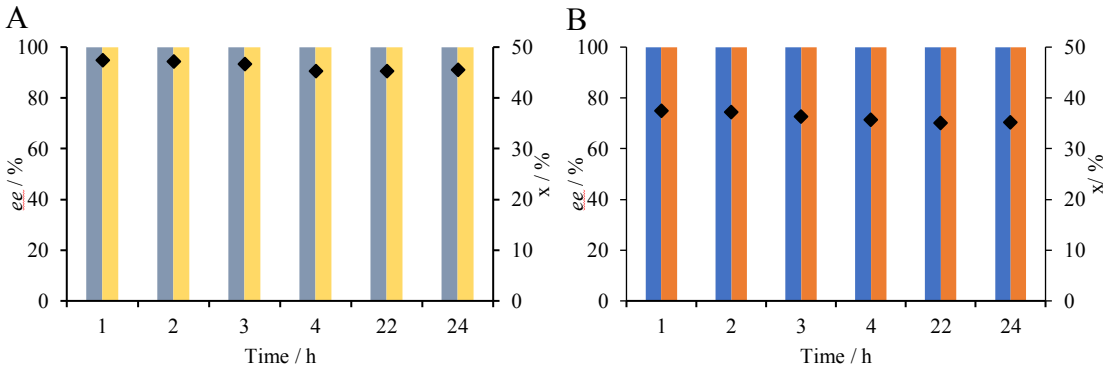


**Figure 6**. Kinetic resolution/bioreduction cascade from (±)-**8f** (■ $ee_{(R)\text{-}\mathbf{8f}}$; ■ $ee_{(S)\text{-}\mathbf{2f}}$; ◆ $c_{(S)\text{-}\mathbf{2f}}$) (Panel A) and (±)-**8b** (■ $ee_{(R)\text{-}\mathbf{8b}}$; ■ $ee_{(S)\text{-}\mathbf{2b}}$; ◆ $x_{(S)\text{-}\mathbf{2b}}$) (Panel B)with CvTA/LeKRED

### *4.4. Kinetic resolution of racemic 1-phenylethanol with immobilized lipase from Rhizomucor miehei or Thermomyces lanuginosus*

Firstly, the biocatalysts were prepared by hydrophobic adsorption of *lipase from Rhizomucor miehei* (*Rm*L) and *Thermomyces lanuginosus* (*Tl*L) on mesoporous silica gel, etched silica gel and nineteen variously grafted silica gel supports. The obtained lipase biocatalyst were investigated in kinetic resolution of racemic 1-phenylethanol (Figure 7.).

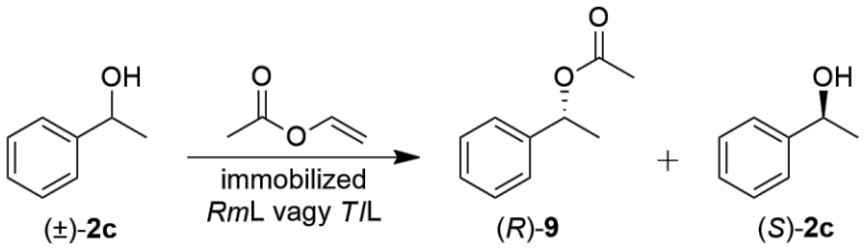


**Figure 7**. Kinetic resolution of racemic 1-phenylethanol (±)-**2c** with immobilized *Rm*L or *Tl*L

We have found that both *Rm*L and *Tl*L can be efficiently immobilized on surface-modified mesoporous silica gel supports (Table 4.). Our results have shown that the optimal method of enzyme immobilization depended both on the nature of the substrate and the reaction conditions. This study indicated that using a broad selection of variously grafted silica gels for the immobilization of *Rm*L and *Tl*L provided a selection of biocatalysts with a wide range of activity and selectivity when applied for the kinetic resolution of racemic 1-phenylethanol *rac*-**2d**.

**Table 4**. Kinetic resolution of (±)-**2c** with lipase from *Rhizomucor miehei* (Panel A) and *Thermomyces lanuginosus* (Panel B) adsorbed on various surface-modified silica gels (in n-hexane:MTBE 2:1 at 1 h).

| | *Rm*L | | | | *Tl*L | | | |
|---|---|---|---|---|---|---|---|---|
| Grafting functon on silica gel | $c_{(\pm)\text{-}2c}$ (%) | $ee_{(R)\text{-}9}$ (%) | $E^{[a]}$ (-) | $U_B^{[b]}$ (μmol $min^{-1}$ $g^{-1}$) | $c_{(\pm)\text{-}2c}$ (%) | $ee_{(R)\text{-}9}$ (%) | $E^{[a]}$ (-) | $U_B^{[b]}$ (μmol $min^{-1}$ $g^{-1}$) |
| - | 0.4 | 74.6 | 6.9 | 1.2 | 1.3 | 97.9 | 93 | 3.6 |
| Methly | 6.4 | 98.4 | 132 | 17.6 | 13.5 | 97.3 | 84 | 37.1 |
| Ethly | 7.8 | 98.3 | 128 | 21.2 | 17.1 | 96.9 | 77 | 46.9 |
| Propyl | 16.3 | 98.5 | 163 | 44.9 | 10.6 | 97.5 | 90 | 29.2 |
| Hexyl | 1.3 | 96.8 | 62 | 3.5 | 6.5 | 98 | 106 | 17.8 |
| Decyl | 1.8 | 97.3 | 76 | 4.9 | 9.7 | 97.6 | 91 | 26.7 |
| Dodecyl | 0.8 | 96.1 | 59 | 2.1 | 6.5 | 98 | 105 | 18.1 |
| Oktadecyl | 1.8 | 97.3 | 74 | 4.9 | 6.8 | 97.8 | 97 | 18.7 |
| Phenyl | 7.8 | 98.6 | 151 | 21.5 | 14 | 97.3 | 85 | 39 |
| Perfluoroktyl | 10.5 | 98.5 | 148 | 29 | 17.8 | 97.1 | 82 | 49.6 |
| Vinyl | 3.9 | 98.3 | 120 | 10.8 | 14.5 | 97 | 78 | 39.7 |
| 2-Cyanoethyl | 3.9 | 98.6 | 147 | 10.7 | 10.4 | 97.5 | 90 | 29 |
| 3-Chloropropyl | 6.3 | 98.9 | 187 | 17.2 | 13.9 | 97.2 | 81 | 38.3 |
| 3-Mercaptopropyl | 5.2 | 98.6 | 152 | 14.3 | 14.8 | 97.1 | 80 | 41.1 |
| Dimethyl | 0.3 | 98.6 | 142 | 0.9 | 13.5 | 97.2 | 82 | 37.2 |
| Phenyl–methyl | 9 | 98.7 | 172 | 24.8 | 20.2 | 96.6 | 73 | 55.9 |
| Cyclohexyl–methyl | 1.6 | 97.9 | 96 | 4.5 | 10.3 | 97.8 | 99 | 28.7 |

## 5. Thesis points

1. We have discovered two novel strains of *Wickerhamomyces subpelliculosus* as efficient biocatalyst for the bioreduction of aromatic and aliphatic ketones in batch mode. [I]

2. We have designed a new cost-effective sol-gel immobilization of the whole-cells from broth by avoiding lyophilization and further purification. This process provides integrated biocatalysts of good storability and operational stability. [I, II, III]

3. We have successfully carried out the immobilized whole-cells mediated bioreductions of ketones under continuous flow conditions. These immobilized yeast strains had earlier been applied scarcely for biotransformations. We have designed recovery process for used NADH cofactor, and the recovered cofactor was successfully applied in further bioreductions. [IV]

4. We have successfully applied the immobilized yeast strains as biocatalysts for acyloin condensation of benzaldehyde. To our best knowledge, these strains have not been studied before in acyloin condensation. We carried out the immobilized whole-cells mediated continuous flow production of L-phenylacetylcarbinol. Compared with the results obtained in batch mode, the use of the same immobilized biocatalysts in flow enhanced their catalytic activity. [III, IV]

5. We have demonstrated that the multifaceted biocatalytic activity of microbial whole cells was preserved by sol-gel entrapment of yeast cells. The immobilized whole cells have successfully been applied as multipurpose "on-demand switchable" biocatalysts which could mediate both the enantiotope selective bioreduction and the acyloin condensation effectively. [IV]

6. We have designed a sol-gel process for co-immobilization of whole-cells of *E. coli* with *Chromobacterium violaceum* ω-transaminase activity and *Lodderomyces elongisporus* with ketoreductase activity. The co-immobilized cells have successfully been applied in the biocatalytic cascade system for the synthesis of chiral compounds (amines and alcohols) in batch and continuous-flow mode. [II]

7. We have demonstrated that the various mesoporous surface grafted silica gels proved to be efficient supporters for the adsorptive immobilization of lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* and they significantly influenced the catalytic activity of the resulting biocatalysts in the kinetic resolution of alcohols. [V]

## 6. Potential application

The yeasts – known for their powerful ketoreductase activity – were advantageously applied for reduction of prochiral ketones to enantiopure alcohols being valuable building blocks in the synthesis of various pharmaceuticals. Moreover the enantiopure acyloins are also important keto alcohols for the synthesis of active pharmaceutical ingredients. Based on our results, *L. elongisporus*, *P. carsonii*, *C. norvegica*, *D. fabryi, W. subpelliculosus WY3* and *W. subpelliculosus WY13* strains can be recommended as an excellent extension to the toolbox of whole-cell biocatalyst with ketoreductase and/or acyloin condensation activity for production of chiral alcohols in high enantiopurity.

Furthermore the biocatalytic cascades often enable proceeding of complex reactions under mild reaction conditions, as an alternative. Our immobilized microbial cells of various origin with different enzyme activities (*E. coli* with *Chromobacterium violaceum* ω-transaminase activity and *Lodderomyces elongisporus* with ketoreductase activity) into a supported sol-gel system could be integrated in one-step enabling creation of an efficient biocatalytic cascade system for synthesis of chiral compounds.

## 7. Publications

**Scientific achievements of the Thesis are based on following articles:**

[I] V. Bódai, L. Nagy-Győr, R. Örkényi, Zs. Molnár, Sz. Kohári, B. Erdélyi, Zs. Nagymáté, Cs. romsics, Cs. Paizs, ,L. Poppe, G. Hornyánszky: *Wickerhamomyces subpelliculosus* as whole-cell biocatalyst for stereoselective bioreduction of ketones, *Journal of Molecular Catalyss B: Enzymatic*, **2016**, *134*, 206–214. DOI: 10.1016/j.molcatb.2016.11.003. IF: 2,269, contribution by the author: 80%; C: 2, IC: 1.

[II] L. Nagy-Győr, E. Abaházi, V. Bódai, P. Sátorhelyi, B. Erdélyi, D. Balogh-Weiser, Cs. Paizs, G. Hornyánszky, L. Poppe: Co-immobilized whole-cells with ω-transaminase and ketoreductase activity for continuous-flow cascade reaction, *ChemBioChem,* **2018**, *19*, 1845–1848. DOI: doi.org/10.1002/cbic.201800286. IF: 2,774, contribution by the author: 51%; C: 2.

[III] L. Nagy-Győr, E. Farkas, G. Tóth, D. Incze, G. Hornyánszky, L. Poppe, D. Balogh-Weiser: Conversation of the biocatalytic activity of whole-cells – 2$^{nd}$ generation sol-gel entrapment of yeast for sustainable acyloin condensation, *Periodica Polytechnica – Chemical Engineering*, **2019**. DOI: 10.3311/PPch.14645. IF: 1,382, contribution by the author: 50%.

[IV] L. Nagy-Győr, M. Lacatus, D. Balogh-Weiser, P. Csuka, V. Bódai, B. Erdélyi, Zs. Molnár, G. Hornyánszky, Cs. Paizs, L. Poppe: How to turn yeast cells into sustainable and switchable biocatalyst? On-demand catalysis of ketone bioreduction or acyloin condensation, *ACS Sustainable Chemistry & Engineering*, **2019**, DOI:10.1021/acssuschemeng.9b03367, IF: 6,97, contribution by the author: 60%.

[V] L. Nagy-Győr, Z. Boros, L. Poppe: Immobilization of lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* by adsorption on variosuly grafted silica gels, *Periodica Polytechnica – Chemical Engineering*, **2013**, *57*, 37–40. DOI: 10.3311/PPch.2168 IF: 0,130, contribution by the author: 95%; C: 5, IC: 2.

**Articles not included in the Thesis:**

Z. Boros, L. Nagy-Győr, K. Kátai-Fadgyas, I. Kőhegyi, I, Ling, T. Nagy, Z. Iványi, M. Oláh, Gy. Ruzsics, O. Temesi, B. Volk, B. *Journal Flow Chemistry*, **2019**, *9*, 101–113. IF: 1,658, contribution by the author: 15%.

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

**Lectures:**

Nagy-Győr L., Boros Z., Poppe L.: Biodízel újszerű felhasználása oldószermentes enzimatikus kinetikus rezolválásokban, *XVIII. Nemzetközi Vegyészkonferencia*, **2012**. november 22–25., Félixfürdő, Románia.

Nagy-Győr L., Boros Z., Kátai-Fadgyas K., Kőhegyi I., Ling I., Nagy T., Iványi Z., Oláh M., Ruzsics Gy., Temesi O., Volk B.: Lombikból áramlásos reaktorba: a technológia váltásának lehetőségei a vortioxetin szintézisének példáján keresztül, *XLII. Kémiai Előadói Napok*, **2019**. október 28–30., Szeged.

**Posters:**

Nagy-Győr L., Boros Z**.**, Poppe L.: Biodízel újszerű alkalmazása oldószermentes enzimkatalizált kinetikus rezolválásokban, *MKE Vegyészkonferencia*, **2013**. június 26–28., Hajdúszoboszló, Magyarország.

L. Nagy-Győr, Cs. Kalinák, V. Bódai, P. Sátorhelyi, B. Erdélyi, Z. Boros, L. Poppe: Novel ketoreductases from yeasts for stereoselective bioreductions of ketones, *COST CM1303 "SysBiocat" Training School*, **2014**. május 28 – június 1., Certosa di Pontignano, Olaszország.

Nagy-Győr L.**,** Bell E., Boros Z., Oláh M., Weiser D., Dr. Hornyánszky G., Dr. Nagy J., Dr. Csajági Cs., Dr. Poppe L.: Új típusú, komplex kódolási eljáráson alapuló biztonsági, eredet- és egyediségjelölő, hamisítás elleni rendszerek kifejlesztése, *Oláh György Doktori Iskola XII. Konferencia*, **2015**. február 5., Budapest, Magyarország.

Nagy-Győr L., Molnár Zs., Dr. Bódai V., Dr. Erdélyi B., Dr. Hornyánszky G., Dr. Poppe L., Dr. Paizs Cs.: *Wickerhamomyces subpelliculosus* felhasználása egészsejtes biokatalizátorként ketonok sztereoszelektív bioredukciójában, *Oláh György Doktori Iskola XIII. Konferencia*, **2016**. február 11., Budapest, Magyarország.

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