Final report on OTKA project SNN 125637

„MIO-enzyme-based multistep syntheses in continuous-flow microfluidic reactor systems"

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Utilization of enzymes as biocatalysts can significantly contribute to the rational and sustainable synthesis of an ever-growing number of complex, optically active natural products and drugs. Nowadays, synthesis of chiral (i.e., one of the two mirror image isomers) building blocks for production of drugs is allowed only in enantiomerically pure form, because the required therapeutic effect is rendered to one of the two enantiomers. Chiral amine or amino acid units are essential structural elements in more than half of the today applied drugs in human therapy. The project aimed rational development of MIO-enzymes with the aid of other enzymes suitable for efficient stereoselective multienzyme synthesis of enantiomerically pure amines and amino acids in an efficient way.

Due to their high stereoselectivity and simple use, phenylalanine ammonia-lyase (PAL) and phenylalanine 2,3-aminomutases (L-PAM or D-PAM) – both being members of to the so-called MIO-enzyme family and acting on amino acids – are suitable biocatalyst. In addition to their utilization as biocatalysts, further aiding enzymes such as transaminases, lipases, decarboxylases were designed in microfluidic systems for multienzyme syntheses of chiral amines and amino acids.

This project utilized the expertise of the participants in substrate engineering, in protein structure analysis and production, in reactor modeling and design. The project aimed to exploit the flexibility and modularity of platforms based on protein functionalized magnetic nanoparticles (MNPs) in microfluidic systems. A key feature of the MNP-based novel platform was that the micro-sized reactors could be filled by different nanoparticles in a selective and addressable way. Although the almost two years long COVID-hampered research environment hindered to achieve the original goal of developing multienzyme cascade systems, we could extend the original goal of utilizing cells with static anchoring of MNPs by exploring a intrinsically new way of MNP-based microreactors in which the efficiency of MNP-catalysis could be significantly enhanced by external magnetic agitation.

In accordance with the competencies of the academic partners participating in the project, two objectives related to the utilization nanosupported proteins – especially on magnetic nanoparticles (MNPs) – in microfluidic systems were selected as subjects of the project.

Briefly, Objective 1 was related to the development of novel enzymes and enzyme immobilization methods using nanosupports. Objective 2 focused on the use of the novel platform for synthetic biotransformations in MIO-enzyme-based multienzyme microfluidic reactor systems.

Objectives 1 and 2 were performed in close co-operation with the research group of Prof. Igor Plazl and Dr. Polona Žnidaršič Plazl (Ljubljana, Slovenia, organizer of the present "Implementation of microreactor technology in biotechnology" IMTB 2017, 2019 and 2022 conferences). The project was also coordinated with the activities of the NEMSyB project in Cluj, Romania.

Based on his activity in flow-biocatalysis, Prof. Poppe has been asked for providing a general overview as co-author of a chapter on flow biocatalysis within a reference book on flow-chemistry including an example of MNP-based PAL-reactor.[1]

The detailed list of results related to each Objective (1 and 2) for the full working period (2017-2022) is detailed below:

1. Development of novel enzymes and enzyme immobilization methods using nanosupports

2017-18 (12 month):

- New MIO enzymes from bacterial and eukaryotic sources were identified and characterized. After gene synthesis - cloning - expression in *E. coli*, the new enzymes were characterized [e.g. PAL from *Pseudozyma antarctica* (*Pa*PAL),[2] HAL or AAL from *Pseudomonas fluorescens* (*Pf*HAL, *Pf*AAL[3])].
- Novel secondary enzymes were identified [transaminases (TA), cinnamate decarboxylase (CD) and phenylalanine N-acetyltransferase (PAT)] and the production and characterization of these enzymes were started.
- Methods were developed to modify the surface of nanoparticles by one or more functional groups (e.g., combined possibility of covalent bonding and metal chelation for selective immobilization of His-tagged proteins). This approach could be applied for the selective immobilization of *Pc*PAL on the MNP surface.[4]
- Additional nanocarriers (nanotubes, nanofibers) were investigated [special carbon nanotubes from the Slovenian partner, carbon fibers from Zoltek]. In addition to our previously implemented amination technique (*Per. Polytechn. Chem. Eng.* **2017**, *61*(1), 59-66), the applicability of the diazotization reaction to the preparation of amine-functionalized carbons was investigated.
- Enzyme immobilization trials were started with *Pc*PAL, *Pa*PAM and *Pf*XAL on surface-modified nanocarriers, and production of *Pa*PAL, *Pf*PAM, *Tc*PAM begun.

2018-19 (12 month):

- The expression / production of several new MIO enzymes from bacterial and eukaryotic sources were solved (*Kangiella koreensis* PAL, *Pa*PAL, *Pf*XAL and *Pf*PAM). Detailed crystal structure data on *Pc*PAL were determined[5,6] and deposited in PDB (PDB ID: 6F6T,[7] 6HQF,[8] and 6H2O[9]).
- Several new secondary enzymes [transaminases from *Pseudomonas psychrotolerans* (*PpS*-TA), and *Sinorhizobium* sp (*SrR*-TA),[10] or *Halolamina sediminis* (*HsS*-TA); ferulic acid decarboxylase (FDC1)[11] from *Saccharomyces cerevisiae*] were characterized.
- Optimized methods were developed for the fabrication and functionalization of non-magnetic silica nanoparticles and MNPs[4] meeting the requirements of microfluidic systems.
- The developed method for selective immobilization of *Pc*PAL on could be applied for covalent attachment of His-tagged proteins without purification.[4] Preliminary investigations were started to adapt this method to immobilize TAs as well as *Pa*PAL, *Pf*XAL, *Pf*PAM, *Tc*PAM.
- The modeling of the effect of the size of the core-porous shell MNP composites on the diffusion properties was started together with the Slovenian partner.

2019-20 (12 month):

- The yeast-derived enzyme *Pa*PAL was produced on a large scale and characterized in detail.[2]
- Based on the detailed crystal structure data *Pc*PAL (PDB 6F6T,[7] 6HQF,[8] 6H2O[9]) mechanistic and molecular dynamics studies were performed on *Pc*PAL and *Tc*PAM and a manuscript was submitted to ACS Catalysis.[12] After the preliminary review, the manuscript has been supplemented with new data on *Pc*PAL and *Tc*PAM mutants.
- The production of several secondary enzymes (*PpS*-TA,[10] *HsS*-TA, *SrR*-TA;[10] FDC1[11]) was solved. Two Cys point mutants of *Halolamina sediminis* S-TA were also generated and characterization of their effect on structural stability started.
- Investigation and characterization of new MNP-based batch reactors[13] have been started using *Pf*AAL-MNP catalysts.[3]
- We continued to model the effect of the size of the core-porous shell composites on the diffusion properties together with the Slovenian partner.

2020-2022 (15 month)

- Based on crystal structure data (PDB ID: 6HQF, 6F6T, 6H2O) on PcPAL, a paper on mechanistic conclusions supporting the *N*-MIO route (**Fig. 1**) was accepted in ACS Catalysis.[12] In addition to structural and mechanistic data, we reported the conversion of the (R)-selective phenylalanine 2,3-aminomutase (TcPAM) by two point mutations to a (S)-selective β-phenylalanine ammonia lyase.

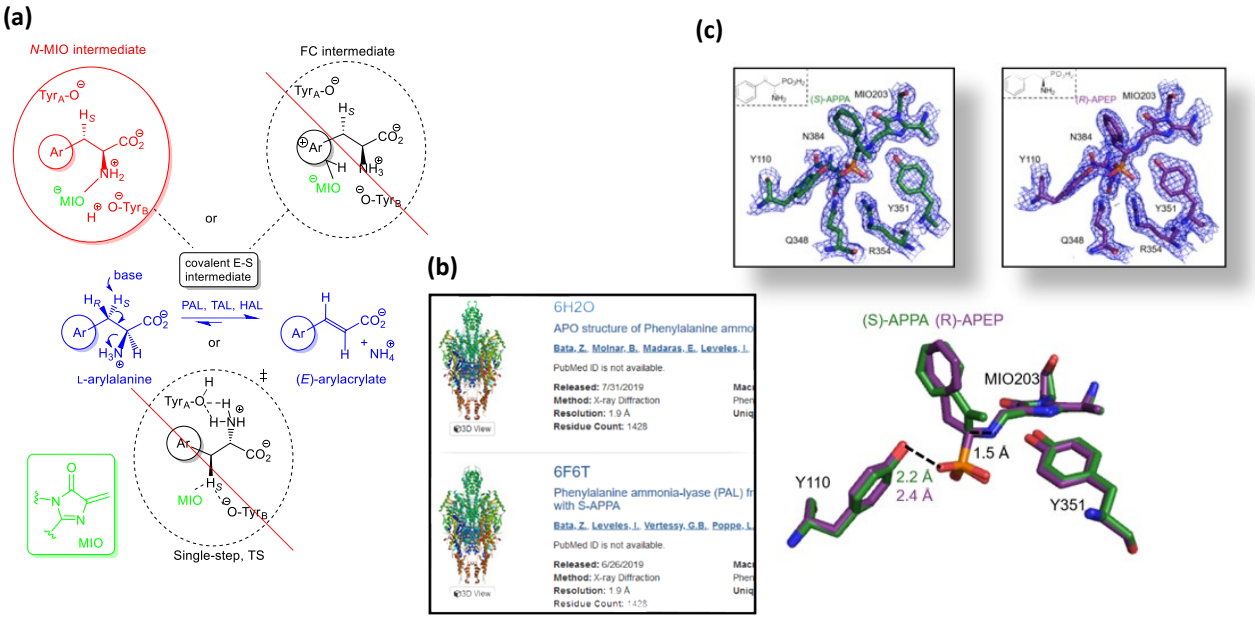

Figure 1. Alternative pathways **(a)** for the reaction catalyzed by the MIO-containing aromatic ammonia-lyases (PAL, TAL and HAL). The X-ray structures of *Pc*PAL **(b)** with substrate-mimicking inhibitors **(c)** indicated covalent *N*-MIO binding.

- Methods to produce PcPAL mutants with enhanced biocatalytic properties were developed.[14]
- The immobilization methods using MNPs meeting the requirements of microfluidic systems were investigated for TAs, *Pa*PAL, *Pf*XAL, *Pf*HAL, *Pf*PAM, *Tc*PAM and *Pf*AAL. Based on the preliminary experiments, the *Pf*HAL and *Pf*AAL enzymes were selected for the characterization of the MNP reactors. The larger amounts these enzymes required for the experiments were produced on a semi-industrial fermentation scale.
- Preparative studies were performed by preparing and characterizing PcPAL enzyme embedded in polylactic acid using electrospinning.[15] The production of MIO enzymes stabilized in the polymer shell allows the preparation of MNP core-polymer shell composites with increased stability.
- Modeling the effect of the size of the core-porous shell composites on the diffusion properties has been continued together with the Slovenian partner.

2. Microfluidic systems for biotransformations

2017-18 (12 month):

- Nano-supported (on halloysite nanoclay) phenylalanine ammonia lyase (*Pc*PAL) and amine transaminase (*Cv*S-TA) enzyme reactions were studied in charged packed bed microreactors

- With the first versions of *Pf*XAL fixed on the surface of MNP, reaction kinetic measurements were performed with in-line UV/Vis reaction monitoring in the basic configurations of the magnetically addressable microfluidic chip reactor arranged in series and in parallel. The *Pf*XAL showed only quite moderate PAL activity.
- We started modeling the particle size and reactor flow conditions and producing particles of the size corresponding to the results of the modeling [the main part of the modeling was performed by BME EET researchers and the Slovenian partner].
- Biotransformations were performed with kinetic resolution of several non-natural amino acids in microfluidic systems using flow-through microreactors (sometimes with in-line UV/Vis reaction monitoring) with *Pc*PAL enzyme immobilized in nanoflower-based systems.
- Integrability study the of the magnetic chip reactor and the microreactors loaded with nanobiocatalysts started by modeling the flow conditions

2018-19 (12 month):

- Optimization of the arrangement and flow parameters of the magnetically addressable microfluidic base cell was continued. At BME EET the geometry of the microfluidic cell inlet and outlet was optimized (**Fig. 2**). [16]

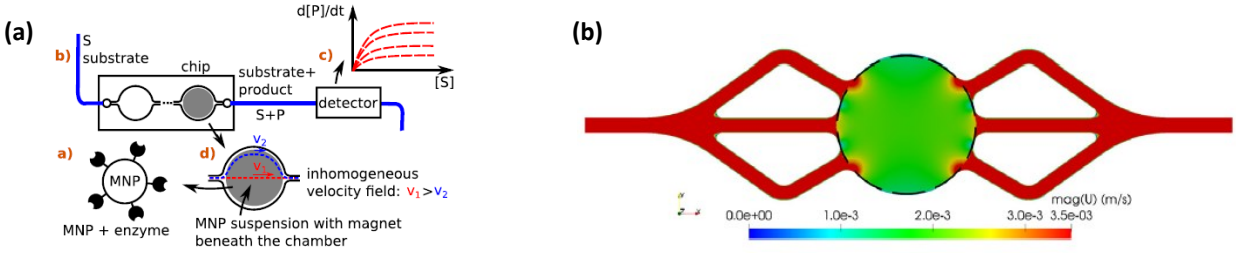

Figure 2. Experimental measurement setup **(a)** for the reaction catalyzed by PcPAL in simplified MagneChip cell.[16] The panel **(b)** shows the magnitude of the velocity field in middle of the a chamber with 3 inlet and 3 outlet channels.

- Preliminary efforts were made for microfluidics studies with MNPs coated with several co-immobilized enzymes (co-immobilized *Pc*PAL / *Tc*PAM-MNPs and *ArS*-TA / *Tc*PAM-MNPs).
- The Slovenian partner prepared a prototype of a flow reactor for the Raman study of MIO-enzyme reactions in the reactor space. Preliminary studies with the Raman spectrometer for these measurements begun.
- BME EET researchers and the Slovenian partner made further progress on modelling the magnetic chip reactor [flow simulations and multiscale modeling].

2019-20 (12 month):

- Researchers at BME EET optimized the geometry of the microfluidic cell inlet and outlet by two-phase CFD model further.[17] The results contribute to the optimization of the arrangement and flow parameters of a magnetically addressable microfluidic basic cell during biotransformations with other immobilized ammonia-lyases (e.g., *Pf*AAL, or *Pf*HAL).
- Preparations with MNPs coated with several immobilized enzymes were characterized for suitability of microfluidics applications (the kinetics of *Pf*AAL[3] and *Pf*HAL enzymes prepared using the established immobilization method[4] were characterized in free and MNP-immobilized form).
- The Slovenian partner prepared a prototype of a flow reactor for the in-line Raman spectrometry study of enzyme reactions in the reactor space. Preliminary experiments were performed with the Raman spectrometer using dissolved *Pc*PAL enzyme and L-Phe as substrate. Due to the significantly increased noise level in the aqueous solutions in addition to the signs of amino acid substrates, we concluded that this class of substrates could not be used analyzed experimentally by Raman methodology. For this reason, studies requiring in-line measurements

on elimination reactions from amino acid substrates were performed by flow-through UV-Vis spectrophotometry further.

2020-2022 (15 month)

- A review on the applicability of biocatalysis in flow systems including an example of MNP-based PAL-reactor has been published as a chapter within a reference book on flow-chemistry.[1]
- The results on the comparative kinetics of *Pf*AAL in free and MNP-fixed form were published in the prestigious ChemBioChem journal.[3] Comparative kinetics of *Pf*HAL in free and MNP-fixed form was also completed.
- The BME EET researchers and the Slovenian partner improved the modeling of the magnetic chip reactor [flow simulations and multiscale modeling].
- Due to the COVID situation improvements towards cascade reactions in MagneChip device were dropped but we ***developed and characterized a set of new MNP-based reactors*** using as model enzyme the *Pf*AAL-MNP catalyst. To date, we have reported the results obtained with *Pf*AAL-MNP-based batch reactors driven by permanent magnets.[13]
- ***New, greatly simplified, tubular reactor-like MNP reactors*** have been developed (**Fig. 3**). The magnetic fields acting on the MNP biocatalysts were successfully modeled in single-layer and double-layer permanent magnet arrays.

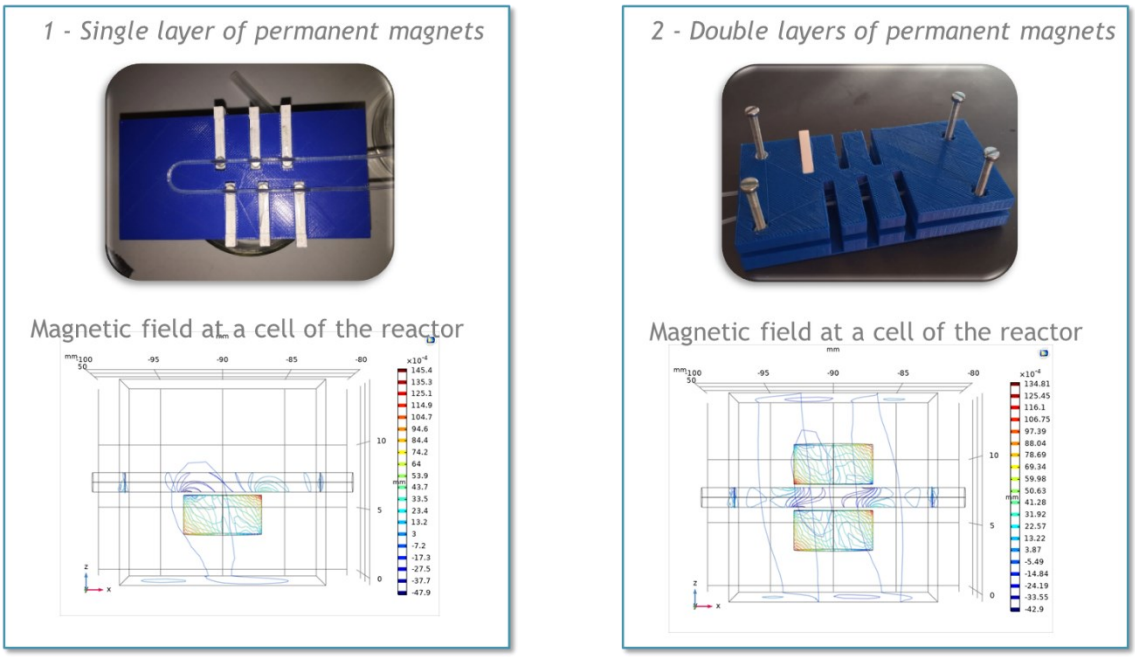

Figure 3. Implementation of a U-shaped tubular MNP microreactor setups **(1)** with a single permanent magnet in one cell and **(2)** with two permanent magnets in attraction arrangement in one cell. The magnetic field models for each kind of cells are depicted below the reactor prototypes.

- MNP-based flow reactors with MNPs agitated by moving permanent magnets based on similar principles as applied for the MNP-based agitated batch reactors were tested with *Pf*AAL and lipase B from *Candida antarctica* (*Ca*LB) enzymes attached to MNPs (**Fig. 4**). With this embodiment we were able to achieve a much higher apparent specific activity of the MNP-based biocatalysts than with the previous batch or flow MNP-based reactors. We intend to publish this work in the prestigious journal Reaction Chemistry and Engineering (D1, Scopus, manuscript in preparation).

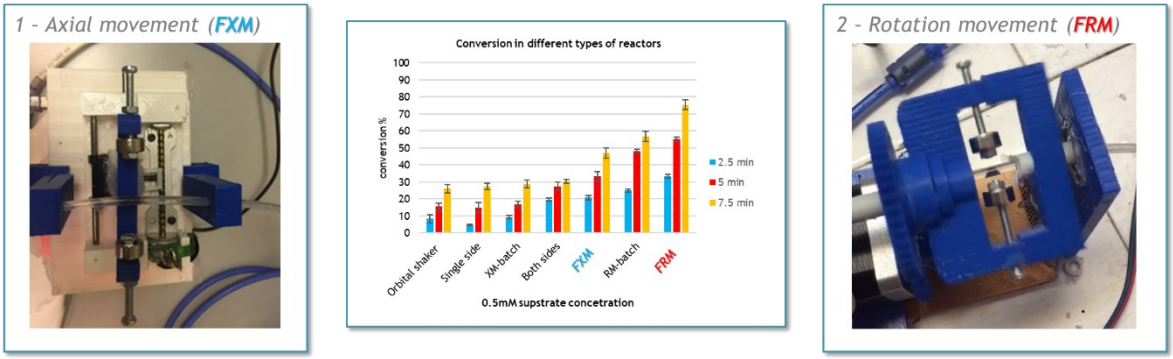

Figure 4. Implementation of tubular MNP microreactor setups **(1)** with axial agitation by two permanent magnets attraction arrangement in one cell and **(2)** with rotating agitation by two permanent magnets in attraction arrangement in one cell. The conversion of the *Pf*AAL-MNP-catalyzed reaction was compared in various reactor setups revealing the FRM mode as the most efficient.

Summary of the results

1. Enzymes and nanocarriers

a Characterization of new PAL from yeast *Pseudozyma antarctica* (*Pa*PAL)[2] and bacterial aspartate and histidine ammonia-lyases from *Pseudomonas fluorescens* (*Pf*AAL)[3] and (*Pf*HAL) as biocatalysts were performed

b Identification and characterization of several secondary enzymes (e.g., *PpS*-TA,[10] *HsS*-TA, *SrR*-TA;[10] FDC1[11]) were accomplished

c Recombinant large-scale bacterial production of PcPAL, PfAAL, PfHAL enzymes was established

d New X-ray structures (PDB: 6F6T,[7] 6HQF,[8] 6H2O[9]) of the known *Petroselinum crispum* PAL (*Pc*PAL) with substrate mimicking phosphonate inhibitors proved the mechanism of PAL reaction (**Fig. 1**)[12]

e Studies on new mutants and of *Pc*PAL[14] and *Taxus canadensis* PAM (TcPAM)[12] led to biocatalysts with improved or altered properties

f Modified silica-shell magnetite-core for magnetic nanoparticles for selective immobilization of PAL,[4] HAL, AAL,[3] TA and lipases

g Electospinning proved to be efficient to create nanofiber-embedded *Pc*PAL as a new nanobiocatalyst[15]

2. Microfluidic systems for biotransformations

a Optimization of the MagneChip cell and flow parameters was performed at various levels of model complexity (**Fig. 2**)[16,17]

b The Raman method in a flow-through reactor was found not sensitive enough for our purposes

c A new, significantly simplified, multicellular MNP tubular reactor was created and the magnetic field acting on the MNPs was modeled in several permanent magnet configurations (**Fig. 3**)

d For the first time, prototypes of the single cells of MNP tubular flow reactors were created in which the MNPs in a flow cell were agitated by external magnetic field (**Fig. 4**). Among all MNP utilization modes, the rotating agitation in flow proved to be the most efficient.

General achievements

- As an important result of the project, a COST Action Proposal OC-2021-1-25449 "Biocatalysis in Continuous-Flow Systems for Bioproduction and Biosensing" has been submitted in October 2021 by the coordination of Prof. Polona Žnidaršič Plazl, with the participation of about 100 researchers from more than 30 European countries. According to the plans, the working group on biocatalyst development and immobilization would be led by Prof. László Poppe.

- The overall results of the project (9 published articles, 2 article prepared for submission, 3 PDB structures deposited and numerous conference presentations) indicated successful research activity involving also international co-operation with the Cluj group (Prof. Csaba Paizs).
- Closely related to the project two young scientists were awarded by PhD degree: Bata Zsófia (Investigation of structure-function relationship in hydroxynitrile lyases and MIO-containing class I lyase like enzymes, 2019) at BME Department of Organic Chemistry and Technology and Pálovics Péter (Numerical modelling of magnetic nanoparticles in microfluidics, 2021) at BME Department of Electron Devices.

Publications derived from the project

1. PARADISI, F.; **POPPE, L.**: "10 Continuous-flow biocatalysis with enzymes and cells" Volume 2, Chapter 10 in *Flow Chemistry – Applications* (Darvas, F.; Dormán, G.; Hessel, V.; Ley, S. V.; Eds.), de Gruyter, Berlin, Boston, **2021**, pp. 277-312. (ISBN: 978-3-110-69361-4) DOI:10.1515/9783110693690-010
2. VARGA, A.; **CSUKA, P.**; SONESOUPHAP, O.; BÁNÓCZI, G.; MOLNÁR, Z.; KATONA, G.; **POPPE, L.**; PAIZS, C.: A novel phenylalanine ammonia-lyase from *Pseudozyma antarctica* for the stereoselective biotransformations of unnatural amino acids, *Cat. Today*, **2021**, *366*, 185–194. DOI:10.1016/j.cattod.2020.04.002 [**IF 6.766**]
3. **CSUKA, P.;** MOLNÁR, Z.; TÓTH, V.; IMARAH, A. O.; **BALOGH-WEISER, D.**; **VÉRTESSY, B. G.**; **POPPE, L.**: Immobilization of the Aspartate Ammonia-lyase from *Pseudomonas fluorescens* R124 on Magnetic Nanoparticles – Characterization and Kinetics, *ChemBioChem*, **2022**, *23*(7), e202100708. DOI:10.1002/cbic.202100708 [**IF 3.164**]
4. **SÁNTA-BELL, E.;** MOLNÁR, Z.; VARGA, A.; **NAGY, F.; HORNYÁNSZKY, G.;** PAIZS, C.; **BALOGH-WEISER, D.; POPPE, L.:** "Fishing and hunting"– Selective immobilization of a recombinant phenylalanine ammonia-lyase from fermentation media. *Molecules* **2019**, *24*(22), 4146. DOI:10.3390/molecules24224146 [**IF 3.060**]
5. **BATA, Z.**; MADARAS, E.; LEVELES, I.; HAMMERSCHMIDT, F.; PAIZS, C.; **POPPE, L.; VÉRTESSY, B. G.**: Bioactive 3D Structure of Phenylalanine Ammonia-Lyase Reveal Key Insights into Ligand Binding Dynamics. *Biophys. J.* **2018**, *114*(3), 406A. DOI:10.1016/j.bpj.2017.11.2248
6. **BATA, Z.**; MOLNÁR, B.; LEVELES, I.; VARGA, A.; PAIZS, C.; **POPPE, L.; VÉRTESSY, B. G.**: Structural snapshots of multiple enzyme–ligand complexes pave the road for semi-rational enzyme engineering. *Acta Crystal. Sect. A* **2018**, *74*(a2), e37-e38. DOI:10.1107/S2053273318094640
7. **BATA, Z.**; LEVELES, I.; **VÉRTESSY, G.B.; POPPE, L.:** Phenylalanine ammonia-lyase (PAL) from *Petroselinum crispum* complexed with (S)-APPA. *Protein DB*, 6F6T, **2019**. DOI:10.2210/pdb6F6T/pdb
8. **BATA, Z.**; MOLNÁR, B.; LEVELES, I.; **VÉRTESSY, G. B.; POPPE, L.:** Structure of Phenylalanine ammonia-lyase from *Petroselinum crispum* in complex with (R)-APEP. *Protein DB*, 6F6T, **2019**. DOI:10.2210/pdb6HQF/pdb
9. MOLNÁR, B.; **BATA, Z.**; LEVELES, I.; **VÉRTESSY, G. B.; POPPE, L.**: APO structure of Phenylalanine ammonia-lyase from *Petroselinum crispum*. *Protein DB*, 6H2O, **2019**. DOI:10.2210/pdb6H2O/pdb
10. GAL, C. A.; BARABÁS, L. E.; VARGA, A.; **CSUKA, P.**; BENCZE, L. C.; TOȘA, M. I.; **POPPE, L.**; PAIZS, C.: How to identify and characterize novel transaminases? Two novel transaminases with opposite enantioselectivity for the synthesis of optically active amines. *Mol. Catal.* **2022**, submitted.
11. NAGY, E. Z. A.; NAGY, C. L; FILIP, A.; NAGY, K.; GÁL, E.; TŐTŐS, R.; **POPPE, L.**; PAIZS, C.; BENCZE, L. C.: Exploring the substrate scope of ferulic acid decarboxylase (FDC1) from *Saccharomyces cerevisiae*. *Sci. Reports* **2019**, *9*, 647. DOI:10.1038/s41598-018-36977-x [**IF 4.122**]
12. **BATA, Z.**; MOLNÁR, Z.; MADARAS-KONCZ, E.; MOLNÁR, B.; **SÁNTA-BELL, E.**; VARGA, A.; LEVELES, I.; QIAN, R.; HAMMERSCHMIDT, F.; PAIZS, C.; **VÉRTESSY, B. G.; POPPE, L.**: Substrate Tunnel Engineering Aided by X-Ray Crystallography and

Functional Dynamics Swaps the Function of MIO-Enzymes. *ACS Catalysis* **2021**, *11*, 4538–4549. DOI:10.1021/acscatal.1c00266 [**IF 13.084**]

13. IMARAH, A. O.; **CSUKA, P.**; BATAA, N.; DECSI, B.; **SÁNTA-BELL, E.**; MOLNÁR, Z.; **BALOGH-WEISER, D.; POPPE, L.**: Magnetically Agitated Nanoparticle-Based Batch Reactors for Biocatalysis with Immobilized Aspartate Ammonia-lyase. *Catalysts* **2021**, *11*(4), 483. DOI:10.3390/catal11040483 [**IF 4.146**]

14. NAGY, E. Z. A.; TORK, S. D.; FILIP, A.; **POPPE, L.**; TOŞA, M. I.; PAIZS, C.; BENCZE, L.C.: "Production of L- and D-phenylalanine analogues using tailored phenylalanine ammonia-lyases" Chapter 5.5 in *Applied Biocatalysis: The Chemist's Enzyme Toolbox* (Whittal, J.; Sutton, J.; Eds.), John Wiley & Sons Inc, Hoboken, **2020**, pp. 216-221. (ISBN: 978-1-119-48703-6) DOI:10.1002/cctc.201800258

15. KOPLÁNYI, G.; **SÁNTA-BELL, E.**; MOLNÁR, Z.; TÓTH, G.; JÓZÓ, M.; SZILÁGYI, A.; **ENDER, F.**; PUKÁNSZKY, B.; **VÉRTESSY, G. B.; POPPE, L.; BALOGH-WEISER, D.**: Entrapment of Recombinant Phenylalanine Ammonia-Lyase in Nanofibrous Polylactic Acid Membrane by Emulsion Electrospinning. *Catalysts* **2021**, *11*(11), 1149. DOI:10.3390/catal11101149 [**IF 4.146**]

16. PÁLOVICS, P.; **ENDER, F.**; RENCZ, M.: Towards the CFD model of flow rate dependent enzyme-substrate reactions in nanoparticle filled flow microreactors. *Microelectron. Reliab.* **2018**, *85*, 84-92. DOI:10.1016/j.microrel.2018.03.035 [**IF 1.483**]

17. PÁLOVICS, P.; NÉMETH, M.; RENCZ, M.: Investigation and Modeling of the Magnetic Nanoparticle Aggregation with a Two-Phase CFD Model. *Energies* **2020**, *13*(18), 4871. DOI:10.3390/en13184871 [**IF 3.004**]