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19-22 MAY·2024
ZADAR • CROATIA

# BOOK OF EXTENDED ABSTRACTS

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Dear Colleagues,

Welcome to the 7nd International Conference Implementation of microreactor technology in biotechnology – IMTB 2024 in Zadar!

The IMTB 2024 Conference provides a forum for people from academia and industry to share and discuss the cutting edge results on implementation of microstructured devices in applied biology involving bioprocesses in engineering, technology and medicine, with the aim of developing new projects and exploiting new technologies for biotechnological applications.

This event is a sequel of the IMTB 2010 Conference held in Ljubljana, Slovenia, in September 2010, the IMTB 2013 Conference, which took place in Cavtat, Croatia, in May 2013, the IMTB 2015 Conference, which was held in Opatija, Croatia, in May 2015, the IMTB 2017 Conference held in Bled, Slovenia, in April 2017, IMTB 2019 Conference, which was performed in Cavtat, Croatia, in May 2019, and IMTB 2022 Conference which took place in Portorož, Slovenia, in June 2022. Organizers of this conference series are collaborative research groups from the Faculty of Chemistry and Chemical Technology of University of Ljubljana, Slovenia, and the Faculty of Chemical Engineering and Technology of University of Zagreb, Croatia.

Invited prominent speakers from leading research institutions and industries together with participants of various backgrounds comprising chemical, mechanical, and electrical engineering, medicine, pharmacy, chemistry, biochemistry, microbiology, and biotechnology, will present their recent achievements in the field of enzymatic microreactors, cells within microdevices, analytical microdevices and bioprocess intensification and integration within microdevices.

We would like to thank all participants and especially members of the Scientific Committee for their contribution. Special thanks go to sponsors and donators who enabled the preparation of this event, as well as the European Society of Applied Biocatalysis and the European Society of Biochemical Sciences for their support.

And we’re looking forward to meeting you at IMTB 2024!

IMTB 2024 Organizing Committee

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P4 *Microfluidics-based generation of crosslinked horseradish peroxidase nanoaggregates and pallidol synthesis from resveratrol*
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University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia

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University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia; Chair of Microprocess Engineering and Technology - COMPETE, Slovenia

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University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia; Chair of Microprocess Engineering and Technology - COMPETE, Slovenia

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**Maria Rodriguez-Torres**, Elif Erdem, Ulrich Krühne and John M. Woodley
Technical University of Denmark, Denmark

P9 *Novel magnetic nanoparticle-based flow reactors for biocatalytic production of enantiopure alcohols and amines*
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Budapest University of Technology and Economics, Hungary

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Technische Universität Braunschweig, Germany

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# Plenary Talk

# Linkage of Biocatalyst Development with Reaction Engineering

Andreas S. Bommarius
School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, 30332-2000, USA

Biotechnological processes to a range of product targets from commodities to life-science industry specialties are held back by issues connected to reactor design, such as an unknown rate-limiting step in a reaction sequence or lack of knowledge about the weakest dimension of merit of a (bio)catalyst, such as activity, stability, or selectivity. Such issues have profound implications for both reaction engineering and (bio)catalyst development. We will cover several situations where improved understanding led to better reactor design and/or catalysts.

Semisynthetic penicillins, essential medicines but often in short supply, have long been synthesized via Pen G acylase (PGA)-catalyzed condensation of appropriate side chains with β-lactam cores. We developed a continuous, enzymatically-catalyzed reactive crystallization process to amoxicillin and cephalexin and will demonstrate the interplay of process modeling and pilot runs for a process with several kinetics (enzyme reaction, pore diffusion, crystallization, and enzyme deactivation).[1] For α-amino ester hydrolase (AEH), an alternative enzyme to the established PGA, we tackle reaction engineering with the base-case enzyme, which we then sought to improve via combined protein engineering and reactor design efforts.[2] Highlights are the detailed kinetic modeling of AEH deactivation [3] and the combination of ML-based tools and time course experiments to establish the best fit between data and kinetic scheme for the process.[4]

Oxidases catalyze a series of interesting reactions, from ring opening of ketones to lactones (BMVOs) to regeneration of $NAD^+$ from NADH (NADH oxidases/nox2). However, they are very often not process-ready owing to instability. Furthermore, they require oxygen as a co-substrate. We will report on successful efforts to tackle both issues. *Acinetobacter calcoaceticus* BMVO was stabilized > $10^3$-fold without even resorting to protein engineering, before stabilizing another $10^3$-fold with it.[5,6] We observed a two-stage, first-order deactivation behavior of *Lactobacillus plantarum* nox2 in a bubble column.[7] Comparing deactivation in the bubble column with that in quiescent solution, we ultimately attributed the two-stage behavior to a sequence of interfacial adsorption followed by deactivation at the interface.[8]

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[3] CE Lagerman et al., *Chem. Eng. Sci*. **2023**, *277*, 118804 (doi.org/10.1016/j.ces.2023.118804)
[4] J Range et al., *to be submitted*
[5] LCP Goncalves et al., *Adv. Synth. Catal.* **2017**, *359*, 2121-2131 (doi: 10.1002/adsc.201700585)
[6] H Mansouri et al., *ACS Catalysis* **2022**, *12*, 11761−11766 (doi.org/10.1021/acscatal.2c03225)
[7] SR Anderson et al., *Chem Eng J* **2021**, *417*, 127909 (doi: 10.1016/j.cej.2020.127909)
[8] AV Høst et al., *in revision*

# Droplet Microreactors for Ultrahigh Throughput Enzyme Discovery

F. *Hollfelder*[a,*]

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**email: fh111@cam.ac.uk*

Functional proteins for a variety of useful applications, as binders and catalysts, are required, but currently not known. Functional metagenomics and directed evolution promise access to such new proteins, but the chances of finding them are low. Therefore high-throughput technologies are crucial to beat the odds: screening in picoliter water-in–oil emulsion droplets produced in microfluidic devices allow screening of >10e7 clones and permit successful selections.[7] While potentially faster, the vastness of sequence space (and the scarcity of 'solutions' in it) require strategies for the identification and interconversion of enzymes[1-6]. In this context the role of 'promiscuous' enzymes, sequencing of full length of genes at high throughput (UMIC-seq)[8], insertion/deletion mutagenesis (using the transposon-based method TRIAD)[5] and the role that machine learning will play in interpreting large datasets obtained in droplet screening will be discussed. Together with a molecular and mechanistic understanding new routes to functional proteins can be charted.

*Keywords*: directed evolution, protein engineering, in vitro compartmentalization, functional metagenomics, droplet microfluidics

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# Shining Light on Microbioreactors: Exploring the Power of Optical Sensing

Torsten Mayr*

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Optical chemical sensors are established in process monitoring tools in industry and research laboratories. They basically comprise of a luminescent indicator dye based in a host polymer. They are easy to integrate, non-invasive, do not need any reference element and can be read-out contactless from outside. However, to fully exploit the potential in microfluidic systems or microreactors, the sensors have to fulfil several demands including high brightness, capability to be applied as thin film, excellent photo stability, cheap and accurate read-out systems, ease in use (simple calibration and drift free), simple mass production compatible preparation steps, compatibility with the chip materials, resistance towards sterilization and no toxicity.

We present sensors for important chemical parameters such as oxygen, pH, glucose, lactate, ammonia and hydrogen peroxide fulfilling these demands. Our sensors can be excited with red-light and emit light in the near infra-red range (<700 nm). This suppresses background fluorescence and scattering from biological material. Sensor can be prepared in different formats including coated fibers, ink-jet printed sensor spots or knife-coated spots. Compact measurement instruments enable the read-out of integrated sensor elements in microreactors in sizes from 300 to 800 micrometers or miniaturized probes.

Oxygen and pH sensor are applied in microbioreactors and microfluidic cell culture systems. The integrated sensors are used to monitor cell culture conditions and the cell metabolism. We demonstrate the influence of model drugs and nanomaterials on the cell respiration and acidification.[1,2] Integrated oxygen sensors are also presented in organ-on-chip to measure the oxygen consumption of cardiac cells during beating motion with electrical stimulation.[3] We also present glucose and lactate sensors in microfluidic format to measure additional important metabolic parameters. We also developed sensors for ammonia and hydrogen peroxide – analytes that were not able to be monitored continuously, so far. These sensors can be applied to monitor ammonia in trace levels for environmental monitoring and high concentrations in flow reactors.[4] We present a new sensing concept for detection of hydrogen peroxide in a microfluidic flow cell[5] and demonstrate the application of this sensors.[6]

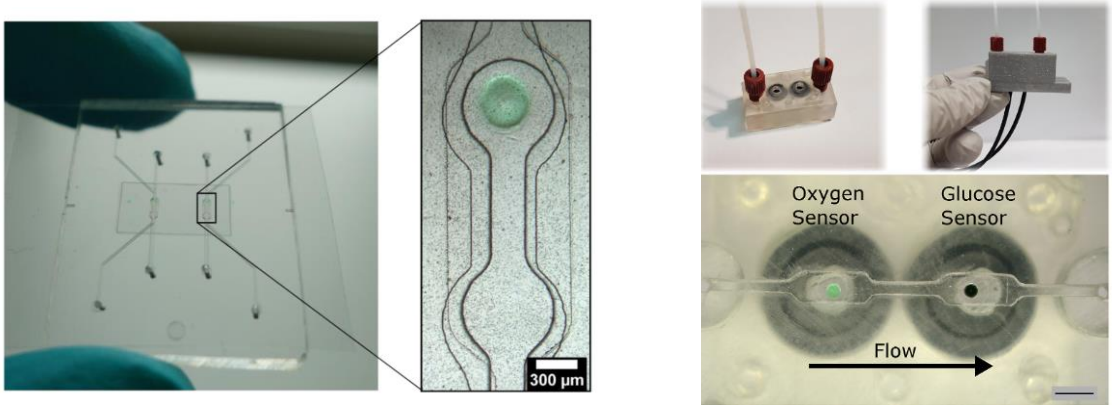


Figure Left: Heart-on-Chip with integrated optical oxygen sensors (green dots). Figure Right: Glucose sensor integrated in a microfluidic flow cell.

*Keywords*: optical sensor, microbioreactor, organ-on-chip, on-line monitoring

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# Lessons learned – Using capillary biofilm reactors in biotechnology

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Capillary biofilm reactors (CBR) (Figure 1) are established in applied biofilm research for many years. With inner diameters in the low mm range these systems benefit from a large surface area to volume ratio. If run in a segmented flow fashion by applying a second, water immiscible phase, CBRs profit from Tylor type flow occurring at the phase boundaries of the different phases enhancing mass transfer. The specific hydrodynamic conditions prevailing in the CBR initiate biofilm formation in many microorganisms. Upon inoculation, the microbes interact with the surface while undergoing significant physiological changes. Finally, they will start to excrete extra polymeric substances and thereby form a mature biofilm inside the CBR. The system is continuously flushed with medium at a low flow rate, ensuring constant cultivation conditions, while the segments keep the biofilm at a fixed thickness and prevent clogging of the capillary.

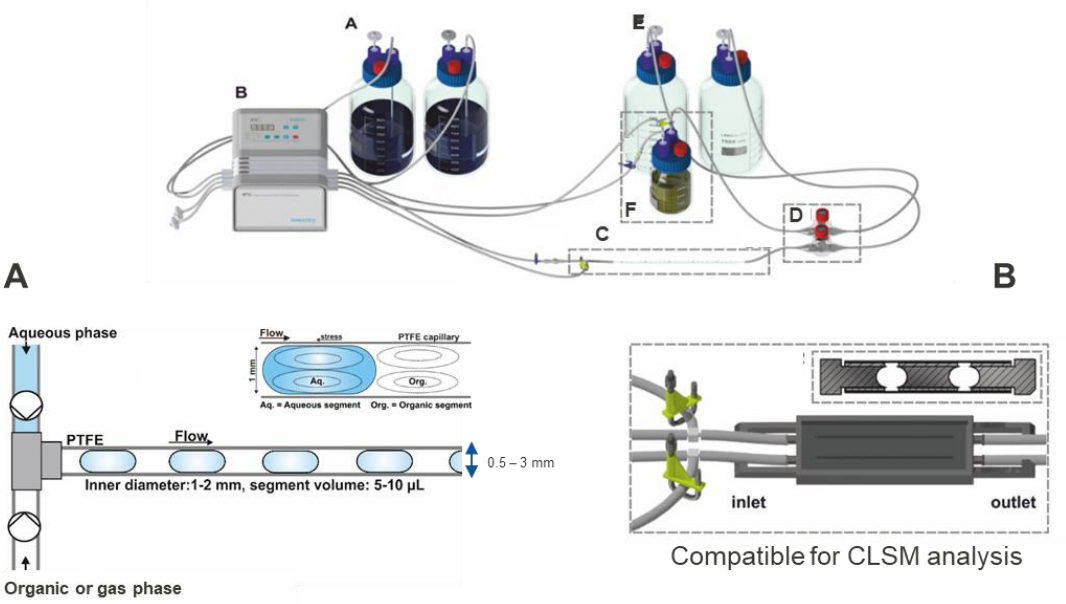


Figure 1: Schematic of the capillary biofilm reactor (CBR). *Top:* Overall system with medium reservoir (A), pump (B), cultivation chamber (C), sampling port (D), and waste (E). Alternative feeding option (F). *Below A*: Concept of a capillary reactor run in segmented flow fashion. *Below B*: Flow cell for confocal laser scanning microscopy adapted to CBR geometries. Figure modified from[1].

The goal of developing alternative production routes with a smaller environmental footprint compared to established processes is driving our projects. In this context, biofilms are an interesting biocatalyst format[2]. Organisms growing in biofilms profit from an extraordinary robustness and resilience, which is beneficial if working with otherwise toxic reactants like organic solvents usually strongly decreasing cellular viability. One of our biofilm workhorses is *Pseudomonas taiwanensis* VLB 120.

It was also one of the first organisms applied in a CBR, catalyzing the challenging reaction of styrene to styrene oxide while growing in a styrene saturated environment[3]. The biotransformation substrate was in this case fed as a pure phase via the segments, which proved to be an elegant way of supplying this otherwise highly volatile compound. The biofilm showed a constant activity but closer investigations showed, that it was strongly oxygen limited. Unexpectedly, cells reacted dramatically to the styrene shock by showing significant membrane damage. However, the biofilm adapted to these toxic environment and recovered to nearly 100 % in course of the experiment and thereby convincingly showing the potential of this catalyst format.

In the recent years we shifted our focus a bit and investigated phototrophic organisms (in particular cyanobacteria and non-sulfur purple bacteria) and their suitability to be applied as biofilms in CB reactors. Especially cyanobacteria are discussed as "high potential future workhorses", as they are independent from any organic carbon and energy source and many of them even fix atmospheric nitrogen. Despite photo-biocatalysis developing remarkably and the huge potential photoautotrophic microorganisms hold for eco-efficient production scenarios, photo-biotechnology is still in its infancy. A key-challenge in this respect is the low cell-density which cyanobacterial culture typically reach due to light limitation in conventional photobioreactors. CBRs proved surprisingly beneficial for the cultivation of light depending microbes (Figure 2). Due to the low diameter, the necessary light penetration depth into the culture is low and the microbes readily form biofilms inside the capillaries of up to 400 µm thickness, reaching up to 60 g biofilm dry weight / L, which is 10 times as much as in common photobioreactors for suspended cell cultivation[4].

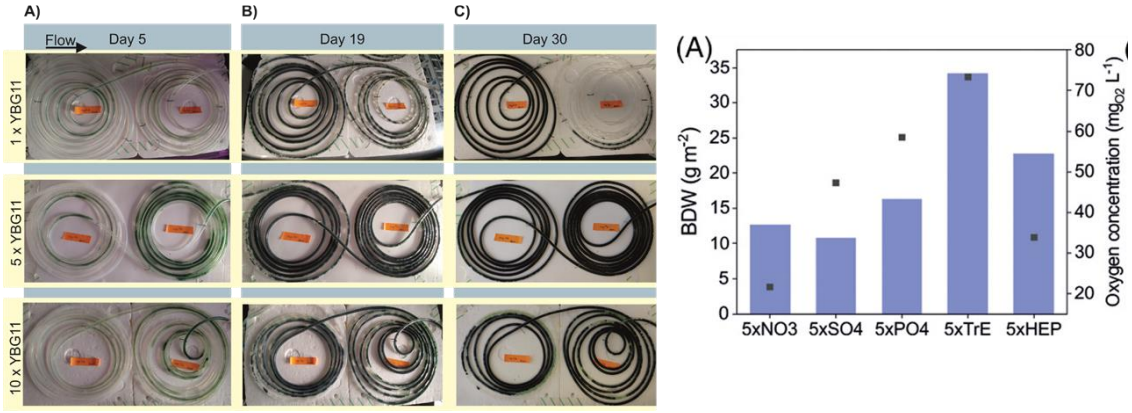


Figure 2: *Synechocystis* sp. PCC 6803 biofilms in CBRs. Experiment on medium composition. Limiting compounds trace elements (A). Total length of CBR 5 m. Figure taken from[5].

In my presentation, I will cover several examples of biofilm cultivation in such CBRs, reaching from axenic organo-heterotrophic Pseudomonas to mixed species biofilms dominated by photo-autotrophic cyanobacteria and discuss benefits and shortcomings of the CBR system in biofilm applications.

*Keywords*: capillary biofilm reactors, cyanobacteria, Pseudomonas, biocatalytic conversion, organic solvent

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# Session A: ENZYMATIC MICROREACTORS

# Identification of opportunities and challenges of immobilized-enzyme miniaturized reactors for reaction intensification

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Biocatalytic transformations have recently transitioned towards operation in continuous flow reactors. This shift to continuous flow is closely associated with the integration of immobilized enzymes in miniaturized reactors under different reaction formats such as wall-coated reactors and fixed-bed reactors. Numerous studies report that continuous-flow reactor offers improved performance in terms of reduced reaction time, higher productivity, and product yield[1]. However, the development and optimization require an identification of the relevant interdependent features of the reactor, reaction and biocatalyst. These factors are essential for a rigorous analysis of the reactor's impact on reaction performance. While aspects like protein engineering, catalyst design, enzyme immobilization, and reaction medium engineering are typically addressed, reaction engineering aspects such as mass transfer resistances and fluid dynamics, which often serve as limiting factors, have received comparatively less attention[1,2].

In this presentation, we aim to show a systematic approach for characterizing immobilized-enzyme reactors based on time-scale analysis and the interplay of diffusion, dispersion, and reaction kinetics. We will highlight how in wall-coated reactors, exploiting microscale effects can lead to the intensification of reactions that are limited by mass transfer. The interplay between surface immobilization, reactor miniaturization, and mass transfer requirements is crucial, and further opportunities for process intensification can therefore be identified[2]. In the fixed-bed format, evidence suggests that using a milli-reactor under laminar flow combined with mesoporous catalyst particles allows for operation in the absence of external and internal mass transfer resistances[3]. Residence time emerges as a key parameter determining an operational window with low axial dispersion, short residence times, and plug flow behavior, while longer residence times transition to backmixing, ultimately affecting the degree of conversion. In conclusion, while the implementation of continuous flow transformations in biocatalysis offers significant potential, clear identification of the reaction-limiting factors connected to reactor design, and optimization of immobilization protocols are essential for realizing this potential. Continued efforts in systematic characterization and optimization are necessary to fully exploit the opportunities presented by enzyme-immobilized reactors for reaction intensification in biocatalysis.

*Keywords*: enzyme microreactors; enzyme immobilization; mass transfer; reactor fluid-dynamics

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# 3D-printed microreactors for enzyme immobilization: A paradigm towards customized microfluidic screening platforms

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Additive manufacturing, referring to tailor-made designs and fabrication techniques, has been claimed as a game-changer in the construction of reactors and peripheral units in the field of flow chemistry[1]. From one point of view, the inherent nature of this enabling technology allows for freedom of design and adjustment according to the specific needs of each system. Adding to this, the ongoing research on 3D-printable materials is providing a variety of options to explore for the ideal reactor material and/or immobilization matrix to incorporate into the flow system[2]. On the other hand, enzyme immobilization for microbioreactor development has long been proven as a highly efficient approach for biocatalyst retainment and reuse[3]. This work explores both aspects of enzymatic microreactor development: i) the 3D printing technique/3D-printed material, and ii) the immobilization method, to achieve a robust, efficient, and reusable microbioreactor system. The proof-of-concept was demonstrated with an enzyme that has not been elaborated so far for microfluidic applications since there are certain challenges for its immobilization and stability.

The enzyme of choice was unspecific peroxygenase (UPO). Fungal UPOs (EC 1.11.2.1), are heme-thiolate enzymes, displaying characteristic peroxidase activity, but also a unique peroxygenase activity. This way, UPOs are versatile biocatalysts with great importance in synthetic chemistry for an ensemble of highly selective C-H oxyfunctionalizations, while engineered UPO variants are currently under intensive study[4]. After research on the 3D printing technique and material investigation, a surface functionalization protocol was developed, leading to enzyme anchoring on the microreactor's internal walls. Process parameters optimization was performed in regards to (i) reactor geometry, (ii) flow rates, and (iii) reaction conditions. Ultimately, the developed systems were evaluated for their performance as microfluidic screening tools, to explore the suitability of different enzyme variants for oxyfunctionalization reactions of interest.

*Keywords*: 3D printing, enzymatic microreactors, enzyme immobilization, oxyfunctionalization

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# U Can Load (UCL): a Universal Microreactor for Flow Biocatalysis

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Microreactors have been successfully applied to execute a broad range of biotransformations in flow. However, microreactors have typically been designed with a specific biotransformation or a specific biocatalyst immobilization method in mind, constraining their wider applicability. Furthermore, their design is typically either applicable for whole-cell or for enzyme biocatalysis, but not for both. We present a novel microreactor design which offers cartridge-like insertion of both immobilised enzymes and cells[1]. A T-shaped lid opens and closes the reaction chamber (whilst leaving the rest of the microreactor unchanged), enables the easy insertion of immobilised biocatalysts, and thus allows the user to configure different reactor types. We demonstrated this novel concept showing three different reactor types: a hydrogel microreactor containing entrapped *E. coli* cells overexpressing transketolase, a packed-bed microreactor containing commercial beads with immobilised *Candida antarctica* lipase B, and a micropillar microreactor containing surface-immobilised ω-transaminase. The proposed design showed consistency and robustness for 10 consecutive T-shaped lid 'open and close' cycles and withstood the pressure of at least 4 bar. Design analysis further included Computational Fluid Dynamics models and Residence Time Distribution measurements[1]. The presented design offers a standardised approach for multiple applications, underpinning process development and paving the way for off-the-shelf microreactor technology for biocatalysis.

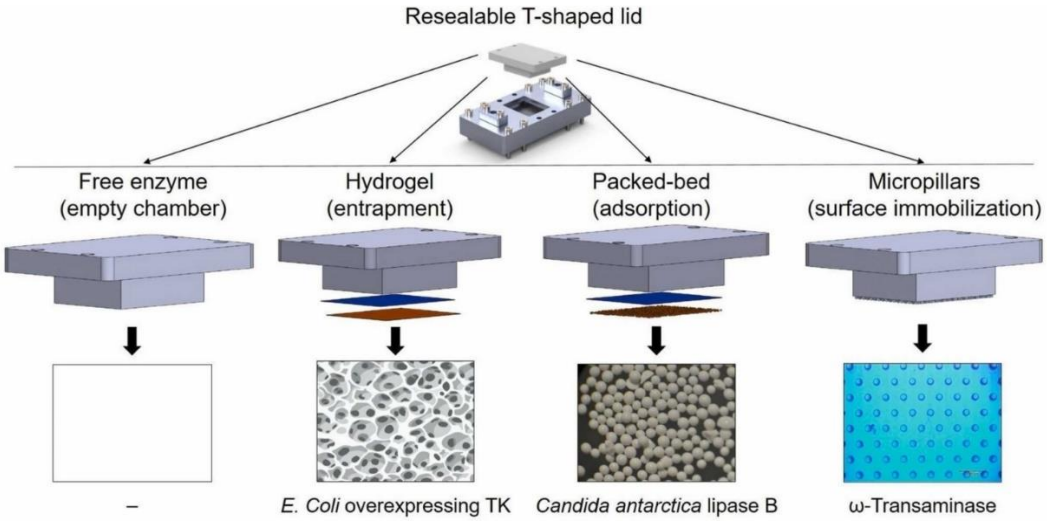


Figure 1: Decoupling application from reactor design for flow biocatalysis applications.

*Keywords*: Standardisation, Enzymes, whole cells, microreactors, residence time distribution

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AO3

# Redesigning a 3D-printed micro bubble column reactor for biocatalytic multiphase reactions

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As screening trials involve an ever-growing number of parameters to be tested, and process development is in need to become more financially efficient, there is an increasing interest in the use of rapidly advancing microbioreactors (MBRs). Developments in biotechnological and biopharmaceutical research benefit from MBRs due to several advantages they offer: small volumes reduce substrate consumption (leading to cost savings and increased safety), parallelization and automation enable higher experimental throughput, and newly developed sensor technologies adapted to the microscale enable continuous online monitoring in realtime. However, challenges that still need to be overcome often arise from the laminar flow regimes in MBRs – especially in terms of mixing and ensuring sufficient mass transfer[1].

This work presents a novel micro bubble column reactor (MBCR) specifically designed to emulate the operating characteristics of a fully functional bubble column. Most of the reactor parts are manufactured using innovative additive manufacturing techniques. The 3D-printed components of the reactor shown in **Fig. 1** include the (**B**) main body, (**E**) the frame, and (**F**) a docking module, all produced with a multijet printing procedure using acrylate polymer. The reactor is (**C**) sealed and closed with a (**D**) sensor slide, made of a glass microscope slide.

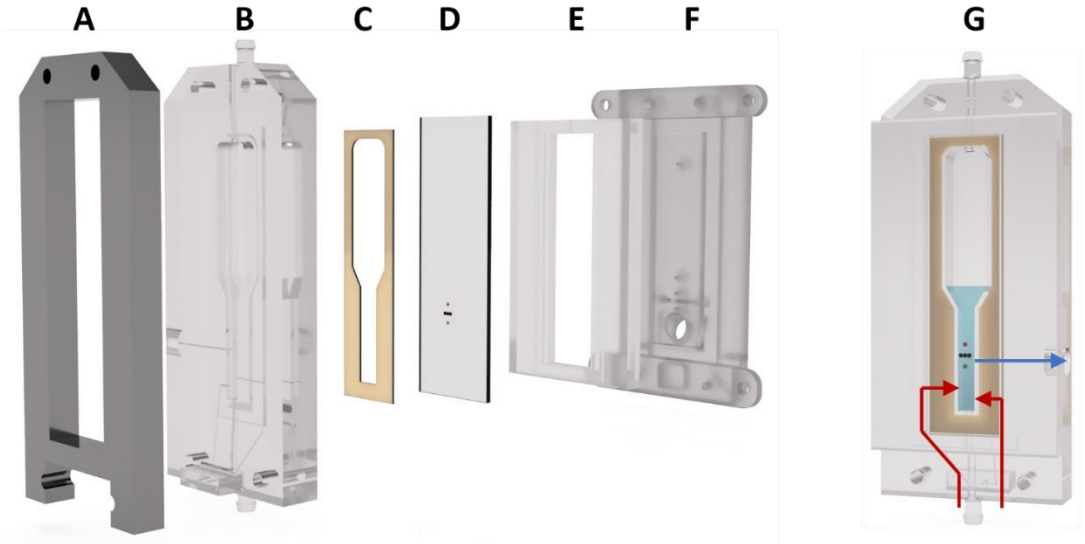


**Figure 1:** 3D-printed micro bubble column reactor (MBCR) setup, exploded-view: (**A**) Connector clip, (**B**) 3D-printed MBCR, (**C**) sealing, (**D**) sensor slide, (**E**) frame for sensor slide, (**F**) docking station, (**G**) assembled MBCR with sensor slide and micro channels for sampling (blue), as well as substrate/media inlets (red).

This slide serves as the backwall of the MBCR and its transparency enables the latest generation of sensor spots to be read out via fiber optics, so that the pH values and the concentration of dissolved oxygen could be determined during the cultivation of microorganisms in applicability tests of the MBCR[2].

The initial experiments have successfully validated the concept as a fully functional reactor system. To broaden the scope of applications, the MBCR was redisigned to mimic a lab-scale bubble column, serving as a scaledown model. As a result, the design of the sparger was thoroughly investigated, resulting in various solutions with defined single- and multi-nozzle spargers, as well as irregular porous filters. The variations in sparger design were investigated with a focus on mass transfer and its potential limitations in order to obtain a broader spectrum of options for specific applications. Furthermore, adjustments were made to the reaction chamber's dimensions to accurately replicate the lab-scale reactor. Microfluid channels were introduced to facilitate the supply of aqueous substrates (**Fig. 1, (G), red arrows**). Modifications to the reactor design were integrated virtually effortlessly due to the system's adaptability and the rapid prototyping capabilities offered by 3D printing. Subsequent changes to the channel positions, aimed at preventing concentration gradients at the inlet, were made possible by analyzing the flow patterns (**Fig. 2**) provided by a three-dimensional and two-phase Computational Fluid Dynamic (CFD) model. These insights were applied in the MBCR to identify areas of enhanced mixing performance and predict improvements in inlet regions.

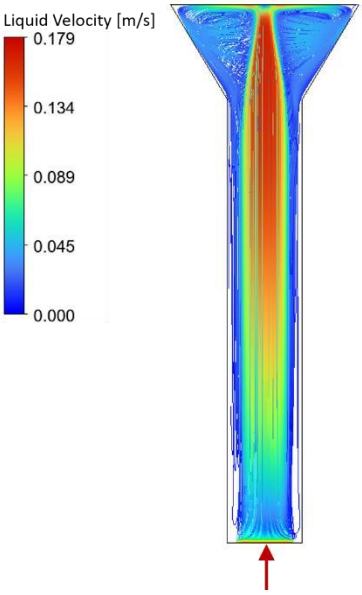


**Figure 2**: Steady-state multiphase model of the MBCR (RANS/Euler-Lagrange-approach), gas inlet (red arrow).

With the completion of the redesigning process, the MBCR now serves as a scale-down model, replicating the existing lab-scale bubble column in the microscale. In this application, the key parameter for downscaling is the volumetric mass transfer coefficient. Mass transfer is particularly crucial for gas-phase fermentations. In reactions where the enzyme is present in the liquid phase, the diffusion of poorly soluble substrates from the gas stream into the liquid becomes the limiting factor. The MBCR is used to simulate the conversion of butane to butanol and its performance is compared to that of the original 2 L bubble column reactor[3]. The advantages of MBRs become apparent as significantly smaller amounts of expensive enzymes are sufficient to perform experiments. Consequently, the MBCR expands the potential for series of experiments and enables the investigation of various process parameters. Overall, it opens up new horizons for experimental research into enzymatic multiphase reactions, thanks to the system's adaptability for use in diverse research applications.

*Keywords*: microbioreactor, bubble column, 3D-printing, biocatalysis, gas phase fermentation

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# Optimized Spatial Configuration of Heterogeneous Biocatalysts intensifies flow bioprocess system to render ω-Hydroxy and ω-Amino Acids

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The employment of flow systems in biocatalysis has undergone a significant development in recent years due to the well-known advantages in biochemical synthesis compared to batch reactions: scalability, process intensification and better specificity and sustainability. Even the final mix product can become a next-mix reaction in a modular telescope reaction system, having interesting industrial applications in biomanufacturing and active pharmaceutical ingredients (APIs) synthesis. However, there are several drawbacks yet to overtake such as low productivity and low enzyme stability.

In previous work, we intensified cell-free biotransformations reaching up to 100% conversion using 100 mM of initial substrate (1); and exploited a heterofunctional support to confine multienzymatic systems (2). Hence, we combined them to develop a stable multifunctional heterogeneous biocatalyst co-immobilizing five enzymes on microparticles to transform 1,ω-diols into 1,ω-hydroxy acids. We improved the operational efficiency and stability of the heterogeneous biocatalyst by fine-tuning enzyme loading and spatial organization. Stability issues are overcome through post-immoblization polymer coating. The general applicability of this heterogeneous biocatalyst is demonstrated by its scale-up in packed bed reactors, allowing a product yield > 80% (3). The continuous process is fed with $H_2O_2$ as oxygen source, reaching a Space-Time Yield (STY) of 0.76 $g \cdot L^{-1} \cdot h^{-1}$, maintained for the first 12 hours. Finally, this flow system is telescoped with a second plug-flow reactor packed with a different heterogeneous biocatalyst. As a result, this 6-enzyme 2reactor system sequentially transforms 1,ω-diols into 1,ω-aminoacids while in-situ recycling $NAD^+$, depleting $H_2O_2$ and generating $O_2$. (Figure 1).

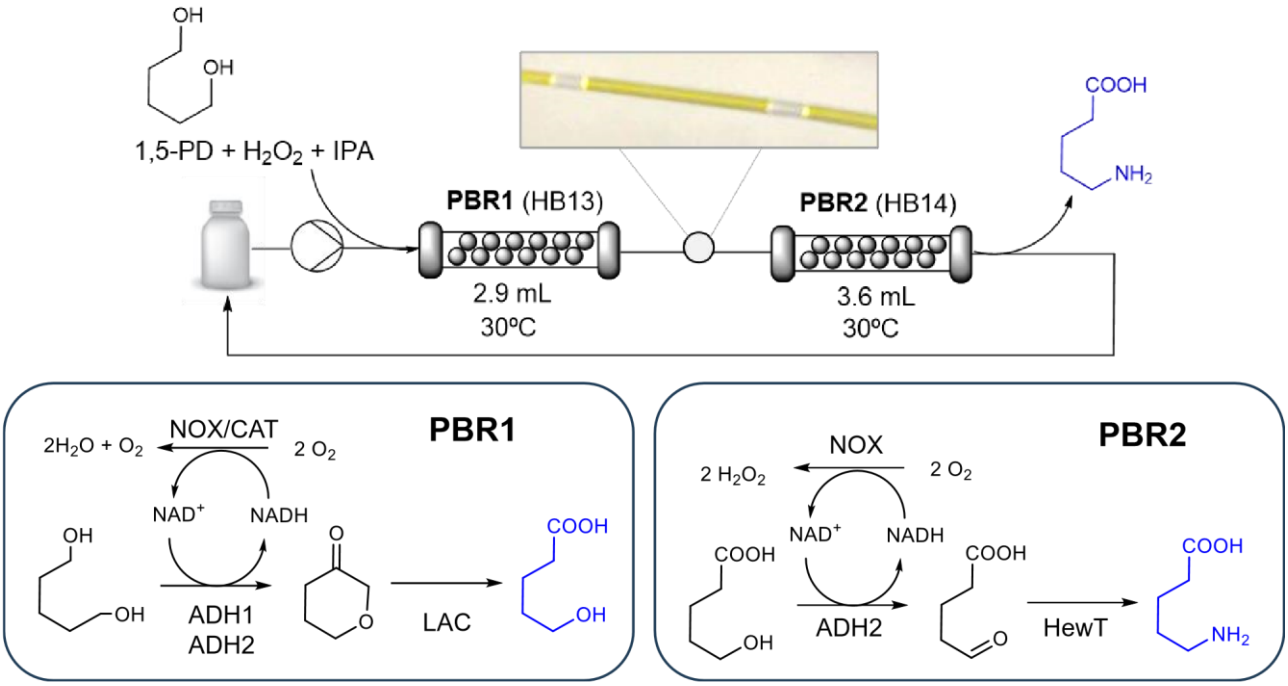


Figure 1: Continuous flow synthesis of 5-AP by telescoped packed-bed reactors.

Our work highlights the potential of strategically arranging immobilized enzymes to create more productive and complex heterogeneous biocatalytic systems, paving the way for the continuous biosynthesis of industrially relevant products. While the current system, with seven reactions catalyzed by six enzymes in two telescoped packed-bed reactors, may not be considered a complete cell-free metabolic pathway, we anticipate that further advancements in rational enzyme co-immobilization will contribute to expanding the capabilities of continuous chemical bio-manufacturing, enriching the bio-manufacturing portfolio.

*Keywords*: enzyme immobilizations, telescope flow systems, bioprocess intensification, continuous bioprocess, enzymatic microreactors.

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# Enzymatic Acetophenone Reduction in Deep Eutectic Solvent: Transitioning from Batch to Continuous System

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Due to their economic efficiency and overall greenness, deep eutectic solvents (DES) are considered potential replacements for conventional solvents in the enzyme-catalyzed synthesis of enantiomeric compounds.[1,2] Therefore, this research aimed to establish an environmentally friendly preparation of chiral pharmaceutical intermediate, (*S*)-1-phenylethanol, using rationally designed DES. Taking into account considerations such as substrate solubility, as well as enzyme stability and activity, betaine:ethylene glycol with 50% (w/w) water was identified as the optimal reaction media in the alcohol dehydrogenase (ADH)-catalyzed acetophenone reduction. Additionally, following the exciting results in nicotinamide adenine dinucleotide (NAD) stabilization in DES[3], the reaction system was designed to regenerate the expensive $NAD^+$ cofactor by adding the second substrate, isopropanol (Figure 1).

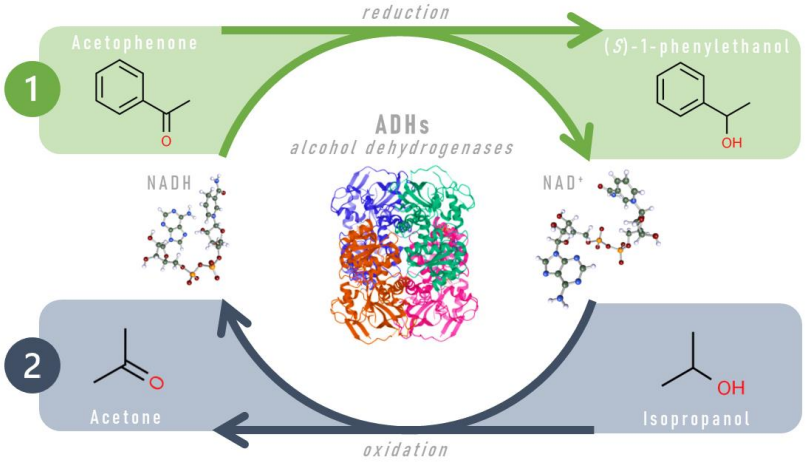


Figure 1: Biotransformation reaction scheme - alcohol dehydrogenase (ADH)-catalyzed reduction of acetophenone to a chiral pharmaceutical intermediate, (*S*)-1-phenylethanol, along with the regeneration of the $NAD^+$ cofactor.

After assessing the Michaelis-Menten reaction kinetics parameters and ensuring sufficient enzyme load in a batch process, the reaction was transferred to a continuous flow system. Continuous biocatalytic processes in miniaturized reactors facilitate process intensification, enhance process control, help the development of multi-step synthetic pathways with compartmentalized biocatalysts and seamless integration with downstream processes, thereby enabling efficient utilization of biocatalysts across various industries.[4] In this research, cross-linked enzyme aggregate (CLEA) particles were generated using a microfluidic system to enhance the stability of ADH in the flow system.[5] Further, a microbioreactor system between two plates, featuring CLEA-ADH immobilized on the membrane surface (Figure 2) was manufactured utilizing 3D printing. In the end, a validated 2D model-based design approach, incorporating time-scale analysis with characteristic times, was employed to identify the optimal process parameters and operational conditions.[6]

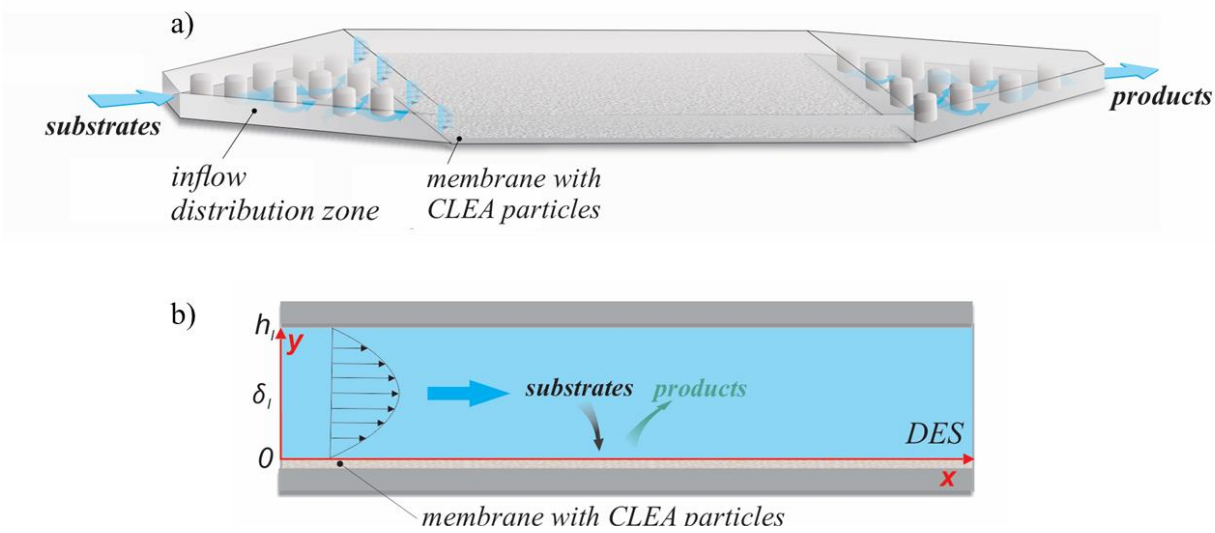


Figure 1: a) scheme of a microbioreactor between two plates with CLEA-ADH immobilized on the membrane surface; b) physical domains of a 2D mathematical model.

*Keywords*: deep eutectic solvent, enzymatic stereoselective reduction, microreactor, enzyme immobilization, crosslinked enzyme aggregate.

## Acknowledgement

This work was supported by the Croatian Science Foundation (HRZZ), grand number IP-2019-04-7712. MR would like to thank HRZZ for the MOBDOK-2023 scholarship. The authors would like to thank prof. Wolfgang Kroutil from the University of Graz for providing the enzyme.

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# Polymerization of apigenin catalysed by horseradish peroxidase in a microreactor

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Flavones, particularly the potent compound apigenin, have emerged as promising candidates for cancer metastasis prevention due to their ability to suppress critical signaling pathways[1,2]. However, their natural form often exhibits poor solubility and stability, limiting their therapeutic efficacy. In order to address these limitations, apigenin can be modified in biflavonoids and polyapigenin derivatives using enzymes such as laccase or horseradish peroxidase (HRP). HRP, a phytochemical enzyme, has already been utilized to catalyze the formation of polyphenols, polyanilines and polyquercetin[3]. These enzymatic reactions offer an efficient and environmentally friendly approach to diversifying the chemical repertoire of apigenin, yielding compounds with different biological activities and pharmacological profiles[4]. Harnessing the catalytic capabilities of HRP opens new possibilities for the scalable production of valuable biflavonoids and polyapigenin, paving the way for their exploration in drug development and biomedical research. Moreover, the integration of microreactor technology holds promise for intensifying the enzymatic transformation process. Microreactors provide precise control over reaction conditions, such as temperature, pressure, and residence time, thereby optimizing enzyme activity and product yield. By leveraging microreactor platforms, the enzymatic synthesis of biflavonoids and polyapigenin can be intensified, leading to higher throughput and efficiency.

In this research, enzyme HRP was used in order to synthase biflavonoids and polyapigenin to discover the novel bioactive compounds. The reaction was performed in biocompatible buffer/ethanol mixtures under mild conditions (0.1 M phosphate buffer pH 7 and 25 °C). In the first step, the reaction was performed in a batch reactor ($V$ = 50 mL) resulting in only 45% conversion of apigenin (Figure 1a) in 1 h. Fourier transform infrared spectroscopy (FTIR) was used to examine the specific chemical groups of obtained product, poly(apigenin) by examine the oscillating spectra in the wavenumber range from 4000 to 650 $cm^{-1}$. Obtained biflavonoids were identified and quantified by the HPLC-DAD analysis. Unfortunately, as it can be seen from Figure 1b, the HRP was completely deactivated ($k_d$ = 1.96 ± 0.36 $h^{-1}$) during the first hour of the process resulting in relatively small conversion.

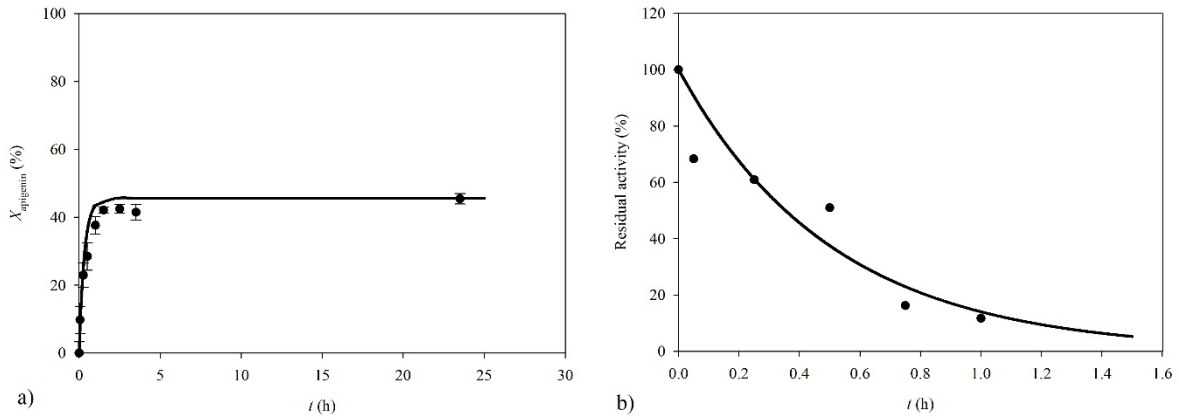


Figure 1: a) Dynamic change of apigenin conversion and b) HRP process stability for experiment performed in a batch reactor (● experimental results, ▬ mathematical model)

In order to better understand the process, kinetic measurements were performed according to the initial reaction rate method. Impact of apigenin concentration on the initial reaction rate of HRP was measured. The dependence of initial reaction rate on apigenin concentration was measured by varying the apigenin concentration (0 – 0.15 mg/ml). The Michaelis-Menten kinetic constants were estimated by non-linear regression using the simplex or least squares method implemented in Scientist software and determined to be $v_{max}$ = 332.76 ± 19.10 U/mg and $K_m$ = 0.04 ± 0.01 mg/ml. The mathematical model for batch reactor was proposed and the obtained results of apigenin polymerisation are presented in Figure 1a.

In the next step, PTFE microreactor (length: width: depth = 30 cm: 1000 mm: 1000 mm, internal volume 235.62 ml) was used to enhance the process.

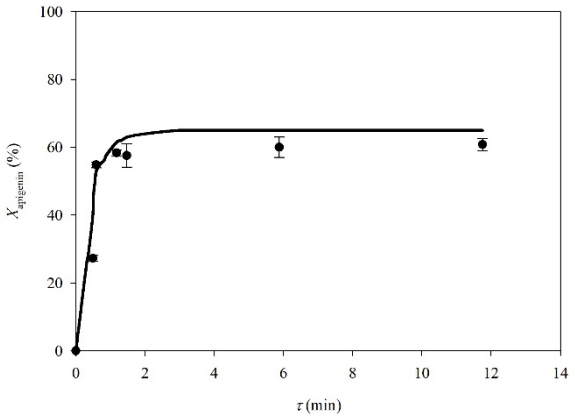


Figure 2: Influence of residence time on apigenin conversion for experiment performed in a microreactor (● experimental results, ▬ mathematical model)

As it can be observed from obtained results, higher conversions for shorter residence times were obtained in microreactor (Figure 2). A model of steady state plug flow reactor with negligible axial dispersion was used to predict apigenin conversion in a microreactor experiment. The same model will be used for further process optimization.

*Keywords*: apigenin, HRP, polymerization, microreactor, mathematical model

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# Session B: CELLS WITHIN MICROSTRUCTURED DEVICES

# Engineering microdevices to recapitulate complex diseases using induced pluripotent stem cells

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Under the influence of increasing environmental pollution and climate change, studies on chronic non-communicable diseases that develop as a result of exposure to different pollutants such as inorganic particles, microplastics have gained importance. Respiratory exposure to inorganic particles, especially particulate matter (PM), <PM2.5 and PM10, has been implicated to have roles in chronic respiratory diseases. Another common global pollutant is microplastics, which have been detected both indoor and outdoor environments[1] and even in human lung tissues.[2] With increase in plastic production and with the lack of global plastic disposal capability, a large amount of plastics is being introduced into the natural environment, which increases exposure to humans through various means. Furthermore, microplastics can decompose into nanoscale fragments and more easily penetrate biological barriers, thereby threatening human health. Among these biological barriers, airway epithelial barrier is the first line of defense against airborne pollutants[3]. Exposure to inorganic particles and microplastics[4] disrupts the integrity and function of the airway epithelial barrier (**Fig. 1**). Therefore, investigating the effect of the deteriorated airway epithelial barrier to understand the etiopathogenesis of respiratory and nervous system neurodegenerative diseases are of prime importance. However, since it is not ethically possible to evaluate the particulate matter that increase with the ever-increased global warming, climate change and natural destructive events, through human exposure in the clinic, reliable and robust preclinical models are needed. The current models span from Transwells to more complex and physiologically relevant organ-on-chip platforms[5], enabling culturing of human small airway epithelial cells and endothelial cells[6], as well as organotypic lung tissue cultures involving both tissue specific and resident immune cells.[7]

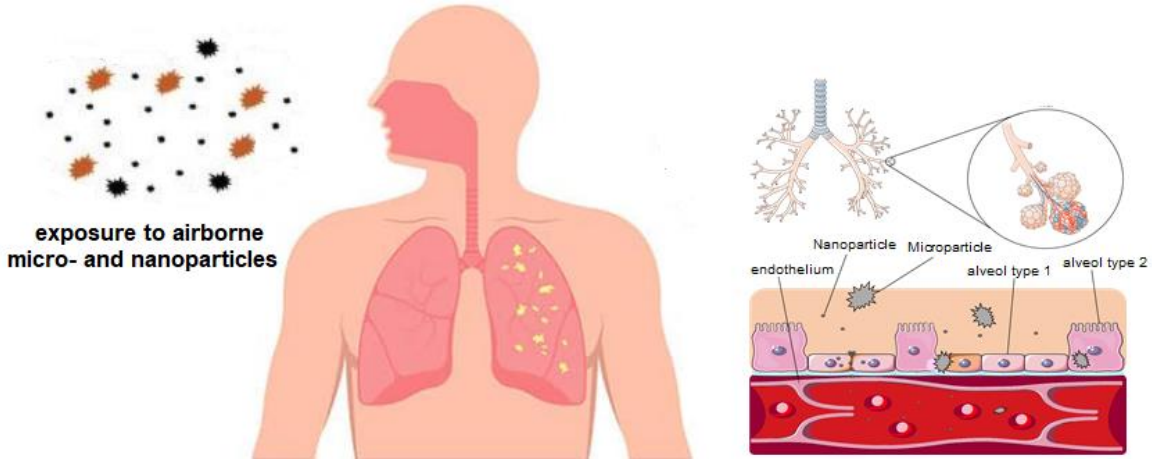


**Figure 1**: Transition of inhaled micro/nanoparticles through the alveolar epithelial barrier into the vascular system. While the majority of microparticles cannot pass through the epithelial barrier, the majority of nanoparticles can (the image has been created using smart.servier.com)

Recently, induced pluripotent stem cells (iPSC) are differentiated to specific cells for physiologically relevant recapitulation of the epithelial barrier and 3D organoids[8] representing the connective tissue. Indeed, one of the groundbreaking biotechnological developments in the world is induced pluripotent stem cell differentiation and 3D organoid culture studies. The differentiated iPSCs are seeded on membranes to emulate barriers in the human body such as lung and blood brain barriers.[9] Porous polyethylene terephthalate[10] and poly(dimethyl siloxane)[11] membranes are widely used allowing optical observation of the cells. However, there is a need for more biomimetic membranes to be integrated to the microfluidic-based organ-on-chip platform, where oxygen and nutrient delivery, natural flow conditions, shear stress and cell-cell/cell-environment interactions can be provided in a physiologically relevant manner. In these models, biomimicry of extracellular matrix (ECM) is imperative to form an appropriate microenvironment to the cells. Decellularization of organs[12] and formulation of the ECM with hydrogels ensure the presence of tissue specific proteins such as laminin, collagen, fibronectin, as well as glycosaminoglycan[13], along with physiologically relevant stiffness and viscoelasticity[14] of the tissue. These microphysiological models provide in-depth information on the pathophysiology of airway remodeling, as well as the genetic and epigenetic characteristics, which reflect the interaction between intrinsic and environmental signals.

In conclusion, airborne pollutants pose a significant future respiratory hazard for human airways under climate change conditions and environmental protection agencies and regulators are facing unprecedented challenges in assessing the potential toxicity of these particles. The organ-on-chip models are envisaged to serve as robust and reliable substitutes in this context. It is also worth to mention the roadmap for potential standardization issues relevant for organ-on-chip prepared by the CEN-CENELEC Focus Group on organ on chip[15], which will ensure the deployment of standards in organ-on-chip industry, enhancing the reproducibility of the data and accelerating the diffusion of the technology.

*Keywords*: lung-on-chip, epithelial barrier, induced pluripotent stem cell, organoid, airborne particles

**Acknowledgement**: The funding provided by TUBITAK through 123M406 project is highly appreciated.

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# Automation of a capillary-wave microbioreactor for conducting viability studies on mammalian cells

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Nowadays, different small-scale cultivation platforms, including microbioreactors (MBRs), are applied for various tasks in biopharmaceutical research. These include screening of bacterial strains or cell cultures, early-stage development of biopharmaceutical production processes, or cell-based assays like toxicity studies. Due to their small size, MBRs can easily be parallelized and automated, facilitating high-throughput experiments with minimal manual lab-work. However, many conventionally available systems contain comparably large cultivation volumes at the milliliter scale, limiting parallelizability and increasing costs for expensive media or testing substances. Furthermore, platforms like microtiter plates (MTPs) often exhibit an insufficient temperature distribution between the different wells [1]. Automation of these systems is often limited to expensive and space-consuming pipetting robots. MBRs at the lower microliter scale ($V < 10$ µL) often lack sensor equipment or flexible automation. To overcome these limitations, a parallelized, automated, and sensor-equipped capillary-wave MBR platform (cwMBR, $V = 7$ µL) with a consistent temperature distribution was developed for conducting viability studies on mammalian cells.

The cwMBR is characterized by a Foturan glass chip featuring a frustum shaped cavity in its center, wherein a sessile droplet of cultivation medium with a volume of 7 µL is contained [2]. To secure its position, the cwMBR is placed within a 3D-printed holder affixed to an exciter platform designed for mixing. The mixing process employs an innovative technique based on vertical oscillation, inducing capillary waves on the droplet's surface. This methodology results in short mixing times below 2 s and high $k_La$ values exceeding 340 $h^{-1}$ [3]. Optical sensors for pH, dissolved oxygen or glucose measurement can be integrated within the cwMBR [4]. For automated liquid addition, a nano dispenser was integrated in a tailor-designed parallelized cwMBR platform, mainly consisting of cost-effective 3D printed components (**Figure 1**). The nano dispenser is affixed to a spindle axle, which is rendered mobile through a stepper motor for accurate positioning. Non-invasive absorbance sensors, utilizing a blue LED and a spectrometer, facilitate the quantification of absorbance-derived parameters such as cell growth or cell viability, rendered accessible through colorimetric assays. The integration of a second spindle axle, connecting optical fibers from the cwMBR chips to the spectrometer, allows for a parallelized sensor read-out of multiple cwMBR chips using a single spectrometer. All devices are controlled through a programmable microcontroller. Real-time monitoring capabilities of the parallelized platform are enhanced through the implementation of an Application Programming Interface of both the spectrometer and the microcontroller. A constant temperature distribution, which is often not feasible in conventional MTPs, was achieved by integration of a heating foil.

To ensure the practical applicability of the aforementioned developed automated and parallelized cwMBR setup, it was applied for viability studies on mammalian cells. For this purpose, the colorimetric XTT assay was employed to quantify disparities in viability of the Chinese Hamster Ovary (CHO) cell line, induced by the cytotoxic metabolic intermediate ammonium chloride. Moreover, results were compared to those of the established MTP. The comparison highlights the advantages of the cwMBR platform compared to MTPs, including the high parallelizability and automatability, capacity for customizable environmental regulation, significant reduction in sample volume requirements, and ability to adequately handle sensitive organisms such as CHO. These attributes position the developed cwMBR platform as a highly efficient and cost-effective choice for not only viability studies, but a variety of biopharmaceutical applications such as phage sensitivity tests called phagograms.

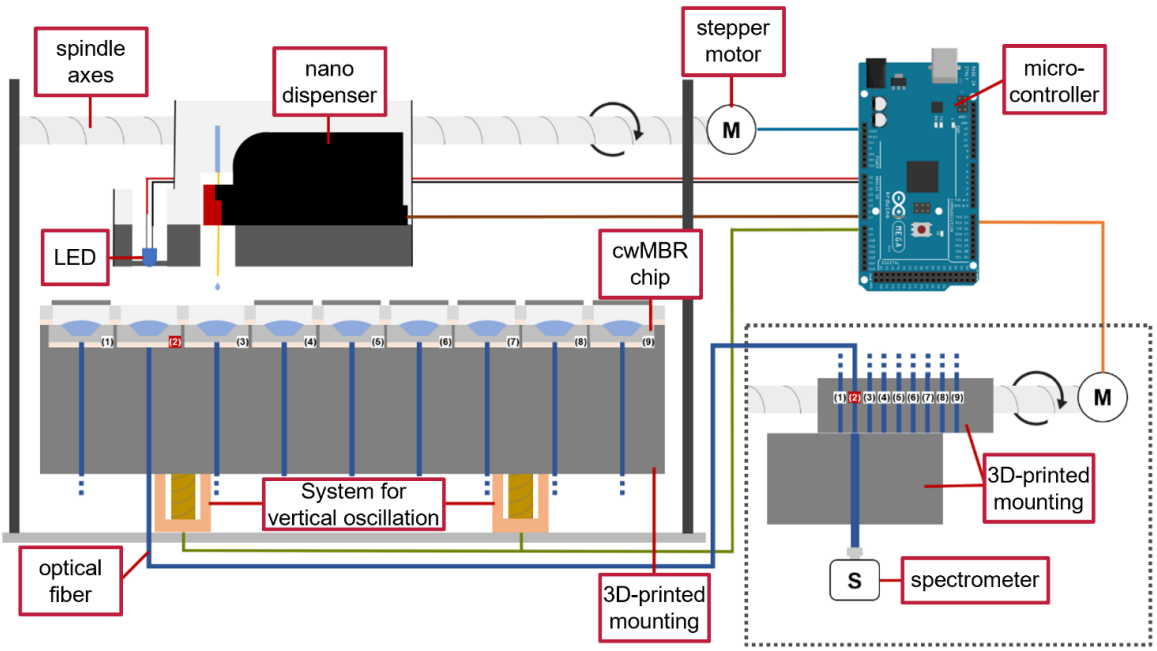


**Figure 1:** Automated cwMBR platform. A nano dispenser is connected to a spindle axes, which renders the nano dispenser mobile above the parallelized cwMBR chips positioned in a 3D-printed mounting. The cultivation medium in the cwMBR chips is mixed by vertical oscillation inducing capillary waves. Sensor-read out is carried out through a spectrometer (S) connected to a movable optical fiber setup ( ). All devices are controlled by a programmable Arduino® micro-controller.

*Keywords*: microbioreactor, automation, mammalian cell line, viability studies, biopharmaceutical applications

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# Probing bacteria-phage interactions at the single cell level using droplet microfluidics

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Bacteriophages (phages) are viruses that are natural killers of bacteria. They are important regulators of microbial populations, microbial diversity, and disease outcomes. Owing to their phenomenal diversity (~$10^7$ phages/ml in oceans), they constitute an untapped resource to fight microbes.[1] However, we are currently very limited in our ability to understand their interactions with bacteria, or fully exploit their genomic diversity. To discover the mechanisms behind host lysis by phages and possible resistance to phages, we need novel tools and methods to study phage-host interactions at high-throughput.

To this aim, we have established droplet microfluidic platforms able to probe these interactions at the singlecell level, enabling us to precisely dissect the dynamics of cell growth in response to exposure to phages. These microreactor formats offer precise control over volume and composition of the droplets and enable long term observation of encapsulated components.[2] We have harnessed the combined potential of object detection oriented deep learning and high-resolution microscopy to study the growth and lysis of individual bacterial cells within microfluidic droplets. Our Z-stack imaging methods can detect individual cell division and lysis events based on either fluorescent labels or cellular morphology (Figure 1).

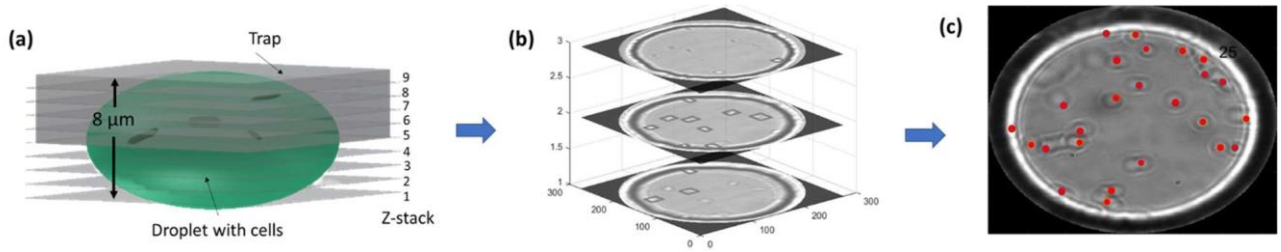


Figure 1: (a) Z-stack imaging of droplet containing cells. (b) YOLO detections for every Z slice. (c) Final position and count of cells represented by red dots.

We have validated the methods by studying the dynamics of lysis of E. coli cells in presence of DNA and RNA bacteriophages (Figure 2).[3, 4]

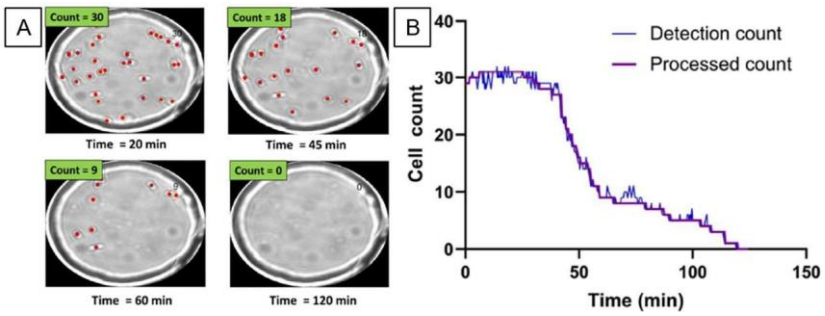


Figure 2: Lysis of E. coli cells in a picolitre droplet in response to T7 phage infection. A. Count of cells over time following exposure to T7. B. Post-processing of bacterial counts to infer accurate lysis times.

We have also extended this work to investigating polymicrobial cultures of the clinically relevant strains of P. aeruginosa and S. aureus. These bacterial strains often occur together in lung infections and might potentially have a synergy in combating the effects of antibiotics. We have evaluated the possible synergetic interactions in presence of phages that effect either strain. We have used them to study the differential lysis of parallel cocultures screened with droplet trapping arrays and infected by bacteriophages (Figure 3).

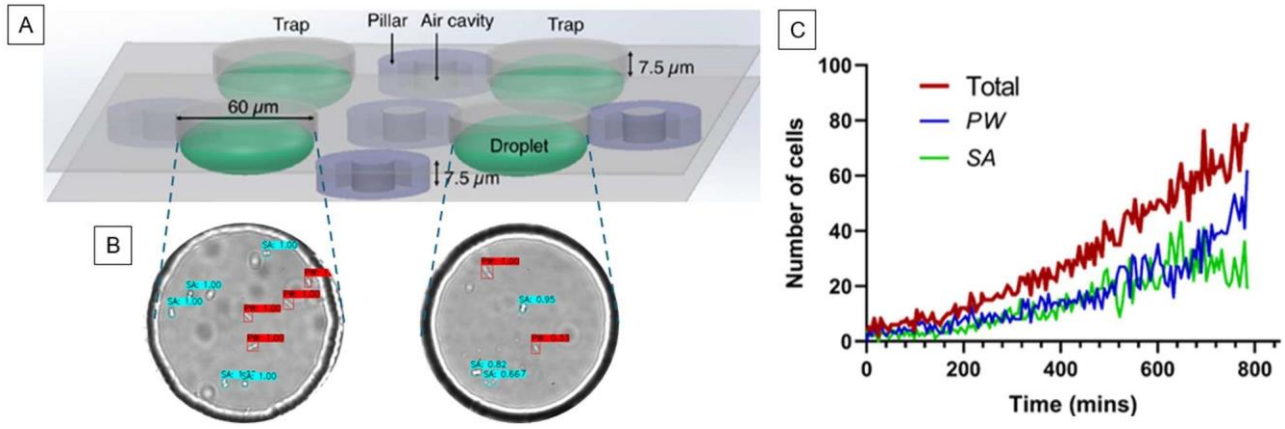


Figure 3: A. Encapsulation and detection of P. aeruginosa (PW) and S. aureus (SA) within anchored droplet arrays. B. Morphology-based detection of both cell types C. Quantification of cell growth for both cell types.

To date, much of our understanding of bacteria-phage interactions is based on interactions between single bacteria and phage, while nearly all microbes exist in complex, dynamic communities in which they influence each other's behaviors.[5] Our work provides high-throughput methods to answer whether phage infections are impeded or exacerbated by microbial diversity with deep implications for the future application of phage therapy. We will be able to form on-demand communities, possibly from clinical isolates and observe interactions with several antimicrobial compounds, with particular focus on antibiotics-phage combinations.[6]

Keywords: microfluidics, single-cell analysis, deep learning, bacteriolysis, phages

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# Microfluidic approaches for plant cell technology

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With the ongoing progression of climate change, agriculture requires innovative and contemporary methods to enhance cultivation efficiency and generate high-quality, safe food, feed, and fiber at reasonable prices. In recent years, plant tissue culture has become increasingly important in the production of fully developed plants.[1] Currently, the screening procedures needed for this process are time-consuming and limited in throughput. This limitation is due to the manual nature of many steps and the significant challenge of maintaining sterile conditions for long-term cultures in plant biotechnology. However, the lack of techniques to systematically optimize cultivation parameters in a high-throughput manner is holding back progress towards industrialization.

To overcome this bottleneck, we introduce a droplet-based microfluidic platform that enables the efficient use of space by employing automated procedures with computer-controlled pumping systems.[2] In our dropletbased microfluidic system, minute populations of protoplasts and microspores are enclosed in nanoliter droplets. This configuration allows the generation of numerous replicates for each condition, ensuring statistical significance. The method facilitates systematic variation of nutrient composition or regulator concentrations for screening programs. Additionally, it also allows the tracking of changes within the culture at a nearly single-cell level.

The analysis is primarily based on imaging method. Through the integration of image and AI-based analysis techniques, different developmental stages of plant cells can be thoroughly examined. Furthermore, an optical micro flow-through photofluorometer has been invented that incorporates live staining.

As a result, in the droplet-based microfluidic system, it is possible to quantify the dose-response function of a chemical regulator on the vitality of plant cells cultured in nanoliter segments over several weeks. Additionally, successful microspore growth can be achieved within nanoliter droplets, leading to the induction of microspore-derived embryo formation which can subsequently be regenerated into whole, fertile plants.

Keywords: droplet-based microfluidics, tobacco protoplast, microspore embryogenesis, dose-response screening, artificial intelligence cell detection

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# Microfluidic cultivation and mechanical testing of fungal hyphae enabled by two photon polymerization

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Filamentous microorganisms are of significant interest in the field of biotechnology due to their role as prolific producers of various compounds, including organic acids, pharmaceuticals, and enzymes.[1] Their morphology in submerged culture profoundly affects productivity[2–5], spanning from dispersed mycelium to dense agglomerates, or pellets.[6,7] Optimal morphology varies by strain and desired product[2,8,9], highlighting the importance of understanding factors influencing growth morphology for tailored process optimization. One major influence on the developed macro-morphology of filamentous microorganisms are the hydrodynamic conditions or the hydrodynamic stress within the reactor volume.[10,11] Agitation leads to relative velocity between mycelial compounds (e.g. pellets) and liquid media, resulting in shear and normal forces acting on the hyphae. An accurate knowledge of their mechanical properties that define the response to these stresses is key to modelling the mechanical behavior of more complex hyphal networks. Related works aim to simulate the mechanical response of fully resolved pellet structures constituted of many branched hyphae. This promises to shed more light on the morphological differentiation processes at given cultivation conditions. As a main input variable for such models, we aim to characterize the mechanical properties of whole hyphae of a representative *Aspergillus niger* strain, which is a filamentous fungus highly relevant and widely used in white biotechnology.[12]

A promising alternative to the regularly applied but very local probing of the elasticity of the hyphal cell wall by atomic force microscopy (AFM)[13–16] is a microfluidic bending test of the whole cell.[17–20] This methodology uses tailored microfluidic devices that serve as micro-bioreactors for cultivation and later testing of single filamentous cells. The system design used in our work is shown in Figure 1a. After spore immobilization and

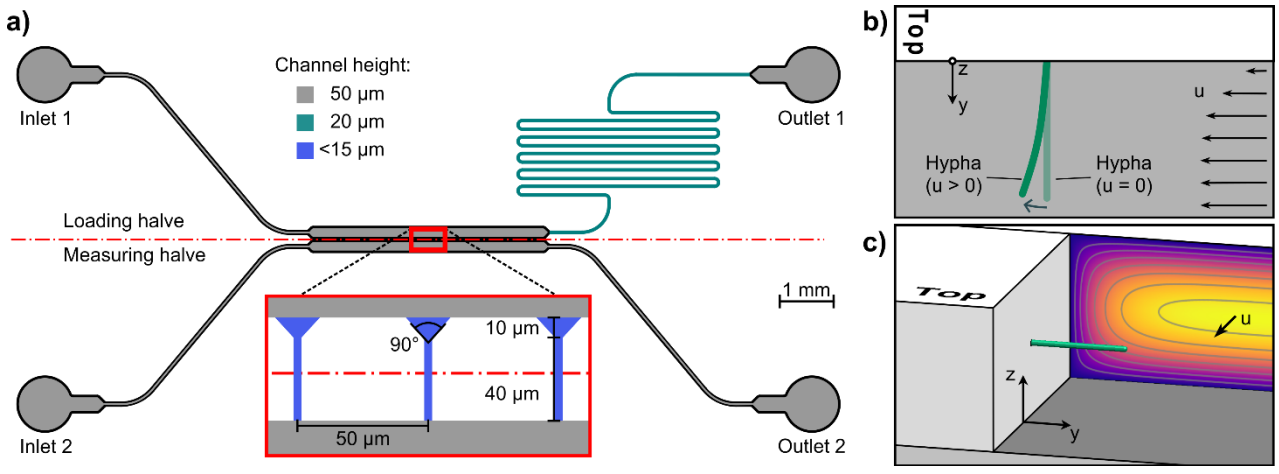


Figure 1: a) Schematic view of microfluidic system design with zoomed in view of the growth channels. Schematic top view (b) and perspective view (c) of the measurement configuration in the measurement chamber (system cover plate not shown). In b) the light and dark green shapes resembles the initial and deflected hyphal shape. The flow velocity is indicated as $u$.

deflection. So far this methodology has been applied to germ tubes[17], bacterial cells[18,19] and recently fungal hyphae of *Candida albicans*.[20] In the latter case the size difference between asexual spore and hyphae germinating from it can be exploited to trap spores at the entrance of narrow growth channels. For the application of this methodology to *A. niger* hyphae we utilized Two-Photon Polymerization (2PP) to incorporate cell traps and growth channels sized for the exact specifications of *A. niger*. As a laser based 3D-printing process for the maskless fabrication of arbitrary microstructures, 2PP enables sub-micron spatial resolution. To take full benefit of this resolution we employed a multi-material approach combined with an advanced method for replication molding of the 2PP structures developed previously.[21] The systems were fabricated out of poly-dimethylsiloxane (PDMS) mold replicas and glass. By altering the conventional system design to change the measurement configuration we could significantly increase the number of successful bending tests per cultivation experiment. At the same time the newly developed design is less prone to measurement errors due to deviations of the calculated fluid-induced forces. Micro-Particle Image Velocimetry (µPIV) measurements of the measurement flow revealed good agreement with the theoretical flow profile. Representative results of a bending test are shown in Figure 2a and 2b while all measured values for the conventional (Floor) and our proposed new (Center) system design are shown in Figure 2c. The measured average bending stiffness for *A. niger* hyphae is about four times the value previously reported for *C. albicans*.[20] At the same time the derived longitudinal Young's Modulus of both organisms' hyphal cell wall derived from their bending stiffness is comparable. Also, the local indentation modulus of the hyphal cell wall of different *A. niger* strains measured by AFM is in the same order of magnitude.[15,16] The presented results demonstrate that the proposed systems are a powerful tool for studying hyphal biomechanics in a very controlled environment. As such it promises to contribute to a better understanding of underlying mechanisms in submerged biotechnological cultivation.

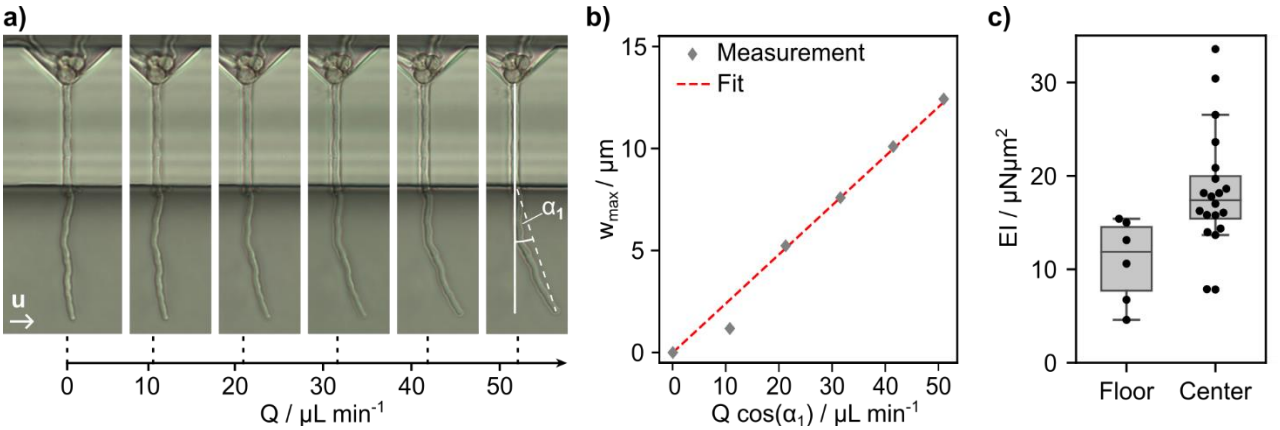


Figure 1: Results of bending tests in both configurations: conventional floor and new center configuration. a) Frames recorded during the bending experiment at increasing flow rates from left to right. b) Plot of tip deflections extracted from the frames in a) used to derive the bending stiffness from a linear fit (red dashed line). The scaled volume flow rate on the x-axis is proportional to the fluid-induced forces. c) Box plot of the measured bending stiffness of all analyzed hyphae in floor and center configuration ($n_{floor} = 6$ from two cultivations, $n_{center} = 20$ from one cultivation).

*Keywords*: *Aspergillus niger*, Two-Photon Polymerization, single-cell mechanics, fluid-induced forces, hyphal bending stiffness

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# Characterization of different biocatalyst formats for BVMO-catalyzed cyclohexanone oxidation

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Cyclohexanone monooxygenase (CHMO), a member of the Baeyer–Villiger monooxygenase family, is a versatile biocatalyst that efficiently catalyzes the conversion of cyclic ketones to lactones. We expressed an *Acidovorax*-derived CHMO gene in *Pseudomonas taiwanensis* VLB120. Upon purification, the enzyme was characterized in vitro and shown to feature a broad substrate spectrum (Figure 1).[1] This enzyme also was used as integral part of multiple in vivo cascades for the production of diverse polymer precursors.[2-5]

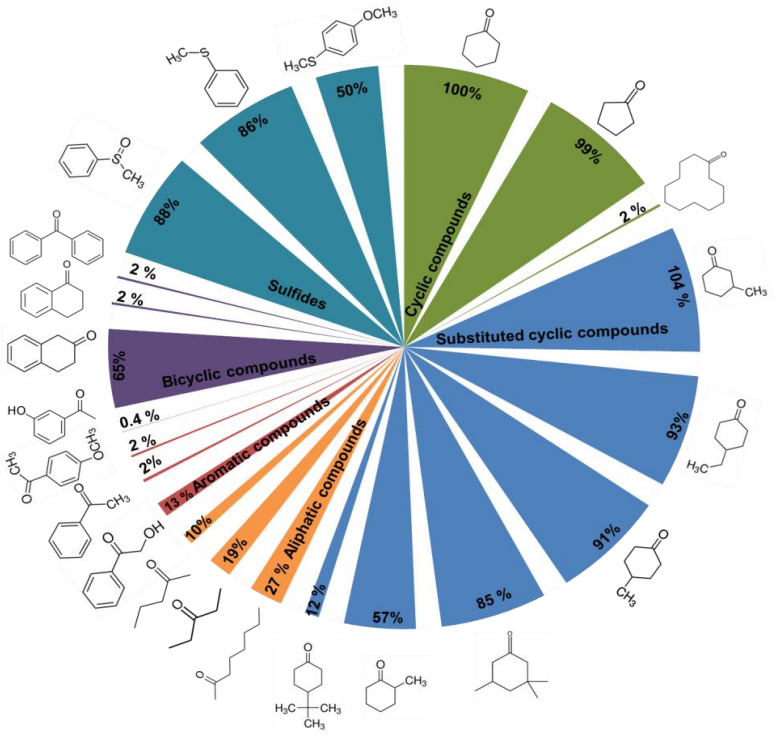


Figure 1: Substrate spectrum of CHMO. Substrates from different compound classes were subjected to activity assays with isolated *Acidovorax* CHMO. Specific activities were determined photometrically and normalized to the activity for cyclohexanone (1.06 ± 0.08 $Umg^{-1}$).

The application of BVMOs in in vivo and/or immobilized formats constitutes a promising strategy to improve biocatalyst stability and total turnover number. However, a change in biocatalyst configuration can affect reaction kinetics and, consequently, reaction performance. Typically, in vitro kinetics are characterized under conditions that do not resemble in vivo environments, and thus reaction kinetics often differ among in vitro and in vivo formats. Conversely, other studies found that in vivo catalytic rates generally concur with in vitro measurements. Such contradictory results also have been reported for the comparison of kinetics for suspended and immobilized microbial cells. These findings indicate that the determination and understanding of differences in kinetics is of significant interest for modeling biological systems and selecting the most promising biocatalyst format for technical applications.

In the present work, we aimed to understand if, to what extent, and why CHMO-reaction kinetics concur or differ among isolated enzyme-, suspended cell-, and biofilm/mircroreactor-based formats. For this purpose, CHMO and respective kinetics also was tested in suspended and biofilm-based whole-cell biocatalyst formats using *P. taiwanensis* VLB120 as host strain, a solvent-tolerant strain and good biofilm former.

Biofilms showed less favorable values for $K_S$ (9.3-fold higher) and $k_{cat}$ (4.8-fold lower) compared with corresponding $K_M$ and $k_{cat}$ values of isolated CHMO, but a favorable $K_I$ for cyclohexanone (5.3-fold higher). The unfavorable $K_S$ and $k_{cat}$ values are related to mass transfer- and possibly heterogeneity issues and deserve further investigation and engineering, to exploit the high potential of biofilms regarding process stability. Suspended cells showed only 1.8-fold higher $K_S$, but 1.3- and 4.2-fold higher $k_{cat}$ and $K_I$ values than isolated CHMO. This together with the efficient NADPH regeneration via glucose metabolism makes this format highly promising from a kinetics perspective.

*Keywords*: Baeyer–Villiger monooxygenase, biofilm kinetics, capillary biofilm reactors, enzyme kinetics, whole-cell kinetics

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# Optimization of continuous L-malic acid production in a microbioreactor through mathematical modeling

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In the realm of process intensification, miniaturized flow reactors with immobilized biocatalysts are gaining atraction for their ability to ensure prolonged biocatalyst activity, surpassing batch processes in continuous operation, and providing precise control over reaction conditions through efficient mass and heat transfer.[1] Despite their increasing application in biocatalytic processes, the absence of mathematical models for optimizing reactor design and operation remains a significant gap. This study aims to bridge this void by devising and validating a mathematical model for the continuous biotransformation process within a microreactor, sandwiched between two plates with whole cells immobilized in hydrogel layers at the reactor's base and top.

Leveraging the hydration of fumaric acid to L-malic acid by permeabilized *Saccharomyces cerevisiae* cells as a model reaction, we conducted preliminary batch experiments to estimate substrate and product diffusivities in both liquid and copolymeric hydrogel layers, alongside reaction kinetics employing Michaelis-Menten kinetics for the reversible enzymatic reaction. The subsequent validation of our model with continuous operation in a microbioreactor, varying fumaric acid concentrations and flow rates, exhibited excellent alignment with model predictions encompassing transport phenomena and reaction kinetics.
Through the integration of Time-scale analysis featuring characteristic times, we determined the optimal process and operating conditions for our developed microbioreactor system. Our model forecasts an equilibrium conversion of fumaric acid at the highest inlet concentration tested, with a liquid height of 200 µm and hydrogel thickness on both sides of the channel at 400 µm, maintaining a residence time of 30 min.[3]

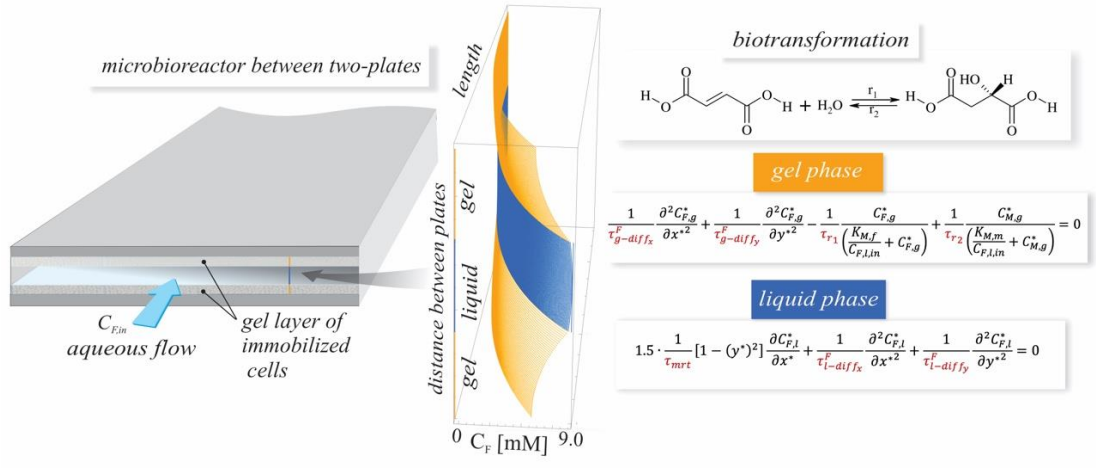


Figure 1: Presentation of L-malic acid production in microbioreactor between two layers of immobilized yeast cells.[3]

*Keywords*: Microreactor, Mathematical model, Immobilization, Flow biocatalysis; Time-scale analysis

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# Session C: ANALYSIS WITHIN MICROSTRUCTURED DEVICES

**CK**

# HD-SACA System for Single CTCs/CTM Rapid Diagnosis/Prognosis and Tumor-organelle-on-a-Chip Drug Screening for AI-Precision Medicine

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Despite the recent advancement of biotechnology and pharmaceutical research, cancers remain the leading cause of human mortality. It is vital to diagnose cancers at an early stage when treatment can dramatically improve prognosis. So far, low-cost and easy to operate devices, which allow efficient isolation and sensitive detection of circulating tumor cells (CTCs) for routine blood screening, remain lacking. This talk will introduce a micro fluidic platform (High-Density Self-Assembled-Cell-Array, HD-SACA system) which can not only isolate single CTCs/CTMs from the real blood sample in one hour for rapid CTCs identification and diagnosis, together with a nano particle sensing system, but also conduct Tumor organelle drug screening for precision medicine. This system includes a high throughput 3D Micro-Dialysis and Self Assembled Cell Array (SACA) chip to quickly self-assemble cells into a dense monolayer ($10^6$ cells/cm$^2$) for rapid staining and in-parallel inspection at high speed ($6x10^6$ cells/1 hour for 4 images) [1]. The distinguished ratio can reach 1 to 1 billion, recovery rate more than 95%, and whole process can be finished in two hours for 4 ml blood sample [2]. In this research, we have successfully enumerated CTCs/CTMs by self-assembled cell array (SACA) chip system for more than 1000 patients with diagnosed colorectal cancers and others. We found that the CTC count in PB but not IMV correlates with disease stages. Neoadjuvant chemotherapy did not lead to decreased CTC count in both types of blood samples [3]. With the combination of CTCs/CTMs and other biomarkers, the detection odds ratio can be higher than 21[4]. Due to the rapid and gentle process, the on-chip isolated CTCs are still in vital and can be further characterized and cultivated for the identification by using nano-particle sensors for further prognosis by reading the information from cancer specific metabolism ingredients [5]. The cultivated/expanded CTCs on chip, when forming Tumor-organelles, can be further used as drug testing targets to screening combinatory drugs for AI assisted precision medicine [6].

*Keywords*: Circulating Tumor Cells, Microfluidic Chips, Self-Assembled Cell Array, Liquid Biopsy, Cancer Diagnosis and Therapy, Precision Medicine, Artificial Intelligence

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CO1

# Glucose and lactate optical biosensors for microfluidic cell culture monitoring

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Organ-on-Chip systems are microfluidic platforms that allow the investigation of human physiology in laboratory conditions and serve as an ethical alternative to animal testing. Monitoring certain parameters of cell cultures is crucial to obtain information about the state of the tissue grown inside the chip and enables to detect early signs of cell damage. Two of those parameters are glucose and lactate concentrations in the effluent cell culture medium.

Most of the available glucose and lactate biosensors are based on enzymatic conversions by the respective oxidase, shown in equations (1) and (2). The analytes can be quantified indirectly from measuring the concentration of produced hydrogen peroxide or consumed oxygen. Despite great accuracy and reversibility that can be achieved with that concept, integration of the sensors into miniature platforms still remains a challenge. Moreover, the majority of presented sensors is based on electrochemical detection, which often calls for complicated sensor fabrication procedures.[1]

$$glucose + O_2 \xrightarrow{glucose\ oxidase} gluconolactone + H_2O_2 \quad (1)$$

$$lactate + O_2 \xrightarrow{lactate\ oxidase} pyruvate + H_2O_2 \quad (2)$$

In 2023, we presented an optical glucose biosensor that can be easily integrated into microfluidic systems.[2] Since then, further characterization of the sensor was conducted and the developed concept was adapted to lactate sensing. The sensor consists of an oxygen sensitive indicator dye and glucose or lactate oxidase, immobilized in a hydrogel matrix. The presence of the analyte causes oxygen depletion on the sensor's surface, which results in a detectable change in the indicator's phosphorescence lifetime. Additionally, a catalyst for the degradation of hydrogen peroxide is added to improve sensor stability. Sensor integration is achieved by spotting the sensor formulations with an automated microdispenser. With this approach, miniaturized sensor spots can be integrated directly into the microfluidic cell and their size can be altered according to the width of the channel (Figure 1).

Influence of various parameters on the sensor's performance was investigated. It was established that the sensitivity and dynamic range of the sensor can be altered by incorporating different diffusion membranes and by modifying the spot size. Eventually, long-term sensor stability was examined and the developed system was tested with effluent cell culture media. The sensors enable detection of glucose and lactate in the concentration range relevant for Organ-on-Chip systems.

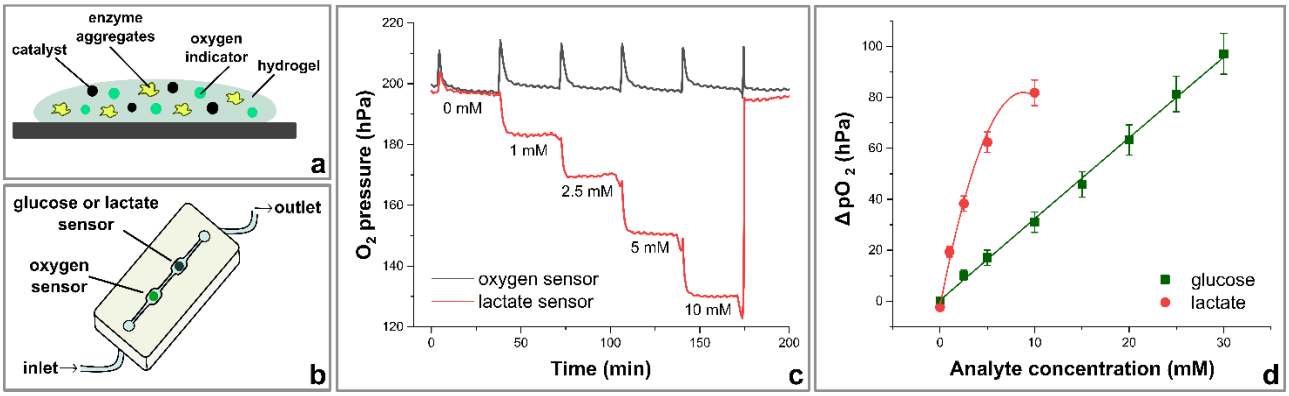


Figure 1: a) composition of the sensor spots; b) placement of the spots in a microfluidic cell used for the sensors' characterization; c) response of the lactate sensor exposed to the analyte in 0-10 mM concentration range; d) response curves for glucose and lactate sensors with diffusion membrane of 0.5% porosity.

*Keywords*: glucose, lactate, cell culture, biosensors, optical sensing

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# Development of an automated platform for the optimization of microfluidic reactors through multi-reactor integration and online (chip-)LC/MS-detection[1]

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The efficient utilization of biocatalysts, such as enzymes, is crucial for realizing the goal of integrating eco-friendly chemistry into industrial processes.[1–3] The use of purified enzymes can facilitate highly selective reactions under milder and greener conditions.[4] Additionally, immobilization of these enzymes, can simplify the enzyme's removal from the reaction bulk, and allow for incorporation into continuous-mode, flow reactors. However, even though immobilization can increase the cost-effectiveness of the process, the overall costs for the preparation of the enzyme are still significant. Moreover, due to their biological nature, enzymes typically cannot function under harsh conditions and require optimization before implementation. Therefore, an optimization process requiring minimal amounts of enzyme is desired. Miniaturization of these reactors, in the form of microfluidic immmobilized enzyme reactors (MIERs) can provide a possible solution to this issue.

We present a novel microfluidic optimization setup with real-time analytics for investigating enzyme catalyzed reactions utilizing multiple MIERs. The setup combines microreactor technology, multi-reactor integration, and online (chip-)LC/MS analysis in a sequential automated workflow. The potential of this setup is demonstrated by optimizing a biocatalytic aromatic bromination using vanadium-dependent haloperoxidase from *Curvularia inaequalis* (*Ci*VHPO), as the first proof of concept. The CiVHPO was immobilized onto chloroalkane-functionalized silica particles using the HaloTag™, a commercially available peptide tag. These silica particles were packed into multiple, in-house manufactured fused-silica glass chips, employed as reusable packed-bed microreactors. Via the integration of two multi-selector valves and further developing the automation principle, multiple microreactors were connected in a parallel configuration allowing for flexible microreactor selection. The biotransformation was then continuously monitored with an automated LC/MS data acquisition.[1]

Figure 1: Overview of the Instrumental setup with LC/MS-detection. A) Photograph of the complete instrumental setup. B) Schematic sketch of the respective valving, and setup structure, including a simplified flow chart for visualization.[1]

This novel setup allows generation of high qualitative data sets on the performance of the immobilized enzyme at different reaction conditions in continuous flow. The generated knowledge has the potential to be further used to upscale or numbering up the continuous process. Finally, a faster, modular chipHPLC solution was also tested as an alternative for the conventional LC to reduce the overall solvent consumption by over 80%. Thus, providing a platform for optimizing biocatalytic reaction conditions using minimal amounts of both enzymes and reactant.[1]

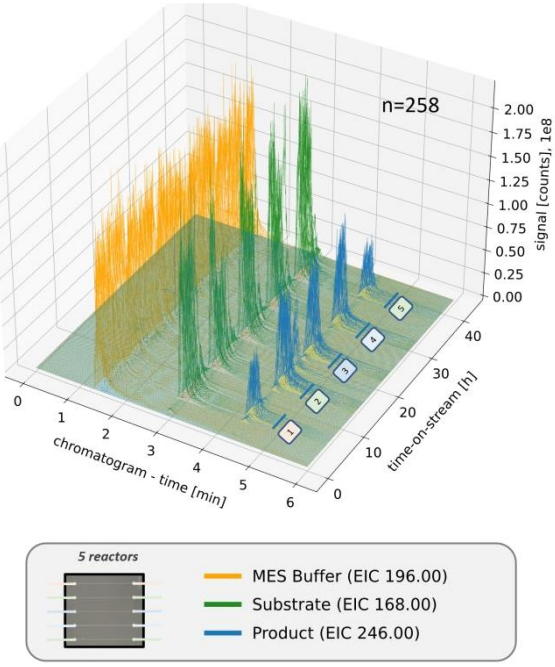


Figure 2: A waterfall diagram of acquired chromatograms of buffer, substrate and product of a model biocatalytic bromination of a run consisting of 5 separate microreactors showcasing the extensive data generation capabilities of this setup.[1]

*Keywords*: optimization, LC/MS, biocatalysis, MIER, chipHPLC/MS

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# Towards rapid, high-throughput and cost-effective evaluation of viral vector efficacy

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Chimeric Antigen Receptor (CAR)-T cells represent an emerging therapeutic modality for complex diseases such as cancer. Success of such approaches relies on the *ex vivo* engineering of patient T cells to stably express a therapeutic transgene. Within this context, the use of viral vectors such as lentivirus is one of the most preferred and efficient methods employed for modifying human cells with the aim of constitutively expressing a protein of interest. Consequently, the infectivity or functional titer characterizing the lentiviral material represents an essential Critical Quality Attribute (CQAs) in the clinical development of CAR-T cells. This is typically identified through the titer assay which measures the proportion of cells transduced by a lentiviral vector material upon interaction under defined conditions *in vitro*[1].

In this context, viral vector transduction is governed in part by diffusion, implying a relationship between the diffusion path length and the efficiency in transducing the target cells. Therefore, this parameter is dictated by the cell type and physical conditions used for the evaluation of functional titer, this assay being typically performed with adherent cell lines such as HEK293T cells. More recently, alternative cell lines such as immortalized T cells (e.g. Jurkat) have been evaluated as a more representative alternative for the downstream application of *ex vivo* engineering. The assay has been explored using either adherent or suspension cell lines, coupled with a microfluidic counterpart to the traditional multi-well plate format, with the potential of achieving a more controlled and relevant interaction between the viral particles and target cells. Consequently, performing testing under such conditions could bring advantages in terms of development for the functional titer assay, its assay intermediate precision and most importantly its ability to predict vector functionality on target cells. Ultimately, the integration of image processing algorithms could streamline the current workflow by eliminating the need for flow cytometry as an additional readout step.

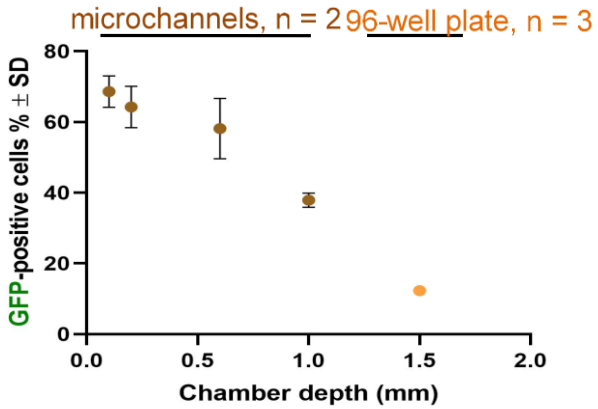


Figure 1: Flow cytometry results outlining the proportion of GFP positive cells as a function of chamber depth characterizing the microchannels used for HEK293T cells transduction. The ratio between viral particles and target cells, a proxy for multiplicity of infection, is maintained constant across the experiment.

We investigated the effect of diffusion distance between the viral particles and target cells by implementing microfluidic channels, with depths between 100 µm and 1000 µm. This parameter becomes particularly relevant for the transduction of adherent cell lines. Lentiviral particles with Green Fluorescent Protein (GFP) as transgene were used in all experiments. An inverse correlation was displayed between the microchannel depth, and transduction efficiency as evaluated by transgene expression. An approximately 2-fold increase in the proportion of GFP-expressing cells is achieved when the transduction is performed in a microchannel of 1000 µm relative to a 96-well plate, with a liquid height of 1500 µm minimum. A further 50 % improvement in the transduction efficiency is obtained by reducing the channel depth from 1000 µm to 100 µm while the Multiplicity of Infection (MOI) remains constant (Figure 1.). This result agrees with previous publications investigating this hypothesis[2,3,4]. The implementation of microfluidic technology therefore brings several advantages such as increased sensitivity, outlining microchannel depth as crucial parameter for transduction efficiency.

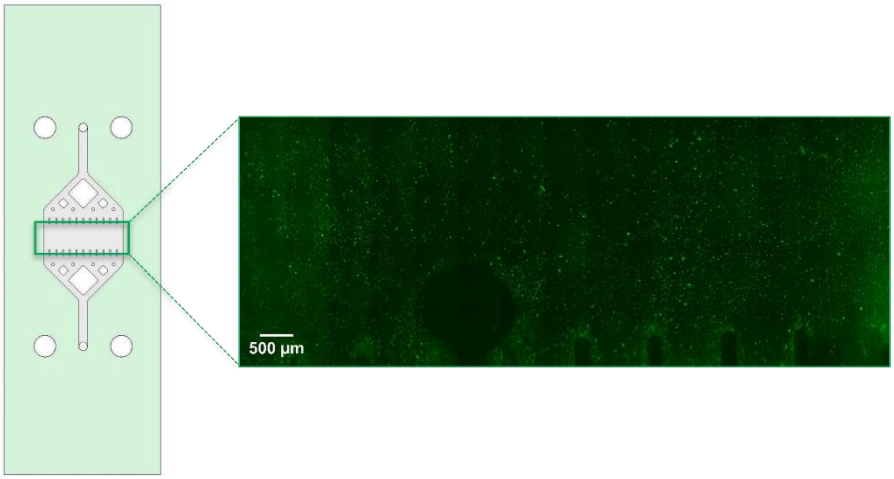


Figure 1: Microfluidic device workflow outlining a homogeneous GFP expression throughout the chamber upon lentiviral transduction of a suspension cell line.

In contrast, for the use of suspension cell lines, different considerations were investigated. One of the challenges in this instance is the identification of an optimal flow rate, allowing for successful perfusion operation while ensuring the cell population is retained and homogenously distributed within the microfluidic device. For this we can use our previously presented microfluidic cell culture device[5] and analyze cells for GFP expression via fluorescence microscopy (Figure 2.). Future steps in developing the microfluidic chip design may include a microfluidic mixer that can be implemented to promote the interaction between cells and viral particles while both being introduced simultaneously on chip.

*Keywords*: microfluidics, lentivirus, functional titer, diffusion, analytical development

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# A Novel Therapeutic Method for Eliminating Amyloid-β in Alzheimer's Disease: Utilizing the iCore Blood Processing Platform

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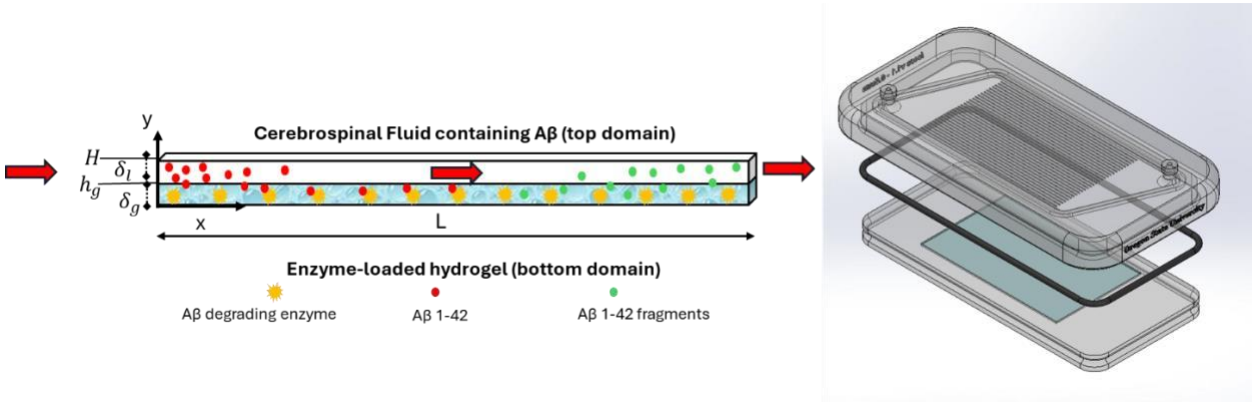
Alzheimer's disease (AD), a progressive neurological disorder that worsens gradually over time, is the most common form of dementia worldwide[1]. The predominant theory for the cause of AD is the formation of neuritic plaques and neurofibrillary tangles (NFTs) in the brain[2]. Neuritic plaques comprise amyloid beta (Aβ) peptides, which play a crucial role in AD development. Accumulation of Aβ peptide in the brain is due to unregulated production, insufficient clearance, and defective proteolytic degradation. Amyloid-beta structures exist in three forms: monomeric, oligomeric, and fibrillar. In contrast to oligomers, which are soluble and able to spread throughout the brain, fibrils are insoluble, large, and form plaques. The accumulation of 42-residue-long Aβ peptides has been proposed to be a major factor in the onset of AD. Two approaches are known to control Aβ levels in the human body: controlling the production and controlling the clearance of Aβ. An imbalance in these processes may lead to Alzheimer's disease.

The long-term goal of this work is to provide a technological solution for the extracorporeal capture & elimination of Aβ, thus reducing the biochemical potential for the formation of fibrils and plucks while eliminating patient exposure to drugs and degradation products.

This research focuses on finding ways to eliminate Aβ molecules using two different concepts. The first involves capturing Aβ 1-42 through polymerization in a hydrogel layer seeded with fibrils. The second approach is the degradation of Aβ 1-40 and Aβ 1-42 by using Amyloid degrading enzymes (ADEs) also entrapped in hydrogels. ADEs are proteases responsible for digesting Aβ peptides into less toxic species[3]. Both approaches use an ***i***Core device to host a *seeded hydrogel film* to eliminate the Aβ molecules.

***i***Core is an extracorporeal blood processing platform that exploits microfluidics' fundamental advantages, such as a high surface-to-volume ratio and intensified mass transfer. The device is comprised of two primary components: an array of microchannels transporting blood sealed against a thin functionalized hydrogel film. A schematic representation of an ***i***Core device and mechanism of Aβ degradation process is shown below:




Schematic representation of ***i***Core (courtesy of Andrea S. Garcia-Ortiz, OSU Honor College Thesis, 2022)

A successful proof of concept experiments was performed, in which Aβ is captured/polymerized in the *hydrogel film seeded* with fibrils. The experimental results show a decisively effective polymerization of Aβ,

resulting in the reduction of Aβ in the solution by ~90% over the five days. The polymerization reaction was supported by a phosphate buffer solution or HFIP (hexafluoro-isopropyl-alcohol). Moreover, the control experiment showed an auspicious elimination level of Aβ with a nonfunctionalized hydrogel (the mechanisms involved in the elimination of Aβ are yet to be determined.)

The second elimination mechanism of Aβ is based on ADEs enzymes that break down Aβ peptides into smaller, less-toxic species through their protease activity. Numerous studies include investigations of effects of insulindegrading enzymes to lower the levels of amyloid protein[4], investigation of Aβ degradation using Neprilysin and Insulysin[5], investigation of the mechanisms of the enzymatic degradation of Aβ peptides[3], investigation of microbial enzyme degradation of Aβ peptides[6], and exploration of the internasal delivery of collagen-loaded neprilysin to clear Aβ plaques[7]. Again, we successfully performed the proof of concept of a functioning nonspecific enzyme entrapment in the iCore device[8].

All these investigations provide hope that the effective enzyme entrapment in various hydrogel films may become a successful method for eliminating Aβ in the ***i***Core device.

We are currently working on the development of a digital tool called Avatar-***i***Core. This tool is a Digital Twin that will enable physicians to use the ***i***Core platform to support their therapeutic decisions. The Avatar-***i***Core model is based on mathematical modeling and numerical simulation, solely relying on first-principles transport and kinetic models.

*Keywords*: Alzheimer's disease, Amyloid-β peptides, Microfluidics, Hydrogels, Aβ degrading enzymes

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# Microfluidic PAT for CAR T Cell Therapy Manufacturing

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Chimeric Antigen Receptor (CAR) T cell therapies have revolutionised the treatment of haematological malignancies, offering high chances of remission for patients with previously incurable cancers. Their wide-spread use, however, is still limited, in part due to lack of reliable manufacturing processes and concerns around cost effectiveness. Currently, the production of such products is complex, with multiple manual steps as well as labour intensive analytics, adding to already high costs[1]. To increase both the cost-efficiency and quality of manufacturing, it is recommended that process analytical technologies (PAT) are implemented for process monitoring and subsequent feedback control. Because of the unique characteristics of CAR T cell therapies, novel PAT systems must be developed to accurately measure relevant critical quality attributes[2].

As a novel and complex product, CAR T cell manufacturing requires monitoring of not only typical process parameters such as temperature or pH, but also cell-specific characteristics, i.e. their phenotype. Due to the rapid translation of pre-clinical discoveries to clinical and even commercial use of CAR T cell therapies, research-grade analytical techniques are used in Good Manufacturing Practice (GMP) environment. However, they are often time-consuming and highly manual and therefore not fit for purpose when manufacturing GMP products at scale. This necessitates the development of new technologies capable of rapid, accurate and automated analysis of critical quality attributes.

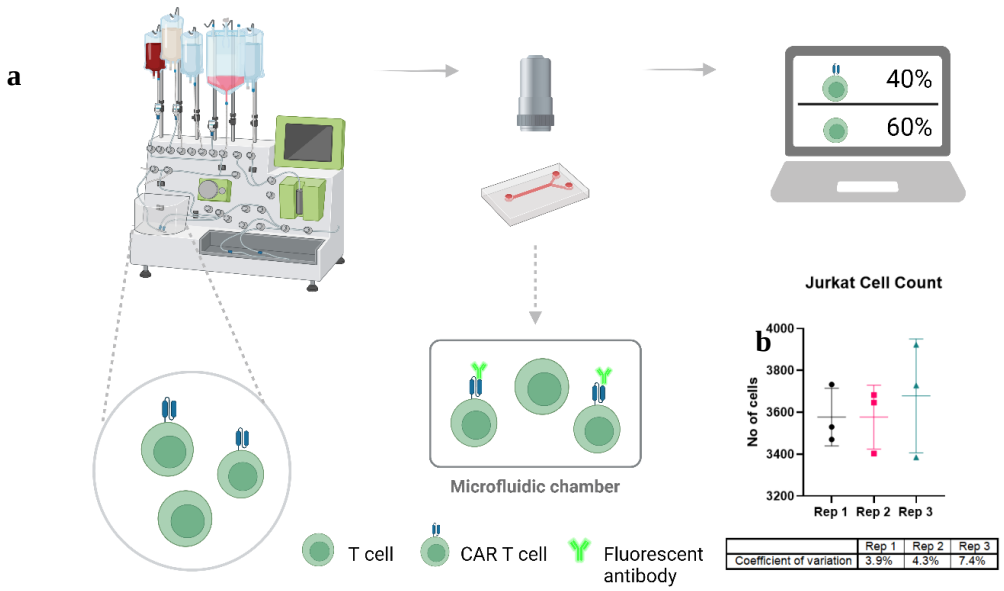


Figure 1: Microfluidic PAT for CAR T cell therapy manufacturing (a) A workflow for microfluidic analytical system integrated with CAR T cell manufacturing technologies such as CliniMACS Prodigy®, aiming to provide quantitative analysis of T cells in process by labelling them with fluorescent antibodies (b) Results from immunofluorescence-based counting of Jurkat cells. This figure was made in Biorender.

Microfluidic technology has had a wide range of applications, with its benefits such as the use of low sample volume and fine control of fluid flow behaviour particularly suitable for development of analytical systems[3]. Here, we aim to utilise microfluidics as a tool for addressing the gap in the development of PAT for CAR T cell therapy processing. The proposed chip was designed to enable mixing of cell sample with the reagent within the channels, and subsequent incubation and visualisation of cells inside the chamber as shown in **Error! Reference source not found.**a. The device can be manufactured within a day, using computer numerical control (CNC) milling of cyclin olefin co-polymer (COC) and thermal bonding. When coupled with immunofluorescence and subsequent image processing it forms the basis of a cell phenotyping PAT.

For the purpose of assay development, Jurkat cell line samples were incubated with fluorescently tagged antibody and detected using fluorescence microscopy. An image processing macro was developed in ImageJ to obtain the number of cells captured in the images collected. As shown in **Error! Reference source not found.**b, this method was able to deliver reproducible results, with coefficient of variation below 10%.

The utilisation of microfluidic technology allows rapid prototyping and therefore testing of chip candidates and the initial results from the immunofluorescence experiments indicate the potential of this approach in detection of T cell phenotype. The use of COC, a hard polymer with low autofluorescence enables the integration of the immunofluorescence assay with the microfluidic device. The chip can be then integrated with common bioreactors used in CAR T cell processing before its performance can be compared to industry-standard analytical methods. This work aims to offer solutions to the analytical bottleneck currently experienced by the cell therapy industry when commercializing ground-breaking cancer therapies.

*Keywords*: microfluidics, CAR T cell therapy, phenotyping, analytics, PAT

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# Flow chemistry monitoring with in-situ React-IR 702L and React-Raman 802L systems

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Flow chemistry is fast groving chemistry area and therefore also in-situ monitoring have become important. Mettler-Toledo React-IR 702L and React-Raman 802L systems are capable to monitor those processes as in-situ measurement devices with high sensitivity and selectivity and with lot of 2- and 3-dimensional data handling tools. These features make the devices very capable to monitor reactions when they happen.[1,2,3]

This presentation will highlight how React-Raman and React-IR data fit to HPLC data in the flow chemistry application and shows literally what is the power of systems which are designed for reaction monitoring. We will show how React-Raman 802L and React-IR 702L data (Figures 1 and 2) were monitoring the process. We also show, how we fit the data with HPLC data. [4]

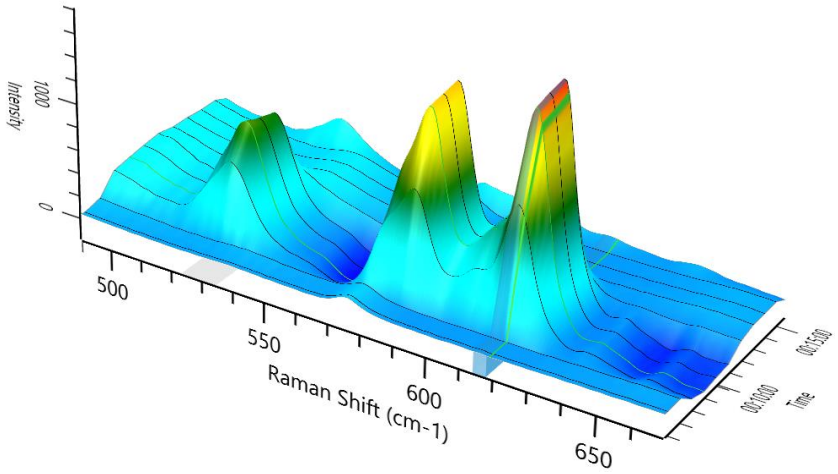


Figure 1: 3-dimensional image of React-Raman measurement.[4]

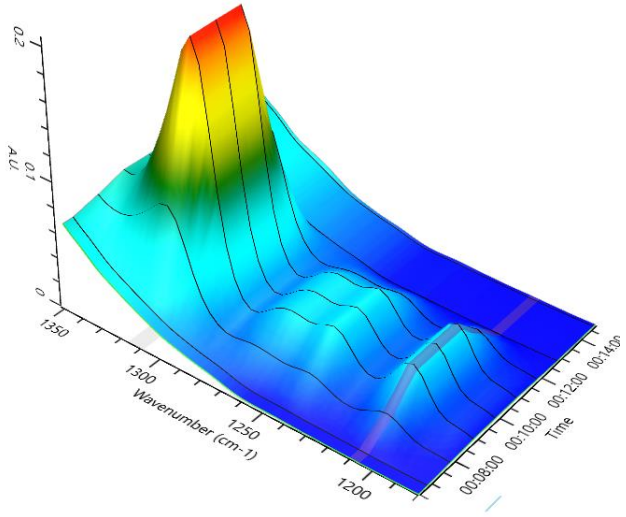


Figure 2: 3-dimensional image of React-IR measurement.[4]

*Keywords*: ReactIR, FlowIR, reaction analysis, Mettler-Toledo, AutoChem

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This work was done with collaboration partner and report presentation without name of the company is allowed

# Session D: PROCESS INTENSIFICATION AND INTEGRATION

# Self-Assembling Biocatalytic Materials and Additive Manufacturing for Flow Biocatalysis

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Applications employing catalysts in fluidic, cascaded setup are becoming increasingly relevant.[1] In order to also incorporate biocatalysts in such setups, efficient immobilization strategies are in demand. Employing the genetically encoded, covalent SpyTag/SpyCatcher (ST/SC) system, we have recently developed an all-enzyme hydrogel, which can be prepared from many enzymes, enabling the continuous conversion of a wide variety of substrates.[2-6] In such carrier-free formulations the enzymes themselves form a material, which for example stabilizes the enzymes and also offer the retention of expensive cofactors such as NAD(P)H. Depending on the application, the same immobilization strategy using the ST/SC system can also be used to immobilize enzymes onto epoxy-modified beads,[7] fully biogenic SC-modified magnetosomes[8] or as highly stable enzyme foams.[9] In all cases the biocatalytic conversions in the resulting flow reactors converted the corresponding substrates continuously for several days with high efficiency. Recently, we have also developed similarly efficient reactors using a flow deposition method in microfluidic reactors that is also highly compatible with whole cell biocatalysts.[10]

As an alternative approach, biocatalysts can also be implemented in flow biocatalysis employing additive manufacturing. Such bioprinting applications call for thermotolerant organisms and proteins. We have recently showed reported how to employ guided protein evolution for engineering biocatalysts for such applications.[11-13] In this context new methods using correlation analysis and machine learning have been developed which greatly aid the identification of thermostable enzymes.[11, 14] With this approach, we have successfully evolved enzyme variants that reveal significantly increased stability and activity at elevated temperatures. These improved variants along with naturally thermostable proteins were then used for direct 3D printing of bioinks to manufacture reaction modules.[12, 15] These modules can be produced on demand from bioinks, which are stable over weeks inside the printing cartridge. Reactor modules containing different enzymes can be arranged into cascades and show a tunable behavior. Different enzyme classes have been successfully utilized including alcohol dehydrogenases, esterases, ketoisovalerate decarboxylases, phenolic acid decarboxylases and benzaldehyde lyases.

*Keywords*: Flow biocatalysis, enzyme immobilization, enzyme engineering, thermostabilization, additive manufacturing

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# Large-scale integrated microfluidic systems for chemical synthesis

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Microfluidic technology offers the superior capability to manipulate fluids in time and space, thus enabling various applications, mostly in biomedical and life science, but chemical synthesis is another target.

To this end, our team at Nation Tsing Hua University (NTHU) has collaborated with Daicel Corporation, a leading Japanese chemical company, to establish the Daicel-NTHU Joint Research and Development Center (JRDC) and the startup company IMT Taiwan (IMTT). This partnership is dedicated to developing large-scale integrated microfluidic systems, which we call "Desktop Chemical Plants (DTPs)." These systems, by linking multiple microfluidic chips in series, carry out complex chemical processes that not only enhance product quality but also increase production volume through large-scale parallel numbering-up, without the need for re-optimization of production conditions. In May 2022, we successfully built and sold a DTP containing 120 microfluidic chips, specifically for the production of high-quality polymers, with an annual production rate of 1.2 tons. This is the world's first successful case and a pioneering innovation in technology, anticipated to provide next-generation high-performance chemicals for the semiconductor and biomedical industries, contributing to green chemistry and the United Nations Sustainable Development Goals (SDGs). The development of a DTP with ten times the production capacity is almost completed this year.

While we have made significant strides in microfluidics, there remains a gap in essential components such as pumps, manifolds, connectors, sensors, microelectromechanical systems (MEMS) components, simulators, and advanced control technologies, including AI-driven system control. These are crucial for the realization of large-scale, multi-device microfluidic systems.

To bridge this gap, we are poised to harness Taiwan's renowned capabilities in MEMS and precision machining. Our goal is to create a “solution center” through the Academia-Industry Research (AIR) center project. This initiative will serve as a nexus for the cutting-edge “microfluidics-MEMS” technology, fostering collaboration with companies to open a new industry in Taiwan.

*Keywords*: microfluidics, micro unit operation, Desktop Chemical Plant, fine chemical, SDG, continuous-flow chemical processing

# Bioprocess Microfluidics 2.0: Towards Standardisation for Bioprocess Microfluidics Applications

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Standardisation facilitates the commercial uptake of new technologies. It is a de-risking approach to reduce failure rates which in turn makes the development of products more economical. Standardisation for example enables the manufacturers to produce off-the-shelf components. These lead to enhanced reliability in the fabrication process, resulting in yield improvement and ultimately in the mentioned cost reductions. Standards facilitate the communication between supplier and end-user, they standardize and thus streamline any testing protocols, and they strengthen manufacturing practices and commerce between different customers and companies around the world [1]. Standards are particularly valuable for low to medium high production volumes, such as those known for microfluidics manufacturers. With the ISO norm 22916:2022, a set of minimum specifications have already been defined for the interoperability of microfluidic components.

Platform technologies relate to the principle of standardization, and quite a few microfluidic platforms have been successfully developed [2]. With these platforms, many possibilities and opportunities exist to manipulate liquids, for example to mix them for a specific reaction, or to separate out compounds, and to integrate detection methods, and to create automated workflows. We have identified two challenges particular to Bioprocess Microfluidics [3], which are not typical to the analysis systems where microfluidic applications originally stem from; hence the name of the most prestigious microfluidic conferences being µTAS (micro total analysis systems). Unless a specific high-throughput screening application or an analytical tool is sought, microfluidic devices for bioprocessing must accommodate comparatively large volumes. For cell therapy and regenerative medicine, it might be chambers where organoids or 3D cell clusters, such as embryoid bodies, can fit. In biopharmaceutical processing and microbial fermentations, large cell densities of cells in suspension facilitate the comparability with larger scales. And for small molecule drugs, a significant number of immobilized biocatalysts is required to avoid variability in biocatalyst load. The second challenge ties in with the first one. It is often cumbersome to reliably and reproducibly insert the bio-material into microfluidic devices. Unlike with larger reactors, it is non-trivial to create access ports into the small structures, and flowing the bio-material from an up- or downstream port can negatively affect either the bio-material itself or induce variability of the amount of bio-material loaded.

We have developed a unique microfluidic design [4] which contains a resealable lid with which the afore-mentioned challenges are addressed. With this device, we have demonstrated the gamut of bioprocessing applications: immobilized enzymes for the synthesis of active pharmaceutical ingredients, mammalian cell culture for the production of monoclonal antibodies, adherent cell transfection, expansion, and differentiation for regenerative medicine, and CAR T cell expansion for the immunotherapies.

*Keywords*: bioprocessing, microfluidics, microreactors, standardization, platform technologies

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# Design and fabrication of a microfluidic device for in-line crosslinked enzyme aggregates purification

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When preparing nanoscale cross linked enzyme aggregates (CLEAs) in a microfluidic system comprising solvent precipitation and subsequent crosslinking[1], a problem of residual solvent arises. Solvent removal is crucial to prevent deactivation of enzymes crosslinked in agglomerates[2]. For this reason, a microfluidic device enabling in-line removal of solvents from CLEAs was designed and fabricated using two-photon polymerization 3D printing (2-PP).

Two-photon polymerization facilitates 3D printing with nanometer-scale feature sizes[3]. With this capability in mind, a microfluidic device was designed. It featured a square channel measuring 290 µm, outfitted with two nozzles on each side. The smaller nozzle with diameter of 30 µm, was placed to introduce CLEAs into the device's core, while the second, a larger 50 µm nozzle, faced in the opposite direction (Figure 1). This larger nozzle was tasked with CLEA particles collection.

The device's separation mechanism relies on dilution, leveraging the difference in diffusion coefficients between 100 nm amine transaminase CLEA particles ($4.28\ 10^{-12}$ $m^2s^{-1}$) and acetone ($0.75\ 10^{-9}$ $m^2s^{-1}$), which was used for enzyme precipitation in this particular case. Acetone, given ample time, diffuses away while the particles remain confined in the channel's central region. Fabricated on a glass substrate using photosensitive resin, the microdevice was complemented by a bespoke holder crafted for its secure positioning.

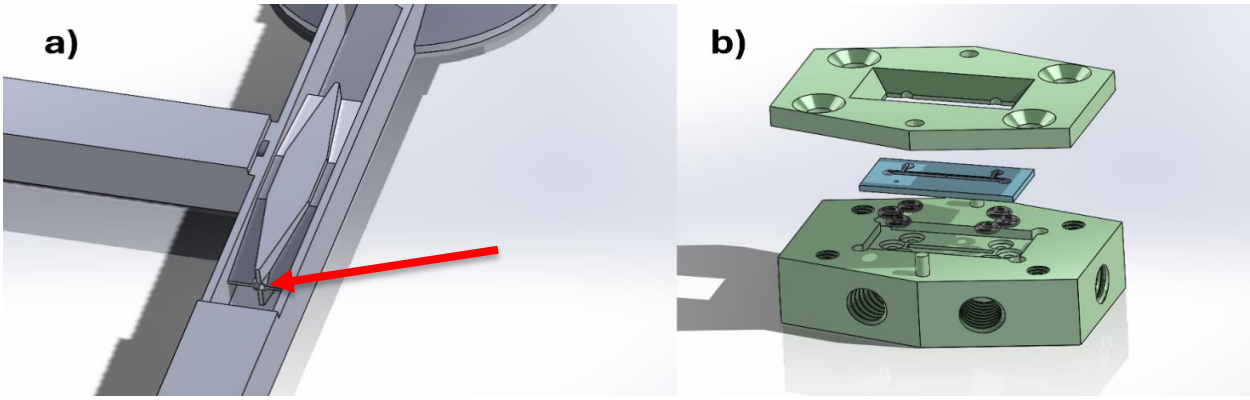


Figure 1: Two renders of the microfluidic device; a) the nozzle designed for the outlet with the same design as the outlet nozzle, and b) a microdevice on a glass substrate (blue) together with the holder (green)

The flow in the device was simulated, exploring various flow ratios between the buffer containing CLEAs and acetone and the pure buffer flowing around the nozzle. The flow rate around the nozzle remained constant at 25 µL/min, while the flow within the nozzle ranged from 1.25 to 10 µL/min. Flow rates of 25 and 2.5 were selected based on the simulation results, yielding a residence time of 1.2 s. Subsequently, the device was then tested with both blue dye and fluorescent nanoparticles to assess the congruence of the flow pattern with expectations. Finally, experiments with buffer and acetone were conducted to ascertain the adequacy of acetone diffusion, alongside tests with CLEA particles to validate the expected separation efficiency.

*Keywords*: two-photon polymerization, solvent removal, microdevice design, particles purification, 3D printing

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# Lipase catalyzed synthesis of enantiomers and their continuous separation in an electric field

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Mandelic acid enantiomers serve as pivotal precursors for synthesizing valuable chiral compounds, particularly active pharmaceutical ingredients. Traditional batch reactor methods for synthesizing and purifying these enantiomers are plagued by high costs, lengthy processing times, and scalability issues. To address these challenges, we developed, investigated, and validated two orthogonal milli-fluidic systems operating in fully continuous flow regimes for the synthesis of mandelic acid enantiomers.

The first system[1] comprises two distinct units: a packed-bed microreactor (PBR) housing immobilized enzyme lipase (*Aspergillus niger*) and a membrane microseparator controlled by a direct-current electric field, see Fig. 1. The immobilized lipase efficiently converts racemic methyl mandelate into mandelic acid enantiomers, primarily yielding (*R*)-(−)-mandelic acid within minutes. Subsequently, the product stream from the PBR undergoes separation in a counter-current membrane microseparator, achieving complete separation of electrically charged enantiomers within 1.5 minutes. This modular system yields approximately 1 gram of (*R*)-(−)-mandelic acid per day with a 60% enantiomeric excess.

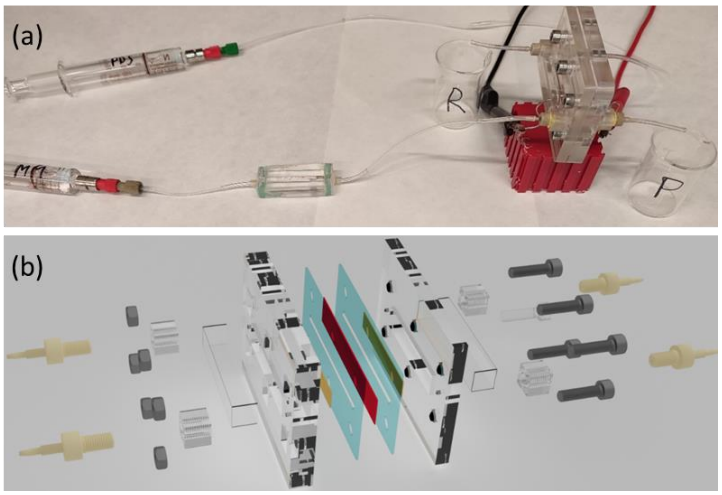


Figure 1: (a) Photo of the modular system for the synthesis of mandelic acid enantiomers; (b) Detail scheme of the membrane separator.

We also explored a fully integrated reactor-separator milli-fluidic system[2], which features a single packed-bed milli-reactor unit, see Fig. 2. An electric field applied orthogonally to the catalytic bed enables *in situ* separation of mandelic acid enantiomers, selectively extracting electrically charged enantiomers while leaving the electroneutral substrate untouched. This selective separation significantly enhances mandelic acid synthesis by favoring product formation, achieving over 90% substrate conversion compared to 30% without current. We identified an optimal electric current yielding nearly 70% enantiomeric excess of (*R*)-(−)-mandelic acid, surpassing the modular system performance.

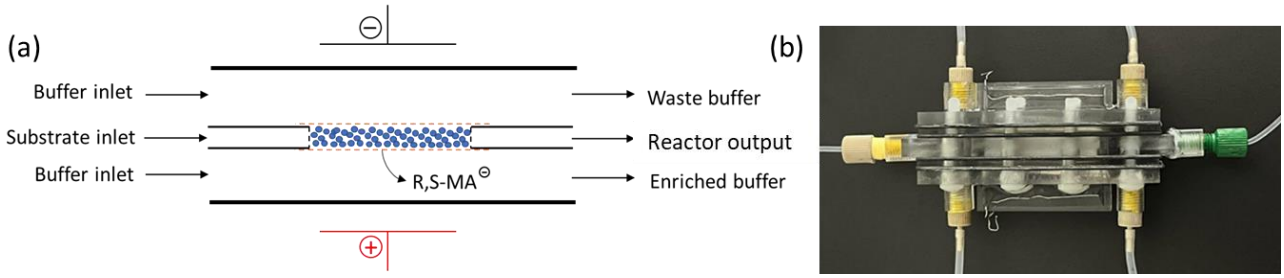


Figure 1: (a) Schema and (b) photo of the fully integrated system for the synthesis of mandelic acid enantiomers.

Both systems offer scalable productivity without the loss of reaction-transport efficiency. Additionally, the enzymatic reaction proceeds under mild conditions without organic solvents, aligning with principles of green chemistry. Our integrated milli-fluidic units provide superior process control and significantly enhance enantiomerically enriched enzymatic synthesis, offering promising ways for efficient and sustainable chiral compound production.

*Keywords*: enantiomer, lipase, mandelic acid, continuous flow synthesis, electric field

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# Advanced methods for continuous chiral separation in modular milli-fluidic systems

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Pharmaceutical companies are increasingly focused on the synthesis of optically pure and enantiomerically enriched chemicals, crucial as active pharmaceutical ingredients (APIs). Traditional methods such as asymmetric synthesis and chromatography are effective but often costly and discontinuous. Thus, there is a growing need for alternative approaches, especially for low-scale production. One such promising alternative is continuous chemistry in milli-fluidic devices, offering enhanced reproducibility, facile prototyping, and adaptable scalability.

Among continuous separation techniques, orthogonal methods play a significant role in separating aqueous and non-aqueous reaction mixtures, including enantiomeric compounds. Free-flow electrophoresis (FFE), for instance, utilizes pressure-controlled convective transport with an orthogonally applied electric field to separate electrically charged molecules in electrolytes. In the context of lipase-catalyzed hydrolytic reactions, FFE can effectively separate ionic chiral products. This separation employs chiral selectors attached to one enantiomeric form of the product. The attached chiral selector slows down the electromigration of a targeted enantiomer in the direction of the electric field, allowing for its separation from the mixture of ionic enantiomers.

Another orthogonal technique, membrane extraction, utilizes pressure-controlled convective transport along a membrane to form a gradient of the chemical potential across a membrane. Green solvents, such as deep eutectic solvents (DES) composed of biodegradable and non-toxic components like choline chloride and glycerol or urea, can be used in the membrane extractors to separate valuable alcohols. In this view, DES offer a sustainable production of pharmaceuticals. We have tested various DES for the separation of chiral alcohols from mixtures with chiral esters. We have found DES with high affinity to alcohols and acceptable viscosity. We will present a pilot milli-extractor for a continuous extraction of chiral product obtained in an enzymatic reactor.

*Keywords*: Enantiomers, Continuous flow separation, Electric field, Deep eutectic solvents

# Modeling and kinetic parameter estimation of glucose dehydrogenase-catalyzed glucose oxidation

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Hydrogen stands out as a leading contender among alternative energy solutions, with biological processes gaining attraction as favorable alternatives to chemical methods for its production. This form of hydrogen, termed biohydrogen ($BioH_2$), has long been recognized for its potential. However, the challenge lies in ensuring its industrial sustainability, given the low substrate conversion, production rates, and yields[1]. Economic viability typically hinges on achieving substrate conversion rates of 60-80%. While conventional pathways for $BioH_2$ production, such as photo-fermentation and anaerobic fermentation, are established[2], novel strategies are emerging to address limitations. These include optimizing light utilization, genetically modifying microorganisms, fine-tuning process parameters, employing enzymes, and even creating synthetic enzymatic pathways for $BioH_2$ synthesis. Additionally, exploring innovative reactor designs and transitioning from macro- to microscale operations offer promising avenues for improvement. Among these advancements, microfluidic systems represent a cutting-edge technology poised to overcome the challenges associated with current $BioH_2$ production methods. In the paper of Woodward and Orr (1998)[3] the authors proposed the simple enzymatic conversion of glucose to gluconic acid with parallel molecular hydrogen production. Enzyme glucose dehydrogenase (GDH) catalyzes the oxidation of glucose to glucono-δ-lactone, which is hydrolyzed to gluconic acid, and the cofactor for this reaction is either $NAD^+$ or $NADP^+$, which is reduced. The second enzyme, hydrogenase, uses cofactor as an electron donor, resulting in molecular hydrogen and the regeneration and recycling of cofactor. Unfortunately, the reaction resulted in low yields. As noted by the authors, a significant drawback of the proposed system is the utilization of two distinct enzymes, each requiring different optimal process conditions. Furthermore, the authors demonstrated that gluconic acid negatively impacts the activity of GDH. They suggest that eliminating gluconic acid and maintaining a constant pH throughout the reaction could enhance stability and potentially yield stoichiometric quantities of $BioH_2$ from higher glucose concentrations. This increase in $BioH_2$ yield may also lead to higher production rates. To address these limitations and enhance the overall process, microtechnology presents a viable solution.

The first step of the proposed system, namely glucose oxidation by GDH, was investigated in this research. GDH was kinetically characterized by the oxidation of glucose to gluconic acid. Kinetic measurements were performed according to the initial reaction rate method in 0.1 M TRIS-HCl buffer pH 7 at 40 °C. The kinetic parameters were estimated by non-linear regression using the simplex or least squares method implemented in Scientist software. The obtained results are presented in Table 1.

Table 1. Kinetic parameters for the glucose oxidation catalyzed by GDH

| **Glucose oxidation parameters** | | **Glucose reduction parameters** | |
|---|---|---|---|
| $V_{m1}$ (U/mg) | 373.64 ± 95.41 | $V_{m2}$ (U/mg) | 70.02 ± 9.76 |
| $K_m^{Glucose}$ (mM) | 103.14 ± 54.62 | $K_m^{Glucono\ -\delta-lactone}$ (mM) | 2.60 ± 0.75 |
| $K_m^{NAD+}$(mM) | 0.0042 ± 0.0012 | $K_m^{NADH}$ (mM) | 0.0044 ± 0.0012 |
| $K_i^{Glucose}$ (mM) | 723.97 ± 423.21 | $K_i^{Glucono\ -\delta-lactone}$ (mM) | 40.65 ± 12.50 |
| $K_i^{Glucono\ -\delta-lactone}$ (mM) | 1.31 ± 0.29 | $K_i^{Glucose}$ (mM) | 10.35 ± 3.06 |
| $K_i^{NADH}$ (mM) | 0.0078 ± 0.0011 | $K_i^{NAD+}$(mM) | 0.0107 ± 0.0013 |
| $K_i^{Gluconic\ acid}$ (mM) | 58.40 ± 22.27 | $K_i^{Gluconic\ acid}$ (mM) | 24.37 ± 3.89 |

The reaction rates of glucose oxidation and reduction catalyzed by GDH were described using double substrate Michaelis–Menten equations with substrate and competitive product inhibition. The product of glucose oxidation catalysed by GDH is glucono-δ-lactone, which spontaneously hydrolyses into gluconic acid. Spontaneous hydrolysis of glucono-δ-lactone is a first-order reaction ($k$ = 0.000776 $min^{-1}$). A mathematical model composed of reaction kinetics and corresponding mass balances was developed. In order to validate the model, two independent experiments were performed in a batch reactor, and the obtained results are presented in Figure 1.

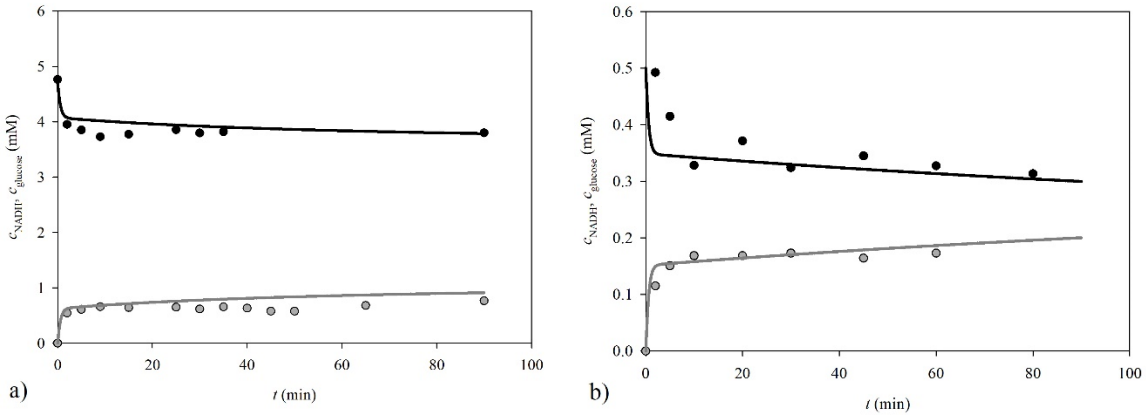


Figure 1: Biocatalytic glucose oxidation by GDH in the batch reactor ($\gamma_{GDH}$ = 0.002 mg/ml; 0.1 mM TRIS-HCl buffer pH 7; $T$ = 40 °C; $V_{reactor}$ = 50 ml) at different initial substrate concentrations: a) $c_{glucose}$ = 4.7 mM, $c_{NAD+}$ = 1.1 mM and b) $c_{glucose}$ = 0.5 mM, $c_{NAD+}$ = 1.1 mM; (●) glucose, (●) NADH, (—) model

In the following step, kinetic parameters were evaluated using results obtained from experiments performed in a microreactor.

*Keywords*: Biohydrogen, GDH, glucose oxidation, kinetic parameters, microreactor

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# POSTER PRESENTATIONS

# Diamin-alkyl derivative functionalized Immobead T2-150 as enzyme carrier for biocatalysis in continuous flow microfluidic system

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As miniature devices, microreactors function as a small vessel designed to contain and facilitate chemical reactions in a confined space. Its configuration is finely tuned to enhance reaction efficiency, aiming to maximize the production of the desired output [1]. It finds utility in both batch mode and continuous flow microfluidic systems, the latter being significant in biocatalysis. Within a microreactor, catalysts such as enzymes in their native or immobilized forms, can be housed in its inner channels, allowing starting materials to flow through for facilitating reactions and enabling parameter optimization control [2].

In this investigation, lipase B from Candida Antarctica (CaL-B) yeast was immobilized onto Immobead T2-150 carrier, featuring epoxy functional groups on its surface [3]. Before immobilization, the carrier underwent functionalization with diverse diamino-alkyl derivatives and glycerol diglycidyl ether to generate spacers of varying lengths between the support and enzyme. This process has the potential to augment the activity and selectivity of the immobilized CaL-B.

The structural characteristics of the prepared biocatalysts were analyzed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), while elemental analysis was conducted to confirm the covalent attachment of the linker molecule on the carrier surface.

The efficacy of these biocatalysts was evaluated within a continuous flow microfluidic system utilizing a self-designed and manufactured aluminum-based microchip filled with the biocatalyst. To assess the selectivity and activity of the enzyme upon immobilization, acylation of rac-1-phenylethanol with vinyl-acetate was carried out at room temperature and at 30°C respectively, using diethyl ether as solvent. Additionally, the acylation reaction was performed in batch mode under the same conditions at room temperature.

Comparison of the suitability of Immobead before and after functionalization as a carrier for enzyme immobilization revealed that diamine moieties with longer carbon chains exhibited higher synthetic activity and stability compared to those with shorter carbon chains. Furthermore, lower activity was observed when using the as-purchased carrier.

The efficiency of the microfluidic system was found to be significantly influenced by parameters such as concentration, flow rate and microchip temperature. The prepared biocatalysts were further tested in acylation reactions with various racemic 1-phenyl-1-ethanol derivatives as substrates to validate their selectivity and activity under previously established optimal conditions.

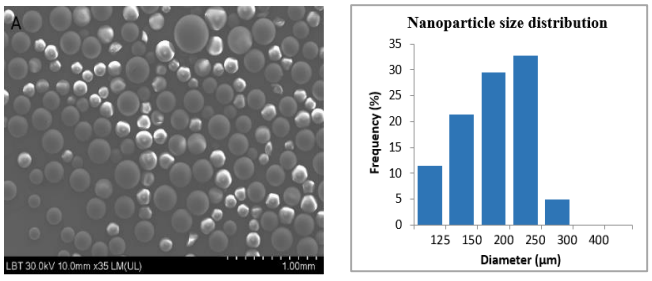


Figure 1: TEM image and nanoparticle size distribution of Immobead T2-150

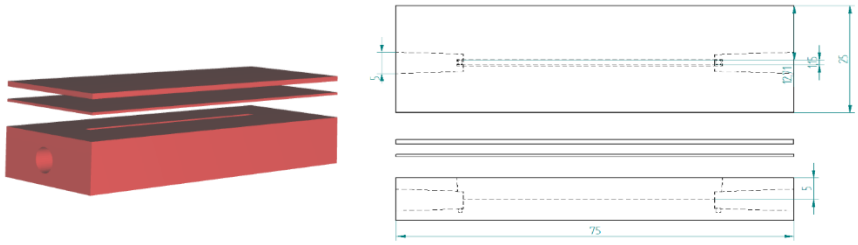


Figure 1: The designed microchip

*Keywords*: Immobead $T_2$-150, CaL-B, microchip, continuous flow microfluidic system

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# Immobilization of phenylalanine ammonia-lyase from *Arabidopsis thaliana* for continuous-flow processes

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Biocatalysis is an established important tool in chemical synthesis, in both academia and industry. The development of biotransformation-based synthetic processes has significantly progressed in recent years, especially due to advancements in protein engineering, enabling technologies with increased efficacy, productivity and selectivity, diminished costs and environmental impact. Besides high catalytic activity and selectivity, key factors for the successful application of enzymes are stability and recyclability, generally achieved through enzyme immobilization.[1] Moreover, flow technology has proven to be an attractive tool for developing efficient immobilized enzyme-based biocatalytic processes with upgraded productivity as compared to the batch systems.[2]

Phenylalanine ammonia lyases (PALs), naturally catalyzing the non-oxidative deamination of L-phenylalanine to *trans*-cinnamic acid, are already well-established biocatalysts for the production of both D- and L-phenylalanine derivatives, useful building blocks in medicinal and synthetic chemistry.[3] PAL from *Arabidopsis thaliana* (*At*PAL) recently proved to be superior to other well-investigated PALs in the biotransformation of several substituted phenylalanine and *trans*-cinnamic acid analogues.[4]

Herein, we explored different immobilization strategies for *At*PAL with the aim of developing robust and active biocatalysts for continuous-flow production of optically pure aromatic amino acids.[5,6] Adsorption-based binding and three covalent immobilization techniques were investigated: (1) non-specific covalent immobilization employing amino-functionalized supports and a bis-epoxide linker, (2) maleimide-thiol coupling of enzyme's native surficial Cys residues to maleimide-derivatized supports and (3) site-specific maleimide-thiol coupling of engineered enzymes with unique cysteine residues introduced at specific positions on the enzyme surface, to maleimide-functionalized supports. The immobilization supports consisted in poly(methacrylic) resins functionalized with spacers of different lengths and amino groups. All developed biocatalysts were studied in both ammonia elimination and addition reaction routes.

Encouraged by our previous successful site-specific immobilization of PAL from *Petroselinum crispum* (*Pc*PAL) based on thiol-maleimide coupling[7], we initially focused on developing the site-specific immobilization of *At*PAL. Sequence alignment of *At*PAL and *Pc*PAL revealed high sequence identity (81%) and identical catalytic sites, but also additional Cys residues supposedly located at the surface of *At*PAL. Since the successful application of the site-specific maleimide-thiol immobilization method is conditioned by the lack of Cys residues at the surface of the enzyme, five surficial Cys residues were replaced to Ser, without significant effect on the enzyme activity. Subsequently, by analogy to *Pc*PAL, two sites for immobilization, S615 and S391, were selected and replaced by Cys, obtaining two site-specifically immobilized biocatalysts.

Similarly to *Pc*PAL the site-specifically immobilized S615C *At*PAL variant outperformed the non-specifically covalently immobilized biocatalyst *via* bis-epoxide binding. However, the adsorbed native enzyme and the covalently attached one through non-specific maleimide-thiol coupling demonstrated improved performance in both ammonia addition and elimination reactions. For the two best performing biocatalysts the enzyme load was optimized, their specific activities were determined and their recyclability was investigated. Moreover, the biocatalysts' operational stability and productivity in a packed-bed continuous-flow mini-reactor was explored.

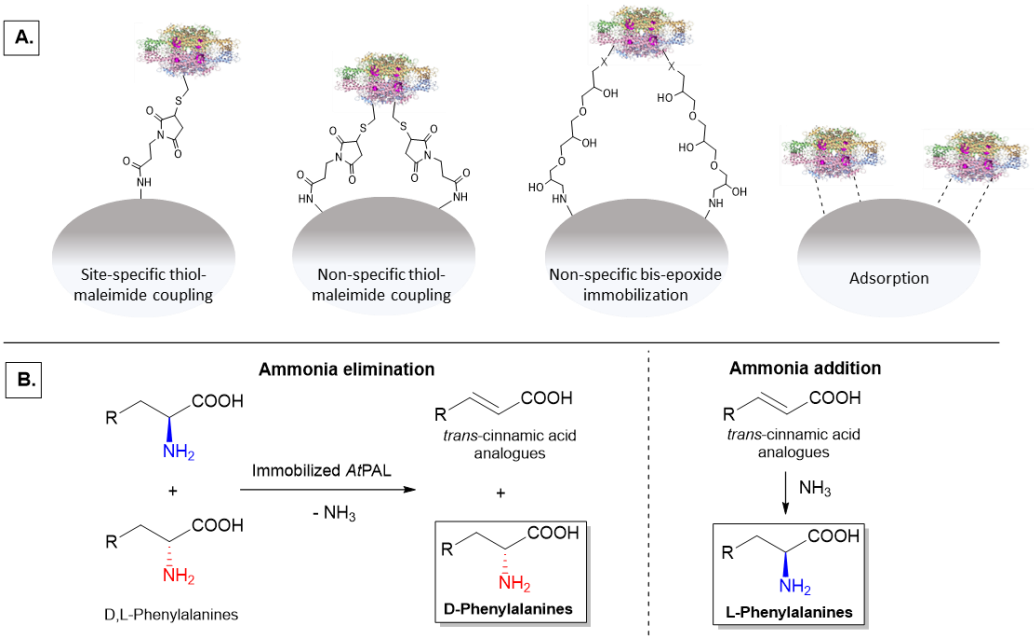


Figure 1: (A) Immobilization strategies for *At*PAL and (B) bioprocesses for the synthesis of both enantiomers of phenylalanines

*Keywords*: enzyme immobilization, maleimide-thiol coupling, phenylalanine ammonia-lyase, phenylalanines, continuous-flow process.

## Acknowledgements

This work was supported by the project "Advanced (multi)-enzymatic synthesis and purification processes for biobased furan derivatives –ASPIRE" funded by European Union – Nextgeneration EU and Romanian Government, under National Recovery and Resilience Plan for Romania, contract no. 760042/23.05.2023, cod PNRR-III-C9-2022-I8-ASPIRE/PNRR2022, through the Romanian Ministry of Research, Innovation and Digitalization, within Component 9, Investment I8.

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# Enhanced Enzyme Immobilization in Agarose-based Hydrogels

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The compartmentalization of chemical reactions is a basic principle in nature, which can be implemented in technical processes by performing reaction cascades with physically separated enzymes. For the development of novel approaches in biocatalysis this principle is a major source for innovations and is therefore mimicked in several ways. The immobilization of biocatalysts in a fluidic setup is one way to achieve compartmentalization. We recently demonstrated the entrapment of unmodified thermostable enzymes in a 3D printed, agarose-based thermoreversible hydrogel (Figure 1) and have successfully utilized this approach in a flow multi-step sequential biotransformation employing naturally thermostable alcohol dehydrogenase and stabilized ketoisovalerate decarboxylase.[1,2] The scope of this technology was further expended by integrating a chemoenzymatic workflow involving phenacrylate decarboxylases (PAD) followed by a palladium(II)-catalyzed Heck reaction into our entrapment approach.[3] Convenient scale-up of the reaction was achieved through reactor numbering-up without additional process optimization. This demonstrates the high modularity and scalability of our immobilization approach. The ease and flexibility of our additive manufacturing process allows for the introduction of new enzyme classes into flow reaction systems with rapid prototyping of suitable hydrogel geometries.

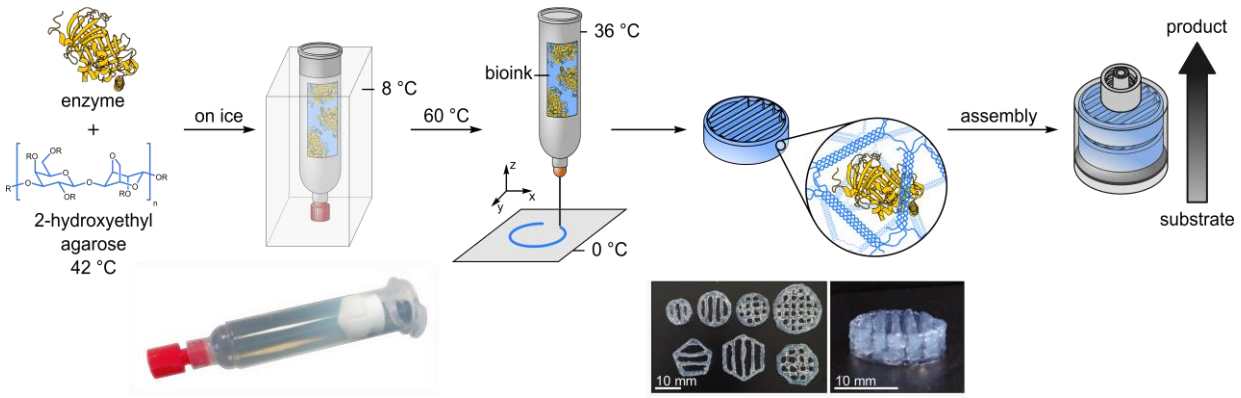


Figure 1: Additive manufacturing process for entrapment of enzymes in 3D printed hydrogel structures.

To enhance enzyme retention in such agarose-based hydrogels, we identified a versatile agarose-binding tag derived from the carbohydrate-binding module (CBM) of *Microbulbifer thermotolerans* thermostable β-agarase I (MtCBM).[4] Among four tested CBMs, MtCBM exhibited superior binding affinity to both solid agarose and cross-linked agarose beads. Employing a newly developed fluidic flat-bed agarose reactor (Figure 2), which enables the direct casting of agarose hydrogel films with defined heights of 200 µm to 600 µm, we demonstrated that fusion proteins incorporating MtCBM demonstrated significantly enhanced retention within agarose hydrogels. Notably, a PAD enzyme tagged with MtCBM yielded nearly six times more product compared to a similarly sized fusion enzyme lacking the agarose-binding tag. Even tripling the hydrogel layer thickness did not compensate for the higher leaching rate of the unlabeled enzyme, underscoring the effectiveness of MtCBM labeling for retention in the gel matrix. These results highlight the promising potential of this innovative approach for enhancing stability and reusability of enzymes in agarose-based carriers, including but not limited to hydrogel structures.

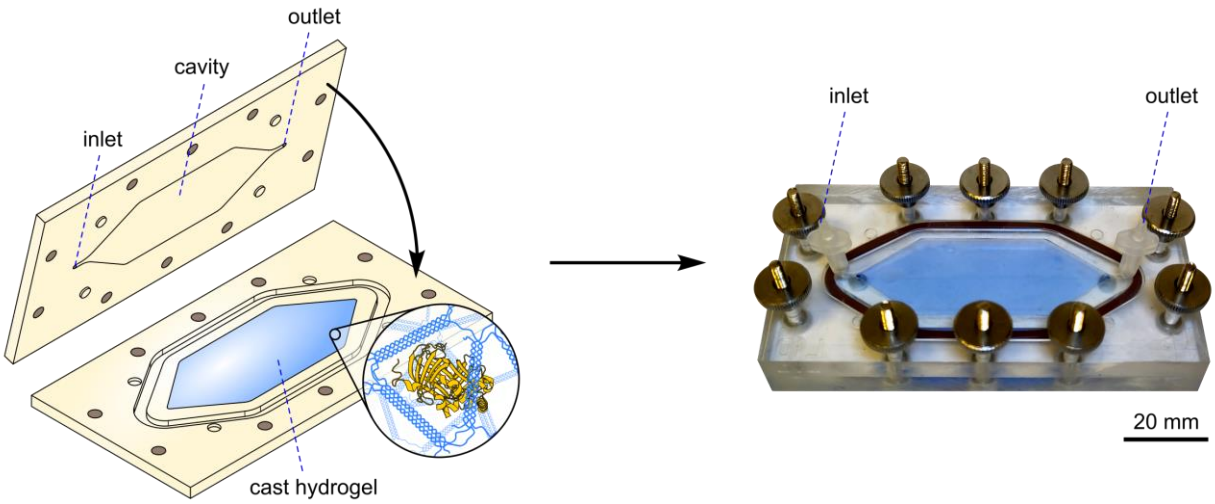


Figure 1: Assembly of the flat-bed agarose reactor for the investigation of enzyme leaching.

*Keywords*: Flow biocatalysis, enzyme immobilization, enzyme engineering, additive manufacturing, hydrogels

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# Microfluidics-based generation of crosslinked horseradish peroxidase nanoaggregates and pallidol synthesis from resveratrol

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Pallidol, a resveratrol dimer, has captured attention within the medical community for its potential therapeutic applications, particularly in neurology and psychiatry. Early research suggests that pallidol possesses antioxidant and neuroprotective properties, indicating its potential efficacy across various conditions. For instance, studies have highlighted its promise in mitigating neurodegenerative diseases like Parkinson's and certain autoimmune conditions.[1,2] Moreover, emerging evidence points towards its anti-cancer properties, offering potential avenues for cancer treatment.[3,4] Additionally, pallidol has shown promising antimicrobial activity against foodborne pathogens.[5] However, the steep cost of pallidol—fetching over 400 euros for just a one milligram—may mean that research efforts undertaken with pallidol are very expensive and limited for global applications.

To address this challenge, this study aimed to develop a cost-effective method for producing pallidol from bio-based materials, using resveratrol as a substrate and horseradish peroxidase as a biocatalyst (Figure 1).

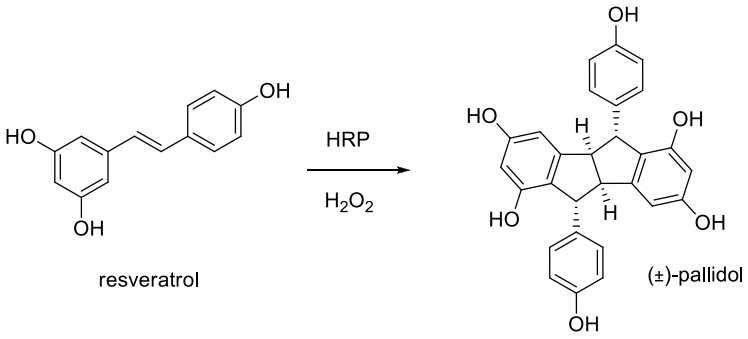


Figure 1: HRP-catalyzed synthesis of pallidol from resveratrol.

The focus shifted towards developing a continuous pallidol production system utilizing a microflow setup. To achieve this, an innovative process was employed to immobilize the enzyme using microfluidics, where the enzyme underwent precipitation and subsequent crosslinking. This method allowed for the generation of highly uniform cross-linked enzyme aggregates (CLEAs) on a nanometer scale.[6] The optimization of CLEA-HRP production took place within a 3D-printed microfluidic system (Figure 2a), involving the determination of the appropriate residence time for precipitation and crosslinking, screening various precipitation solvents, and setting the glutaraldehyde concentration for cross-linking. The size of CLEA-HRP was evaluated using dynamic light scattering analysis. The primary objective was to maximize the recovered activity of the enzyme, defined as its activity post-immobilization compared to its free form. The best recovered activity of 99 % was achieved at a residence time of 0.34 min with acetone and glutaraldehyde concentrations of 90 % (v/v) and 1 mM, respectively. The resulting CLEA-HRP exhibited an average particle radius of 150 nm.

The reactor, featuring CLEA-HRP immobilized on the membrane surface, was specifically designed for this biotransformation, and subsequently fabricated using a 3D printer (Figure 2b).

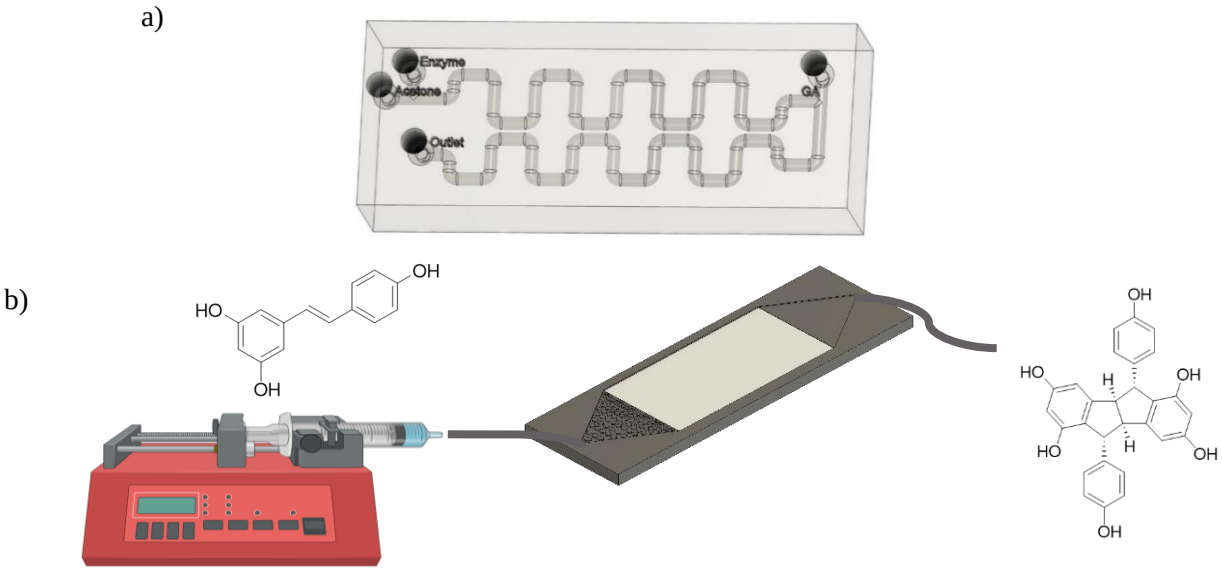


Figure 2: a) 3D-printed microflow system for CLEA-HRP production; b) continuous production of pallidol utilizing immobilized CLEA-HRP on a membrane integrated in a 3D-printed microreactor.

*Keywords*: pallidol, resveratrol, cross-linked enzyme aggregate, horseradish peroxidase, biomass valorization

## Acknowledgement:

This work was supported by Slovenian Research and Innovation Agency (ARIS) through Grants J4-4562 and P2-0191. MB was financed through Horizon Europe MSCA Doctoral Network project GreenDigiPharma (Grant 101073089), while FA was supported by National Recovery and Resilience Plan (NRRP), Mission4 Component 2 Investment 1.3 -Call for tender No. 341 of 15/03/2022 of Italian Ministry of University and by the European Union–NextGenerationEU, in the frame of the project Research and innovation network on food and nutrition Sustainability, Safety and Security (ON Foods).

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# Development of a continuous δ-viniferin synthesis in a microreactor with immobilized horseradish peroxidase

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δ-viniferin, a resveratrol dehydrodimer, exhibits diverse biological activities, including antiviral, anti-inflammatory, antibacterial, anticancer, and antioxidant properties. It possesses strong antioxidant properties, which can help protect the body against free radicals and oxidative stress. Additionally, δ-viniferin has been found to have anti-inflammatory properties, aiding in reducing inflammation in the body. Some researchers have also suggested that δ-viniferin could have the potential to fight various diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders.[1,2] However, at a cost often exceeding 300 euros for just 1 milligram, the expense associated with δ-viniferin may severely restrict research efforts and its global applicability.

This study aimed to develop a cost-effective method for synthesizing δ-viniferin from bio-derived materials, employing resveratrol as a substrate and horseradish peroxidase (HRP) as a biocatalyst (Figure 1). The enzyme was immobilized to enhance stability, facilitate reusability, boost productivity, enable continuous processes, and ensure compatibility with diverse environments. To achieve this, the enzyme was precipitated and further crosslinked in an innovative process employing microfluidics. This enables the production of very uniform cross-linked enzyme aggregates (CLEAs) in the nanometer range.[3]

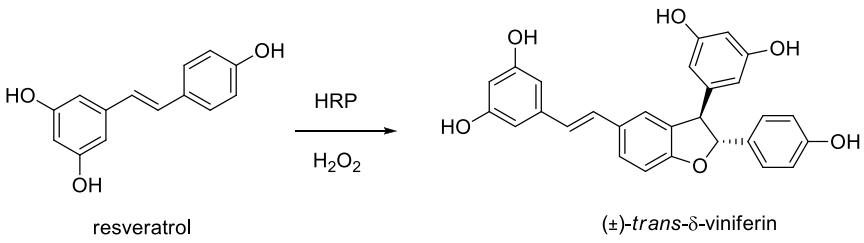


Figure 1: HRP-catalyzed synthesis of δ-viniferin from resveratrol.

Optimization of CLEA-HRP production focused on determining the optimal residence time for precipitation and crosslinking, screening various precipitation solvents, screening different precipitation solvents, and adjusting the glutaraldehyde concentration for crosslinking (Figure 2). The size of CLEA-HRP was assessed through dynamic light scattering analysis. The objective was to maximize the recovered activity of the enzyme, i.e. the activity after immobilization compared to that of the free enzyme. The best recovered activity of 99 % was achieved at a residence time of 0.34 min with acetone and glutaraldehyde concentrations of 90 % (v/v) and 1 mM, respectively. The resulting CLEA-HRP exhibited an average particle radius of 150 nm.

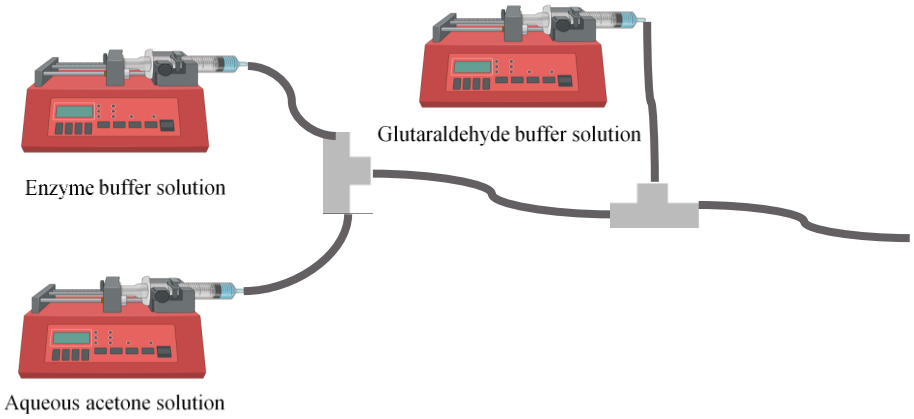


Figure 2: Scheme of a continuous CLEA-HRP production

The produced and immobilized CLEA-HRP was evaluated in the continuous biocatalytic process for synthesizing δ-viniferin from resveratrol (Figure 3).

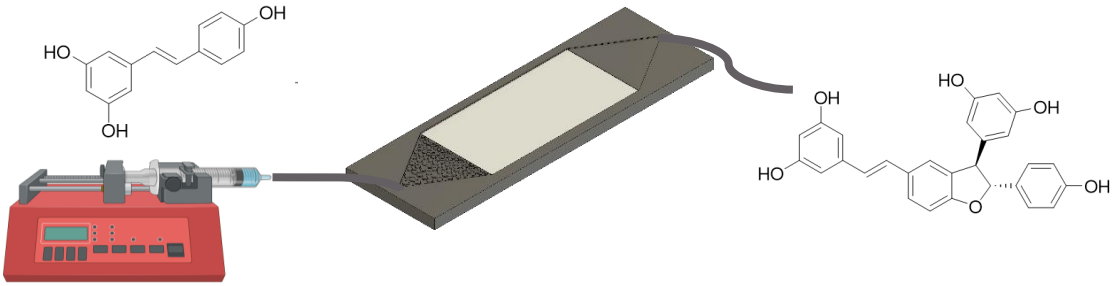


Figure 3: Scheme of a continuous δ-viniferin production in a microreactor with CLEA-HRP immobilized on the membrane surface

*Keywords*: δ-viniferin, resveratrol, cross-linked enzyme aggregate, horseradish peroxidase

## Acknowledgement:

This work was supported by Slovenian Research and Innovation Agency (ARIS) through Grants J4-4562 and P2-0191. MB was financed through Horizon Europe MSCA Doctoral Network project GreenDigiPharma (Grant 101073089), while FA was supported by National Recovery and Resilience Plan (NRRP), Mission4 Component 2 Investment 1.3 -Call for tender No. 341 of 15/03/2022 of Italian Ministry of University and by the European Union–NextGenerationEU, in the frame of the project Research and innovation network on food and nutrition Sustainability, Safety and Security (ON Foods).

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# Membrane microreactor with immobilized His-tagged enzymes for continuous transamination

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The use of microfluidic systems in the field of biocatalysis has several advantages leading to intensification of processes[1]. High surface to volume ratio enables efficient mass and heat transport while integrated materials (e.g. monolyths, membranes, sol-gels, magnetic particles) support immobilization of biocatalysts[2,3]. Enzyme immobilizations are crucial for industrial processes as they enable re-use of the enzyme as well as potentially improve their operational stability[4].

Immobilization of various amine transaminases (ATAs) with hexahistidine ($His_6$) tags on functionalized nonwoven nanofiber membranes was studied in a microreactor. The microreactor consisted of two poly(methyl methacrylate) (PMMA) plates and non-compressible polytetrafluoroethylene gasket (PTFE) that formed a hexagonal channel[5] and the nonwoven nanofiber mat was integrated by means of a double sided tape (Figure 1). Evaluation of biocatalyst immobilization efficiency, optimal temperature, and stability was done *in operando* in this microreactor setup. Nanofiber mats with various functional groups, namely Tiss-Link, Tiss-NH2, Tiss-IMAC-Fe and Tiss-IMAC-Cu (NanoMyP, Grenada, Spain) were tested. A wild type ATA-wt[6] with $His_6$ tag at the N-terminus (N-$His_6$-ATA-wt), and at the C-terminus (C-$His_6$-ATA-wt), and a genetically improved ATA-v1 with $His_6$ tag at the N-terminus (N-$His_6$-ATA-v1) were expressed as soluble proteins in *Escherichia coli* BL21(DE3) and immobilized on selected nanofiber mat. The enzyme activity and volumetric productivity of the reactor (space-time yield, STY) were monitored by generation of acetophenone (ACP) and L-alanine (L-ALA) from (*S*)-α-methylbenzylamine ((*S*)-α-MBA) with pyruvate (PYR) as the amine acceptor substrate molecule and pyridoxal-5'-phosphate (PLP) as the cofactor[5].

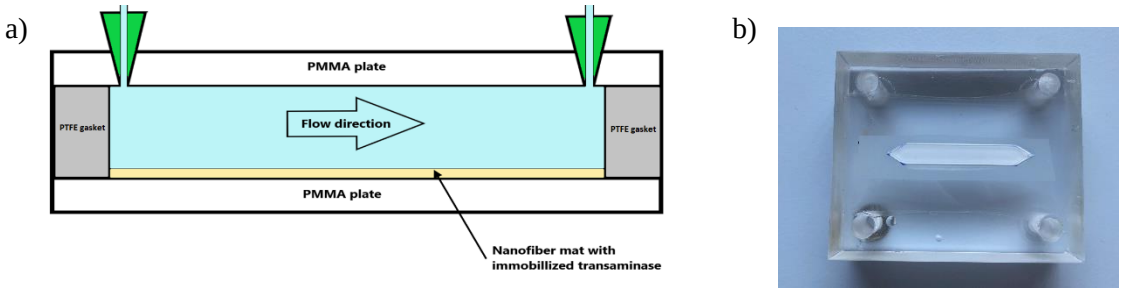


Figure 1: a) A scheme of a microreactor between two plates with an integrated nanofiber mat and b) an open reactor assembly without the PTFE gasket.

Among the tested supports for immobilization of selected enzymes, Tiss-IMAC-Cu utilizing the coordinative bonding of $His_6$ tag and $Cu^{2+}$ ion was found as the best, yielding up to 14 mg $mL^{-1}$ of immobilized enzyme in a microreactor (Figure 2). Additionally, no PLP was required in the inflow stream, confirming that the cofactor was efficiently retained in the tetrameric structure of the enzyme. N-$His_6$-ATA-wt gave the highest enzyme activity and STY for the selected reaction. The highest gross yield reached was 78.8 % at the residence time of 3.3 min. The highest space-time yield was >244 gACP $L^{-1}$ $h^{-1}$ and was obtained at the shortest residence time as shown in figure 2. 50˚C was found as the optimal temperature for all immobilized enzymes. The stability of immobilized N-$His_6$-ATA-wt and N-$His_6$-ATA-v1 in the microreactor with Tiss-IMAC-Cu was also very high over several days of continuous microreactor operation.

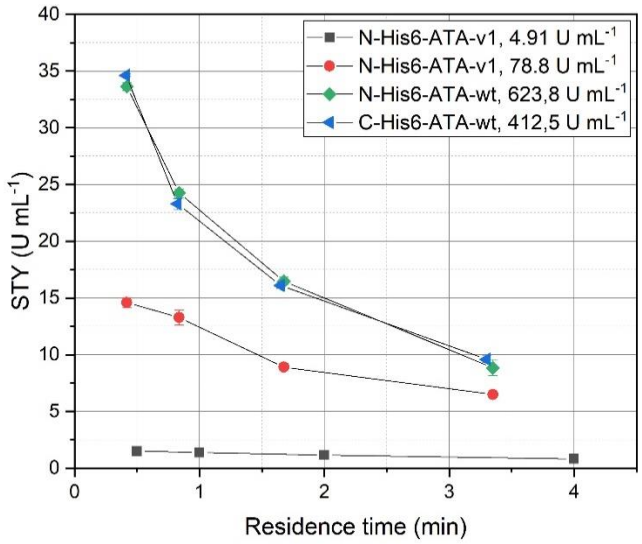


Figure 2: Space time yields (STY) obtained in the microreactor with Tiss®-IMAC-Cu using various enzymes and enzyme loads as specified in the legend. The biotransformations were performed at 30°C with 40 mM (S)-α-MBA and PYR in 20 mM phosphate buffer, pH 8, without added PLP.

*Keywords*: amine transaminase, microreactor, microflow system, immobilization, biotransformation.

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# Simultaneous amine transaminase aggregation and immobilization from cell lysate in a microfluidic system

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Enzyme immobilization plays a crucial role in the profitability of industrial use of enzymes. Immobilization of enzymes allows them to be reused, used in a continuous system, or more easily separated from the product. Immobilization can improve the stability of enzymes, increasing their long-term usability. Among the techniques for enzyme immobilization, preparation of crosslinked enzyme aggregates is considered as simple and cost-efficient approach[1]. It comprises enzyme precipitation using various salts, organic solvents, or non-ionic polymers, and further crosslinking with agents such as glutaraldehyde.

However, one step immobilization from cell lysate further simplifies the process by eliminating the need for expensive enzyme purification. This approach reduces the amount of waste, further decreasing the effects of this technology on the environment[2]. The main disadvantage of the batch process for crosslinked enzyme aggregates preparation is a relatively large scale (several micrometres) and non-uniformity of the produced aggregates. Recently, a microfluidics-based preparation of enzyme aggregates was reported by our group yielding highly uniform particles of down to 100 nm diameter[3]. Among enzymes tested by this approach are amine transaminases that have a wide range of industrial applications[2]. This is due to their ability to produce optically pure amines from ketones that can be used for valuable chemicals production.

In this work, a step further with microfluidics-based preparation of enzyme aggregates was done by starting from the *Escherichia coli* lysate containing overexpressed enzyme instead of a purified enzyme. A selected amine transaminase was precipitated and *in situ* immobilized in a microfluidic device based on a membrane. The comparison of purification and immobilization efficiency of both, i.e. chromatography-purified enzyme and the same enzyme from the cell lysate was done based on immobilization yield and retained activity. The selected biotransformation process was evaluated in a microfluidic system with integrated in line Fourier Transform Infrared Spectroscopy (FT-IR) analytics.

*Keywords*: amine transaminase, microreactor, microflow system, immobilization, biotransformation.

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# Microfluidic devices for scaling-down biocatalysis and enzyme stability studies

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Biocatalysis has gained a relevant role as a prominent green alternative to replace conventional petrochemical processes, primarily for its capacity for cost process reduction and its contribution to minimising the impact of climate change and energy resources. Therefore, optimising the performance of a biocatalyst is crucial to guarantee competitive processes from an economical and sustainable point of view[1]. In recent years, microfluidic devices have emerged as a promising and sustainable approach for biocatalysis[2]. These devices offer several advantages over traditional methods[3,4], e. g., high mass and heat transfer rate, reduced cost and time-product development with low waste generation, improved yields, and usage for enzyme screening. However, despite these advantages, few published studies have reported using microfluidic devices to scale down biocatalytic processes, for the collection of performance data.

On this basis our objective is to develop new methods, which involve designing microfluidic devices that enable enzyme stability studies under specific lab reaction conditions in bioreactors at a smaller scale, with lower cost, and in a much shorter time. In this sense, by creating controlled environments using different enzyme variants as testing systems, such a platform will allow us to model the enzyme performance, improve enzyme stability, and facilitate scale-down trials for process optimization. In this poster several examples will be given of the use of microfluidics for the scale-down of biocatalytic reactions.

*Keywords*: microfluidic device, enzyme stability, scale-down, biocatalysis processes

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# Novel magnetic nanoparticle-based flow reactors for biocatalytic production of enantiopure alcohols and amines

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The production of active pharmaceutical ingredients (APIs) and fine chemicals is accelerating due to the advent of novel microreactors and new materials for immobilizing customized biocatalysts that permit long-term use in continuous-flow reactors. In a recent study, the elimination of ammonia from L-aspartic acid utilizing aspartate ammonia-lyase from *Pseudomonas fluorescens* R 124 immobilized covalently onto magnetic nanoparticles (MNPs) was explored. Different types of continuous flow reactors were compared (Figure 1), and the $U_b$ value in a magnetically fixed cell could be enhanced further by three-four times in an agitated MNP flow reactor (AFR) cell with rotational movement of two magnets ($AFR_{RM}$) when compared to orbital shaker processes. However, due to the difficulty in the upscalability of AFR, static flow reactors were an easier and more suitable option for this purpose.[1]

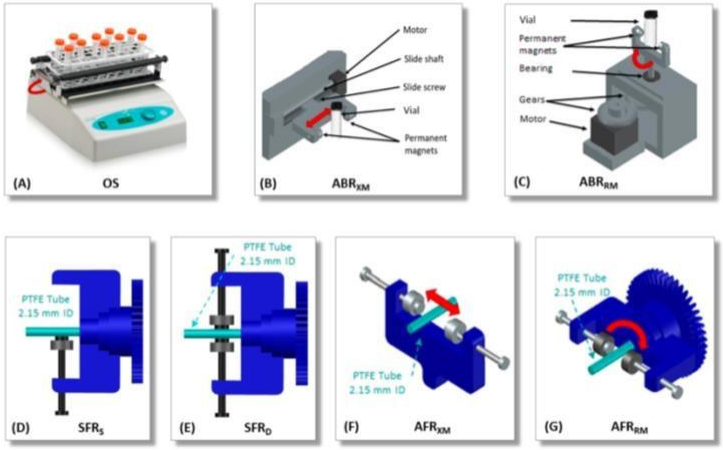


Figure 1: Modes of the reactors for the MNPs-catalyzed biotransformations

This work then aimed to implement and study the scalability process of a convenient microreactor for biocatalysis with Lipase B from *Candida antarctica* (CaLB) covalently immobilized on MNPs and its usage in the kinetic resolution of racemic alcohols and amines. The resulting biocatalysts (CaLB-MNPs) were tested in the continuous flow system, created by 3D printing to hold six adjustable permanent magnets beneath a polytetrafluoroethylene tube (PTFE) to anchor the MNP biocatalyst inside the tube reactor. The anchored CaLB-MNPs formed reaction chambers in the tube to pass the fluid through and above the MNP biocatalysts, thus increasing the mixing during the fluid flow and resulting in enhanced CaLB activity on MNPs. (Figure 2)

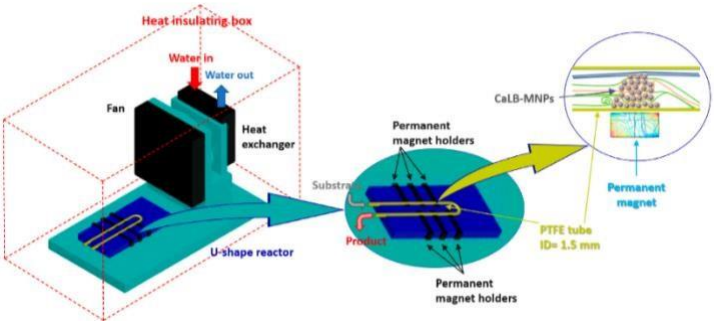


Figure 2: Schematic illustration of the U-shape reactor

Initially, the enantiomer selective acylation of 4-(morpholin-4-yl)butan-2-ol (±)-**1a**, being the chiral alcohol constituent of the mucolytic drug Fedrilate, was carried out by CaLB-MNPs in the U-shape reactor. The reactions in flow mode were compared in batch reactions to the lyophilized CaLB and to the CaLB-MNPs using the same reaction composition, and the same amounts of CaLB showed similar or higher activity in flow mode and superior activity as compared to the lyophilized powder form.[2]

To study the scalability of the tunable U-shape MNPs-based microreactor, a second study was performed investigating the influence of various inner diameters (ID = 0.75 mm, 1.50 mm, or 2.15 mm) in the conversion. This work tested the preparative scale kinetic resolution of the drug-like alcohols 4-(3,4-dihydroisoquinolin2(1*H*)-yl)butan-2-ol (±)-**1b** and 4-(3,4-dihydroquinolin-1(2*H*)-yl)butan-2-ol (±)-**1c**, utilizing CaLB-MNPs, leading to highly enantioenriched products [(*R*)-**2b,c** and (*S*)-**1b,c**]. Of the three different systems, the one with ID = 1.50 mm showed the best balance between the maximum loading capacity of biocatalysts in the reactor and the most effective cross-section area. The results showed that this U-shaped tubular microreactor might be a simple and flexible instrument for many processes in biocatalysis, providing an easy-to-set-up alternative to existing techniques and reducing costly and time-consuming downstream processes.[3]

To broaden the spectrum of substrates evaluated in this system, in a U-shape reactor, reactions with selected chiral amines – important building blocks pharmacologically – were performed aided by the same biocatalysts (CaLB-MNPs). The complete work utilized different acylating agents with isopropyl leaving groups in batch and continuous flow modes.[4] Therefore, future steps of the research are to apply different enzymes and/or different substrates, focusing on the study of heterocyclic racemic alcohols and amines towards the next steps of scalability of the microreactors proposed.

*Keywords*: magnetic nanoparticles, microreactor, biocatalysis, continuous-flow, chiral compounds

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# Application of cross-linked enzyme crystals of halohydrin dehalogenase HheG D114C in microfluidics

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Halohydrin dehalogenase HheG from *Ilumatobacter coccineus* shows high industrial potential because of its acceptance of sterically demanding internal epoxides in ring opening reactions [1,2]. However, its industrial application is limited by its low stability with an apparent melting temperature of only 38°C [3]. To increase stability, HheG was previously immobilized as cross-linked enzyme crystals (CLECs) [3,4]. These CLECs were successfully produced in volumes up to 50 mL and enabled the continuous operation of a packed-bed reactor and a fluidized-bed reactor over several weeks [5].

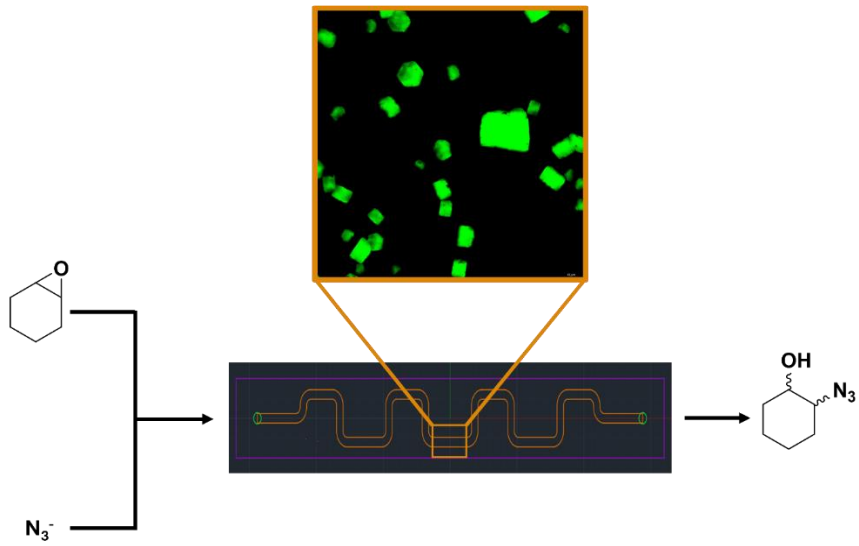


Figure 1: HheG D114C CLECs (stained with fluorescein isothiocyanate) on a glass microchip catalyzing the ring opening of cyclohexene oxide with azide as a model reaction.

Now, downscaling of the crystallization and cross-linking of the HheG variant D114C in glass microchips with volumes up to 8 µl was intended. In this context, the influence of different channel geometries and surface properties on the crystallization efficiency was investigated. Furthermore, the catalytic efficiency of these microreactors in epoxide ring opening reactions was studied. Overall, a high loading of the HheG D114C CLECs in the microchannels was achieved. Furthermore, the CLECs were catalytically active and a high productivity of the microreactors was determined in the continuous transformation of cyclohexene oxide with azide. Interestingly, the crystallization efficiency was influenced only by the surface properties of the microchannel, while the overall performance of the microreactors depended on the channel geometry. In conclusion, HheG D114C CLECs can also be generated and applied in glass microchips with volumes up to 8 µl to perform continuous epoxide ring opening reactions with high enzyme loadings and productivities.

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*Keywords*: Enzymatic bioreactor, enzyme immobilization, biocatalysis in flow, cross-linked enzyme crystals, halohydrin dehalogenase

P11

# Design of Novel Pathways for Production of High Value-added Chemicals in Multi-Enzyme Cascades

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Multi-enzymatic cascade (MEC) reactions, i.e., the combination of several enzymatic transformations in one pot, have proven to have considerable advantages compared to classical synthesis. Such reactions, often called biocatalytic reaction cascades because they mimic Nature's approach to synthesize chemical compounds, have unique properties. There is increasing interest in the production of chemicals from renewable bio-based feedstocks in the transition to a circular economy. Great advances have been made in the use of biological pathways to replace traditional chemical routes, for example, in the production of polylactic acid from lactic acid bacteria. However, there are limitations in exploiting living organisms and some processes remain uneconomical. Transformations may be achieved under wider conditions using non-natural cascade reactions involving multiple enzymes and efforts have been made to split reactions into small functional units, which can be recombined into production pipelines for various applications.

With the help of mathematical modeling, it is possible to directly reduce costs, to allow for overall reactions that would otherwise not be possible, and the concentration of harmful or unstable compounds can be kept to a minimum. For example, declarative approaches to solve design and optimization problems are well established in computer science: one does not aim at specifying how a solution is implemented, but one rather specifies what an overall goal should be and the solution is then automatically computed. Such approaches have an immense potential in general for chemistry, and more specifically for the design of multienzyme cascades: conceptually enzymes define graph transformation rules, which can be used to expand chemical spaces. The declarative approaches can subsequently be used to find efficient reaction cascades (i.e. sequences of graph transformation rules). Specially, the design of novel pathways can be completed by MØD to give the best solution of MEC from feedstock to product. The design focus lies on local and global properties of the solutions such as allosteric regulation of enzymes, competitive inhibition of the active sites, cofactor recycling, switching between different operation modes, or overall autocatalytic behavior. MØD is a software package developed for graph-based cheminformatics[1]. It includes a general graph transformation system for automatically generating reaction networks from graph grammar formulations of chemistries. The package includes a large visualization module that makes it possible to automatically visualize molecules, reactions, and reaction networks. Computational methods allow us to explore the diversity of the MEC design space in-silico prior to wet-lab implementation and will thus hugely speed up the development process of productive MECs.

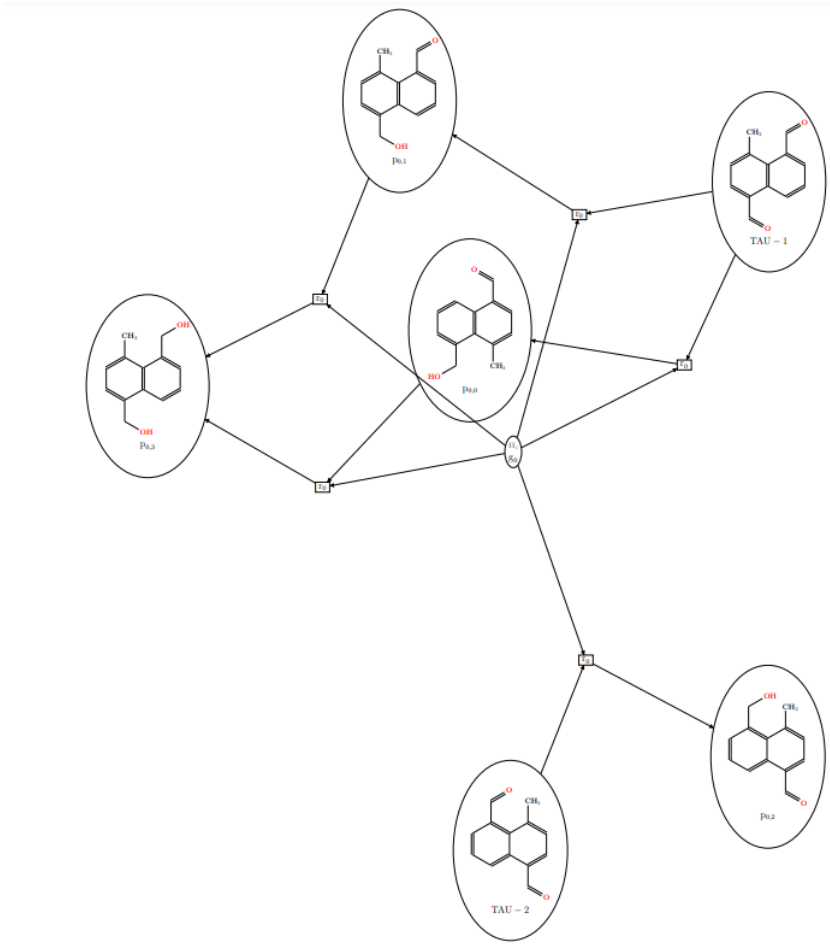


Figure 1: Derivation graph produced by mød. While for molecule TAU-1 both 6-rings of the dekalin system match the rule, for molecule TAU-2 only the left 6-ring matches the pattern, the right one does not and is therefore left untouched by the rule.

### 0.0.15 acetyl-CoA + 4-amimophenol = CoA + paracetamol

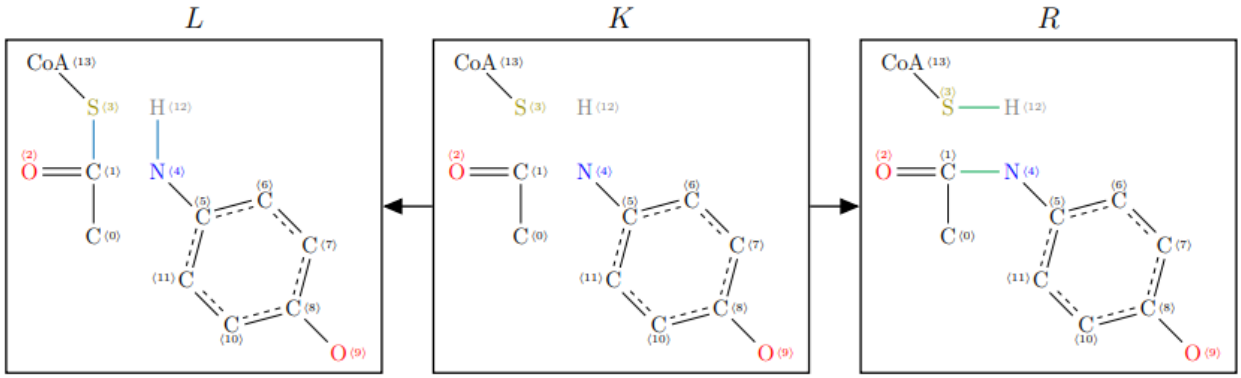


Figure 2: One example of the rules generated, cofactor is substituted with the symbol

*Keywords*: muti-enzyme cascades, grapy theory, mød

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P12

# Rapid discovery and development of enzymes for novel and greener consumer products (RadicalZ)

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There is an increasing demand to move away from non-renewable resources as starting material for chemical production. This can be achieved by using enzymes that are an environmentally friendly alternative to oil-based chemistry in industrial processes. To further advance this transition, in RadicalZ we aim to reduce the time required for enzyme discovery and development which is currently often a limiting factor for a biocatalytic approach. Enzymes that can decompose synthetic plastics such as polyethylene terephthalate (PET) are urgently needed to remove environmental microplastic pollution. However, a bottleneck remains due to a lack of techniques for efficient screening of good enzyme candidates. The project RadicalZ aims to reduce the time required for enzyme discovery and development using ultrahigh-throughput screening methodologies, where enzyme libraries, compartmentalized in water-in-oil emulsion droplets are assayed for required activity.

Here we describe a pipeline for the ultrahigh-throughput screening of evolved thermophilic enzymes using fluorescence activated droplet sorting, in which we have selected fluorescein dibenzoate (FDBz) as the fluorogenic probe. FDBz has PET-like ester bonds linked with a benzene group and can be hydrolysed by PETases to generate fluorescein monobenzoate [1]. FDBz is not a reactive substrate of common esterases, hence it has a low fluorescence background in cell lysates. The pipeline comprises four steps: 1) generation of a library of PETase mutants by error-prone PCR. 2) Incubation of droplets encapsulating single cells and FDBz. 3) screening and sorting of droplets to obtain improved PET-degrading enzymes. 4) Sequencing of the evolved enzyme and further evolution cycles to improve activity.

Thermogutta terrifontis esterase 2 (TtEST2) [2], is being used as a test system to establish the micro-fluidic experiments. TtEST2 differs from most enzymes of the α/β-hydrolase family 3 as it lacks most of the 'cap' domain and its active site cavity is exposed to the solvent allowing bulkier substrate - like PET - to be accepted. TtEST2 is active against FDBz which is a good indication of its PET digesting capability and is currently being evolved to improve its activity to bulkier substrates.

Furthermore, five additional potential PETase sequences have been identified in the HotZyme Exeter DNA database (containing novel thermophilic genomes and metagenomes) and from other public databases, that also have been shown to have activity against FDBz. This work serves as a foundation for the evaluation of PETase activity, using PET film and fibres, with the goal to develop a label free microfluidic screening methods for directed evolution of these industrially useful enzymes.

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Keywords: Biocatalysis, microplastic degradation, enzyme evolution, microfluidic techniques.

# Use of cpGFP to monitor the real-time signal response of bacterial stress during fermentation processes

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This study addresses challenges in bacterial fermentation, including monitoring the accumulation of cAMP, indicative of glucose availability. Through real-time measurements, we anticipate gaining insights into fermentation dynamics and facilitating the timely adjustment of process parameters.

Glucose is often used in these processes as a carbon source being readily metabolized by bacteria. Limitation of glucose in *E. coli* triggers the synthesis of cyclic AMP (cAMP) which serves as an activator for the transcription of other-sugar genes in the carbon catabolite regulation. Alterations in glucose availability and consequent shifts in gene regulation during the fermentation processes might decrease the productivity and product yield.

In this work, we make use of a biosensor based on a circularly permuted fluorescence protein (cpFP)[1] previously developed for real-time monitoring of cAMP in living eukaryotic organisms[2]. The biosensor comprises a rearranged fluorescent protein (cpGFP) fused to a cAMP-binding domain, the cyclic nucleotide-binding domain (CNBD) derived from a gram-negative bacterium, *Mesorhizobium loti*. Upon binding to cAMP, CNBD changes conformation and enhances fluorescence emission, enabling real-time detection of cAMP concentration in the cell.

Circularly permuted fluorescent proteins represent a class of rearranged proteins that maintain their native fold and function while offering versatility in sensor design. Although extensively studied in eukaryotic cells, particularly mammalian systems, our work introduces the application of cpFP-based biosensors in bacteria. The cpFP-based biosensor is therefore expressed in a low copy plasmid vector suitable for *E. coli*, *B. subtilis* and lactic acid bacteria, and the detectable shifts in fluorescence intensity were observed from a constitutively high transcribed promoter.

Quantification of fluorescence intensity (or cAMP synthesis) was followed during exponential growth in micro bioreactors (Biolector II) in a population of cells exposed to different growth conditions, with or without glucose. Our preliminary results focus on the functionality of cpFP-based biosensors in *E. coli*. These biosensors were expressed through highly expressed-inducible promoters. Further enhancements to the biosensors involve identifying bacterial promoters that alleviate cellular burden and adjusting the metabolite binding site to match the range of intracellular metabolite levels. Once optimized, these biosensors hold the potential to monitor and provide insights into cellular stress at a single-cell level.

*Keywords*: Biosensor, circularly permuted fluorescence protein, carbon catabolite regulation.

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# Enzymatic dimerization of 4-hydroxyphenethyl acetate with different co-solvents

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Expanding on previous studies that showed the dimerization of tyrosol acetate (**1)** to compounds **2** and **4** in a biphasic system, catalyzed by laccase from *Trametes versicolor* in the presence of oxygen[1], we explored this reaction in various single and two-phase solvent systems. The aim was to find a solvent suitable for continuous dimerization in a microreactor having high surface-to-volume ratio and thereby enables efficient mass and heat transfer.

The solvent systems we investigated were acetate buffer with 20 % ethyl acetate, 20 % propylene glycol, 10 % acetone, and 25 % acetonitrile. The product compositions obtained in batch reactions differed depending on the solvent systems used. NMR analysis revealed the presence of a diphenylether dimer (**2**), 1,1´-dityrosol-8,8´-diacetate (**3**), and a Pummerer ketone (**4**).

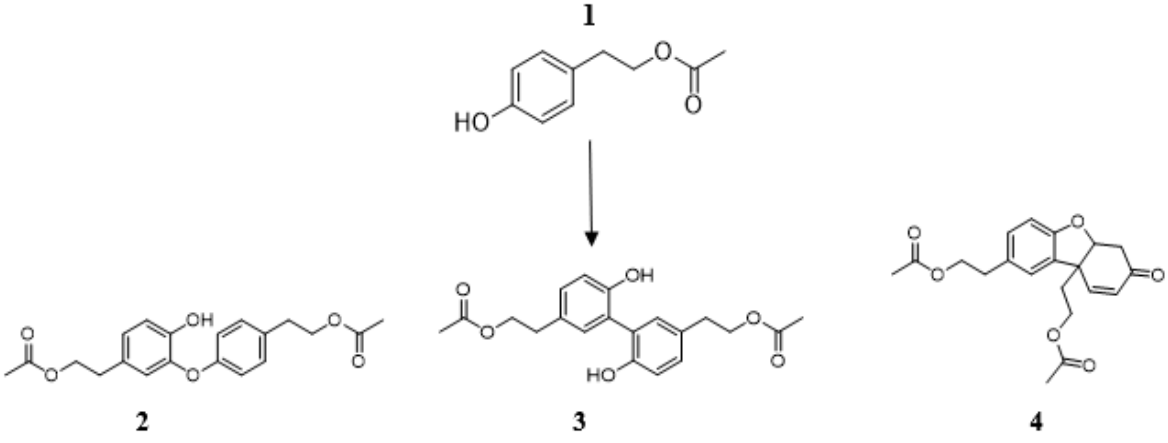


Figure 1: Dimerization of tyrosol acetate (**1**) to diphenylether dimer (**2**), 1,1`-dityrosol-8,8`-diacetate (**3**) and a Pummerer ketone (**4**).

Besides laccases, peroxidases are known to catalyze oxidation of simple phenol derivatives using $H_2O_2$, reporting also the generation of compound **3**[1,2]. In our study, reaction of tyrosol acetate catalyzed with horseradish peroxidase (HRP) in a batch biphasic ethyl acetate/acetate buffer system resulted in a 100 % conversion after approximately 4 hours. The primary product that emerged was compound **3** (Figure 1), recognized for its bioactive properties[3]. Both products and substrate were soluble in this biphasic system.

On the other hand, when the same reaction was performed in a monophasic system comprising acetate buffer and acetone, the product precipitated and the reaction mixture turned white (Figure 2), while the substrate remained soluble. NMR analysis of the precipitate showed that only compound **4** was present in this reaction mixture. This result highlights the opportunity to direct the reaction to individual product by carefully choosing a solvent system.

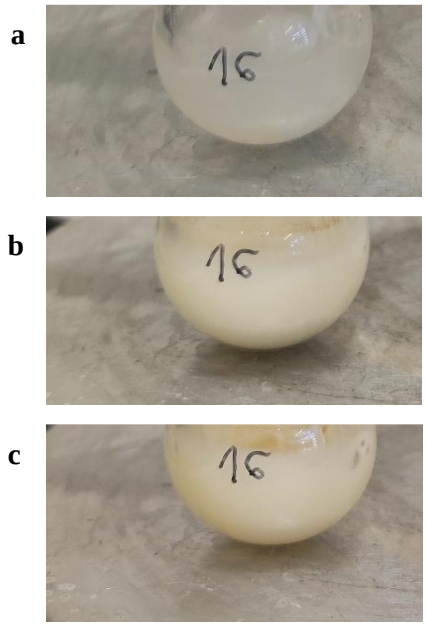


Figure 2: Reaction mixture during the reaction conducted in 20 mM acetate buffer with 10 % acetone, pH 3.5, at reaction time a) 0, b) 1, and c) 5 h at 500 rpm and room temperature.

*Keywords*: laccase, horseradish peroxidase, tyrosol acetate, biotransformation, solvent selection

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# *Bacillus* sp. endospore assisted biosensor for fast antioxidant capacity measurement in a microfluidic device

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The development of efficient biosensors to monitor bioactive molecules such as antioxidants and phenols in food is crucial. Conventional techniques such as HPLC detection are often time-consuming, and determining the concentration of a single component may not accurately reflect the total antioxidant capacity of a biological sample due to synergistic effects. Biosensors offer a portable and user-friendly alternative for measuring the cumulative capacity of antioxidants in food[1].

Genetically engineered bacteria-based sensor systems have shown promise in various analyzes due to their selectivity, sensitivity and ease of use. However, their widespread use is hampered by the limited survivability of the bacteria, especially under harsh environmental conditions. The spores produced by *Bacillus* species exhibit high resistance to environmental stressors and long-term stability, making them excellent candidates for biosensor development[2].

Recent advances include a batch antioxidant assay based on *Bacillus* sp. spores, which streamlines the process by eliminating the need to remove the spores. This assay is based on the oxidation of the colourless compound 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) to form a coloured radical cation ($ABTS^{\bullet+}$) in the presence of dissolved oxygen. The catalytic activity of the CotA protein, found in the endospore coat of *B. subtilis*, facilitates this oxidation reaction. Antioxidants added to the system reduce the $ABTS^{\bullet+}$ radical back to its colourless form, allowing the determination of antioxidant capacity based on the observed colour change. The use of endospores instead of purified enzymes reduces process costs and time[3].

In this study, the biosensor system is further improved by development of a continuous flow-through system using immobilized endospores. Endospores of *B. subtilis* were immobilized using magnetic microparticles within magnetic field-assisted microreactors. Functionalized magnetic microparticles were mixed with an endospore suspension in a citrate-phosphate buffer and introduced into a microfluidic device equipped with permanent magnets. Two syringe pumps provided a continuous flow of ABTS and samples. Filtered samples of various commercially available fruit juices and green teas were injected into a 15 µL loop and flushed into the system with the ABTS buffer solution. The combined streams were then analyzed in a flow-through spectrophotometer to detect changes in absorbance. The proposed antioxidant capacity assay compares the ability of a sample to scavenge $ABTS^{\bullet+}$ with that of a standard antioxidant (Trolox) using a calibration curve.

*Keywords*: endospores, *Bacillus subtilis*, whole-cell biosensor, antioxidant capacity

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**Design of biocatalytic oxidative reactions with deep-eutectic solvents**

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In a bio-based economy, the sustainability of commodity chemicals production could substantially benefit form the usage of second-generation renewable biomass, such as lignocellulosic and lignin derived compounds. To fully harvest these resources in the spirit of green chemistry a biocatalytic approach is desirable. Laccase, a copper-dependent oxidase, exhibits considerable commercial interest for its broad substrate specificity and use of dioxyge ($O_2$) to oxidise a wide spectrum of compounds, among which lignin-derived phenols play an important role. Currently, the main challenges yet to be overcome are the solubility of phenolic compounds in water and the supply of molecular oxygen for the reoxidation of laccases. To better handle substrate solubility, the introduction of non-conventional media to enhance substrate loadings may become a straightforward solution for process intensification. Deep eutectic solvents (DES) have recently emerged as an alternative to conventional solvents, offering cost effectiveness, biodegradability, biocompatibility, and sustainability. With this aim DECADES (DEsign of CAtalytic processes with Deep-Eutectic-Solvents) was conceived doctoral network project funded by the European Union, dedicated to exploring the potential of DESs in biocatalysis.

Although the usage of DES/DES-water media is promising regarding substrate solubility and solvent design, it seems to magnify issues related to mass transfer rather than resolving them. Hence, solutions and ideas are to be explored in advanced reactor designs which engage in facilitating oxygen mass transfer in non-aqueous and relatively viscous media. DES-based processes could be augmented and intensified by the usage of microreactor technology, with their potential to work under continuous operational mode, higher surface-to-volume, mass transfer rates and the ease of throughput increasing. Combining this reaction setup with active and stable heterogeneous catalyst could prove to be the right approach towards intensified biocatalytic processes in DESs. With the knowledge of mass transfer characteristics and reaction kinetics one can then judge whether a use of microreactor is justified and to what extent when designing DES-based biocatalytic oxidative reactions.

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