FINAL REPORT

1 General Information

DFG reference number: **HO 5667/10-1 (Dirk Holtmann), LI 899/18-1 (Andreas Liese)**

Project number: **465474720**

Project title: **Enzyme and reaction engineering of unspecific peroxygenase driven hydroxylation of butane (BUPOx)**

Name(s) of the applicant(s): **Prof. Dr. rer. nat. Andreas Liese, Prof. Dr.-Ing. Dirk Holtmann**

Official address(es):

Dirk Holtmann, Karlsruher Institut für Technologie (KIT) Institut für Bio- und Lebensmitteltechnik (BLT) Bereich Elektrobiotechnologie, Fritz-Haber-Weg 4, 76131 Karlsruhe, Tel.: 721 608 421 31

Andreas Liese, Technische Universität Hamburg, Institut für Technische Biokatalyse, Denickestr. 15, 21073 Hamburg, Tel.: 040-42878-3018

Reporting period (entire funding period):
1.10.2021 - 31.3.2025 (incl. cost-neutral extension)

2 Summary

2.1 Zusammenfassung

In diesem Projekt wurden skalierbare biokatalytische Prozesse entwickelt, bei denen Unspezifische Peroxygenase (UPO) zur selektiven Oxyfunktionalisierung eingesetzt wird. Dabei wurden die wichtigsten Herausforderungen in Bezug auf Enzymproduktion, und Stabilität, kontrollierte, elektrochemische Erzeugung von Co-Substraten, *in situ* Produktentfernung und halbkontinuierliche Operabilität adressiert. Eine robuste UPO-Expression und Downstream-Strategien ermöglichten einen kontinuierlichen Betrieb, während eine detaillierte kinetische Charakterisierung zeigte, dass ein präzises Gleichgewicht zwischen Substrat- und Wasserstoffperoxidkonzentrationen erforderlich ist, um eine oxidative Inaktivierung zu verhindern. Immobilisierte UPOs wie His-Tag und Twin-Strep-markierte sowie YSD-Varianten (yeast surface display), erreichten eine hohe Aktivitätserhaltung und

Betriebsstabilität in Wirbelschichtsystemen, was längere Reaktionszyklen und eine vereinfachte Produktrückgewinnung ermöglicht.

Eine geregelte *in situ* Erzeugung von Wasserstoffperoxid (H_2O_2) wurde durch optimierte elektrochemische Systeme mit Gasdiffusionselektroden etabliert, die hohe Faraday Effizienzen (>95 %) erzielten. Die im Projekt etablierte *inline* Peroxidmessung in Kombination mit einer Closed-Loop-Steuerung ermöglichte niedrige konstante H_2O_2-Konzentrationen, wodurch eine hohe Enzymstabilität erzielt wurde. Eine *in situ* Produktabtrennung mithilfe einer Festphasenadsorption auf Aktivkohle führte zu einer skalierbaren Rückgewinnung ohne deaktivierenden Einfluss auf das Enzym. Tests zur Enzymstabilität zeigten eine starke Abhängigkeit von der Immobilisierungsumgebung und der Lösungsmittelexposition, wobei einige YSD-Systeme sogar eine lösungsmittelinduzierte Aktivierung zeigten. Die Integration dieser Ansätze führte zu deutlich erhöhten *total turnover numbers* (TTN), volumetrischer Aktivität und Prozessrobustheit. Die mechanistischen Erkenntnisse, kombiniert mit elektrochemischer Co-Substratversorgung, Immobilisierungstechniken und Produktabtrennung, bieten einen Rahmen für skalierbare oxidative Biotransformationen und zeigen die Machbarkeit der UPO-Katalyse in industriellen Prozessumgebungen.

2.2 Summary

In this study, scalable biocatalytic processes were developed in which unspecific peroxygenase (UPO) is used for selective oxyfunctionalization. The most important challenges in terms of enzyme production and stability, controlled electrochemical generation of co-substrates, *in situ* product removal and semi-continuous operability were addressed. Robust UPO expression and downstream strategies enabled continuous operation, while detailed kinetic characterisation showed that a precise balance between substrate and hydrogen peroxide concentrations is required to prevent oxidative inactivation. Immobilised UPO such as His-tag, Twin-Strep-labelled and YSD (yeast surface display) variants, achieved high activity retention and operational stability in fluidised bed systems, enabling longer reaction cycles and simplified product recovery.

Controlled *in situ* production of hydrogen peroxide (H_2O_2) was established using optimised electrochemical systems with gas diffusion electrodes, which achieved high Faraday efficiencies (>95%). Inline peroxide measurement combined with closed-loop control ensured low, stable concentrations that maintained enzyme activity over a long period of time. In situ product removal using solid-phase adsorption on activated carbon resulted in scalable recovery without deactivating the enzyme. Enzyme stability tests showed a strong dependence on the immobilisation environment and solvent exposure, with some YSD systems even

showing solvent-induced activation. The integration of these approaches resulted in significantly improved total turnover numbers (TTN), volumetric activity and process robustness. The mechanistic insights, combined with electrochemical co-substrate supply, immobilisation techniques and product separation, provide a framework for scalable oxidative biotransformations and demonstrate the feasibility of UPO catalysis to industrial process environments.

3 Progress Report

Work Package 1 (WP1) focused on establishing robust production methods and thoroughly characterizing the catalytic behavior and stability of the unspecific peroxygenase (UPO) for selective oxyfunctionalization. Recent studies revealed that proton transfer from H_2O_2 triggers active site rearrangement for catalysis, while substrate binding slows formation of the reactive Compound I intermediate. Thus, balancing substrate and H_2O_2 availability is key to effective UPO catalysis and high total turnover numbers (TTN), beyond simply controlling peroxide levels. WP1 therefore optimized continuous UPO catalysis under controlled, stable H_2O_2 supply based on this mechanistic understanding. In collaboration with the Franzreb group at KIT, the twin-Strep-tagged® UPO immobilized on solid carriers (see also WP5) was utilized in a fluidized packed-bed reactor for the hydroxylation of ethyl benzoic acid. Enhancing enzyme load and substrate feed rate substantially improved process metrics, extending catalytic operation from 12 hours to over 30 hours, increasing Faraday efficiency from ~64% to ~72%, and achieving TTNs exceeding 740,000. Productivity (~2 mM h^{-1}) and space-time yields (~60 g L^{-1} d^{-1}) remained constant, limited by upstream electrochemical peroxide generation rates. To support these aims, WP1 developed scalable enzyme production. A 2.4 L fine-bubble fermenter was retrofitted with controlled feed strategies, inspired by earlier pilot fermentations . The standard microbial fermentation comprised three stages of in total about 170 h including high-cell-density cultivation, as well as enzyme induction phase lasting. Recognizing the lengthy process and relatively constant productivity after initial phases, a repetitive batch fermentation strategy was developed. Parallel investigations across multiple institutions (KIT, THM, TUHH) and scales (shaking flasks to bioreactors) revealed that although absolute productivity varied between systems (~0.05 to ~0.2 nmol r*Aae*UPO/(g_{cdw}·h)), cumulative productivity remained stable over multiple cycles, only declining significantly after approximately 28 days. This approach promises savings in time and resources while reducing contamination risk and process complexity. Following centrifugation of the fermentation broth, sterile filtration was implemented using a 0.2 µm cross-flow membrane, succeeded by volume reduction through ultrafiltration (10 kDa cut-off) to concentrate the UPO fraction to roughly 10% of initial volume. Isochoric diafiltration with sterile water removed residual contaminants

(organic acids, salts), and final rebuffering stabilized the enzyme in 50 mM potassium phosphate buffer (pH 7). The cumulative process entailed an average activity loss of ~40%, yielding a stable crude extract suitable for downstream applications. A defining challenge addressed by WP1 was the enzyme's pronounced sensitivity to oxidative inactivation by H_2O_2. Prior studies revealed that unregulated peroxide feeding engendered abrupt enzyme deactivation beyond poorly defined thresholds. This motivated development of a custom electrochemical H_2O_2 generation and monitoring system integrated within a bubble column reactor, combining a proprietary GDE, an *in situ* Prominent Perox H3E sensor, and a programmable logic controller with a controlled feedback loop. This setup enabled precise, real-time peroxide dosing matched to enzymatic demand. Batch-mode stability assays demonstrated a steep peroxide-dependent decline in activity, with half-life decrease from 472 minutes without peroxide to 47 minutes at 10 µM H_2O_2 and 6.8 minutes at 100 µM. Critically, peroxide concentrations above ~50 µM markedly reduced TTN, establishing ~10 µM as a technical lower bound for stable operation. Considering the concurrent substrate limitations imposed by the poor aqueous solubility of gaseous *n*-butane (~0.0011 mol/(kg·bar)), WP1 investigated gas-liquid mass transfer properties. Bubble size (Sauter diameter) and gas holdup were studied under varying buffer strength, pH, temperature, pressure, gas flow rates, and product concentrations (2-butanol, butanone) using optical probes and image analysis. Both *n*-butane and air showed comparable trends, with buffer concentration and product levels positively correlating with interfacial area (a). Additional significant influences included pressure and butanone concentration for *n*-butane, and pH and temperature for air. Due to similar Henry's constants for butane and oxygen, volumetric oxygen transfer coefficient ($k_L\alpha$) trends measured with air were deemed transferable to butane systems. The process window analysis examined the effects of buffer concentration, pH, gas flow rate, temperature, and enzyme loading on key metrics: TTN, turnover frequency (TOF), and selectivities for peroxygenase vs. catalase and hydroxylation vs. over-oxidation. Temperature enhanced TOF, peaking near 40 °C, but caused TTN decline due to thermal inactivation, while catalase selectivity remained stable. Buffer concentration optimized TTN at ~50 mM by balancing substrate availability and ionic strength-related deactivation. pH effects were minor, with a slight TOF decrease between pH 6.0 and 6.5. Gas flow rate linearly increased TTN, especially at low enzyme loading, highlighting substrate limitation; catalase selectivity peaked at moderate flow rates. Selectivity for secondary oxidation remained low under all conditions. Overall, optimal conditions balancing turnover, stability, and selectivity involve moderate temperature (~25 °C), high gas flow (0.75 L/min), pH 6.5, buffer around 50 mM, low enzyme concentration (~0.075 µM), and low peroxide levels (~10 µM). To dissect molecular

determinants of substrate binding and regioselectivity, in silico docking studies employed *Aae*UPO (PDB 5OXU) and multiple substrate classes: linear alkanes (butane, pentane, hexane), cyclic alkanes (cyclohexane), diamondoids (adamantane, diamantane), aromatic derivatives (benzene, ethylbenzene), and surrogate substrates (5-nitro-1,3-benzodioxole, NBD). Docking identified three phenylalanines (F69, F121, F199) near the active site coordinating substrate positioning, and four lining phenylalanines (F76, F188, F191, F274) in the substrate access channel, consistent with literature correlating access channel composition with substrate preference. Notably, “short” UPOs prefer aliphatic substrates and possess aliphatic residues in equivalent positions. Mutagenesis targeted active site phenylalanines, substituting them by aliphatic residues (glycine, alanine, leucine, isoleucine, valine) to shift substrate affinity. Docked variants predicted altered substrate affinity constants (ka), with, for example, F69L anticipated to reduce aromatic affinity and enhance aliphatic binding. Experimental validation via yeast surface display (YSD) and screening for hexane hydroxylation revealed low or no hexane activity, contrary to predictions. Six top variants were produced at scale and kinetically characterized, showing decreased hexane activity relative to wildtype and unchanged stereoselectivity. Nonetheless, select variants exhibited increased activity towards NBD and ABTS substrates, with some demonstrating altered substrate preferences. These findings elucidate structure-function relationships between specific amino acids near the heme center and substrate affinity, providing a foundation for future enzyme engineering despite incomplete validation of initial hypotheses. WP2 and WP3 focused on developing and integrating an efficient product removal strategy within the bubble column process. The enzyme activity and stability in organic solvents typically used to dissolve UPO substrates, such as acetone, acetonitrile (ACN), and dimethyl sulfoxide (DMSO). Seven different YSD UPO systems were subjected to varying solvent concentrations to measure residual enzyme activity (with solvent present during assay) and stability (post-incubation in 50% solvent, assayed without solvent). Free PaDa-I enzyme exhibited a concentration-dependent activity decrease with acetone, ACN, and DMSO, achieving 50% relative activity (C50) at approximately 9%, 7%, and 1% (v/v), respectively, consistent with prior literature. Among the YSD systems, SAG1 showed slightly improved tolerance with C50 values of 9%, 9%, and 1.5%. Other systems displayed varied solvent tolerances: FLO1-1447 was sensitive to acetone and ACN but tolerated up to 10% DMSO; FLO1-428 tolerated acetone concentrations up to 15% with a C50 of 18%, performing comparably to SAG1 in ACN. Systems SAG1, FLO1-428, and PIR1 P41 exhibited high stability in acetone, whereas ACN resulted in poor stability for most YSD systems, except SAG1 and PIR1 P41, which retained 15% and 37% activity after 24 hours, respectively. Remarkably, all YSD systems maintained or exceeded free enzyme stability in DMSO, with PIR1 P41 reaching 200% residual activity

after 48 hours. During stability assessment, baseline activity was defined using immediate mixing with 50% organic solvent to standardize starting conditions. Minor solvent concentrations (0.75% v/v) introduced by sampling affected activities differently across systems: while SAG1 remained unaffected, free PaDa-I was slightly inhibited, and other YSD systems showed activation, particularly PIR1 P41, which increased activity approximately sixfold at 0.75% acetone. The observed activation of PIR1 systems by organic cosolvents, alongside moderate temperature and low pH stimuli, is notable. Possible mechanisms include stress-induced refolding of misfolded UPO, leading to actual enzyme activation, or apparent activation via the anchor system unique to PIR1. PIR1 enzymes are attached to β-1,6-glucan, dominating yeast cell wall glucans, potentially limiting diffusion. These comprehensive studies on extraction strategy and enzyme solvent tolerance informed the product removal integration and catalyst development within the bioprocess. The solvent stability results have been published (2024, https://doi.org/10.1002/cctc.202400908). The criteria for solvent evaluation included solubility and selectivity for 2-butanol over substrates and enzymes, aqueous phase compatibility, and ease of subsequent separation steps. Screening of representative solvents revealed that they showed better solubility and binding affinity onto the enzymes active side than *n*-butane. Additionally, solvents with amphiphilic or protic character negatively interacted with the enzyme active site, potentially displacing the gaseous substrate and impairing catalysis. Consequently, organic solvents were deemed unsuitable as extraction media for maintaining enzymatic process integrity. To address these limitations, adsorption-based extraction was investigated, with activated carbon identified as a highly promising sorbent due to its large specific surface area, chemical inertness, and granular form facilitating reactor integration. Adsorption isotherms of 2-butanol onto activated carbon were measured under varying pH and temperature conditions, exhibiting fits to both Langmuir ($R^2 \approx 0.98$) and Freundlich ($R^2 \approx 0.93$) models. These results suggested complex adsorption behavior with a predominance of monolayer adsorption over the tested parameter range. Notably, low product concentrations (<5 mM) showed negligible effects of temperature or buffer strength on adsorption capacity, favorable for steady-state bioreactor operation. Thermogravimetric analysis of 2-butanol-loaded carbon revealed a bimodal desorption profile with distinct peaks at 87 °C and 135 °C, corresponding to water and azeotropic desorption of 2-butanol and water, respectively. This differentiation allowed for selective product recovery post-adsorption. Subsequent headspace gas chromatography and Karl-Fischer titration confirmed significant water removal during heating, yielding desorbed fractions enriched in 2-butanol. Kinetic studies demonstrated rapid uptake of 2-butanol by activated carbon within 15–45 minutes, reaching equilibrium around 90 minutes. Breakthrough experiments established a linear relationship between feed flow rates and product removal, with a slope of approximately 0.404 µmol/mL.

Process simulations under continuous production at varying rates (2, 6, and 18 mmol/(L·h)) indicated efficient extraction at lower production rates, maintaining reduced reactor product concentrations and high extraction efficiencies over one hour. However, higher production rates led to decreased efficiency due to sorbent saturation. Integration of the activated carbon adsorption unit with the bubble column reactor was straightforward, showing no measurable enzyme loss or deactivation and minimal pressure drop, thereby supporting scalability. These findings led to the selection of adsorption-based extraction as the preferred method, effectively balancing product recovery, enzyme stability, and reactor compatibility, with potential future improvements involving regenerable adsorption columns and continuous in-line desorption.

WP4 developed a precise, scalable electrochemical method for *in situ* H_2O_2 generation using gas diffusion electrodes (GDEs) integrated into a bubble column reactor to maintain low, steady peroxide levels compatible with UPO biocatalysts. A first-generation flow-cell GDE module showed peroxide production rates of 0.14–1.47 µmol/min but suffered from spatial inhomogeneity, large loop volumes, and concentration gradients. To overcome these issues, a second-generation integrated cylindrical GDE reactor was designed and optimized via central composite design, assessing variables including membrane area, voltage, buffer pH/concentration, and temperature over 63 experiments. Optimal conditions (2.4–2.5 V, >30 °C, 100–200 mM buffer) achieved peroxide generation rates >2.3 µmol/s and Faraday efficiencies up to 96.9%, closely matching UPO requirements. The integrated design improved flow uniformity, minimized dead volume, and enabled closed-loop peroxide control using real-time sensing and PI feedback regulation. Validation with a prototype electrode (1750 mm² membrane area) confirmed high Faraday efficiencies (>95%) and stable peroxide production (>1.3 µmol/s) across tested conditions. Higher temperatures enhanced performance, while buffer concentrations above 200 mM reduced efficiency, defining an optimal operating window for robust, controllable H_2O_2 supply. Complementary mixing studies employed a tracer-based method using bromothymol blue and hydrochloric acid, analyzed via a computer vision algorithm, to assess macroscopic transport effects within the bubble column. Measured mixing times of 4.5 to 6.5 seconds at gas flow rates of 0.5 to 1.5 L/min confirmed adequate convective mixing to prevent peroxide accumulation and associated enzyme deactivation hotspots.

WP5 focused on enzyme immobilization strategies to enable in situ product separation and stabilize long-term operation of biocatalytic processes using unspecific peroxygenases (UPOs). The objective was to improve enzyme reusability and process economics by enhancing enzyme stability while maintaining catalytic performance. Three immobilization methods, covalent, adsorptive, and affinity-based, were tested using SUNRESIN carriers with tailored surface functionalities. Covalent immobilization on Seplife EMC7225M achieved the highest

enzyme loading per gram of carrier under dilute conditions (weight wet carrier per enzyme vector of 1:71), but with a modest yield of approximately 39%, indicating binding site saturation near 50 mg enzyme per gram of wet carrier. Adsorptive immobilization on Seplife EMC1020 yielded significantly lower loading and was discarded. In contrast, affinity immobilization leveraging a HIS-tagged UPO on Seplife 7350 resin achieved high yield (>80%) and enabled enzyme recovery, making it the preferred approach for application in bubble column reactors. Despite improved stability, immobilization caused a notable decline in catalytic activity: the immobilized enzyme exhibited only 39% of the TTN and 9% of the tTOF compared to the free enzyme, primarily due to mass transfer limitations imposed by the carrier matrix. Nonetheless, the immobilized UPO retained approximately 72.3% residual ABTS activity after 166 minutes, confirming substantially enhanced operational stability. Importantly, product selectivity towards 2-butanol was maintained without overoxidation, indicating immobilization did not alter the reaction profile. These observations highlight the classical trade-off between enzyme stability and activity. Although immobilization improved robustness and enabled potential enzyme reuse, kinetic performance was sacrificed due to diffusion constraints, underscoring the need for improved carrier designs optimizing mass transfer. Enzyme cost represents a major barrier to industrial UPO applications, especially for lower-value products. Maximizing product yield per enzyme mass combined with minimizing enzyme production expenses is essential for economic viability. Immobilization can increase product per enzyme via stabilization and enzyme recycling in continuous processes, but often incurs added downstream processing costs, e.g., purification, concentration, and carrier expenses, while potentially reducing enzyme activity and immobilization efficiency. Previously published immobilization approaches for *Aae*UPO generally yielded less than 10% activity recovery, questioning their practical applicability. Addressing this, WP5 explored YSD of UPO PaDa-I on the cell wall of its production host *Komagataella phaffii* (*Pichia pastoris*) as a cost-effective immobilization strategy without additional downstream burden. Seven anchoring systems were engineered and expressed in *K. phaffii*; their activities and stabilities were evaluated under diverse temperatures, pH values, and organic cosolvent conditions typical for UPO catalysis. Among these, a fusion with the C-terminal half of α-agglutinin from *Saccharomyces cerevisiae* emerged as a superior immobilization approach, delivering activity yields of approximately 80%, a significant improvement compared to prior methods. The YSD-UPO approach offers streamlined downstream processing, as cells can be separated from culture broth directly post-fermentation, arguably simplifying processing relative to free enzyme production. The immobilized cells demonstrated remarkable operational stability during repeated fed-batch catalysis over 13 cycles totalling over 200 hours, achieving TTNs exceeding 300,000. These promising results were published (doi.org/10.1002/cctc.202400908). Subsequently, production

optimization focused on the YSD-UPO system, employing both shaking flask and bioreactor cultivations. A one-factor-at-a-time approach improved volumetric activity by 60% in shaking flasks by reducing expression temperature and inducing protein production at lower optical densities. In 5 L stirred bioreactors, volumetric yields more than doubled when complex media replaced minimal media recommended by Invitrogen's guidelines. For high-throughput screening, YSD-UPO production was successfully adapted to parallelized micro-bioreactors (Biolector XT®), applying design-of-experiments approaches to optimize protein expression. Storage stability under various conditions was characterized, and an effective sterilization method preserving enzyme activity was developed, involving resuspension and inversion of YSD-UPOs in isopropanol. These production optimizations are currently being prepared for publication. During dissemination, a notable challenge was the absence of a universal metric to compare immobilized enzyme processes (IEPs) effectively. Published research typically focuses on isolated IEP aspects, such as immobilization efficiency, neglecting upstream enzyme production or its inherent specific activity. To address this, WP5 devised a comprehensive evaluation methodology dividing IEPs into seven distinct process phases from enzyme modification and production to immobilization and catalysis, each characterized by key performance indicators (KPIs). Data from these phases are condensed into four "meta KPIs," providing simplified but holistic process comparisons. This framework was validated by case studies comparing IEPs for alcohol dehydrogenases and UPOs (https://doi.org/10.1002/cctc.202500699). In another immobilization approach exploiting streptavidin biotin affinity, the strongest known non-covalent protein interaction a UPO variant (PaDa-I) was cloned with a twin-Strep-tag® and expressed as a secreted enzyme. Immobilization employed commercial Strep-Tactin®XT 4Flow® resin. Various purification levels were tested prior to immobilization, from crude culture broth to affinity-purified enzyme, identifying the optimal step balancing yield and activity. The tagged enzyme retained high specific activity (714 U/mg, 86% of wild type), and immobilized preparations maintained 685 U/mg (83%). Total immobilization yield reached 12%, the highest reported besides the YSD immobilization. This immobilized enzyme was applied in a fluidized bed reactor coupled with electrochemical *in situ* H_2O_2 generation in collaboration with the Franzreb group. Continuous catalysis of ethyl benzoic acid hydroxylation showed increased productivity and TTN relative to batch modes with free and immobilized enzyme. These findings are currently being prepared for publication.

In summary, BUPOx met all objectives, establishing scalable biocatalytic protocols and reactor concepts. The project generated key datasets and revealed mechanistic insights into UPO stability, substrate interactions, and the role of immobilization in process performance.

The research data is stored at the TUHH or at the KIT in a redundant manner on a network drive and a backed-up server at the computer centre for the required period of 10 years. In addition, published data will be made available upon request.

4 Published Project Results

4.1 Category A – Articles in peer-reviewed journals, contributions to peer-reviewed conferences or to anthology volumes, and book publications

1) Stöckl, M., Lange, T., Izadi, P., Bolat, S., Teetz, N., Harnisch, F., & Holtmann, D. (2023). Application of gas diffusion electrodes in bioeconomy: An update. Biotechnology and Bioengineering, 120, 1465–1477. https://doi.org/10.1002/bit.28383
2) G. Sayoga, M. Abt, N. Teetz, V. Bueschler, A. Liese, M. Franzreb, D. Holtmann, Quantitative and Non-Quantitative Assessments of Enzymatic Electrosynthesis: A Case Study of Parameter Requirements. ChemElectroChem 2023, 10, e202300226. https://doi.org/10.1002/celc.202300226
3) N. Teetz, S. Lang, A. Liese, D. Holtmann, Yeast Surface Display Enables One-Step Production and Immobilization of Unspecific Peroxygenases. ChemCatChem 2024, 16, e202400908. https://doi.org/10.1002/cctc.202400908
4) N. Teetz, A.-L. Drommershausen, L. Gebele, D. Holtmann, Holistic Evaluation of Enzyme Immobilization Processes: A Method for Evaluating the Entire Production Process. *ChemCatChem* 2025, 17, e00699. https://doi.org/10.1002/cctc.202500699
5) N. Teetz, L. Zuhse, D. Holtmann, Optimizing Yeast Surface Displayed Unspecific Peroxygenase Production from Screening to Lab Scale. *Bioengineering* 2025, 12(8), 822;

4.2 Category B – Any other form of published results

1) *Poster:* F., Kelsch, N., Teetz, P., Bubenheim, D., Holtmann and A., Liese; Enzymatic oxidation of butane; International Congress on Biocatalysis (2022); Hamburg
2) *Poster:* F., Kelsch, V., S., Bueschler, N., Teetz, G., V., Sayoga, D., Ohde, P., Bubenheim, F., Hollmann, D., Holtmann, A., Liese; Production Improvement of Unspecific Peroxygenase by Repetitive Batch Fermentation of Pichia pastoris; Federation of European Microbiological Societies Congress (2023); Hamburg
3) *Poster:* F., Kelsch, V., S., Bueschler, L. Klose, N., Teetz, G., V., Sayoga, D., Ohde, P., Bubenheim, F., Hollmann, D., Holtmann, A., Liese; Production Improvement of Unspecific Peroxygenase by Repetitive Batch Fermentation of *Pichia pastoris*; FSP Biobased Process and Reactor Technologies (2023); Hamburg
4) *Lightning Lecture and Poster:* N., Teetz, S., Lang, D., Holtmann; One-step production and immobilization approach for unspecific peroxygenases; 16th International Symposium on Biocatalysis and Biotransformations (2023); La Rochelle
5) *Poster:* F., Kelsch, G., Schultz, K., Roth, L., Schumann, P., Bubenheim, R., Krull and A., Liese; Investigating Phase Interface Dynamics in a Bubble Column Reactor; DECHEMA Himmelfahrtstagung on Bioprocess Engineering (2024); Regensburg
6) *Lecture:* N., Teetz, S., Lang, A., Liese, D., Holtmann; One-step production and immobilization approach for unspecific peroxygenases; DECHEMA Himmelfahrtstagung on Bioprocess Engineering (2024); Regensburg
7) *Lecture:* F., Kelsch, L. Klose, K., Roth, N., Teetz, G., Schultz, R., Krull, D., Holtmann, P., Bubenheim, A., Liese; FSP Biobased Process and Reactor Technologies (2024); Hamburg
8) *Lightning Lecture and Poster:* F., Kelsch, G., Schultz, K., Roth, M., Steffen, P., Bubenheim, R., Krull and A., Liese; Reaction Dynamic of Enzymatic Butane Hydroxylation in a Bubble Column Reactor; International Congress on Biocatalysis (2024); Hamburg
9) *Lecture:* N., Teetz, S., Schönrock, D., Holtmann; Engineering the Access Channel of an Unspecific Peroxygenase from *Agrocybe aegerita*; International Congress on Biocatalysis (2024); Hamburg
10) *Lightning Lecture and Poster:* F., Kelsch, G., Schultz, K., Roth, L., Schumann, P., Bubenheim, R., Krull and A., Liese; Investigating Gas-Liquid Mass Transfer for Biocatalytic Processes in Bubble Column Reactors; Jahrestreffen der Dechema / VDI-Fachgruppen Mischvorgänge, Hochdruckverfahrenstechnik und Mehrphasenströmungen (2025); Hamburg

4.3 Patents (applied for and granted)

1) F., Kelsch, A., Liese, P., Bubenheim, A., Solovey, Hamburg University of Technology (TUHH), Germany; *Dosiervorrichtungs-Gasdiffusionselektrode, zugehöriges Betriebsverfahren sowie Dosiervorrichtungs-*

Gasdiffusionselektrode-Reaktor-System und zugehöriges Betriebsverfahren; DE 10 2025 124 633.5; June 25, 2025; submitted and received by the German Patent and Trade Mark Office (DPMA)