Final Report under the Individual Grants Programme: Research Grants Programme

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FINAL REPORT

1 General Information

DFG reference number: LI 899/16-1 and GR 3461/10-1

Project number: LI 899/16-1 and GR 3461/10-1

Project title: Überwindung der Limitierungen von L-Threoninaldolasen in der Biokatalyse: Durch Reaktionstechnik zu hocheffizienten Anwendungen

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2 Summary

Threonine aldolases (LTA) have been demonstrated to be a synthetically valuable enzyme class for performing diastereoselective C-C forming reactions. Such biotransformations are of interest for the preparation of pharmaceuticals and fine chemicals as they catalyze asymmetric aldol reactions with glycine as readily available aldol donor, thus resulting in the formation of α-amino-β-hydroxycarboxylic acids. So far, a major limitation has been the non-favourable diastereoselectivity due to the switch from a kinetic to thermodynamic control of the biocatalytic reaction system. This project aimed for understanding and overcoming such process limitations.

At the start of this project, a comprehensive enzyme library of wild-type and mutant LTAs was constructed, and bioinformatic analysis confirmed structural conservation across variants. Several mutants exhibited enhanced *threo*-selectivity relative to wild-type enzymes, with the literature-known BnLTA-6xMut emerging as a lead candidate. The structural alignment of active sites indicated that catalytic cores were preserved, while mutations introduced localized changes that improved diastereoselectivity. To enable reliable process analytics, both off-line and in-line methods were established. Off-line HPLC with a chiral column and GC for benzaldehyde quantification complemented a novel in-line benchtop NMR system integrated with complemental hard modeling. This setup enabled continuous, non-invasive quantification of substrates and products, achieving agreement with high-field NMR and chromatographic methods. Kinetic characterization combined initial rate measurements with progress curve analysis (PCA). PCA allowed robust estimation of forward and reverse rate constants, even without pure standards, and was validated against experimental data and molecular docking simulations. These studies revealed the limits imposed by thermodynamic control, showing that mutants, despite their improved selectivity, cannot surpass equilibrium constraints and require operation under kinetic control with excess glycine. At the same time, a process window and optimized reaction conditions could be identified which enabled high conversion (>80%) in combination with high diastereoselectivity (>90% de). In addition, immobilization strategies were explored to improve enzyme reusability and process stability. Screening of 14 commercial carriers identified several supports with >90% immobilization efficiency, high relative activity, and recyclability over multiple cycles. In reaction engineering studies, free and immobilized enzymes were tested in stirred-tank, fed-batch, enzymatic membrane, and magnetically stirred rotating bed reactors. Fed-batch operation mitigated substrate inhibition and, together with immobilization, offered improved productivity, although further optimization of feeding strategies is required. Beyond process intensification, relaxometry (TD-NMR) was

introduced as a new, label-free method for quantifying enzyme immobilization within porous carriers. This provided insight into adsorption behaviour and pore filling beyond conventional photometric assays and formed the basis of a follow-up DFG proposal with the Institute of Process Imaging.

3 Progress Report

3.1 Identification and Library Construction of L-Threonine Aldolases (LTAs)

This section aimed to construct an enzyme library as a basis for selecting a biocatalyst with high diastereoselectivity. Based on literature data, an enzyme pool comprising 11 wild-type and 9 mutant LTAs was created. Recombinant expression in *E. coli* was established, and enzyme activity was proven with a spectrophotometric activity assay. Bioinformatic analysis confirmed overall structural conservation within the library. Sequence similarity and E-value matrices revealed scores ≥0.4 for all enzymes, suggesting at least moderate similarity, with most variants scoring higher. Active site conservation was further validated by 3D alignment using SWISS-MODEL and PyMOL. Mutant active site residues aligned well with the wild-type catalytic core, indicating that the overall framework remained intact.

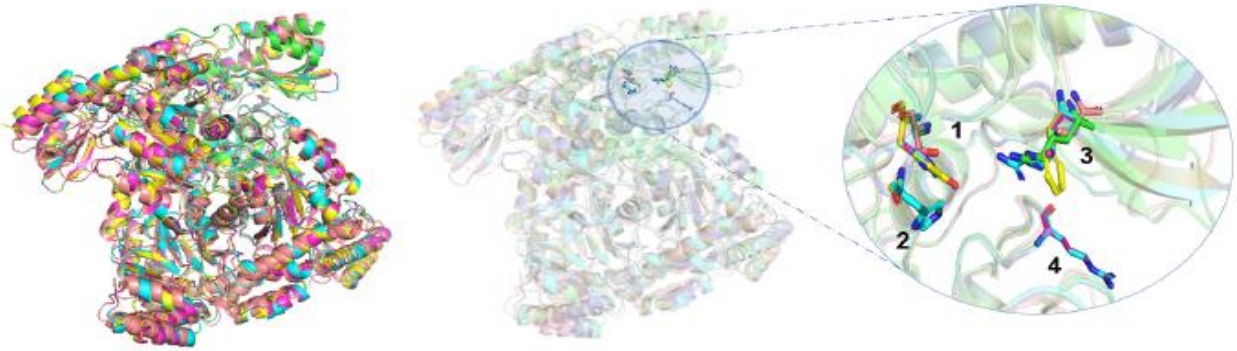


Figure 1. Structural alignment (left) of selected mutants: LmLTA-3xMut (yellow), BnLTA-6xMut (cyan), AhLTA-3xMut (green), CpLTA-Mut1 (magenta), and NmLTA-3xMut (wheat). Conservative active site marked with circle (center) and focused (right).

In conclusion, all candidates exhibited moderate to high structural and functional similarity. Mutant variants preserved the catalytic architecture while introduced changes might affect substrate recognition and binding kinetics. The alterations appear to underline the observed improvements in diastereoselectivity compared with wild-type enzymes, most likely through local conformational adjustments or altered electrostatic interactions in the active site.

3.2 In- and Offline Analytical Methods

A central objective was the development of a robust and process-compatible analytical platform for the real-time monitoring of enzymatic α-amino acid synthesis. To this end, a novel analytical approach based on in-line benchtop NMR spectroscopy was implemented and validated. The system was constructed by integrating a flow-through NMR cell into a recirculating loop connected to a stirred tank reactor (STR). This configuration enabled continuous acquisition of low-field 1H NMR spectra (80 MHz) throughout the course of the enzymatic reaction without requiring sampling. To extract quantitative information from the NMR data, the spectroscopic analysis was coupled with Complementary Hard Modeling (CHM), a chemometric deconvolution method that allows robust and accurate quantification of individual components in overlapping multiplet regions. The CHM model was built using reference spectra of all relevant species.

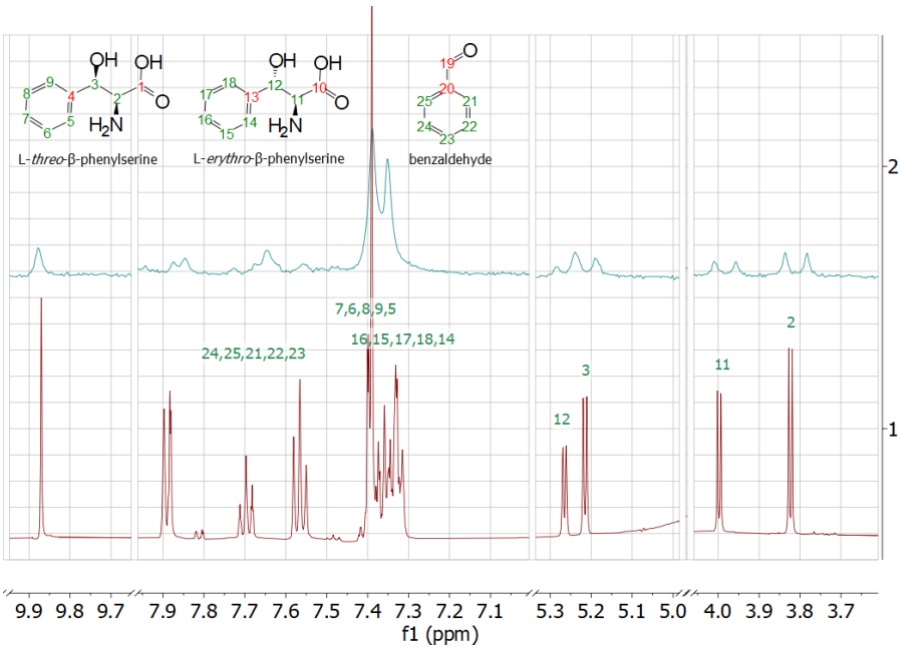


Figure 2: 1H NMR Spectrum of the aldol reaction. Starting conditions: 1 mol/L glycine; 100 mmol/L benzaldehyde in 20 vol% v/v DMSO in kalium phosphate buffer pH 8.0 V = 30 mL; T = 30 °C; sample taken after 5 h reaction time and top) measured by low-field NMR Spinsolve 80 MHz ULTRA bottom) measured by Bruker Avance I 600 MHz (AV700)

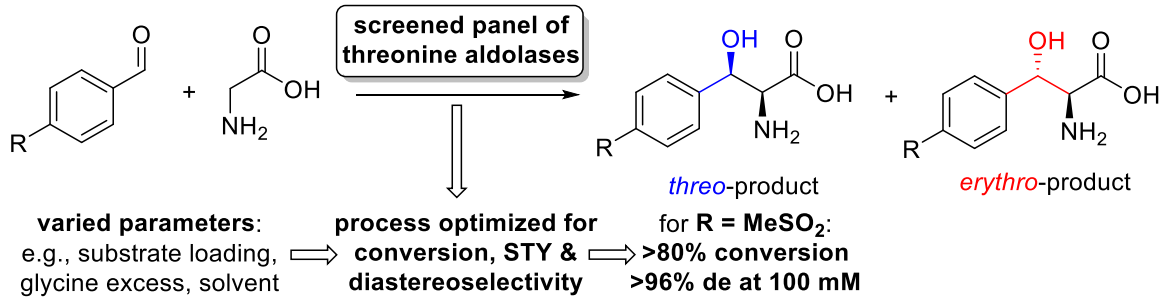
The in-line analytical system was capable of distinguishing between the two diastereomeric products, L-*threo*- and L-*erythro*-β-phenylserine, based on their chemical shift patterns. A spectrum recorded using the low-field NMR system Spinsolve 80 MHz ULTRA, which was employed to monitor the reaction, is presented in Figure 2 (top). For comparison, a spectrum acquired using the high-field NMR system Bruker Avance I 600 MHz is illustrated in Figure 2

(bottom). The low-field NMR spectrum was collected after 5 hours of reaction at a flow rate of 0.3 mL/min. This enabled the precise determination of diastereomeric ratios and allowed for continuous monitoring of reaction selectivity during process development and optimization. The analytical performance of the in-line NMR–CHM system was thoroughly validated using orthogonal methods. Quantitative comparisons with high-field NMR, HPLC, GC, and GC-MS revealed deviations of less than 5%, confirming the reliability and accuracy of the benchtop setup for process analytical applications.

This methodology provides high-resolution, quantitative data on complex enzymatic systems in a fully integrated format and eliminates the need for invasive sampling, derivatization, or offline sample handling.

3.3 Kinetic Characterization and Initial Rate Measurements

This work package investigated the sequence–activity relationship of LTAs by assessing conversion and diastereoselectivity for the addition of glycine to a range of aromatic aldehydes (4-methylsulfonylbenzaldehyde, 4-nitrobenzaldehyde, 4-fluorobenzaldehyde, and benzaldehyde) as shown in Scheme 1. Initial rate measurements revealed that mutants displayed significantly higher *threo*-selectivity than wild-type enzymes. For example, in the presence of BnLTA-6xMut excellent *threo*-values of up to 99% *de* were achieved compared with ~54% for the wild-type enzyme across substrates, confirming mutational hot spots that improve diastereoselectivity. Based on these results, BnLTA-4xMut, BnLTA-6xMut, AhLTA-3xMut, and CpLTA-Mut1 were selected for further studies.



Scheme 1. Reaction scheme for the target biotransformation utilizing a panel of recombinant threonine aldolases.

Due to the low water solubility of aromatic aldehydes, co-solvents were tested. At 10%(v/v) DMSO, conversion improved, whereas higher co-solvent loads (20–30%) reduced activity. Screening with DMSO, DMF, MeOH, and MeCN confirmed that 10% (v/v) was optimal. Stirring speed also affected mass transfer, with 800 rpm improving substrate dispersion compared to

300 rpm. Subsequent detailed experimental studies explored kinetic *versus* thermodynamic control. With BnLTA-6xMut, lowering the aldehyde-to-glycine ratio decreased both conversion and selectivity, indicating that the reaction is still thermodynamically controlled. High substrate concentrations (>100 mM) further reduced conversion and accelerated the loss of selectivity, likely due to aggregation, substrate inhibition, and enzyme inhibition. Based on the intensive parameter screening and characterization of the reaction in terms of kinetic *versus* thermodynamic control, finally optimized reaction conditions and a promising process window were found which enabled the formation of the pharmaceutically interesting amino acid target product at 100 mM substrate concentration with a conversion exceeding 80% and a high diastereoselectivity of >96% *de*.

In conclusion, although LTA mutants achieve excellent diastereoselectivity under kinetic control in the intíal phase of the reaction, such biotransformations later proceed under thermodynamic control. Thus, in contrast to many other enzymatic transformations, the use of threonine aldolases leads to a thermodynamic equilibrium even when using such engineered mutants. Thus, for an efficient synthetic application furnishing high conversion, diastereo- and enantioselectivity, an excess of glycine and a termination of reactions in due time before the system shifts to thermodynamic control is required.

3.4 Kinetic Analysis by Progress Curve

In this work package, the kinetics of threonine-aldolase-catalyzed synthesis of L-*threo*-β-phenylserine and L-*erythro*-β-phenylserine from glycine and benzaldehyde were investigated. An 80 MHz benchtop NMR spectrometer enabled in-line monitoring of batch reactions, and the resulting data were analyzed by progress curve analysis (PCA) to extract kinetic parameters. This approach provided forward and reverse rate constants even without pure standards for all components. Initial rate measurements revealed inhibition of product formation by benzaldehyde, while glycine followed Michaelis–Menten kinetics without inhibition. No product inhibition was observed for L-*threo*-β-phenylserine; studies with L-*erythro*-β-phenylserine were not possible due to its unavailability. To address these limitations, a kinetic model based on ordinary differential equations was fitted to experimental NMR data using numerical optimization in Python. The model delivered robust kinetic parameters: v_{max} for L-*threo*- and L-*erythro*-β-phenylserine formation was ~0.12 ± 0.048 and 0.15 ± 0.03 U/mL, respectively; corresponding K_M values for benzaldehyde and glycine matched literature ranges. For the reverse reactions, inaccessible experimentally, the model predicted lower v_{max} values (0.01–0.03 U/mL) and K_M values of 60–75 mmol/L. Simulated concentration–time profiles agreed

with batch experiments across varying substrate concentrations, confirming the predictive power of the model. Molecular docking with SwissDock further supported reverse kinetics: L-*threo*-β-phenylserine exhibited lower binding affinity to the active site compared with L-threonine, consistent with its higher K_M . Diastereoselectivity analysis showed a slight preference for L-*erythro*-β-phenylserine at high benzaldehyde concentrations, whereas glycine had little effect.

In summary, benchtop NMR combined with PCA proved highly effective for characterizing complex reversible, multi-substrate enzyme systems, providing kinetic and selectivity insights beyond traditional initial rate analysis.

3.5 Immobilization of the Best Mutant Variant

This work package focused on immobilization studies with purified BnLTA-6xMut to facilitate process optimization and improve control over *threo*-preference in biotransformations. Purification yielded 90–92% recovery with a 14-fold increase in purity, providing material for immobilization trials. Given the hydrophobic benzaldehyde derivatives and hydrophilic glycine involved, carrier selection was critical. Fourteen commercial supports (adsorptive, covalent, and ionic) from Chiral Vision were screened. Immobilization efficiency exceeded 90% within 24 h for IB-ANI-14, IB-COV-8, and IB-ADS-11, while all carriers except IB-CAT-1 reached high efficiency within 42–66 h. A 24-day leaching study confirmed carrier stability, with only IB-CAT-1 showing significant protein release. Based on these results, IB-ANI-14, IB-COV-8, and IB-ADS-11 were chosen for further work. Relative activity assays compared immobilized and free enzymes. IB-COV-8 retained ≥90% conversion with 89 ± 2% immobilization efficiency and 18 ± 2 mg/g enzyme load. IB-ANI-14 and IB-ADS-11 maintained ≥70% relative conversion with similar immobilization efficiencies and enzyme loads. Recycling studies demonstrated ≥80% activity retention across at least seven cycles.

	Immobilization %	**Relative Conversion %**	**Enzyme load (mg/g)**
IB-ANI-14	**99±2**	**≥70**	**21±2**
IB-COV-8	**89±2**	**≥90**	**18±2**
IB-ADS-II	**90±2**	**≥70**	**18±2**

In conclusion, immobilization with selected carriers showed high efficiency, stability, and reusability, demonstrating strong potential for further application in threonine aldolase-catalyzed biotransformations.

3.3 **Reaction Engineering and Reactor System Evaluation**

The reaction engineering studies evaluated threonine-aldolase-catalyzed reactions in different reactor formats and assessed the benefits of enzyme immobilization. Stirred-tank reactors (STR), fed-batch reactors, enzymatic membrane reactors (EMR), and a magnetically stirred rotating bed reactor (Mag-RBR) with immobilized enzyme were tested. In STRs with free enzyme, a fed-batch process mitigated substrate inhibition but limited enzyme recovery. Immobilization on epoxy-functionalized carriers addressed this issue and enabled reuse. Immobilized threonine aldolase retained high activity and was successfully applied in a Mag-RBR, showing stable operation across multiple runs, negligible enzyme loss, and unchanged diastereoselectivity. Although diffusion effects reduced initial rates, immobilization improved process productivity through recyclability, continuous operation, and simplified downstream processing. In the EMR, space–time yield (STY) was analyzed at varying residence times. Higher residence times increased benzaldehyde conversion but decreased STY, with an optimum at ~30 minutes. Enzyme stability was further investigated by repeated EMR operation at a 15-minute residence time. Over 73.5 hours of continuous use, a half-life of ~63.5 hours was determined, lower than literature values, likely due to immobilization or shear stress. Selectivity analysis showed only a minor shift from *threo*- to *erythro*-products at extended residence times.

3.4 Scale up and product isolation

Based on the successful identification of a suitable “process window” within the process development study described in chapter 3.3., the objective of this working package was to perform the biotransformation under optimized conditions at an elevated lab scale, followed by an isolation of the product. Toward this end, the reaction was carried out at a 100 mL scale using the following conditions: 100 mM 4-methylsulfonylbenzaldehyde, 1 M glycine, 50 µM PLP, 10% DMF, and 5% (v/v) BnLTA-6x Mut in 100 mL of 100 mM Tris/HCl buffer (pH 8.0) at 25 °C, for 12 hours. The reaction was terminated by adding 10 volumes of methanol, centrifuged to remove precipitated glycine, and reduced to approximately 1 mL via vacuum concentration. For purification, the resulting reaction mixture was subjected to a C18 semi-preparative column. Fractions containing the product were identified through NMR, LC-MS analysis, and dried through vacuum concentration, yielding a solid product of 350 mg (99%

purity), which corresponds to an isolated, yet non-optimized yield of 18%. As for the diastereoselectivity of this reaction, the NMR and HPLC showed a ratio of the *threo*- and *erythro*-diastereomer of 88:12 in combination with excellent enantiomeric excess of ≥99% *ee*.

3.4 **Relaxometry**

The initial aim was to explore *in situ* product removal (ISPR) strategies to influence the diastereoselectivity of threonine aldolase reactions under process conditions. However, recent work by Zheng et al. (ACS Catalysis, 2021, DOI: https://doi.org/10.1021/acscatal.0c04949) showed that protein engineering alone could drastically alter selectivity. Accordingly, the work package was refocused to address an alternative goal, namely the NMR-based determination of enzyme loading in immobilized enzyme systems.

Conventional quantification of immobilized enzymes relies on indirect assays, such as photometric detection of unbound proteins, which provide little insight into adsorption within carrier pores. To address this, a TD-NMR method was developed and validated. By monitoring proton transverse relaxation times (T_2) in hydrated carriers, enzyme binding could be quantified: adsorption restricts water mobility, decreasing T_2. From these data, a pore-filling ratio (f_{nmr}) was defined as a measure of enzyme loading. A geometric model was introduced to relate T_2 reduction to effective pore radius and enzyme concentration. Comparison with Bradford assays showed that NMR-derived adsorption curves mirrored classical isotherms during monolayer formation but also captured deviations at higher enzyme concentrations due to multilayer adsorption and mass transfer limitations. Derivative analysis of f_{nmr} enabled precise identification of the monolayer-to-multilayer transition, providing a robust tool for optimizing immobilization protocols.

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

L. F. Schmidt; L. Jolly; L. Hennecke; F. Lopez Haro; H. Gröger; A. Liese; Benchtop NMR-based in-line analysis of diastereoselective enzymatic α-amino acid; synthesis: quantification and validation, Org. Proc. Res. Dev. 28 (10) (2024) 3791-3800; DOI: 10.1021/acs.oprd.4c00076

M. R. Serial, L. Schmidt, A. Muhammad, G. Brauckmann, S. Benders, V. Bueschler, A. Liese, A. Penn; A novel method for quantifying enzyme immobilization in porous carriers using simple NMR relaxometry; Biochem. Eng. J. 225 (2025) 109909; DOI: 10.1016/j.bej.2025.109909