Final Report

1 General Information

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Project title: "Multienzymkaskade im 2-Phasensystem - Prozessintegration"

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01.03.2021 – 15.06.2025 (incl. cost-neutral extension)

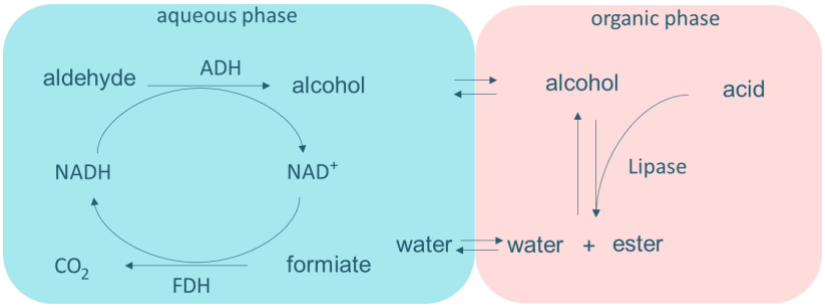
2 Zusammenfassung / Summary

Die in dieser Studie untersuchte biokatalytische Reaktion ermöglicht die nachhaltige Synthese des natürlichen Aromastoffs Zimtsäurezimtester über eine multienzymatische Kaskadenreaktion in einem Zweiphasensystem. Die Reaktionskaskade besteht aus drei Reaktionsschritten – zwei in der wässrigen Phase und einem in der organischen Phase –, die durch einen integrierten reaktiven Extraktionsschritt miteinander verbunden sind. Das Reaktionssystem ist in Abbildung 1a dargestellt und lässt sich wie folgt zusammenfassen: Die Ausgangsreaktion umfasst die Reduktion von Zimtaldehyd zu Zimtalkohol, katalysiert durch eine Alkoholdehydrogenase (ADH) in Gegenwart des Cofaktors NADH. Da NADH stöchiometrisch verbraucht wird, wird in einem parallelen Cofaktor-Regenerationsschritt eine Formiatdehydrogenase (FDH) eingesetzt, die NAD^+ durch Oxidation von Formiat zu CO_2 zu NADH reduziert. Der derzeitige Prozess ist eine Miniplant-Anlage, wie in Abbildung 1b dargestellt.
In der ersten Projektphase wurde die Enzymkaskade erfolgreich im Zweiphasensystem etabliert und ein Modell entwickelt. Auf der Grundlage dieses Modells wurden Sensitivitäts- und Optimierungsstudien mit dem Ziel der Prozessverbesserung durchgeführt. In der zweiten Phase wurde die Prozessintensivierung in Form einer Prozessintegration erfolgreich umgesetzt. Das Modell wurde erweitert, und insgesamt konnte die Effizienz des Prozesses in der zweiten Phase um das Zehnfache gesteigert werden.

The biocatalytical reaction under investigation in this study enables the sustainable synthesis of the natural flavour compound cinnamyl cinnamate via a multi-enzymatic cascade reaction in a two-phase system. The reaction cascade consists of three reaction steps - two in the aqueous phase and one in the organic phase - coupled by an integrated reactive extraction step. The reaction system is shown in Figure 1a and can be summarized as follows: the initial reaction involves the reduction of cinnamyl aldehyde to cinnamyl alcohol, catalysed by an alcohol dehydrogenase (ADH) in the presence of the cofactor NADH. Since NADH is consumed stoichiometrically, a formate dehydrogenase (FDH) is employed in a parallel cofactor regeneration step, reducing NAD^+ to NADH by oxidizing formate to CO_2. The current process is a miniplant setup as shown in Figure 1b.
During the first project phase, the enzyme cascade was successfully established in the two-phase system, and a model was developed. Based on this model, sensitivity and optimization studies were carried out with the aim of improving the process. In the second phase, process intensification in the form of process integration was successfully

implemented. The model was expanded, and overall, the efficiency of the process was increased tenfold during the second phase.

a)



b)

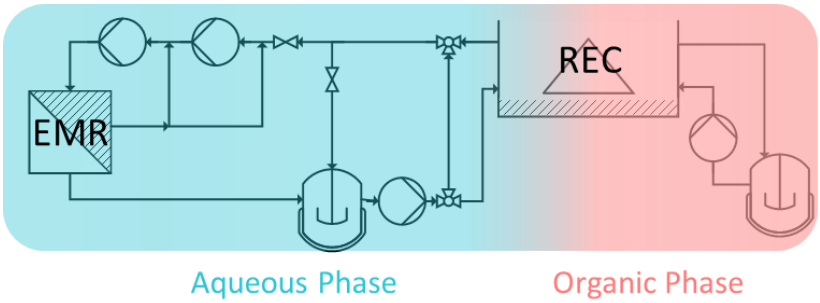


Figure 1: a) Sequence of the reaction cascade of the whole process. b) Scheme of the miniplant for the synthesis of cinnamyl cinnamate by a multi-enzymatic cascade. In the aqueous phase with the Enzyme Membrane Reactor (EMR), cinnamyl aldehyde is converted to cinnamyl alcohol by the use of an alcohol dehydrogenase and formate dehydrogenase for cofactor regeneration. In the organic phase the alcohol is esterified with cinnamic acid by a lipase to form cinnamyl cinnamate in a Reactive Extraction Centrifuge (REC).

3 Progress Report

The present project was funded in two phases. The first phase focused on successfully operating the multienzyme cascade in a two-phase system. The following objectives were defined in the research proposal:

1. Complete characterization of the three enzymatic kinetics.
2. Identification of a suitable solvent that meets the requirements of the process.
3. Establishment of the enzyme cascade.
4. Implementation and modelling of the overall process.

All objectives were successfully achieved. The results are summarized in four different publications and were described in detail in the subsequent proposal. Therefore, this report now focuses primarily on the second funding phase. This second phase aimed at process integration, with a particular emphasis on integrating the esterification reaction into the centrifuge.

1. The first commissioning of a three-phase enzymatic reactive extraction centrifuge.
2. Modelling of the whole multi enzyme reaction cascade.
3. Mathematical optimization of the plant.

In the evaluation of our proposal for the second phase, the reviewer noted that only the lipase reaction is being considered. We took this comment into account and changed the third aim into an intensification of the reactions of the dehydrogenases. The decision was taken to utilize an enzyme membrane reactor (EMR), enabling the use of native enzymes, which have been shown to exhibit significantly higher activity in comparison to immobilised enzymes employed in the process that was developed in the first phase of the project. The other aims remained unaffected.

The three objectives were successfully achieved in the second project phase. The results have so far been summarized in four different publications. Another publication is currently in preparation. The following report provides an overview of the key results from the second project phase.

Both dehydrogenase reactions are conducted in the EMR, a pressurized system that retains the freely dissolved enzymes. The EMR is operated at 35 °C, pH 8.0, and 20 bar. A modular envelope membrane unit (798 cm² membrane area, 1.2 L hold-up) facilitates the retenton of the enzyme. A PEBAX on PAN membrane is used. Circulation is driven by a centrifugal pump in order to prevent depositing of particles on the membrane. A piston pump is used to ensure stable operating pressure. The remaining aqueous volume is stored in a heated tank. The intermediate cinnamyl alcohol is transferred into the organic phase via the reactive extraction centrifuge (REC). The REC is based on a CINC V02 extraction centrifuge as it was used in the first phase of the project. It has a liquid hold-up of 140 mL and allows a rotational speed of up to 6000 rpm. The remaining volume of the organic phase is stored in a heated tank.

Compared to the classical CINC V02 centrifuge, the REC raises new operational challenges. The reactive zone in the rotor increases the pressure drop, limiting the operating window to avoid bypass flows. Reaction and extraction strongly depend on certain conditions, particularly residence time, which is determined by the volume flow. Three parameters define the operating window: organic flow, aqueous flow, and rotational speed. Both phases can be pumped at up to 800 mL/min. Figure 2 illustrates conditions that allow reaction, extraction, and separation in one unit. Rotational speed is varied between 7.5–13.5% of 6000 rpm. Higher speeds cause both phases to leave via the aqueous outlet; lower speeds force both phases through the organic outlet. Organic flows of 200 - 500 mL min-1 with aqueous flows of 180 - 250 mL min-1 are examined. In addition to that, a non-homogenous flow through the packing with short circuits, a non-homogeneous enzyme distribution, and wall effects cannot be prevented.

The operating window is constrained by pressure drop and phase ratio. Excessive pressure drop causes bypass streams, preventing reaction and separation. A too low flow in one

phase leads to backflow at the inlet. At speeds above the stable range, phase separation fails and accumulates in the aqueous tank; at lower speeds, centrifugal force is insufficient, again causing bypass streams.

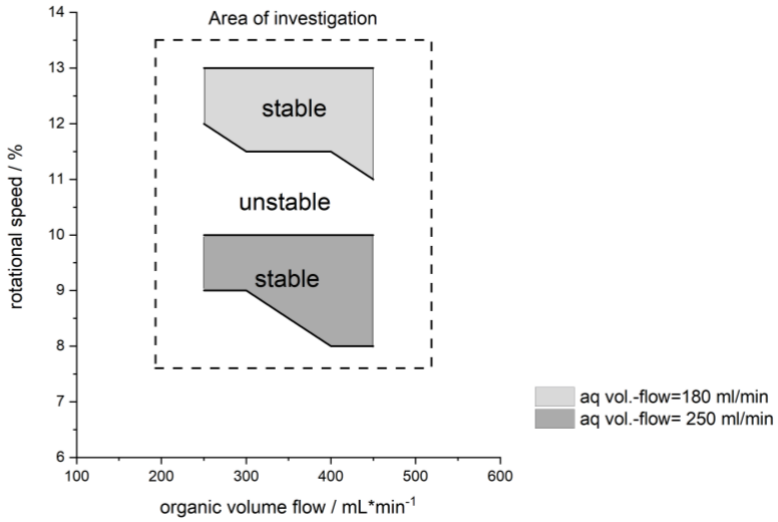


Figure 2: Operation window depending on the rotational speed of the rotor and the volume flow of the organic and aqueous phase.

For both dehydrogenases an irreversible Michaelis–Menten kinetics involving two substrates and product inhibition was applied. For parameter estimation a spline interpolation approach of progress curve analysis was used, as this methodology is particularly well-suited for complex enzymatic systems and provides robust results with less dependence on initial guess when estimating parameters from progress curves. The resulting parameters for a temperature of 35°C are shown in Table 1.

Table 1: Kinetic parameters with standard errors for the ADH and FDH catalysed reactions

Kinetic parameter ADH		**Kinetic parameter FDH**	
$\mathbf{v_{max,ADH}}$	4.8 ± 122 mmol L^{-1}·min^{-1}·mg_{ADH}^{-1}	$v_{max,FDH}$	5.17 ± 1.55 mmol L^{-1}·min^{-1}·mg_{FDH}^{-1}
$\mathbf{K_{mNADH}}$	0.2 ± 1.39 $mmol^{-1}$·L	K_{mNAD}	5.3 10^{-2} ± 0.45 10^{-2} $mmol^{-1}$· L
$\mathbf{K_{maldehyde}}$	4.2 ± 115.7 mmol L^{-1}	$K_{mformate}$	112.4 ± 89.3 mmol L^{-1}
$\mathbf{K_{ialcohol}}$	1.5 ± 16.9 mmol L^{-1}	K_{iNADH}	2.7 10^{-2} ± 3.9 10^{-2} mmol L^{-1}

The enzymatic conversion of the lipase catalysed reaction follows a bi–uni mechanism, with water present in excess and at constant saturation, thus not suitable as an educt. Its influence on the kinetics is treated as a competitive inhibition. However, under constant concentration and isothermal conditions, the inhibition can be implemented into the apparent Michaelis-Menten constants $K_{m,j,app}$. The resulting kinetic parameters are shown in Table 2.

Table 2: Kinetic parameters with standard errors for the lipase catalysed reaction

Kinetic parameter	
$v_{max,f}$	3.6 10^{-4} ± 0.19 10^{-4} mmol L^{-1}·min^{-1}·g_{Lipase}^{-1}
K_{Eq}	25.9 ± 1.1 10^{3} $mmol^{-1}$·L
K_{iCAC}	1.1 ± 0.3 mmol L^{-1}
$K_{m,appCOH}$	4.5 ± 0.6 mmol L^{-1}
$K_{m,appCAC}$	10.4 ± 2.6 mmol L^{-1}
$K_{m,appCCI}$	0.16 ± 0.04 mmol L^{-1}
K_{iCOH}	4.1 ± 5.5 mmol L^{-1}

The resulting parameters indicate a random mechanism [1]. The literature reports on a wide variety of reaction mechanisms [2-6]. However, it has to be considered, that the reaction medium can have a strong effect on the reaction mechanism itself [2,7,8]. The parameters equilibrium constant K_{eq} and the dissociation constant K_{iCOH} showed higher relative standard errors, this is attributed to their limited occurrence within the rate expression, reducing identifiability. Nevertheless, their magnitudes remain consistent with the expectations and literature published in the area of biocatalysis [9,10].

In order to study the given process deeply, a dynamic process model was developed. The model, implemented in Python, is based on ordinary differential equations that describe the molar balances of all components. The molar balances are solved in discrete time steps of one minute using the odeint solver from scipy. The validity of the developed model was assessed by comparing the simulation results with experimental data, as illustrated in Figure 3. The left side of the figure shows the concentration profiles in the aqueous phase, while the right side shows the results for the organic phase. Experimental data points are represented as dots, and simulation results are shown as lines. The shaded regions around the simulation curves represent the uncertainty based on the standard error of the kinetic parameters. In the aqueous phase, the experimental data shows an initial decrease in aldehyde concentration due to its partitioning into the organic phase. Over the course of the reaction, the expected increase in alcohol concentration and corresponding decrease in aldehyde concentration was observed. The simulation closely replicates this behaviour, with all experimental data points falling within the shaded error bounds of the model.

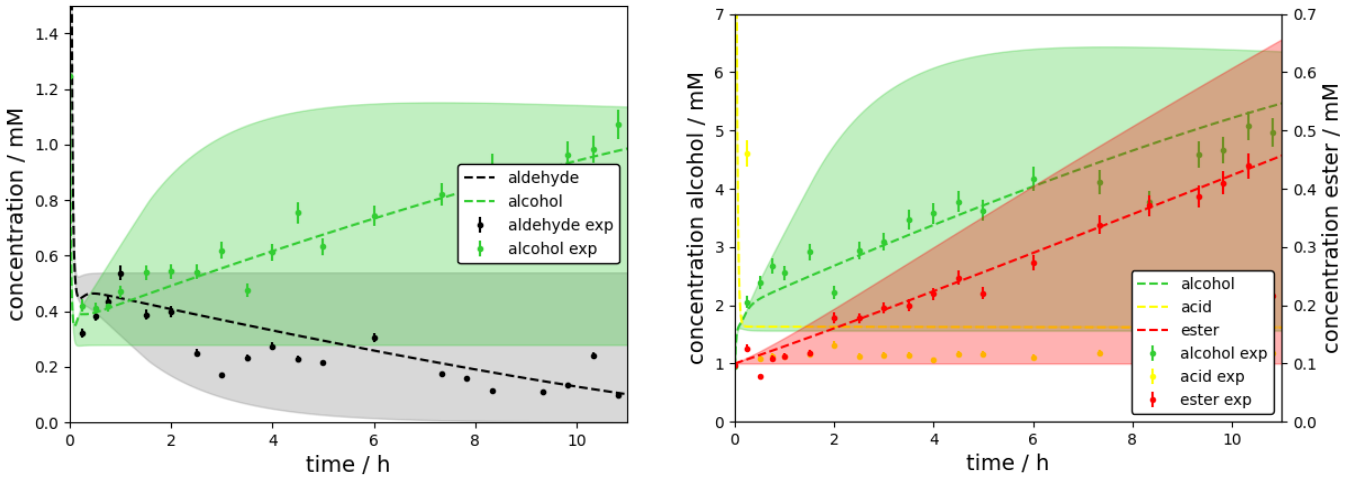


Figure 3: left: aqueous phase concentrations of cinnamyl alcohol and cinnamyl aldehyde with experimental and simulative results, right: organic phase concentrations of cinnamyl alcohol, cinnamic acid and cinnamyl cinnamate with experimental and simulative results at 35 °C, initial conditions: m_{ADH} = 10 mg, m_{FDH} = 20 mg, $m_{Novozym}$ = 10.2 g, $c_{NADH,int,aq}$ = 4.9 mmol L^{-1}, $c_{Aldehyde,int,aq}$ = 6.1 mmol L^{-1}, $c_{Acid,int,org}$ = 31 mmol L^{-1}

The normalized root mean squared error (nRMSE) between experimental and simulated data is 16.8% for the aldehyde and 6.8% for the alcohol. In the organic phase, the concentration of the final product, cinnamyl cinnamate, increases steadily, accompanied by an accumulation of the intermediate cinnamyl alcohol. This indicates that the lipase-catalysed esterification is the rate-limiting step of the enzymatic cascade. The second substrate, cinnamic acid, shows an initial rapid decrease followed by a plateau, consistent with its distribution equilibrium between the two liquid phases and relatively low conversion due to its involvement in only one reaction step. The simulation results agree very well with the experimental data, particularly for cinnamyl cinnamate and cinnamyl alcohol, with most of experimental values lying within the error bounds of the simulation. For cinnamic acid, the shaded area is not visible due to the relatively small conversion on the absolute scale. The simulation slightly overestimates the experimental data, but the simulation still captures its trend accurately. This may be caused by the mass transfer, which is currently considered to be instantaneous. The calculated nRMSE values for cinnamyl alcohol, cinnamic acid, and cinnamyl cinnamate are 11.6%, 30.5%, and 18.6%, respectively. It shows a particularly strong prediction for cinnamyl alcohol in both phases as well as cinnamyl cinnamate with nRSME of below 13% on average. These components are valuable indicators for the model quality as they are the intermediate and product of the cascade reaction. A good match for these components validates the implementation of the reaction kinetics, which were up to this point only validated individually. The highest nRSME is shown for the cinnamic acid. This matches with the observed systematic deviation from the experimental result. The reason for

that is probably the inaccuracy of the partition coefficient. The nRSME for all experiments and components is 18.1%, which is in the range of expectation for a biocatalytic model on a miniplant scale. Compared to literature many biocatalytic models are unfortunately only validated based on a qualitative match of experimental and simulative result, which applies for this model as well [10,11].

The operability together with the validation of the model were under detailed investigation. It is important to note that in highly integrated processes, the sensitivity of process parameters is an important factor. Minor changes to a process parameter can lead to significant changes in the product flow. Therefore, the operating window of the centrifuge and its limitations were investigated in detail. The primary objective of the second project phase was to intensify the process established during the first phase. To this end, development of a reactive extraction centrifuge was advanced, and, in addition, a membrane reactor was implemented as a unit operation. Taken together, these measures led to a pronounced intensification of the process. Figure 4 reports the yield of the intermediate cinnamyl alcohol in respect to the initial cinnamyl aldehyde concentration. Whereas in the first project phase the synthesis of the intermediate required several days to reach completion, an equivalent conversion was achieved within 6 hours in the intensified process. The same is true for the final product cinnamyl cinnamate. While it took several days to produce up to 1 mM cinnamyl cinnamate in the first phase of the project, this magnitude of concentration was achieved by the use of the intensified process within 8 – 10 hours. Consequently, the process productivity was increased approximately by a factor of ten. At the same time, the model quality was improved. While the overall mean error of the model across all measured components was 24.3% in the first project phase, it has now been reduced to 18.1%. The project thus successfully accomplished a process intensification.

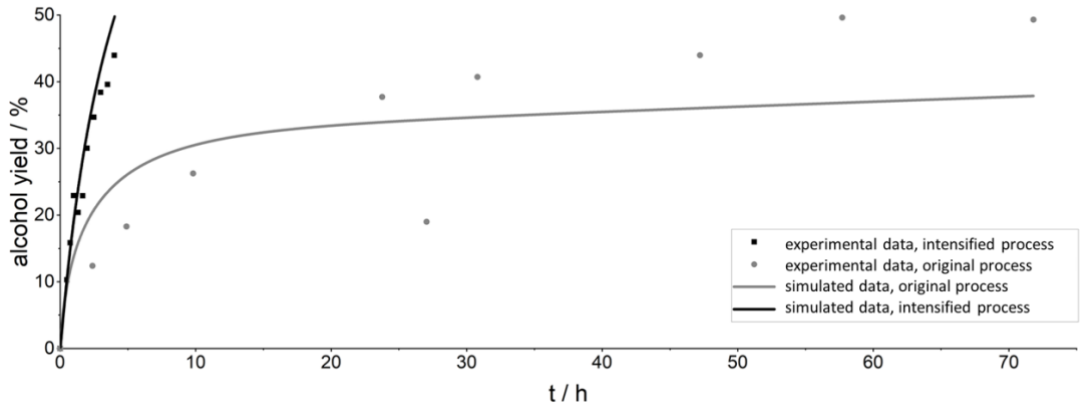


Figure 4: Experimental data and simulation of the mini plant. Black coloured data for the second phase of the project (intensified process). Grey coloured data for the first phase of the project (original process).

Handling of Research Data:

Particularly during the second project phase, we increasingly relied on the use of repositories. The data from the publications were made available either in a GitLab repository or in the TORE (TUHH Open Research) repository. This ensures that interested researchers can directly access our data, use our models, and apply them for their own research purposes. Furthermore, it is guaranteed that the data will be stored for at least 10 years.

Measures for Science Communication:

The results of the first project phase were presented in 18 contributions at international, national, and local events. The presenting researchers received two poster awards during this phase. The results of the second project phase were presented at seven international and national conferences. An overview of this can be found in Chapter 4.2.

Literature:

[1] H. Bisswanger, Enzyme kinetics: principles and methods, 3rd, enlarged and improved ed ed., Wiley-VCH, Weinheim, Germany, 2017.
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4 Published Project Results

4.1 Category A

First project phase:

[A1] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Pilot-scale Operation of a Multi-Enzymatic Cascade Reaction in a Multiphase System" Chem. Eng. Trans., 79, (2020), pp. 25-30, doi: 10.3303/CET2079005
[A2] C. Engelmann, N. Ekambaram, J. Johannsen, O. Fellechner, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: „Enzyme immobilization on nanoporous silica particles and their application in a bi-enzymatic reaction" ChemCatChem, 12, (2020), pp. 1-9, doi: 10.1002/cctc.201902293
[A3] C. Engelmann, J. Johannsen, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: " A Multi-Enzyme Cascade for the Production of High-Value Aromatic Compounds" Catalysts, 10, 1216 (2020), doi: 10.3390/catal10101216
[A4] J. Johannsen, F. Meyer, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Multi-Enzyme Cascade Reaction in a Miniplant Tw-Phase-System: Model Validation and Mathematical Optimization" AIChE Journal, 67, 4, (2021), e17158 doi: 10.1002/aic.17158

Second project phase:

[A5] F. Meyer, J. Johannsen, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Evaluation of process integration for the intensification of a biotechnological process" Chemical Engineering and Processing: Process Intensification, 167, (2021), 108506, doi: 10.1016/j.cep.2021.108506
[A6] F. Meyer, N. Gasimov, P. Bubenheim, T. Waluga: "Concept of an enzymatic reactive extraction centrifuge" Processes 10 (10): 2137 (2022), 10.3390/pr10102137
[A7] T. Waluga, F. von Ziegner, M. Skiborowski: "Analytical and Numerical Approaches to the Analysis of Progress Curves: A Methodological Comparison" Process Biochemistry 151, (2025), 1359-5113, doi: 10.1016/j.procbio.2025.01.029.
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4.2 Category B

First project phase

[B1] C. Engelmann, J. Johannsen, T. Waluga, G. Fieg, P. Bubenheim, A. Liese: „Multienzyme Cascade in 2-Phases" 5. Workshop des FSP Integrierte Biotechnologie und Prozesstechnik, Hamburg, 08.06.2017
[B2] J. Johannsen, G. Fieg, T. Waluga: „Enzymatic Cascade Reactions for Synthesis of High Value Products in a Multiphase System" AIChE Annual Meeting, Minneapolis, USA, 29.10.-03.11.2017
[B3] C. Engelmann, J. Johannsen, T.Waluga, G. Fieg, A. Liese, P.Bubenheim: „Production of flavours via a 2-phase multienzyme cascade" Himmelfahrtstagung 2018, Magdeburg, 07.-09.05.2018
[B4] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Multienzymatic cascade reactions in a 2-phase system", 9th International Congress on Biocatalysis, Hamburg, 26.-30.08.2018
[B5] C. Engelmann, J. Johannsen, G. Fieg, A. Liese, T. Waluga, P. Bubenheim: „Enzyme Cascades for Flavor Synthesis" 9th International Congress on Biocatalysis, Hamburg, 26.-30.08.2018
[B6] J. Johannsen, G. Fieg, T. Waluga: „Synthese von Spezialchemikalien durch Multienzymkaskaden im 2-Phasensystem" ProcessNet-Jahrestagung und 33. DECHEMA-Jahrestagung der Biotechnologen, Aachen, 10.-13.09.2018
[B7] C. Engelmann, J. Johannsen, P. Bubenheim, T. Waluga, G. Fieg, A. Liese: „Flavor Synthesis via a 2-Phase Enzyme Cascade" ProcessNet-Jahrestagung und 33. DECHEMA-Jahrestagung der Biotechnologen, Aachen, 10.-13.09.2018
[B8] C. Engelmann, C. Wallner, J. Johannsen, T. Waluga, G. Fieg, P. Bubenheim, A. Liese: „Multienzyme Cascade in 2-Phases" 6. Workshop des FSP Integrierte Biotechnologie und Prozesstechnik, Hamburg, 25.10.2018
[B9] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Multienzymatic Cascade Reactions in a Multiphase System" 6. Workshop des FSP Integrierte Biotechnologie und Prozesstechnik, Hamburg, 25.10.2018
[B10] C. Engelmann, N. Ekambaram, J. Johannsen, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: „Enzyme Cascade for Flavor Synthesis via Process Intensification" Himmelfahrtstagung 2019, Hamburg, 27.-29.05.2019
[B11] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Integration of Separation and Reaction for a Complex 2-phase Multienzymatic Cascade" Himmelfahrtstagung 2019, Hamburg, 27.-29.05.2019

[B12] C. Engelmann, J. Johannsen, O. Fellechner, I. Smirnova, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: „Enzyme cascades with integrated process intensification for flavor synthesis“ 14th International Symposium on Biocatalysis and Biotransformation, Groningen, Niederlande, 07.-11. Juli 2019
[B13] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Integrated Separation in a Complex 2-Phase Multienzymatic Cascade Reaction System“ 5th European Congress of Applied Biotechnology, Florenz, Italien, 15.-19.09.2019
[B14] C. Engelmann, E. Narendhiran, J. Johannsen, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: „An Enzymatic Cascade with Integrated Process Intensification for Synthesis of Natural Flavors“ 5th European Congress of Applied Biotechnology, Florenz, Italien, 15.-19.09.2019
[b16] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „Multienzymatic Cascade Reactions with Integrated Separation in a Complex 2-Phase System“ AIChE Annual Meeting, Orlando, USA, 10.11.-15.11.2019
[B17] J. Johannsen, C. Engelmann, A. Liese, G. Fieg, P. Bubenheim, T. Waluga: „3-Enzymatic Cascade Reaction Sequence in a 2-Phase-System“ 7. Workshop des FSP Integrierte Biotechnologie und Prozesstechnik, Hamburg, 28.11.2019
[B18] C. Engelmann, C. Wallner, J. Johannsen, T. Waluga, G. Fieg, A. Liese, P. Bubenheim: „A Promising Immobilization Technique Applied In An Enzyme Cascade“ 7. Workshop des FSP Integrierte Biotechnologie und Prozesstechnik, Hamburg, 28.11.2019

Second project phase

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[B20] F. Meyer, A. Liese, M. Skiborowski, P. Bubenheim, T. Waluga: “Commissioning and operation of an enzymatic reactive extraction centrifuge” 10th International Congress on Biocatalysis, Hamburg, Germany, 28.09.-01.09.2022
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[B24] F. Meyer, T.J. Brandt, M. Skiborowski, A. Liese, P. Bubenheim, T. Waluga: „Multifunctional Reactor for Process Intensification of a Multi-Enzymatic Cascade” AIChE Annual Meeting, Orlando, USA, 05.11.-10.11.2023
[B25] G. Brauckmann, F. von Ziegner, T. Waluga, P. Bubenheim: „ Investigation of an Enzyme Cascade Reaction in a Miniplant for Flavor Synthesis” 11th International Congress on Biocatalysis, Hamburg, Germany, 25.-29.08.2024

4.3 Patente (angemeldete und erteilte)

none